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Diagnosis and epidemiology of animal diseases in Latin America

Proceedings of the final Research Co-ordination Meetings of FAO/IAEA/SIDA co-ordinated research projects on

Immunoassay Methods for the Diagnosis and Epidemiology of Animal Diseases in Latin America held in Guadeloupe, Lesser Antilles, France, 13–17 June 1994 and

The Use of ELISA for Epidemiology and Control of Foot and Mouth Disease and Bovine Brucellosis in Latin America held in Vienna, Austria, 14–18 April 1997

organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture

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FOREWORD

In 1986 the Animal Production and Health Section of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture embarked on a programme of support to scientists in developing countries focused on improving animal disease diagnosis through the use of nuclear and related technologies. As part of this programme the Swedish International Development Authority (SIDA) agreed to provide support for a FAO/IAEA Co-ordinated Research Project (CRP) concerned with the introduction and use of such technologies in Latin America. Through this programme, which was entitled Regional Network for Latin America on Animal Disease Diagnosis Using Immunoassays and Labeled DNA Probe Techniques, studies were supported on a number of diseases considered to be of substantial economic and social importance to the region, including brucellosis, tuberculosis, babesiosis, leukosis, bluetongue and chlamydia infections in cattle and pseudorabies in pigs. The results obtained were published in 1992 as IAEA-TECDOC-657.

One significant conclusion was that the large number of diseases studied limited research findings owing to the lack of a critical mass of scientists studying any one specific disease problem. Thus when in 1991, SIDA agreed to support a follow-up CRP on Immunoassay Methods for the Diagnosis and Epidemiology of Animal Diseases in Latin America, the work was restricted to three diseases, i.e. foot-and-mouth disease (FMD), bovine brucellosis and bovine babesiosis. In 1994 results were presented in Guadeloupe, Lesser Antilles, France. The outcome of this meeting was the validation of ELISAs for the above mentioned diseases and a recommendation that future research should focus on diagnosis and epidemiology to support existing control and eradication campaigns against the two diseases of major importance in the region (FMD and Brucellosis).

A follow-up CRP (1994–1997) entitled The Use of ELISA for Epidemiology and Control of Foot-and-Mouth Disease and Bovine Brucellosis in Latin America focused on the further validation and subsequent use of a competitive brucellosis ELISA and a FMD antibody liquid phase blocking ELISA.

This TECDOC records the scientific achievements (39 papers) of these last two research projects (1991–1997) in a number of countries in Latin America. The report details the validation of standardized assays for the three diseases studied and recounts national surveys and their implications.

Closely related to national or regional animal health campaigns these efforts are expected to have a sustainable impact not only on health and production but also on improvement of international trade of livestock in Latin America. The expected eradication of FMD and brucellosis from the continent by the year 2009 from the majority of countries can be considered as milestones in that process.

FAO and IAEA wish to acknowledge the generous support provided to these programmes by the Government of Sweden through SIDA. The enthusiastic collaboration and assistance of all research contract and agreement holders during the programmes is also acknowledged. Particular thanks are due to R.H. Jacobson and K.H. Nielsen for their commitment in editing and revising the manuscripts. The officer responsible for this publication is A. Colling of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

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CONTENTS

Part B: FOOT-AND-MOUTH DISEASE

FMD antigen ELISA

FMD antibody ELISA

PART C: BABESIOSIS

Babesiosis antibody ELISA

INTRODUCTION

DIAGNOSIS AND EPIDEMIOLOGY OF ANIMAL DISEASES IN LATIN AMERICA

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1. BACKGROUND

Support for scientists and their endeavours in developing countries by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture is provided through FAO/IAEA Co-ordinated Research Projects (CRP) and IAEA Technical Co-operation Projects (TCPs). Using these mechanisms the Animal Production and Health Section of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture aims to encourage and improve the capacity of national institutions in developing countries to identify and resolve problems connected with improving livestock productivity and health. In 1986, the Section introduced an animal health component into its Project. The initial support was for five years but in 1991 this was extended for a further three years and linked with the support available from the IAEA's Technical Co-operation Project through national and regional TCPs and ARCAL* activities in Latin America dealing with diagnosis of animal diseases. Central to this overall project was the use of ELISA for the diagnosis and control of livestock diseases.

FAO/IAEA CRPs are developed around a well defined research topic on which between 15 and 20 national institutes collaborate — the topic itself being defined through consultation with national authorities in developing and developed countries and international agricultural research centers and organizations. The primary role of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture in such programmes is to ensure that the inputs and efforts under these programmes are co-ordinated and that the results are published.

The studies being reported in this IAEA TECDOC were initiated in 1991 and whilst the focus was on three major disease affecting livestock in the region (foot-and-mouth disease (FMD), brucellosis and babesiosis) the approach taken by individual Research Contract holders was different and thus in some cases research concentrated on assay validation whilst in other cases the focus was on the disease itself and its importance within the country in question.

Although this publication contains details of research work conducted under two CRPs, the papers are essentially a compilation of data presented at final Research Co-ordination Meetings (RCM) of the two CRPs, held in Guadeloupe, Lesser Antilles, June 1994 and in Vienna, Austria, April 1997.

2. FAO/IAEA CO-ORDINATED RESEARCH PROJECTS ON DIAGNOSIS AND EPIDEMIOLOGY OF ANIMAL DISEASES IN LATIN AMERICA.

Both CRPs were concerned with supporting scientists in the Latin American region wishing to use ELISA for improving the diagnosis and control of animal diseases, such as FMD, brucellosis and babesiosis. Although the first CRP was funded primarily by SIDA and co-ordinated by staff of the Animal Production and Health Section, substantial additional support for the introduction and use of the ELISA was provided through the IAEA's Technical Co-operation (TC) Project in Latin America. This allowed for the provision of ancillary equipment through national and regional TCPs operating at institutes where individual research contract holders were located as well as the provision of experts to visit laboratories and training through fellowships and scientific visits. The follow-up CRP was funded through the regular budget of the Joint FAO/IAEA Division of Nuclear Techniques in Food and

* ARCAL = Acuerdos Regionales Cooperativos para la Promoción de la Ciencia y la Tecnología Nucleares en Latino-América.

Agriculture but again activities were further supported through the Technical Co-operation Project of the IAEA.

2.1. Research Contracts and Agreements

2.1.1. Research contract holders

Under the CRP entitled Immunoassay Methods for the Diagnosis and Epidemiology of Animal Diseases in Latin America 1991–1994, 22 Research Contracts were awarded to scientists from Latin America (Figure 1). Three diseases e.g. brucellosis, FMD and babesiosis were covered by the project. Seven contracts were concerned with brucellosis, seven with FMD and eight with babesiosis.

FIG. 1. FAO/IAEA/SIDA Co-ordinated Research Project, 1991–1994, Immunoassay Methods for the Diagnosis and Epidemiology of Animal Diseases in Latin America.

A follow-up CRP (1994–1997) entitled: The Use of ELISA for Epidemiology and Control of foot-and-mouth Disease and Bovine Brucellosis in Latin America focused on the international standardization and validation of a competitive brucellosis ELISA and a FMD antibody liquid phase blocking ELISA. 10 Research Contracts (5 for each disease under study) were awarded to scientists in eight countries and 10 institutions (Figure 2).

Research Contract funds were used primarily to purchase ELISA equipment, reagents, FAO/IAEA ELISA kits and microtitre plates. In several cases a portion of the research grant was made available locally to provide funds for sample collection.

At each RCM each principal investigator provided a detailed report of the work carried out and an account of the workplan for the following year. The country reports, presented at the final RCMs in Guadeloupe, Lesser Antilles, and Vienna, Austria contained in addition a summation of these individual reports and constitute an account of the activities and results carried out between 1991 and 1997. For the

FIG. 2. FAO/IAEA Co-ordinated Research Project, 1994–1997, The Use of ELISA for Epidemiology and Control of foot-and-mouth Disease and Bovine Brucellosis in Latin America.

most part, these reports detail the introduction of the ELISA into a laboratory, the establishment of local "cut-off" values for the assay, the establishment of the test as routine and the collection and testing of samples initially for assay validation and subsequently as part of a national or regional survey on the occurrence of the disease in question and/or the monitoring control and eradication programmes.

2.2. Research co-ordination meetings

Under each Project 3 RCMs were held. The first of these was held at PANAFTOSA, Rio de Janeiro, Brazil in November 1991. The second meeting was held at ICA-CEISA, Bogota, Colombia in November-December 1992. The final meeting of the first Project was held at the IEMVT-CIRAD Guadeloupe, Lesser Antilles in June 1994.

The first meeting of the second Project was held in Buenos Aires, Argentina in November, 1994, the second meeting at PANAFTOSA, Rio de Janeiro, Brazil in September, 1995 and the final meeting in Vienna, Austria in April, 1997.

These meetings provided a platform for wide-ranging discussions on problems and experiences. Solutions to individual or common problems were thus shared amongst the group. During these meetings, Agreement holders were able to provide overall guidance and to offer advice at both the individual and group level. These meetings also offered the opportunity to provide training in both ELISA and in the use of computer software for data analysis and epidemiology.

2.3. Support activities

2.3.1. FAO/IAEA ELISA kits

Central to supporting the Latin American project was the development and introduction of standardized ELISA kits specifically suited to the types of conditions found in laboratories in this region.

A full report of the ELISA systems for the various diseases and their field validation is contained in these proceedings. However, crucial to their design was the use of a standard format and protocol and wherever possible standard reagents, thus ensuring that once a laboratory had established an FAO/IAEA ELISA kit for one particular disease, it would be a simple matter to introduce similar kits for the study of other epizootics. In principle the approach was to use an indirect assay utilizing Ortho-phenylenediamine (OPD) or 2,2'-azino bis (3-ethyl-benzthiazoline sulfonic acid) (ABTS) as the substrate, samples tested in 100µl amounts and in duplicate, a 96-well plate format, and one hour incubation steps at 37°C with three plate washes between each step.

The cut-off value for the assay was determined using 2–3 standard deviations of the mean value of the local negative population and in comparison to the local positive population. The internal controls included a strong positive (C++), moderate or weak positive (C+), a negative (C-) reference serum and a conjugate control (Cc) in quadruplicate. Robustness of the kit was considerably improved through the use of freeze-dried reagents.

The FAO/IAEA ELISA kits were designed to contain all the necessary reagents, be robust enough to withstand extremes of temperature and contain sufficient reagents to test 4000 sera in duplicate. ELISA plates were purchased in bulk to avoid batch to batch variation and shipped together with the kit. To ensure further standardization, the equipment supplied was primarily from the same manufacturer.

2.3.2. Training

Adequate and appropriate training was considered a key element in this Project. At the first RCM in Brazil a one-week course introduced the FAO/IAEA ELISA kits for the diagnosis of FMD, brucellosis and babesiosis to the Contract holders. During the following 12 months several of the Contract holders were visited by FAO/IAEA experts and thus further training provided at the national level.

FIG. 3. FAO/IAEA Technical Co-operation Projects 1993, Animal Disease Diagnosis in Latin America.

The second RCM of the project was preceded by a one-week FAO/IAEA/ARCAL regional training course in epidemiology and data analysis held at the ICA-CEISA, in Bogota, Colombia in November, 1992. Also scientists, who were not involved in this CRP were able to attend this training course. During the subsequent three years of this Project and the follow-on CRP further local training was provided through visits to the region by FAO/IAEA and PANAFTOSA experts and Agreement holders in the Project.

Activities were complemented by several IAEA TC national and regional training courses (Chile, 1991; Ecuador, 1991; Paraguay, 1992; Peru, 1992; Costa Rica, 1993; Cuba 1993; El Salvador, 1994; Mexico, 1994; Chile, 1994; Paraguay, 1994; Argentina, 1994; Chile, 1997).

3. CONCLUSIONS AND RECOMMENDATIONS

3.1. General conclusions

3.1.1. Support activities

Both CRPs and the various training activities associated with them were concerned with introducing, validating and using FAO/IAEA ELISA kits for the diagnosis and monitoring of animal diseases in support of studies undertaken at national research institutes in Latin America. This approach was augmented by inputs from a number of IAEA national TCPs and one regional TCP (ARCAL III, RLA/5/028). For the most part this approach proved highly successful and the ELISA was shown to be both an appropriate and effective system for diagnosing and monitoring these diseases. In the majority of countries the clear advantage of ELISA over existing methods was demonstrated.

3.1.2. Reagents

The development and provision of standardized reagents and protocols for the ELISA offered considerable advantages, particularly with respect to international assay validation, inter-laboratory comparison of results, trouble-shooting and assessment of the reliability of results through external quality assurance.

3.1.3. Standardized approach

Difficulties were highlighted that can be encountered both in introducing and maintaining an ELISA system. Nevertheless, it was clear that a standardized approach to the diagnosis and control of a particular disease was possible and enabled several countries in the region to undertake co-operative control Projects. Central to these was assurance that ELISA results from individual laboratories participating in such projects were reliable and comparable.

3.1.4. Establishing ELISA laboratories

Under the CRPs and the linked national/regional IAEA TCPs, the necessary equipment and training to carry out the ELISA were provided to many national research institutes. Over 50 ELISA laboratories in Latin America were established and strengthened through these activities.

3.1.5. FAO/IAEA ELISA software program EDI (ELISA Data Interchange)

Initially individual ELISA software programs (e.g. BREIA) were developed and distributed for each assay. The new ELISA software package EDI was found to be suitable for all FAO/IAEA ELISAs and is crucial for the adequate handling and storage of results. Through the eqstat.qc file, results were retrieved and easily copied on a diskette for further analysis e.g. for the EQA project to monitor internal

controls Initial communication problems between ELISA reader and computer were solved using a "smart cable"

3.2 General recommendations

3 2 1 Further use of ELISA

ELISA should be recommended as the test of choice for the diagnosis and monitoring of FMD, brucellosis and babesiosis in the developing country situation The further development and routine use of internationally standardized and validated ELISA kits against the major epizootics should further be encouraged and supported

3 2 2 FAO/IAEA external quality assurance programme

The FAO/IAEA external quality assurance programme needs to develop towards a generic veterinary diagnostic testing laboratory accreditation scheme with a focus on quality management and documentation of specific laboratory activities through standard operating procedures (SOPs) Participation in an EQA programme will assist in creating a quality management working environment, which will assist laboratories - especially from developing countries, which do not have a national accreditation body and thus bridge the gap between what they have now and formal national or international recognition of Quality Management and Technical Competence

3 2 3 Further supply of crucial reagents

Mechanisms should be developed to ensure the long term supply of crucial reagents for use in the ELISA For the majority of diseases, laboratories should be encouraged to use internationally standardized and validated kits Support should not be provided for individual laboratories in the region to develop their own kits but a move towards regional laboratories providing specific kits should be encouraged

3.3. General considerations and future activities

The reports contained in these proceedings clearly indicate that the FAO/IAEA CRPs and Technical Co-operation activity succeeded in introducing into the participating Latin American institutes an ability to carry out epidemiological studies using ELISA technology.

The first goal of the programme was to evaluate the usefulness of ELISA methods for the diagnosis of three diseases as compared with existing conventional methods.

The second goal was to use these test, once validated, for the conduct of serological surveys of the incidence/prevalence of the diseases under consideration and to monitor the effectiveness of national programmes aimed at disease control e.g through seromonitoring studies. ELISA can also subsequently be used as a tool for surveillance to demonstrate freedom and provide evidence for international recognition of eradication

The third objective was to generate the data necessary to enable international acceptance of test reagents and protocols in order that these may subsequently be used in other countries and sectors of the world to improve the diagnosis and control of diseases considered to be of importance in the region

It is clear though that the task of establishing these techniques in most countries in the region and for these three epizootics is almost complete Over 50 ELISA laboratories were established in Latin America FAO/IAEA ELISA kits for these three diseases are now available and for the most part have now been standardized and fully validated Sustainability of utilizing ELISA-based systems can be assured through cost-benefit studies, which have shown that ELISA kits are only a minor component in the overall expenditure of animal health activities Future support in the Animal Production and Health Sub-programme of the Joint FAO/IAEA Division will move away from kit development and standardization to the use of the ELISA system to carry out epidemiological studies on the occurrence of

such disease and the monitoring of control and eradication programmes. Central to the use of ELISA will be participation in the FAO/IAEA external quality assurance programme.

Specific Conclusions and Recommendations

3.4. Indirect Brucellosis ELISA

3.4.1. Conclusions

3.4.1.1. Cut-off determination

Considerable problems were experienced in initially establishing a universal "cut-off" for this assay and it became clear that it is necessary to undertake this exercise for each defined cattle population.

3.4.1.2. Use of indirect brucellosis ELISA

The indirect ELISA has been fully validated and standardized through the studies conducted. It has clearly been shown that it does not separate vaccinated from naturally infected animals but that variations in the "cut-off" can be used to alter sensitivity and specificity in a defined manner.

3.4.2. Recommendations

3.4.2.1. Selection of an appropriate cattle population for cut-off determination

At a minimum each national laboratory should establish a set of known brucellosis antibody negative sera and use these to determine its own "cut-off" value for the indirect brucellosis ELISA. Care should be taken that this set of sera are typical of the national cattle population. Studies may be necessary to ensure that a national "cut-off" is appropriate.

3.4.2.2. Appropriate cut-off for different stages of brucellosis campaign

The FAO/IAEA standardized ELISA is a valuable addition to the diagnosis and control of brucellosis. By altering the "cut-off" it can be used in a fully defined manner through the various stages of brucellosis control and eradication.

Competitive brucellosis ELISA

3.4.3. Conclusions

3.4.3.1. ELISA in comparison with other diagnostic techniques

In general ELISA was more accurate than the conventional tests e.g. buffered plate antigen test (BPAT), Rose bengal test (RBT) and confirmatory tests e.g. complement fixation test (CFT), 2-mercaptoethanol, (2-ME) and Radial immunodiffusion (RID). This study included 30 000 individual samples that were tested in seven different assays. The total number of individual tests performed was more than 200 000. Furthermore, the sera tested were from throughout the continent from various countries and different bovine breeds. This individual work has been the largest serological study ever done involving testing for brucellosis in the Americas.

3.4.3.2. Performance of C-ELISA II

Competitive ELISA II (which uses LPS as antigen and monoclonal antibody Mab 84 as competing reagent) is preferred because the LPS antigen is relatively simpler to prepare, the antibody is

directed against a defined epitope and possesses high affinity. In addition the test has high sensitivity and specificity, is useful for differentiating infected from vaccinated cattle and resolves cross reactions due to infections with *Yersinia enterocolitica*. Even though the competitive ELISA I (O-chain antigen) also performed well, it has the disadvantage that the antigen and conjugate preparation are more troublesome.

3.4.3.3. Versatility of C-ELISA

One of the major potentials of the competitive ELISA is that it can be used for diagnosis in different species of animals, including humans.

3.4.3.4. Limitations of ELISA in general

One of the limitations of all the current ELISA techniques is that they are not suitable for testing animals on the farm since they require adequate laboratory facilities and trained personnel for their use.

3.4.3.5. Restrictions of indirect ELISA

Although the indirect ELISA possesses good sensitivity and specificity, it is useful only for testing a limited number of species and does not distinguish vaccinal antibody from that due to infection.

3.4.3.6. Cut-off selection

Cut-off values must be established for the different countries and regions, and may depend on factors such as prevalence and vaccination status.

3.4.3.7. Other important serological diagnostic techniques

BPAT and RBT are useful diagnostic tests for screening sera, specially in laboratories where the capability to effectively use the ELISA has not yet been developed.

3.4.4. Recommendations

3.4.4.1. OIE Approval

OIE approval should be sought for ADRI/ELISA, C-ELISA I and C-ELISA II as OIE prescribed tests for the diagnosis of brucellosis and the separation of vaccinated from naturally infected animals.

3.4.4.2. Publication of results

The compiled validation data generated by all the participant countries should be published in a refereed scientific journal.

3.4.5. Recommendations for future projects

3.4.5.1. Alternative diagnostic techniques

Evaluation of the fluorescence polarization assay (FPA) and radial immunodiffusion (RID) test for the field diagnosis of brucellosis and comparison of the performance, characteristics of these assays with the standardised ELISAs, in particular the C-ELISA should be undertaken.

3.4.5.2. Further investigation

Evaluation of the competitive ELISA II and other assays in calfhood, adult and revaccinated herds.

3.4.5.3. Milk-ELISA

Validation of the I-ELISA(ADRI) for the detection of antibodies (anti-*brucella* LPS) in milk.

3.4.5.4. C-ELISA II

Application of competitive ELISA II for the diagnosis of brucellosis.

3.4.5.5. Quality Assurance

Any country that uses FAO/IAEA ELISA kits should participate in the FAO/IAEA external quality assurance programme.

3.5. FMD group

Antigen liquid phase blocking ELISA

3.5.1. Conclusions

3.5.1.1. Sensitivity

The FMD antigen detection ELISA is a more sensitive test than CFT for the primary diagnosis of FMD.

3.5.1.2. Standardization

Reagents for the ELISA can be more easily standardized and stored than those for the CFT.

3.5.1.3. Monitoring

Monitoring of field strains using ELISA provide valuable data for epidemiological surveillance and assessment of suitable vaccine strains.

3.5.2. Recommendations

3.5.2.1. Superiority of ELISA

The higher detection rate of the ELISA over the CFT and the ease of performance strongly recommends its use for fast and reliable diagnosis of FMD.

3.5.2.2. Standard Tests

The ELISA and tissue culture isolation should be the OIE prescribed tests for primary diagnosis of FMD.

Antibody liquid phase blocking ELISA

3.5.3. Conclusions

3.5.3.1. Interlaboratory comparison

One of the central observations was that the intra- and interlaboratory variation of the test was too high and that the range for the C++ (=strong positive control serum) and (Ca) antigen control were

too narrow. In Brazil and Venezuela the values for the C++ sometimes exceeded the upper control limit (too high) or were below the lower control limit (too low). In Argentina and Colombia only too high C++ values were observed in some cases. In Paraguay the general tendency was towards too low C++ values with the exception for the antigen of serotype A, where both too low and too high C++ values were observed. The reason for these differences may be due to different pipetting techniques which may become important when glycerinated antigen is used. Nevertheless the predictive value of the test was good.

3.5.3.2. Reproducibility

The evaluation of reproducibility of the liquid phase ELISA for FMD revealed that the C++, C+, and C- controls were reasonably consistent between laboratories.

3.5.3.3. Upper and lower control limits (UCL, LCL)

The pre-determined UCL and LCL values for the C++ were too narrow (3 PI units) to be useful in making decisions about this parameter. These limits were recalculated using data from all laboratories.

3.5.3.4. Antigen control

The antigen control (Ca) proved to be variable between laboratories. The accuracy of the assay (Ca values falling within control values) was lacking in some laboratories; either the data exceeded the upper control limits or were less than the lower control limits. The precision of the assay within a laboratory also varied between laboratories.

3.5.3.5. Accuracy

The accuracy for the Ca was assessed in relation to the UCL and LCL as originally defined by PANAFTOSA. These limits were too narrow. The UCL and LCL were thus re-defined based on the data collected for the Ca control from all laboratories.

3.5.3.6. Pipetting

Because of the general consistency of value for all Ca samples within and between plates in a run, it was concluded that the probable cause of the problem was with pipetting of the glycerinated antigen stock solution.

3.5.4. Recommendations

3.5.4.1. Consistency of Ca values

The laboratories involved should retest the serum panels using a more explicitly defined procedure for pipetting the glycerinated antigen. It is essential that this reagent (Ca) gives uniform, reproducible, and accurate results, because it is used for calculation of the PI values for all control and test sera.

3.5.4.2. Field use

Application of the assay for routine diagnosis will require resolution of this problem.

3.5.4.3. Training

Once these problems are resolved, training will be needed for interpretation of FMD ELISA results in control and eradication campaigns.

3.5.4.4. FAO/IAEA external quality assurance programme (EQAP)

An external quality assurance programme is essential to assess proficiency in conducting the FMD ELISA within and between laboratories.

3.5.4.5. Laboratory accreditation

An international harmonized set of principles for the quality management of veterinary diagnostic testing laboratories and a process for monitoring compliance with these principles is needed to establish a common ground for understanding and evaluating the reliability of the management, operations and outputs of these laboratories.

3.5.4.6. Interregional comparison

The reproducibility and repeatability of the data should be compared with the same data from an FAO/IAEA Co-ordinated Research Project on FMD diagnosis in Asia CRP (D3.20.14). Reagents and protocols for these two FMD assays differ: the current study was done with PANAFTOSA-specific reagents while the reagents for the Asian study are from Pirbright laboratories, UK. Such an analysis may clarify which assay format is most appropriate for use in developing countries.

3.5.4.7. FAO/IAEA ELISA software

The FAO/IAEA ELISA software program "EDI" rejects a plate when the value of a control serum is a border line value. EDI should be modified so that border line values are still regarded as "within limits".

3.5.4.8. Use of antibody ELISA

The FMD virus antibody ELISA should be routinely used for surveillance, import/export testing and for assessing protection in vaccinated populations. It can also be used in combination with cattle challenge for measuring FMD vaccine potency.

3.5.4.9. Non-structural-protein antibody ELISA (NSP)

Research should be undertaken preferably through a CRP to adapt the FMD ELISA for detection of antibodies to non-structural proteins for separating vaccinated from infected animals and a CRP.

3.6. Babesiosis group

3.6.1. Conclusions

3.6.1.1. Use of ELISA

The ELISA kit for the determination of antibodies to *Babesia bovis* proved to be appropriate for use in Latin American countries although the "cut-off" point needed to be determined for each country. This assay is particularly appropriate for epidemiological studies to determine conditions for establishing enzootic stability and the most appropriate control/intervention strategy.

3.6.1.2. Epidemiology

Surveys carried out by the Contract holders showed that the transmission of *Babesia bovis* varied not only from country to country but also from regions within most countries and depended on the geography, climate, breed of cattle, acaricide treatment and infection rate of *Boophilus microplus,* the vector of *Babesia bovis.* Some Contract holders had difficulties in obtaining or selecting sample sub-populations of cattle that were truly representative of the whole population.

3.6.1.3. Standardization

These studies highlighted the need for standardization and international validation of such assays if conclusions are to be meaningful.

3.6.2. Recommendations

3.6.2.1. Promotion of a standardized assay

The use of an internationally validated and standardized assay and protocol to obtain international recognition for the ELISA as a standard assay for the diagnosis of babesiosis should be promoted.

3.6.2.2. Establishing a serum bank

Every effort should be made to expand national banks of sera by sampling regions where *Boophilus microplus* is present. In addition to serum collection, each serum should be identified according to the following criteria: age, sex, breed, geography, climate, acaricide treatment and *Babesia bovis* vaccination status. When analyzing data all parameters should be assessed in determining the risk of babesiosis outbreaks. On this basis meaningful recommendations for appropriate babesiosis control strategies can be made to national authorities.

3.6.2.3. ELISA for control programme

The use of ELISA to improve control programmes against *Babesia bovis* should be promoted.

PRINCIPLES OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES[1]

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Abstract

PRINCIPLES OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES

Assay validation requires a series of inter-related processes Assay validation is an *experimental process* reagents and protocols are optimized by experimentation to detect the analyte with accuracy and precision Assay validation is a *relative process* its diagnostic sensitivity and diagnostic specificity are calculated relative to test results obtained from reference animal populations of known infection/exposure status Assay validation is a *conditional process* classification of animals in the target population as infected or uninfected is conditional upon how well the reference animal population used to validate the assay represents the target population, accurate predictions of the infection status of animals from test results (PV+ and PV–) are conditional upon the estimated prevalence of disease/infection in the target population Assay validation is an *incremental* process confidence in the validity of an assay increases over time when use confirms that it is robust as demonstrated by accurate and precise results, the assay may also achieve increasing levels of validity as it is upgraded and extended by adding reference populations of known infection status Assay validation is a *continuous process* the assay remains valid only insofar as it continues to provide accurate and precise results as proven through statistical verification Therefore, the work required for validation of diagnostic assays for infectious diseases does not end with a time-limited series of experiments based on a few reference samples rather, to assure valid test results from an assay requires constant vigilance and maintenance of the assay, along with reassessment of its performance characteristics for each unique population of animals to which it is applied

INTRODUCTION

Validation is the evaluation of a process to determine its fitness for a particular use A validated assay yields test results that identify the presence of a particular analyte (e g an antibody) and allows predictions to be made about the status of the test subject However, for infectious disease diagnostic assays, the identity and definition of the criteria required for assay validation are elusive, and the process leading to a validated assay is not standardised

By considering the variables that affect an assay's performance, the criteria that must be addressed in assay validation become clearer. The variables can be grouped into three categories (a) the sample — host/organism interactions affecting the analyte composition and concentration in the serum sample, (b) the assay system — physical, chemical, biological and technician-related factors affecting the capacity of the assay to detect a specific analyte in the sample, and (c) the test result — the capacity of a test result, derived from the assay system, to predict accurately the status of the host relative to the analyte in question

Factors that affect the concentration and composition of analyte in the serum sample are mainly attributable to the host and are either inherent (e g. age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e g passively acquired antibody, active immunity elicited by vaccination or infection). Non-host factors, such as contamination or deterioration of the sample, may also affect the analyte in the sample

Factors that interfere with the analytical accuracy of the assay system are instrumentation and technician error, reagent choice and calibration, accuracy of controls, reaction vessels, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and error introduced by detection of closely related analytes, such as antibody to cross-reactive organisms, rheumatoid factor, or heterophile antibody.

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[1] This paper has been published in the "Manual of Standards for Diagnostic Tests and Vaccines", p 8-15 by OIE, France 1996 and is included as an excerpt in this TECDOC with the permission of the OIE

Factors that influence the capacity of the test result to predict accurately the infection or analyte status of the host[2] are diagnostic sensitivity (D-SN), diagnostic specificity (D-SP), and prevalence of the disease in the population targeted by the assay D-SN and D-SP are derived from test results on samples obtained from selected reference animals The degree to which the reference animals represent all of the host and environmental variables in the population targeted by the assay has a major impact on the accuracy of test result interpretation For example, experienced diagnosticians are aware that an assay, validated by using samples from northern European cattle, may not give valid results for the distinctive populations of cattle in Africa

The capacity of a positive or negative test result to predict accurately the infection status of the animal is the most important consideration of assay validation This capacity is not only dependent on a highly precise and accurate assay and carefully derived estimates of D-SN and D-SP, but is heavily influenced by prevalence of the infection in the targeted population Without a current estimate of the disease prevalence in that population, the interpretation of a positive or negative test result may be compromised

Many variables obviously must be addressed before an assay can be considered 'validated' [1] However, there is no consensus whether the concept of assay validation is a time-limited process during which only those factors intrinsic to the assay are optimised and standardised, or whether the concept includes an ongoing validation of assay performance for as long as the assay is used Accordingly, the term 'validated assay' elicits various interpretations among laboratory diagnosticians and veterinary clinicians Therefore, a working definition of assay validation is offered as a context for the guidelines outlined below

A. DEFINITION OF ASSAY VALIDATION

A validated assay consistently provides test results that identify animals as positive or negative for an analyse or process (e g antibody, antigen, or induration at skin test site) and, by inference, accurately predicts the infection status of animals with a predetermined degree of statistical certainty This chapter will focus on the principles underlying development and maintenance of a validated assay Guidelines for the initial stages in assay development are included because they constitute part of the validation process How this early process is carried out heavily influences the capacity of the eventual test result to provide diagnostic accuracy

B. STAGES OF ASSAY VALIDATION

Development and validation of an assay is an incremental process consisting of at least five stages 1) Determination of the feasibility of the method for a particular use, 2) Choice, optimisation, and standardisation of reagents and protocols, 3) Determination of the assay's performance characteristics, 4) Continuous monitoring of assay performance, and 5) Maintenance and enhancement of validation criteria during routine use of the assay (Figure 1) Although some scientists may question the relevance of the fourth and fifth stages as validation criteria, they are included here because an assay can be considered valid only to the extent that test results are valid, i e whether they fall within statistically defined limits and provide accurate inferences

An indirect enzyme-linked immunosorbent assay (ELISA) test for detection of antibody will be used in this chapter to illustrate the principles of assay validation It is a test format that can be difficult to validate because of signal amplification of both specific and non-specific components [2] This methodology serves to highlight the problems that need to be addressed in any assay validation process The same basic principles are used in validation of other complex or simple assay formats

Because of space limitations, this introductory chapter provides only basic guidelines for the principles concerned with assay validation It is derived from a more detailed treatment of the subject [3]

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[2] In this paper, the terms positive and negative have been reserved for test result and never refer to infection or antibody/antigen status of the host Whenever reference is made to 'infection' or 'analyte', any method of exposure to an infectious agent that could be detected directly (e g antigen) or indirectly (e g antibody) by an assay, should be inferred

STAGE 1. FEASIBILITY STUDIES

In the ELISA example, feasibility studies are the first stage in validating a new assay They are carried out in order to determine if the selected reagents and protocol have the capacity to distinguish between a range of antibody concentrations to an infectious agent while providing minimal background activity Feasibility studies also give initial estimates of repeatability, and of analytical sensitivity and specificity.

1. *Control samples*

It is useful to select four or five samples (serum in our example) that range from high to low levels of antibodies against the infectious agent in question. In addition, a sample containing no antibody is required. These samples will be used to optimise the assay reagents and protocol during feasibility studies, and later as control samples The samples ideally should represent known infected and uninfected animals from the population that eventually will become the target of the validated assay. The samples should have given expected results in one or more serological assays other than the one being validated. The samples are preferably derived from individual animals, but they may represent pools of samples from several animals. A good practice is to prepare a large volume (e.g 10 ml) of each sample and divide it into 0.1 ml aliquots for storage at -20°C One aliquot of each sample is thawed, used for experiments, and held at 4°C between experiments until depleted Then, another is thawed for further experimentation This method provides the same source of serum with the same number of freeze-thaw cycles for all experiments (repeated freezing and thawing of serum can denature antibodies so should be avoided) Also, variation is reduced when the experimenter uses the same source of serum for all experiments rather than switching among various sera between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples After the initial stages of assay validation are completed, one or more of the samples can become the serum control(s) that are the basis for data expression and repeatability assessments both within and between runs of the assay They may also serve as standards if their activity has been predetermined; such standards provide assurance that runs of the assay are producing accurate data [1].

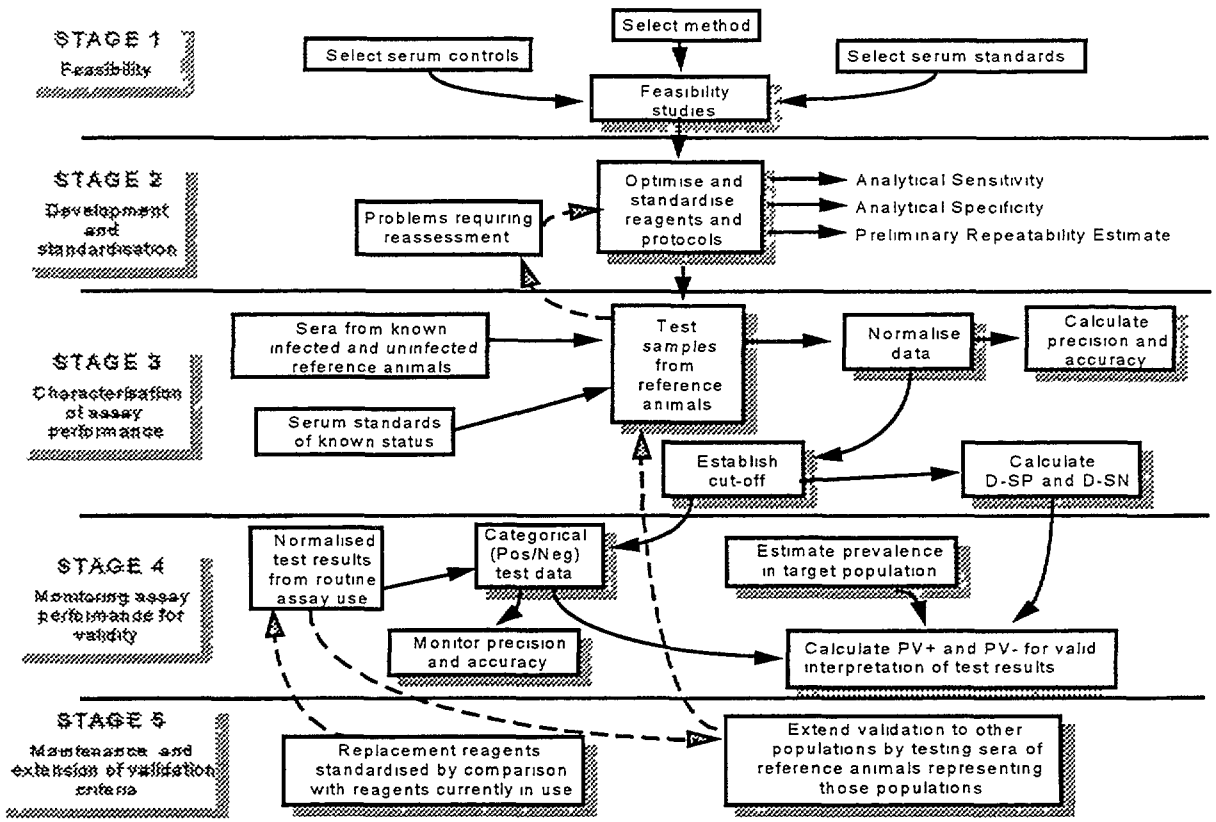

FIG. 1. Stages in the incremental process of assay validation. Shadowed boxes indicate action points within each stage in the process.

2. Selection of method to achieve normalised results

Normalisation adjusts raw test results of all samples relative to values of controls included in each run of the assay (not to be confused with transformation of data to achieve a 'normal' [Gausian] distribution). The method of normalisation and expression of data should be determined preferably no later than at the end of the feasibility studies. Comparisons of results from day to day and between laboratories are most accurate when normalised data are used. For example, in ELISA systems, raw optical density (absorbance) values are absolute measurements that are influenced by ambient temperatures, test parameters, and photometric instrumentation. To account for this variability, results are expressed as a function of the reactivity of one or more serum control samples that are included in each run of the assay. Data normalisation is accomplished in the indirect ELISA by expressing absorbance values in one of several ways [1]. A simple and useful method is to express all absorbance values as a percentage of a single high-positive serum control that is included on each plate. This method is adequate for most applications. More rigour can be brought to the normalisation procedure by calculating results from a standard curve generated by several serum controls. It requires a more sophisticated algorithm, such as linear regression or log-logit analysis. This approach is more precise because it does not rely on only one high-positive control sample for data normalisation, but rather uses several serum controls, adjusted to expected values, to plot a standard curve from which the sample value is extrapolated. This method also allows for exclusion of a control value that may fall outside expected confidence limits.

For assays that are end-pointed by sample titration, such as serum (viral) neutralisation, each run of the assay is accepted or rejected based on whether control values fall within predetermined limits. Because sample values usually are not adjusted to a control value, the data are not normalised by the strict definition of the term.

Whatever method is used for normalisation of the data, it is essential to include additional controls for any reagent that may introduce variability and thus undermine attempts to achieve a validated assay. The normalised values for those controls need to fall within predetermined limits (e.g. within ±2 or ±3 standard deviations of the mean of many runs of each control).

STAGE 2. ASSAY DEVELOPMENT AND STANDARDISATION

When the feasibility of the method has been established the next step is to proceed with development of the assay and standardise the selected reagents and protocols.

1. Selection of optimal reagent concentrations and protocol parameters

Optimal concentrations/dilutions of the antigen adsorbed to the plate, serum, enzyme-antibody conjugate, and substrate solution are determined through 'checkerboard' titrations of each reagent against all other reagents, following confirmation of the best choice of reaction vessels (usually evaluation of two or three types of microtitre plates, each with its different binding characteristics, to minimise background activity while achieving the maximum spread in activity between negative and high-positive samples). Additional experiments determine the optimal temporal, chemical, and physical variables in the protocol, including incubation temperatures and durations; the type, pH, and molarity of diluent, washing, and blocking buffers; and equipment used in each step of the assay (for instance pipettes and washers that give the best reproducibility).

Optimisation of the reagents and protocol includes an assessment of accuracy by inclusion, in each run of the assay, one or more serum standards of a known level of activity for the analyte in question. An optimised assay that repeatedly achieves the same results for a serum standard and the serum controls may be designated as a standardised assay.

2. Repeatability - preliminary estimates

Preliminary evidence of repeatability (agreement between replicates within and between runs of the assay) is necessary to warrant further development of the assay. This is accomplished by evaluating results from replicates of all samples in each plate (intra-plate variation), and inter-plate variation using the same samples run in different plates within a run and between runs of the assay. For ELISA, raw absorbance values are usually used at this stage of validation because it is uncertain whether the results of the high-positive control serum, which could be used for calculating normalised values, are reproducible in early runs of the assay format. Also, expected values for the controls have not yet been established. Three-to-four replicates of each sample run in at least five plates on five separate occasions are sufficient to provide preliminary estimates of repeatability. Coefficients of variation (standard deviation of replicates/mean of replicates), generally less than 20% for raw absorbance values, indicates adequate repeatability at this stage of assay development. However, if evidence of excessive variation (>30%) is apparent for most samples within and/or between runs of the assay, more preliminary studies should be done to determine whether stabilisation of the assay is possible, or whether the test format should be abandoned. This is important because an assay that is inherently variable has a high probability of not withstanding the rigours of day-to-day testing on samples from the targeted population of animals.

3. Determination of analytical sensitivity and specificity

The analytical sensitivity of the assay is the smallest detectable amount of the analyte in question, and analytical specificity is the degree to which the assay does not cross-react with other analytes. These parameters are distinguished from diagnostic sensitivity and specificity as defined below. Analytical sensitivity can be assessed by end-point dilution analysis, which indicates the dilution of serum in which antibody is no longer detectable. Analytical specificity is assessed by use of a panel of sera derived from animals that have experienced related infections that may stimulate cross-reactive antibodies. If, for instance, the assay does not detect antibody in limiting dilutions of serum with the same efficiency as other assays, or cross-reactivity is common when sera from animals with closely related infections are tested, the reagents need to be recalibrated or replaced, or the assay should be abandoned.

STAGE 3. DETERMINING ASSAY PERFORMANCE CHARACTERISTICS

If the feasibility and initial development and standardisation studies indicate that the assay has potential for field application, the next step is to identify the assay's performance characteristics.

1. Diagnostic sensitivity and specificity

a) Principles and definitions

Estimates of diagnostic sensitivity (D-SN) and diagnostic specificity (D-SP) are the primary parameters obtained during validation of an assay. They are the basis for calculation of other parameters from which inferences are made about test results. Therefore, it is imperative that estimates of D-SN and D-SP are as accurate as possible. Ideally, they are derived from testing a series of samples from reference animals of known history and infection status relative to the disease/infection in question. Diagnostic sensitivity is the proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false negative results. Diagnostic specificity is the proportion of uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results. The number and source of reference samples used to derive DSN and D-SP are of paramount importance if the assay is ever to be properly validated for use in the general population of animals targeted by the assay.

It is possible to calculate the number of reference samples, from animals of known infection/exposure status, required for determinations of D-SN and D-SP that will have statistically defined limits. Formulae for these calculations, their limitations, and a discussion of the selection criteria for standard sera are detailed elsewhere [3]. Because of the many variables that must be accounted for, at least 300 reference samples from known-infected animals, and no fewer than 1,000 samples from known-uninfected animals, should be included to determine initial estimates of D-SN and D-SP, respectively. The number of samples should be expanded to approximately 1,000 and 5,000, respectively, to establish precise estimates of D-SN and D-SP [3].

b) Standards of comparison for the new assay

In serology, the 'standard of comparison' is the results of a method or combination of methods with which the new assay is compared. Although the term 'gold standard' is commonly used to describe any standard of comparison, it should be limited to methods that unequivocally classify animals as infected or uninfected. Some isolation methods themselves have problems of repeatability and sensitivity. Gold standard methods include unequivocal isolation of the agent or pathognomonic histopathological criteria. Because the gold standard is difficult to achieve, relative standards of comparison are often necessary; these include results from other serological assays and from experimentally infected or vaccinated animals. Calculations of D-SN and D-SP are most reliable when the gold standard of comparison is available. When only relative standards of comparison are available, estimates of D-SN and D-SP for the new assay may be compromised because the error in the estimates of D-SN and D-SP for the relative standard is carried over into those estimates for the new assay.

c) Precision, repeatability, reproducibility, and accuracy

Repeatability and reproducibility are estimates of precision in the assay. Precision is a measure of dispersion of results for a repeatedly tested sample; a small amount of dispersion indicates a precise assay. Repeatability in a diagnostic assay has two elements: the amount of agreement between replicates (usually two or three) of each sample within a run of the assay, and the amount of between run agreement for the normalised values of each control sample. Reproducibility is the amount of agreement between results of samples tested in different laboratories. Accuracy is the amount of agreement between a test value and the expected value for an analyte in a standard sample of known activity (e.g., titre or concentration). An assay system may be precise, but not accurate, if the test results do not agree with the expected value for the standard.

Reliable estiates of repeatability and accuracy, both within and between runs of the assay, can be obtained by use of normalised results from the many runs of the new assay that were required to assess the sera of reference animals (less reliable estimates were obtained from preliminary data using raw absorbance values). At least 10, and preferably 20 runs of the assay will give reasonable initial estimates of these parameters. Methods for evaluating these parameters have been described in detail [3].

Accuracy can be assessed by inclusion of one or more standards (samples of known titre, concentration, etc.) in each run of the assay. The standards may be control sera provided that the amount of analyte (e.g. titre, concentration) in each one has been previously determined by comparison with primary or secondary reference standards [1], and the control sera are not used in the data normalisation process.

Reproducibility of the assay is determined in several laboratories using the identical assay (protocol, reagents, and controls) on a group of at least 10 samples, preferably duplicated to a total of 20 samples. These samples need to represent the full range of expected analyte concentrations in samples from the target population. The extent to which the collective results for each sample deviate from expected values is a measure of assay reproducibility. The degree of concordance of between laboratory data is one more basis for determining whether the assay's performance characteristics are adequate to constitute a validated assay.

2. Selection of the cut-off (positive/negative threshold)

To achieve estimates of D-SN and D-SP of the new assay, the test results first must be reduced to positive or negative categories. This is accomplished by insertion of a cut-off point (threshold or decision limit) on the continuous scale of test results. Although many methods have been described for this purpose, three examples will illustrate different approaches, together with their advantages and disadvantages. The first is a cut-off based on the frequency distributions [3] of test results from uninfected and infected reference animals. This cut-off can be established by visual inspection of the frequency distributions, by receiver-operator characteristics (ROC) analysis [4, 5], or by selection that favours either D-SN or D-SP, whichever is required for a given assay [6]. A second approach is establishing a cut-off based only on uninfected reference animals; this provides an estimate of DSP but not D-SN. The third method provides an 'intrinsic cut-off' based on test results from sera drawn randomly from within the target population with. no prior knowledge of the animals' infection status [7]. Although no estimates of D-SN and D-SP are obtained by this method, they can be determined as confirmatory data are accumulated.

If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is difficult to select a cut-off that will accurately classify these animals according to their infection status. Rather than a single cut-off, two cut-offs can be selected that define a high D-SN (e.g. inclusion of 99% of the values from infected animals), and a high D-SP (e.g. 99% of the values from uninfected animals). The values that fall between these percentiles would then be classified as suspicious or equivocal, and would require testing by a confirmatory assay or retesting for detection of seroconversion.

3. Calculation of diagnostic sensitivity and specificity

The selection of a cut-off allows classification of test results into positive or negative categories. Calculation of D-SN and D-SP are aided by associating the positive/negative categorical data with the known infection status for each animal using a two-way (2 x 2) table (Table 1). After the cut-off is established, results of tests on standard sera can be classified as true positive (TP) or true negative (TN) if they are in agreement with those of the gold standard (or other standard of comparison). Alternatively, they are classified as false positive (FP) or false negative (FN) if they disagree with the standard. Diagnostic sensitivity is calculated as TP/(TP + FN) whereas diagnostic specificity is f TN/(TN + FP); the results of both calculations are usually expressed as percentages (Table I).

TABLE I. CALCULATIONS OF D-SN AND D-SP AIDED BY A 2 X 2 TABLE THAT ASSOCIATES INFECTION STATUS WITH TEST RESULTS FROM 2000 REFERENCE ANIMALS

		Reference animals of known infection status	
		Infected	**Uninfected**
Test result	**Positive**	**570** **TP**	**FP** **46**
	Negative	**30** **FN**	**TN** **1354**
		Diagnostic sensitivity $\frac{TP}{TP+FN} = \frac{570}{600} = 95\%$	**Diagnostic specificity** $\frac{TN}{TN+FP} = \frac{1354}{1400} = 96{,}7\%$

STAGE 4. MONITORING VALIDITY OF ASSAY PERFORMANCE

1. Interpretation of test results - factors affecting assay validity

An assay's test results are useful only if the inferences made from them are accurate. A common error is to assume that an assay with 99% D-SN and 99% D-SP will generate one false-positive and one false-negative result for approximately every 100tests on animals from the target population. Such an assay may be precise and accurate, but produce test results that do not accurately predict infection status. For example, if the prevalence of disease in a population targeted by the assay is only 1 per 1,000 animals, and the false positive test rate is 1 per 100 animals (99% D-SP), for every 1,000 tests on that population, ten will be false positive and one will be true positive. Hence, only approximately 9% of positive test results will accurately predict the infection status of the animal; the test result will be wrong 91% of the time. This illustrates that the capacity of a positive or negative test result to predict infection status is dependent on the prevalence of the infection in the target population [8]. Of course, the prevalence will probably have been determined by use of a serological test.

An estimate of prevalence in the target population is necessary for calculation of the predictive values of positive (PV+) or negative (PV-) test results. When test values are reported without providing estimates of the assay's D-SP and D-SN, it is not possible to make informed predictions of infection status from test results [8]. It is, therefore, highly desirable to provide an interpretation statement with test results accompanied by a small table indicating PV+ and PV- for a range of expected prevalences of infection in the target population. Without provision of such information, test results from the assay may have failed to accurately classify the infection status of animals, and thus do not reflect a fully validated assay.

STAGE 5. MAINTENANCE AND ENHANCEMENT OF VALIDATION CRITERIA

A validated assay needs constant monitoring and maintenance to retain that designation. Once the assay is put into routine use, internal quality control is accomplished by consistently monitoring the assay for assessment of repeatability and accuracy [9].

Reproducibility between laboratories should be assessed at least twice each year. It is useful to volunteer membership in a consortium of laboratories that are interested in evaluating their output. In the near future, good laboratory practice, including implementation of a total quality assurance programme, will become essential for laboratories seeking to meet national and international certification requirements.

Proficiency testing is a form of external quality control for an assay. It is usually administered by a reference laboratory that distributes panels of samples, receives the results from the laboratories, analyses the data, and reports the results back to the laboratories. If results from an assay at a given laboratory remain within acceptable limits and show evidence of accuracy and reproducibility, the laboratory may be certified by government agencies or reference laboratories as an official laboratory for that assay. Panels of sera for proficiency testing should contain a full representation of an analyte's concentration in animals of the target population. If the panels only have high-positive and low-positive sera (with none near the assay's cut-off), the exercise will only give evidence of reproducibility at the extremes of analyte concentration, and will not clarify whether routine test results on the target population property classify infection status of animals.

Because of the extraordinary set of variables that impact on the performance of serodiagnostic assays, it is highly desirability to expand the number of standard sera from animals of known infection status because of the principle that error in the estimates of D-SN and D-SP is reduced with increasing sample size. Furthermore. when the assay is to be transferred to a completely different geographic region, it is essential to re-validate the assay by subjecting it to sera from populations of animals that reside under local conditions.

When serum control samples are nearing depletion, it is essential to prepare and repeatedly test the replacement samples. When other reagents, such as antigen for capture of antibody, must be replaced, they should be produced using the same criteria as for the original reagents, and tested in at least five runs of the assay. Whenever possible, it is important to change only one reagent at a time to avoid the compound problem of evaluating more than one variable at a time

C. VALIDATION OF ASSAYS OTHER THAN ENZYME-LINKED IMMUNOSORBENT ASSAY

Although the example used has been an indirect ELISA test, the same principles apply to the validation of any diagnostic assay. It is of utmost importance not to stop after the first two stages of assay validation - that does not constitute a validated assay for diagnostic use. Although reagent and protocol refinement are important, the selection of the reference populations is probably the most critical factor. it is no surprise when reviewing the literature to find a wide range of estimates for D-SN and D-SP for the same basic assay. Although part of the variation may be attributed to the reagents chosen, it is likely that the variation in estimates of D-SN and D-SP is due to biased selection of sera on which the test was 'validated'. This stage in assay validation needs more attention than it has been given previously. This is particularly true in the current atmosphere of international trade agreements and all their implications with regard to movement of animals and animal products.

REFERENCES

[1] WRIGHT, P.F., NILSSON, E., VAN ROOIJ, E.M.A., LELENTA, M., JEGGO, M.H., Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis, Rev. sci. tech. Off. int Epiz. **12** (1993) 435-450.

[2] CROWTHER, J.R., ELISA theory and practice. In: Methods in Molecular Biology. Humana Press, Totowa, NJ, USA, (1995) 1-256.

[3] JACOBSON, R.H., Validation of serological assays for diagnosis of infectious diseases, Manual of Standards for Diagnostic Tests and Vaccines, OIE, France (1996) 8-15.

[4] GREINER, M., SOHR D., GOBEL P., A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests, J. Immunol Methods **185** (1995) 123-132.

[5] ZWEIG, M.H., CAMPBELL, G., Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine, Clin. Chem. **39** (1993) 561-577.

[6] SMITH, R.D., Clinical Veterinary Epidemiology, Butterworth-Heinemann, Stoneham, MA, USA, (1991) 1-223.

[7] GREINER, M., FRANAKE, C.R., BOHNING, D., SCHLATTMANN, P., Construction of an intrinsic cut-off value for the sero-epidemiological study of *Trypanosoma evansi* infections in a canine population in Brazil: a new approach towards unbiased estimation of prevalence, Acta Trop. **56** (1994) 97-109.

[8] JACOBSON, R.H., How well do serodiagnostic tests predict the infection of disease status of cats, J. Am. Vet. Med. Assoc. **199** (1991) 1343-1347.

[9] CEMBROWSKI, G.S., SULLIVAN, A.M., Quality control and statistics, In: An Introduction to Clinical Chemistry, Bishop, M.L., Duben-Engelkirk, J.L., Fody, E.P., eds. Lippincott, Philadelphia, USA, (1992) 63-101.

FACTORS IN SELECTING SERUM SAMPLES FOR USE IN DETERMINING THE POSITIVE/NEGATIVE THRESHOLD (CUT-OFF) IN ELISA

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Abstract

FACTORS IN SELECTING SERUM SAMPLES FOR USE IN DETERMINING THE POSITIVE/NEGATIVE THRESHOLD (CUT-OFF) IN ELISA

The threshold (cut-off) that defines whether a test result is seropositive or seronegative is calculated by testing serum samples from a subpopulation of animals that is assumed to represent the target population in all aspects For this proposition to be true, it is essential to consider the variables in the target population that must be represented in the subpopulation Without representation of the variables in the subpopulation, it is likely that the cut-off selected for the test will be errant and will misclassify animals as to their infection status The purpose of this paper is to identify a few of the principal variables that need to be taken into account when selecting a subpopulation of animals for test validation

1 INTRODUCTION

The threshold (also known as the cut-off) is the unit of activity in a serodiagnostic test above which animals are classified as positive and below which they are considered negative Serologic antibody activity is often used to infer whether an animal is infected or uninfected with a particular agent of disease The consequences for an animal or herd may be quarantine or depopulation of the herd as occurs in disease eradication programs or the data may be used in disease control and herd management strategies The accuracy of the cut-off, therefore, has considerable herd health and economic implications Placement of the cut-off on the scale of antibody activity for a disease agent is the basis for calculation of diagnostic sensitivity and specificity [1,2] Diagnostic sensitivity and specificity are usually viewed as evidence of the test's performance when it is being considered for diagnostic uses The selection of the cut-off and the ability to determine the appropriateness of the data underlying the cut-off have highly consequential ramifications Cut-offs must be selected with utmost care

2 FACTORS IN SELECTING A CUT-OFF

The cut-off is determined by applying the test to a selected subpopulation of animals of known infection and vaccinal status The underlying assumption in any assay is that the subpopulation of animals upon which the cut-off was based is representative in every respect of the targeted population of animals for which the assay is intended Because the targeted population for the test may be all of the cattle in a given country, it is apparent that these animals may represent a wide variety of genetic, environmental, and nutritional variables which may impact on a test's sensitivity, specificity, accuracy and precision The first consideration in selecting a subpopulation for determining the cut-off in an assay is to obtain a group of animals that is unequivocally infected and another group that has never experienced an infection of the disease agent in question

2.1. Infected animal group

Historically, the infection status of animals used as subjects in assay validation has been either known or infered Isolation of the organism from the animals that make up the infected group is proof that they are experiencing the infection at the time the serum sample was obtained A second indication of infection is histopathology, which may be pathognomonic resulting in a strong inference that the animal was infected with the agent in question, for other agents, however, histopathology renders only a presumptive diagnosis A third and less compelling inference of infection is the positive result of another

serological test This method is among the least useful because all of the error in determining the sensitivity and specificity of this so-called "gold standard" test is carried over to the test being validated A fourth method is clinical diagnosis without any laboratory evidence of infection, this is the least useful in designating an animal as infected or not

Experimental infection of animals with the agent in question is another means of assuring exposure to the agent Route of administration and dose of the inoculum are variables that may elicit antibody responses that are qualitatively and/or quantitatively much different that those elicited by natural infection For instance, inoculation of massive numbers of organisms intramuscularly may result in a completely different antibody response than when a tick vector introduces a few organisms into the host percutaneously Conversely, if prior studies indicate that experimental and natural infections elicit antibodies in ways that are indistinguishable from natural infections, experimental infections may be the method of choice to develop a subpopulation of animals for test validation It would be important, however that environmental and genetic considerations not be overlooked in the choice of such experimental animals because these factors can have a profound effect on antibody responses or, through non-specific factors, a test's performance

Among infected animals, the antibody response can be considerably different between individuals depending upon the stage of infection, the species of organism that elicits that response, and the age of the host If the serum sample is taken at two to three weeks after exposure of adult animals to an acute infection that is known to elicit a strong antibody response, a relatively high level of antibody would be expected However, if the sample was taken from such animals just after exposure to the organism or at 6 months after recovery from the infection, the antibody level may be much lower Cattle exposed as neonates to the causative agent of paratuberculosis, Mycobacterium paratuberculosis, may not develop detectable antibody responses for several months or even years despite isolation of the organism from the feces Indeed, even adult animals will be anergic for months to this organism after initial exposure The dynamics of exposure to an agent and antibody levels in serum that the agent elicits require that animals selected for the subpopulation be representative of all stages of the targeted infection in order to reflect the realities of the population at large This requires knowledge of the host-parasite relationship at both the individual animal and population levels

2.2. The uninfected group

Selection of a group of organism-negative animals as determined by isolation procedures, is not sufficient to assure that antibody to the agent is not present in the animal The animal may have eliminated the organism and still have varying amounts of specific antibody in its serum, or the method to detect the organism may have lacked sensitivity Animals negative on other antibody tests are only presumptively uninfected if they do not have detectable antibody, it is possible that the reference test is analytically less sensitive than the test being validated Correlative test results by themselves, therefore, are not the best means of determining the animal's prior infection history and may be misleading in classifying animals as uninfected

Animals from geographical areas that are known to be free of the disease in question are usually good candidates for the "uninfected group" in assay validation assuming that 1) there is no history of disease in these animals clinically, 2) laboratory tests have always been negative during the last several years, 3) the herds are "closed" so that no movement of infected animals into the herds has occurred, 4) adjacent farms are also known to be infection/disease free, and 5) the environment, genetics, and nutrition of the animals is similar to that of the target population This combination of criteria has a high likelihood of providing an uninfected group of animals that can be used to establish the specificity of the assay

An additional factor that must be considered is the possibility that closely related organisms, endemic in the area, may induce antibody that is cross-reactive in the test being validated The uninfected animals may then give results that raise the cut-off artificially and make the test not as diagnositcally sensitive as it could have been were cross-reactive antibodies considered in validation of the assay

3 CUT-OFF SELECTION

Assuming that the subpopulations of infected and uninfected animals have been properly selected, samples from these animals have been run in the ELISA, and variation within and between runs of the assay were minimal, the normalized data for the test samples then can be evaluated for selection of the optimal cut-off that will minimize false responses (Normalization in this case means that optical density readings of the test samples are converted to percent of the high positive serum control in each plate as indicated in the FAO/IAEA kit instructions)

Diagnostic sensitivity and specificity in any assay is a trade-off for a range of ELISA values such as percent of positive control (PP) As the location of the PP cut-off point increases on the continuum from 0 to 100%, the sensitivity decreases and the specificity increases If one sums the sensitivity and specificity values for each interval of PP points on the continuum of cut-off points (such as for PPs of 10%, 20%, 30%, etc), one of the cut-off points will give the greatest sum of the sensitivity and specificity values this is considered the most accurate cut-off point for that assay [1] A receiver operator characteristic (ROC) curve is a similar way of describing the accuracy of a test over a range of cut-off points [3, 4] It serves as a nomogram for reading off the specificity that corresponds to a given sensitivity for the test From this curve, a point can be found that describes the greatest accuracy for the assay and allows the optimal sensitivity and specificity to be read from the x and y axes These methods provide a statistically sound means of determining a cut-off for an assay

A commonly used method for establishing the cut-off is to select the mean plus two or three standard deviations of PPs from all uninfected animals This method assumes that the PPs for all uninfected sera will form a Gausian (normal) curve when plotted as a frequency distribution of PP values Because such data are virtually never normally distributed, this method lacks validity statistically Alternatively, the non-parametric method of two times the mean of PPs of the sera from uninfected animals has been used as the cut-off which is a reasonable way to establish that approximately 95% to 99% of the uninfected animals in the population will fall in the test negative group A limitation of methods that determine cut-off only on the basis of the sera from uninfected animals is that they do not take into consideration the degree of overlap of PPs between uninfected and infected animals

The actual cut-off selected is usually mandated by practical realities and not statistical optimization Using ROC curves may give the statistically optimal cut-off for an assay for FMD In practice, however, the cut-off may need to be lowered to assure that the test sensitivity approaches 100% in order to not misclassify any infected animals as negative-false negative results for a disease with devastating economic and animal health consequences are not acceptable With such a cut-off, test results for some uninfected animals will be interpreted as positive, but will be falsely positive because the specificity of the test has been lowered as a consequence of lowering the cut-off Alternatively, for diseases of low morbidity and low mortality, the consequences of false positive results, and the slaughter of non-infected animals, is not acceptable In this case, the cut-off may be raised to assure that uninfected animals are not mis-classified serologically The result of raising the cut-off is that some infected animals in the population will be misclassified as uninfected

If a test is unable to detect antibody in about 40% - 50% of the infected animals when the specificity of the test is set at 99%, another strategy for interpretation of results is needed This is the case with paratuberculosis in cattle It may be necessary to test entire herds and determine the percentage of animals that fall into low, moderate, and high risk categories based on multiple cut-offs Based on faecal culture data, all animals that fall below a selected low PP cut-off would be considered at low risk of having an infection whereas animals with PP values above a high PP cut-off would be considered as having a high risk of being infected, between the two cut-offs would be the animals of moderate risk of infection Using multiple cut-offs to define risk levels gives the producer an indication of the status of the herd if, for instance, 98% of the animals fall into the "low risk" category, and only 2% in the moderate risk category, the probability that this herd has paratuberculosis is very low Alternatively, if 60% of the animals are in the low risk but 40% are evenly distributed among the moderate and high risk categories, the chances of paratuberculosis in this herd is very high So, even for a test with poor performance characteristics, useful data can be obtained in the quest to classify herd status

4. SIZE OF SUBPOPULATION REQUIRED FOR DETERMINING THE CUT-OFF

Standard tables are published in many epidemiology and statistical texts that give the sample size required to detect various minimum levels of infection in different sized herds at various confidence levels. These tables give some guidance in selecting the size of the known uninfected and known infected groups of animals for establishing a cut-off that will be valid for the entire target population. The guidance is imperfect because sampling theory assumes that the number and effects of unknown variables that contribute to the continuum of PP values for infected and uninfected populations of animals is fully represented in the sample. Indeed, when one tries to list the variables and the variation within each variable that must be considered in selecting the reference animals, the task becomes daunting. For instance, an infection process usually results in antibody production that is a dynamic variable as a function of time. Similarly, the antigen used in the test system may appear to be specific for a group of animals but then another group may give consistently elevated values attributable to some external unknown factor. These two examples represent but two of many such variables, all of which may affect PP values. For this reason, it is not possible to establish the set of underlying assumptions for a test's probable performance when applied to a subpopulation for the purpose of establishing a valid cut-off. It is, therefore, desirable to make an error on the high side when determining the number of infected and uninfected animals by which the test will be evaluated. Generally, the experience of ELISA developers has shown that several thousand samples from uninfected animals and at least several hundred from known infected animals, that are good representatives of the population at large, will allow development of a cut-off that "will stand the test of time" [4] .

REFERENCES

[1] SMITH, R.D., Clinical Veterinary Epidemiology, Butterworth-Heinemann, Stoneham, MA, USA, (1991).

[2] CROWTHER J.R., ELISA theory and practice, In Methods in Molecular Biology, Humana Press, Totowa, NJ, USA, (1995).

[3] ZWEIG M.H. & CAMPBELL G., Receiver-operating characteristics (ROC) plots: a fundamental evaluation tool in clinical medicine, Clin. Chem. **29** (1993) 561-577.

[4] JACOBSON, R.H., Validation of serological assays for diagnosis of infectious diseases, Manual of Standards for Diagnostic Tests and Vaccines, OIE, France (1996) 8-15.

COUNTRY REPORTS

Part A: BRUCELLOSIS

Indirect brucellosis ELISA

COMPARISON OF ENZYME IMMUNOASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS

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Abstract

COMPARISON OF ENZYME IMMUNOASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS

The indirect enzyme immunoassay for measurement of bovine antibody to *Brucella abortus* was tested on 15,716 Canadian sera to assess the specificity These sera were also tested by the buffered plate antigen test Two ELISA formats were used for assessment of data the targeting procedure using a positive control serum allowed to develop to an optical density of 1 0 and the use of a positive control serum to determine relative positivity at a set time Two different cut-off values were also assessed for each assay

A total of 763 sera gave reactions above established cut-off values in the ELISA while 216 were positive in the buffered plate antigen test (BPAT) A modification of the indirect ELISA employed divalent cation chelating agents (EDTA/EGTA) incorporated into the serum incubation stage to eliminate some non-specific reactions This method was applied only to the 763 indirect ELISA reactor sera and it eliminated all but 93 or 37, depending on the cut-off selected, of the reactions Sensitivity was assessed by testing 424 sera from *Brucella abortus* culture positive cattle The indirect ELISA classified all 424 sera as positive by either method of data handling and with or without addition of EDTA/EGTA for a specificity estimate of 100% In the BPAT, 412 sera gave a positive agglutination reaction Ten percent of the 15,716 sera were randomly selected and tested by two different competitive ELISAs and by the complement fixation test (CFT) One competitive ELISA used *Brucella abortus* O-polysaccharide as the antigen and an enzyme conjugated monoclonal antibody to the O-polysaccharide for competition and detection Of the sera tested, 34 gave false positive reactions On a retest, the false positive reactions were reduced to 2 The second competitive ELISA used lipopolysaccharide as the antigen, a different monoclonal antibody but also specific for the O-polysaccharide for competition and commercially available goat anti-mouse IgG enzyme conjugate for detection In the initial assessment, this test gave rise to 5 false positive reactions This number was reduced to 2 when retesting the sera The CFT used was a micro format test and 3 sera gave false positive reactions A total of 654 sera from animals from which *Brucella abortus* was isolated were tested by the first competitive ELISA Of these, 9 sera were negative on the initial test This number was reduced to 3 on repeat testing All 636 sera tested by the second competitive ELISA were positive Fifteen of 636 sera gave sufficient prozoning in the complement fixation test to be considered diagnostically negative and 59 sera were anticomplementary Sensitivity and specificity are summarized in Table I Cut-off values for each assay were initially established by visual observation of frequency distributions of positive and negative serum samples These cut-off values were confirmed by receiver operating characteristics (ROC) analysis In addition, an index of performance (accuracy) was established for each assay in order to allow direct comparison Accuracy estimates were based on the sensitivity, specificity and disease prevalence for the data

TABLE I. SENSITIVITY AND SPECIFICITY FOR DIFFERENT TESTS

Test	Sensitivity %	Specificity %
BPAT	97 9	98 6
CFT (AC reactors +)	97 1	93 1
CFT (AC reactors -)	87 9	99 8
I-ELISA-T 0 460 cut-off	100	96 0
I-ELISA-T 0 607 cut-off	100	98 5
I-ELISA-%P 46% cut-off	100	95 9
I-ELISA-%P 62% cut-off	100	98 5
I-ELISA EDTA/EGTA 0 460 cut-off	100	99 4
I-ELISA EDTA/EGTA 0 607 cut-off	100	99 8
C-ELISA (O-polysac)	98 6	97 7
C-ELISA (LPS)	100	99 7

1. INTRODUCTION

A number of enzyme immunoassays have been applied to the diagnosis of bovine brucellosis. Two main types of immunoassays used for these purposes, the indirect and the competitive formats, have recently been reviewed [1, 2]. The most commonly used methods of controlling bovine brucellosis has been vaccination with *Brucella abortus* strain 19 [3] and the serological detection of exposure to the organism, followed by removal of the animal from the herd or depopulation of the herd. In a number of eradication programmes, conventional serological tests were used for detection of infection. In these assays, the antibody to be assessed must be capable of a secondary function, such as agglutination, activation of complement or precipitation, to be measured.

Therefore these assays measure some of the isotypes of antibody and not others [2] leading to the use of panels of tests for more accurate diagnosis. This led to the development of serological screening tests of high sensitivity and lower specificity, such as the buffered plate antigen test - BPAT [4]. A positive reaction in the screening test led to the serum being tested in a number of confirmatory tests, such as the complement fixation tests [5] or the 2-mercaptoethanol modification of the tube agglutination test [6]. This arrangement is costly and the time between submission and output of results is long. An additional problem with these assays is that with the exception of an immunodiffusion test using a polysaccharide antigen [7], none could distinguish the antibody response due to vaccination from that resulting from infection. It was hoped that introduction of the indirect enzyme immunoassay would overcome some of these problems but in reality, until recently [8], the indirect ELISA, while more sensitive that the conventional test, has been less specific, even using highly specific monoclonal antibodies to bovine immunoglobulins as detection reagents. Similarly, the indirect ELISA could not distinguish vaccinal antibody from that arising from infection. For this reason there has been reluctance in accepting the indirect ELISA as a diagnostic test.

An additional problem has been the cost per test which was considerably higher than that of the BPAT. A competitive ELISA was developed [9] which was more specific than the indirect ELISA and which could discriminate vaccinal antibody from antibody induced by infection. This assay used O-polysaccharide prepared from lipopolysaccharide from *Brucella abortus* as the antigen and a monoclonal antibody specific for an O-polysaccharide epitope for competition. A problem with this assay was the difficulties in preparing and standardizing the antigen. This led to modifications of the antigen to make it passively absorption more uniformly to the polystyrene matrix [10]. A second approach to improving the competitive ELISA has been to attempt to use lipopolysaccharide as the antigen. Lipopolysaccharide, because of the hydrophobic lipid A portion of the core region, attaches readily to polystyrene. Such a test was developed [11]. This communication compares the sensitivities and specificities of two conventional tests, the BPAT (screening test) and the complement fixation test (confirmatory test) and the indirect ELISA, with or without addition of divalent chelating agent and with data expressed by two methods and two competitive ELISAs, one using O-polysaccharide as the antigen and the other using lipopolysaccharide.

2. MATERIAL AND METHODS

2.1. Sera

The following serum samples were selected for testing as shown in Table II: Fifteen thousand seven hundred sixteen Canadian serum samples submitted for routine diagnostic testing for brucellosis were tested by the two indirect ELISA formats and by the BPAT. Of these sera, 763 gave positive reactions in the indirect ELISAs and these sera were retested using added divalent cation chelators. From the 15,716 sera, some were randomly selected for testing by the competitive ELISAs and by the complement fixation test. A number of sera from cattle from which *Brucella abortus* had been isolated from secretions or tissues were tested in all assays. Sera tested in each assay are shown in Table II. Sera that were depleted or became contaminated with bacteria resulted in different numbers of sera being tested in some of the assays.

TABLE II. NUMBER OF SERA TESTED AND RESULTS PER TEST

Test	Negative sera	Positive sera
Indirect ELISAs	15.716	424
Indirect ELISA (EDTA/EGTA)	763	636
BPAT	15.716	424
Competitive ELISA (O-polysac)	1508	654
Competitive ELISA (LPS)	1446	636
Complement fixation test	1508	654

2.2. Serological test

The BPAT was performed as described by Anderson et al., 1994 [6]. Briefly, 30 µl of antigen was mixed with 80 µl of serum and incubated at 37°C for 8 min. Agglutination was read by observation. The complement fixation test was described by Samagh and Boulanger [12].

All ELISA procedures used NUNC 2-69620 polystyrene plates (CIBCO-BRL, Burlington, Ontario, Canada) and unless otherwise indicated, horseradish peroxidase (type VI, Sigma Chem. Corp., St. Louis, Missouri, USA) was used to conjugate to the monoclonal antibodies by the method of Henning et al. [13]. All spectrophotometer readings were at 414 nm. The indirect ELISA format was described by Nielsen [2]. This format is a rapid screening procedure that used lipopolysaccharide [14] as the antigen, 1 µg/ml in carbonate buffer, pH 9.6. An amount of 200 µl of antigen was dispensed into each well of a 96 well polystyrene plate and incubated for 18 hours at 20°C. The plates were then frozen at -20°C. After thawing for 30 min. at 37°C, the plates were washed four times in 0.01M phosphate buffer, pH 7.2 and containing 0.15M NaCl and 0.05% tween 20 (PBS/tween buffer).

Two hundred microtitre of test serum, diluted 1:50 in PBS/tween buffer was added to individual wells. Control sera including serum from an infected animal (prediluted to give an optical density value of approximately 1.0 after 10 min. of development); serum that gave an optical density near the cut-off value after 10 min. of development; a negative serum and a buffer control, all set up in quadruplicate. After 30 min. of incubation at 20°C, the plates were again washed four times in PBS/tween and 200 µl of a monoclonal antibody to bovine IgG1, conjugated with horseradish peroxidase, was added. After 30 min. of incubation at 20°C, the plates were washed as above and 200 µl substrate (1mM H_2O_2) and chromogen (4mM ABTS) in 0.05 M citrate buffer, pH 4.5, was added to each well. Optical density readings are taken after 10 min. of incubation with the substrate/chromogen and data were presented as a percentage of the positive control serum included in each plate and 46% or 62% positivity were considered a positive reaction. A second method of data assessment used the targeting procedure [15] in which the optical density of the positive control serum was measured after 4 min. of substrate/chromogen interaction. The length of time required for the positive control serum to achieve an optical density value of 1.0 was calculated and the plate was re-evaluated for color development at that time. Cut-off values of 0.460 and 0.607 optical density values were used for data interpretation. A modification of the indirect ELISA (EDTA/EGTA ELISA) used PBS/tween to which 7.5mM of each of ethylene diaminetetra-acetic acid disodium salt (EGTA) and ethylene glycol-bis-(B-aminoethyl ether) N,N,N',N'.-tetraacetic acid (EDTA) [both from Sigma Chem. Corp., St. Louis, Missouri, USA] were added as the serum diluent buffer. At all other stages, PBS/tween buffer was used. One competitive ELISA [9] used 100 µl purified O-polysaccharide at 2.0 µg/ml, covalently linked to poly-L-lysine [10] and dissolved in 0.06M carbonate buffer, pH 9.6 as the antigen, passively attached to polystyrene 96-well plates for 18 hours at 20°C. The sealed plates were then frozen at -20°C. After thawing at 37°C for 30 min., the plate was washed four times in 0.1M tris buffer pH 8.0 containing 0.15M NaCl and 0.05% tween 20 (tris/tween buffer).

Test serum (50 µl) diluted 1:50 in tris/tween buffer was added to the wells in duplicate followed by appropriately diluted mouse monoclonal antibody YsT9-2 (specific for an O-polysaccharide epitope), conjugated with horseradish peroxidase (50 µl). After mixing on a rotary shaker for 3 min., the plate was incubated at 20°C for 2 hours. After four further washes in tris/tween buffer, bound enzyme was measured by the targeting procedure using H_2O_2 and ABTS. Results were expressed as percent

inhibition of an uninhibited control to which tris/tween buffer was added instead of serum. Additional controls included serum from a *Brucella abortus* infected cow, serum from a *Brucella abortus* immunized animal and a negative serum. The second competitive ELISA [11] used 1.0 μg/ml lipopolysaccharide as the antigen at 100 μl per well, incubated for 18 hours at 20°C. The plates were then frozen at -20°C. After thawing at 37°C, the plates were washed with tris/tween buffer. Fifty microliter of serum, diluted 1:10 in tris/tween buffer containing 7.5 mM of each of EDTA and EGTA followed by 50 μl of monoclonal antibody, M84, also diluted in tris/tween buffer with EDTA/EGTA were added to duplicate wells. Controls (in quadruplicate) included a high titered serum from an infected cow, a low titered serum from an immunized cow, serum from a negative animal and a buffer control (only monoclonal antibody and buffer). The plate was incubated for 30 min. at 20°C and washed four times in tris/tween buffer. Goat anti-mouse IgG (H & L chain specific) affinity purified antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Labs. Inc., West Grove, Pennsylvania 19390, USA) was diluted in tris/tween and 100 μl was added to each well for 30 min. After four additional washes, substrate/chromogen (1.0 mM H_2O_2 and 4 mM ABTS) was added for 10 min. with continuous orbital shaking. Optical density readings were obtained after 10 min. and the data presented as % inhibition relative to the buffer (uninhibited control). Sera that gave an value of 30% inhibition or greater were considered positive and were retested.

2.3. Statistical analysis

A combination of statistical approaches were used to compare the data from the various assays. The initial estimates of cut-off values were derived using negative serum samples from the population of animals under study [2]. The data was sorted in ascending order and divided into 100 equal percentiles. The mean of the 99th percentile was calculated and used as the initial cut-off value. The diagnostic sensitivity and specificity could then be optimized by plotting the sorted data for defined negative and positive sera using a frequency histogram. The initial cut-off values were used in 2x2 tables to calculate specificity and sensitivity. Confidence limits (95%) were calculated.

The data was also analyzed using signal detection analysis (receiver operating characteristics ROC, analysis) [16, 17]. This analysis confirmed the proximity of the initial cut-off estimate by comparing sensitivities and specificities using a range of cut-off values. An index of comparison (accuracy) was used to compare the performance of different assays with respect to actual positive and negative data [17]. Accuracy estimates were obtained using the relationships summarized below:

Definitions:
TPF = Sensitivity
TNF = Specificity
P(D+) = Disease prevalence from data
P(D-) = 1-P(D+)

Relationships:
Accuracy = TPF x P(D+) + TNF x P(D-)

3. RESULTS

Of the 15,716 sera tested, 216 agglutinated the BPAT antigen, resulting in a specificity estimate of 98.6%. Nine of the sera from the *Brucella abortus* infected animals did not agglutinate the BPAT antigen resulting in a sensitivity estimate of 97.9%. These data are included in Table III along with similar calculations for the various data manipulations of the indirect ELISA data. There was no significant difference between data obtained for the indirect ELISA by the targeting procedure or by the percent positivity method and clearly the data indicates that the higher the cut-off value used, the higher the specificity of the indirect ELISA, however, even at the higher cut-off values, the specificity was marginally less than that of the BPAT.

The frequency distributions and the ROC analysis for the targeted and the percent positivity methods of expressing data are depicted in Figures 1 and 2, respectively. The accuracy estimates are presented in Table IV. As expected, increasing the cut-off values increased the accuracy from 0.96 to 0.98. If cut-off values of 0.460 or 46% positivity were used, 763 of the negative sera gave positive earlier study using approximately 1000 serum samples. The cut-off represents the 99th percentile of the mean of the values obtained with negative sera (unpublished data) The higher cut-off values, 0 607 or 62% positivity were derived by the same calculation from the current study.

The 763 serum samples that gave false positive reactions were tested in a modified indirect ELISA (targeted) in which 7.5 mM EDTA/EGTA were included in the serum diluent. This resulted in 93 sera remaining positive at the 0.460 cut-off value and 37 giving optical density values over 0.607 In both instances, the specificity of the assay was increased over that of the BPAT (Table III) The 424 sera from infected cattle gave positive reactions when either cut-off was used and addition of chelating agents did not alter these results. These data are summarized in Table III. Because 91 of the 1508 negative sera and 59 of the sera from confirmed positive animals (an additional 15 positive sera prozoned sufficiently to be diagnostically negative and 6 sera gave incomplete hemolysis) selected for testing by the complement fixation test gave anticomplementary reactions, one set of calculations considered these sera as negative and one set as positive.

If the anticomplementary reactions were considered as positive, the specificity of the complement fixation test was 93.1%, the sensitivity was 97.1% and the accuracy 0.95 A specificity value of 99.8%, test sensitivity of 87.9% and an accuracy estimate of 0.96 were obtained if the sera causing anticomplementary reactions were considered as negative. These data are presented in Tables III and II and frequency distributions and ROC analysis are presented in Figure 3. In testing 1508 negative sera, selected randomly, in the competitive ELISA with O-polysaccharide antigen, the initial specificity was 97 7%. When the 34 false positive reactor sera were retested, the specificity increased to 99.9%

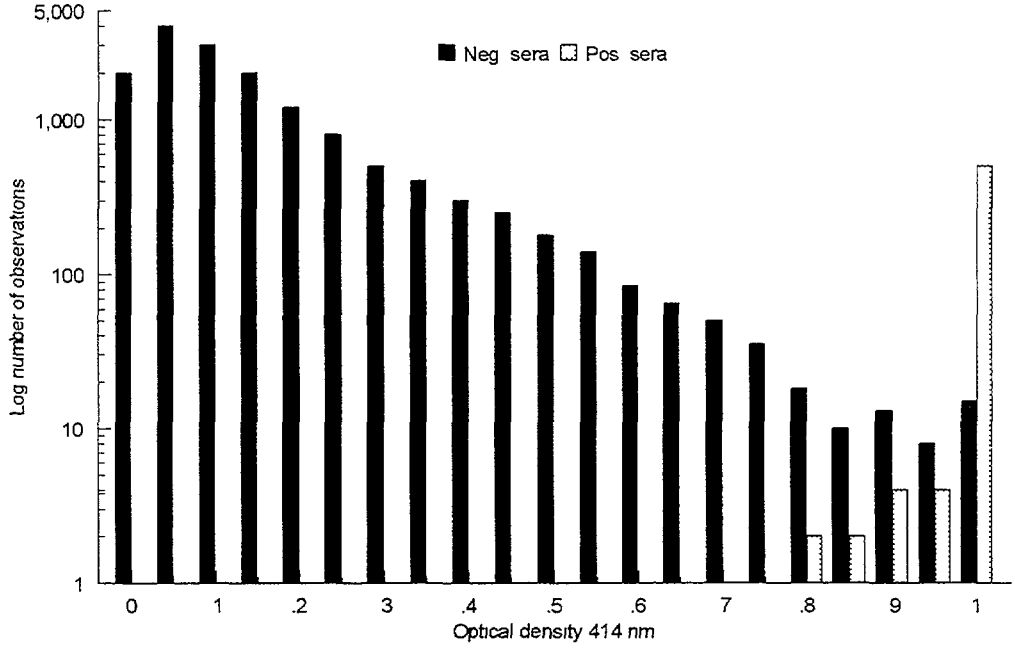

FIG.1. Frequency distribution of negative and positive sera selected for testing in the targeted indirect ELISA. The Y-axis represents the log10 number of sera in each 0.05 optical density interval on the X-axis. The open bars represent negative sera and the closed bars positive sera. From this graph, cut-off values of 0.460 and 0.607 were selected for data analysis. The former cut-off represents 99th percentile of the mean of the negative samples tested previously (small sample) while the latter cut-off represents the 99th percentile of the mean of the current data. Theoretically the cut-off could be set as high as 0.700 optical density units and still retain a sensitivity estimate of 100%.

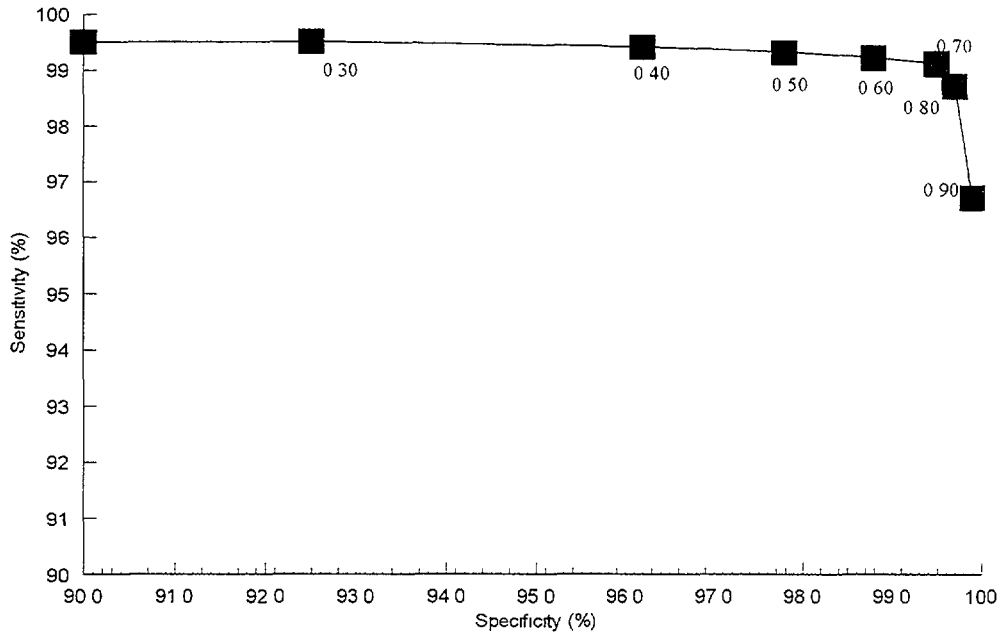

FIG 2 Receiver operating characteristic (ROC) analysis of the targeted indirect ELISA data (optical density values are included with the graph) The assay sensitivity in percent is plotted on the Y-axis and the specificity (%) is plotted on the X-axis. From this plot, a sensitivity may be selected to reflect a desired specificity value For instance, if a sensitivity value of 99% is selected, the specificity will be 99 5% and a cut-off value of 0 700 would be used

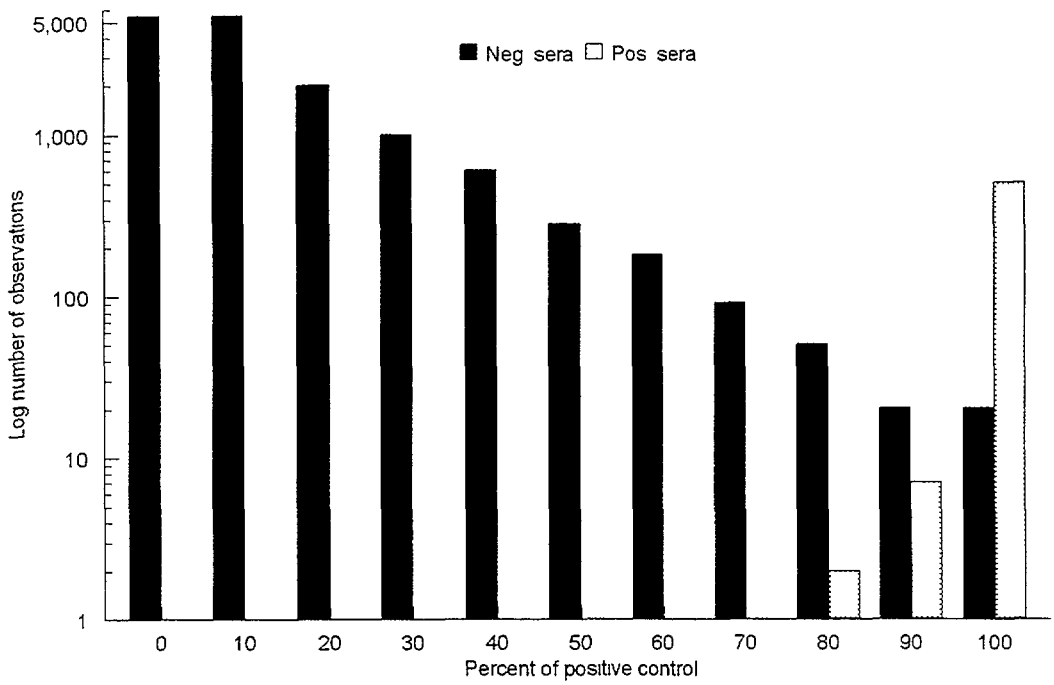

FIG.3. Frequency distribution for the same sera represented in Figure 1 in an indirect ELISA but using percent positivity of a positive control serum for data handling (plotted along the X-axis) Cut-offs of 46% (old value) and 62% (from the current data), representing the 99th percentile of the mean were used for data analysis

The sensitivity of this assay of 98 6% was enhanced to 99 5% by repeat testing of false negative samples (Table III) The accuracies before and after retesting were 0 98 and 0 998, respectively (Table IV) Graphs representing the frequency distributions and the ROC analysis before and after retesting are presented in Figures 5 and 6 Using lipopolysaccharide antigen and M84

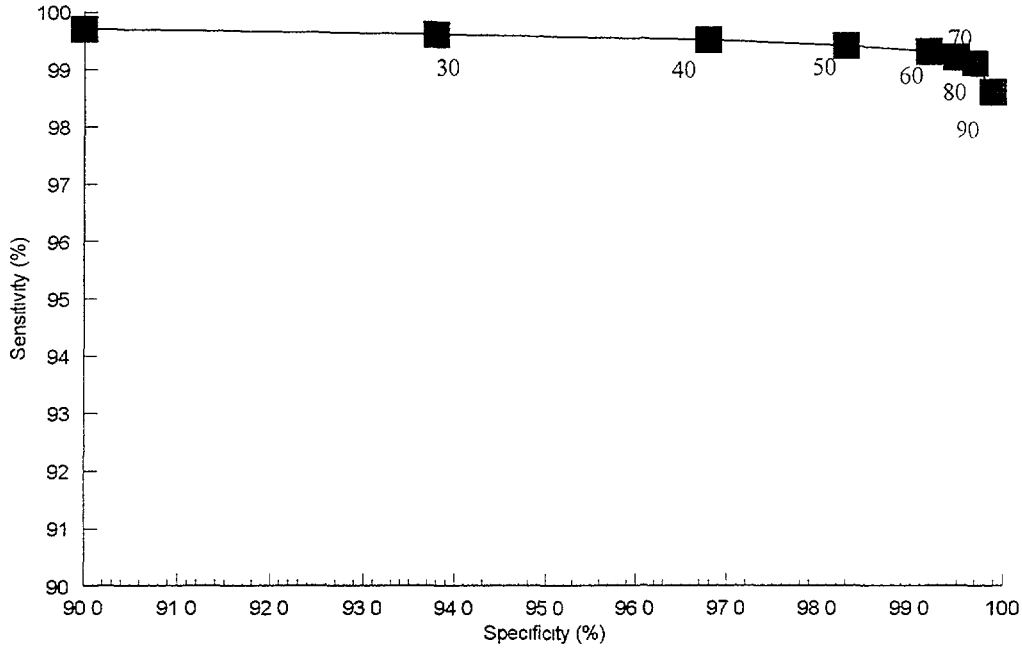

FIG 4 ROC analysis of the data from the indirect ELISA using percent positivity (numbers included in graph) for data analysis Based on this analysis, the optimum cut-off value would be approximately 70% which corresponds with the value of 0 700 optical density units established in Figure 2

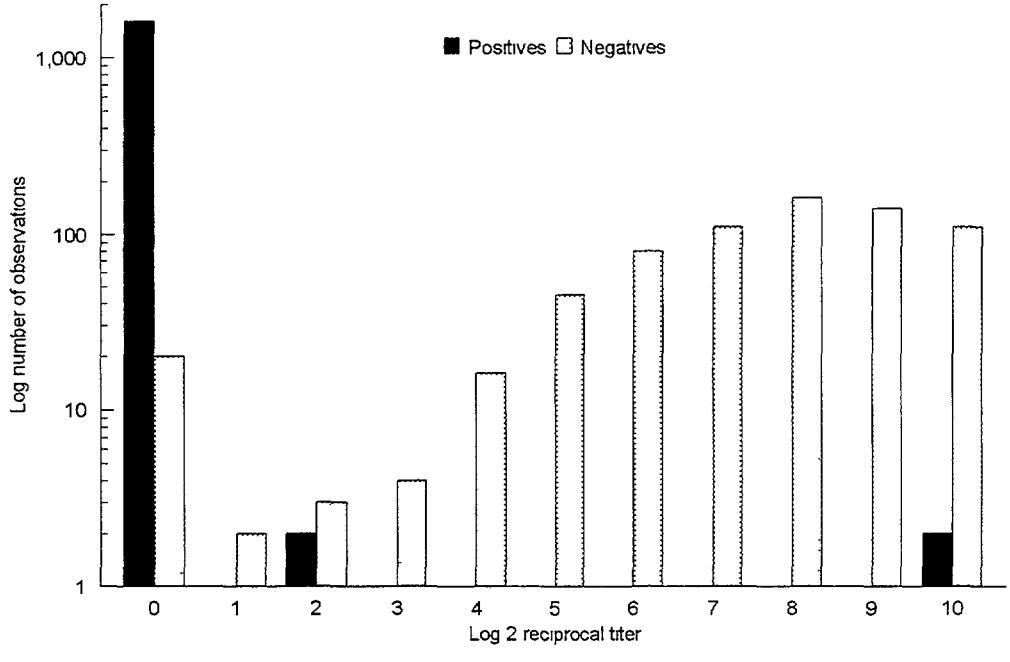

FIG 5 Frequency distribution of sera tested by the complement fixation test for antibody to Brucella abortus The log10 number of observations are plotted on the Y-axis and reciprocal titers (log2) are plotted on the X-axis Three sera gave false positive reaction (open bars) and 19 sera gave false negative reactions Anticomplementary serum samples were assigned to their respective status From this graph, it would appear that the cut-off for the complement fixation test should be a titer of zero

monoclonal antibody in a competitive ELISA enabled detection of significant antibody in all 629 serum samples from proven infected animals giving an assay sensitivity of 100% The specificity before testing false positive sera was 99 6% and this increased to 99 9% after retesting (Table III) The accuracy of this assay was 1 00 before and after retesting (Table IV) Frequency distributions and ROC analysis for data before retesting are presented in Figure 7 Data after retesting was not included due to the minor changes (the number of false positive reactions were reduced from 5 to 2 There were no false negative reactions)

TABLE III SENSITIVITY AND SPECIFICITY ESTIMATES FOR THE BUFFERED PLATE ANTIGEN TEST THE COMPLEMENT FIXATION TEST THE VARIOUS FORMATS OF THE INDIRECT ELISA AND THE COMPETITIVE ELISAS, BEFORE AND AFTER RETESTING SERA THAT GAVE UNEXPECTED RESULTS (CONFIDENCE LIMITS OF 95% ARE INCLUDED)

Test	Sensitivity %	CL[1] %	Specificity %	CL %
BPAT[2]	97 9	1 4	98 6	0 18
CFT (AC reactors +)[3]	97 1	1 3	93 1	1 2
CFT (AC reactors -)	87 9	2 5	99 8	0 20
I-ELISA-T (0 460 cut-off)[4]	100		96 0	0 31
I-ELISA-T (0 607 cut-off)	100		98 5	0 19
I-ELISA-%P (46% cut-off)[5]	100		95 9	0 31
I-ELISA-%P (62% cut-off)	100		98 5	0 19
I-ELISA EDTA (0 460 cut-off)[6]	100		99 4	NA
I-ELISA EDTA (0 607 cut-off)	100		99 8	NA
C-ELISA C-ELISA (O-poly-before)[7]	98 6	0 90	97 7	0 76
C-ELISA (O-poly-after)	99 5	0 50	99 9	0 10
C-ELISA (LPS-before)[8]	100		99 7	0 18
C-ELISA (LPS-after)	100		99 9	0 10

1 confidence limits
2 buffered plate antigen test
3 complement fixation test with anticomplementary sera considered as positive or negative
4 targeted indirect ELISA using cut-off values of 0 460 or 0 607 optical density units
5 indirect ELISA data based on percent positivity relative to a positive serum control Cut-off values of 46% or 62% positivity were selected
6 targeted indirect ELISA into which divalent cation chelating agents (EDTA and EGTA) were incorporated into the serum dilution tested Two cut-off values 0 460 and 0 607 were selected Confidence limits for this modification were not calculated
7 competitive ELISA format using O-polysaccharide from *Brucella abortus* as the antigen and an enzyme conjugated monoclonal antibody for competition and detection Results before and after retesting sera that gave aberrant results are included
8 competitive ELISA format using lipopolysaccharide from *Brucella abortus* as the antigen a monoclonal antibody for competition and enzyme conjugated anti mouse IgG antibody for detection Results before and after retesting sera that gave aberrant results are included

4 DISCUSSION

It is of interest to note that the screening test for bovine antibody to *Brucella abortus* used in Canada identified 216 sera positive of the 15 716 sera from negative herds tested in this study The specificity, 98 6%, found in this study agrees with that observed in other Canadian studies [18] of 98 7% and 98 9% found by Stemshorn et al [19] The sensitivity of the BPAT was 97 9%, a little higher than the figures reported previously [18] and Stemshorn et al [19] of 95 4 and 96 9%, respectively The accuracy of the BPAT was 0 98

TABLE IV ACCURACY ESTIMATES FOR EACH TEST WERE BASED ON THE SENSITIVITY, THE SPECIFICITY AND THE DISEASE PREVALENCE FOR THE DATA USED BY THE EQUATION BELOW

Test	Accuracy [1]
BPAT [2]	0 98
CFT (AC reactors +)[3]	0 95
CFT (AC reactors -)	0 96
I-ELISA-T (0 460 cut-off)	0 96
I-ELISA-T (0 607 cut-off)	0 98
I-ELISA-%P (46% cut-off)	0 96
I-ELISA-%P (62% cut-off)	0 98
C-ELISA (O-poly-before)	0 98
C-ELISA (O-poly-after)	0 998
C-ELISA (LPS-before)	1 00
C-ELISA (LPS-after)	1 00

1 Accuracy =Test sensitivity x disease prevalence for data + test specificity x (1-disease prevalence for data)
2 the legend is as for Table III
3 the legend is as for Table III

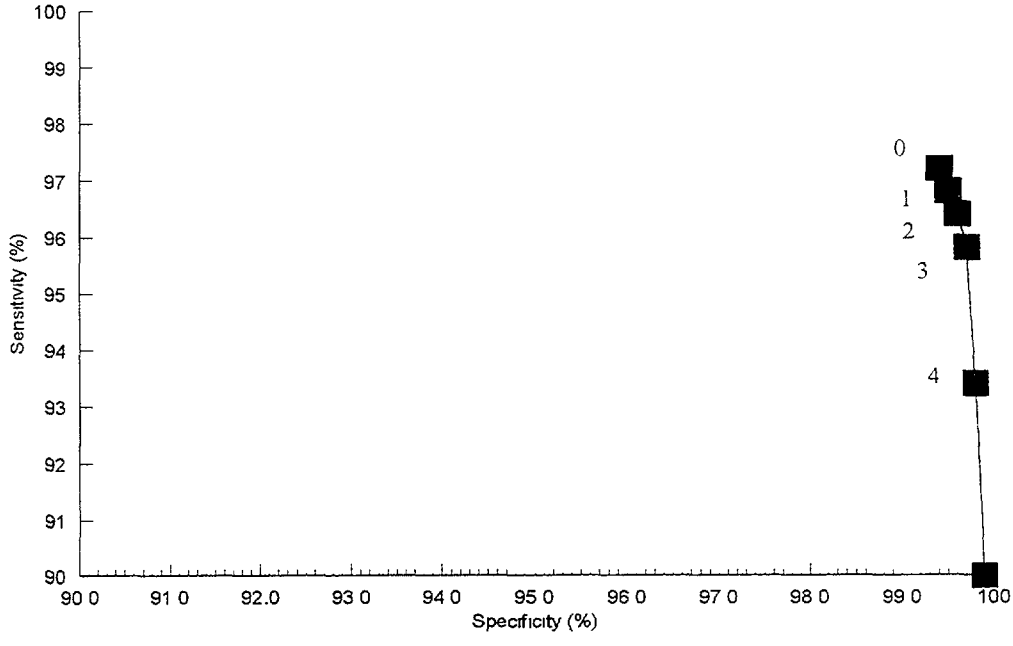

FIG 6. ROC analysis of the complement fixation test data The cut-off value of 0 gives the highest sensitivity (97%) while the specificity did not change a great deal irregardless of the titer selected for the cut-off

These data are summarized in Tables III and IV. Sera from proven *Brucella abortus* infected cattle that were negative in the BPAT were unable to agglutinate at dilutions of 1:10, 1:50 and 1:100 eliminating prozoning as an explanation for the negative results. Since all sera reacted in the indirect ELISAs, an assay that measures only IgG1, lack of agglutination could not be a result of lack of antibody capable of reacting, as IgG1 has been shown to agglutinate efficiently at the lower pH [2]. Therefore, the inability of the antibody of the 9 negative serum samples to agglutinate the BPAT antigen remains unexplained.

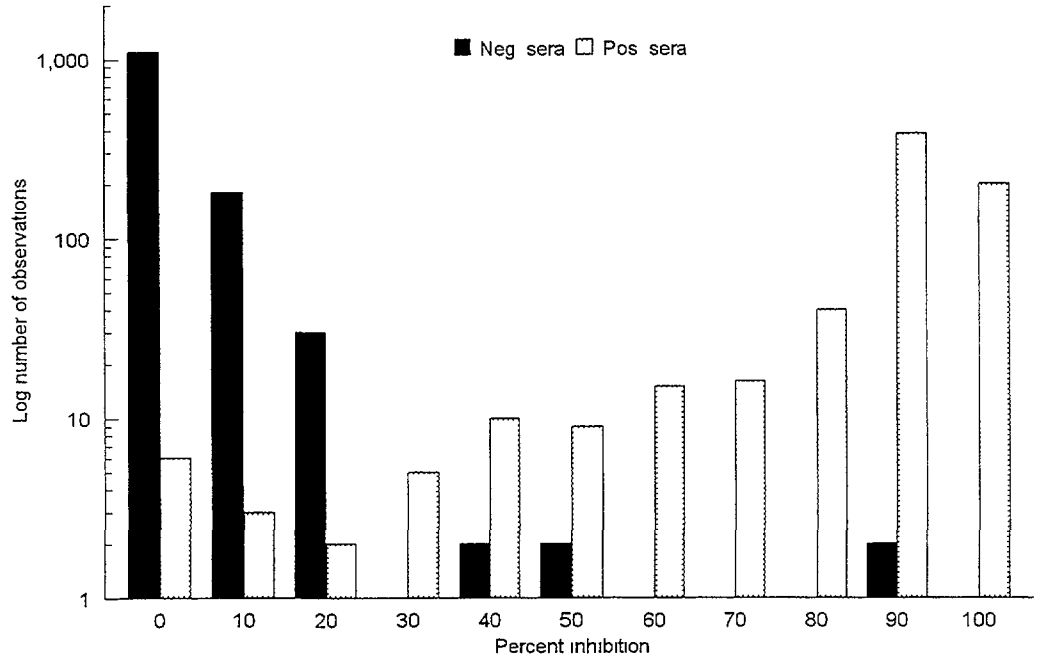

FIG 7 Frequency distribution of negative and positive sera randomly selected for testing in a competitive ELISA before sample that gave unexpected results were retested in the assay This competitive ELISA used the O-polysaccharide of Brucella abortus as the antigen and an enzyme conjugated monoclonal antibody, specific for the O-polysaccharide, as the competing and detection antibody The Y-axis represents the log10 number of observations and the X-axis indicates the percent inhibition in increments of 10% From the graph is appears that the most suitable cut-off value for this assay is 20% inhibition.

The complement fixation test, the current Canadian confirmatory test, cannot be used to make a definite diagnosis if the serum sample activates the complement cascade in the absence of antigen. Such sera are deemed anticomplementary. In the current study, a number sera selected from negative herds and from the proven infected animals were found to be anticomplementary and therefore, the data obtained was analyzed twice, considering all anticomplementary reactions as positive reactions or as negative reactions. If the anticomplementary sera are considered as positive, the specificity of the test was 93.1%, the sensitivity was 97.1% and the accuracy was 0.95. If the anticomplementary reactions were considered negative, the specificity was 99.8%, the sensitivity 87.9 and the accuracy was 0.96. Dohoo et al. [18] found the specificity of the complement fixation test to be 100% and the sensitivity to be 92.9%, very similar to the latter interpretation of the current data. The data from the current study are presented in Tables III and IV.

The indirect ELISA detected antibody in all sera from infected animals regardless of the data interpretation used in this study. The specificity was 96% using a cut-off of 46% or a targeted cut-off value of 0.460 optical density units. If the cut-off values were increased to 62% or 0.602, the specificity increased to 98.5%. Again, the former values are similar to those published earlier [2], demonstrating the consistency of this test. The accuracy was determined to be 0.96 and 0.98 for the lower and higher threshold values, respectively. Since the specificity estimates for the indirect ELISA were lower than those observed for the BPAT, a modification utilizing divalent cation chelating agents as part of the serum diluent buffer was attempted. This modification was based on an earlier observation in which non-specific interaction in the tube agglutination test was reduced by addition of ethylenediaminetetraacetic acid disodium salt (EDTA) and ethylene glycol-bis-(B-aminoethyl ether) N,N,N',N' tetraacetic acid (EGTA) [20]. It was found that addition of EDTA and EGTA decreased the number of false positive reactions in the indirect ELISA from 763 to 93 or 37, using the 0.460 or the 0.602 cut-offs, respectively. This increased the specificity to 99.4% or 99.8%, both of which exceed that of the BPAT, without any reduction in sensitivity.

These data are summarized in Tables III and IV The higher cut-off value used in this study may not be useful in a diagnostic context as it would only be suitable in surveillance situations where brucellosis is not expected to be found, as is the case in Canada A high cut-off value in the indirect ELISA would not be suitable for testing imported animals, especially from areas where bovine brucellosis was in evidence as two test criteria are not advantageous However, it would markedly decrease the number of confirmatory tests or trace-backs required While the sensitivity and the specificity of the indirect ELISA appear to be excellent, this type of assay cannot distinguish between the antibody response induced by vaccination with *Brucella abortus* strain 19 and that resulting from infection with pathogenic strains

This led to the development of competitive ELISAs[9, 21] which could differentiate between the induced antibody responses The initial competitive ELISA developed at Agriculture Canada utilized purified O-polysaccharide as the antigen and a mouse monoclonal antibody, produced with O-polysaccharide from *Yersinia enterocolitica* serotype 0 9 and conjugated with horseradish peroxidase as the competing antibody and detection reagent [9] The O-polysaccharide was subsequently modified by covalently linking it with poly-L-lysine to improve its binding characteristics to polystyrene [10] This assay was also used to test the 1508 randomly selected sera from Canadian herds and 654 sera from animals from which *Brucella abortus* was isolated

Two sets of data are included for this assay Firstly, the data obtained on the initial test and then data obtained after serum samples that gave unexpected results were retested The sensitivity of the test with the initial data and based on a cut-off value of 20% inhibition was 98 6% the specificity was 97 7% and the accuracy was 0 98 After repeat testing of false positive and false negative samples, the overall sensitivity increased to 99 5%, the specificity increased to 99 9% and the accuracy was 0 998 These data are presented in Tables III and IV A second version of the competitive ELISA [11] using lipopolysaccharide as the antigen and a different monoclonal antibody for competition in a buffer containing EDTA and EGTA and goat anti-mouse IgG antibody-horseradish peroxidase as the detection reagent was also used on the same sera

Again, the data is presented before retesting of serum samples giving aberrant results Initial test sensitivity, based on a cut-off value of 30% inhibition, was 100% sensitivity, test specificity was 99 7% and the accuracy was 1 00 After retesting five false positive samples, two were eliminated raising the specificity to 99 9% It is of interest to note that one of the three samples that gave false positive reactions was obtained from a recently vaccinated animals and this sample was positive on all the tests A second sample was contaminated with bacteria and was positive on both competitive ELISAs and anticomplementary in the complement fixation test The third positive sample was different for the two competitive ELISAs and the complement fixation test and its reactivity remain unexplained

These data are summarized in Tables III and IV Initially, the cut-off values may be approximated by visual inspection of the frequency distribution graphs (Figures1,3 5 7,9) as the point that gives maximum distinction between positive and negative samples For example, in Figure 9, it would appear that the cut-off between positivity and negativity should be 20% to accommodate the most negative samples and the least number of positive samples To obtain a more accurate estimate of the cut-off values for the assay, ROC analysis (Figures 2,4,6,8,10) allowed the determination of sensitivity and specificity values at different points and therefore a decision could be made to maximize the sensitivity or the specificity and the reciprocated decrease in the other Thus, for instance in Figure 10, if an acceptable false positive rate was 0 36% (a cut-off value of 20% inhibition), then the specificity will be 99 64% and the sensitivity will be 98 47%

Alternately, if a specificity value of 99 80 is required (at a cut-off of 50% inhibition), the sensitivity will decrease to 94 80% Accuracy estimates allow direct comparison of the performance of each assay in that the false positive and the false negatives rates are considered in the context of disease prevalence for the specific data set From Table IV, it is clear that the competitive ELISA using LPS antigen is the most accurate (1 0) but the older version of the competitive ELISA after repeat testing comes very close (0 998) A number of technical improvements have been made to the current competitive ELISA compared to that reported previously [9] Thus lipopolysaccharide antigen is used rather than the O-polysaccharide, simplifying antigen production A serum dilution of 1 10 was chosen to allow dilution directly into the wells of a 96-well plate, obviating the requirement for a tube for preparing the dilution

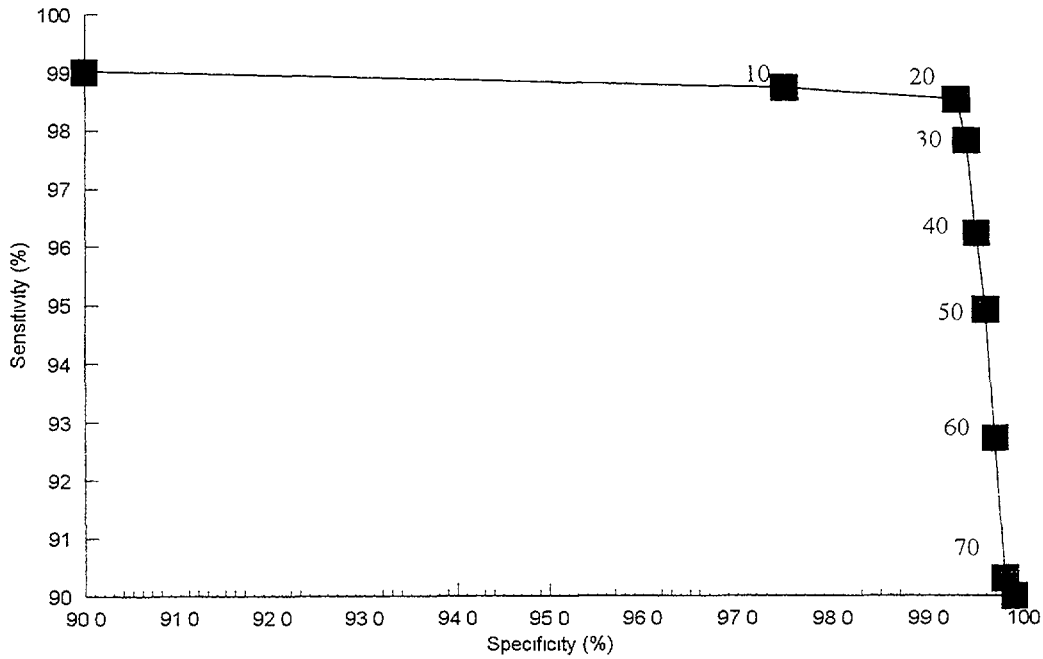

FIG 8 ROC analysis of the competitive ELISA data The most suitable cut-off value is 20% inhibition giving a sensitivity of 98 6% and a specificity estimate of 99 6%

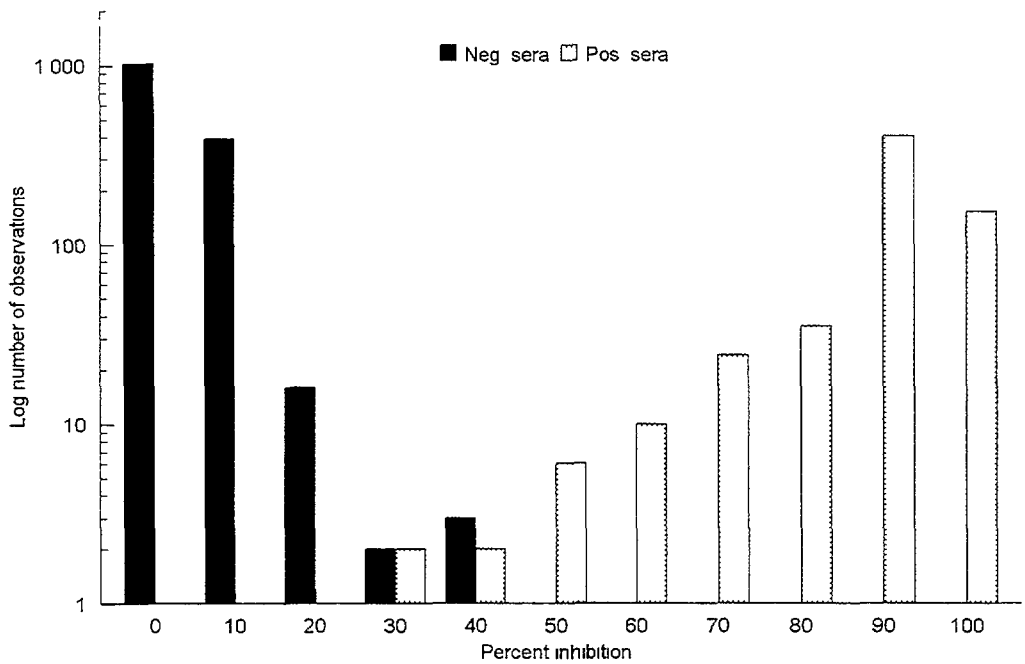

FIG 9 Same as Figure 7 but data after aberrant samples were retested in the competitive ELISA It is clear that the cut-off value did not change with repeat testing Data obtained when testing randomly selected negative and positive sera in a second competitive ELISA after initial testing This assay used Brucella abortus lipopolysaccharide as the antigen a different monoclonal antibody (but also specific for the O-polysaccharide) for competition and an enzyme conjugated anti-mouse IgG antibody for detection The log10 number of observations are plotted along the Y-axis and the percent inhibition in increments of 10% is plotted on the X-axis The cut-off value selected from this graph was 30% inhibition Retesting sera that gave unexpected results eliminated all but 3 false positive reactions (data not shown)

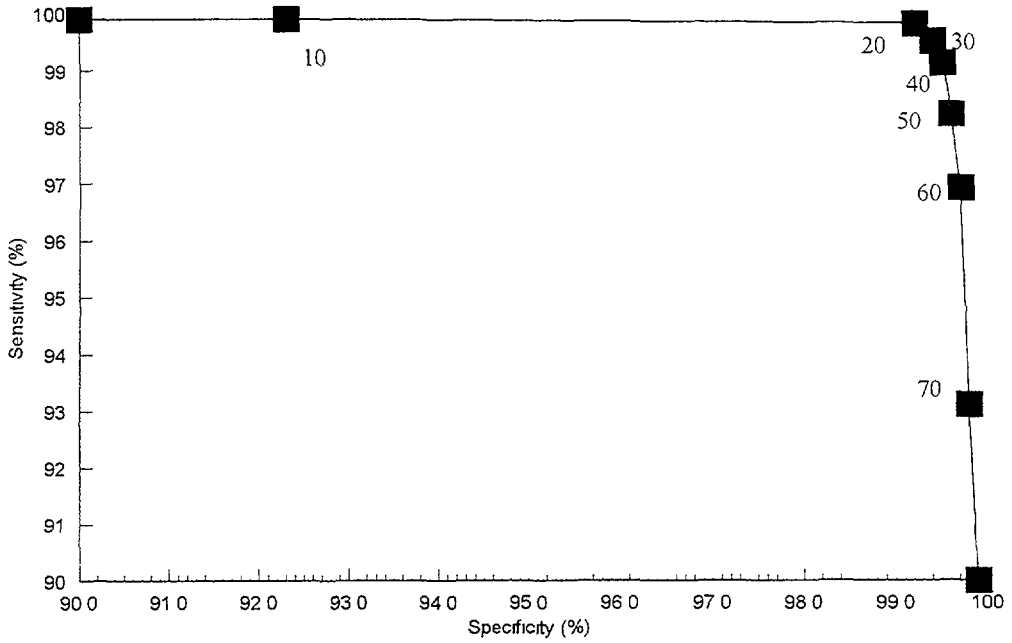

FIG 10 ROC analysis of the data from the second competitive ELISA If a cut-off of 30% inhibition is selected the sensitivity estimate is 99 9% and the specificity is 99 8% Retesting of samples that gave unexpected reactions increased the specificity to 99 9% (data not shown) The ROC analysis revealed an increase in sensitivity to 99 5% and an increase in specificity 99 8%

Addition of divalent cation chelating agents reduced the non-specific serum protein interactions, however, because EDTA/EGTA are incompatible with horseradish peroxidase, the additional step of using a goat anti-mouse IgG antibody reagent conjugated with enzyme was included This inclusion also allowed for minor adjustments in optical density without altering the assay sensitivity as was the case when the competing monoclonal antibody was conjugated directly with enzyme Both incubation periods have been decreased to 30 min each, compared to a single stage of two hours previously and each sample may be set up once rather than in duplicate Based on the specificity and sensitivity of the LPS-based competitive ELISA reported here, it would appear that this procedure may replace not only the currently used confirmatory test, the complement fixation test, but also the in-use screening test, the BPAT Little or no extra cost is involved since although that it may be slightly more expensive to perform, there would be no requirement for additional expensive tests and 90% of the trace-backs would be eliminated An additional saving can be made by reusing the antigen coated polystyrene plates for as many as five assays [22], making it possible to perform 400 assays per plate

REFERENCES

[1] WRIGHT, P F , NIELSEN, K H . KELLY, W . Primary binding techniques for the serodiagnosis of bovine brucellosis enzyme immunoassay in animal brucellosis Ed Nielsen, K H . Duncan, J R CRC Press, Boca Raton, Florida (1990) 199-235

[2] NIELSEN, K H , GALL, D , KELLY, W . HENNING, D . GARCIA, M M . Enzyme immunoassay Application to diagnosis of bovine brucellosis, Agriculture Canada monograph, ISBN 0-662-19838-7, (1992)

[3] NICOLETTI, P . Vaccination in Animal Brucellosis, Eds Nielsen, K H . Duncan, J R . CRC Press, Boca Raton, Florida, (1990) 283-300

[4] ANGUS, R D , BARTON, C E . The production and evaluation of a buffered plate antigen for use in the presumptive test for brucellosis, Dev Biol Standard **56** (1984) 349-356

[5] HILL, W K . Standardization of the complement fixation test for brucellosis, Bull Off Internat Epizool **60** (1963) 401-419

[6] ANDERSON, R K, JENNESS, R, BRUMFIELD, H P , GOUGH, P , Brucella agglutination antibodies, Relationship of mercaptoethanol stability to complement fixation, Science **143** (1964) 1334-1335

[7] DIAZ, R , GARATEA, P , JONES, L M , MORIYON, I, Radial immunodiffusion test with *Brucella* polysaccharide antigen for differentiating vaccinated from infected cattle, J Clin Microbiol **10** (1979) 37-41

[8] NIELSEN, K H , KELLY, L , GALL, D , SMITH, P , BOSSE, J , NICOLETTI, P , KELLY, W , Use of divalent cation chelating agents (EDTA/EGTA) for reduction of non-specific serum protein interaction in enzyme immunoassay, Vet Res Comm **18** (1994) 433-437

[9] NIELSEN, K H , CHERWONOGRODZKY, J , DUNCAN, J R , BUNDLE, D R , Enzyme linked immunosorbent assay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19, Am J Vet Res **50** (1989) 5-9

[10] GALL, D , NIELSEN, K H , Improvements to the competitive ELISA for detection of antibody to *Brucella abortus* in cattle sera, J Immunoassay **15** (1994) 277-291

[11] NIELSEN, K H , KELLY, L , GALL, D , BALSEVICIUS S , NICOLETTI, P , KELLY, W , Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis, Vet Immunol Immunopathol **46** (1995) 285-291

[12] SAMAGH, B S , BOULANGER, P , Adaptation of the Technicon Autoanalyzer II for an automated complement fixation test for detection of antibodies to *Brucella abortus* in bovine serums Proc 21st Ann Meet Am Assoc Vet Lab Diagnost (1978) 347-366

[13] HENNING, D M , NIELSEN, K H , Peroxidase labeled monoclonal antibody for use in enzyme immunoassay, J Immunoassay **8** (1987) 297-305

[14] BAKER, P J , WILSON, J B , Hypoferremia in mice and its application to the bioassay for endotoxin, J Bacteriol **90** (1965) 903-910

[15] WRIGHT, P F , KELLY, W , GALL, D E , Application of a timing protocol to the reduction of inter-plate variability in the indirect enzyme immunoassay for detection of anti-Brucella antibody, J Immunoassay **6** (1985) 189-199

[16] STENSON, H , SIGNAL, A , Supplementary module for SYSTAT and SYSGRAPH, SYSTAT Inc Evanston IL (1988)

[17] METZ, C E , Basic principles of ROC analysis, Seminars in Nuclear Medicine **8** (1978) 283

[18] DOHOO, I R , WRIGHT, P F , RUCKERBAUER, G M , SAMAGH, B S , ROBERTSON, F J , FORBES, L B , A comparison of five serological tests for brucellosis, Can J Vet Res **50** (1986) 485-493

[19] STEMSHORN, B W., FORBES, L B , EAGLESOME, M D , NIELSEN, K H , ROBERTSON, F J , SAMAGH, B S., A comparison of standard serological tests for the diagnosis of bovine brucellosis in Canada, Can J Comp Med **49** (1985) 391-394

[20] NIELSEN, K H , SAMAGH, B S , SPECKMANN, G , STEMSHORN, B W , The bovine immune response to *Brucella abortus*, Elimination of some sporadic serological reactions by the chelation of divalent cations, Can J Comp Med **43** (1979) 40-47

[21] MACMILLAN, A P , GREISER-WILKE, I , MOENNIG, V , MATHIAS, L A , A competitive enzyme immunoassay for brucellosis diagnosis Dtsch Tierarztl Wochenschr **97** (1990) 83-85

[22] NIELSEN, K H , KWOK, A , Reuse of polystyrene 96 well plates for indirect enzyme immunoassay for detection of antibody to *Brucella abortus*, Arch Med Vet **27** (1995) 39

VALIDATING A BOVINE BRUCELLOSIS ELISA TEST FOR APPLICATION IN URUGUAY

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Abstract

VALIDATING A BOVINE BRUCELLOSIS ELISA TEST FOR APPLICATION IN URUGUAY

Sera from 600 cattle on Rio Negro Island, known to be free of brucellosis, and 400 sera from vaccinated cattle but known to be negative in the Rose-Bengal test were selected for validation of the FAO/IAEA test kit for detection of antibody to *Brucella abortus* Two conjugates, one a polyclonal antiserum and the other a monoclonal antibody, were evaluated When evaluated for reproducibility using the sera from uninfected cattle, the average coefficient of variation for duplicate samples was 7 1% ± 5 5 The serum control samples did not exceed OD limits as established for the kit, for any of the 15 plates evaluated When evaluated by regression analysis, the control sera had an average correlation coefficient of 0 996, indicating a high degree of agreement between the observed OD values of controls on each plate vs the expected values for those controls Specificity in the assay was >98% as calculated by the PP or regression methods Comparison of the monoclonal and polyclonal conjugates using sera from vaccinated cattle indicated that many of the cattle must have been vaccinated as adults because of high antibody levels detected by both conjugates Before this assay can be used on vaccinated animals, the kit will have to be evaluated using sera from animals of known age of vaccination

1 INTRODUCTION

As in many Latin American countries, brucellosis was a major problem in Uruguay [1, 2] In 1965 animal health authorities established a National Control Plan based on vaccination, serodiagnosis, and slaughter of seropositive animals Strain 19 vaccination (9×10^9 organisms per dose) was used in heifer calves and serodiagnosis was accomplished using the Rose-Bengal test (RBT) as the screening test followed by the 2-mercaptoethanol (2ME) and the Complement Fixation test (CFT) as a confirmatory tests Currently, RBT and the rivanol confirmatory test are being used Positive reactors on the screening and confirmatory tests continue to be systematically removed from the farms and slaughtered Because of this intensive program during the past 30 years there have been neither abortions nor bacteriological isolation of *Brucella abortus* in the country

Currently, the estimated prevalence of bovine brucellosis in dairy cattle is 0 2% and in beef cattle is 0 5% [3] An efficient serodiagnostic assay applied to a disease of such low prevalence requires that the test be highly sensitive if the predictive value of a positive test result is to be of any value Non-specific responses that give false-positive results must be avoided However, antibody from vaccination in heifers may interfere with the test This is particularly true when the vaccine is given to animals older than 9 months of age which occurs in an estimated 10% of heifers [4] Authorities in Uruguay are considering eliminating vaccination because of its interference in seroassays and therefore its detrimental effects in attempts to certify the country free of brucellosis The objective of this work was to confirm a highly specific and sensitive test that may be used either as a screening test or a confirmatory test for brucellosis We evaluated the FAO/IAEA ELISA kit for use under Uruguayan epidemiological conditions

2 MATERIALS AND METHODS

Sera from dairy cattle heifers of known *Brucella abortus* infection/vaccination status were selected for use in assessing an ELISA (FAO/IAEA test kit) for bovine brucellosis Six hundred sera of non-infected cattle from the non-endemic Rio Negro island were selected at random and 400 sera from vaccinated cattle, negative on the Rose-Bengal test, were also evaluated The age of vaccination of these cattle was unknown

Two versions of the ELISA test kit were used The first employed a polyclonal conjugate whereas the second utilized a monoclonal conjugate with specificity for bovine IgG_1 The kits were used as described in the kit instructions with the exception that the monoclonal conjugate had to be retitrated to achieve the desired reactivity it was used at 1 4000 rather than the recommended 1 11000 as specified in the instructions The polyclonal test kit was assessed for reproducibility between wells of the microlitre plate and between plates Cut-offs were determined and compared for non-infected/non-vaccinated and vaccinated animals for the polyclonal version of the kit, and for only vaccinated animals on the monoclonal-based kit

The data were analyzed following normalization by two methods The ODs of each sample were converted to percent of the high positive control (PP) as described in the test kit This method relies on a control against which tested samples on the plate are normalized The data were also normalized by regression analysis which uses a standard curve and the weight of all 4 control samples as the basis for normalizing all test sera For regression analysis, the mean OD (n = 60) for each control serum, derived from all 15 plates, was determined and designated the "expected OD value" for that control A standard curve for each plate was determined by plotting the ODs of the control samples for that plate against their expected OD values by linear regression analysis All test sample ODs for that plate were then normalized by extrapolation from the standard curve Data normalized by regression analysis and "percent of positive control" were then compared

3 RESULTS

Reproducibility in the assay was based on 300 duplicates from 15 plates for the sera from uninfected non-vaccinated cattle, the coefficient of variation (CV) exceeded 25% an average of 7 1 $\pm$ 5 5 (SD) times per 40 duplicates on each plate The high positive, low positive, and negative control sera, as well as the conjugate control did not exceed the upper and lower OD limits, as prescribed in the kit instructions, in any of the 15 plates When evaluated by regression analysis of observed versus expected ODs for the serum controls within each plate, the average correlation coefficient for all plates was 0 996 (range of 0 990 - 1 000), indicating that the controls performed as expected Comparison of the PP values for each serum control in all plates confirmed this high degree of reproducibility between plates with the high positive control set at 100%, the low positive and the negative controls had mean PPs of 53 3% $\pm$ 4 1 and 5 0% $\pm$ 1 5, respectively Visual inspection of plate data using sera from vaccinated and seropositive (by other tests) animals did not reveal any unexpected aberrations

The cut-off for the sera from uninfected animals was determined for data normalized by regression analysis and by PP Two samples had PPs of 173% and 89%, which were clearly outliers and were removed from the data base Of the remaining 598 samples, only 19 had PPs greater than 20%

The mean plus 3 SD of the normalized ODs for these sera was 244 OD units At this cut-off, the calculated specificity by was 98 3% Similarly, when calculated using the mean PP + 3SD, the cut-off was 26% resulting in a specificity of 98 5% (one less animal was classified as "false positive" using the PP method than in the regression method of normalization)

The sera from vaccinated animals were evaluated to establish a cut-off reflective of antibody that might be detected as a result of vaccination Of 272 sera, 6 exceeded the 100% PP of the positive control Likewise, 13 (4 7%) of the animals exceeded PP of 70%, and 21 (7 7% of 272) exceeded a PP of 50% These data represent a severe skew to the right in the frequency distribution of PP values For this reason, it is not possible to use parametric statistics (Standard Deviation) that assume a normal distribution in determining the cutoff Rather, 2 times the mean PP of all samples, including those exceeding PP of 100%, was 42 9% If the PPs for the 6 samples exceeding 100% PP were eliminated, then the cut-off was calculated at 38 3% If only those samples having PPs of <70% were included in the calculation, the cut-off would be 35 1%

When these sera plus an additional 128 sera from vaccinated animals (n = 400) were evaluated using the monoclonal sera, the same skewing to the right occurred in the frequency distribution of PP values Twice the mean of all of the PPs for these vaccinated animals resulted in a calculated cut-off of 34 4% as compared to 42 9% for the comparable samples using the polyclonal conjugate

4. CONCLUSIONS

Reproducibility within the assay was acceptable with a minimum of variation between the plates. The only limitation was that CVs for the duplicates tended to be high with over 7% of the samples exceeding a CV of 25%. This occurred even when the mean of the duplicates exceeded 20 OD units.

Plate-to-plate variation as determined by reproducibility of the serum controls was minimal. All of the ODs for the controls fell within the upper and lower limits as indicated in the kit instructions. Also, the mean correlation coefficient of 0.996 for the regression of the controls versus their expected values was excellent. When the data normalized by the PP or regression method were compared, little difference was noted. The regression method has the advantage of normalizing all samples to a standard curve created by 4 samples rather than only the high-positive control as in the PP method. This indicated that the high positive control did not vary significantly between plates.

The cut-off (mean plus 3 times the SD) calculated by use of the panel of known uninfected and non-vaccinated animals, was 24 PP and 26 PP when data were normalized by the regression or the PP method, respectively. The calculated specificity at these cut-offs were 98.3% and 98.5%, respectively. The cut-off was only 14 PP when the convention of two times the mean of all samples was used. Using the mean + 3 x SD as the cut-off, the difference between data normalized by regression and PP resulted in a change in classification in only one of 598 animals. In contrast, attempts to calculate a cut-off for vaccinated animals that were RBT negative met with difficulties. Extreme skewing of the frequency distribution of PP values to the right precluded use of the mean plus three standard deviations convention in determining specificity. When twice the mean of all samples was used, the cut-off was 43%. This extreme difference (PP of 14 versus 43) between non-vaccinated/non-infected and vaccinated animals is probably due to vaccination of animals later than in their calfhood. We have no explanation of why the samples with high PPs are negative on Rose-Bengal; it was expected that the samples with high PP values would also have been positive on the RB test.

When the monoclonal and polyclonal conjugates were compared, the same skewing of the frequency distribution occurred for the monoclonal as for the polyclonal conjugate. Although the two times the mean PP value for the monoclonal conjugate was somewhat lower (34.4% versus 42.9%), the same trend occurred. This further suggests that the sera from vaccinated animals represented recent vaccinations for which antibody titers had not yet waned.

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REFERENCES

[1] CASAS OLASCOAGA, R., Algunos aspectos de Brucelosis Bovina. Banco de Seguros del Estado, (1969) 147.

[2] BERMUDEZ, J. BARRIOLA, J., DEL BAGLIVI, L., Brucelosis Bovina en el Uruguay. Veterinaria (ROU) **14** (1977) 33.

[3] PROYECTO DE FACTIBILIDAD DE ERRADICACION DE BRUCELOSIS Y TUBERCULOSIS. (MGAP-FONPLATA).

[4] SILVA, M., Control y erradicacion de brucelosis, Disertacion en Panel sobre, Congreso Internacional Veterinario, Facultad de Ciencias Veterinaria (1993) Argentina.

[5] NIELSEN, K., GALL, D., KELLY, W. HENNING, D., GARCIA, M., Enzyme immunoassay application to diagnosis of bovine brucellosis. Agricultural Canada Monograph (1992) 203.

USE OF AN INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS AND EPIDEMIOLOGICAL STUDIES OF *BRUCELLA ABORTUS* IN CHILE

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Abstract

USE OF AN INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS AND EPIDEMIOLOGICAL STUDIES OF *BRUCELLA ABORTUS* IN CHILE

It is well known that traditional brucellosis diagnostic tests have a lack of sensitivity and specificity depending on the area or country where they have been used Seeking for better alternatives, we undertook an evaluation of an ELISA test for bovine brucellosis diagnosis, for its possible use in Chile

We collected 2 groups of sera from negative animals one from an area free of S19 vaccination (n=491) and a second from vaccinated herds (n=349) These groups were used for determination of cut-offs and diagnostic specificity calculations A positive group of sera was collected from animals (n=186) from infected herds which were all reactors in the Rose Bengal (RB) and Rivanol (RIV) tests, some were also bacteriological culture positive and positive to an immunodiffusion test This group was used for sensitivity calculation A final test group of 385 sera from herds with brucellosis, either negative or positive in the RB or RIV tests, were used for relative sensitivity and specificity calculations

The calculated cut-off values were 26% and 61% of Percentage Positivity (PP) for the free area and vaccinated negative groups, respectively Real sensitivity was 100% and real specificity was 98 6% when a 26% cut-off was used Using the 61% cut-off value, the relative sensitivity respect of the RB and RIV tests were 95,7% and 100%, respectively In addition, relative specificity with respect to the RB and RIV tests were 82 4% and 81 8%, respectively

1 INTRODUCTION

Conventional brucellosis diagnostic tests have achieved success in many control and eradication programs. However, there are problems with their diagnostic performance, depending on the cattle population in which they are applied and the epidemiological circumstances of the area or country where they are in use [1, 2]

Among the classical serological tests for brucellosis diagnosis, the agglutination tests have been used extensively and today they are still playing an important role. The standard agglutination test (SAT) performs relatively poorly resulting in several modifications; in particular, in the buffered plate antigen test (BPAT), the card and rose bengal tests (RBT), the rivanol test (RIV) and 2-mercaptoethanol (2-ME) tests. The complement fixation test (CFT), also considered as a classical test, is undoubtedly one of the better performing diagnostic tests [2].

The use of soluble extracts of *Brucella sp* as antigens instead of whole cells has made it possible to develop other conventional tests such as the indirect hemolysis test (IHLT), hemolysis-in-gel test (HIGT), radial immunodiffusion (RID) and immunodiffusion (ID) tests [2]. Although these tests do not enhance substantially the diagnostic performance, some of them were useful for differentiating S19 vaccinated animals from *Brucella* field strain infected cattle and have been used for the evaluation of reduced dose S19 re-vaccination schemes [3, 4, 5, 6, 7]. These antigens have also been used in primary bindings assays developed to achieve better diagnostic performances in brucellosis serology [8, 9, 10, 11].

This work was conducted to validate an indirect ELISA and to evaluate this test as a future diagnostic test for use in Chile.

2 MATERIAL AND METHODS

Bovine sera from different groups of animals were collected for use in determining the cut-off and real and relative sensitivity and specificity values of the ELISA All sera were obtained from adult cattle by the method recommended by MacMillan [12]

- Brucellosis Free Area-Negative group (A) consisted on 491 sera from cattle in a geographic areas free from brucellosis and where S19 vaccine was never used All sera were negative in the RB and RIV tests They were used to determine a cut-off value and were considered as true negatives for real specificity calculations
- Brucellosis Vaccinated Herd-Negative group (B) 349 sera were collected from herds certified by the Ministry of Agriculture as "free herds" The sampled animals had received S19 vaccine between 3 to 8 months of age and were negative in the RB and RIV tests This group was used for a second cut-off determination
- Brucellosis Positive group (C) consisted of 186 sera from cattle raised in herds with more of 2% of prevalence and where brucellosis had been diagnosed by bacteriological culture All these sera were positive in the RB and RIV tests and a considerable number were cultured positive for *B abortus* and gave positive immunodiffusion test (ID) reactions This group was considered as true positive animals and was used for real sensitivity calculation
- Test group (D) 385 sera were collected from herds with brucellosis diagnosed by serological, bacteriological and epidemiological means These sera were either negative or positive in the RB and RIV tests and were used to calculate the relative sensitivity and specificity of the ELISA test

2.1. RV and RIV tests

Classical serological tests such as the RB and RIV tests were performed as described by Alton et al [13]

2.2. ELISA

The indirect ELISA test was performed as recommended by FAO/IAEA [14] Briefly, the indirect ELISA used a s-LPS antigen diluted in carbonate buffer pH 9 6 for coating 96-wells polystyrene plates (NUNC 69620) A volume of 100 ul per well was used and incubated for a period of 18 hours at 4°C After washing the wells three times, tests sera, diluted 1 200 in described PBS, were added in duplicate and incubated for one hour at 37°C Quadruplicate wells of strong positive, weak positive and negative control sera diluted 1 200 were also included A conjugate control (in quadruplicate) with PBS instead of serum was included After incubation and another, wash cycle, rabbit anti-IgG peroxidase conjugate with H+L activity, diluted in PBS, was added to each well and incubated at 37°C for one hour After incubation and an additional wash cycle, 100 µl of a substrate solution, containing hydrogen peroxide and ABTS in citrate buffer pH 4 5 were added to each well and color was allowed to develop for 10 minutes with continuous shaking At ten minutes a stopping solution (4% SDS) was added to each well and the plate was read immediately in an Immunoskan Plus ELISA reader using the BREIA 1 1 software This program calculates the mean OD of serum duplicates and also calculates the Percent Positivity (PP) value for each serum relative to the strong positive control sera of each plate With this method it is possible to compare all sera on the same basis The PP values were used for the cut-off estimations and the positive and negative results obtained using these cut-offs were used for sensitivity and specificity determinations

3 RESULTS

3.1. Cut-off determination

Initially, the cut-off values were determined for the two negative groups Analysis of variance showed that a significant difference existed ($p<0\ 05$) between the two groups The statistical

calculations of the PP values from the Free Area Negative and Vaccinated Herd Negative groups and their respective cut-offs (cut-off = negative group PP mean + 3D) are shown in Table I and Figure 1.

TABLE I. INDIRECT ELISA PP AND CUT-OFF VALUES CALCULATED FROM TWO DIFFERENT POPULATIONS OF BRUCELLLOSIS NEGATIVE ANIMALS (VACCINATED AND NON-VACCINATED)

		PP%		
Negative group	n'	mean	SD	cut-off
Free area	491	12.1	4.7	26.3
Vaccinated herd	349	23.6	12.5	61.1

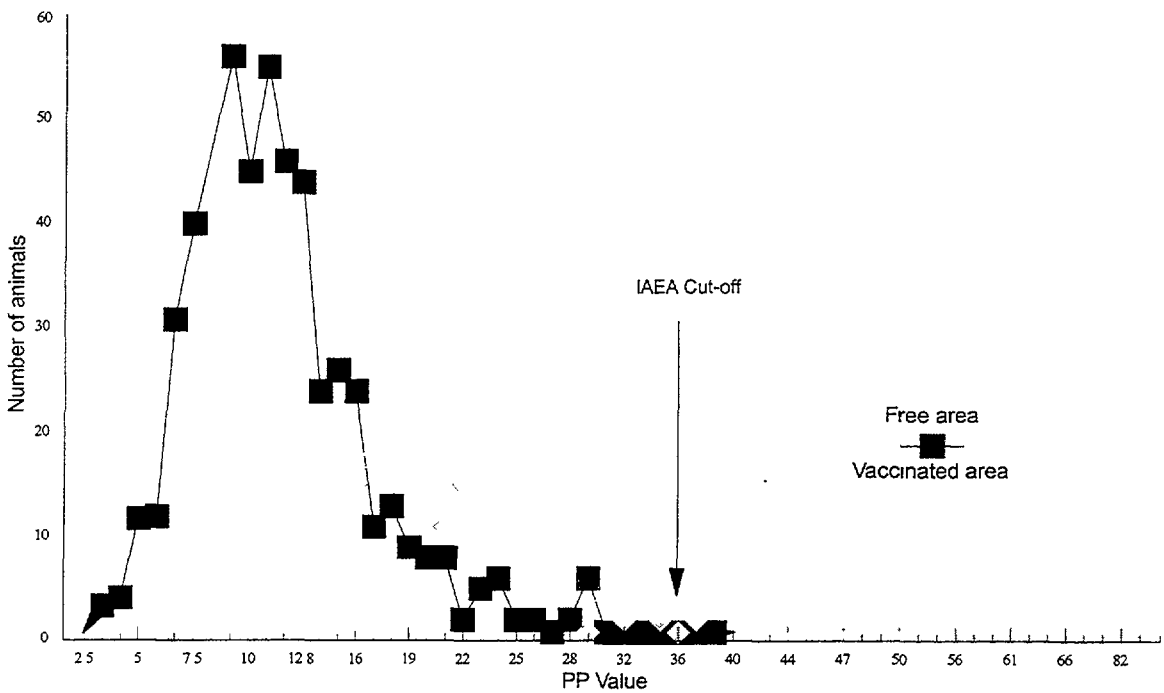

FIG. 1. Frequency of ELISA PP values in two brucellosis negative cattle populations in Chile.

3.2. Calculation of real sensitivity and specificity values

Real sensitivity and specificity values were calculated with the IAEA 35% proposed cut-off and with our own, 26% and 61%, proposed cut-offs values calculated for negative groups from Free Area and Vaccinated Herds. These results are presented in Table II.

TABLE II REAL SENSITIVITY AND SPECIFICITY OF THE FAO/IAEA INDIRECT ELISA KIT FOR BRUCELLOSIS DIAGNOSIS IN CHILE, IN TWO EPIDEMIOLOGICAL CATEGORIES OF ANIMAL POPULATIONS USING DIFFERENT CUT-OFF VALUES

		Sensitivity			Specificity
Cut-off		Positives		Negatives	(Free area)
26%	Number	186	100%	491	98 6%
	Positive	186		7	
	Negative	0		484	
35%	Number	186	100%	491	99 8%
	Positive	186		1	
	Negative	0		490	
61%	Number	186	100%	349	(vaccinated herd)
	Positive	186		9	97 4%
	Negative	0		340	

3.3. Relative sensitivity and specificity

The relative sensitivity and specificity of the ELISA kit was calculated with respect to the RB and RIV tests, using the FAO/IAEA cut-off of 35% and our calculated cut-off of 61% These data are presented in Table III We did not use the other calculated cut-off of 26% because all animals of group D were vaccinated with S19 at 3 to 8 months of age and this cut-off was calculated using sera from non-vaccinated cattle

TABLE III RELATIVE SENSITIVITY AND SPECIFICITY OF THE FAO/IAEA INDIRECT ELISA WITH RESPECT TO THE RB AND RIV TESTS IN CHILE, USING TWO CUT-OFF VALUES (THE CUT-OFF RECOMMENDED BY FAO/IAEA AND THAT CALCULATED FOR A VACCINATED CATTLE POPULATION)

Test	Cut-off	Sensitivity %	Specificity %
Rose Bengal	35	100	62 7
	61	95 7	82 4
Rivanol	35	100	72 5
	61	100	81 8

4 CONCLUSIONS

Prior to the establishment of the diagnostic usefulness of a serological test, it is necessary to determine the diagnostic threshold or cut-off value which will separate the positive from negative animals for that particular test The indirect ELISA was used to determine the cut-off values for the negative control readings of OD or Percentage Positivity (PP) [15, 14] For this reason, depending of the population, cut-off values could vary from one area to another depending on the vaccination status of the animals

Theoretically, to calculate the real diagnostic specificity of a serological test in a specific area, sera from a true negative group of animals are required This group ideally should never have had contact with the etiological agent of the disease [16] For this, sera from cattle in a geographically

isolated zone where cattle never have been vaccinated with *Brucella abortus* S19 were collected Also of importance is that brucellosis had only been diagnosed serologically and then sporadically in this area However, when required to test populations with different epidemiological characteristic, sera from negative animals in that area should be collected and negative sera should be used to determine a "local" cut-off value In our country, the official brucellosis control programme recommends the use of S19 calfhood vaccination with a standard dose and a large proportion of the bovine population has been vaccinated between 3 to 8 months of age Therefore, we collected sera from negative animals that had received the vaccine but belonged to officially-declared brucellosis-free herds for cut-off determinations

The difference in PP values between these two negative groups is statistically significant and clearly demonstrates that different cut-off value should be used depending on whether the population to be tested has been vaccinated or not

This ELISA kit gave a 100% sensitivity independently of the cut-offs values used (Table II) and this indicates that all positive animals will be detected by this test When using a 61% cut-off the sensitivity was 100% although this cut-off seems rather high When relative sensitivities were calculated it was clear that ELISA had a lower sensitivity (95 7%) with respect to the RB test which traditionally is considered as being too sensitive i e giving too many false positives

Analyzing the real specificity, the ELISA is apparently quite specific, (98 9% and 99 8) regardless of the cut-off value used (26% or 35%), which were calculated based on reactivity of the true negative population or provided by the FAO/IAEA, respectively However, this specificity decreased to 97 4% when the cut-off calculated from the negative vaccinated population was used This is undoubtedly due to the effect of vaccination and the high analytical sensitivity of the indirect ELISA It appears that due to the analytical sensitivity of the ELISAs, this test can detect lower levels of persistent antibodies resulting from vaccination than the traditional tests are capable of Moreover the H&L chain specificity of the conjugate causes enhanced detection of IgM and for this reason the specificity of the ELISA decreased when compared to the RB and RIV tests which predominantly detect IgG antibody

REFERENCES

[1] ALTON, G G , Development and evaluation of serological tests, In An international symposium Crawford, R P and Hidalgo, R J Ed Texas A&M University Press College Station, Texas USA (1977) 61-71

[2] WRIGHT, P F , NIELSEN, K H , Primary binding techniques for the serodiagnosis of bovine brucellosis enzyme immuno assay, In Advances in brucellosis research, Adams, L G Ed Texas A&M University Press, College Station, Texas, USA (1990) 305-320

[3] DIAZ, R , GARATEA, P , JONES, L M , MORIYON, I Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle, J Clin Microbiol **10** (1979) 37-41

[4] CHERWONOGRODZKY, J W , NIELSEN, K , *Brucella abortus* 1119-3 O-chain polysaccharide to differentiate sera from *B abortus* S19 vaccinated and field-strain-infected cattle by agar gel immunodiffusion, J Clin Microbiol **26** (1988) 1120-1123

[5] PINOCHET, L , ABALOS, P , STEPHENS, L , DURAN, M , Normalizacion de un antigeno soluble para descartar respuesta postvacunal a *Brucella abortus* Cepa 19 Av Cs Vet **5** (1990) 119-123

[6] PINOCHET, L , ABALOS, P , BEST, A , LOPEZ, J , VERGARA, C , Proteccion contra brucelosis bovina en hembras adultas mediante revacunacion con dosis reducida de Cepa 19, Av Cs Vet **6** (1991) 152-157

[7] ABALOS, P , PINOCHET, L ,BEST, A , LOPEZ, J , VERGARA, C , PALAVICINO, I Respuesta serologica y proteccion en terneras revacunadas con Cepa 19 en dosis reducida Av Cs Vet 7 (1992) 165-170

[8] ALONSO-URMENETA, B , MORIYON, I , DIAZ, R , BLASCO, J M , Enzyme linked immunosorbent assay with *Brucella* native hapten and smooth lipopolysaccharide, J Clin Microbiol **26** (1988) 2642-2646

[9] NIELSEN, K WRIGHT, P F KELLY, W A , CHERWONOGRODZKY, J W , A review of enzyme immunoassay for detection of antibody to *Brucella abortus* in cattle, Vet Immunol Immunopathol **18** (1988) 331-347

[10] NIELSEN, K , CHERWONOGRODZKY, J W , DUNCAN, J R , BUNDLE, D R , Enzyme-linked immunosorbent assay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19, Am J Vet Res **50** (1989) 5-9

[11] ABALOS, P , PINOCHET, L , FABREGA, F , Desarrollo de una prueba de ELISA para descartar respuesta post-vacunal con Cepa 19, Av Cs Vet **8** (1993) 138-143

[12] MACMILLAN, A , Conventional serological tests, In Bovine brucellosis, Nielsen, K and Duncan, J R , Ed CRC Press, Inc Florida, USA (1991) 153-198

[13] ALTON, G G , JONES, L M , ANGUS, R D , VERGER, J M , Techniques for the brucellosis laboratory, INRA, Paris (1988) 81-136

[14] FAO/IAEA , Brucellosis ELISA kit manual Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Austria (1991)

[15] FAO/IAEA , Brucellosis ELISA kit manual, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency Austria (1989)

[16] JACOBSON, R H , How well do diagnostic tests predict the infection or disease status of animals? Proc of a Regional network for Latin America on animal disease diagnosis using immunoassay and labeled DNA probe techniques, Vienna IAEA-TECDOC-657, (1992) 31-42

USE OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DIAGNOSIS OF BRUCELLOSIS IN CATTLE IN YUCATAN, MEXICO

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Abstract

USE 0F ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DIAGNOSIS OF BRUCELLOSIS IN CATTLE IN YUCATAN, MEXICO

Sera (247) from non-vaccinated brucellosis negative herds, 328 negative sera from *Brucella abortus* strain 19 vaccinated herds (brucellosis free), and 95 sera positive to the Rose Bengal test (RBT) and Complement Fixation test (CFT) from *Brucella abortus*-infected herds, were used to determine the relative sensitivity and specificity of a FAO/IAEA I-ELISA kit and the Rivanol Agglutination Test (RAT), using the CFT as a "gold standard" A threshold value for the I-ELISA was determined to be 37 PP using the mean plus 3 standard deviations of the negative sera from vaccinated animals The I-ELISA showed a high relative sensitivity (100%) and a good relative specificity (92 5%), using the threshold determined for local conditions The RAT gave a lower sensitivity value than the CFT (97 8%) and good specificity (99 3%) The I-ELISA could be used as a screening test under Yucatan conditions or as a confirmatory test in places where vaccination is not carried out The RAT lacks sensitivity and is therefore not recommended for use in final stages of eradication programs but could be used in control programmes or early stages of eradication campaigns as a confirmatory test

1 INTRODUCTION

Yucatán is a brucellosis endemic area, and vaccination with the full dose of S-19 is a common practice Vaccination practices are not uniform, and for the most cases there are no records, making considerations in diagnostic serology difficult. In Latin America, Mexico has one of the highest incidences of brucellosis. In México the estimate of annual losses due to this infectious disease are thought to amount US$ 350,000,000 [1]. An important aspect of brucellosis is its strong zoonotic potential In 1988, México reported 6303 cases of brucellosis in humans [2]

Although several seroprevalence studies have been done, different diagnostic techniques have been used and the results vary from zone to zone and for different laboratories within the country Yucatán is not an exeption and none of the studies performed have been carried out following a reliable sampling design and using a reliable diagnostic protocol designed for conditions of the area In previous studies, the Rose Bengal Plate Test (RBT), the Rivanol Agglutination test (RAT), the 2-mercaptoethanol agglutination test (2-ME), and an indirect ELISA (I-ELISA) were compared to Complement Fixation Test (CFT) in order to check their sensitivity and specificity However, not enough samples were tested and the results were therefore of limited value [3].

Although the definitive diagnosis of infectious disease can be accomplish only through the direct demonstration and identification of the causative agent(s) by culture and isolation procedures, sometimes this may be difficult and beyond the expertise and capabilities of diagnostic laboratories, particularly those in developing countries However, accurate presumptive diagnosis can be achieved from serological techniques used in combination with clinical observations and case histories [4].

Classical serological techniques (i e agglutination, precipitation, complement fixation and virus neutralization tests) have proved useful but they suffer from several drawbacks such as poor performance and lack of standardization ELISA techniques have the potential to solve many of these problems [4]. ELISA has become widely used for both antigen and antibody detection in animal disease diagnosis. Unfortunately, little has been done concerning internationally

acceptable reference reagents or protocols, with a negative impact on international control of animal diseases and inter-country trade in livestock [5].

The objectives of the present study were to establish a serum bank and to use it to validate the FAO/IAEA I-ELISA kit, determining the threshold value for local conditions; to calculate the relative sensitivity and specificity of the FAO/IAEA I-ELISA test; to determine the relative specificity of other tests used, considering the CFT as the " gold standard"; to start a seroprevalence survey using the I-ELISA and to catalogue and analyze the data using computer facilities.

2. MATERIAL AND METHODS

For the comparison of the serological tests a serum bank was established using 247 sera from non-vaccinated negative brucellosis free herds, 328 negative sera from *Brucella abortus* strain 19 vaccinated herds (brucellosis free), and 95 sera positive to RBT and CFT from infected herds (at least 2% seropositivity in the herd using the above tests and showing clinical signs).

The method described by Alton et al. [6] and Morgan et al. [7] was used for the RBT. The antigen was supplied by Productora Nacional de Biológicos Veterinarios (PRONABIVE). For the RAT, the method described by Morilla and Bautista was used [8]). The antigen was provided by PRONABIVE. Serum dilutions used were 1:25, 1:50, 1:100 and 1:200. The CFT was the microtiter method as used at the Central Veterinary Laboratory (CVL), Weybridge, England [9], except that samples were run at doubling dilutions up to 1:64, due to strong prozone effect found previously at lower dilutions. The concentration of International Units (I.U.) of antibody were derived from standard tables. The positive and negative controls and antigen were supplied by the CVL, Weybridge, UK.

An ELISA kit developed by the Joint FAO/IAEA Division was used, following the procedure exactly as indicated in the protocol provided. The antigen was a hot water/hot phenol extract from *Brucella abortus* and the conjugate was horseradish peroxidase-labeled mouse monoclonal anti-bovine IgG1. The substrate was H_2O_2 and the chromogen was ABTS. The optical density (OD) of each well was measured using a Immunoskan Plus automatic reader (BDSL) linked to a computer using the FAO/IAEA BREIA 1.01 program to interpret results. The reader, the program and the computer were provided under an FAO/IAEA Research Contract.

The basis of interpretation of each test is shown in Table I. For the RBT and RAT, this is provided in the papers, which describe the techniques. For the CFT, the antibody levels used were calculated using a table supplied by the Center for Tropical Veterinary Medicine, Edinburgh, U.K.

The I-ELISA threshold was determined using the sera negative to all tests from vaccinated animals, then calculating the mean (x) of the percentage positivity (PP) of the animals and adding three standard deviations (SD).

TABLE I. ANTIBODY LEVELS FOR INTERPRETATION OF TESTS

	Vaccinated	Non-vaccinated
RBT	reaction	reaction
RAT	1:50	1:25
CFT	50 i.u.	20 i.u.
I-ELISA*	37%	28%

* These values are the cut-off point calculated using the kit for each of the groups

The relative sensitivity and specificity of each test compared to the others and the Predictive Value for the I-ELISA were calculated using the methods described by Thrusfield [10]. The Epi-Info version 5.01b software [11] was used for sorting and analyzing the data.

3. RESULTS

The cut-off point for the I-ELISA was found to be 37 PP of the strong positive control. Tables II-V show the results for the RAT and I-ELISA tests compared to those for the CFT using sera from vaccinated, non-vaccinated cattle and overall. Sensitivity and specificity of all the tests compared to CFT are also shown.

TABLE II. SENSITIVITY AND SPECIFICITY OF RIVANOL TEST COMPARED TO CFT (n=688)

	CFT positive	CFT negative	Sensitivity (%)	Specificity (%)
Non-vaccinated Riv. +ve	88	4	97.8	98.5
Non-vaccinated Riv. -ve	2	271	-	-
Vaccinated Riv. +ve	-	-	-	-
Vaccinated Riv. -ve	-	323	-	-
Overall Riv. +ve	88	4	97.8	99.3
Overall Riv. -ve	2	594	-	-

Riv = Rivanol Agglutination test

TABLE III. SENSITIVITY AND SPECIFICITY OF I-ELISA TEST COMPARED TO CFT (n=681).

	CFT positive	CFT negative	Sensitivity (%)	Specificity (%)
Non-vaccinated E. +ve	83	14	100	94.9
Non-vaccinated E. -ve	0	261	-	-
Vaccinated E. +ve	-	31	-	90.4
Vaccinated E. -ve	-	292	-	-
Overall E. +ve	83	45	100	92.5
Overall E -ve	0	553	-	-

TABLE IV. SENSITIVITY AND SPECIFICITY OF I-ELISA TEST COMPARED TO RIVANOL TEST (n=691).

	Riv. +ve	Riv. -ve	Sensitivity (%)	Specificity (%)
Non-vaccinated E. +ve	89	6	98.9	97.8
Non-vaccinated E. -ve	1	271	-	-
Vaccinated E. +ve	-	31	-	90.4
Vaccinated E. -ve	-	293	-	-
Overall E. +ve	89	37	98.9	93.8
Overall E. -ve	1	564	-	-

Riv = Rivanol Agglutination test

TABLE V. SENSITIVITY AND SPECIFICITY OF RIVANOL TEST COMPARED TO I-ELISA TEST (n=691).

	E +	E -	Sensitivity (%)	Specificity (%)
Non-vaccinated Riv. +ve	89	1	86.4	99.6
Non-vaccinated Riv. -ve	14	263	-	-
Vaccinated Riv. +ve	39	0	100.	100
Vaccinated Riv. -ve	0	285	-	-
Overall Riv. +ve	128	1	90.1	99.8
Overall Riv. -ve	14	548	-	-

Riv. = Rivanol Agglutination test

The predictive value of the I-ELISA, was 34% and 100% for the positive and negative results respectively, using a calculated prevalence of 4% for the Yucatán state, a sensitivity and specificity of 100% and 92% respectively.

4. DISCUSSION

The cut-off point (37 PP) was determined using the negative vaccinated population because it improved the specificity of the test by 0.7% (from 91.6% to 92.3%) over the cut-off determined using the negative non-vaccinated population (28 PP). This is comparable to the 35 PP recommended in the kit protocol, without loss of sensitivity. The reason for considering the vaccinated negative population instead of the negative non-vaccinated population was because vaccination is a common practice in Yucatán and a large number of negative vaccinated animals would have been misclassified as positive, thereby decreasing assay specificity. Sensitivity of the I-ELISA was 100% relative to the CFT (gold standard). Specificity was lower in the overall population due to the inability of the test to differentiate vaccinated from infected animals, however, the specificity for the different groups (94.9% for non-vaccinated and 90.4% for the vaccinated animals) indicates the possibility of using the I- ELISA test as a confirmatory test perhaps as a replacement for the more complicated CFT or the rivanol agglutination test, especially in areas where vaccination with *Brucella abortus* S-19 is not carried out. The lower specificity of the I-ELISA compared to CFT, although the former uses a Mab specific to detect IgG1 could be explained by the fact that only capture enzyme immuno assays permit precise isotype analysis of antibodies of a distinct isotype (12).

The RAT showed a relative sensitivity of 97.8% compared to CFT. This makes the RAT less desirable to use as a confirmatory test in the final stages of eradication programmes, because of the danger of leaving false negative animals in the herd. This finding agrees with previous studies performed in Yucatan [3], however, the relative specificity of the RAT compared to CFT was good (99.3%) giving comparable results to other studies which indicated that the RAT could be used to differentiate between vaccination and infection titers [7,13] therefore it could be used as a confirmatory test during control of brucellosis and early stages of eradication campaigns.

The relative sensitivity and specificity of the I-ELISA compared to RAT test were also calculated. The results show that the I-ELISA has a comparable performance to the RAT in both aspects. However, when the comparison of the relative sensitivity and specificity of the RAT to I-ELISA was done, it showed that the relative sensitivity of the RAT was low, (90.1%), although the specificity was high (99.8%). This indicates that the I-ELISA is more sensitive than the RAT as performed in Yucatán. However, both tests demonstrated specificities which were comparable.

The I-ELISA Predictive Value for the positive (34 %) and negative (100 %) results, confirmed that this is a highly sensitive and but less specific test for our conditions, so it can be used as a screening test, but the positive results would have to be confirmed with a more specific laboratory test. A more realistic picture of the utility of the I-ELISA in comparison with other serological tests, will be obtained when it is field tested, and the prevalence survey in Yucatán is completed.

From the results presented here the following conclusion may be drawn. It is recommended that under the Yucatan conditions when possible the CFT should be used for identification of reactors after initial screening with a highly sensitive test (i.e. RBT or I-ELISA) which is more simple and quicker than CFT. The RAT seems to have a high specificity but a lower sensitivity compared to the CFT and might therefore not identify some reactors.

REFERENCES

[1] SECRETARIA DE AGRICULTURA Y RECURSOS HIDRAULICOS, Primer Simposium Internacional de Actualización en Brucelosis, México, D.F., 10 y 11 de Marzo de 1994.

[2] LOPEZ-MERINO, A., LOPEZ-SANTIAGO, R., ANTONIO-OCAMPO, D., HERNANDEZ-MONROY, I., GONZALEZ-DOMINGUEZ, F., Brucelosis, Avances y Perspectivas. Publicación Técnica del INDRE No. 6. Secretaría de Salud, México, D.F. (1991) 202-205.

[3] DAJER, A., GUTIERREZ, E., HONHOLD, N., ZAPATA, D., VILLEGAS, S., Comparison of Five Serological Tests to Detect *Brucella abortus* Antibodies and a Report on Prevalence of the disease in Livestock in the State of Yucatán, México, Proceedings of a Final Research Co-ordination Meeting of an FAO/IAEA/SIDA Research Co-ordination Programme, FAO/IAEA, Heredia, Costa Rica (1990) 131-137.

[4] WRIGHT, P.F., NILSSON, E., VAN ROOIJ, E. M. A., LELENTA, M., JEGGO, M. H., Standardization and Validation of Enzyme-Linked Immunosorbent Assay Techniques for the Detection of Antibody in Infectious Disease Diagnosis, Rev. Sci. Tech. Off. Int. Epiz.**12** (1993) 435-440.

[5] JACOBSON, R.J., SPENCER, T., EDWARDS, S., JEGGO, M.H., WRIGHT, P., Expression and Evaluation of ELISA data in Serological Tests Used for Infectious Disease Control in Animals, Report of a Joint FAO/IAEA Consultants Meeting on ELISA Data Evaluation and Expression, FAO/IAEA, Vienna, Austria (1992) 1-22.

[6] ALTON, G.G., JONES, L.M., PIETZ, D.E., In: Laboratory Techniques in Brucellosis, 2nd. ed. Pub: WHO, Geneva (1975) 82-85.

[7] MORGAN, B.W.J., MacKINNON, D.J., GILL, K.P.W., GOWER, S.G.M., NORRIS, P.I.W., In: Brucellosis Diagnosis: Standard Laboratory Techniques, M.A.F.F., HMSO, London (1987) 5-30.

[8] MORILLA, G.A., BAUTISTA, C.R., Manual de Inmunología, Editorial Diana, México, (1986) 218-220.

[9] CENTRAL VETERINARY LABORATORY (CVL), Standard Laboratory Techniques for Brucellosis Diagnosis, M.A.F.F., Weybridge (1991) 23-35.

[10] THRUSFIELD, M., Epidemiología Veterinaria, Acribia S.A., Zaragoza, Spain **130** (1990) 228-230.

[11] EPI INFO Ver.5.01b, Public Domain Software for Epidemiology and Disease Surveillance, Center for Disease Control, Epidemiology Programme Office, Atlanta, Georgia/World Health Organization, Global Programme on AIDS, Geneva (1991).

[12] PORTSMANN, T., KIESSIG, ST., Enzyme Immunoassay Techniques, J. Immunol. Methods, **150** *(1992) 5-21.*

[13] METCALF, H.E., Control of bovine brucellosis in the United States, In: Practices in Veterinary Public Health and Preventive Medicine in the United States, Ed. George T. Woods, Pub. Iowa State University Press, Iowa (1986) 97-100.

APPLICATION OF ENZYME LINKED IMMUNOSORBENT ASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN RIO GRANDE DO SUL, BRAZIL

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Abstract

APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN RIO GRANDE DO SUL, BRAZIL

The results of an indirect ELISA (I-ELISA) and a competitive ELISA (C-ELISA) for detection of antibody to *Brucella abortus* in cattle were compared with those of conventional serological tests The sensitivity of I-ELISA using 230 sera from infected animals was 98 6% and the specificity in 720 sera from brucellosis-free animals was 98 2% The C-ELISA when tested in 94 positive sera and 91 negative sera was 100% sensitive and specific The relative sensitivity and specificity of I-ELISA compared to C-ELISA in 582 sera from groups of animals vaccinated with S19 by different routes and doses was respectively 64% and 100% When comparing five serological tests on sera from vaccinated animals, the specificity was 94% for C-ELISA, 93% for 2-mercaptoethanol (2ME), 88% for Rose Bengal Plate test (RBPT), 84% for Slow Tube Agglutination test (SAT) and 34% for I-ELISA It can be concluded from this study that the RBPT gave less false positive results than the I-ELISA in vaccinated animals and the C-ELISA is a good confirmatory test with the advantage of distinguishing the antibody response due to vaccination from that resulting from infection with *Brucella abortus*

1 INTRODUCTION

The diagnosis of brucellosis in cattle is frequently complicated particularly when live vaccines such as strain 19 of *Brucella abortus* are used on a large scale The antibody response induced by these vaccines is difficult to distinguish from that of natural infection by conventional serological tests [1].

Several supplementary serological tests such the agglutination with 2 mercaptoethanol, complement fixation and agar-gel immunodiffusion containing a soluble polysaccharide antigen have been shown to differentiate to some extent the antibody response of vaccinated from infected animals [2,3]

In recent years, the enzyme-linked immunosorbent assay (ELISA) using well characterized smooth lipopolysaccharide of *Brucella abortus* in an indirect ELISA or the O-polysaccharide in a competitive ELISA, has been shown to be a very sensitive and specific method for measuring antibody responses [4,5]. On the other hand, brucellosis can be controlled by the use of a reduced dose of S19 either by subcutaneous or conjunctival routes in young or adult females [6,7] In controlled conditions, these methods resulted in good protection against exposure with a minimum of serological responses [8] In order to minimize the problems of agglutinin titers that complicate the diagnosis, the use of reduced doses of S19 vaccine and more specific serological tests such enzyme immunoassays have been investigated [5, 9,10]

In the State of Rio Grande do Sul, Brazil, after a period of 20 years of vaccination with *Brucella abortus* S19, the prevalence of brucellosis decreased from 5 2% in 1965 [11] to 0.33% in 1986 [12] In this situation, the possibility of starting a program of eradication of the disease is envisaged The objective of the present study was to compare ELISA tests (indirect and competitive) with conventional tests on sera from negative, infected and S19 vaccinated cattle.

2 MATERIAL AND METHODS

2.1. Test sera

Negative sera: a total of 720 sera were obtained from herds in an area without recent history of infection or vaccination against brucellosis.

Positive sera a total of 230 sera from three herds were collected These herds presented reproductive problems, abortions, several positive bacteriological and serological results for *Brucella abortus*

2.2. Vaccinated animals

Group 1 16 heifers (3-6 months) were vaccinated with standard dose ($6x10^{10}$) of *Brucella abortus* S19 by subcutaneous route

Group 2 20 adult females (over 2 years) were vaccinated with reduced dose ($3\text{-}5x10^{9}$) of *Brucella abortus* by conjunctival route and revaccinated four months later

Group 3 21 heifers (3-6 month) were vaccinated with reduced dose ($3\text{-}5x10^{9}$) of *Brucella abortus* by conjunctival route and revaccinated four months later

All animals were tested in all serological tests before vaccination and bled monthly for up to 300 days after vaccination

2.3. Serological tests

2 3 1 Conventional tests

The tests used were the Rose Bengal Plate Test (RBPT), the Slow Tube agglutination test (SAT) and the 2 mercaptoethanol (2ME) as described by Alton et al [2] In the RBPT any degree of agglutination was considered to be positive For the SAT, visible agglutination at the dilution of 1/100 was considered to be positive and for the 2ME, visible agglutination at the dilution of 1/25 was considered to be positive

2 3 2 Indirect ELISA

An ELISA kit provided by the FAO/IAEA, which contained all the necessary reagents was used The test was performed according the manual which accompanied the kit [13] Briefly, a 1 µg/ml dilution of smooth lipopolysaccharide (S-LPS) was prepared in 0 05M carbonate buffer (pH 9 6) and 100 µl were added to wells of 96-well polystyrene plates (NUNC 2-69620) and incubated covered overnight at 4° C

After three wash cycles (Handiwasher, BDSL) with 0 01M phosphate buffered saline (pH 7 4) containing 0 05% Tween 20 (PBST), 100 µl of test sera (in duplicate) and control sera (in quadruplicate) diluted 1/200 in PBST were added to the wells and incubated for 1h at 37°C with continuous shaking on an orbital shaker

Four controls were included a strong positive serum, a weak positive serum, a negative serum and a buffer control After three more wash cycles with PBST, 100 µl of a horseradish peroxidase (HRPO) conjugated with rabbit anti-bovine IgG (H+L), diluted in PBST, were added to each well and the plates incubated again as described above

After three final wash cycles, 100 µl of 4mM hydrogen peroxide (H_2O_2) and 1mM [2,2-azinobis (3-ethyl-benzthiazoline sulfuric acid)] (ABTS) dissolved in 0 05M sodium citrate/citric acid (pH 4 5) were added to all wells The plates were incubated for 10 min at 37°C with continuous shaking for color development and to stop the reaction 100 µl of sodium dodecyl sulphate (SDS) were added to all wells

Optical density readings were obtained using a spectrophotometer (Titertek Multiskan Plus) at 405 nm The reader was linked to a computer and the results expressed as percent positivity (PP), calculated by means of the software BREIA 1 02, supplied by the FAO/IAEA with the kit Any serum which gave 35% or higher positivity (PP) was considered as positive

2 3 3 Competitive ELISA

The technique adopted was described by Nielsen et al [14] Briefly, the O-polysaccharide (PS) was diluted in coating buffer (carbonate/bicarbonate, pH 9 6), and used at 2µg/ml The plates were coated with 100 µl/well of diluted antigen and incubated at 25°C overnight and again for 18-20 hours at -20°C After thawing at 37°C in water bath, the plates were washed 5 times as described for I-ELISA and test sera and control a buffer control, a serum from vaccinated animal, a negative serum and a strongly positive serum were added

All sera were diluted 1/50 in PBST and 50 µl of each dispensed in duplicate and the controls in quadruplicate. Immediately, 50 µl of prediluted horseradish peroxidase conjugated monoclonal antibody was dispensed into all wells.

The plates were sealed and incubated for 2 hours at 25°C. After 5 wash cycles, H_2O_2-ABTS was added as described for I-ELISA and color was developed for 10 minutes, when 100 µl of stopping solution were added to all wells.

The readings were performed on the same equipment as for I-ELISA and the results expressed as percent inhibition, calculated by mean of the software ELISA 2.11. Any serum which gave over 20% inhibition was considered as positive.

2.4. Data analysis

Indirect ELISA - provided the controls were within acceptable limits, the results were expressed in percent positivity (PP) which was calculated as follows [13]:

$$PP = \frac{\text{Mean OD of duplicate tests}}{\text{Mean OD of C++ control}} \times 100$$

Competitive ELISA - the results were expressed as percent inhibition (PI) of the monoclonal antibody activity against O-polysaccharide antigen,(buffer control) and was calculated by the formula [14]:

$$PI = 1 - \frac{\text{Mean OD of replicate tests}}{\text{Mean OD of buffercontrol}} \times 100$$

Sensitivity and Specificity - the sensitivity of tests used on sera from infected cattle and the specificity in brucellosis free animals was calculated according to Thrusfield [15] and followed the formulae for each test:

$$\text{Sensitivity} = \frac{\text{Total N° of positive results}}{\text{Total N° of positive animals sampled}}$$

$$\text{Specificity} = \frac{\text{Total N° of negative results}}{\text{Total N° of negative animals sampled}}$$

3. RESULTS

3.1. Negative sera

The specificities of the four tests performed on serum from 720 brucellosis-free animals are presented in Table I. Additionally the competitive ELISA was done on 91 sera from the negative group.

3.2. Positive sera

The sensitivities of the four serologic tests performed on 230 *Brucella*-infected animals are presented in Table II. Additionally competitive ELISA data for 94 sera are included.

3.3. Vaccinated animals

The specificities of the serologic tests on serum from 452 vaccinated animals are presented in Table III. Additionally competitive ELISA results on 291 sera are also included.

The comparison between C-ELISA and I-ELISA on 582 sera from S19 vaccinated animals is shown in Table IV.

TABLE I. THE SPECIFICITY OF DIFFERENT TESTS ON SERA FROM BRUCELLOSIS-FREE ANIMALS

Test	Neg	Pos	Total	Specificity
RBPT	720	0	720	100.0
SAT	719	1	720	99.8
2ME	720	0	720	100.0
I-ELISA	707	13	720	98.2
C-ELISA	91	0	91	100.0

RBPT-Rose Bengal Plate Test
SAT-Serum Agglutination Test in tubes
2ME-2 Mercaptoethanol
I-ELISA-Indirect Elisa
C-ELISA-Competitive ELISA

TABLE II. THE SENSITIVITY OF DIFFERENT SEROLOGIC TESTS ON SERA FROM *BRUCELLA*-INFECTED ANIMALS

Test	Pos	Neg	Total	Sensitivity
RBPT	230	0	230	100.0
SAT	195	35	230	84.8
2ME	206	24	230	89.6
I-ELISA	227	3	230	98.6
C-ELISA	94	0	94	100.0

(See Table 1 for key)

TABLE III. THE SPECIFICITY OF DIFFERENT SEROLOGICAL TESTS IN SERA FROM S19-VACCINATED ANIMALS

Test	Pos	Neg	Total	Specificity
RBPT	54	398	452	88.0
SAT	72	380	452	84.0
2ME	31	421	452	93.1
I-ELISA	299	153.0	452	34
C-ELISA	18	273	291	94.0

(See Table 1 for key)

TABLE IV. COMPARISON BETWEEN C-ELISA AND I-ELISA ON 582 SERA FROM S19-VACCINATED ANIMALS

	Competitive ELISA		
	Positive	Negative	Total
Indirect ELISA			
Positive	18	191	209
Negative	0	373	373
Total	18	564	582

Relative sensitivity = 100%, Relative specificity = 64%

The serological response of heifers and adult cattle vaccinated with a standard dose subcutaneously and reduced doses conjunctivally of S19 vaccine in five serological tests is shown in Figures 1–3. All animals were bled at an interval of approximately 1 month until 300 days after vaccination.

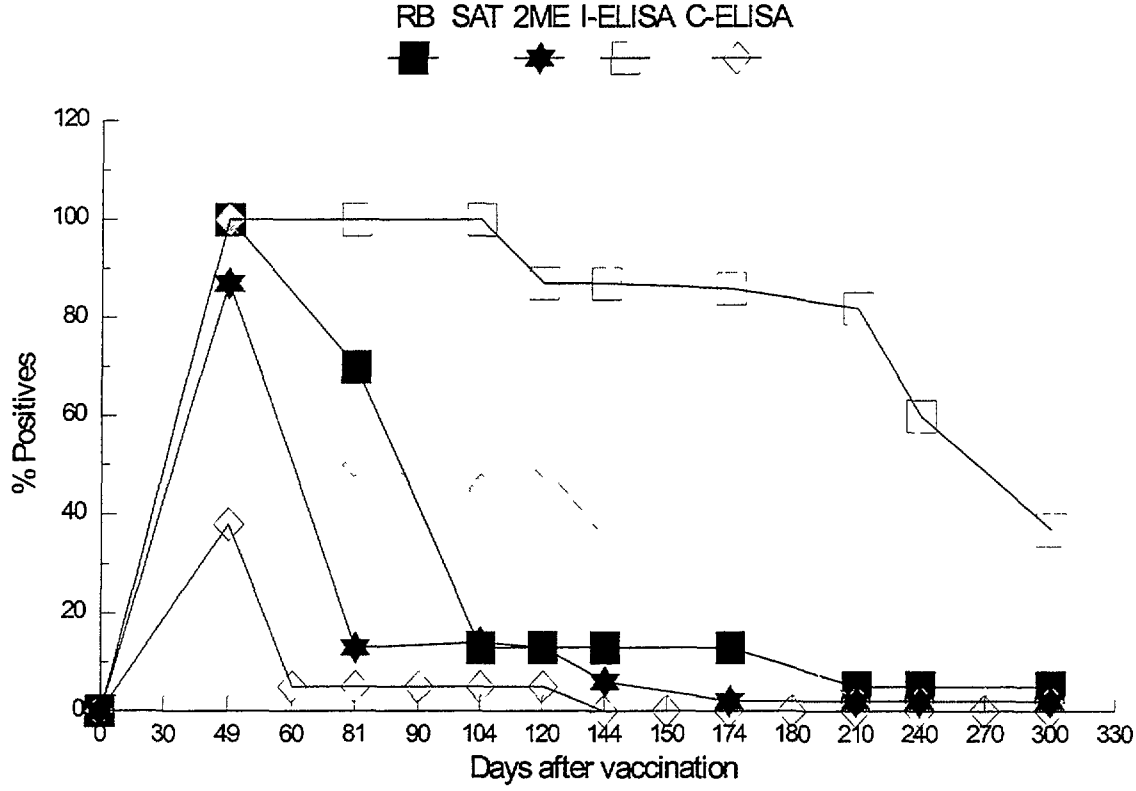

FIG. 1. Serological response of heifers vaccinated with subcutaneous standard dose of S-19 in five serological tests (n=16).

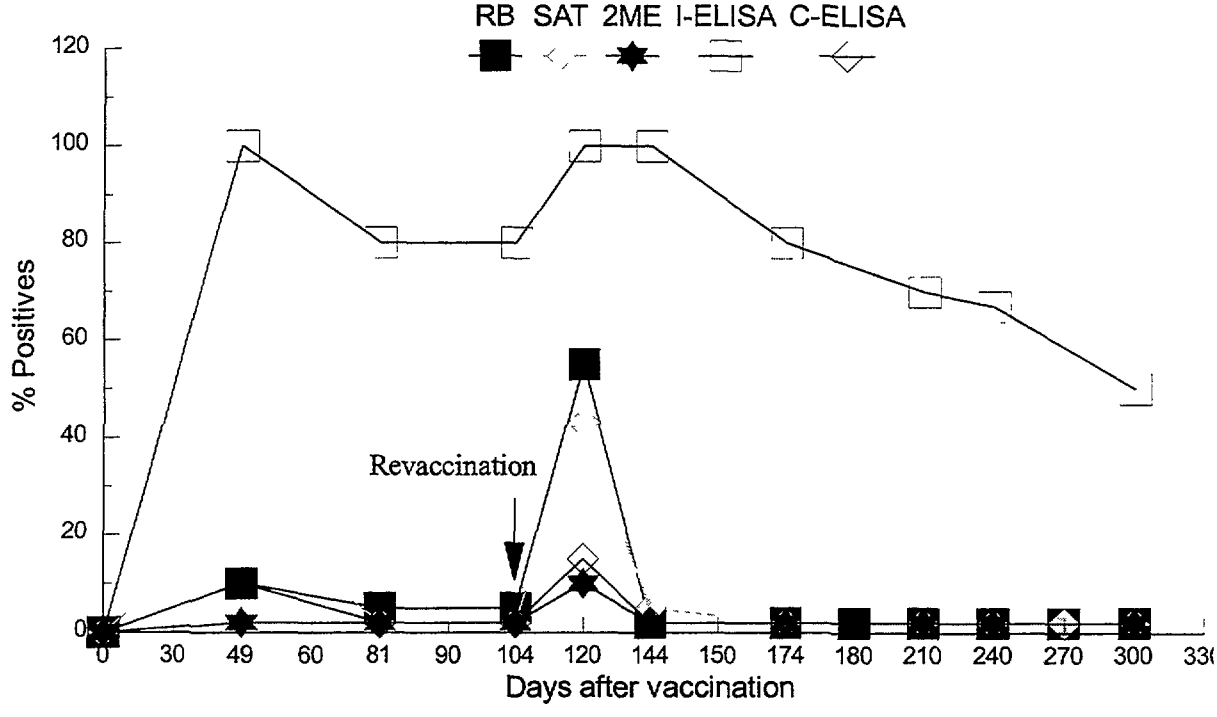

FIG. 2. Serological response in adult cattle vaccinated with reduced dose of S 19 in five serological tests (n=20).

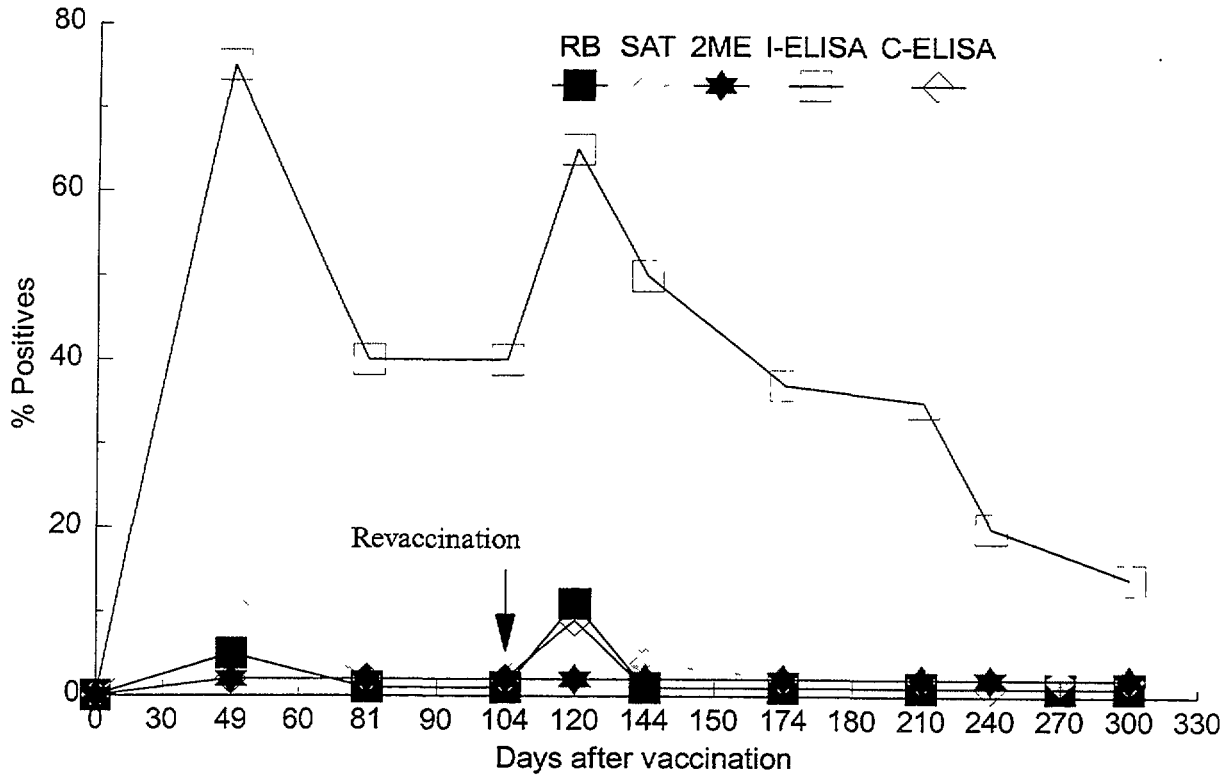

FIG. 3. Serological response of heifers vaccinated with conjunctival reduced dose of S19 in five serological tests (n=21).

4. DISCUSSION

A simple, rapid and inexpensive serological test that will detect infected animals early in the incubation period and at all stages of the disease and that does not detect antibody in vaccinated animals is still to be found. Nevertheless, a great deal of improvement was achieved recently either by the introduction of enzyme immunoassays [16] or by vaccination of animals with reduced dose of S19 *Brucella abortus* vaccine [8].

In the present study two different enzyme immunoassays were compared with conventional tests on sera of negative, infected and vaccinated animals. The specificity of the enzyme immunoassays was high, 98.2% for the I-ELISA and 100% for the C-ELISA on sera from brucellosis-free animals. In this situation, the I-ELISA was the test which gave the highest rate of false positive reactions. Those positive sera gave a PP just over the threshold value and the sera could be classified as suspicious.

The sensitivities of the ELISAs were 98.6% for the indirect and 100% for the competitive. Except for the RBPT, the two ELISAs were more sensitive than the other tests. These findings are in accordance with others [17,18] in that RBPT was found to be a good screening test, although some authors [19] have found an unacceptable false negative rate with the RBPT.

In the vaccinated groups, the animals vaccinated with a standard dose subcutaneously (Figure 1) gave 100% of positive results in RBPT, I-ELISA and SAT, 90% in 2ME and less than 40% in C-ELISA at 50 days after vaccination. Except for the SAT and I-ELISA, most sera were negative 6 months after vaccination, which is in accordance with the literature [10-20]. The I-ELISA was the most sensitive test, giving a high percentage of positive results until the end of the experiment (300 days). This may be explained by the use of a polyclonal anti bovine IgG (H+L) conjugate which measures all isotypes present in the sera. This stresses the usefulness of the test as a screening test among vaccinated animals [14].

The competitive ELISA gave fewer positive results in the vaccinated groups It is known that this test may be unable to differentiate about 15% of sera from S19 vaccinated animals, and it is not uncommon to observe positive results at the peak of the antibody response [16] Furthermore, one animal remained positive in all tests throughout the experiment This could be due to a transient S19 infection making this serum behave as sera from infected animals [5]

In the groups of animals vaccinated with a reduced conjunctival dose (Figures 2-3) except for I-ELISA most animals became negative to all tests in less than 3 months after vaccination with a peak of antibodies just after revaccinations These rapid decreases in the antibody suggest that this practice could be adopted in campaigns against brucellosis The I-ELISA gave a high percentage of positive results which could be explained by the different antibody affinity of the test [4]

The degree of immunity induced by a reduced dose of S19 dose was not determined Nevertheless protective immunity is well documented in the literature [8,21]

When the specificity of all tests was determined for vaccinated (Table III), the C-ELISA was the most specific (94%), followed by 2ME (93%), RBPT (88%), SAT (84%) and I-ELISA (34%) The relative sensitivity and specificity of I-ELISA when compared with C-ELISA (Table IV) was 64% and 100% respectively This low specificity of I-ELISA may account by the fact that only vaccinated animals were compared in both tests

From these results it can be concluded that C-ELISA can be used as confirmatory test The actual mechanism by which C-ELISA can differentiate vaccinated from infected animals is not fully understood Some authors speculate that it can be a result of antigen presentation on the polystyrene matrix, duration of exposure to the bacterium and antibody affinity [14-16]

In spite of the low specificity of the I-ELISA and the relatively high cost of this test, it has the advantage of being highly sensitive, that only small amounts of reagents are required and that it can be readily adapted to large scale screening

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REFERENCES

[1] FAO/WHO Expert Committee on Brucellosis, Fifth Report, WHO Technical Report, Series 464, Agricultural Studies, Geneva, N **85,** (1971)

[2] ALTON, G G , JONES, L M , ANGUS, L D , VERGER, J M , Techniques for the brucellosis laboratory, Paris, Institut National de la Recherche Agronomique, (1988)

[3] DIAZ, R , GARATEA, P , JONES, L M , MORIYON, I , Radial immunodiffusion with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle, J Clin Microbiol **10** (1979) 37-41

[4] WRIGHT, P F , NIELSEN, K H , Application of enzyme immunoassay in Veterinary Medicine Serodiagnosis of bovine brucellosis, In "Non isotopic immunoassay", Ngo, T T , Ed Plenum Press (1988) 129-145

[5] NIELSEN, K H , CHERWONOGRODZKY, J , DUNCAN, J R , BUNDLE, D R Enzyme immunoassay for differentiation of the antibody response of *Brucella abortus* infected or strain 19 vaccinated cattle, Am J Vet Res **50** (1989) 5-9

[6] NICOLETTI, P , JONES, L M , BERMAN, D T , Adult vaccination with standard and reduced doses of *Brucella abortus* strain 19 vaccine in a dairy herd infected with brucellosis, J Am Vet Med Assoc **173** (1978) 1445-1449

[7] CRAWFORD, R P , HECK, R C , WILLIAMS, J D , Experiences with *Brucella abortus* strain 19 vaccine in adult Texas cattle, J Am Vet Med Asoc **173** (1978) 1457-1461

[8] PLOMMET, M , FENSTERBANK, R , La vaccination antibrucellique administree par voie conjonctivale, Dev Biol Stand , Paris **56** (1984) 681-687

[9] NICOLETTI, P , A preliminary report on the efficacy of adult cattle vaccination using strain 19 in selected dairy herds in Florida, Proc 80th Ann Meet US An Hlth Assoc (1976) 91-106

[10] WORTHINGTON, R W , MULDERS, M S , MCFARLANE, I S , BECKER, D A , Serological investigation on adult cattle vaccinated with *Brucella abortus* strain 19 Onderstepoort, J Vet Res , **40** (1973) 7-12

[11] CARRILLO, C G , La Brucelosis de los animales en America y su relación con la infeccion humana, Office Int des Epizooties, Paris, (1987)

[12] Diagnosticos efetuados durante o periodo de Set 86 a Ago 87, (1987)

[13] FAO/IAEA , Indirect Brucellosis ELISA kit manual, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Austria, (1992)

[14] NIELSEN, K H , GALL, D , KELLY, W , HENNING, D , GARCIA, M , Enzyme immunoassay, Application to diagnosis of bovine brucellosis, ADRI, Agriculture Canada, (1992)

[15] THRUSFIELD, M , Veterinary Epidemiology, Butterworth, London, (1986)

[16] WRIGHT, P , NIELSEN, K H , KELLY, W , Primary binding techniques for the serodiagnosis of bovine brucellosis, In Animal Brucellosis, Nielsen, K H and Duncan, J R Ed CRC Press, Boca Raton, (1990) 199-235

[17] DOHOO, I R , WRIGHT, P F , RUCKERBAUER, G M , SAMAGH, B S , ROBERTSON, F J , FORBES, L B , A comparison of five serological tests for bovine brucellosis, Can J Vet Res , **50** (1986) 485-493

[18] SUTHERLAND, S S , Evaluation of the Enzyme-Linked Immunosorbent Assay in the detection of cattle infected with *Brucella abortus*, Veterinary Microbiology **10** (1984) 23-32

[19] SARAVI, M , GREGORET, R J , WRIGHT, P F , GALL, D , Comparison of an indirect enzyme immunoassay and conventional serological techniques for the detection of bovine antibody for *Brucella abortus*, Proc Final Res Coord Meet FAO/IAEA/SIDA, Austria, (1990) 151-162

[20] Joint FAO/WHO Expert Committee on Brucellosis, 6th Report, World Health Organization, Technical Report, Series 740, (1986)

[21] NICOLETTI, P , JONES, L M ,BERMAN, D T , Comparison of subcutaneous and conjunctival route of vaccination with *Brucella abortus* strain 19 vaccine in adult cattle J A V M A **173** (1978) 1450-1456

EVALUATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN PATAGONIA, ARGENTINA

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Abstract

EVALUATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS IN PATAGONIA, ARGENTINA.

Control and eradication of bovine brucellosis is usually based on the serological detection of antibodies. In Argentina, the Rose Bengal test (RB) and the Buffered Plate antigen test (BPA) are the two screening test officially recognized, while the 2-mercaptoethanol test (2ME) and the Tube Agglutination test (SAT) are the confirmatory assays currently in use. In order to improve the serological diagnosis of bovine brucellosis in Patagonia, Argentina, an indirect ELISA kit produced by the Joint FAO/IAEA Division was evaluated. Sera from negative non-vaccinated, negative but vaccinated and positive animals were tested by all the above techniques. The specificity of the I-ELISA (99.6% and 99.7%) was similar to that of the BPA, RB, 2ME and Complement Fixation test (CF) when used to test sera from non-vaccinated, negative and vaccinated, negative animals, respectively. The sensitivity of the I-ELISA (98%) was higher than the BPA test (96%) and the CF test (95,2%). The I-ELISA kit evaluated in this study was thought to be a valuable tool for the diagnosis of bovine brucellosis in Patagonia region where little epidemiological information is available about this disease and where large numbers of sera should be tested to obtain such information.

1. INTRODUCTION

Serological detection of antibodies is usually the method of choice for control and eradication of bovine brucellosis. Several conventional serological tests have been used singly or in combination for the serological diagnosis of this disease [1].

Usually, a rapid screening test of high sensitivity is applied initially in testing of sera in control programmes. A positive reaction in the screening test would result in the serum being subjected to a confirmatory test of high specificity. In Argentina, the rose bengal test (RB) and the buffered plate antigen test (BPA) are the two screening tests officially recognized, with the 2-mercaptoethanol test (2ME) and the tube agglutination test (SAT) used as the confirmatory tests (Resolución 1269/93. Servicio Nacional de Sanidad Animal, 16-11-93). However, the agglutination techniques may have limitations in sensitivity due the to prozone phenomena and may result in non-specific agglutination reactions due to the presence of antibodies against bacteria with antigenic determinants common with *Brucella abortus* such as *Yersinia enterocolitica, Escherichia coli, Salmonella urbana* and *Campylobacter fetus* [2, 3]. The complement fixation test (CF) is a highly sensitive and specific technique, but is a cumbersome, time consuming and difficult to standardize test. The indirect enzyme linked immunosorbent assay (I-ELISA), on the other hand, has less of these problems, is highly sensitive and specific and detects all the isotypes of IgG and IgM in serum [4]. In addition, this test requires a minimum volume of serum and other reagents to be performed.

The aim of the present study was to evaluate an indirect enzyme linked immunosorbent assay for the diagnosis of bovine brucellosis in the Patagonia region, southern Argentina.

2. MATERIAL AND METHODS

2.1. Sera

The following groups of Patagonic sera were processed by the RB, BPA, 2ME, CF and I-ELISA tests.

2.1.1. Group 1

Sera from 286 cows, older than 24 months, from 13 herds free from bovine brucellosis. The herds were serologically negative (to RB and 2ME) for bovine brucellosis in two consecutive tests and had no history of abortions or other signs of this disease in at least the previous 5 years. These animals had never been vaccinated against bovine brucellosis.

2.1.2. Group 2

Sera from 459 cows, older than 24 months, vaccinated against bovine brucellosis (strain 19, standard dose) between 3 and 8 months of age that were negative to RB, BPA and 2ME originated from 11 farms.

2.1.3. Group 3

Sera from 156 cows, older than 24 months, that reacted positively in the RB and 2ME tests. These cows originated from 10 herds with at least 2% of animals positive to the RB and 2ME tests.

2.1.4. Group 4

1309 sera originated from 17 farms with at least 2% positive reactors to the RB and 2ME tests.

2.2. Serological techniques

Most of the sera were tested by the RB, BPA, 2ME and I-ELISA tests. A selected group of sera were also processed by the CF test. The antigens for the conventional tests were purchased from the Research Center on Veterinary Sciences, The National Institute of Agricultural Technology (INTA), Castelar, Argentina.

2.2.1. Rose Bengal test

This technique was performed as previously described [5,6]. Briefly, a dilution of serum was obtained by mixing 30 µl of serum and 30 µl of rose bengal antigen on a glass plate. The reaction was incubated for 4 minutes at room temperature applying rotatory movements to the plate (approximately 12 rotations per minute). The reaction was interpreted as positive when agglutination was visible at 4 minutes and negative when the mixture was homogeneous at this time.

2.2.2. Buffered Plate Agglutination test

This technique was also performed as previously described [5,7]. In brief, a dilution of serum was obtained mixing 80 µl of serum and 30 µl of antigen on a glass plate. The reaction was incubated for 8 minutes at room temperature. Four rotatory movements were applied to the plate after the first 4 minutes of incubation. The reaction was interpreted as positive when agglutination was visible at 8 minutes and negative if the mixture was homogeneous at this time.

2.2.3. 2-mercaptoethanol test

This technique was performed according to Alton et al. (1988). Dilutions of serum (1:25, 1:50, 1:100 and 1:200) were obtained by mixing 0.08 ml, 0.04 ml, 0.02 ml and 0.01 ml, respectively, with 1ml of a 1% solution of 2 mercaptoethanol followed by addition of 1ml of a 2% antigen suspension after 30-60 minutes of incubation at room temperature. The mixture was then incubated for 48 ± 6 h at 37 °C. The reaction was considered positive when the supernatant was transparent and there was an agglutinate in the bottom of the tubes and negative if the supernatant was turbid and no agglutinated cells were observed.

2.2.4. Complement Fixation test

This technique was performed according to Alton et al. (1975), using haemagglutination plates incubated at 37°C for 30 minutes. The antigen was standardized to give 50% fixation of complement with a dilution of 1/256 of the second international standard anti-*Brucella abortus* serum. The sera were

tested up to a 1/256 dilution. The sera that did not reach a final titer were retested at higher dilutions. Fifty percent fixation of complement at 1/8 dilution was considered as the positive threshold.

2.2.5. Indirect enzyme linked immunosorbent assay, ELISA

The I-ELISA was performed using an Indirect ELISA Brucellosis Kit provided by the Joint FAO/IAEA Division, International Atomic Energy Agency (IAEA, Vienna, Austria) and following the recommendations of the manual supplied with the kit. Briefly, medium binding capacity, 96 wells polystyrene plates (Flat bottom, Nunc, cat.#2-69620), were coated with 100 µl of hot water/hot phenol extracted *Brucella abortus* smooth lipopolysacharide at a dilution of 10 ug/ml in a 0.06 M carbonate buffer pH 9.6 and incubated overnight at 4°C. The plates were then washed three times and test and control sera were added to the wells of microplates at a dilution of 1:200. The plates were incubated for 1 hr at 37°C. All the test sera were tested in duplicate, while control sera were tested in quadruplicate. Controls consisted of a conjugated antiglobulin control with no sera being added to the wells, a strong positive control serum, a weak positive control serum and a negative control serum. After further washing cycles, 100 µl of a 1:12,000 dilution of a rabbit anti-bovine IgG (H+L) conjugated to horseradish peroxidase serum was added to all the wells followed by another 1 hr period of incubation at 37°C. Finally, and after another wash cycle, ABTS/H_2O_2/citrate buffer substrate/chromogen solution was added and incubated for 10 minutes at 37°C with shaking. The reaction was stopped by addition of 100 µl of 4% sodium dodecyl sulphate solution. Plates were read in a Multiskan Plus ELISA reader using the software provided with the kit. Optical density values were converted to percentages of the strong positive control serum (pp). The threshold was determined by adding 3 SD to the mean of the pp values of the negative non-vaccinated animals (Group 1). Values below this threshold were considered to be negative.

2.2.6. Specificity

Specificity was defined as the ability of a given technique to correctly identify negative cattle as negative. The diagnostic specificity of each test was calculated for both non-vaccinated negative (Group 1) and vaccinated negative animals (Group 2).

Diagnostic specificity was calculated as follows:

$$\frac{\text{No. of test negative}}{\text{No. of negative cattle tested}} \times 100$$

The relative specificity of each test relative to the 2 screening tests used (RB and BPA) was calculated for the sera from infected herds (Group 4), as follows:

$$\frac{\text{No. of comparative test negative}}{\text{No. of screening test negative}} \times 100$$

2.2.7. Sensitivity

Sensitivity was defined as the ability of a technique to correctly identify positive cattle as positive. It was calculated with sera from Group 3 for each test as:

$$\frac{\text{No. of test positive}}{\text{No. of positive cattle tested}} \times 100$$

The sensitivity of the test in relation to each other was calculated using sera from infected herds (Group 4) as:

$$\frac{\text{No. of comparative test positive}}{\text{No. of relative test positive}} \times 100$$

3. RESULTS

The pp threshold for the ELISA technique was 49% positivity. Therefore, for further calculations sera with pp higher than 49% was considered to be positive, while sera with pp below this value was considered as negative. The diagnostic specificity of all the tests used for non-vaccinated negative herds is shown in Table I. The distribution of I-ELISA pp values of the 286 non-vaccinated negative sera is shown in Figure 1. Only 1 out of the 286 sera tested was positive by I-ELISA.

The diagnostic specificity of all the tests used for vaccinated negative herds is depicted in Table II. The distribution of I-ELISA pp values of the 459 vaccinated negative sera is displayed in Figure 2. Only 1 of the sera gave a positive reaction in the I-ELISA.

The sensitivity of all tests estimated with sera positive to the RB and 2ME tests is shown in Table III. The distribution of I-ELISA pp values of the 156 positive sera from infected herds is plotted in Figure 3. Three of the 156 sera gave pp values below the threshold of 49.9% pp. The comparative distribution of I-ELISA pp values of sera from Groups 1 and 3 is plotted in Figure 4.

The specificity relative to the two screening tests used (BPA and RB) in sera from *Brucella* infected herds is shown in Table IV.

The sensitivity relative to both screening and confirmatory tests positive reactors in *Brucella* infected herds, is shown in Table V.

TABLE I. DIAGNOSTIC SPECIFICITY OF ALL SEROLOGICAL TESTS CALCULATED USING SERA FROM NON-VACCINATED, NEGATIVE HERDS

Test	No. of sera tested	Negative	Positive	Specificity
BPA	286	286	0	100.0%
RB	286	286	0	100.0%
2ME	286	286	0	100.0%
CF	70	70	0	100.0%
ELISA	286	285	1	99.6%

TABLE II. DIAGNOSTIC SPECIFICITY OF ALL SEROLOGICAL TESTS ESTIMATED WITH SERA FROM VACCINATED, NEGATIVE HERDS

Test	No. of sera tested	Negative	Positive	Specificity
BPA	459	448	11	97.6%
RB	459	459	0	100.0%
2ME	459	459	0	100.0%
CF	72	72	0	100.0%
ELISA	459	458	1	99.7%

TABLE III. SENSITIVITY OF ALL THE SEROLOGICAL TESTS ESTIMATED USING SERA POSITIVE TO THE RB AND THE 2ME

Test	No. of sera tested	Positive	Negative	Specificity
BPA	153	147	6	96.0%
RB	-	-	-	-
2ME	-	-	-	-
CF	42	40	2	95.2%
ELISA	156	153	3	98.0%

TABLE IV. SPECIFICITY RELATIVE TO THE BPA AND RB TESTS NEGATIVE SERA IN *Brucella abortus* INFECTED HERDS

Test	Specificity relative to BPA %	Specificity relative to RB %
BPA	-	83.91
RB	97.28	-
2ME	94.31	89.37
ELISA	93.82	91.54

TABLE V. SENSITIVITY OF THE BPA, RB, 2ME AND I-ELISA TESTS RELATIVE TO BOTH SCREENING AND CONFIRMATORY TEST

Test	BPA 279*	RB 208*	2ME 259*	ELISA 260*
BPA	-	84.6%	79.45%	73.84%
RB	63.08%	-	61.62%	70.38%
2ME	77.47%	76.44	-	70.38
ELISA	68.81%	87.98%	70.93%	-

* Positive reactors out of 1309 cattle tested

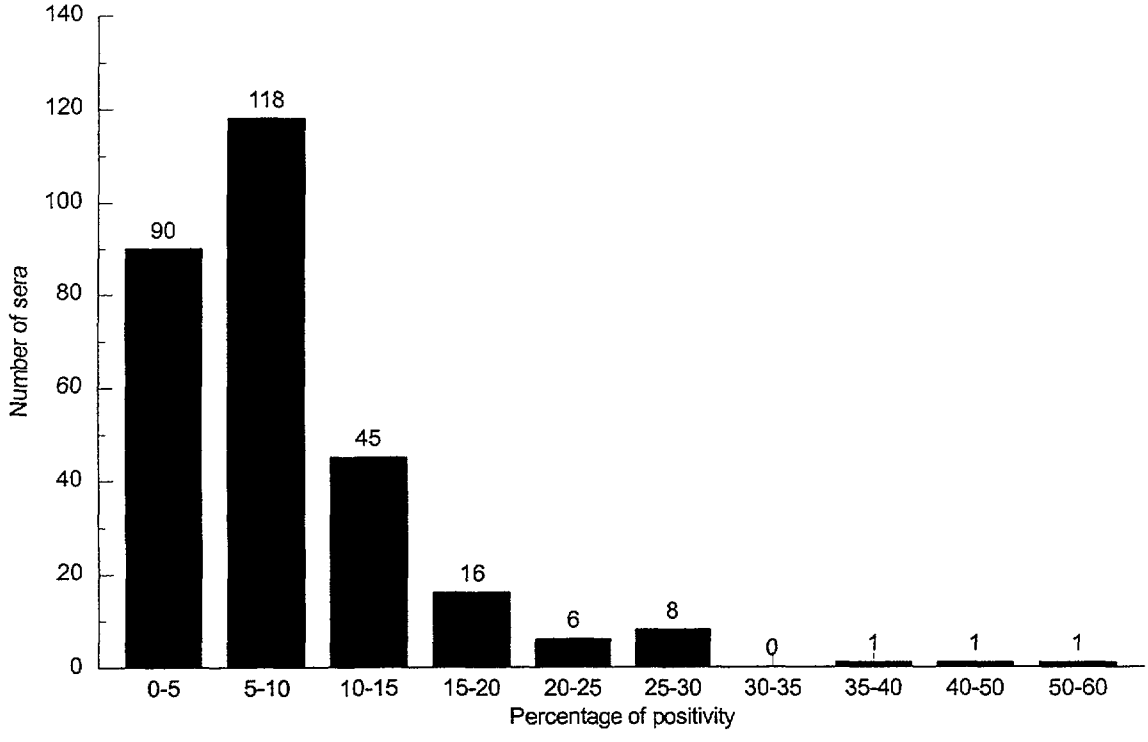

FIG. 1. Distribution of I-ELISA pp values of 286 non-vaccinated, negative sera.

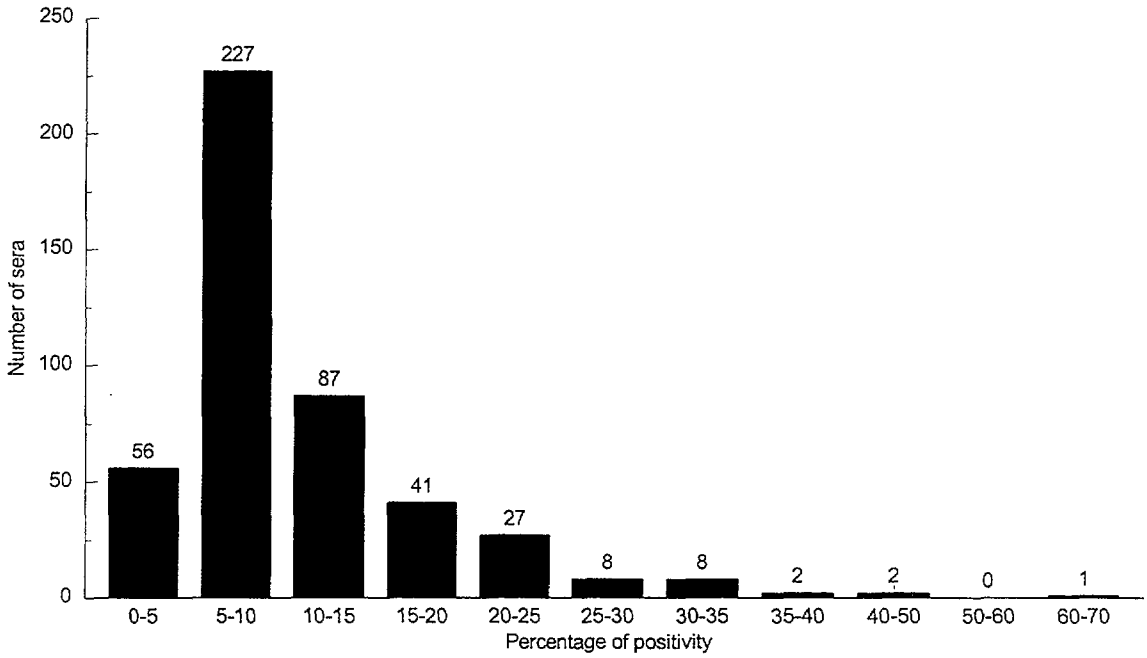

FIG. 2. Distribution of I-ELISA pp values of 459 vaccinated, negative sera.

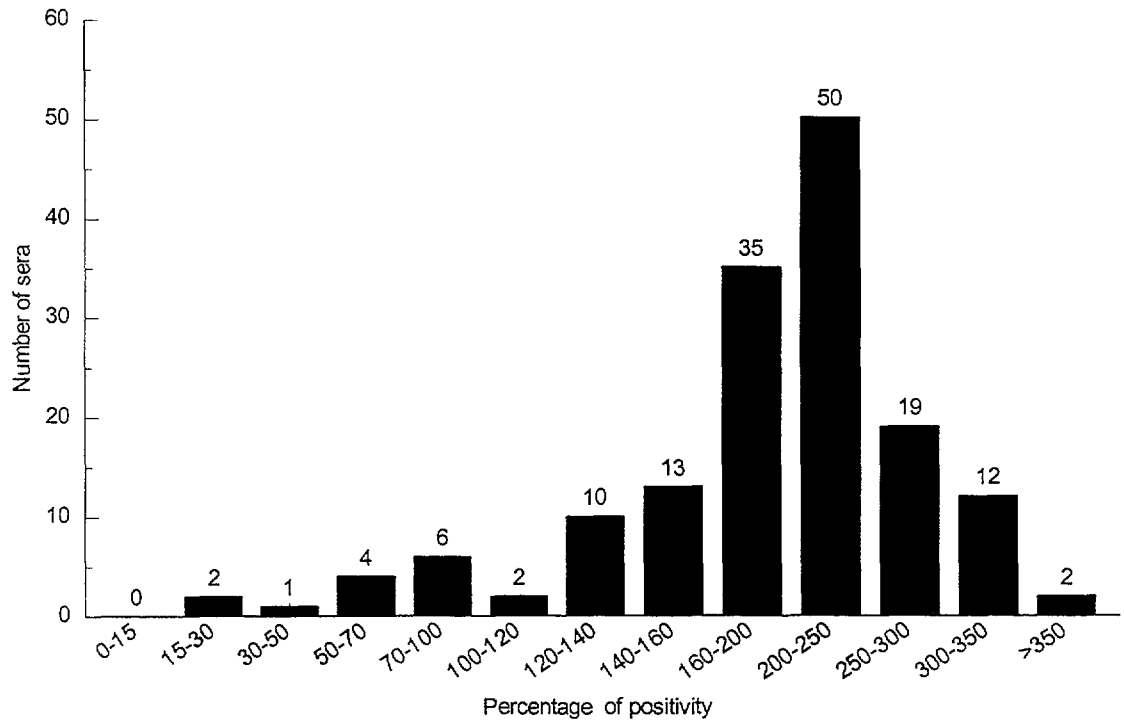

FIG. 3. Distribution of I-ELISA pp values of 156 sera positive to RB and 2ME.

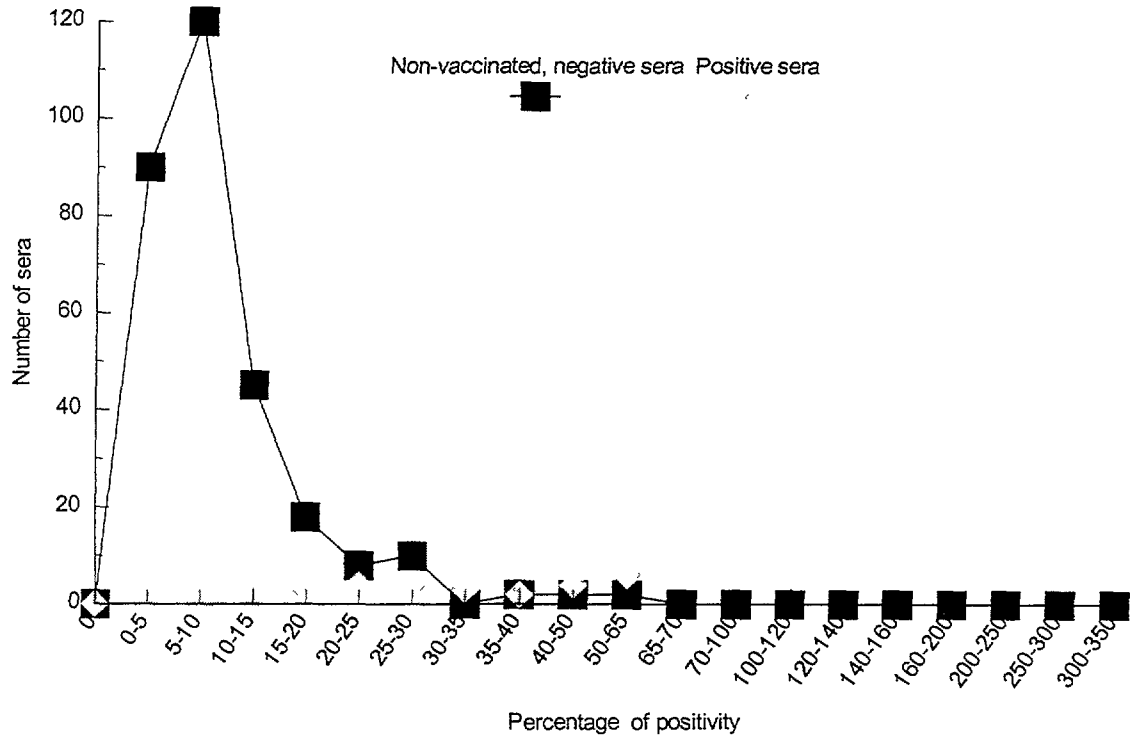

FIG. 4. Comparative distribution of I-ELISA pp-values of 286 non-vaccinated, negative and of 156 positive sera.

4. DISCUSSION

The objective of this study was to evaluate the diagnostic performance of an indirect ELISA kit produced by the Joint FAO/IAEA Division for diagnosis of bovine brucellosis, in Patagonia, Argentina.

The results presented in this communication for the conventional techniques are similar to those cited by the literature [9]. Stemshorn et al (1985), based on 1051 sera from brucellosis free herds (both vaccinated and non-vaccinated) found a specificity of 98.9% for the BPA, 99.8% for the 2ME and 100% for the RB and the CF. These results are quite similar to those presented here, however, Stemshorn et al. (1985) observed no improvement of BPA specificity when only non-vaccinated herds were considered in contrast with our results which show that BPA specificity was higher for non-vaccinated (100%) than for vaccinated (97.6%) cattle. Calfhood (3-8 months) vaccination with the standard dose of strain 19 is compulsory in Argentina. A possible explanation for this difference could be that in Patagonia, the age of vaccination for heifers (3-8 months) is not always observed and some animals may be vaccinated later than 8 months of age. This could result in production of antibodies that persist for a longer period.

The specificity of the I-ELISA was only slightly lower than the BPA, RB, 2ME, and CF tests with sera from non vaccinated animals. However for negative, vaccinated animals, I-ELISA specificity was higher than BPA. This result is encouraging as the most frequent situation in Patagonia is to ignore the vaccinal status of the cattle and therefore, a technique of high sensitivity with vaccinated animals is desirable. Nevertheless, a large sample would be required to estimate the specificity of the techniques with more precision.

In the study by Stemshorn et al (1985) only 82.0% of 167 culture positive cattle were detected by any of the serological methods used. The authors suggested that rapid spread of infection in the herds may have contributed to some of these failures, the cattle not having time to develop serological responses. In our case, the higher sensitivity demonstrated by all the techniques may be due to chronic infection with good antibody response in most infected animals. A difference in sensitivity was observed between RB and BPA. The later technique was more sensitive. However, the specificity of both

techniques was similar. The higher sensitivity of the BPA is in agreement with previous reports. The I-ELISA detected more infected cattle than any other test.

The I-ELISA resulted in specificity estimates approximating that of the other tests used when evaluated with sera from negative, non-vaccinated herds.

The I-ELISA showed a good diagnostic performance. In addition, this technique offers several major advantages, e.g. sera need not to be heat inactivated as for the CF test or treated as for the 2ME test. This technique also requires fewer complex standardization processes than the CF test The I-ELISA measures reactivity objectively which reduces reading errors and it allows greater number of samples to be processed at one time.

From the results obtained, the I-ELISA kit appeared to be a very useful tool in the diagnosis of bovine brucellosis in the Patagonia region. The technique seems to be particularly useful for this region, where little epidemiological information is available about this disease and where large numbers of sera should be tested to obtain such information. The conventional diagnostic tests for bovine brucellosis are time consuming and not sensitive and reliable enough to be used in a large scale survey. The I-ELISA kit assay evaluated in our lab seemed to be rapid, simple, sensitive and specific for detecting antibodies to *Brucella abortus*.

The I-ELISA should be further evaluated as a diagnostic tool in control programmes in the Patagonia region.

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REFERENCES

[1] FAO/OMS, Comité Mixto de expertos en brucelosis 1986, Sexto informe, OMS, Geneve, pp 149.

[2] ALTON, G.G., Recent developments in vaccination against bovine brucellosis, Aust. Vet. J. **54** (1978) 551-557.

[3] MARIÑO, O., Gallego, M.M.I., de Leon, L.S., Almansa, M.J., Comparación de técnicas serológicas en la evaluacíon de los bovinos infectados naturalmente por *Brucella abortus* In: J.F. Frank (Ed.), Networking in Brucellosis Research, The United Nations University, Tokyo (1991) 120-130.

[4] NIELSEN, K.H., WRIGHT, P.F., KELLY, W.A., CHERWONOGRODZKY, J.H., A review of enzyme immunoassay for detection of antibody to *Brucella abortus* in cattle, Vet. Immunol. Immunopath. **18** (1988) 331-347.

[5] ALTON, G.G., JONES, L.M., ANGUS, R.D., VERGER, J.M., Techniques for the Brucellosis Laboratory, INRA, Paris (1988) 190.

[6] MACMILLAN, A., Conventional Serological test, In: Nielsen, K. & Duncan, J.R. Animal brucellosis, CRC Press, Boca Raton, U.S.A. (1990) 153-197.

[7] ANGUS, R.D., BARTON, C.E., The production and evaluation of buffered plate antigen for use in a presumptive test for brucellosis, Develop. Biol. Standard. **56** (1984) 349-356.

[8] ALTON, G.G., JONES, L.M., PIETZ, D.E., Laboratory techniques in brucellosis, 2nd. edn. FAO-WHO, Geneve, Series de monografía **55** (1975).

[9] STEMSHORN, B.W., FORBES, L.B., EAGLESOME, M.D., NIELSEN, K.H., ROBERTSON, F.J., SAMAGH, B.S., A comparison of standard serological test for the diagnosis of bovine brucellosis in Canadá, Can. J. Comp. Med. **49** (1985) 391-394.

ELISA FOR THE DIAGNOSIS AND EPIDEMIOLOGY OF *BRUCELLA ABORTUS* INFECTION IN CATTLE IN CHILE

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Abstract

ELISA FOR THE DIAGNOSIS AND EPIDEMIOLOGY OF *BRUCELLA ABORTUS* INFECTION IN CATTLE IN CHILE

A serum bank of 1251 adult cows sera was prepared The sera originated from animals of three different epidemiological groups 1) 244 from infected cows, strain 19 vaccinated when calves, 2) 507 from herds free of infection but all cows were strain 19 vaccinated when calves and 3) the last group, 500 sera from cows free of infection and non-vaccinated

All the sera where tested with the routine Rose Bengal (RB) Rivanol (RIV) and Complement Fixation (CF) tests and additionally three enzyme immunoassays were performed They included two indirect ELISA both using the kit from the Joint FAO/IAEA Division, Vienna, Austria One assay used a polyclonal conjugated antibody (I-ELISAp) and the other a monoclonal conjugated antibody (I-ELISAm) The third assay was a competitive ELISA (C-ELISA) performed with sLPS, plus monoclonal antibody, M84, and goat anti-mouse antibody-HRPO Using the CFT as "gold standard" the sensitivities of all the methods were RB 87 1%, RIV 87 1%, I-ELISAp 100%, I-ELISAm 100% The calculated specificity was RB 100%, RIV 100%, I-ELISAp 96 4% and I-ELISAm 100%

In the group of infected animals (244) the following results were obtained RB 13 5%, RIV 11 9%, CF 12 7%, I-ELISAp 50 8% and I-ELISAm 22 9% Results for the non-vaccinated group were RB 0 2%, RIV 0%, CFT 0 2%, I-ELISAp 6 9% and I-ELISAm 2 9%

The C-ELISA was performed on samples from the positive group or with positivity values close to the cut-off value in the I-ELISAm In the infected group 28 out of 63 animals were detected as infected and from the non-vaccinated herds none of 15 I-ELISAm positive samples were detected as infected in the C-ELISA

1 INTRODUCTION

In Chile like in other Latin American countries brucellosis is one of the most important infectious diseases mainly due to the economic losses it causes This is the main reason why animal health authorities wish to improve the diagnostic and vaccination schemes to decrease the prevalence of the disease

For that purpose the following control plan was applied from 1968 until June 1997

a) strain 19 vaccination, using doses of 10 to 20 x 10^9 cells/ml for female calves between 3 to 8 month of age

b) diagnosis using the Rose Bengal test as screening test and Rivanol and Complement Fixation tests as the confirmatory tests

c) to remove all positive reactors from the farm for slaughtering

Unfortunately due to budget limitations this plan was not compulsory and farmers participated on a voluntary basis. As a result there are some brucellosis-free farms but brucellosis-infected farms may still exist This aspect is closely related with the efficacy of the diagnostic methods for epidemiological surveillance. It is crucial to have a highly sensitive and specific test to detect animals recently infected and furthermore differentiate between infected and vaccinated animals as the risk of infection makes it necessary to keep the strain-19 vaccinated cattle in the herd.

Other farms with a high prevalence of infection could be included in the control plan by increasing the vaccine protection using strain-19 in reduced doses for adult cows In this case it is very important to have a diagnostic method which is able to accurately differentiate between vaccinated and infected animals.

Based on the aspects mentioned above the purpose of this research was to compare the diagnostics methods included in the brucellosis control plan with two indirect ELISAs and the positive reactors in a competitive ELISA.

2. MATERIAL AND METHODS

2.1. Serum Bank

The serum bank included a total of 1251 adult cow sera divided in three categories according the farm status:

a. From positive herds, with *Brucella abortus* isolation from aborted fetuses and some level of strain 19 vaccination(N = 244).

b. From *Brucella* free cows, strain 19 vaccinated with complete dose when calves between 3 to 8 month of age and located in areas free of brucellosis(N = 507).

c. From free herds, located in non vaccinated areas(N = 500).

2.2. Diagnostic tests

All the sera were tested by the following methods:

2.2.1. The Rose Bengal test (R.B.T)

This was done according to Alton et al. [1]. The antigen was prepared at the Instituto de Microbiologia following the CEPANZO standardization procedure and officially accepted by the Chilean Department of Agriculture (S.A.G.).

2.2.2. The Rivanol (RIV) test

The antigen and Rivanol solution were produced in the Instituto de Microbiologia as mentioned above, and the test was performed according to Alton et al. [1].

2.2.3. The Complement Fixation test (CF)

The cold method was used [1] and the antigen prepared at the Institute.

2.2.4. Indirect ELISA (I-ELISA) with a polyclonal antibody

The brucellosis ELISA kit was provided by the Joint FAO/IAEA Division of the International Atomic Energy Agency (IAEA) and the procedure was in according to the manual included in the kit using a conjugated polyclonal antibody.

2.2.5. Indirect ELISA (I-ELISA) with a monoclonal antibody

Kit and methodology provided by IAEA with conjugated monoclonal antibody.

2.2.6. Competitive ELISA (C-ELISA)

The sLPS antigen, the M84 monoclonal antibody and the goat anti-mouse HRPO conjugate (Jackson Laboratories) were kindly provided by Dr. Klaus Nielsen from A.D.R.I., Canada. The methodology for this test, also provided by Dr. Nielsen was followed with some modification [2]. Briefly, NUNC polystyrene plates were coated with 100 µl per well of sLPS, 1ug/ml in carbonate buffer pH 9.6, at 20°C. overnight and frozen until used or used immediately. After 3 washes using washing buffer plus 0.05% tween 20 the control and sample sera plus the previous diluted M84 were added to each well, e.g. 95 µl diluted M84 plus 5 µl of undiluted serum. Each control was added to four wells and each sample separately only to one well. After shaking for 3 Min. the plate was incubated for 30 Min. at room temperature and after washing 3 times 100 µl of the previously titrated goat anti-mouse conjugate were added. The plate was incubated for 30 Min. at 20°C. and after 3 washes ABTS and H_2O_2 were added as in the I-ELISA method. After 10 minutes at 20°C. the plate was read at 405nm and percentage of inhibition (%I) was calculated using the conjugate control as 0 % inhibition (about OD=1.0) in the formula:

$$\%\,I = 100 - \frac{\text{OD sample}}{\text{OD conjugate control}} \times 100$$

Sera ranging from complete inhibition (no color) to 30% inhibition were considered as originating from infected cattle while less than 30% I the sera were thought to come from vaccinated or brucellosis free animals.

The modifications introduced to this technique were the antigen incubation temperature and the use of stopping solution (SDS). For the antigen coating the plates were coated and incubated at 4°C. overnight. Some of them were maintained at this temperature until further use and others were frozen at -20°C. For stopping 100 µl of SDS were added to each well after the substrate-chromogen incubation period.

3. RESULTS AND DISCUSSION

The sera tested by six methods previously mentioned and divided according to their serological status gave the results shown in Tables I , II and III.

TABLE I. ANTI-*BRUCELLA* ANTIBODIES DETECTED BY SIX DIAGNOSTIC METHODS IN SERA FROM A HERD WITH *B. ABORTUS* INFECTION AND STRAIN-19 VACCINATION

Tests	Number (+)	%	Number (-)	%
RB	33	13.5	211	86.5
RIV	29	11.9	215	88.1
CF	31	12.7	213	87.3
I-ELISAp	124	50.8	120	49.2
I-ELISAm	56	22.9	188	77.0
C-ELISA	28	44.4	35	55.5

Cut-off value for I-ELISAs was 35% of Positivity (P) and 30% of Inhibition (I) for the C-ELISA

TABLE II. ANTI-*BRUCELLA* ANTIBODIES DETECTED BY SIX DIAGNOSTIC METHODS USING SERA FROM A BRUCELLOSIS-FREE, STRAIN-19 VACCINATED HERD

Tests	N (+)	%	N (-)	%
RB	1	.2	506	99.8
RIV	0	0	507	100
CF	1	2	506	99.8
I-ELISAp	35	6.9	472	93.1
I-ELISAm	15	2.9	492	97.0
C-ELISA	0	0	15	100

Cut-off values as for Table I

TABLE III. ANTI-*BRUCELLA* ANTIBODIES DETECTED BY SIX DIAGNOSTIC METHODS, SERA FROM A BRUCELLOSIS-FREE, NON-VACCINATED HERD

Tests	N (+)	%	N (-)	%
RB	0	0	500	100
RIV	0	0	500	100
CF	0	0	500	100
I-ELISAp	8	1.6	492	98.4
	2	0.4	498	99.6
I-ELISAAm	0	0	500	100

I-ELISA cut-off as for Table I

The I-ELISA results were obtained using an FAO/IAEA defined cut-off of PP 35% being considered as positive. From an epidemiological point of view our samples could be considered as belonging to at least two categories: the first one including those samples from cattle free of infection and not vaccinated (non-exposed) and the second group made up of samples from a negative but calfhood vaccinated population. With these groups the threshold value was calculated for each non-infected group resulting from I-ELISAm and the data are presented in Table IV.

TABLE IV. THRESHOLD FOR THE NEGATIVE GROUPS

	(a)	(b)
2 x X	3%	15%
X + 3 S.D.	10%	26%
Median of 100 percentile	18%	30%

a) Brucellosis-free/not vaccinated
b) Brucellosis-free/vaccinated

If we consider, for example the threshold from (a) 10% (Mean + 3 S.D.), 148 (29.19%) serum samples will be positive from the samples belonging to a *Brucella*-free, vaccinated herd but the same threshold from (b) decreased the number of positive reactors to 27 (5.3%). Therefore, it seems advisable to calculate different thresholds for the different epidemiological states of the population.

From the I-ELISAp data it is clear that there is an increased number of reactors compared to the CF. This could be due to the detection of all four immunoglobulin isotypes instead of only IgG1 detected by the CF. In the same way, the I-ELISAm also increased the number of positive samples in comparison to CF but this could be due to the fact that I-ELISAm detects IgG1 in lower amounts than the CF. This is very important in areas where this immunoglobulin can be related to infection because earlier stages of infection can be detected. However, in herds were strain-19 vaccination is carried out this IgG1 antibody could be the remainder of the antibody due to vaccination. This may be the IgG1 antibody detected in the samples from cattle free of infection but strain 19 vaccinated, as is apparent in Table II. In this case all the sera detected in the I-ELISAm were negatives in the C-ELISA.

The C-ELISA appears to be promising because it is quite easy to perform less time consuming, repeatable and from a practical point of view can differentiate infected from vaccinated animals. The sensitivity and specificity estimates of the *B. abortus*-infected group (a) and the brucellosis free group (c) respectively using the CF test as the "gold standard" are shown in Table V.

TABLE V. SENSITIVITY AND SPECIFICITY OF FOUR DIAGNOSTIC TESTS IN THE DETECTION OF *Brucella abortus* ANTIBODIES USING THE COMPLEMENT FIXATION TEST AS THE "GOLD STANDARD" USING 244 SERA FROM *B. abortus* INFECTED AND STRAIN 19 VACCINATED HERDS AND 500 SERA FROM A BRUCELLOSIS FREE NON-VACCINATED HERD

Tests	(a) Sensitivity %	(c) Specificity %
RB	87.1	100
RIV	87.1	100
I-ELISA(p)	100	96.4
I-ELISA(m)	100	100

The correlation between the RB and Rivanol test in comparison to the CF test was in the range observed by others [3], who reported a sensitivity of 92% compared to *brucella* isolation. Dajer et al. [4] obtained 100% sensitivity and 83% specificity in a group of non vaccinated cattle with the RB test. For

infected cattle 80% and 100% sensitivity and specificity values respectively were obtained with the RIV test.

The sensitivity of both I-ELISAs were high but the specificity of I-ELISAp was relatively low. As may be seen in Table III, repeated testing of some of the positive samples in this test, when the result were close to the threshold gave some negative results, improving the specificity. Perhaps results near the cut-off value routinely should be considered for retesting to confirm that the serum was obtained from an infected cow.

The results presented above raise the question: Would it be advisable to use RB as screening test and C-ELISA a the confirmative test in those areas where strain 19 vaccination is routine ? On the other hand in areas free of vaccination perhaps the most advisable test as the confirmative one could be the I-ELISAm because of its ability to detect small amount of IgG1.

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REFERENCES

[1] ALTON, G., JONES, L., ANGUS, R., VERGER, J., Techniques for the Brucellosis Laboratory, I.N.R.A. (1988).

[2] NIELSEN, K., GALL, D., KELLY, W., HENNING, D., GARCIA, M., Enzyme Immunoassay, Application to diagnosis of bovine brucellosis, Agriculture Canada Monograph (1992) 10 - 11.

[3] HORNITZKY, M., SEARSON, J., The relationship between the isolation of *Brucella abortus* and serological status of infected non vaccinated cattle, Aust. Vet. J. **63** (1986) 172-174.

[4] DAJER, A., GUTIERREZ, E., HONHOLD, N., ZAPATA, D., VILLEGAS, S., Comparison of five serological test to detect *B. abortus* antibodies and a report on prevalence of the disease in livestock in the state of Yucatan, Mexico, IAEA TECDOC 657, Vienna Austria (1992) 131-137.

SEROLOGICAL RESPONSE TO AN INDIRECT AND A COMPETITIVE ELISA IN HEIFERS VACCINATED WITH *BRUCELLA ABORTUS* STRAIN 19

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Abstract

SEROLOGICAL RESPONSE TO AN INDIRECT AND A COMPETITIVE ELISA IN HEIFERS VACCINATED WITH *BRUCELLA ABORTUS* STRAIN 19

The different serologic techniques for bovine brucellosis diagnosis have different abilities to detect antibodies after vaccination with *Brucella abortus* strain 19 The humoral response in heifers vaccinated with *Brucella abortus* strain 19 was evaluated by using several serologic techniques In the experimental field of INTA, Pilcaniyeu, Rio Negro province, sixteen 5 months old heifers were vaccinated subcutaneously with a standard dose (2ml, containing $20x10^9$ to $10x10^9$ living organisms) of *Brucella abortus* strain 19 Sera from all the heifers were obtained on 18 occasions (one 87 days before vaccination, one immediately before vaccination and on 16 occasions after vaccination, during 488 days) and analyzed by buffered plate antigen test, rose bengal test, standard tube agglutination test, 2-mercaptoetanol test, complement fixation test, indirect ELISA, and competitive ELISA Prior vaccination, 100% of the heifers gave negative results in all the techniques used, while 100% of them gave positive reaction in the first sampling after vaccination to all the techniques, with the exception of standard tube agglutination test that showed agglutinating titters of 1/100 or higher (positive threshold) in only 71 4% of the heifers The indirect ELISA technique showed a reducing percentage of positive animals up until 316 days after vaccination, after which positive results were obtained

The competitive ELISA gave positive results in a variable number of heifers up to 253 days after vaccination when 100% of the sera were negative to this technique Buffered plate antigen test was the technique that gave positive results for a longest period, being 100% of the animals negative to this technique at 450 days after vaccination The other serological techniques assayed gave positive results during variable periods of time, intermediate between standard tube agglutination test and buffered plate antigen test Although the present results were obtained from a limited number of animals, they clearly show that the diagnosis of bovine brucellosis in animals older than 18 months, with the techniques used here is not interfered by the residual antibodies after vaccination with strain 19 at 5 months of age

1 INTRODUCTION

Bovine brucellosis is a zoonotic infectious disease produced by *Brucella abortus* [1] Although the resistance to bovine brucellosis is essentially cellular (Sutherland, 1980), the disease generates the production of antibodies that albeit do not protect against the infection, are very useful for the diagnosis of the disease These antibodies are of the type IgG_1, IgG_2 and IgM and are detectable by different serological techniques [2].

In Argentina, the serologic techniques more frequently used for diagnosis of bovine brucellosis are rose bengal test (RB), buffered plate antigen test (BPA), tube agglutination test (SAT), 2- mercapto ethanol test (2ME) and, less frequently, complement fixation test (CF). Calfhood vaccination with strain 19 (S-19) is compulsory between the age of 3 and 10 months of age (Resolución 1269/93 del Servicio Nacional de Sanidad Animal, 16-11-93).

The S-19 is a live vaccine which may produce persistent antibodies that make difficult the interpretation of diagnostic serological tests [3] The different serologic techniques for diagnosis of bovine brucellosis have different abilities to detect antibodies after vaccination with *B abortus* S-19 [3, 4]

The information available about the persistence of antibodies after vaccination with S-19 in heifers, measured with different serologic techniques (particularly with ELISA) is scanty and frequently

contradictory. The objective of this study, therefore, was to evaluate the serological response in heifers with different serological techniques after S-19 vaccination

2 MATERIAL AND METHODS

2.1. Animals

Sixteen Hereford heifers born and reared under field conditions on a farm without clinical and serological evidence of bovine brucellosis during the last 10 years in the Pilcaniyeu Department, Rio Negro Province, Patagonia, Argentina, were used.

2.2. Vaccination

All the heifers were subcutaneously vaccinated at 5 months of age with standard dose (10×10^9 to 20×10^9 living organisms per dose of 2 ml) of a commercial S-19 vaccine (Laboratorios Newton Buenos Aires, Argentina)

2.3. Serum samples

The heifers were bled 87 days prior to, and at vaccination and then monthly on 16 occasions during 488 days after vaccination.

The blood was obtained by jugular punction in glass tubes and allowed to clot during 24 hours at room temperature. Then, the serum was separated and stored at -20° C until processed

2.4. Serologic techniques

2 4 1 Conventional techniques

All the sera were analyzed by RB, BPA, 2ME, SAT and CF. These techniques were performed according to previous descriptions [5-8]. The antigens for all the tests were purchased from the Research Center on Veterinary Sciences, The National Institute of Agricultural Technology (INTA), Castelar, Argentina.

2 4 2 Indirect ELISA (I-ELISA)

This technique was performed using an FAO/IAEA ELISA. The procedures for this technique were published elsewhere [9].

2 4 3 Competitive ELISA (C-ELISA)

This technique was performed according to [10]. Briefly, 96 flat-bottom wells plates (Nunc MicroWell, cat # 2-69620. Denmark) were coated with O-polysaccharide at a concentration of 2 µg/ml After an overnight period of incubation at room temperature, the plates were washed. Diluted test and control sera (1:50 dilution) were added, to the plates immediately before that a HRP conjugate monoclonal mouse anti O-polysaccharide at a dilution of 1:2500 was added and the plates were incubated for 2 has at room temperature. Then, the plates were washed again and developed using an ABTS/H_2O_2/citrate buffer substrate solution. The reaction was stopped with a 4% sodium dodecyl sulphate solution and the plates were read in a Multiskan Plus ELISA reader using a 405nm filter

Controls consisted of a buffer control, a negative serum, a vaccinated control on a strong positive reactor. Every control was run in 4 replicates while every test sera was analyzed in duplicate.

The degree of competition was calculated as a proportion of the conjugate control and expressed as percentage inhibition. Values greater than 30% were considered to be positive, while values less than 30% were considered negative. The degree of inhibition was calculated as:

$$\% \text{ inhibition} = 100 - \frac{\text{X OD test serum}}{\text{X OD conjugate control}} \times 100$$

The O-polysaccharide and the anti-O-polysaccharide mouse monoclonal conjugate were provided by the Animal Disease Research Institute, Nepean, Canada.

3. RESULTS

The number and percentage of positive animals to every test before and after vaccination are shown in Table I and Figures 1 to 2. In the two samplings prior vaccination (87 and 0 days before vaccination), 100% of the heifers were negative to all the techniques, while 28 days after vaccination, all the animals were positive reactors to BPA, RB, 2ME, CF, I-ELISA and C-ELISA. The SAT showed agglutinating titers of 1/100 (positive threshold) or higher only in the 71.4% of the animals, wile 28.6% of the heifers showed diverse titers lower than 1/100. This technique gave agglutinating titers at intervals, though titers of 1/100 or higher were observed only until the 148 days after vaccination.

The test that gave positive results for a longest period was BPA that at 419 days after vaccination still detected 6.2% of the animals as positive reactors. At 450 days after vaccination 100% of the heifers were negative to BPA. On the other hand the test that gave positive results for the shortest period was the SAT, which at 148 days after vaccination detected only 6.2 of the heifers as positive reactors, and in the following sampling (190 days post-vaccination) gave negative results in 100% of animals. The 2ME and CF gave 100% of negative results at 235 days after vaccination.

With RB, 100% of the animals were negative at 214 days after vaccination. The I-ELISA technique showed a decreasing percentage of positive animals, with 100% of heifers being negative at 316 days after vaccination. The C-ELISA gave also a decreasing percentage of positive animals, being 100% of heifers negative to this technique at 253 days after vaccination.

TABLE I. NUMBERS AND PERCENTAGES OF POSITIVE ANIMALS TO SEVERAL SEROLOGICAL TESTS BEFORE AND AFTER VACCINATION WITH *BRUCELLA ABORTUS* S-19

Positive to														
	BPA		RB		SAT		2ME		CFT		I-ELISA		C-ELISA	
(*)	+/T	%	+/T	%	+/T	%	+/T	%	+/T	%	+/T	%	+/T	%
(**)		0	0/13	0	0/13	0	0/13	0	0/13	0	0/13	0	0/14	0
0	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/15	0	0/14	0
28	14/14	100	14/14	100	10/14	100	14/14	100	9/9	100	14/14	100	14/15	93
62	16/16	100	8/16	50	3/16	19	13/16	81	3/16	25	13/15	87	5/16	31
90	16/16	100	3/16	19	2/16	12	6/16	37	3/16	19	8/14	57	2/17	12
118	14/15	93	1/15	7	2/15	13	1/15	7	1/14	7	4/15	27	1/14	7
148	14/16	87	1/16	6	1/16	6	1/16	6	1/16	6	2/15	13	1/16	6
190	11/16	69	1/16	6	0/16	0	1/16	6	1/16	6	2/15	13	1/16	6
214	5/16	31	0/16	0	0/16	0	1/16	6	1/16	6	2/16	12	1/15	6
253	4/15	27	0/15	0	0/15	0	0/15	0	0/15	0	1/15	7	0/15	0
289	4/15	27	0/15	0	0/15	0	0/15	0	0/15	0	1/15	7	0/9	0
316	4/16	25	0/16	0	0/16	0	0/16	0	0/16	0	0/15	0	0/16	0
328	2/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/11	0
363	1/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/13	0	0/16	0
393	1/15	0	0/15	0	0/15	0	0/15	0	0/15	0	0/14	0	0/8	0
419	1/16	6	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0
450	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/9	0
488	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/16	0	0/15	0

(*) Days after vaccination
(**) Sampling 87 days after vaccination
+/T number of positive reactors/number of animals tested
% percentage of positive animals

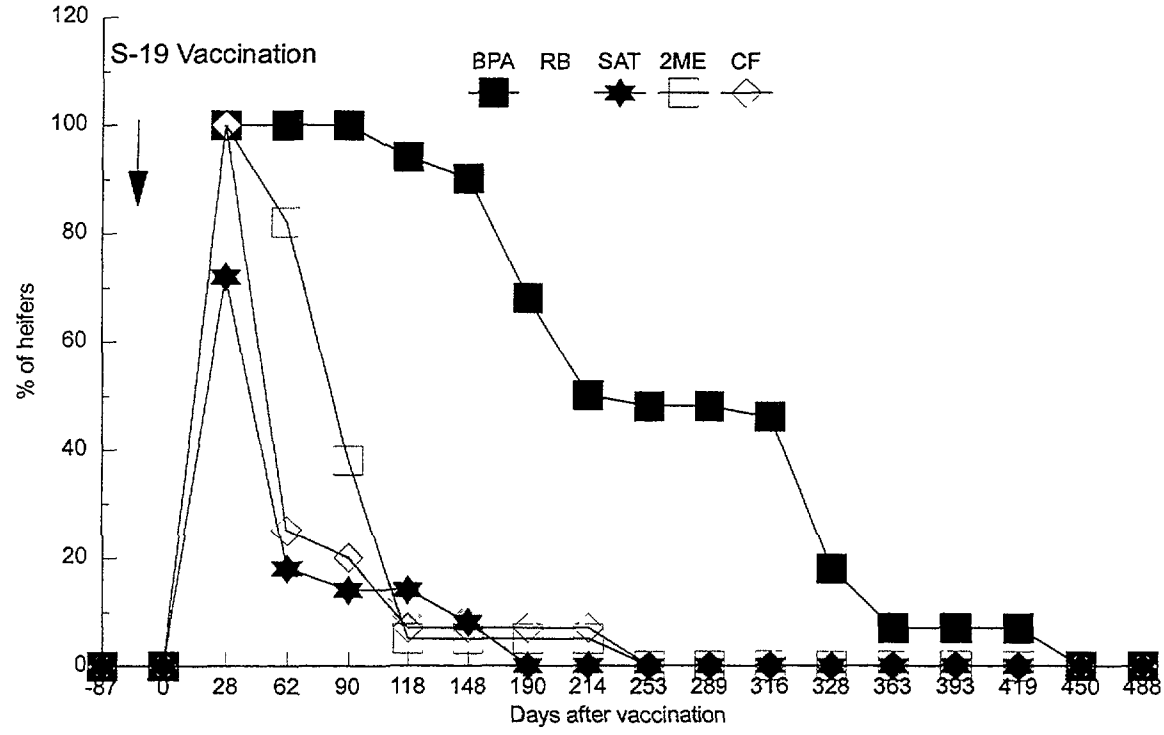

FIG. 1. Percentage of heifers positive to different conventional tests after S-19 vaccination.

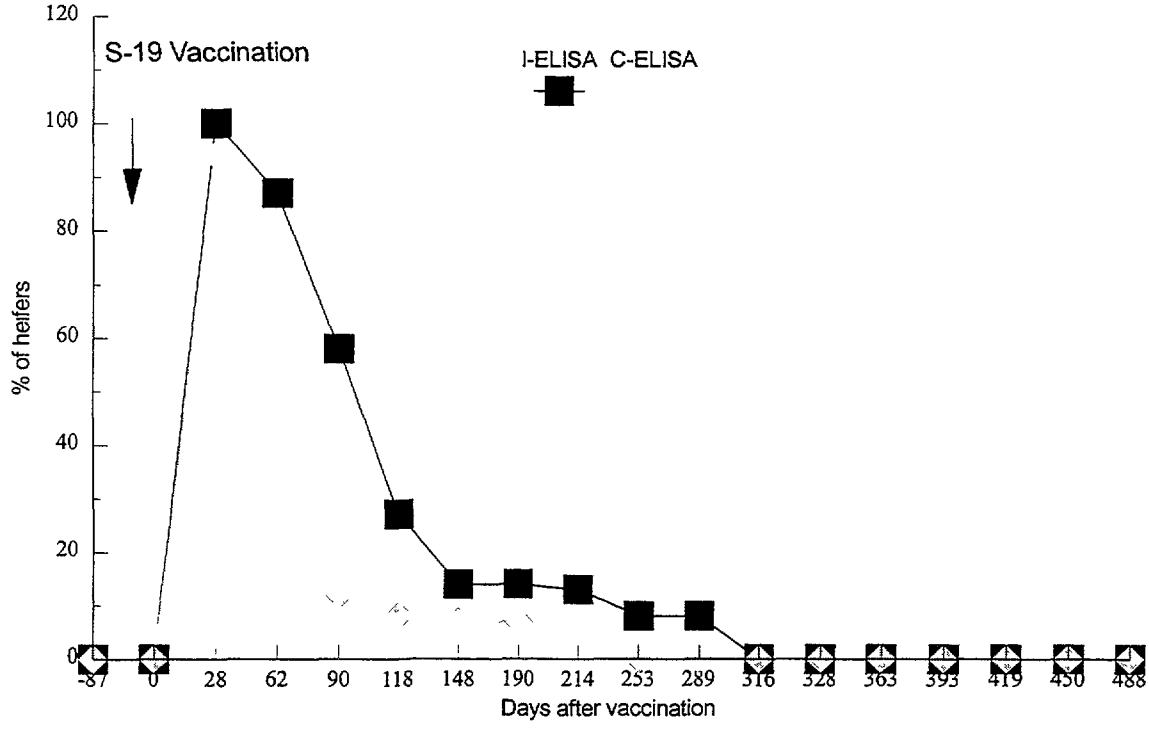

FIG. 2. Percentage of heifers positive to I-ELISA and C-ELISA after S-19 vaccination.

4. DISCUSSION

We evaluated the humoral immune response to eight serologic techniques of 16 heifers vaccinated with standard dose of B. abortus S-19. The S-19 is a live, attenuated vaccine that protects cattle against infection with B. abortus. However, since the 1950s it is known that vaccination of cattle with S-19 produces persistent antibodies that make difficult the interpretation of diagnostic serological test [3]. It has also been shown proved that the younger the heifers are vaccinated, the less persistent are the antibodies [4, 10, 11, 17].

Sutherland [12], vaccinated 24 heifers between 3 and 6 months with standard dose of strain 19 and evaluated the sera at 2 and 3 months after vaccination by RB, SAT, CF and I-ELISA. The RB detected as positive reactors 100% of the animals until 3 months after vaccination, which differs from the results showed here that indicated that at 3 months after vaccination only 19% of the animals were positive to RB. In the study referred to, the positivity to SAT descended from 100% to 50% between the second and third month, and the CF descended from 87% to 37% in the same period, while the I-ELISA remained positive in 87% for the two both months. In the present study, excluding the results of I-ELISA, which agreed with those of Sutherland (1984/85) at 2 months post-vaccination, all the other values were lower than those found by that author.

Cunningham and O'Reilly [4] vaccinated heifers from 3 to 6 months of age with strain 19. Sera were analyzed weekly by SAT for 8 months. They obtained titers lower than 1/100 prior to 2 months post-vaccination which indicates that the decline in antibody of all the animals occurred earlier than in our study. Casaro [13] vaccinated heifers between 5 and 7 months of age with S-19 and measured antibodies until 362 days post-vaccination by SAT and RB. He found that 100% of the animals were negative at 105 days with SAT and at 158 days with RB. These results differ moderately from those obtained in our assay (100% negative to SAT and to RB at 190 days and at 214 days after vaccination, respectively). Nagy et al. [3] reported that 100% of the animals were negative to SAT at 180 days after vaccination in heifers vaccinated between 4 and 8 months of age with strain 19. Those results agree with ours.

A possible explanation for the difference in results between different authors could be the use of different vaccines, doses and age in the immunization of the animal and the use of different antigens and other reagents. Finally, a different immunologic status of the animals can not be ruled out.

Traditionally, the CF has been used as a definitive test of high sensitivity and specificity for reactors in agglutination tests [12, 14]. In the present study, the CF did not detect any positive reactors at 253 days after vaccination (approximately 13.5 months of age) which provides a broad margin of security in the serological testing at 18 months of age. However, in spite of its great sensitivity and specificity, this is a cumbersome, time consuming technique which is also difficult to standardize.

The ELISA, on the other hand, is free from all the problems of the agglutination and CF technique. This technique is highly sensitive and specific and detects all the isotypes of IgG and IgM present in serum [15]. In addition, it is rapid and requires a minimum amount of serum.

In our study, the I-ELISA detected 100% of the animals positive at 28 days after vaccination, decreasing this percentage until the 148 days after vaccination when only 13.3% of the animals were positive, 100 % of the heifers being negative at 316 days after vaccination (approximately 15.5 month of age). The C-ELISA here used was developed to differentiate vaccinated from infected cattle [10]. In our study, we found positive animals to C-ELISA until 214 days after vaccination. Nielsen et al [16], found that antibodies produced to B. abortus strain 19 cannot compete in the C-ELISA, with two major exceptions: animals persistently infected with B. abortus, and occasionally, antibody at the peak of the primary anti-strain 19 antibody response 4-8 weeks post vaccination, the latter competition being weak at best. In our case, persistent infection with S-19 can be ruled out as at 450 days after vaccination, all the heifers became negative in all the techniques. The difference in duration of antibodies found in our study with that stated by Nielsen et al [16] could, at least partially, be explained by the different doses of S-19 used in different countries. The C-ELISA improved the result, of the I-ELISA as at 253 days after vaccination it did not detect any positive reactor. These results make the I-ELISA and C-ELISA useful for diagnosis of bovine brucellosis, even according to the new Argentine regulations that establish compulsory serological testing from the 18 month of age (Resolución 1269/93 del Servicio Nacional de Sanidad Animal, 16-11-93).

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REFERENCES

[1] BLOOD, D.C., RADOSTITS, O.M., HENDERSON, J.A., Medicina Veterinaria, 6th. ed., Nueva Editorial Interamericana, Mexico, (1986) 1441 pp..

[2] BLASCO, J.M., Estado actual de la brucelosis en España. Tratado de veterinaria práctica, Bovis, Serie nacional de monografías sobre la patología y producción del ganado vacuno para la formación continuada del pre y posgraduado veterinario, Madrid, Spain **9** (1986) 19-25.

[3] NAGY, L.K., HIGNETT, P.G., IRONSIDE, C.J.T., Bovine brucellosis: a study of an adult vaccinated, *Brucella* infected herd, Vet. Rec. **81** (1967) 140-144.

[4] CUNNINGHAM, B., O'REILLY, D.J., *Brucella abortus* vaccines, Agglutination in blood serum and milk following vaccination of cattle of various ages with live S-19 and killed 45/20adjuvant *B. abortus* vaccine, Vet. Rec. **82** (1968) 678-689.

[5] ANGUS, R.D., BARTON, C.E., The production and evaluation of buffered plate antigen for use in a presumptive test for brucellosis, Develop. Biol. Standard. **56** (1984) 349-356.

[6] ALTON, G.G., JONES, L.M., ANGUS, R.D., VERGER, J.M., Techniques for the brucellosis laboratory, INRA, Paris (1988) 190 pp.

[7] GONZALEZ TOME, J.S., VILLA, L.J., DEL PALACIO, E., GREGORET, R., El test de Angus y Barton (BPA) como prueba tamiz en el diagnóstico de la brucelosis bovina, Rev. Med. Vet. (Buenos Aires) **70** (1989) 34-36.

[8] MACMILLAN, A., Conventional Serological test, In: Nielsen, K. & Duncan, J.R. Animal brucellosis, CRC Press, Boca Raton, U.S.A. (1990) 153-197.

[9] UZAL, F.A., CARRASCO, E.A., ECHAIDE, S., ROBLES, C.A., Application of an indirect ELISA for the diagnosis of bovine brucellosis in Patagonia, Argentina, (to be published).

[10] NIELSEN, K., CHERWONOGRODZKY, J.W., DUNCAN, R.J., BUNDLE, D.R., Enzyme-linked inmunosorbent assay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19, Am.J. Vet. Res. **50** (1989) 5-9.

[11]]SZYFRES, B., El diagnóstico de la brucelosis bovina en el contexto de un programa de lucha, IICA - SENASA - Ministerio de Economía, Secretaría de Agricultura y Ganadería, Buenos Aires, (Boletín Técnico 2) (1983).

[12] SUTHERLAND, S.S., Immunology of bovine brucellosis, Vet. Rec. **50** (1980) 359-368.

[13] CASARO, A., Títulos de vacunación residual en animales vacunados con cepa 19, A.A.P.A. Santa Rosa, La Pampa, Argentina (1976).

[14] DIAZ, R., BLASCO, J.M., Diagnóstico inmunológico, Tratado de veterinaria práctica, Bovis, Serie nacional de nonografía sobre la patología y producción del ganado vacuno para la formación continuada del pre y posgraduado veterinario, Madrid, Spain **9** (1986) 55-69.

[15] SUTHERLAND, S.S., Evaluation of the enzyme linked immunosorbent assay in the detection of cattle infected with *Brucella abortus,* Vet. Microbiol. **10** (1984/85) 23-32.

[16] NIELSEN, K., GALL, D., KELLY, W., HENNING, D., GARCIA, M., Enzyme Immunoassay. Application to diagnosis of bovine brucellosis, Agriculture Canada, (1992) 203 pp.

[17] NICOLETTI, P., Vaccination, bovine brucellosis, In: Networking in brucellosis research, Ed. J.F. Frank, The United Nations University (1991) 85-92.

USE OF AN INDIRECT ELISA FOR *BRUCELLA ABORTUS* DIAGNOSIS IN CUBA

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Abstract

USE OF AN INDIRECT ELISA FOR *BRUCELLA ABORTUS* DIAGNOSIS IN CUBA

Introducing immunoassays in *Brucella* diagnosis requires a comparative study with reference techniques such as the complemente fixation reaction (CFR) Sensitivity and relative specificity studies allowed us to observe the behaviour of this immunoassay, using samples from free of disease, free by vaccination and affected areas Sensitivity results for a cut-off point of 40PP and a confidence interval of 95% ranged from 94 8 to 99 5% and the specificity between 94 1 and 97 5% For free of disease areas a cut-off point of 22PP was calculated that reached a 99% specificity This immunoassay for the detection of antibodies against *Brucella abortus* must be used with two different cut-off points, depending on the epidemiologic conditions of the country, with CFR in affected or vaccinated areas as a confirmative method

1. INTRODUCTION

Serological diagnosis of brucellosis is used in many countries as the criteria for control and eradication of this disease. Several conventional techniques are used for this, and although each one characteristically detects different antibody isotypes, to determine an animal seropositive to brucellosis it is necessary to use techniques such as Bengal Rose (BR), agglutination in buffered plate (ABP) or Slow tube agglutination (SAT). The 2-mercaptoethanol (2-Me) and Complement fixation test (CFT) are used as confirmatory techniques. [1].

The introduction of indirect immunoenzymatic techniques (ELISA) in serological diagnosis has allowed the achievement of higher sensitivity and specificity levels than most commonly used conventional techniques [2,3]. These assays have already been approved by the Office Internationale des Epizootics (OIE) [4]. Nevertheless a correct diagnosis requires adjusting the technique according to the epidemiological situations existent in each region [3,5,6,7]. These studies require negative samples from disease free animals and positive samples from animals with clinical, serological or epidemiological evidences of the disease [6,8].

In Cuba, for more than 30 years, a programme for the control and eradication of brucellosis in cattle has been carried out by slaughter of all serologically positive animals. A decrease of 0.045% in the incidence of the disease has been achieved. Presently to assure an efficient diagnosis in the affected areas, as well as an adequate monitoring of disease-free areas it is necessary to use a highly sensitive and specific diagnostic method.

This study details the results obtained with an indirect ELISA provided by the Joint FAO/IAEA Division of the International Atomic Energy Agency (IAEA), for the serological diagnosis of brucellosis in sera of disease-free, vaccinated disease-free and affected animals, using the complement fixation test as a reference technique.

2 MATERIALS AND METHODS

2.1. Sera

2 1 1. Brucellosis-free area

Sera samples from 2125 non-vaccinated animals from areas free of brucellosis were collected. The studied herds had not presented clinical, bacteriological or serological evidences of disease during the last 20 years.

2.1.2. Vaccinated area

Sera samples from 1313 animals vaccinated with *Brucella abortus* strain 19, from areas without clinical, bacteriological or serological evidences of the disease for the last 2 years.

2.1.3. Brucellosis affected area

Sera samples from 1278 animals from brucellosis affected areas. These herds were vaccinated with strain 19 and animals with clinical, bacteriological and serological evidences of brucellosis infection have been found. In these areas the incidence is low thanks to the adequate control and eradication program carried out in the country.

2.2. Serological test

2.2.1. Complement Fixation Test

The antigen used in the CFT was produced in Cuba by Laboratorios Biológicos Farmaceúticos (LABIOFAM, Biological Pharmaceutical Laboratories). All sera were evaluated by 50% hemolysis CFT according to the protocol of Alton and co-workers (1988).

2.2.2. Indirect ELISA

This ELISA was carried out using an indirect ELISA kit for the diagnosis of brucellosis, following the instructionsdescribed elsewhere [10]. In NUNC polystyrene microplates 100 µl of 1 µ g/ml concentrated smooth lipopolysaccharide (SLPS) in 0.05M pH 9.6 carbonate buffer were added, and incubated overnight at 4°C. The plates were washed three times in PBS-Tween-20 and samples or controls diluted 1:200 were added and incubated for 1 hour at 37°C. All samples were tested in duplicate, while the control sera were tested four times. Controls: strongly positive, weakly positive, negative and a conjugate control, where no serum was added. After another washing, a peroxidase conjugated anti bovine IgG monoclonal antibody was added. While using the kit, conjugate dilutions varied; they were adjusted to an O.D. values of 1.000 The incubation time was 1 hour at 37°C. Finally, after another washing, 100 µl of 3% hydrogen peroxide plus 1 mM [2,2 azinobis (3-ethyl-benzthiazoline sulfonic acid)] ABTS dissolved in 0.05M citric acid/sodium citrate buffer (PH-4,5) were added and incubated 10 min. at 37°C. The reaction was stopped by adding 100 µl of 4% solution of sodium dodecyl sulphate and the plate was read at 405 nm using the software supplied with the kit.

All the data were stored in Microsoft Excel and the cut-off point calculations, as well as the analysis of the samples were carried out using EPI INFO-6.0 and the Statistical Package Program.

The cut-off point for areas free of disease was calculated organizing all the results in increasing order and dividing them in one hundred equal percentiles. The average of the 99 percentile for each population was calculated and this value used as the cut-off point for areas free of disease. For the affected areas the Receiver Operating Characteristics Analysis (ROC-analysis) was used [2,7]. To select the cut-off value, the point where the specificity of the assay assures a minimum of false positive samples and a higher positive predictive value without affecting the sensitivity of the technique was determined.

3. RESULTS AND DISCUSSION

Of the 2125 samples tested in areas free of disease, 37 reacted positively when the cut-off point was established at a 22 positivity percent (PP) reaching a specificity of 99 %. This sera were reevaluated and negative results obtained. In the samples from vaccinated disease free areas only 25 sera were positive at a 44 PP cut-off point with specificity ranging between 97.3 and 98.8 %. Among the sera from affected areas the results for 58 samples were not in accordance with the CFT. In this population sensitivity values reached 99 to 99.5 % with a positive predictive value between 66 and 78 % while specificity reached 94 to 96.3 % and the negative predictive value was 99 - 100 %. All these data are shown in Table I.

TABLE I. SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE AND NEGATIVE PREDICTIVE VALUES OF INDIRECT ELISA USING CFT AS A REFERENCE FOR A 95% CONFIDENCE INTERVAL

Area	Sensitivity	PPV	Specificity	NPV
Free areas 35PP	-	-	99 5%	-
Free areas 22PP	-	-	99%	-
Vaccinated areas 35PP	-	-	96.5-98 2%	-
Vaccinated areas 44PP	-	-	97.3-98 8%	-
Affected areas 35PP	95-99.8%	59-71%	92-94%	99-100%
Affected areas 44PP	94-99 5%	66-78%	94-96 3%	99-100%

PPV Positive predictive values
NPV Negative predictive values
PP Percent positivity

Figure 1 shows the behaviour of the sensitivity and specificity using different cut-off points in animals from affected areas. It can be observed that when the specificity of the assay increases, the sensitivity decreases [2,3]. The 44 PP cut-off point results in a specificity of 95 2 % with a sensitivity of 98 %

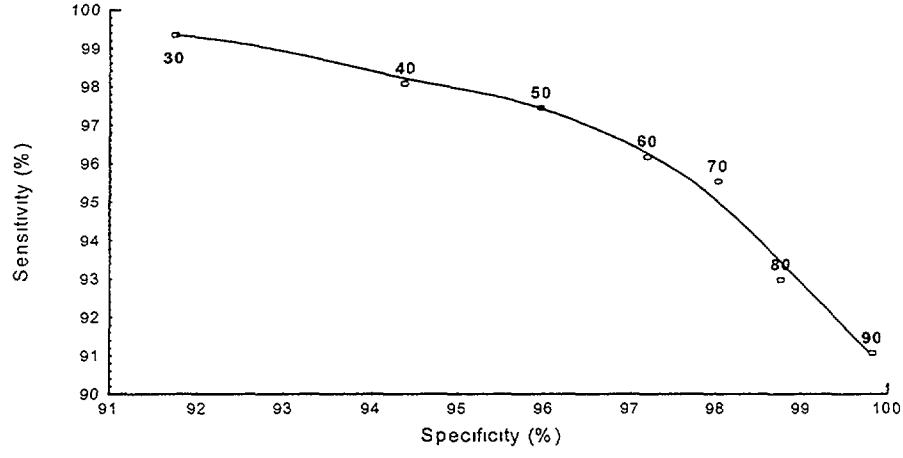


FIG 1 Analysis of the sensitivity and specificity using different cut-off points in animal from affected areas

The frequency distribution of the samples from areas free of disease (Figure 2) shows that the maximum dispersion of samples is found between 0 and 20 PP, which proves that there are no specific antibodies against *brucella sp.* Samples over 22 PP in a first analysis showed non-specific reactions and resulted negative upon reevaluation, as expected This indicates that samples in these areas showing unexpected results should be retested.

In vaccinated free-of-disease and affected areas the frequency distribution for negative samples (Figures 3 and 4) showed dispersion in a higher PP range than the one observed for disease free areas, because the levels of antibodies in healthy animals in these areas are higher due to circulation of the vaccine strain [2,5]. In the affected areas non-specific reactions were found in 58 samples, since the assay can not distinguish vaccinated from infected animals and because beyond the selected cut-off point (44 PP) there are samples with positive and negative results for the CFT For better diagnostic assurance Jacobson (1990) recommends confirmation of the ELISA positive results by a reference technique (CFT) when the disease prevalence is low [6]

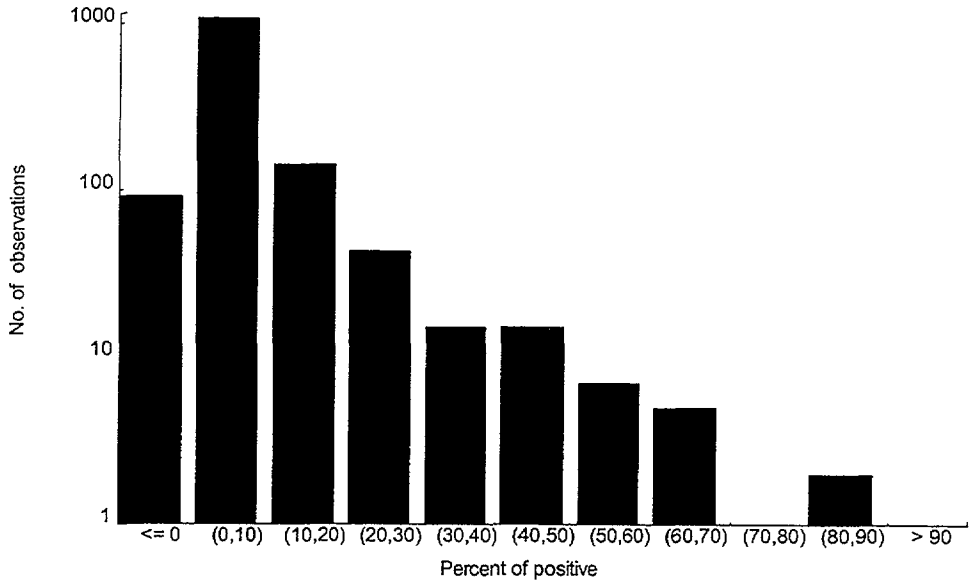


FIG. 2. Frequency distribution of the samples from areas free of disease.

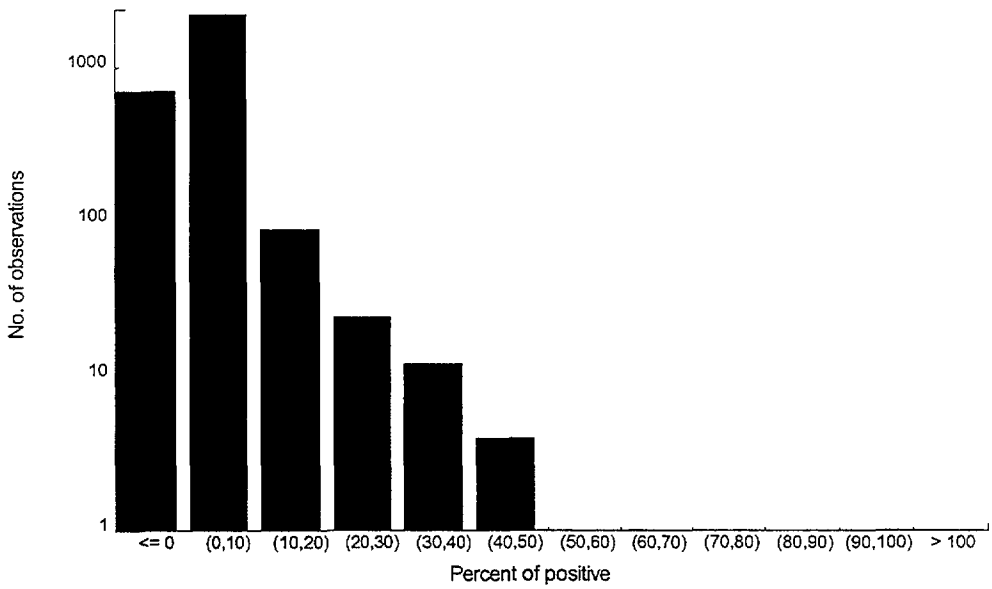


FIG. 3. Frequency distribution of the samples from vaccinated free of disease areas.

As can be appreciated in Figure 4, for a cut-off point of 44 PP the positive predictive value of this immunoassay is affected by false positive samples. Jacobson [7] establishes the need for increasing the specificity of the assay in these cases, nevertheless in our conditions this is not posible because we would miss some of the true positives. In Cuba, thanks to the control and eradication program, there is a low incidence of this disease. At present it is considered preferable to slaughter an animal suspicious of being positive than missing a seropositive animal. The use of two cut-off points depending on the epidemiological situation found in different area has been established before by Jacobson [7] and Uzal and co-workers [11]. In Cuba this allows using same assay for the serological study of any herd by just changing the cut-off value. Due to the disease control in the country these areas are well defined geographically, which makes laboratory diagnosis easier.

An analysis of the sample distribution in the three evaluated categories is shown in Figure 5. It can be appreciated that for negative samples from the affected areas the maximum PP values are higher than those obtained in vaccinated areas and both, taken separately, are higher than those found in free of disease areas. These results are justified by the circulation of the vaccine strain in those areas, that maintains detectable antibody titers [4]. It is also observed that most of the negative samples from these populations are under the 44 PP cut-off point for affected and free by vaccination areas and 22 PP for disease free areas.

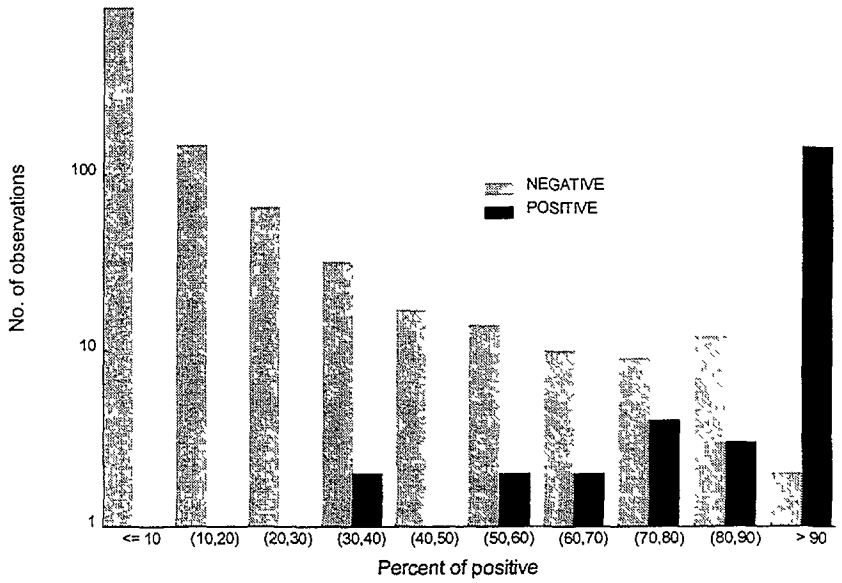
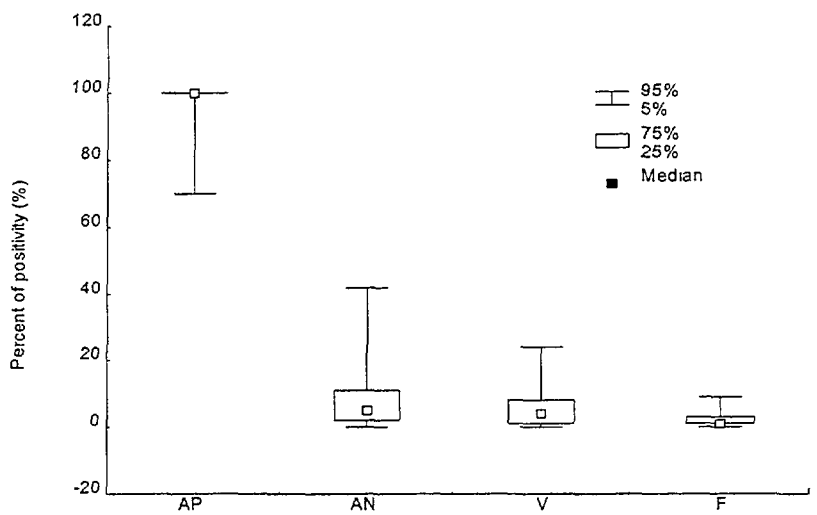


FIG. 4. Frequency distribution of samples from affected areas.



AP Positive animals from affected areas
AN Negative animals from affected areas
V Animals from vaccinated areas
F Animals from free areas

FIG. 5. Analysis of the samples dispersion in the three evaluated categories.

4. CONCLUSIONS

In the indirect ELISA, changing the cut-off point to 22 PP for free-of-disease areas does not affect the specificity of the technique and allows a much more strict control for monitoring these areas, where antibody titres are low.

Using a 44 PP cut-off point instead of the 35 PP recommended by the IAEA gives a better assay specificity in affected areas, as well as a better positive predictive value. Although this assay does not distinguish vaccinated from infected animals, it can also be used for monitoring free by vaccination areas and for diagnosing affected areas where animals were vaccinated more than one year before. For that purpose an appropriate cut-off has to be determined.

Due to its high sensitivity CFT can be used as a confirmatory diagnostic test to eliminate false positive samples.

ELISA technique is a fast diagnostic method that enables a large number of samples to be tested at a relatively low cost.

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REFERENCES

[1] Norma Ramal 586, Diagnóstico Veterinario, Brucelosis, Método de ensayo, Ministerio de la Agricultura, República de Cuba.

[2] NIELSEN, K.H., GALL, D E., KELLY, W.A., VIGLIOCCO, A M , HENNING, M.D., GARCIA, M.M ,Immunoassay development, Application to Enzyme Immunoassay for the Diagnosis of Brucellosis, Copyright, Agriculture and Agri-Food Canada (1996) ISBN 0-662-24163-0.

[3] NIELSEN, K.H., KELLY, L., GALL, D., BALSEVICIUS, S.J., BOSSE, NICOLETTI, P , KELLY, W., Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis, Preventive Veterinary Medicine **26** (1996) 17-32.

[4] FAO/OMS, Comité Mixto de expertos en brucelosis, Sexto informe, OMS, Ginebra, Suiza, (1986) 149 pp.

[5] ABALOS P., IBARRA, L., PINOCHET, L., NAVIA, F., BOISIER, X., Residual anti-*Brucella abortus* strain 19 antibodies detected in adult cattle by two indirect-ELISA tests, Veterinary Record **138** (1996), 140.

[6] JACOBSON, R H. How well do serodiagnostic tests predict the infection or disease status of an animal? Regional network for Latin America on animal disease diagnosis using immunoassay and labelled DNA probe techniques, Proceeding of a Final Research Co-ordination meeting of a FAO/IAEA/SIDA Co-ordinated Research Program organised by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Heredia, Costa Rica, 22-26 October 1990, IAEA-TEC DOC-657.

[7] JACOBSON, R.H., Factors in selecting serum samples for use in determining the positive/negative threshold (cut-off) in ELISA, IAEA TECDOC (in preparation).

[8] WRIGTH, P. F., NILSSON, E , VAN ROOIJ, E.M.A., LELENTA, M., JEGGO M. H., Rev Sci. Tech. Off. Epiz. **12** (2) (1993) 435-450.

[9] ALTON, G.G., JONES, L.M., ANGUS;, R.D., VERGER., J.M., Techniques for the brucellosis laboratory, INRA, Paris, (1988).

[10] FAO/IAEA, Brucellosis ELISA kit manual, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Austria.

[11] UZAL, F.A., ABALOS, P., PADILLA POESTER, F., ROJAS, X., DAJER, A., SILVA, M , NIELSEN, K.H., WRIGTH, P.F., Evaluation of an indirect ELISA kit for the diagnosis of bovine brucellosis in Latin America, Arch. Med. Vet. XXVII, No Extraordinario, (1995) 59-63.

USE OF NON-CONVENTIONAL TESTS FOR THE DIAGNOSIS OF BRUCELLOSIS

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Abstract

USE OF NON-CONVENTIONAL TESTS FOR THE DIAGNOSIS OF BRUCELLOSIS

A number of non-conventional tests to complement traditional diagnostic methods for Brucellosis were established and assessed in order to verify whether the adoption of a panel of methods combined to alternative sampling strategies would increase the possibility of detecting low levels of *Brucella* spp antibodies or microrganisms The diagnostic performance of each test was established by means of reference standards and compared with conventional screening and confirmatory tests under field conditions Non-conventional tests assessed for detecting *Brucella* organisms included an agglutination method using a monoclonal antibody for an early and specific detection of *Brucella* spp from colonies, polymerase chain reaction (PCR) for detection of *Brucella* spp in raw milk Methods for detecting *Brucella* spp antibodies included an ELISA test applied to cow milk, evaluation of milk-ELISA test through repeated sampling and ELISA in milk for the diagnosis of ovine brucellosis The adopted strategy of repeated milk testing in dairy cows using ELISA increased the chance of identification of positive animals

1 INTRODUCTION

Following the eradication strategy for brucellosis adopted by the EU countries, prevalences of infection in cattle, sheep and goat populations are expected to decrease to very low levels. The percentage of false positive results of any test increases considerably with the decrease in the prevalence of the disease [1]. Likewise, when prevalence of brucellosis decreases, the predictive value of a positive test result decreases as well. The acceptable prevalence of infection established by the EU for a country to be considered officially free of brucellosis is 0.2%. Consequently, a test having a specificity of ≥99.8% should be adopted. Unfortunately, most of the tests commonly employed for the surveillance of brucellosis do not reach such degree of reliability [2,3,4]. A possible strategy to overcome the analytical limitation of individual tests could be the adoption of a panel of complementary tests and a multiple testing scheme at herd/flock level.

In this report results on the establishment and validation of a number of non-conventional tests for brucellosis are shown and the advantages of a multiple testing strategy applied to a dairy herd with known prevalence of infection are evaluated.

2. MATERIAL AND METHODS

2.1. Direct tests

2 1 1 A rapid and direct method for identification of Brucella spp. using monoclonal antibodies

A rapid and simple method to perform colony agglutination assay using a monoclonal antibody was developed for a specific and early identification of *Brucella species* from colonies, thus reducing the time usually required for identification of isolates from pathologic materials [5]. Immunization of BALB c mice with inactivated whole *Brucella* permitted the derivation of a collection of hybridomas among which one IgM MAb (H11B) could be isolated.

The *brucella* strains examined, obtained from CVL, Weybridge, U.K. are listed in Table I. The Mab proved capable of producing a fast and strong agglutination with a number of *Brucella* smooth biovars tested and expressing A and M epitopes at different ratios, while it failed to recognize *B ovis* naturally occurring under rough phase. The Mab was tested with a number of other organisms likely to share common epitopes with *Brucella spp.* Strains of *Escherichia coli, Yersinia enterocolitica, Salmonella urbana, Salmonella enteritidis, Salmonella thyphimurium, Campylobacter foetus, Vibrio chlerae suis, Proteus mirabilis* from our collection of domestic isolates, were tested for

cross-reactivity with the Mab as shown in Table II. Only *Yersinia enterocolitica* evoked a weak agglutination within 60 Min..

The agglutination method developed in this study was validated under field conditions using 8 plates from pathologic materials and organs and containing *Brucella spp.* in mixed or contaminated cultures. Colonies from all plates produced strong agglutination within 30 Min. with Mab H11B regardless of the purity of the cultures tested.

TABLE I. PANEL OF *BRUCELLA* STRAINS TESTED FOR AGGLUTINATION WITH MAB H11B

Type of Bateria	Biovar	Strain Form	Colony	Agglutination
B. abortus	1	99W	S	+++(10 Min.)
B. abortus	1	544	S	+++(10 Min.)
B. abortus	1	S19	S	+++(10 Min.)
B. abortus	2	86/8/59 S	S	+++(10 Min.)
B. abortus	3	Tulya	S	+++(10 Min.)
B. abortus	6	B70	S	+++(10 Min.)
B. mellitensis	1	16M	S	+++(10 Min.)
B. mellitensis	1	Rev1	S	+++(10 Min.)
B. mellitensis	2	63/9	S	+++(10 Min.)
B. mellitensis		Ether	S	+++(10 Min.)
B.suis	1	1330	S	+++(10 Min.)
B. ovis		63/290	R	-

TABLE II. PANEL OF OTHER BACTERIA TESTED FOR CROSS-REACTION WITH MAB H11B

Type of Bacteria	Strain	Agglutination
Y. enterocolitica	09(C1)	+ (60 Min.)
E. coli	0157-H7	-
Salmonella urbana		-
Salmonella enteritidis (pt 4)		-
Salmonella typhymurium		-
Campylobacter jejuni		-
Campylobacter foetus		-
Vibrio cholerae		-
Proteus mirabilis		-

Because of its unique reactivity in agglutination assay, the developed Mab was found to be useful for the rapid and preliminary detection of *Brucella spp.* in culture plates and it was adopted within the scheme of bacteriological tests for Brucellosis in our laboratories.

2.1.2. Detection of Brucella spp. in raw milk by PCR

Milk is the foremost source of *Brucella* infection in humans and its testing by bacteriological culture is made difficult by the low analytical test sensitivity as well as by the erratic shedding of the organism. Therefore, there is a need for the development of more accurate and sensitive tests capable of detecting lower number of *Brucella* organisms in infected milk. Molecular reagents like a DNA probe, made from the whole genomic DNA, which are able to detect *B. abortus* genomes at 10/5 organisms/g of spiked tissue and 10/7 organisms/g of tissue in experimentally infected mice have been described [6,7,8].

In this study a number of PCR systems were tested on milk [9]. All sets of primers used were specific but showed different analytical sensitivities ranging from 20 to 7000 microrganisms. Some of the primers proved unable to detect *brucella* organisms in milk. No system of primers alone could detect and differentiate all *Brucella spp.* and biovars used in the study. Only one system using the primers B4/B5 (Baily) showed enough analytical sensitivity for application as diagnostic tool for the presence of *brucella* organisms in milk. Groups of primers used were: AMOS/IS711 , provided by B. Bricker [7] B4/B5, provided by G.G. Baily [8] 1,2 and 3 provided by G. Adams (Texas A&M University, USA)

All sets of primers used were specific but showed different analytical sensitivities ranging from 20 to 7000 microrganisms (Table III). Some of the primers proved unable to detect *Brucella* organisms in milk. No system of primers alone could detect and differentiate all *Brucella spp.* and biovars used in the study. Only one system using the primers B4/B5 (Baily) showed enough analytical sensitivity for application as diagnostic tool for the presence of *brucella* organisms in milk.

TABLE III. COMPARISON BETWEEN PCR (BP)

Brucella spp.	AMOS/ IS711 PCR	Adams (expected) PCR	Pst 1	Adams (Teramo) PCR	Pst 1
B. abortus bv. 1	498	800	500, 300	800	500, 300
B. abortus bv. 2	498	n	--	800	500, 300
B. abortus bv. 3	np	n	--	n	--
B. abortus bv. 4	498	n	--	n	--
B. abortus bv. 5	np	940	400, 300, 200	940	400, 300, 200
B. abortus bv. 6	np	n	--	940	400, 300, 200
B. abortus bv. 7	np	n	--	n	--
B. abortus bv. 9	np	n	--	n	--
B. melitensis bv. 1	731	940*	700, 200	940	700, 200
B. melitensis bv. 2	731	n	--	940	400, 300, 200
B. melitensis bv. 3	731	n	--	n	--
B. ovis	976	440	--	n	--
B. suis bv. 1	285	440	--	n	--

bv not specified
np no product
n unknown data

15 field isolates were tested and 11 were identified as belonging to *B. melitensis*, 2 as belonging to *B. abortus* and 2 as belonging to *Brucella spp.* in accordance with AMOS/IS711 primers. Using B4/B5 primers, all positive samples produced, after amplification, DNA fragments of the expected length. Primers provided by Adams allowed us to distinguish between *B. abortus* bv1 (800bp), *B. abortus* bv.5 (949 bp), *B. melitensis* bv 1 and 2 (940 bp). *B. abortus* bv5 could be distinguished from *B. melitensis* through enzyme restriction analysis. The primers B4/B5 were tested with a standard *B. melitensis* and the conditions optimized for use with whole inactivated organisms. About 20 *Brucella* organisms could be detected.

TABLE IV. COMPARISON BETWEEN PCR DETECTION AND BACTERIOLOGICAL ISOLATION

Result	Serology	Bacteriology	PCR
Positive	90	9	18
Negative	47	0	0
Total	137	9	18

All the standard strains and the field isolates produced the expected band with the optimized PCR. The test was compared with the bacteriological culture on 137 sheep milks 90 of which were from sero-positive animals. Relative to the bacterial culture, an increased sensitivity (50%) was recorded using this method (Table IV)

2.2. Indirect tests

2 2 1 An ELISA method for detection of Brucella antibodies in cow milk

In this study an indirect ELISA method, using a sLPS antigen, was developed for detection of *Brucella* antibodies in cow milk (milk-ELISA) and compared to MRT for relative performance and to CFT for diagnostic performance [10]. Parameters established included· reference positive and negative standards, study of the effect of milk fat globules on test performance, positive/negative threshold by using the end point titration of a reference sample and by verifying the signal to noise ratio calculated over a statistically relevant number of milk samples from dairy cow populations from herds officially free from brucellosis.

Reference positive standards were constructed using a national anti-*brucella* standard serum having 1 000 CFT I U and dilute 1:50 to 1:51.200 into negative milk. Strong (100%), medium (30%) and empirical negative (10%) standards were established. Thirthy-five measurements of the OD of the selected reference standards were performed to determine mean values and standard deviations Such numbers of measurements allowed the estimate of the mean values for each standard with a 95% confidence interval of ± 0.33OD units or a 99% conficidence interval of ± 0 44 SD units A pool of 24 milks from 5 brucellosis-free dairy herds was taken for a preliminary establishment of the positive/negative ratio Thirthy-five measurements of the OD of the pool were performed to determine its mean value and standard deviation Such numbers of measurements allowed the estimate of the mean values with a 95% confidence interval of ± 0.34OD units or a 99% confidence interval of ± 0.45 SD units

The ELISA cut-off value for the situation under study was assessed by testing a total of 826 field milks from 35 unvaccinated and brucellosis-free herds. Field milks were classified as negative when their OD was lower than the 90% upper limit of the sample distribution of ODs plus 3SD of the reference negative standard The expected number of false-positives following the adoption of the above defined threshold was 0.5%. Statistically, a sample of 826 milks should be able to detect a percentage of false-positive milks of 0.5% with 95% confidence limits of ± 0.48%.

The mean value of the field milks OD was 0.056 (standard error =0.001) and its SD was 0 032. Minimum and maximum measured values were 0.00 and 0.18 respectively. Using the threshold value based on the negative reference standard OD distribution, 822 out of 826 field milk samples (99.5%) were correctly classified while 4 milks (0.5%) were misclassified. The observed rate of correct classification fell within the expected values since 0.5% is the area under 99% upper tail of the frequency distribution of ODs for negative milks (Fig. 1).

A total of 36 milk samples from CFT positive cows were tested with ELISA to establish the diagnostic sensitivity of the test. Samples were considered positive when their OD was greater then the established cut-off OD. Relative to CFT in serum, the sensitivity of ELISA in milk was 94 4%. Results of 34 animals were in agreement while 2 milk samples gave ELISA negative.

ELISA and MRT elicited different performance characteristics relative to each as shown in Table V. MRT titers ranged from 1:5 to 1:80 while ELISA titers ranged from 1:200 to 1·600 Therefore, ELISA appeared to be 20 to 80 times more sensitive than MRT.

The cut-off values established with the reference standard were not truly representative for the negative dairy cow population under study. For diagnostic purposes and in order to reduce as far as possible the range of false positive results without affecting the test sensitivity, the original cut-off value had to be adjusted. By means of such adjustment 100% of the negative samples were correctly classified.

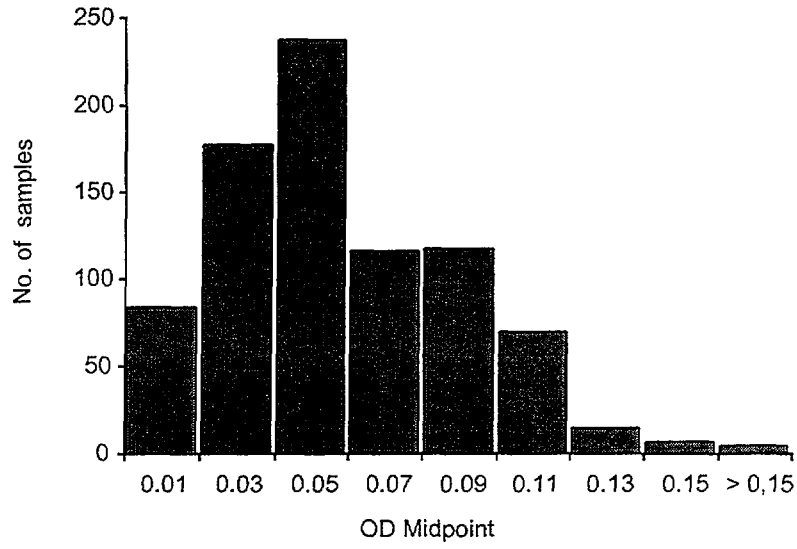

FIG. 1. Frequency distribution of 826 milk samples from brucella-free herds.

TABLE V. COMPARISON OF MRT AND ELISA TITERS ON INDIVIDUAL POSITIVE MILK SAMPLES

Cow No.	MRT	Milk-ELISA
IS62	5	400
IS58	10	200
IS31	10	400
AV1288	20	400
PE29/1	20	400
IS42	20	800
IS43	40	800
PE29/2	80	1600
MRT control (Mab)	160	=

The reference positive and negative values established were still reproducible even when milk samples with different fat content were used as diluent. It means that undiluted milk samples can be used in our system during mass screening and field surveillance without affecting sensitivity and specificity of the test. Preliminary tedious and time consuming steps required for skimming or prediluting milk samples can be avoided, hense reducing the degree of risk to the operator when handling samples from infected herds.

The expected higher sensitivity of the milk-ELISA over the MRT may be accounted for by the physiological dominance of IgG1 over IgA and IgM in mature milk as well as by the analytical higher sensitivity of the test. Relatively low titers provided by the MRT pose once again the question as to whether a MRT run on large tank-milk can still detect weak-positive samples. Milk-ELISA showed a final 99.5% diagnostic specificity and only a 94.4% sensitivity relative to 36 CFT positive animals. It can be speculated that a test having such degree of sensitivity is likely to be a poor predictor of infection in a situation of low prevalence. On the other hand the advantage of testing milk for *Brucella* antibodies in dairy herds to meet requirements for frequent testing for maintenance of brucellosis-free status at herd level.

2 2.2 Evaluation of a milk-ELISA test for Brucellosis through repeated sampling on an infected dairy herd

The aim of the study was:

- to evaluate the ability of milk-ELISA to detect infected animals in comparison to RBPT, CFT, and MRT
- to estimate the probability of milk-ELISA and MRT to detect infected animals in three susbsequent sampling and testing in relation to directives 64/432/EEC and 91/499/EEC regarding application of MRT and milk-ELISA respectively and stating that countries, which have been declared to be free from *Brucella* during the last four years, serological tests may be substituted by three milk-ELISA or three MRT.

Applied tests:
RBPT, CFT, MRT and milk-ELISA. The milk-ELISA was standardized to have a specificity of 99.5%.

Rationale:
Case definition: any animal from an infected herd that proved positive in at least one of the four tests performed or culture in at least one of the four samplings performed.
Herd: a herd of 77 cows where 3 abortions were reported and *B melitensis* biotype 2 was isolated from aborted material.
Sampling. all animals were bleed and milk sampled at the start of the study. Remaining cows after culling of the CFT reactors were tested every 45 days for a period of 135 days.

The initial prevalence of infection was 57.1% (44 infected cows, 30 of which were CFT positive) on the basis of the adopted case definition (Table VI)

TABLE VI. SENSITIVITY (%) OF EACH TEST RELATIVE TO THE CASE DEFINITION

Total number of infected animals	RBT		CFT		MRT (*)		m-EIA(*)	
	Pos.	Se.	Pos.	Se.	Pos.	Se.	Pos	Se.
71	50	70,4	36	50,7	32	68,1	63	96,9

(*) based on a lower number of tested animals

As a whole, 71 cows fulfilled the case definition during the whole study period. The sensitivites recorded for each test were: RBPT= 59.0%, CFT= 36.0%, MRT on individual samples= 47.5%, milk-ELISA on individual samples= 92.3% (Table VII).

TABLE VII. TOTAL NUMBER OF POSITIVES PER SAMPLING

Sam-pling	Infected (*)		RBT			CFT			MRT			m-EIA		
	No	%	Pos.	Exam	%	Pos.	Exam	%	Pos.	Exam	%	Pos.	Exam	%
I	44	57,1	35	77	45,5	30	77	39,0	19	73	26,0	36	72	50,0
II	37	78,7	19	47	40,4	5	47	10,6	1	45	2,2	32	43	74,4
III	18	42,9	4	42	9,5	0	42	0,0	6	31	19,4	15	36	41,7
IV	1	2,4	1	41	2,4	1	40	2,5	--	--		--	--	

(*) According to case definition

Regarding the use of the test on bulk milk, MRT was able to detect infected milk diluted up to 1:80 in negative milk, milk-ELISA was able to detect infected milk diluted 1:1600 in negative

milk. The sensitivities established after repeated samplings were: RBPT= 70.4% (4 samplings), CFT= 50.7% (4 samplings), MRT= 68.1% (3 samplings) and milk-ELISA= 96.9% (3 samplings) (TableVIII).

TABLE VIII. SENSITIVITY OF MILK-ELISA AND MRT RELATIVE TO THE NUMBER OF TESTING ROUNDS

Test	Number of testing rounds		
	1	2	3
m- EIA	92,3%	96,8%	96,9%
MRT	47,5%	51,9%	68,1%

Sensitivity of RBPT and CFT in the case study was lower than the values normally reported in the literature [11.12]. Likewise, the sensitivity of MRT was lower than the values normally observed in infected herds [13]. The milk-ELISA was the most sensitive test when performed on individual milks. Based on the test sensitivities estimated in this study and on tests specificity reported in the literature [11,12,13], the expected predictive value for positive results of the adopted case definition would have been 94.6% (equivalent to 2 false positives) after the first sampling and the predictive value for negative results would have been 99.2% (equivalent to 0 false negatives). The use of CFT as confirmatory test implies a predictive value for positivie results of 98.4% (equivalent to 1 false positive) and a predictive value for negative results of 60.1% (equivalent to 13 false positives). In fact, 6 more animals positive to CFT have been detected in the following samplings. The higher sensitivity of milk-ELISA in three subsequent samplings (96.9%) and its ability to detect positive milk diluted up to 1:1600 indicates that the testing of bulk milk as indicated in EEC directives is a valid and inexpensive method to detect the infection. Conversely, the lower sensitivity of MRT and its ability to detect positive milk diluted 1:5/1:80 only, indicates that the use of MRT, as indicated in EEC directives may be useful only in the case of small sized dairy herds. Repeated milk testing by milk-ELISA would certainly increase the chance of identification of positive animals.

2.2.3. Assessment of an Indirect ELISA in Milk for the Diagnosis of Ovine Brucellosis

The possibility of using an ELISA for the diagnosis of ovine brucellosis in milk was investigated. The aim of the study was to establish whether the specificity and sensitivity of milk-ELISA would be high enough to detect low levels of *brucella* antibodies in ewe milk. The diagnostic performances of the test under study were established by means of reference standard and compared with conventional screening and confirmatory tests under field conditions (Table IX).

TABLE IX. ESTIMATES OF MILK-ELISA DIAGNOSTIC SENSITIVITY RELATIVE TO DIFFERENT TESTS ESTABLISHED ON 48 ANIMALS FROM AN INFECTED FLOCK

Test	Positive	Negative
Culture	13 (27%)	35 (73%)
RBT	43 (89,5%)	5 (10,5%)
CFT	35 (73%)	13 (27%)
M-ELISA	29 (60%)	19 (40%)

The diagnostic specificity of the milk-ELISA established on a number of samples from *brucella*-free flocks was 100% while relative to RBPT and CFT positive reactors the milk-ELISA demonstrated sensitivity of 65% and 83% respectively. No linear correlation was appreciated between titers expressed by CFT and milk-ELISA (Table X). Its sensitivity relative to culture positive animals was 92%.

The course of *brucella* antibodies in milk of positive sheep was evaluated in colostrum and in mature milk for a period of 30 days after delivery and it appeared that concentration of immunoglobulins in milk tend to sharply decrease soon after parturition while in blood serum these remain constantly high.

TABLE X. COMPARISON OF CFT AND MILK-ELISA TITERS

CFT								
≥851.2					+++++	++	+++	+++
425.6			+	+			++	
212.8		+++	++++		+			
106.4		++			+			
50.0								
25.0	+++++	+						
20.0	+							
<20.0	+++++							
	+++++							
	+							
	0	10	20	40	80	160	320	640
	Milk-ELISA							

It was concluded that the milk-ELISA for brucella antibodies in ewe milk can be regarded as a useful complementary diagnostic tool for individual testing but it is poorly viable as a screening test applied to pooled flock milks (15).

REFERENCES

[1] JACOBSON, R.H., How well do serodiagnostic tests predict the infection or disease status of animals? Proceedings of Final Research Co-orditation Meeting of FAO/IAEA/SIDA Co-ordinated Research Program, Costa Rica, IAEA (1990) 31-42.

[2] SUTHERLAND, S.S., Evaluation of the enzyme-linked immunosorbent assay in the detection of cattle infected with *B. abortus,* Vet. Microbiol. **10** (1984) 23-32.

[3] SARAVI, M.A.and coll., Comparison of an indirect enzyme immunoassay and conventional serological techniques for the detection of bovine antibody to *Brucella abortus*. Regional network for Latin America on animal disease diagnosis using immunoassay and labelled DNA probe techniques. IAEA TECDOC- 657 International Atomic Energy Agency (1992) 151-162.

[4] NIELSEN, K.H. and coll., A review of Enzyme immunoassay for detection of antibody to *Brucella abortus* in cattle, Vet. Immunol. & Immunopath.,**18** (1988) 331-347.

[5] BIANCIFIORI, F. and coll., A simple and direct method for identification of *Brucella spp* using monoclonal antibodies, Vet. Ital. **29** (1994) 8-14.

[6] HOPPER, B.R. and coll., Detection of *Brucella abortus* in mammalian tissue, using biotynnilated whole genomic DNA as a molecular probe, Am J. Vet. Res., **50** (1989) 2064-2068.

[7] BRICKER B. and coll., (1994) Differentiation of *Brucella abortus* bv. 1, 2 and 4, *Brucella melitensis*, *Brucella ovis* and *Brucella suis* bv1 by PCR. J. Clin. Microb. 32: 2660-2666.

[8] BAILY, G.G. and coll., Detection of *Brucella meliensis* and *Brucella abortus* by DNA amplification, J. Tropical Med. Hyg. **95** (1992) 271-275.

[9] PAGANICO, G. and coll., Detection of *Brucella spp.* in raw milk by PCR: Proceedings of the First Meeting on Applied Research of IIZZSS, ISS, 9-10 September 1996; Vet. Ital. in press. (1996).

[10] BIANCIFIORI, F. and coll., An ELISA method for detection of *Brucella* antibodies in cow milk, Vet. Ital. **30** (1995) 16-24.

[11] KERKHOFS, P. and coll., Le dépistage de la brucellose bovine par ELISA sur le lait, Ann. Méd. Vét. **133** (1989) 663-672.

[12] BERCOVICH, Z. and TAAIJKE R., ELISA using Mouse anti-bovine antibodies for the detection of *Brucella abortus* antibodies in cow milk, J. Vet. Med. B **37** (1990) 753-759.

[13] BORAKER, D.K. and coll., BrucELISA: an ELISA for detection of *Brucella abortus* antibodies in milk: correlation with the *Brucella* ring test and with the shedding of viable organisms, J. Clin. Microbiol. **14** (1981) 396-403.

[14] BIANCIFIORI, F. and coll., Evaluation of a Milk-ELISA test for Brucellosis through repeated sampling on an infected dairy herd: Proceedings of the First Meeting on applied research of IIZZSS, ISS, 9-10 September 1996; Vet. Ital. in press (1996).

[15] BIANCIFIORI, F. and coll., Assessment of an indirect ELISA in milk for the diagnosis of ovine brucellosis: Com. Immunol. Microbiol. Infect. Dis. 19 1 (1996) 17-24.

COUNTRY REPORTS

Part A: BRUCELLOSIS

Competitive brucellosis ELISA

SUMMARY OF FIELD TRIALS USING THE INDIRECT AND COMPETITIVE ENZYME IMMUNOASSAYS FOR DETECTION OF ANTIBODY TO *BRUCELLA ABORTUS*

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Abstract

SUMMARY OF FIELD TRIALS USING THE INDIRECT AND COMPETITIVE ENZYME IMMUNOASSAYS FOR DETECTION OF ANTIBODY TO *BRUCELLA ABORTUS*.

Two indirect and two competitive enzyme immunoassays for detection of antibody to Brucella abortus, validated elsewhere, were field tested in five different Latin American laboratories. Testing was performed according to standardised protocols using sera obtained in each area. Sera from *B. abortus* infected herds, from vaccinated (but serologically negative in a screening test) and non-vaccinated cattle were tested in each assay and compared to the results obtained with conventional diagnostic tests used for diagnosis of brucellosis in each country. Relative sensitivity and specificity values were calculated for each country as well as a weighted summary combining the data from all the participating laboratories. The result demonstrate that all ELISAs performed as well as, or better than, the conventional serological tests. Given the inherent errors in the use of the latter in the diagnosis of brucellosis, it is recommended that the ELISAs described here be considered as replacements for the conventional tests. The CELISA using the lipopolysaccharide antigen with the competing monoclonal antibody M84, should be considered as the most useful because of cross-species and vaccination considerations.

1. INTRODUCTION

Indirect enzyme immunoassays (IELISA) were developed to increase diagnostic sensitivity, diagnostic specificity and accuracy of diagnostic tests in use for detection of antibody to Brucella abortus. However, because of the increase in sensitivity of the IELISA there was a small decrease in assay specificity. In addition, it became apparent that antibody resulting from vaccination of cattle with *B. abortus* strain 19 sometimes contributed to the decrease in specificity. The specificity of the IELISA was markedly enhanced in early trial incorporation divalent cation chelating agents into the serum incubation step [2]; however, the assay could still not differentiate some cases of vaccinal antibody from antibody resulting from infection. Competitive enzyme immunoassays (CELISA) were developed because the affinity of the competing monoclonal antibody could be selected to exclude residual vaccinal antibody thus creating assays that could distinguish field infection from vaccination [1,3]. An additional advantage of the CELISA is its use for detection of antibody in various species. Unfortunately the I- and CELISAs were developed and validated under conditions where no bovine brucellosis was in evidence and where vaccination with strain 19 had not been practised for a number of years. All sera from infected and vaccinated cattle used in the initial studies were derived from banks of frozen serum. Because of this, it was decided that an extensive trial of these assays should be undertaken in countries where various levels of brucellosis were present in the bovine population and where vaccination with strain 19 was practised. This decision resulted in Research Contracts between five Latin American laboratories and the Joint FAO/IAEA Division of the International Atomic Energy Agency. These contracts allowed for the transfer of technologies to the laboratories, a supply of all necessary biological reagents from the Animal Diseases Research Institute (ADRI), a research grant and advice and backstopping by ADRI personnel.

2. MATERIALS AND METHODS

2.1. Participating laboratories

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2.2. Serological tests

The protocols for performing the Rose Bengal (RBT), buffered plate antigen (BPAT), confirmatory test and the ELISAs (the Joint FAO/IAEA Division International Atomic Energy Agency, OIE approved) version of the IELISA (ELISA1), a modified screening version of the IELISA (ELISA2), a CELISA using O-polysaccharide as the antigen (ELISA3) and a CELISA using smooth lipopolysaccharide (SLPS) as the antigen (ELISA4)) are described elsewhere in this report. In each case, sera were tested by the RBT using antigen purchased from Mérieux; BPAT with antigen purchased from USDA; the regularly used confirmatory test of each country; the OIE approved IAEA IELISA kit; the ADRI modified IELISA; the CELISA using O-polysaccharide antigen and by the CELISA using SLPS antigen.

2.3. Sera

Sera used for this study are described in the contributions by each individual laboratory. The initial premises of the field trials were that each laboratory would test a minimum of 1000 serum samples, from serum banks or obtained from diagnostic services, of the following groups:

2.3.1. *Sera from non-vaccinated, non-exposed cattle*
All sera were BRBT and CFT negative.

2.3.2. *Sera from cattle receiving an approved schedule of B. abortus strain 19 vaccine:*
obtained at the normal testing interval. All sera were RBT and CFT negative.

2.3.3. *Sera from herds with proven B. abortus infection:*
at a prevalence of not less than 5% of the animals. Sera selected were RBT and CFT positive.

2.4. Data

The data from each laboratory is presented in the individual reports.

2.5. Statistical analysis

Where possible, each group of cattle was treated separately. Frequency distributions and receiver operator characteristic (ROC) analysis were used to determine the most suitable cut-off values

between negative and positive results [4]. From these data, relative diagnostic sensitivity and specificity values were calculated for each country.

3. RESULTS

The in-use diagnostic test combination for each country was used as the criteria to establish whether individual serum samples were positive or negative. These data were compared to the values obtained with each of the ELISA tests to determine the most appropriate cut-off value (Table I) and the relative diagnostic sensitivity and specificity for each ELISA for each country was calculated. These calculations are presented in Tables II (sensitivity) III and IV (specificity for vaccinated and non-vaccinated cattle). For comparison, values, where available, for Canada were included [3]. The overall relative diagnostic sensitivity and specificity values were calculated using analysis weighted for the number of samples tested in each location. These data are presented in Table V.

TABLE I. CUT-OFF VALUES DETERMINED BY FREQUENCY DISTRIBUTIONS AND ROC ANALYSIS OF THE DATA FROM EACH LABORATORY OF THE FOUR ELISAS FOR DETECTION OF ANTIBODY TO *BRUCELLA ABORTUS*

	ELISA1 %P	ELISA2 %P	ELISA3 %I	ELISA4 %I
Argentina	40	67	35	44
Chile	16	21	18	27
Colombia	14	40	30	29
Costa Rica	70	73	20	18
Cuba	38	38	28	21
Canada	na	46	30	30

na - data not available.

TABLE II. RELATIVE SENSITIVITY VALUES CALCULATED FOR EACH COUNTRY BASED ON CONVENTIONAL SEROLOGICAL TEST RESULTS ON SERA FROM HERDS INFECTED WITH *BRUCELLA ABORTUS*

	ELISA1 %	ELISA2 %	ELISA3 %	ELISA4 %
Argentina	97.9	92.7	96.9	97.7
Chile	98.9	97.1	98.9	100
Colombia	100	100	99.3	98.2
Costa Rica	100	98.4	92.1	93.2
Cuba	89.7	94.9	94.9	71.8
Canada	na	100	na	100

TABLE III. RELATIVE SPECIFICITY OF TESTS USING CATTLE VACCINATED WITH *BRUCELLA ABORTUS* STRAIN 19 BUT SEROLOGICALLY NEGATIVE BY CONVENTIONAL TESTS

	ELISA1 %	ELISA2 %	ELISA3 %	ELISA4 %
Argentina	98.1	96.3	98.1	98.1
Chile	96.9	78.8	94.4	100
Colombia	86.8	87.6	95.6	92.3
Costa Rica	91.8	94.6	93.8	96.0
Cuba	90.1	94.1	99.6	100
Canada	na	94.8	na	97.7

TABLE IV. RELATIVE SPECIFICITY OF REACTIONS OF SERA FROM NON-VACCINATED CATTLE BASED ON REACTIVITY IN CONVENTIONAL SEROLOGICAL TESTS

	ELISA1 %	ELISA2 %	ELISA3 %	ELISA4 %
Argentina	na	na	na	na
Chile	99.3	99.6	99.7	99.6
Colombia	99.8	99.8	99.8	97.1
Costa Rica	95.8	95.0	93.4	95.8
Cuba	99.7	99.7	100	100
Canada	na	99.4	na	99.9

TABLE V. MEANS (WEIGHTED BASED ON SAMPLE NUMBERS) OF RELATIVE SENSITIVITIES AND SPECIFICITIES OF THE ENZYME IMMUNOASSAYS

	ELISA1	ELISA2	ELISA3	ELISA4
Cut-off value	41%P	41%P	26%I	29%I
Sensitivity %	96.3	96.7	96.9	97.3
Specificity %	97.0	93.8	98.4	98.8

4. DISCUSSION

As the enzyme immunoassays are currently being considered for use as confirmatory tests, all the data were calculated to try to maximise diagnostic specificity. In most of the countries involved in this study, vaccination with *B. abortus* strain 19 is widely practised in both calves and adult cattle. It is therefore debatable if the data for diagnostic specificity obtained with non-vaccinated cattle is of current interest. In other circumstances, such as areas where brucellosis has been eradicated and vaccination is decreased or stopped, this data will be more useful. All enzyme immunoassay data are based on the premise that the screening test followed by the confirmatory test for antibody are absolute. That is, if the screening and confirmatory tests are positive, it was assumed that the serum originated from an infected animal. Similarly, if both tests were negative, the serum was considered as originating from a non-exposed animal. If the screening or the confirmatory tests did not agree, the data was not considered. Because of these selection parameters, the ELISAs will not appear to function as well as the conventional tests. For instance, if a vaccinated animal is positive in the screening and confirmatory tests but negative in one or more of the ELISAs, it is assumed that the ELISAs failed to diagnose brucellosis. This reaction could equally well be due to residual antibody from *B. abortus* strain 19 vaccination. Alternately, if an animal from an infected herd gave a negative result in the conventional tests but was positive in one or more ELISA, it was assumed to be a false positive reaction where in fact it may be brucellosis in early stages of incubation, detected by the ELISAs due to their higher analytical sensitivities. It is of interest to note the considerable variation in the cut-off values established for the IELISAs (ELISA1 and ELISA2) while there were only minor variations in the cut-off values for the CELISAs (ELISA3 and ELISA4). These data are presented in Table 1. The reasons for the variations in the IELISA are not understood but may be a result of cross-reacting antigens present in localised cattle populations. Alternately, vaccination status of animals may be incorrect, especially where adult vaccination is practised. From Table 2, it appears that ELISA4, the CELISA that uses SLPS antigen, is marginally more sensitive than the other three ELISAs, all of which have nearly identical sensitivities, relative to the diagnostic serology tests used in each laboratory. The one aberrant value for the ELISA4 sensitivity is due to testing of sera grossly contaminated with bacteria, a factor known to interfere with M84 monoclonal antibody binding to the SLPS. This data should therefore be omitted in the analysis. The relative specificity in non-vaccinated or vaccinated but diagnostically negative cattle are very similar for all the ELISAs (Tables 3 and 4). It is of interest to note that using these samples, the

IELISAs performed nearly as well as the CELISAs. This is not surprising as the samples were selected based on negativity or lack of exposure to *B. abortus*. From the weighted summary (Table 5), ELISA4 slightly outperforms the other ELISAs for relative sensitivity while its relative specificity is very similar to those of the other ELISAs. From the data presented, the ELISAs perform as well or better than the combination of conventional serological tests used for diagnosis of brucellosis. Because of the inherent advantages of primary binding assay and in particular, the ability to perform assay quality control on an international scale and the ability of the CELISAs to eliminate residual antibody from *B. abortus* strain 19 vaccination, it is envisaged that these assay will replace the in-use serological tests.

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REFERENCES

[1] NIELSEN K, CHERWONOGRODZKY J, DUNCAN JR, BUNDLE D.R., Enzyme immunoassay for the differentiation of the antibody response of *Brucella abortus* infected or strain 19 vaccinated cattle, An. J. Vet. Res. **50** (1989) 5-9.

[2] NIELSEN K, KELLY L, GALL D, SMITH P, BOSSE J, NICOLETTI P, KELLY W., The use of divalent cation chelating agents (EDTA/EGTA) to reduce non-specific serum protein interaction in enzyme immunoassay, Vet. Res. Comm. **18** (1994) 433-437.

[3] NIELSEN K, GALL D, KELLY W, VIGLIOCCO A, HENNING D, GARCIA M., Immunoassay development: application to enzyme immunoassay for the diagnosis of brucellosis, Agriculture and Agri-Food Canada Monograph. ISBN 0-662-24163-0, (1996) (available free of charge).

[4] SCHOONJANS F, ZALATA A, DEPUYDT CE, COMHAIRE FH., MedCalc: a new computer program for medical statistics, Comp. Meth. and Progr. Biomed. **48** (1995) 257pp.

ENZYME IMMUNOASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS

Trial in Latin America

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Abstract

ENZYME IMMUNOASSAYS FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS TRIAL IN LATIN AMERICA

The results of a field trail conducted in Latin America with two indirect (IELISA) and two competitive (CELISA) enzyme immunoassays for the detection of bovine antibody to *Brucella abortus* are reported One of the CELISA formats performed most accurately The relative sensitivity of this assay was 97 47%, the relative specificity for unexposed cattle was 98 32% and the specificity in cattle vaccinated with *B. abortus* strain 19 was 96 51% The same assay format under Canadian conditions had a sensitivity of 100%, a specificity of 99 90% and a specificity of 97 7% in a strain 19 vaccinated population Overall, the CELISA performed as expected and the results were not dissimilar to the results obtained in the Canadian study thus providing further evidence that this CELISA can in many instances differentiate infected cattle from those that are vaccinated or infected with a cross-reacting organism while still giving very low false positive or false negative results.

1. INTRODUCTION

The indirect enzyme immunoassay (IELISA) for detection of antibody to *Brucella abortus* was introduced by Carlsson et al. [1]. The reasons for using IELISAs were firstly, to replace conventional serological tests that in many ways did not perform well and frequently required a panel of tests for diagnosis and secondly, to introduce an assay which could be standardised, quality controlled and automated [2]. A large number of IELISAs have been described in the literature [3] but in spite of the numerous modifications, the specificity of these assays were less than expected. The reason for this is partly because antibody resulting from *B. abortus* strain 19 vaccination or from exposure to cross-reacting antigens is detected by this procedure.

To increase specificity, competitive enzyme immunoassays were developed [4,5,6,7,8]. By selecting a suitable monoclonal antibody to compete with antibody present in test serum, reactivity resulting from the vaccine or cross-reacting antigens could be virtually eliminated Two of these assays were developed and validated largely in circumstances where brucellosis had been eradicated (Canada) using sera from animals in which *B abortus* infection was confirmed by culture as reference sera. It was therefore necessary to field test these assays in areas with brucellosis and vaccination programs. For these purposes, four laboratories in Latin America were selected These laboratories were selected based on the incidence of brucellosis in each area. Chile had a relatively low incident while higher incidences were found in Costa Rica, Colombia and Argentina.

This communication describes the results obtained with two indirect and two competitive enzyme immunoassays compared to the diagnostic serological tests in use in each laboratory.

2. MATERIALS AND METHODS

2.1. Test Samples:

Samples were defined on the basis of their serological reaction on both the Rose Bengal Agglutination Test (RBT) and Complement Fixation Test (CFT) using the official criteria for positive results as determined by each country for the CFT.

Negative samples were defined as those primarily from regions that had no history or serological evidence of *Brucella abortus* infection and were negative on both the RBT and the CFT. Some animals in the negative population were vaccinated with*B. abortus* strain 19.

Positive sera were defined as those samples from infected herds which were positive on both the RBT and the CFT. This population was thought to include cattle with residual vaccinal antibody or antibody resulting from exposure to cross reacting antigens.

2.2. Control Sera:

Control sera were supplied by the Animal Diseases Research Institute (ADRI) for the IELISA and both CELISAs from ADRI. These consisted of a strong positive control serum from a cow from which *Brucella abortus* had been isolated: a weakly positive control for the IELISA that was from a cow inoculated with *B. abortus* strain 19 and negative on the CELISA and a negative control from a pool of cattle with no history of *B. abortus* infection. Separate controls were supplied by the International Atomic Energy Agency (IAEA) for the IAEA IELISA kit.

2.3. Test Procedures:

The RBT antigen was prepared by Rhône Mérieux and the assay performed as described in the NADL Diagnostic Reagents Manual [9].

The CFT reagents were prepared and the assay performed as described in the Public Health Monograph N74 [10].

The IELISA supplied by the Food and Agricultural Organisation, International Atomic Energy Agency was performed as described in the IAEA kit. The basic reagents and protocol for this kit have been adapted from Nielsen et al. [11].

The IELISA supplied by the Agriculture Canada, Animal Diseases Research Institute (ADRI) was performed as described by Nielsen et al. [7]. The CELISA, using smooth lipopolysaccharide (CELISA-sLPS) as the antigen, was performed as described by Nielsen et al. [8]. The CELISA, using o-polysaccharide of sLPS (CELISA-OC) as the antigen, was performed as described by Gall and Nielsen [12]. The procedures for each assay are summarised in Table I.

2.4. Data Handling and Statistical Analysis:

The data for each country was compiled in a database and further divided into negative or positive results according to serological reactions on both the RBT and the CFT.

After the results were defined into negative and positive populations, initial optimal estimates of the criteria between positive and negative reactions (the cut-off values) were determined using receiver operating characteristics (ROC) analysis [13].

Using the initial estimates of cut-off values the diagnostic relative sensitivity and diagnostic relative specificity were calculated and the frequency distributions were plotted to provide a visual confirmation that the cut-off value was applicable. Finally, assays were compared to each other for agreement and a kappa statistic calculated [13].

TABLE I. COMPARISON OF PARAMETERS OF FOUR ELISAs

Parameters	Assay			
	IAEA-IELISA	ADRI-IELISA	CELISA-OC	CELISA-sLPS
Microplate	Nunc Polysorb	Nunc 69620	Nunc 69620	Nunc 69620
Antigen	sLPS	sLPS	O-Chain	sLPS
Concentration	1.0 ug/ml	1.0 ug/ml	2.0 ug/ml	1.0 ug/ml
Buffer	0.05M CO_3	0.05M CO_3	0.05M CO_3	0.05M CO_3
Incubation Temp.	4°C	20°C	4°C (frozen)	20°C
Incubation Time	≥18 hrs.	≥18 hrs.	≥18 hrs	≥18 hrs
Wash Buffer	0.002M PO_4, 0.15M NaCl, 0.05% Tween 20, pH 7.4	0.01M PO_4, 0.15M NaCl 0.05% Tween 20, pH 7.2	0.01M PO_4, 0.15M NaCl 0.05% Tween 20, pH 7.2	0.01M PO_4, 0.15M NaCl 0.05% Tween 20, pH 7.2
Wash Cycles	3	4	4	4
Serum Diluent	Same as Wash Buffer	Wash Buffer + EDTA/EGTA pH 6.3	Same as Wash Buffer	Wash Buffer + EDTA/EGTA pH 6.3
Serum (Controls/Test)				
Serum Dilution	1:200	1:50	1:50	1:20
Incubation Temp.	37°C	20°C	20°C	20°C
Incubation Time	60 min.	30 min.	120 min.	30 min.
Agitation	Yes	No	3 min.	3 min.
Competing Antibody	Not Applicable	Not Applicable	YsT9-HRPo	M84
Detecting Antibody	McAb to bov. IgG_1-HRPo	McAb to bov. IgG_1-HRPo	Same as Competing	GaMIgG-HRPo (diluted in wash buffer)
Incubation Time	60 min.	30 min.	Not Applicable	30 min.
Incubation Temp.	37°C	20°C	Not Applicable	20°C
Agitation	Yes	No	Not Applicable	No
Substrate/Chromogen				
Incubation Time	10 min.	10 min.	10 min.	10 min.
Incubation Temp.	37°C	20°C	20°C	20°C
Agitation	Yes	Yes	Yes	Yes
Wavelength (nm)	405	414	414	414

3. RESULTS

The number of samples used in this study are presented in Table II.

TABLE II. NUMBER OF OBSERVATIONS PER COUNTRY AND COMBINED

	Number of observations percountry and combined[1]				
Status[2]	Argentina	Chile	Colombia	Costa Rica	Combined
Negatives	215	972	554	872	2613
Positives	709	692	266	190	1857
Vaccinates	na[3]	954	1110	1002	3066

1. Except for Cuba (data was not available).
2. All samples were defined relative to their RBT and CFT results.
3. Data is not available (na). There was an insufficient number of vaccinates to be a separate category so the data was combined with negatives.

The samples were divided into three populations. The negative population was defined as those primarily from regions that had no history of *Brucella abortus* infection and had negative reactions on both the RBT and the CFT. The positive population was defined as those samples from infected herds which had positive reactions on both the RBT and the CFT. The vaccinated population was defined as those animals that had been vaccinated with *B. abortus* Strain 19 according to the regulations in each country. The exception to this was Argentina where vaccination is routinely practised and it was difficult to collect samples that were defined as from unexposed cattle. Consequently, the data for the negative category and the vaccinated category for Argentina were combined into the negative category.

The sensitivity data presented in Table III is defined in two ways. The data of the positive population from Argentina, Chile, Colombia, Costa Rica and the combined data is relative sensitivity or the sensitivity of each ELISA relative to the RBT and CFT reactions from cattle in infected herds. The sensitivity for the Canadian data is actual sensitivity since the results were derived from animals in which *Brucella abortus* had been isolated. The highest relative sensitivity of 100% for both IELISAs from Colombia and for the IELISA-ADRI from Costa Rica indicate that it is comparable to the actual sensitivity achieved by the IELISA-ADRI in the Canadian study. Data for the IELISA-IAEA and the CELISA-OC for Canada was not part of the original Canadian study and consequently is not available.

TABLE III. SENSITIVITY COMPARISON

	IELISA-ADRI	IELISA-IAEA	CELISA-OC	CELISA- sLPS
Argentina	92.66	97.88	96.90	97.74
Canada	100	na[2]	na[2]	100
Chile	98.99	97.11	98.84	100
Colombia	100	100	99.25	98.12
Costa Rica	100	98.42	92.10	93.16
Combined[1]	96.77	96.28	97.04	97.47

1. Combined data for all the countries except Canada (included for comparison).
2. This data was not part of the original Canadian study.

Similarly, the specificity of negative populations presented in Table IV were defined. The data of the negative population from Argentina, Chile, Colombia, Costa Rica and the combined data is relative specificity or the specificity of each ELISA relative to the RBT and CFT reactions primarily from regions with no history of *Brucella abortus* infection. The specificity for the Canadian data is actual specificity since the results were derived from cattle in Canada. Canada has been free of *Brucella abortus* infection in cattle since 1982.

TABLE IV. SPECIFICITY COMPARISON

	IELISA-ADRI	IELISA-IAEA	CELISA-OC	CELISA- sLPS
Argentina	96.28	98.14	98.14	98.14
Canada	99.40	na[2]	na[2]	99.90
Chile	99.28	99.59	99.69	99.59
Colombia	99.82	99.82	99.82	97.11
Costa Rica	95.76	94.95	93.35	95.76
Combined[1]	93.57	97.01	98.05	98.32

1. Combined data for all the countries except Canada. This data was not part of the original Canadian study.

The highest relative specificity of 99.82% for both IELISAs from Colombia is comparable to the actual specificity of 99.40% achieved by the IELISA-ADRI in the Canadian study. Data for the IELISA-IAEA and the CELISA-OC for Canada was not part of the original Canadian study and consequently is not available.

The specificity of the IELISAs and CELISAs relative to the RBT and CFT for the vaccinated population are presented in Table V. The relative specificity for each country and for the combined data of Chile, Colombia and Costa Rica are compared to each other and to the Canadian data. The largest difference in specificity was between the IELISA-ADRI and the CELISA-sLPS in the Canadian study. This was 41.4%. The difference between the IELISA-ADRI and the CELISA-sLPS for the data from Chile was 21.2%. In all cases, the specificity for the vaccinated population was greater for the CELISA-sLPS than for the IELISA-ADRI or the IELISA-IAEA, although for the IELISA-IAEA the differences were smaller. Similarly, the specificity of the CELISA-OC was greater than the IELISA-ADRI in all cases. However, the specificity of the IELISA-IAEA was greater than the CELISA-OC for Chile and for the data from Costa Rica with calf-hood vaccination. The maximum difference was 2.4%.

TABLE V. SPECIFICITY COMPARISON FOR VACCINATES

	IELISA-ADRI	IELISA-IAEA	CELISA-OC	CELISA- sLPS
Argentina	na[4]	na	na	na
Canada	56.30	na	na	97.7
Chile	78.82	96.85	94.44	100
Colombia	86.76	87.57	95.50	92.25
Costa Rica[2]	91.80	94.58	93.12	96.03
Costa Rica[3]	95.53	97.56	97.56	97.97
Combined[1]	90.53	94.55	96.08	96.51

1. Combined data for all the countries except Canada, Argentina
2. Calf-hood vaccination
3. Adult Vaccination
4. A separate vaccinated population for Argentina was not available

Cut-off values for each ELISA by country are presented in Table VI. The IELISA data is expressed as percent positivity. The CELISA data is expressed as percent inhibition. For example, the cut-off value for the IELISA-ADRI for Argentina is 67 percent positivity. Samples greater than or

equal to 67% are positive and samples less than 67% are negative on this IELISA. The lowest cut-off value for the IELISA-ADRI was 16%. The highest cut-off value for the IELISA-ADRI was 70%, a difference of 54%. Similarly, the lowest cut-off value for the IELISA-IAEA was 14% and the highest cut-off value was 73%, a difference of 59%. The difference for the CELISA-OC and the CELISA-sLPS were 17% and 26% respectively, indicating that the CELISAs were more specific for the negative population.

TABLE VI. COMPARISON OF CUT-OFF VALUES BY COUNTRY

	IELISA-ADRI	IELISA-IAEA	CELISA-OC	CELISA- sLPS
Argentina	67[1]	40	35	44[2]
Canada	30	na	na	30
Chile	16	21	18	27
Colombia	40	14	30	29
Costa Rica	70	73	20	18
Combined	41	41	26	29

1. Cut-off value is denoted as percent positivity
2. Cut-off values denoted as percent inhibition

Agreement between assays is compared in Table VII. The kappa statistic for each ELISA by country is presented. For example, the kappa indices of Argentina, Chile, Colombia and Costa Rica for the IELISA-ADRI and the IELISA-IAEA are 0.824, 0.963, 0.994 and 0.850, respectively, indicating good agreement between the IELISA-ADRI and the IELISA-IAEA despite the differences in the cut-off values. Except for Costa Rica, the kappa statistic for all assays indicated good agreement between assays. It is generally accepted that a kappa statistic greater than or equal to 0.8 indicates good agreement between assays. The kappa results for Costa Rica were not much lower than 0.8 and were all greater than 0.5 indicating agreement beyond chance.

TABLE VII. COMPARISON OF AGREEMENT BETWEEN ASSAYS

	IELISA-ADRI	IELISA-IAEA	CELISA-OC
	.850 Costa Rica		
	.994 Columbia		
	.963 Chile		
IELISA-IAEA	.824 Argentina		
	.756	.796	
	.989	.989	
	.972	.964	
CELISA-OC	.812	.910	
	.825	.793	.720
	.939	.939	.939
	.978	.965	.981
CELISA-sLPS	.855	.927	.931

The cut-off values of each ELISA by country were determined using a combination ROC analysis and frequency distributions. The ROC analyses are presented in Figures 1 to 5, along with the respective areas under the curve (AUC). For example, in Figure 1a, the optimal cut-off value for the IELISA-ADRI is 67%. In Figure 1b, the optimal cut-off value for the IELISA-IAEA is 40%. In Figure 1c, the optimal cut-off value for the CELISA-sLPS is 44% while an optimal cut-off value for the CELISA-OC in Figure 1d is 35%.

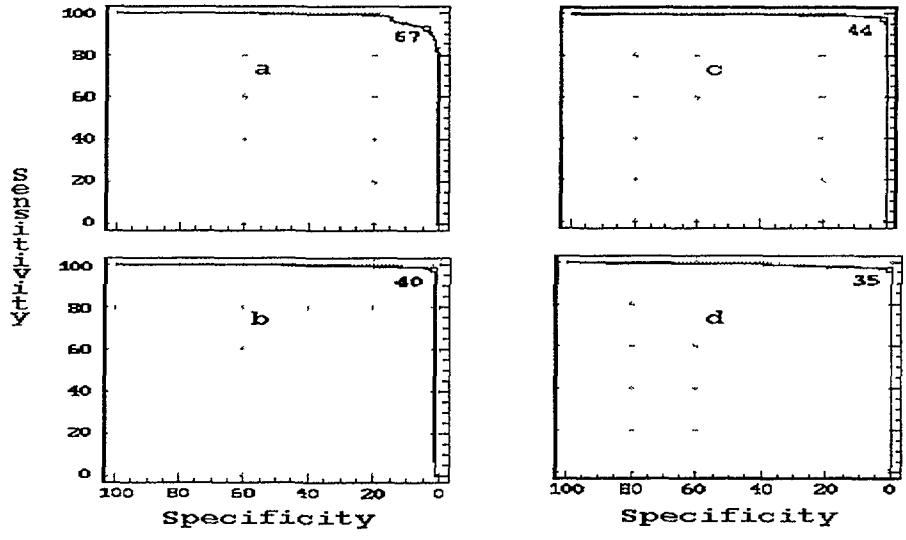

FIG.1 ROC-analysis for determination of most suitable cut-off for IELISA ADRI (a), IELISA IAEA (b), CELISA-sLPS (c), CELISA-OC (d) for Argentina

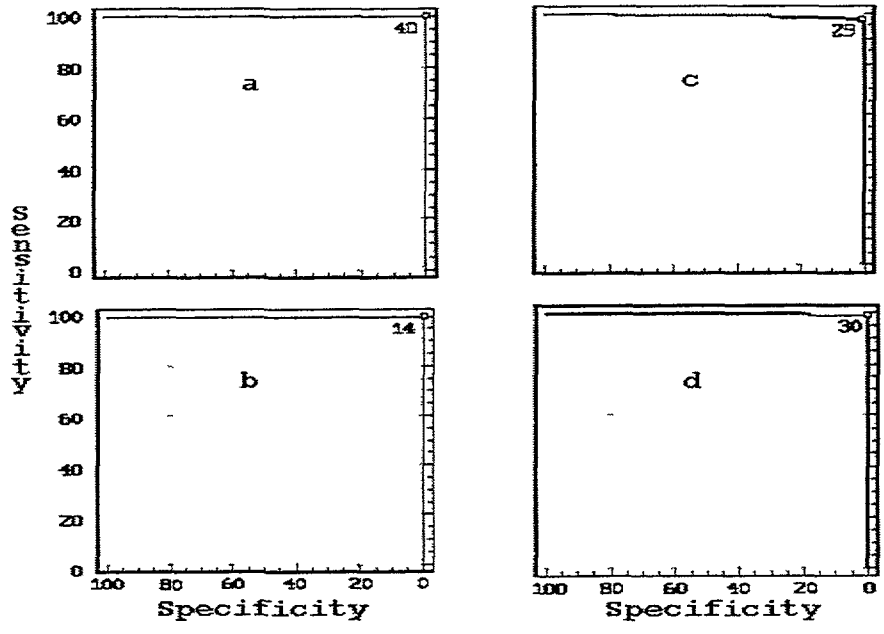

FIG 2. ROC-analysis for determination of most suitable cut-off for IELISA ADRI (a), IELISA IAEA (b), CELISA-sLPS (c), CELISA-OC (d) for Chile.

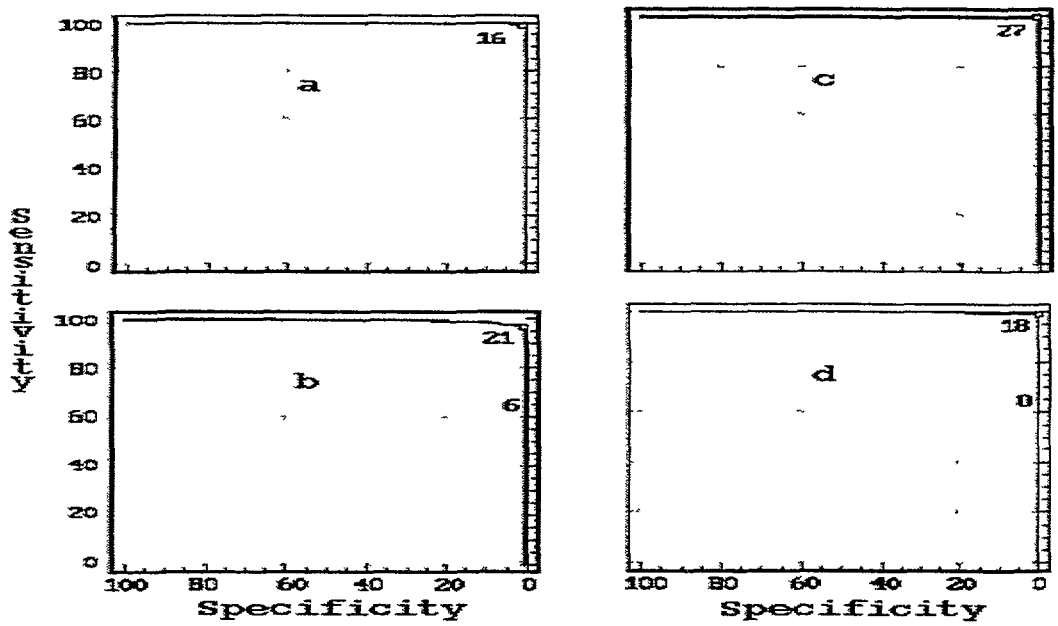

FIG 3. ROC-analysis for determination of most suitable cut-off for IELISA ADRI (a), IELISA IAEA (b), CELISA-sLPS (c), CELISA-OC (d) for Colombia.

The frequency distributions are presented in Figures 6 to 10. The frequency distribution for the IELISA-ADRI in 6a shows considerable overlap between the negative and positive populations. Using the cut-off as determined by ROC analysis, it is much easier to identify the false negatives. The

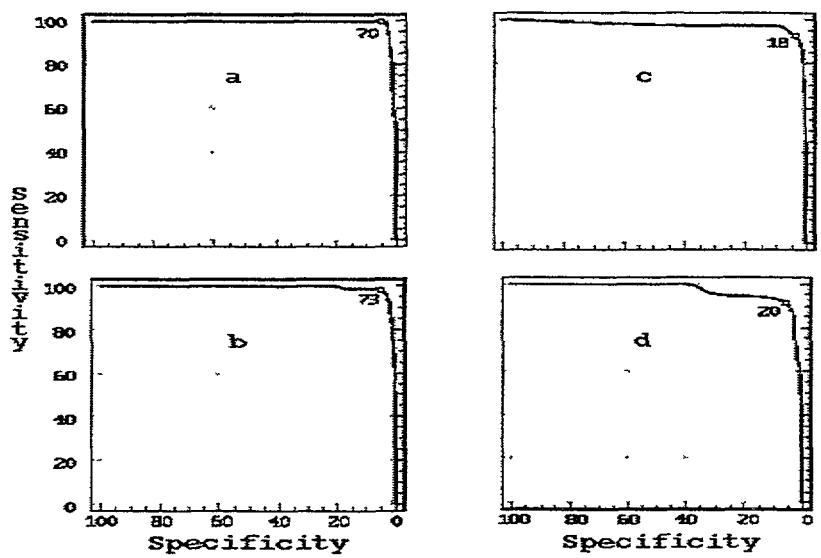

FIG.4. ROC-analysis for determination of most suitable cut-off for IELISA ADRI (a), IELISA IAEA (b), CELISA-sLPS (c), CELISA-OC (d) for Costa Rica.

same is true of the IELISA-IAEA, CELISA-OC and the CELISA-sLPS presented in figures 6b, 6c and 6d. The other frequency distributions for the other countries can be interpreted in similar fashion. *B. abortus* had been isolated. The highest relative sensitivity of 100% for both IELISAs from Colombia and for the IELISA-ADRI from Costa Rica indicate that it is comparable to the actual sensitivity achieved by the IELISA-ADRI in the Canadian study. Data for the IELISA-IAEA and the CELISA-OC for Canada was not part of the original Canadian study and consequently is not available.

Similarly, the specificity of negative populations presented in Table IV were defined. The data of the negative population from Argentina, Chile, Colombia, Costa Rica and the combined data is relative specificity or the specificity of each ELISA relative to the RBT and CFT reactions primarily from regions

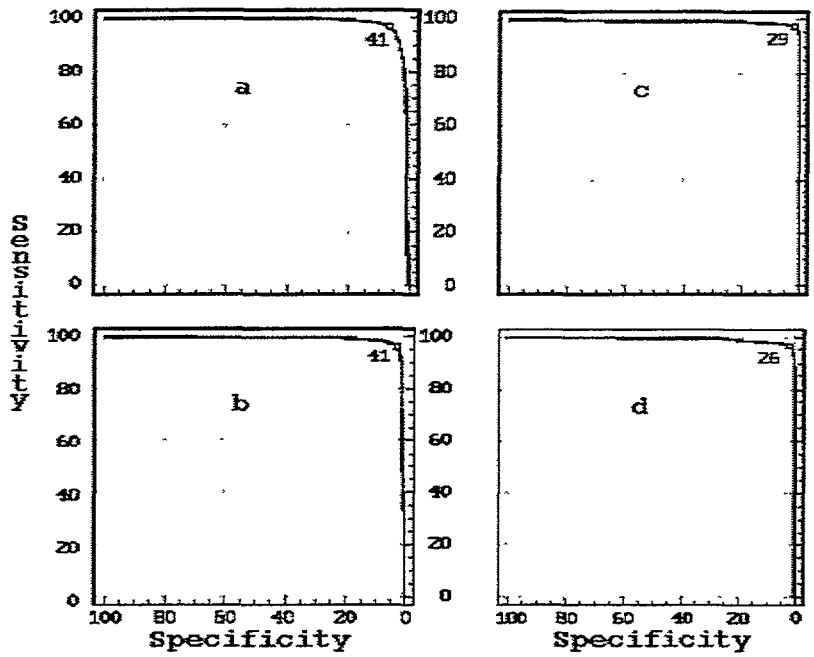

FIG.5. ROC-analysis for determination of most suitable cut-off for IELISA ADRI (a), IELISA IAEA (b), CELISA-sLPS (c), CELISA-OC (d) for Argentina, Chile, Colombia and Costa Rica combined.

The frequency distributions are presented in Figures 6 to 10. The frequency distribution for the IELISA-ADRI in 6a shows considerable overlap between the negative and positive populations. Using the cut-off as determined by ROC analysis, it is much easier to identify the false negatives. The same is true of the IELISA-IAEA, CELISA-OC and the CELISA-sLPS presented in figures 6b, 6c and 6d. The other frequency distributions for the other countries can be interpreted in similar fashion.

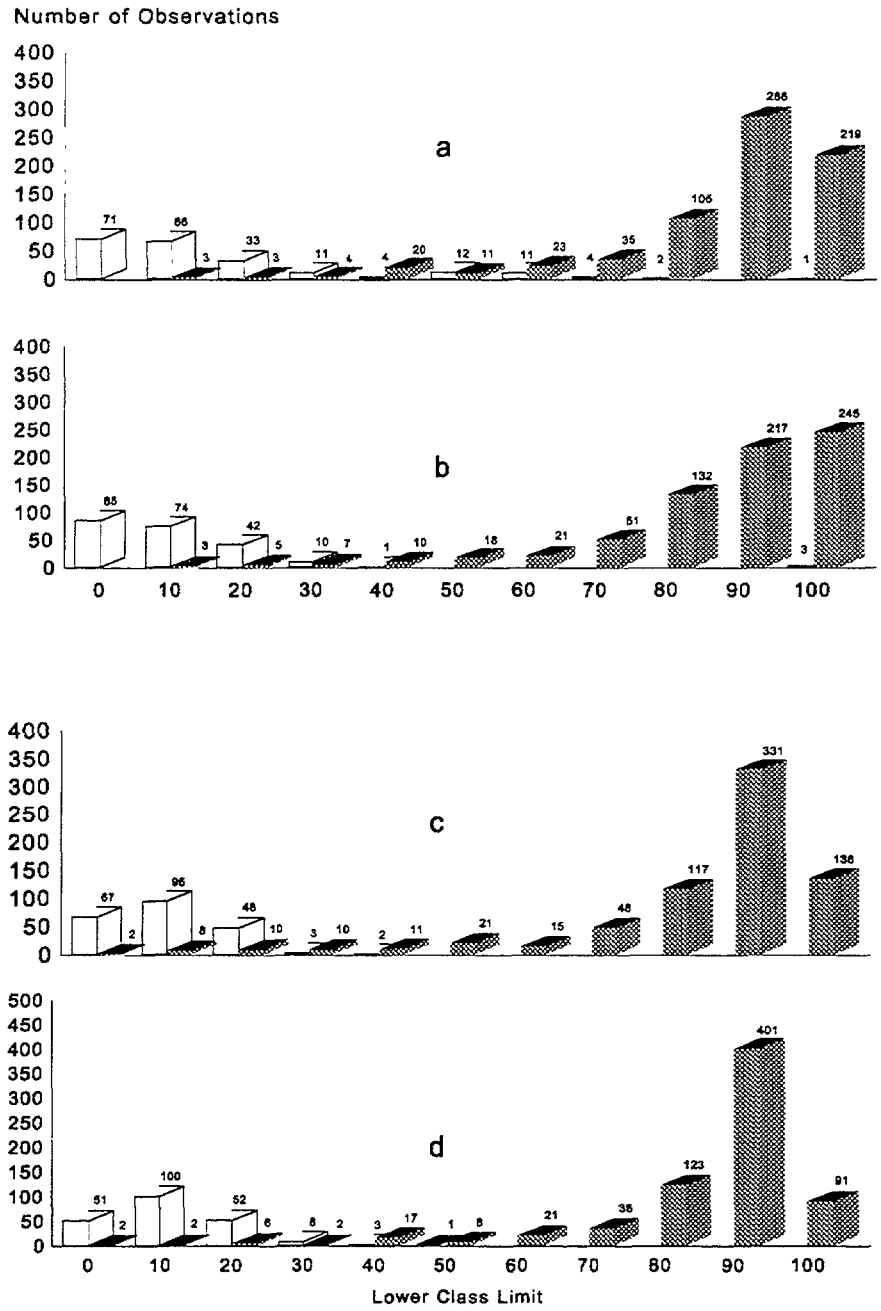

FIG.6. Frequency distribution of ELISA data from Argentina.

a. IELISA-ADRI
b. IELISA-IAEA
c. CELISA-OC
d. CELISA-sLPS

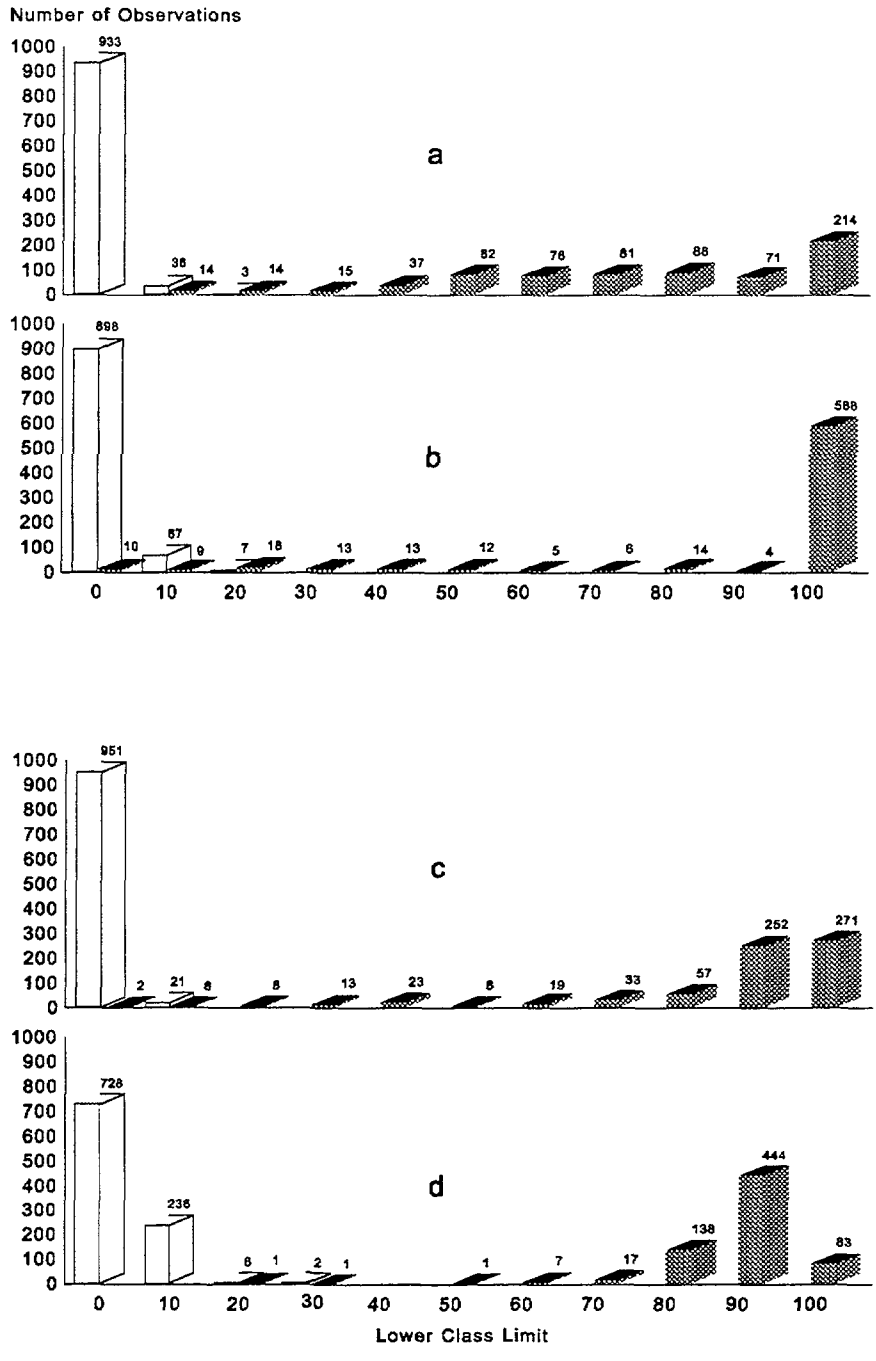

FIG. 7. Frequency distribution of ELISA data from Chile

a. IELISA-ADRI
b. IELISA-IAEA
c. CELISA-OC
d. CELISA-sLPS

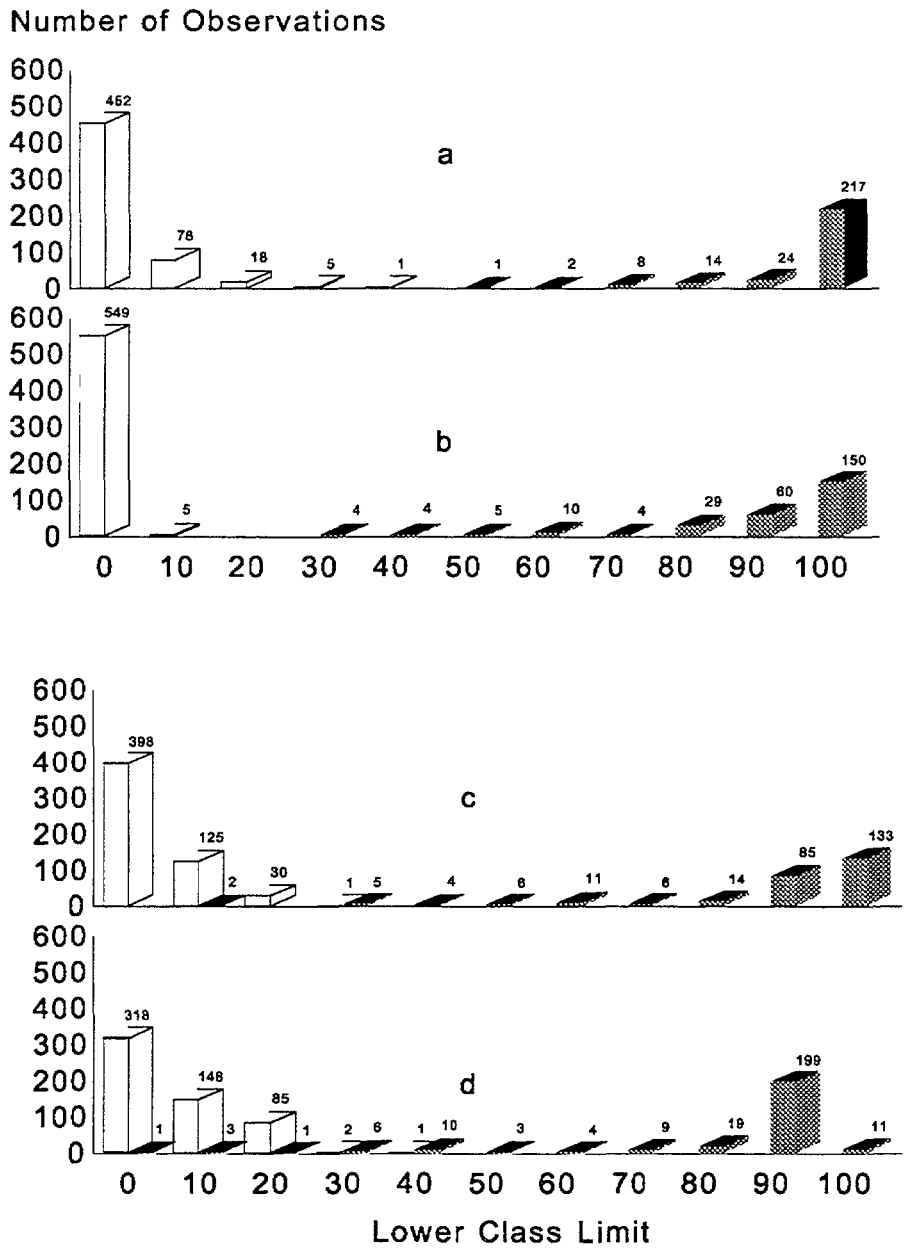

FIG.8 Frequency distribution of ELISA data from Colombia

a. IELISA-ADRI
b. IELISA-IAEA
c. CELISA-OC
d. CELISA-sLPS

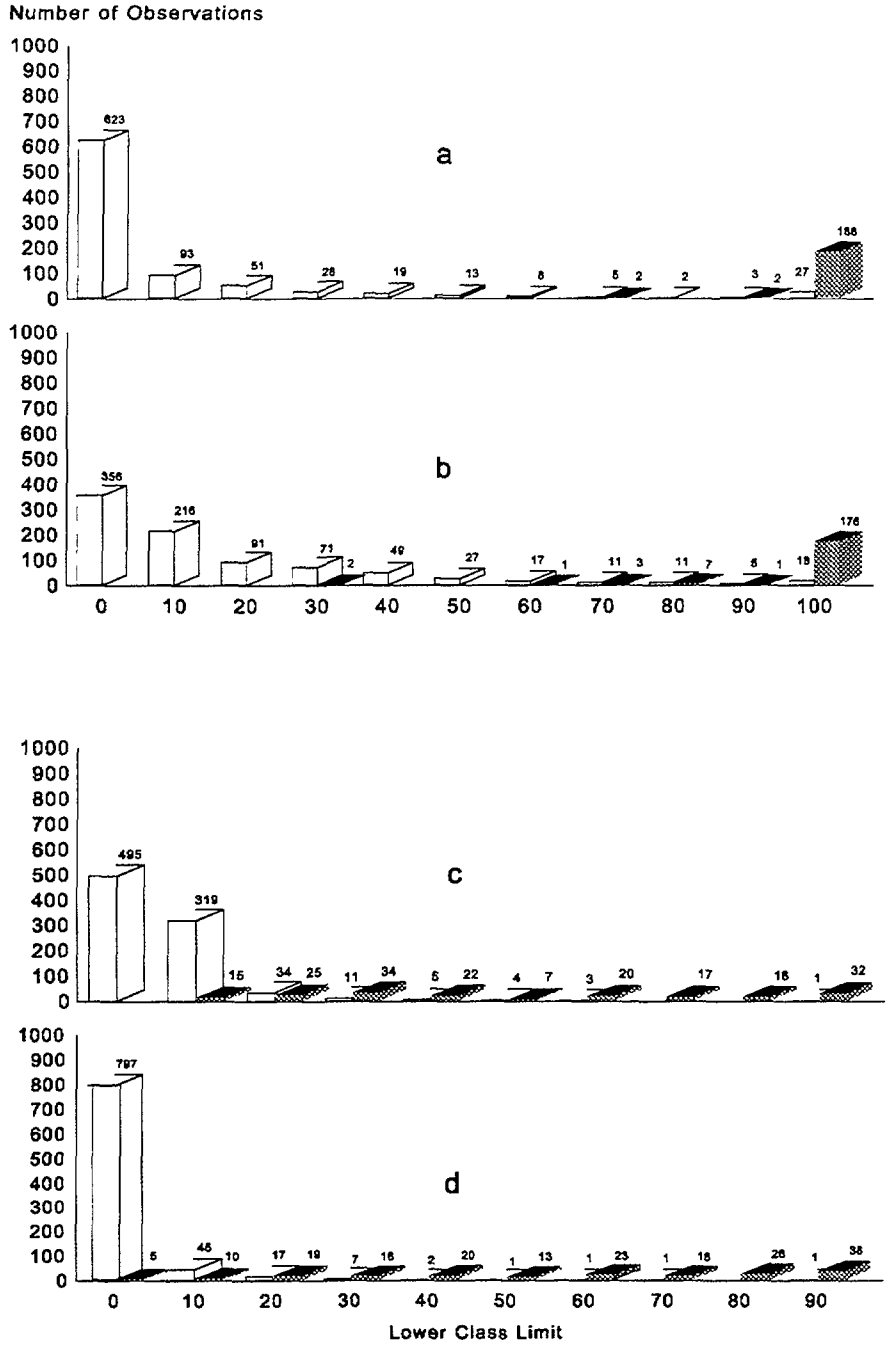

FIG. 9. Frequency distribution of ELISA data from Costa Rica.

a. IELISA-ADRI
b. IELISA-IAEA
c. CELISA-OC
d. CELISA-sLPS

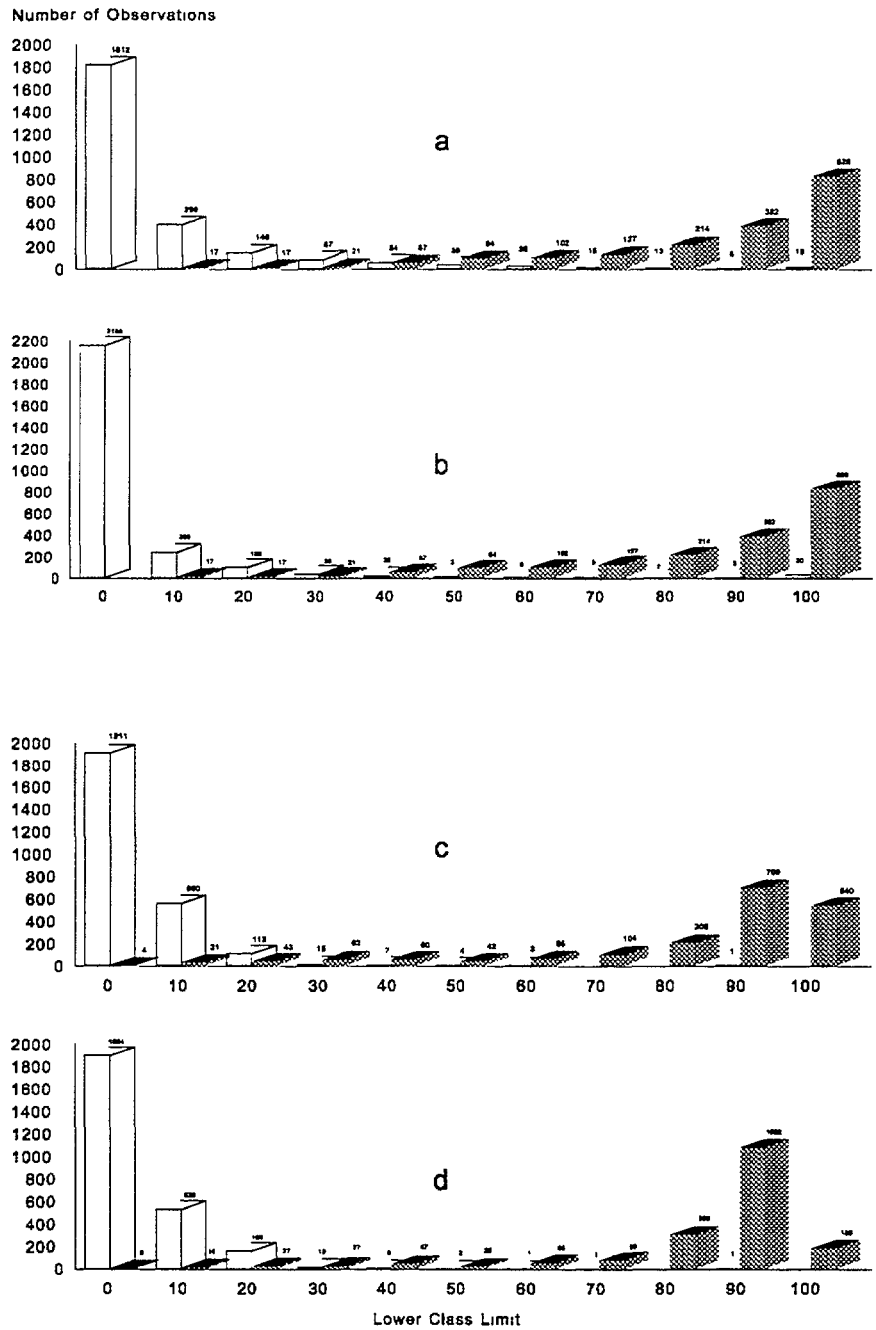

FIG 10 Frequency distribution of combined ELISA data from Argentina, Chile, Colombia and Costa Rica combined

a IELISA-ADRI
b IELISA-IAEA
c CELISA-OC
d CELISA-sLPS

4. DISCUSSION

Enzyme immunoassays (ELISA) have a distinct advantage over conventional serological tests, in that they are primary binding assays that do not rely on secondary properties of antibodies such as their ability to agglutinate or fix complement. Secondly, ELISAs can be tailored by using highly purified reagents such as antigens and monoclonal antibodies to be more specific.

In Canada, which is free of brucellosis in domestic animals, both the IELISA and the CELISA were recently validated [8]. Approximately, 8000 samples from cattle with no evidence of *B. abortus* infection were collected and tested in both the IELISA and CELISA. Similarly, 692 samples from cattle from which *B. abortus* was isolated from milk or tissues were also tested. Another 261 samples from cattle that were vaccinated with*B. abortus* strain 19 and contained residual antibodies were tested as well.

Unlike Canada, conditions in Latin America for validation of assays are different. It is more difficult to define negative and positive sera because diagnosis is based on the isolation of *B. abortus* from herds rather than from individual cattle. In most countries, areas overlap between regions free of *B. abortus* and regions that contain infected herds and strain 19 vaccination is widely practised. For these reasons and for consistency, the negative and positive sera were defined based on the RBT and the CFT reaction in each country under study. As well, determining the *B. abortus* strain 19 vaccination statuses of cattle is sometimes difficult due insufficient data being available including the time of vaccination, the number of times cattle were vaccinated and identification of cattle that were vaccinated. The number of samples defined as positive, negative and vaccinated are tabulated in Table 2.

Comparison of relative assay sensitivities are summarised in Table III. The results are not dissimilar to the results obtained in the Canadian study [8]. Both the IELISA and the CELISA achieved a sensitivity estimate of 100% in Canada. The results obtained in Latin America were comparable. Sensitivity values obtained, ranged from 92.10% for the CELISA-OC in Costa Rica, to 100% for the CELISA-sLPS in Chile, the IELISA-ADRI in Colombia, the IELISA-IAEA in Colombia and the IELISA-ADRI in Costa Rica. When the data was combined for all countries (except Canada) the performance of both CELISAs was marginally better than the IELISAs (presented in Table III). The maximum difference between the CELISAs and the IELISAs for the combined data is 1.19%. The CELISA-sLPS at 97.47% detects 11.9 more positives per 1000 animals than the IELISA-IAEA at 96.28%.

Comparison of relative assay specificities are presented in Table IV. The specificity for the IELISA in Canada was 99.40%, while the specificity for the CELISA was 99.90% [7,8]. The results obtained in Latin America were similar. The lowest specificity achieved was 93.35% for the CELISA-OC in Costa Rica. The highest specificity achieved was 99.82% for the IELISA-ADRI, IELISA-IAEA and the CELISA-OC in Colombia. When the data was combined for all the countries (except Canada), it is obvious that the overall performance of both CELISAs is better than the IELISAs presented in Table IV. The maximum difference between the CELISAs and the IELISAs for the combined data is 4.75%. The CELISA-sLPS at 98.32% is more specific than the IELISA-ADRI at 93.57%. Thus the IELISA-ADRI detected 47.5 more animals per 1000 animals than the CELISA-sLPS.

Comparison of the relative assay specificities for vaccinated cattle is tabulated in Table V. The results of the Canadian study indicated that the CELISA-sLPS was capable of distinguishing animals that were vaccinated or negative from those that were infected in the majority of the cases. In the Canadian study, the specificity of the IELISA-ADRI was 56.30% while the specificity for the CELISA-sLPS was 97.70%. Similar results were achieved in Latin America. In Chile, the specificity for the IELISA-ADRI was 78.82% while the specificity for both CELISAs were 94.44% and 100%. In Colombia, the specificity for both IELISAs was 86.76% and 87.57%, respectively. The specificity for both CELISAs was 95.50% and 92.25%. The combined data clearly indicates that the specificity of the CELISAs as presented in Table V are better than the IELISAs for distinguishing vaccinal antibody. The maximum difference between the CELISAs and the IELISAs for the combined data is 5.98%. The CELISA-sLPS for the combined data at 96.51% is more specific than the IELISA-ADRI at 90.53%. The CELISA-sLPS detects 59.8 fewer vaccinated animals per 1000 animals than the IELISA-ADRI.

Ideally, harmonisation of cut-off values should be the same in each country for the Ileitis or for the Celsius. However, analysis of data indicated that this was not possible. The cut-off values for each country and for the combined data were determined using ROC analysis presented in Figures 1 to 5 and tabulated in Table VI. From the Table, the only assay that had cut-off values approximating the 30% chosen for Canada was the CELISA-slaps, except for Costa Rica. The frequency distributions presented

in Figures 6 to 10 show the difficulty in choosing an optimal cut-off value for each assay For instance, most of the frequency distributions for the IELISA have some overlap between the negative and the positive population The exceptions to this were the frequency distributions from Colombia The reason for the binomial distribution is due to better separation of the negative and positive sera The sera were from definite areas free from *B aborts* infection and from areas of relatively high prevalence of infection Despite the differences in how the IELISA-ADRI and IELISA-IAEA were performed, the distribution patterns were very similar This became quite evident when examining the frequency distributions of the combined data for the ileitis presented in Figure 10 The distribution patterns of the celsius, although different from the IELISAs, were similar to each other and again the similarity was quite evident from the frequency distribution of the combined data presented in Figure 10 Choosing a cut-off value solely on the basis of frequency distribution could give erroneous relative sensitivity and specificity values The frequency distributions of the CELISAs were marginally better than the IELISAs due to less overlap between the negative and the positive populations However, obtaining the optimal sensitivity and specificity for each assay in each country was best determined using ROC analysis and frequency distributions together to get a clearer picture in each instance

The ROC curves presented in Figures 1 to 5 all had areas under the curves (AUC) greater than 0 95 An AUC of 0 95 indicates that a randomly selected individual animal from a positive population will have a test value greater than that of a randomly selected individual animal from the negative population 95% of the time The lowest AUC was 0 969 for the CELISA-OC in Costa Rica, while the highest AUC was 1 000 for the IELISA-ADRI, the CELISA-sLPS, the CELISA-OC in Chile and the IELISA-ADRI and the IELISA-IAEA in Colombia Both CELISAs for the combined data had an AUC of 0 995 which was approximately 1% better than the IELISAs

Finally, a comparison of agreement between assays was calculated and presented in Table VII A kappa statistic of 1 indicates perfect agreement between assays A kappa of 0 5 indicates agreement beyond chance It is generally accepted that kappa indices greater than or equal to 0 8 indicate good agreement between tests The best agreement was 0 994 between the IELISAs in Colombia Again this is probably due to better separation of the negative and positive populations The lowest kappa statistic was 0 720 between the CELISAs from Costa Rica where separation of negative and positive populations was more difficult The highest kappa for both CELISAs was 0 981 from Chile Overall, the kappa statistic for all the assays were good indicating good agreement among all assays

Generally, the technical performance of the assays were good and the results were similar to results obtained in the Canadian study However, there are some reasons why the results could be improved Firstly, a bias was introduced in the study The negative and positive sera were defined according to the RBT and CFT reactions The RBT can produce false positive results, which when used to define sera can affect the sensitivity of the assay being validated Secondly, a better separation of the negative and positive population would have produced better results For example, if individual animals with proven infection based on isolation of the organism had been selected instead of positive animals from infected herds, the sensitivity values should have been higher Thirdly, the RBT and the CFT both detect antibody resulting from *B abortus* strain 19 vaccination or from exposure to cross-reacting antigens Therefore, the results are biased against the CELISAs which eliminate many such reactions

Sensitivity is defined as the ability on an assay to detect a true positive in a diseased population, while specificity is defined as the ability to detect a true negative in a non-diseased population Based on the combined data the CELISA-sLPS was the best performing ELISA It detected 1 19% more positives in the positive population, 4 75% fewer positives in the negative population and 5 98% fewer positives in the vaccinated population The implication of this is important For example, in a population of 15,000,000 animals with a high incidence of brucellosis the CELISA-sLPS would detect 712,500 fewer false positives and 897,000 fewer false positives if vaccination were part of the control program By using the CELISA-sLPS as the primary screening assay in an eradication and control program significant savings in repeat testing and elimination of other conventional assays can be realised In addition, the CELISA-sLPS is less costly in reagents than conventional assays and has excellent quality control leading to additional savings

ACKNOWLEDGEMENTS

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REFERENCES

[1] CARLSSON, H.E., HURVELL, B. AND LINDBERG, A.A., Enzyme linked immunosorbent assay (ELISA) for titration of antibodies against *Brucella abortus* and *Yersinia enterocolitica,* Acta Path. Micro. Scand. **84** (1976) 168-176.

[2] MACMILLAN, A., Conventional serological tests, In Animal Brucellosis, Ed. Nielsen, K.and Duncan J.R. CRC Press, Boca Raton, Fl. (1990) 153-197.

[3] WRIGHT, P.F., NIELSEN, K. AND KELLY W., Primary binding assay techniques for the serodiagnosis of bovine brucellosis - enzyme immunoassay, In Animal Brucellosis, Eds. Nielsen, K. and Duncan J.R. CRC Press, Boca Raton, Fl. (1990) pp 199-235.

[4] NIELSEN, K., CHERWONOGRODZKY, J., DUNCAN, J.R. AND BUNDLE D.R., Enzyme immunoassay for the differentiation of the antibody response of *Brucella abortus* infected or strain 19 vaccinated cattle, Am. J. Vet. Res. **50** (1989) 5-9.

[5] MACMILLAN, A., GREISER-WILKE, I., MOENNIG, V. AND MATHIAS, L., A competitive enzyme immunoassay for brucellosis diagnosis, Dtsch. Tierarztl. Wochenschr. **97** (1990) 83-85.

[6] NIELSEN, K.H., GALL, D., KELLY, W., HENNING, D. AND GARCIA, M.M., Enzyme immunoassay: application to diagnosis of bovine brucellosis, Monograph, Agriculture Canada, Nepean, Ontario, (1992) ISBN Number 0-662-19838-7.

[7] NIELSEN, K., GALL, D., KELLY, W., VIGLIOCCO, A., HENNING, D. AND GARCIA, M., Immunoassay development: Application to enzyme immunoassay for diagnosis of Brucellosis. Monograph, Agriculture and Agri-Food Canada, Animal Diseases Research Institute, Nepean, Ontario, (1996) ISBN 0-662-24163-0.

[8] NIELSEN, K.H., KELLY, L., GALL, D., BALSEVICIUS, S., BOSSÉ, J., NICOLETTI, P., AND KELLY, W., Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis. Preventive Veterinary Medicine **26** (1996) 17-32.

[9] USDA, Supplementary test procedures for the diagnosis of brucellosis. In: NADL Diagnostic Reagents Manual, US Department of Agriculture, National Animal Diseases Laboratory, Diagnostic Reagents Division, Ames, IA, (1965) 65C-65E.

[10] US DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, Standardised diagnostic complement fixation and adaptation to the microtest, Public Health Monograph N74, US Department of Health, Education and Welfare (1965).

[11] NIELSEN, K.H., WRIGHT, P.F., KELLY, W.A. AND CHERWONOGRODZKY, J.H., A review of enzyme immunoassay for the detection of antibody to *Brucella abortus* in cattle, Vet. Immunol. Immunopathol. **18** (1988) 331-347.

[12] GALL, D. AND NIELSEN, K., Improvements to the competitive ELISA for the detection of antibody to *Brucella abortus* in cattle, J. Immunoassay **15** (1994) 277-291.

[13] SCHOOJANS, F., ZALATA, A., DEPUYDT, C.E. AND COMHAIRE, F.H., MedCalc: a new computer program for medical statistics, Computer Methods and Programs in Biomedicine **48** (1995) 257-262

COMPARATIVE EVALUATION OF COMPETITIVE ELISA TEST IN COLOMBIAN CATTLE

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Abstract

COMPARATIVE EVALUATION OF COMPETITIVE ELISA TEST IN COLOMBIAN CATTLE

In order to contribute to the definition of the best ELISA test for screening and differential diagnosis of *Brucella abortus* to be applied for control programmes, a total of 2971 sera from Colombian cattle were tested for brucellosis Conventional agglutination tests, Buffered Plate antigen test (BPAT) and Rose Bengal (RB) as well as Complement Fixation test (CFT) (Alton, et al 1988) were used comparatively Radial immunodiffusion test (RID) was also performed to all sera The sera were also tested using four different ELISAs indirect ELISA from FAO/IAEA and the indirect ELISA modified by Nielsen, et al 1992 as well as two competitive ELISAs one competitive ELISA used *B abortus* O-polysaccharide antigen and an enzyme conjugated monoclonal to the O-polysaccharide for competition and detection The second competitive ELISA used lipopolysaccharide (sLPS) antigen, a different monoclonal antibody for competition but also specific for the O-polysaccharide and a commercially available goat anti-mouse IgG enzyme conjugate for detection The sera were analyzed based on its population status, 987 positive obtained from *Brucella abortus* infected herds based on clinical and/or bacteriological evidence and a high prevalence of brucellosis, CFT percentage of positive animals in the herd was greater than 5% Eight hundred sixty six (866) negative sera from non-vaccinated cattle from a brucellosis free area and 1118 negative sera obtained from reglamentary vaccinated areas under a free herd program Initial cut-off values were derived using negative serum samples The diagnostic sensitivity and specificity was defined from frequency histograms based on this cut-off values and using 2x2 tables, corresponding confidence limits (95%) were calculated The data were also analysed using signal detection analysis (ROC) Kappa statistics was determined for all tests and populations, accuracy was used as index of comparison to evaluate different assays The data support the initial hypothesis that the ELISA methodology designed for brucellosis will provide more precise and standardised method for diagnosis and for the support of control and eradication campaigns

1. INTRODUCTION

Animal diseases affect directly health and economy in all the countries of the world In order to eliminate them it is important to develop control programs based on specific and opportune diagnosis [1]. To obtain this goal very precise diagnostic tests have been developed. Enzyme linked immunosorbent assays (ELISA) have the characteristics of high sensitivity and specificity, they are quick and economic. In the vast majority of its applications they are comparable and superior to most of the conventional diagnostic tests. They are versatile, permit mass screening of livestock, have become simple and objective results are obtained based on computerised programs [2,3,]. Since 1987, the Joint FAO/IAEA Division of the International Atomic Energy Agency has initiated a Coordinated Research Programme to evaluate the use of ELISA as diagnostic support for diseases of mayor importance in livestock production in Latin America [4,5].

In Brucellosis diagnosis, basically two main types of immunoassays are used for these purposes, the indirect and the competitive formats [6,7] It was hoped that the introduction of the indirect ELISA for brucelosis [8] would overcome some of the problems with conventional tests, but the indirect ELISA while more sensitive than the conventional tests, has been less specific than expected, even using highly specific monoclonal antibodies as detection reagent for bovine IgG1 Similarly the indirect ELISA can not distinguish vaccinal antibody from that arising from infection [9,10].

The competitive ELISA, O-chain [11,12] proved more specificity than the indirect [13] and apparently discriminates between vaccinal and infection antibodies. The sLPS antigen modification of the competitive assay attempts to reduce cost and time to obtain results with similar sensibility and specificity [14,15].

The purpose of this study is to contribute to the validation of immunoassays for brucellosis diagnosis and control programs.

2. MATERIALS AND METHODS

2.1. Sera

Three different kind of animal populations were selected to provide validation:

2.1.1. Negative population: 866 sera collected from an area of the country in which no clinical disease have been detected.

2.1.2. Vaccinated negative: In an area of low prevalence, 1,76% (ICA, 1994), 1118 sera were obtained from randomly selected herds under a free herd control program established by the Animal Health authorities. The animals are vaccinated between 3 to 9 months of age and the herd maintained under control and considered free after two consecutive years of negative serology by the conventional tests.

2.1.3. Positive population: A total of 987 sera were obtained from herds with clinical signs of the disease and from which isolation of *Brucella abortus* biotype 1 was performed or herds with clinical signs and greater than 5% percent of CFT positive serology. The whole herd was bled after the positive isolation was confirmed and considered as positive

2.2. Serological Tests

Buffered plate antigen test (BPAT), Rose Bengal (RBT) and Complement Fixation Test (CFT) were performed as described by Alton et al., 1988. CFT was considered as reference test for comparative studies of sensibility and specificity. Radial immunodiffusion using O-chain from *Brucella mellitensis* antigen prepared as reported by Díaz et al., 1976, was used.

Four different ELISA tests were used in order to make the validation of the competitive assay. Indirect ELISA both FAO/IAEA kit [18] as well as ADRI modification [13] were performed on all sera collected.

Briefly the test uses *B. abortus* purified sLPS [13] antigen and monoclonal anti-bovine-IgG1 conjugate labelled with Horseradish peroxidase to detect the reactive sera. The difference between the two versions is essentially sera concentration and volume of reagents. The FAO/IAEA tests is stopped while ADRI is read at 10 minutes without stopping and the buffer uses EDTA/EGTA to reduce non-specific binding [19].

A targeted competitive ELISA [11], which was more specific than the indirect ELISA and which discriminate vaccinal from infection antibodies using O-chain as antigen [20] was performed. A second approach using sLPS as antigen and competition between the sera and the non-labelled anti-O-chain monoclonal [15] was also used to run all the sera.

All the ELISA plates were read at 405-414nm, using Multiskan Mark Plus II, under the computer control of the respective FAO/IAEA BRELISA and C-ELISA programs. Only plates which were accepted by the program were considered for analysis.

2.3. Statistical Analysis

Different statistical analysis were used to compare the data from the various assays [21]. Based on previously defined cut-off values for each ELISA, calculated on the negative population [13], 2x2 tables were used to evaluate diagnostic relative specificity and sensitivity using CFT as reference test. Sorted data were plotted for defined negative and positive sera for frequency histograms. Confidence limits (95%) were calculated. ROC analysis was performed by statistics program, to confirm and optimise the cut-off point definition. Kappa statistics was determined for all the tests and populations, index of comparison, accuracy, were used to compare the different assays [22,23].

3. RESULTS AND DISCUSSION

From the programmed 3000 sera to be tested, a total of 2971 were evaluated for the final report. All ELISA tests, as well as the conventional BPAT, RB and CFT were performed to this sera Table I shows the relative sensitivity and specificity obtained for the infected herds confirming the higher sensitivity of the ELISA tests to detect the infection as compared with the other conventional tests used in the analysis [24,25]. Low values of specificity observed for ADRI ELISA indirect and Competitive ELISA version II were not expected based on the improved characteristics of this tests regarding non-specific reactions [15], but Complement fixation test could not detect positive sera with low level of antibodies [26].

TABLE I. RELATIVE SENSITIVITY AND SPECIFICITY AS COMPARED WITH COMPLEMENT FIXATION TEST IN POSITIVE POPULATION , N=987

Test	Sensitivity %	CL* %	Specificity %	CL* %
Rose Bengal, AT	87.00	2.0	87.00	2.0
BPAT	78.17	2.5	82.10	2.3
I-ELISA-IAEA	95.81	1.2	81.80	2.4
I-ELISA-ADRI	98.07	0.8	71.16	2.8
C-ELISA-1	95.81	1.2	83.57	2.3
C-ELISA-2	93.24	1.5	77.07	2.6
RID	85.40	1.4	99.90	0.2

* CL= 95%Confidence limits

When the analysis included the negative population, (Table II) higher specificity was observed and the capacity of the ELISA tests to detect the positive animals in regard to Complement Fixation Test could be evaluated more clearly. From Table II, it is also important to consider that competitive ELISAs versions 1 (O-chain) and 2, appears to be good assays for the differentiation between vaccinated and infected animals due to their high sensitivity and specificity compared with the low sensitivity of the differential RID test [17, 19].

TABLE II RELATIVE SENSITIVITY AND SPECIFICITY AS COMPARED WITH COMPLEMENT FIXATION TEST IN POSITIVE AND NEGATIVE POPULATION , N=2971

Test	Sensitivity %	CL* %	Specificity %	CL* %
Rose Bengal, AT	86.83	1.2	96.56	0.65
BPAT	78.13	1.4	83.97	1.31
I-ELISA-IAEA	95.81	0.72	94.62	0.81
I-ELISA-ADRI	98.07	0.50	89.28	1.1
C-ELISA-1	95.81	0.72	93.87	0.86
C-ELISA-2	93024	0.90	90.82	1.03
RID	85.40	1.4	99.90	0.2

* CL= 95%Confidence limits

When the analysis was done on population selected based on the conventional tests, (Table III) the competitive ELISA tests proved to have higher performance [15].

TABLE III. RELATIVE SENSITIVITY AND SPECIFICITY AS COMPARED WITH COMPLEMENT FIXATION TEST IN POSITIVE AND NEGATIVE SELECTED SERA, N=2137

Test	Sensitivity %	CL* %	Specificity %	CL* %
I-ELISA-IAEA	100		99.11	0.3
I-ELISA-ADRI	100		96.08	0.8
C-ELISA-1	99.55	0.3	97.39	0.6
C-ELISA-2	99.54	0.3	95.56	0.8
RID	93.4	0.5	99.9	0.2

* CL= 95%Confidence limits

Table IV shows the different cut-off values [27] calculated and defined using different approaches and no significant differences could be seen for the different tests except for the CELISA2.

TABLE IV. CUT-OFF VALUES OR DECISION LIMITS (POSITIVE/NEGATIVE THRESHOLD) BASED ON NEGATIVE "FREE AREA" POPULATION

ELISA Test	Pre-established [1]	Local [2]	ADRI selected [3]	ROC [4]
IELISA-IAEA	35	30	30	20
IELISA-ADRI	46	44	50	50
CELISA1	30	33	30	30
CELISA2	30	37	29	29

1 Defined by the IAEA and ADRI
2 Defined based on the entire uninfected population from San Andrés. Colombia, N=866
3 Defined based on selected (BPTA/RB/CFT (-)) negative population, N=842
4 Defined based on Received Operation Characteristic (ROC) analysis, N=1064

Figures 1, 3, 5 and 7 show the frequency distribution of the selected sera evaluated for each of the ELISA tests under study. In Figures 2,4,6 and 8 values for optimisation of the cut-off are plotted as ROC analysis.

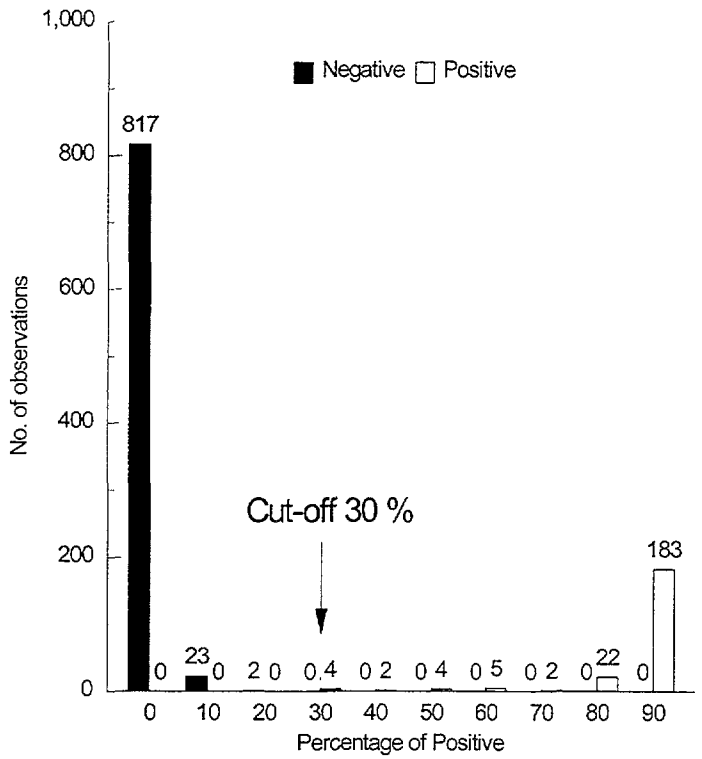

FIG. 1. Frequency distribution Brucella abortus I-ELISA-IAEA (BPRT/RB/CFT N=1064).

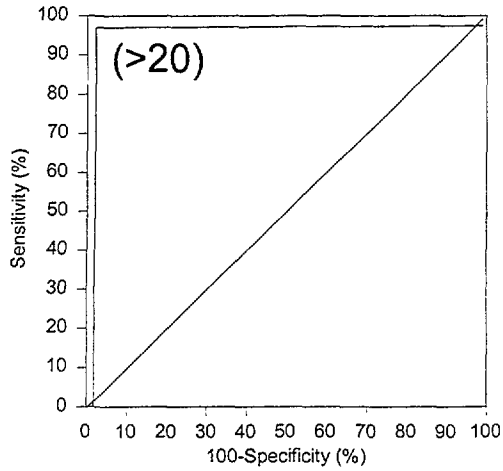

FIG. 2. ROC curve I-ELISA-IAEA.

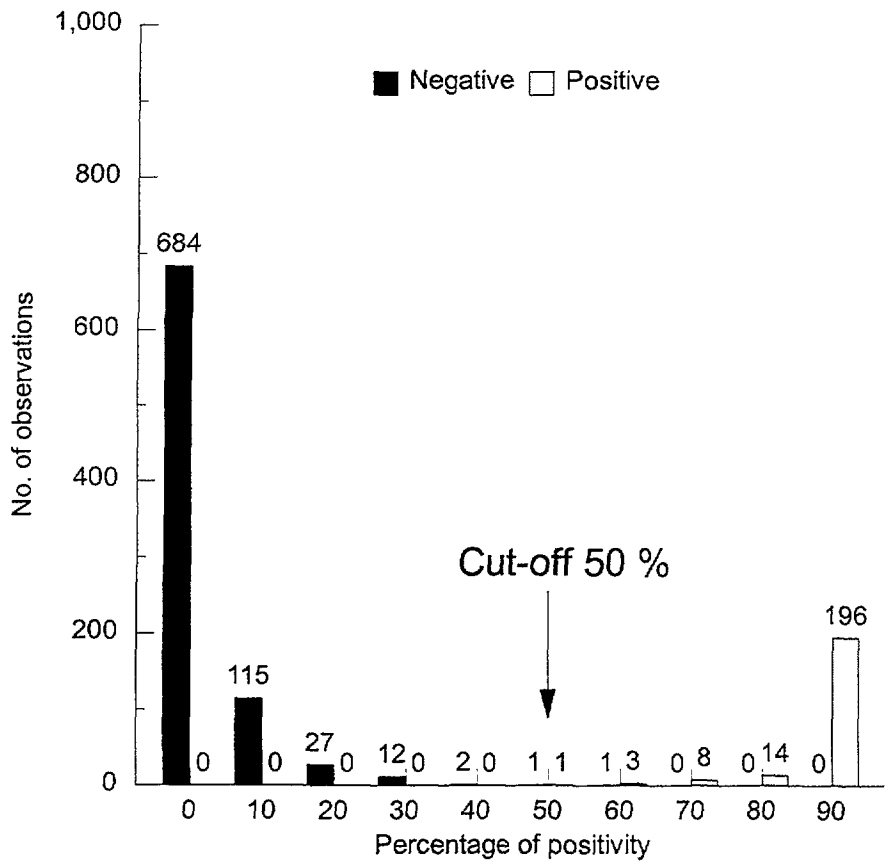

FIG. 3. Frequency distribution Brucella abortus I-ELISA-ADRI (BPRT/RB/CFT N=1064).

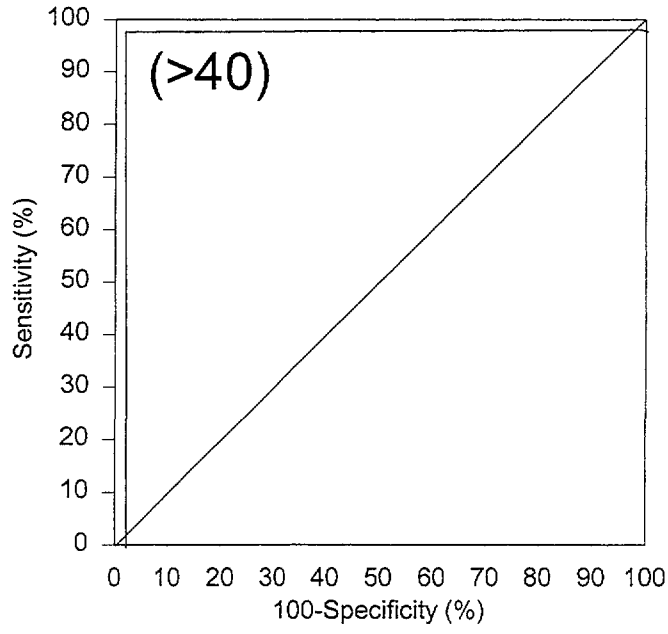

FIG.4. ROC curve I-ELISA-ADRI.

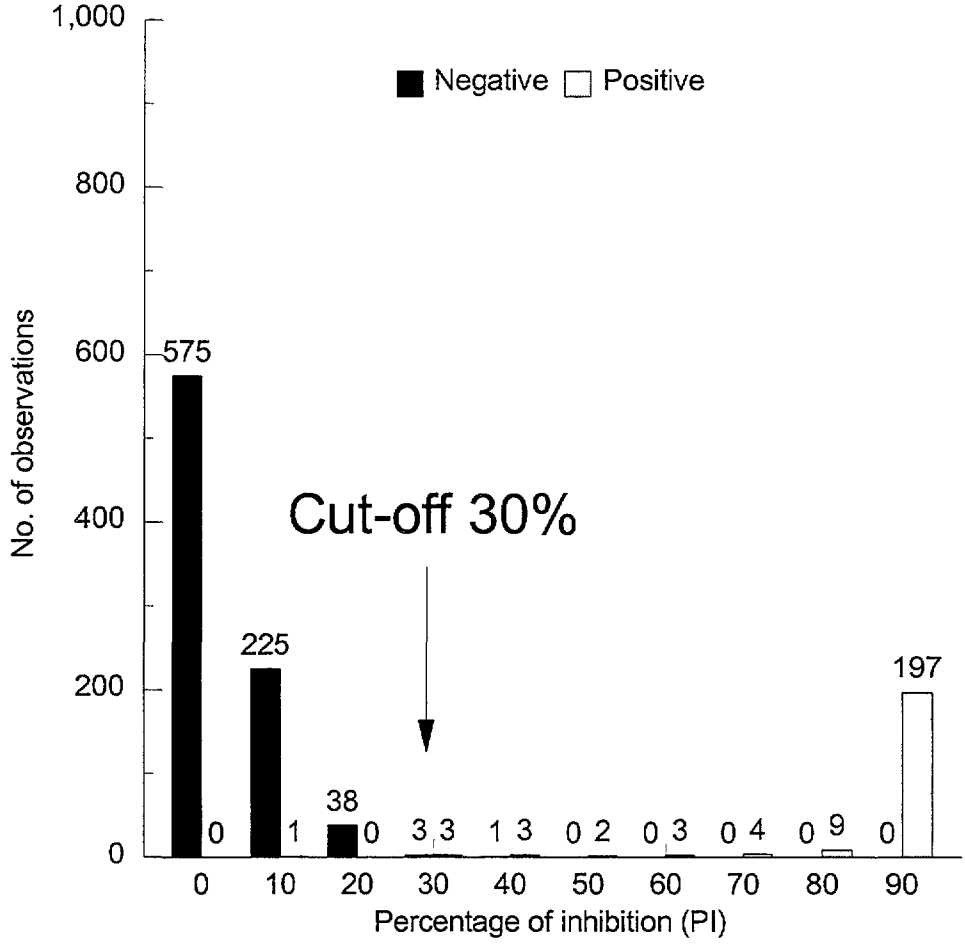

FIG. 5. Frequency distribution Brucella abortus C-ELISA-1 (BPRT/RB/CFT N=1064).

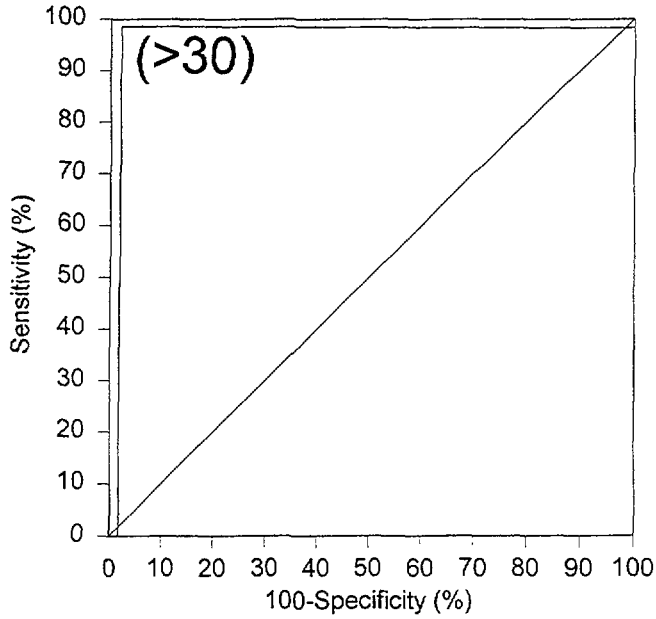

FIG. 6. ROC curve C-ELISA-1.

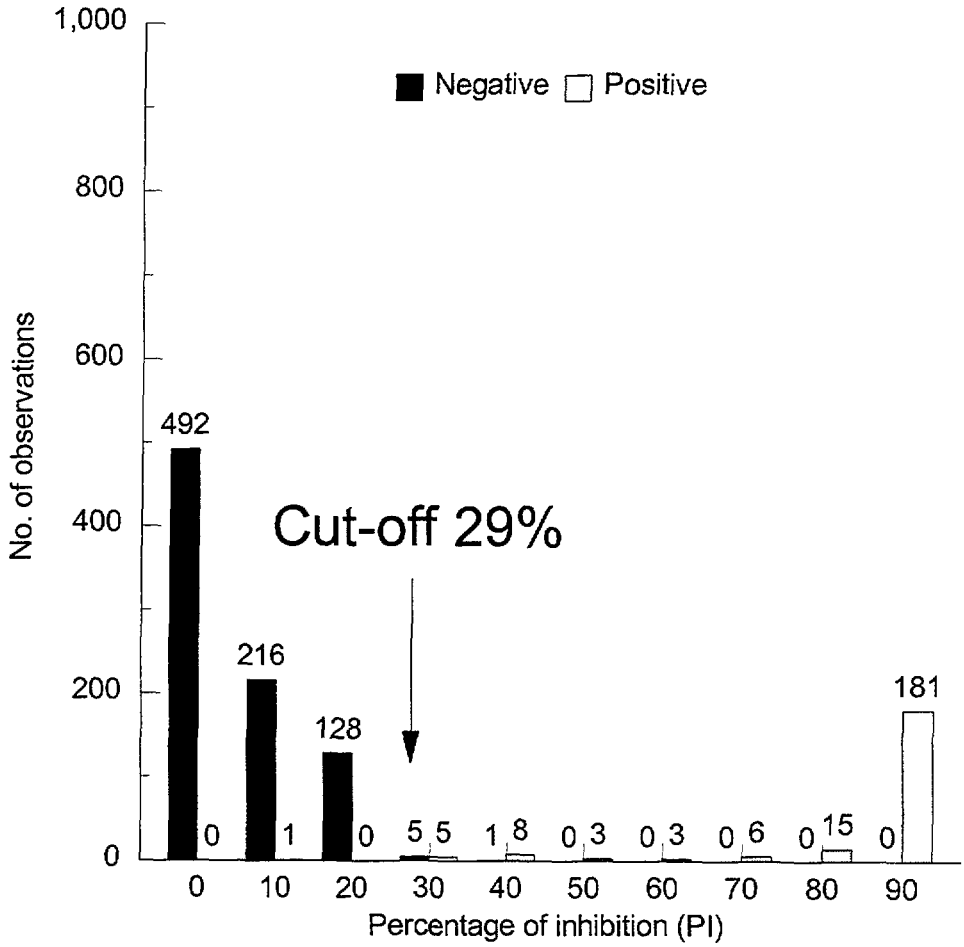

FIG. 7. Frequency distribution Brucella abortus C-ELISA-2 (BRT/RB/CFT N=1064).

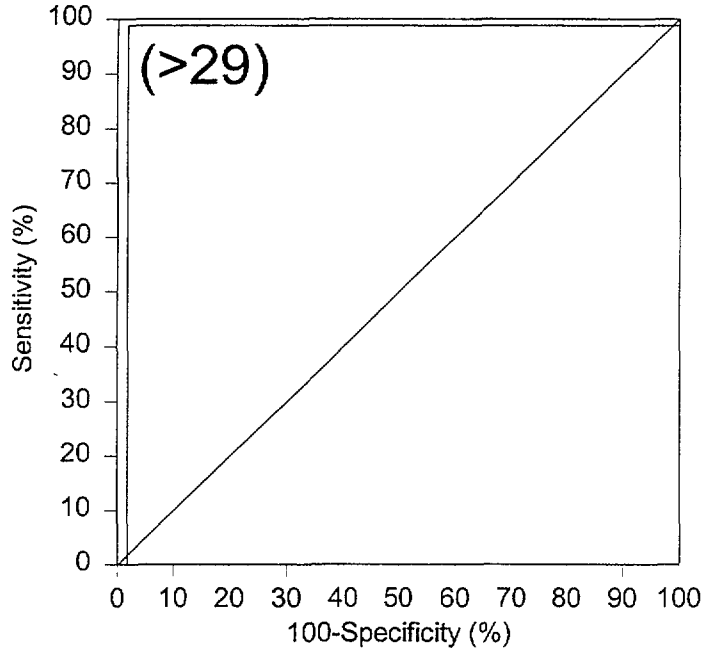

FIG. 8. ROC curve C-ELISA-2.

In our hands the indirect ELISAs performed as well as conventional screening tests but with a higher specificity for FAO/IAEA indirect ELISA. Regarding competitive tests the cut-off values were 30 and 29% for CELISA1 and CELISA2 respectively. As expected for vaccinated population (Table V) in areas were vaccination is commonplace or mandatory the specificity values were lower for all tests, since ELISA detects more positives than CFT. CELISA1, demonstrated higher specificity than CELISA2 and the local cut-off for this test was higher than the one obtained by the ROC analysis. The differences were reduced significantly when the analysis was performed on selected populations. Comparison between ELISAs gave high Kappa agreement and acceptable confidence limits as shown in Table VI . Accuracy estimates were over 0.95 for all ELISA tests Table VII confirming the quality of the assays.

TABLE V. RELATIVE DIAGNOSTIC VALUES FOR SENSITIVITY AND SPECIFICITY IN SELECTED POPULATION

ELISA (cut-off)	Sensitivity[1]	Specificity[2]	Specificity vacc.[3]
IELISA IAEA (30%)	100% (97.84 to 100)	100% (99.11 to 100)	98.70% +/-0.76%
IELISA ADRI (50%)	100% (97.89 to 100)	99.76% (99.46 to 100)	95.51% +/-1.39
CELISA1 (30%)	99.08% (96.37-99.84)	99.81% (98.79-99.99)	96.34% +/-1.26%
CELISA2 (29%)	98.62% +/-1.55%	97.00% +/-1.45%	94.08% +/-1.59%

1 ELISA positives defined (BPTA/RB/CFT(+)) N=222 RID positives N=103
2 ELISA negatives defined (BPTA/RB/CFT(-)) N=842
3 ELISA negatives defined (BPTA/RB/CFT (-)) N=1039

TABLE VI. MEASURE OF AGREEMENT BETWEEN ELISAs: KAPPA VALUES

Test	IAEA	C.L.* %	ADRI	C.L. %	C-ELISA1	C.L. %
I-IAEA						
I-ADRI	0.9954	0.3				
C-ELISA1	0.8707	0.9	0.7766	1.7		
C-ELISA2	0.8031	0.9	0.7454	1.7	0.9475	1.4

Kappa=observed proportion agreement-total chance proportion agreement /1-total chance proportion agreement
*C.L. 95% Confidence limits
BPTA/RB/CFT selected sera, N=2137

TABLE VII. ACCURACY ESTIMATES BASED ON SENSITIVITY, SPECIFICITY AND DISEASE PREVALENCE FOR THE DATA

TEST	(Cut-off)	Accuracy*
I-ELISA IAEA	(30%)	0.9920
I-ELISA ADRI	(50%)	0.9648
I-ELISA ADRI	(46%)	0.9632
C-ELISA1	(30%)	0.9761
C-ELISA2	(29%)	0.9597
C-ELISA2	(30%)	0.9636

*Accuracy TPF x P(D +) + TNF x p(D -) Where: TPF= sensitivity, TNF= specificity, P(D+)= disease prevalence for data and P(D-)= 1-P(D+), BPTA/RB/CFT selected sera, N=2137

4. CONCLUSIONS

The ELISA tests were standardised and validated, and the cut-off values defined for the local conditions. The indirect ELISA demonstrated a higher diagnostic specificity than does the BPAT, RB and CFT without compromising diagnostic sensitivity. The test offer a distinct diagnostic advantage as a laboratory based screening assay.

The competitive ELISA is capable of discriminate between infected cattle and those who have been vaccinated or exposed to a cross reactive organism. Lower values than those observed were expected for CELISA2. This could be explained based on aberrant results from sera of animals from recently vaccinated animals from areas in which adult vaccination could not be excluded.

The data presented continue to support the initial hypothesis that the ELISA methodology designed for Brucellosis will provide more precise and standardised method for diagnosis, and for the support of the control and eradication campaigns.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Isaac Gallego M. and the field veterinarians of ICA, Animal Health Unit for their support provided in the sampling at the different regions involved in the study. A special sentiment of gratitude to bacteriologists Adriana Quevedo and Sandra Rozo for their temporal technical assistance.

REFERENCES

[1] McCULLOUGH, The application of biotechnology to the diagnosis and control of animal diseases, Rev. Sci. Tech.Off. int. Epiz. **12** (1993) 325-353.

[2] GARDNER, Y.A., HOLMES, J.C., Testview: a spreadsheet program for evaluation and interpretation of diagnostic tests, Preventive Veterinary Medicine **17** (1993) 9-18.

[3] FAO/IAEA, ELISA Data Interchange, EDI. User Manual (1994).

[4] International Atomic Energy Agency, Regional network for Latin America on animal disease diagnosis and labelled DNA probe techniques, IAEA TECDOC-657 (1992) Costa Rica.

[5] WRIGHT, P.F., NILSSON, E., VAN ROOIJ, E.M.A., JEGGO, M.H., Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis, Rev. Sci. Tech. Off. Int. Epiz. **12** (1993) 435-450.

[6] LAMB, V.L., JONES, L.M., SCHURIG, G.G., BERMAN, D.T., Enzyme-linked immunosorbent assay for bovine immunoglobulin subclass-specific response to *Brucella abortus* lipopolysaccharide, Infect. Immun. **26** (1979) 240-247.

[7] MACMILLAN, A.P., GREISER-WILKE, I., MOENNIG, V., MATHIAS, L.A., A competition enzyme immunoassy for brucellosis diagnosis, Dtsch. Tierarztl. Wschr. **97** (1990) 83-85.

[8] GODFROID, J., Le diagnostic de la brucellose bovine dans le cadre d'un programme d'eradication de la maladie, Ann. Med. Vet. **136** (1992) 429-434.

[9] YONG, W.M., EDWARDS, L.D., SEARSON, J.E., Evaluation of three LPS-specific monoclonal antibodies for the immunodiagnosis of bovine brucellosis, Res. Vet. Sci. **46** (1989) 413-415.

[10] CHIN, J., DANIELS, J., BUNDESEN, P., Bovine Brucellosis: evaluation of field sera by competitive and superimposable ELISA utilising a monoclonal antibody against *Brucella abortus* lipopolysaccharide, Vet. Immunol. Immunopathol. **20** (1989) 109-118.

[11] NIELSEN, K., CHERWONOGRODZKY, J.W., DUNCAN, J.R., BUNDLE, D.R., Enzyme-linked immunosorbent assay for differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19, Amer. J. Vet. Res. **50** (1989) 5-9.

[12] OOMEN, R.P., YOUNG, N.M.D., BUNDLE, R., Molecular modelling of antibody-antigen complexes between the *Brucella abortus* O-chain polysaccharide and a specific monoclonal antibody, Protein Eng. **4** (1991) 427-433.

[13] NIELSEN, K.H., GALL, D., KELLY, W. HENNING, D. AND GARCÍA, M.M., Enzyme immunoassay: Application to diagnosis of bovine brucellosis. Monograph, Agriculture Canada. ISBN 0-662-19838 (1992).

[14] MEIKLE, P.J., YOUNG, N.M., BUNDLE, D.R., O-antigen biotin conjugates. Preparation and use in direct competitive enzyme immunoassays, J. Immunol. Methods. **132** (1990) 255-261.

[15] NIELSEN, K.H., KELLY, L., GALL, D., BALSEVICIUS, S., NICOLETTI, P., KELLY, W., Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis, Vet. Immunol. Immunopathol. **46** (1995) 285-292.

[16] ALTON, G.G., JONES, L.M., ANGUS, R.D., VERGER, J.M., Techniques for the Brucellosis laboratory. INRA. Paris, (1988) pp.190.

[17] DIAZ, R, GARATEA, P., JONES, L.M., MORIYON, Y., Radial immunodiffusion test with *Brucella* polisaccharide antigen for differentiating infected from vaccinated, J. Clin Microbiol. **10** (1979) 37-41.

[18] WRIGHT, P.F., NIELSEN, K.H., Current and future serological methods, In: Advances in Brucellosis Research. Adams, L. G. Ed. Texas A&M University Press, College Station, Texas (1990) 305-320.

[19] NIELSEN, K.H., KELLY, L., GALL, D., BALSEVICIUS, P., BOSSE, J., NICOLETTI, P., KELLY, W., Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis, Prev. Vet. Med. **26** (1994) 17-22.

[20] NIELSEN, K.H., KELLY, L., GALL, D., SMITH, P., BOSSE, J., NICOLETTI, P., KELLY, W., The use of divalent cation chelating agents (EDTA/EGTA) to reduce non-specific serum protein interaction in enzyme immunoassay, Vet. Res. Comm. **18** (1994) 433-437.

[21] BAILEY, N. T., Statistical Methods im Biology. Second Ed. Cambridge University Press (1992).

[22] JACOBSON, R.A., Guidelines for validation of serological assay for diagnosis of infectious diseases. Off. Int. Epiz., Manual of standards for diagnostic tests and vaccines, 3rd Ed, (1996).

[23] JACOBSON, R.A. AND J. ROMATWOSKY, Assessing the validity of serodiagnostic test results. Seminars in Vet. Med. & Surgery (Small Animal) **11** (1996) 135-143.

[24] NIELSEN, K., The serological response of cattle immunized *with Yersinia enterocolitica* O:9 or O:16 to *Yersinia* and *Brucella abortus* antigens in enzyme immunoassays, Vet Immunol Immunopathol. **24** (1990) 373-382.

[25] MARIÑO, O.C., RUEDA, E., DE DELEON, L.S., RAMIREZ, C., The use of Elisa to evaluate antibodies in *Brucella abortus* naturally infected bovines, In: Regional Network for Latin América on animal disease diagnosis using immunoassay and labelled DNA probe techniques, IAEA-TECDOC 657 (1990) 139-149.

[26] WRIGHT, P.F., NIELSEN, K.H., KELLY, W., Primary binding techniques for the serodiagnosis of bovine brucellosis: Enzyme immunoassay, In: Animal Brucellosis, Nielsen K. And J. R. Duncan Eds. CDR Press, Boca Raton, (1990) 199.

[27] VIZARD, A.L., ANDERSON, G.A., GASER, R.B., Determination of the optimum cut-off value of a diagnostic test, Preventive Vet. Med. **10** (1990) 137-143.

[28] ICA, Instituto Colombiano Agropecuario, Reporte anual, División de Sanidad Animal. Documento, (1994).

[29] METZ, C. E., Basic principles of ROC analysis, Seminars in Nuclear Medicine, **8** (1978) 283-298.

EVALUATION OF FOUR IMMUNOASSAYS FOR DIAGNOSIS OF BRUCELLOSIS IN CUBA

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Abstract

EVALUATION OF FOUR IMMUNOASSAYS FOR DIAGNOSIS OF BRUCELLOSIS IN CUBA.

Four immunoassays (two indirect and two competitive ones) were evaluated by samples from areas free of disease, free by vaccination and affected areas using as reference techniques the Bengal Rose Tests, the Antigen in Buffered Plate Tests and the Complement Fixation Reaction Test. The evaluated samples demonstrated that the competitive assays (ELISAC-1 and ELISAC-2) detected less false positives than the indirect ones (ELISAI-1 and ELISAI-2). Of the competitive ELISAs, version 2 presented better sensitivity and specificity results in affected areas for 95% confidence: 80.9 - 96.9 % and 97.5 - 99.4% respectively with positive predictive value in the range of 76 to 94% and negative predictive one between 98.1 and 99.7%. It was concluded that this assay can be used for brucellosis control because it gives higher assurance than the other evaluated immunoassays and it can discriminate infected from vaccinated animals.

1. INTRODUCTION

Bovine brucellosis is an infectious disease affecting animals of all ages. Its wide distribution throughout the American continent makes this disease an endemic problem for many countries in South America. Cuba belongs to a group of countries where it has been controlled as a result of the Control and Eradication Program introduced since 1964 and a large part of the countries territory is free of brucellosis [1].

In Cuba the disease is restricted to areas of extensive cattle breeding and difficult access for vaccination with *Brucella abortus* strain 19. The methods commonly used for serological diagnosis are: the Bengal Rose Test (BR), Buffered Plate Agglutination (BPA), Slow Tube Agglutination (STA) 2-Mercaptoethanol (2-Me) and the Complement Fixation Test (CFT), although these conventional techniques do not distinguish vaccinated from naturally infected animals [2].

Immunoassays of two different kinds have been designed for brucellosis diagnosis: indirect [3] and competitive [4] ELISA. The first does not displace some of the conventional techniques, as for example CFT, for confirmatory diagnosis, since it does not achieve the necessary degree of specificity (Peraza et al., unpublished results). The competitive ELISA using a specific monoclonal antibody does not only meet better specificity criteria, but also solve the major problem of serological diagnosis of this disease: discriminating between infected and vaccinated animals. Many authors as Wright et al. [5] and Jacobson [6], consider that immunoassays should be evaluated under the epidemiological conditions of each region before being used as diagnostic tools.

The aim of this work is to evaluate two competitive ELISAs and two indirect ELISAs compared with conventional serological techniques as BR, ABP and CFT in sera from cattle free of disease, free by vaccination and affected by brucellosis.

2. MATERIALS Y METHODS

2.1. The following groups of sera were used:

2.1.1. Brucellosis free area

Sera samples from 1019 non-vaccinated animals from areas free of brucelosis were collected. The herds under study did not present clinical, bacteriological or serological evidence of disease during the last 20 years.

2.1.2. Vaccinated area

Sera samples from 927 animals vaccinated with *Brucella abortus* strain 19, from areas without clinical, bacteriological or serological evidence of disease for the last 2 years.

2.1.3. Brucellosis affected area

Sera samples from 726 animals from brucellosis affected areas. These herds were vaccinated with strain19 and animals with clinical, bacteriological and serological evidences of brucellosis infection have been found. In these areas the incidence is low due to the control and eradication program carried out in the country.

2.2. Serological tests

The antigens used in the BPA, and BR tests were provided by Biomerieux, the CFT antigen was produced in Cuba by Laboratorios Biológicos Farmaceúticos (LABIOFAM, Biological Pharmaceutical Laboratories)

The protocols used for the BR, BPA and 50% hemolysis CFT were described elsewhere [7]. These techniques were used as reference tests for evaluation and only those samples showing identical results were taken into consideration.

The immunoassays used in this study: Indirect ELISA 1and 2 (ELISAI-1, ELISAI-2) and competitive ELISA (CELISA-1, CELISA-2) were kindly provided by the Joint FAO/IAEA Division of the International Atomic Energy Agency (IAEA), Vienna, Austria and each assay was carried out according to the protocol provided with the kit.

2.3. Data analysis

All data were stored in Microsoft Excel and the cut-off point calculations, as well as the sample analysis were done in EPI-INFO-6.0 and the Statistical Package Program.

For calculating the cut-off point in affected areas the Receiver Operating Characteristics Analysis (ROC-analysis) was used [2,6]. To select the cut-off values for each test, the point where the specificity of the assay assures a minimum of false positive samples and a higher positive predictive value without affecting the sensitivity of the technique was determined.

3. RESULTS

Samples from disease free areas that gave false positive results were re-evaluated by each one of the techniques for a final negative result

Tables I and II show the specificity of the ELISAI-1 in the free areas (96.3 - 98.7%), with a negative predictive value of 100 %. In the 927 samples from the free-by-vaccination areas 22 false positive samples were found giving a specificity of 99.2 - 99.9 % and a negative predictive value of 100 %. In affected areas the technique detected 59 of the 60 CFT positive samples and 635 of the 666 negative giving a sensitivity of 89.9 - 99.9 %, specificity 93.4-96.8%, positive predictive value of 54.7-75.1% and a negative predictive value of 99 - 100 %. All these results were obtained using a 44 percent

positivity (PP) cut-off point. The ROC analysis for this assay, the distribution frequency analysis as well as the dispersion of samples for each population under evaluation are presented in Figures 1, 2, and 3.

TABLE I. RESULTS OF THE EVALUATED SAMPLES IN EACH IMMUNOASSAY

Sample	ELISAI-1				ELISAI-2				CELISA-1				CELISA-2			
	TP	TN	FP	FN	TP	TN	FP	FN	TP	TN	FP	FN	TP	TN	FP	FN
Affect	59	635	31	1	57	641	25	3	57	651	15	3	55	658	8	5
Vac	-	905	22	-	-	914	13	-	-	925	2	-	-	926	1	-
Free	-	1017	2	-	-	1017	2	-	-	1018	1	-	-	1016	3	-

TP true positives FP false positives
TN true negatives FN false negatives

TABLE II. SENSITIVITY, SPECIFICITY, POSITIVE PREDICTIVE VALUE, NEGATIVE PREDICTIVE VALUE FOR A 95% CONFIDENCE INTERVAL USING THE CONVENTIONAL SEROLOGICAL TESTS AS REFERENCE

Tests	Sensitivity	PPV	Specificity	NPV
ELISAI-1 (44PP)				
Affected	89.9-99.9%	54.7-75.1%	93.4-96.8%	99-100%
Vaccinated			99.2-99.9%	100%
Free			96.3-98.7%	100%
ELISAI-2 (55PP)				
Affected	85.2-98.7%	58.2-79.9%	94.4-97.5%	98.5-99.9%
Vaccinated			99.2-99.9%	100%
Free			97.5-99.2%	100%
ELISAC-1 (40PI)				
Affected	85.2-98.7%	67.7-87.5%	96.2-98.7%	98.5-99.9%
Vaccinated			99.1-99.9%	100%
Free			99.4-99.9%	100%
ELISAC-2 (35PI)				
Affected	80.9-96.9%	76-94%	97.5-99.4%	98.1-99.7%
Vaccinated			99.3-99.9%	100%
Free			99-99.9%	100%

NPV Negative Predictive Value
PPV Positive Predictive Value

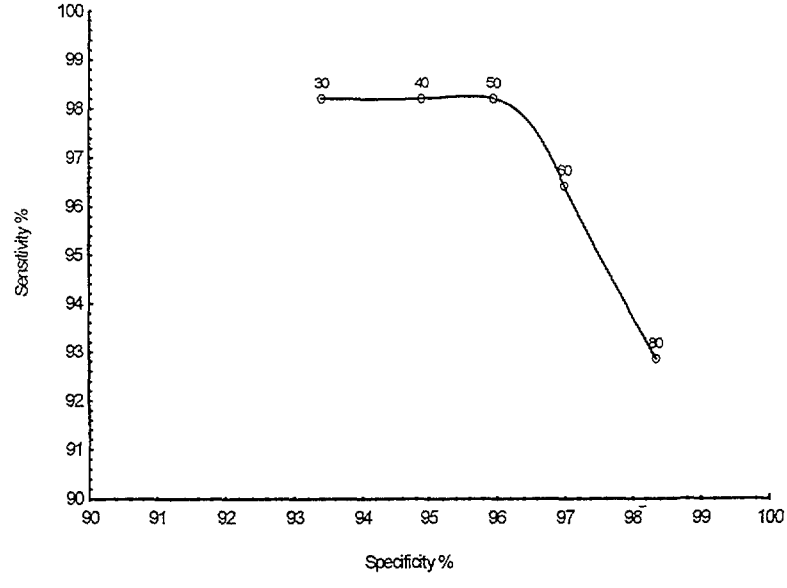
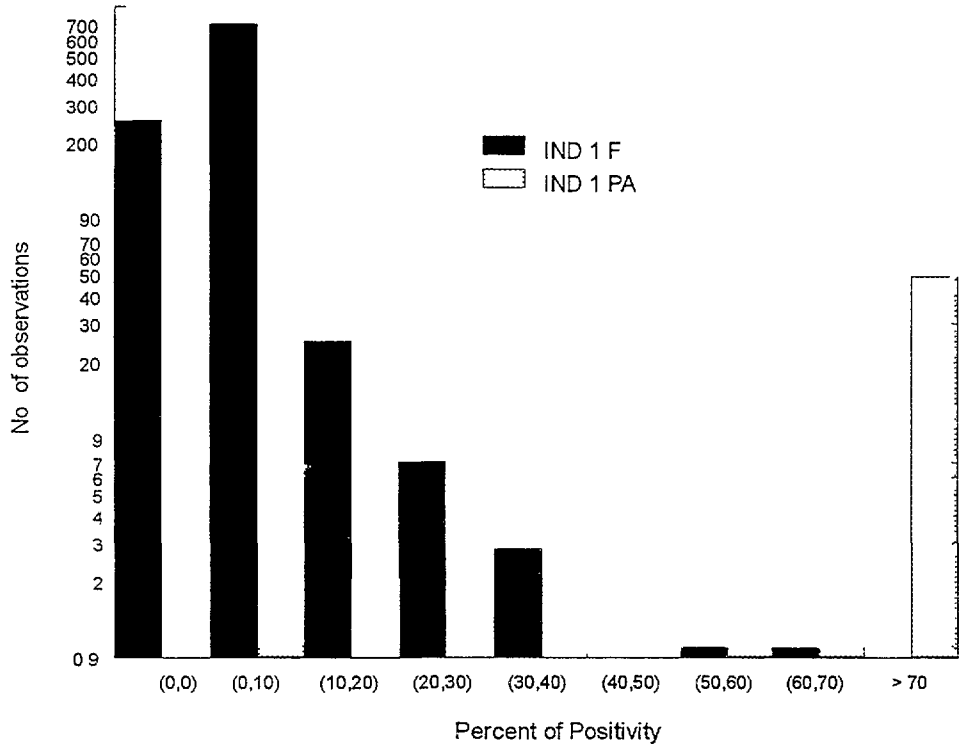
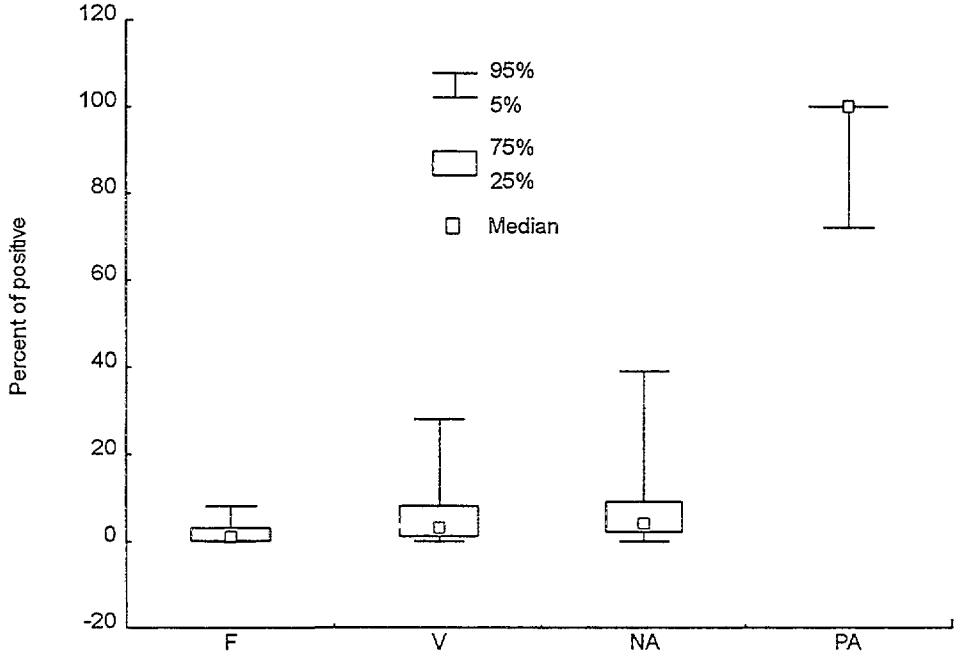


FIG. 1. Frequency distribution of the samples from affected areas of ELISAI-1 using ROC analysis.



IND 1 F Indirect ELISA1, animals from free areas
IND 1 PA Indirect ELISA1, positive animals from affected areas

FIG. 2. Frequency distribution of the samples from affected areas of ELISAI-1.



PA Positive animals from affected areas
NA Negative animals from affected areas
V Animals from vaccine areas
F Animals from free areas

FIG. 3. Analysis of the sample dispersion in the three evaluated categories ELISAI-1.

ELISAI-2 showing a specificity of 97.5 - 99.2% in free areas and a negative predictive value of 100 %. In vaccinated areas 13 samples were found to be false positives giving a specificity of 99.2 -

99.9% and a negative predictive value of 100 %. The sensitivity in affected areas was 85.2 -98.7%, with a positive predictive value of 58.2 - 79.9%, while specificity reached values between 94.4 - 97.5%, with a negative predictive value of 98.5-99.9 % due to 25 false positive and 3 false negative samples. The cut-off point for this analysis was 56 PP. The ROC-analysis results, the frequency diagram for affected areas and the dispersion of the evaluated populations can be appreciated in graphics 4, 5 and 6.

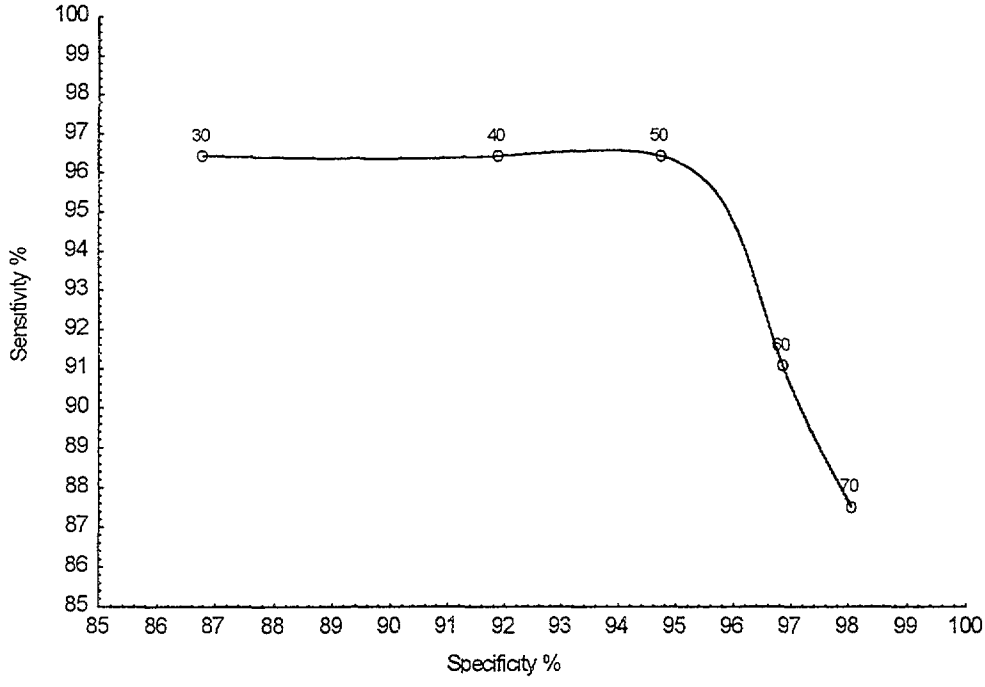
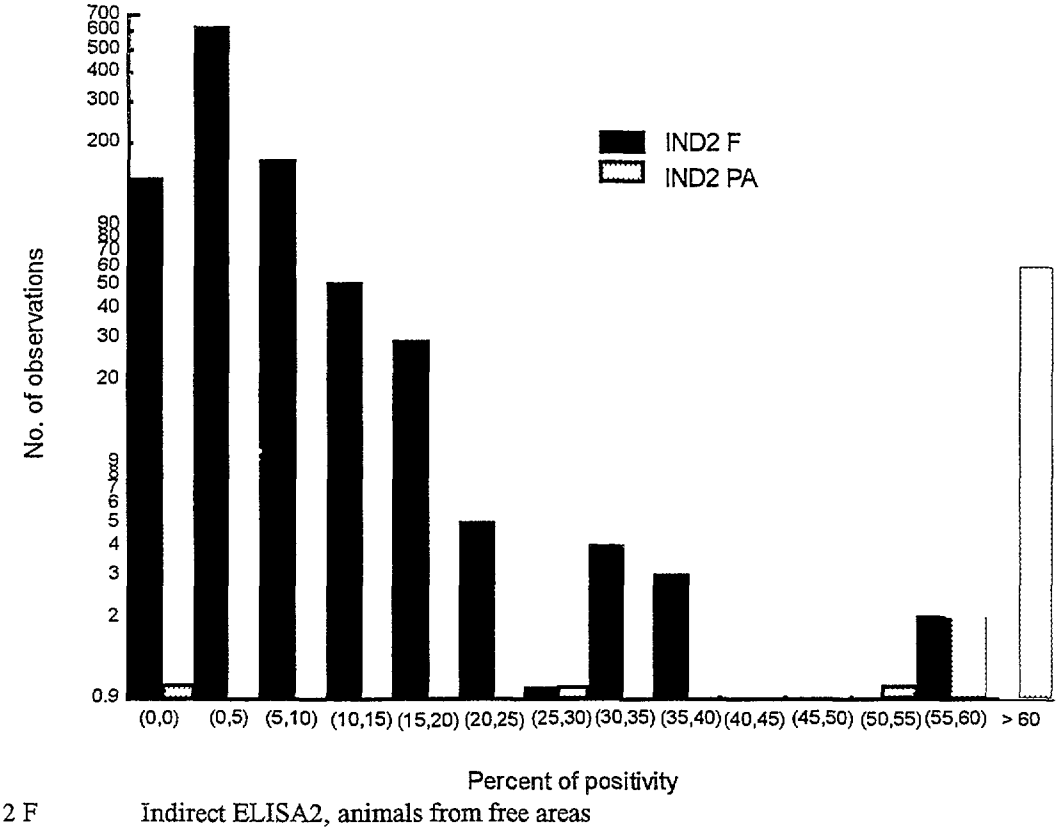
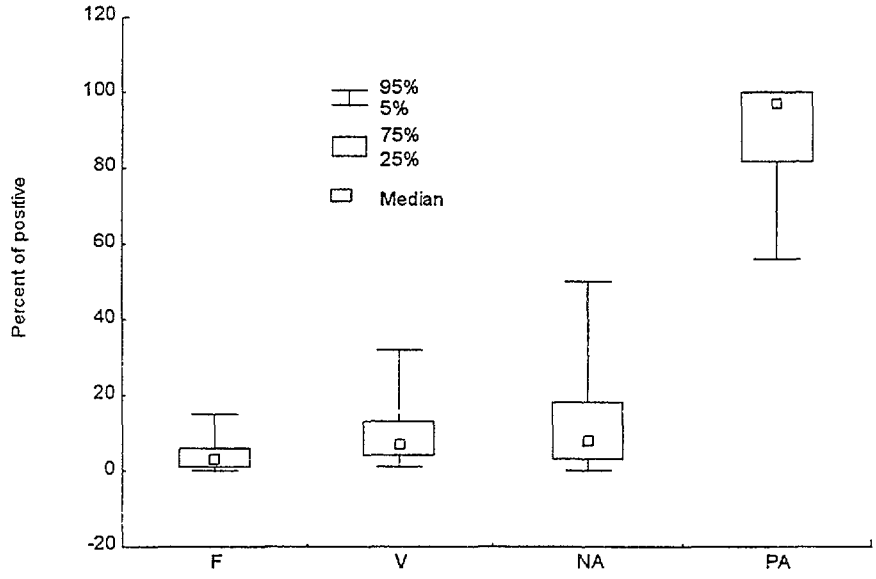


FIG. 4. Analysis of the sensitivity and specificity using different cut-off points in animals from affected areas ELISAI-2 using ROC analysis.



IND 2 F Indirect ELISA2, animals from free areas
IND 2 PA Indirect ELISA2, positive animals from affected areas

FIG. 5. Frequency distribution of the sample dispersion in the three evaluated categories ELISAI-2.



PA Positive animals from Affect areas
NA Negative animals from affect areas
V Animals from vaccine areas
F Animals from free areas

FIG. 6. Analysis of the sample dispersion in the three evaluated categories ELISAI-2.

CELISA-1, presented specificity values of 99.4 - 99.9% in disease free areas and displayed a negative predictive value of 100 %. In vaccinated areas two false positive samples were found giving a specificity of 99.1 - 99.9% and a negative predictive value of 100 %. The immunoassay gave 3 false negative results in samples from affected areas giving a sensitivity of 85.2 - 98.7% and a positive predictive value of 67.7 - 87.5%, while 651 negative samples gave a specificity of 96.2 - 98.7% and a negative predictive value of 98.5 - 99.9%. The cut-off point for this analysis was 40 percent of inhibition (IP). The ROC-analysis results, the frequency distribution and dispersion for each population are displayed in graphics 7, 8 and 9.

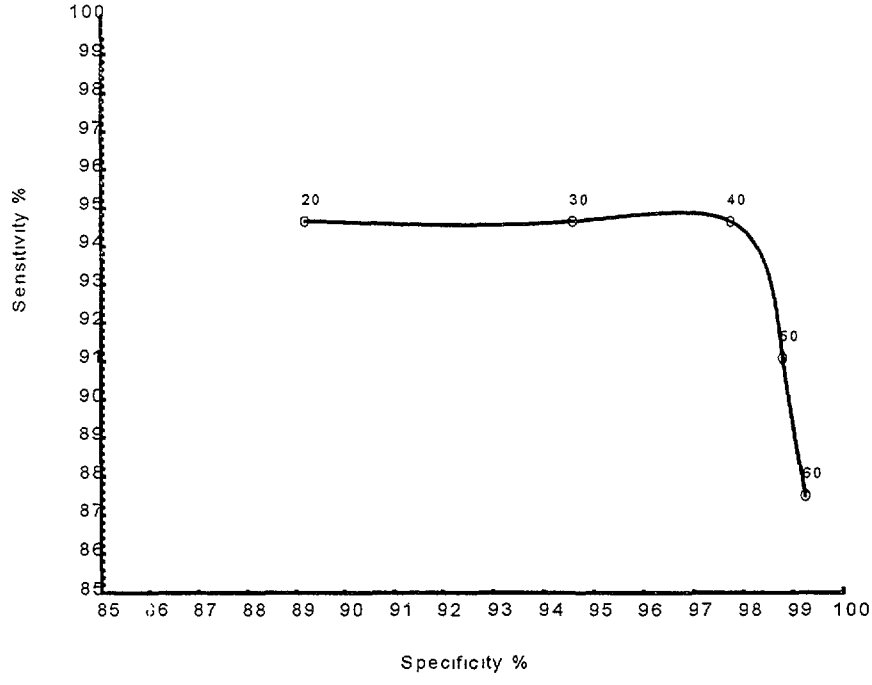
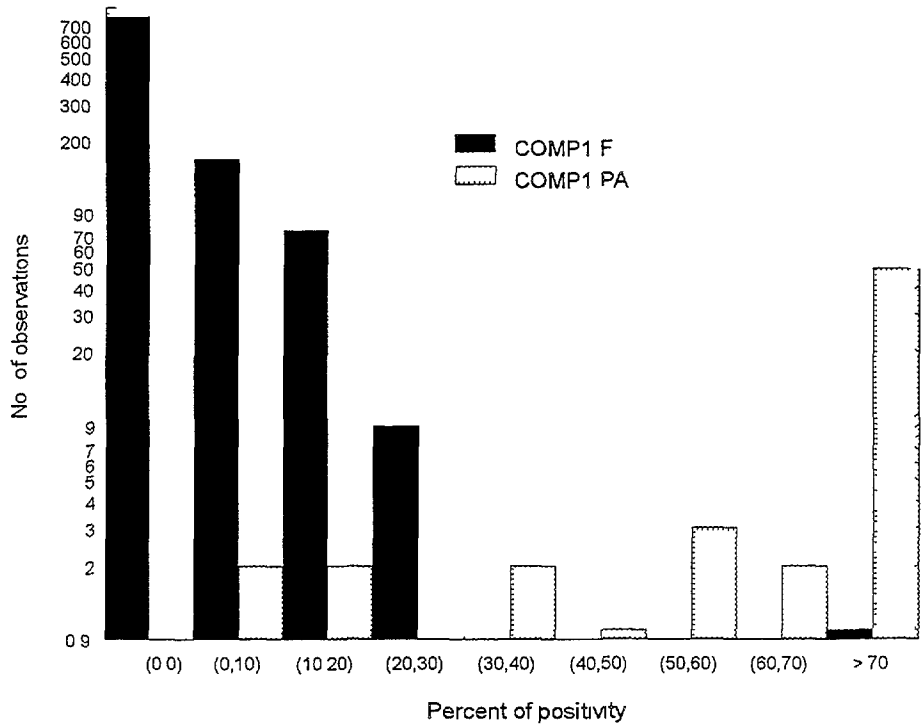
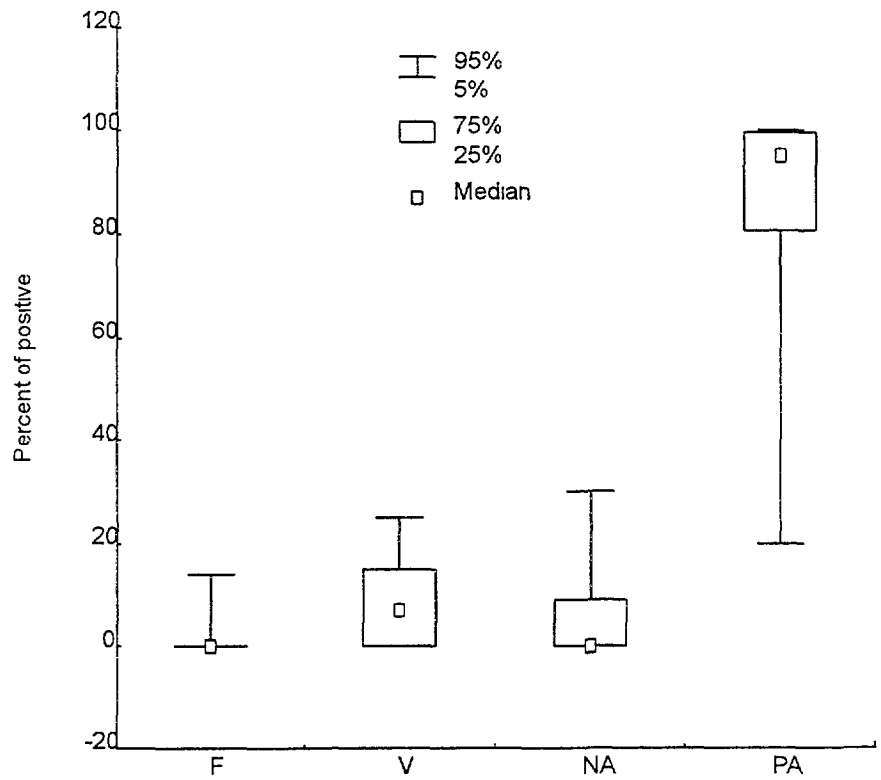


FIG. 7. Analysis of the sensitivity and specificity using different cut-off points in animals from affected areas CELISA-1 using ROC analysis.



COMP 1 F Competitive ELISA1, animals from free areas.
COMP 1 PA Competitive ELISA1, positive animals from affected areas.

FIG. 8. Frequency distribution of the samples from affected areas of CELISA-1



PA Positive animals from Affect areas
NA Negative animals from affect areas
V Animals from vaccine areas
F Animals from free areas

FIG. 9. Analysis of the sample dispersion in the three evaluated categories CELISA-1.

CELISA-2, showed in free areas a specificity of 99 - 99.9% and a negative predictive value of 100 %. In vaccinated areas the specificity ranged between 99.3 - 99.9% with a negative predictive value of 100 %. In the affected areas 5 false negative results gave a sensitivity of 80.9 - 96.9%, with a positive predictive value of 76-94%. The specificity was calculated between 97.5 - 99.4% because of 8 false positive results and the negative predictive value was 97.5 - 99.4%. The cut-off value used in this analysis was 35 IP. The ROC-analysis, frequency distribution for affected areas and dispersion of the evaluated populations are shown in graphics 10, 11 and 12.

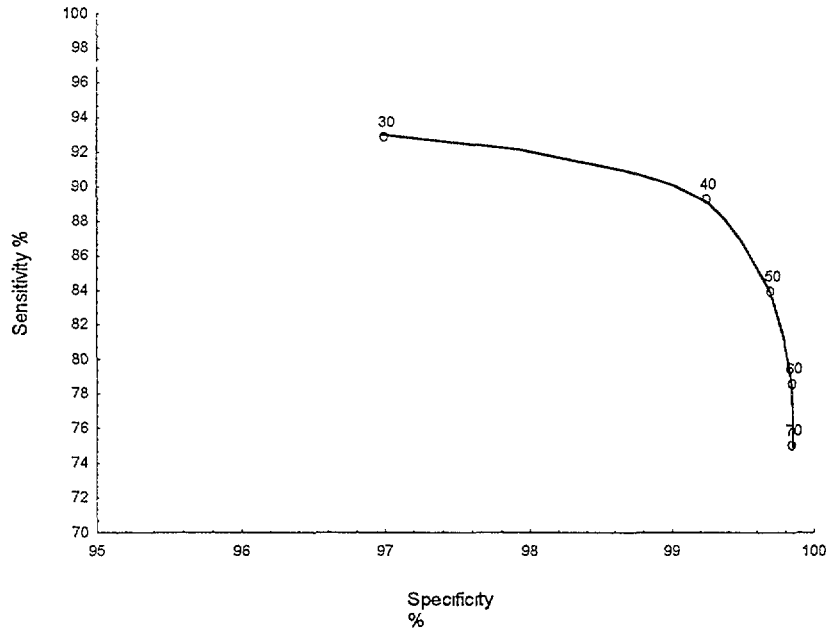
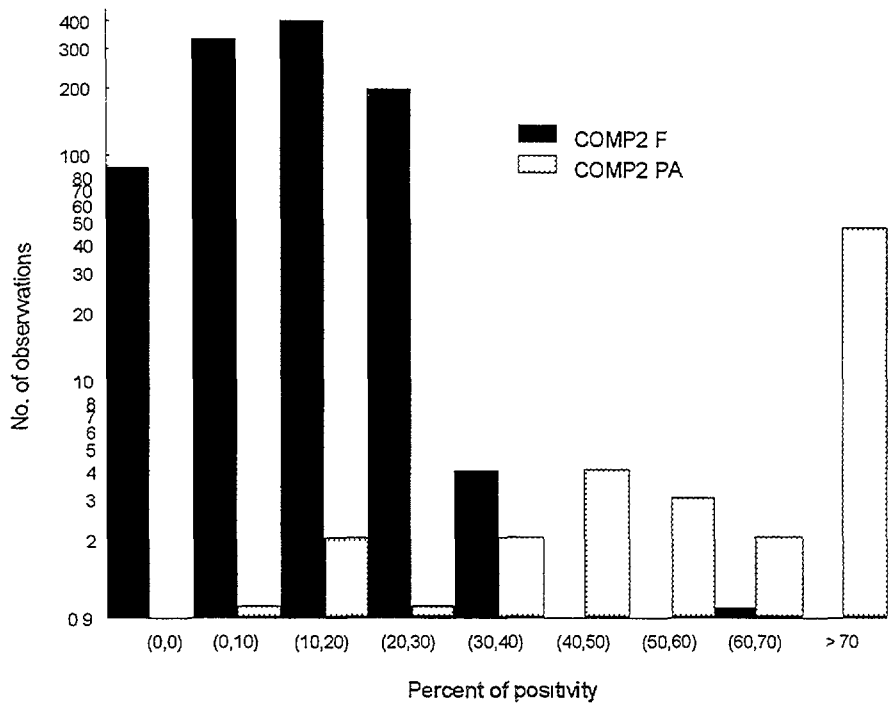
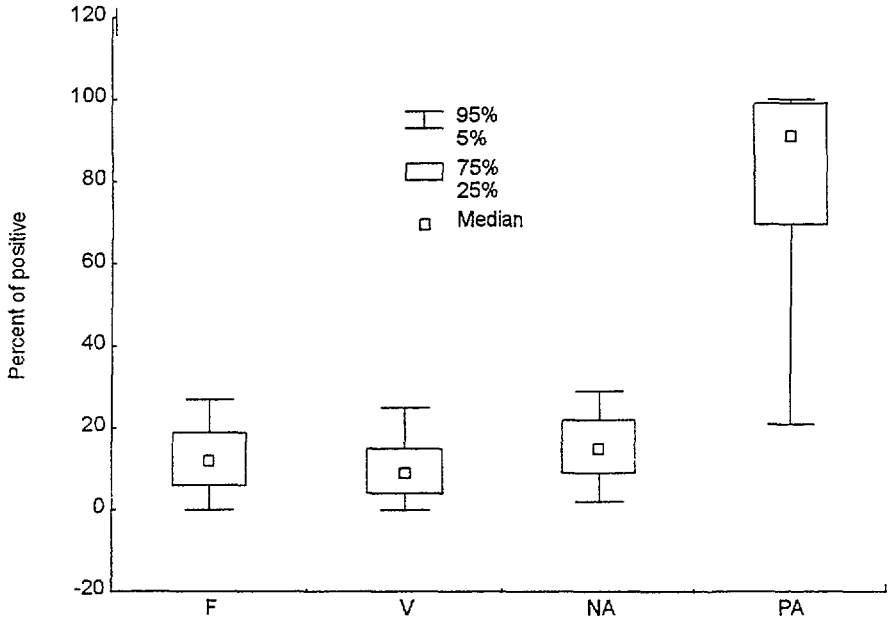


FIG. 10. Analysis of the sensitivity and specificity using different cut-off points in animals from affected areas CELISA-2 using ROC analysis.



COMP 2 F Competitive ELISA2, animals from free areas
COMP 2 PA Competitive ELISA2, positive animals from affected areas

FIG. 11. Frequency distribution of the samples from affected areas of CELISA-2.



PA Positive animals from Affect areas
NA Negative animals from affect areas
V Animals from vaccine areas
F Animals from free areas

FIG. 12. Analysis of the sample dispersion in the three evaluated categories CELISA-2.

4. DISCUSSION

The results obtained in the indirect tests demonstrated that ELISAI-1 shows a higher sensitivity than the rest of the evaluated immunoassays, since it detected all the samples that were positive by the conventional techniques. The only false negative sample was also negative for the rest of the immunoassays which could be due to a specificity problem of the reference techniques. Nevertheless, the specificity of the assay was not good because 26 of the positive samples by this test were negative if tested by ELISAI-2 which uses EDTA/EGTA for the sample buffer to eliminate non-specific reactions [8]. It was also found that 14 sera were positive to both indirect tests and negative for the competitive ones. Since these techniques are not able of differentiate between vaccinated and infected animals, this could be due to the vaccine antibodies in these animals [9,10]. The sensitivity and specificity results for ELISAI-1 and ELISAI-2 do not show differences.

The results for the CELISA-1, developed by Nielsen et al. [4], modified later by Gall et al. [11] detected 3 false negative samples, apparently animals with vaccine antibodies, and 17 false positive samples. CELISA-2 developed afterwards [8,12], and incorporated EDTA/EGTA in the sample buffer and used a different monoclonal antibody, was more specific than the first it did not detect 9 of the false positive samples, confirming the findings in the indirect assays when chelating agents were used. The remaining false positive samples found in both tests were bacteria contaminated sera [13]. The possible cause of the false negative samples in the ELISAC-2 test were the same as for CELISA-1. The sensitivity and specificity found for these immunoassays were not very different, although CELISA-2 showed better positive predictive value (76 -9 4%). These results are shown in Tables I and II.

To determine the cut-off value for each immunoassay with confidence the ROC-analysis program was used (Figures 1, 4, 7 and 10) analysing the sensitivity and specificity for a range of cut-off points. The value was selected taking into account the point where the highest specificity was obtained, without affecting the sensitivity of the assays. As can be observed in each of the Figures starting with the selected value when the specificity increases the sensitivity decreases.

In Figures 3 and 6 the distribution of the populations evaluated in the ELISAI-1 and ELISAI-2 is shown. If the sample dispersion in areas free of disease is analysed it is much lower than from the areas free of disease by vaccination and again these are much weaker than the ones derived from affected negative samples. This occurs because in vaccinated populations vaccine-strain-antibody-titers are higher [9]. These results are characteristic for indirect immunoassays which can not distinguish vaccine antibodies from post-infection antibodies. It could be observed that in affected population ELISAI-1 distinguishes the maximum negative sample values and the minimum positive sample values better than ELISAI-2 and it is concluded that ELISAI-1 is the safer test for diagnostic use.

For the competitive immunoassays the distribution of the negative samples in the three categories is different from the indirect immunoassays (Figures 9 and 12). CELISA-2 requires EDTA/EGTA for the sample buffer. All groups present the same distribution, as expected for this assay, because sera from the vaccinated animales, which contain antibodies against strain 19, do not displace the monoclonal antibody used. CELISA-1 does not show a homogenous distribution for all groups, probably due to non-specific reactions. In the positive samples from affected areas the IP values are lower than the cut-off values for both assays. These sera must come from vaccinated animals which result positive in the indirect and reference tests, but have negative values in the competitive ones.

We may conclude that the addition of divalent cations (EDTA/EGTA), as chelating agents, reduces non-specific reactions improving the specificity of the technique without affecting the sensitivity.

The use of immunoassays for the diagnosis of brucellosis demands their evaluation with respect to conventional diagnostic tests, using different animal populations for adjusting the cut-off point in relation to the sensitivity and specificity criteria required for each area.

In this study CELISA-2 showed better positive predictive value than the rest of the immunoassays and a high specificity. This minimises the possibility of giving false positive animals in comparison with any other of the evaluated immunoassays. This technique, unlike conventional tests and the indirect immunoassays [8,12], allows differentiation between vaccinated and infected animals. It is necessary to bear in mind that this test is very sensitive to bacterially contaminated sera, giving false positive [13]. Its characteristics permit fast processing of a big number of samples (1 hour) and the computerised analysis of the results. Bearing in mind the aforementioned, this technique can be used for screening purposes and as a confirmatory test in the serological diagnosis of brucellosis [13,14].

ACKNOWLEDGEMENTS

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REFERENCES

[1] OPS/OMS, Situación de la brucelosis bovina en las Américas, IX Reunión Interamericana de Salud Animal a Nivel Ministerial, Washington, D.C., EUA, 25 a 27 de abril de 1995.

[2] NIELSEN, K.H., GALL, D. E., KELLY, W.A., VIGLIOCCO, A. M., HENNING, M. D., GARCIA, M.M., Immunoassay development, Application to Enzyme Immunoassay for the Diagnosis of Brucellosis, Copyright, Agriculture and Agri-Food Canada, 1996a. ISBN 0-662-24163-0.

[3] WRIGHT, P.F., KELLY, W., GALL, D.E., Application of a timing protocol to the reduction of inter-plate variability in the indirect enzyme immunoassay for the detection of anti-*Brucella* antibody, J. Immunoassay 6 (1985) 189-199.

[4] NIELSEN, K. H., CHERWONOGRODZKY, J., DUNCAN, J. R. AND BUNDLE, D. R., Enzyme-linked immunosorbent assay for differentiation of the antibody response of cattle

naturally infected with *Brucella abortus* or vaccinated with strain 19, Am. J. Vet. Res., **50** (1989) 5-9.

[5] WRIGHT, P.F., NILSSON, E., VAN ROIJ, E.M.A., LELENTA, M., JEGGO, M.H., Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis, Rev. Sci. Tech. Off. Int. Epiz. **12** (1993) 435-450.

[6] JACOBSON, R.H., Factors in selecting serum samples for use in determining the positive/negative threshold (cut-off) in ELISA, IAEA TECDOC, (in press).

[7] ALTON, G.G., JONES, L. M., ANGUS, R. D. AND VERGER, J. M., Techniques for the brucellosis laboratory, INRA, Paris (1988) 190 pp.

[8] NIELSEN, K. H., KELLY, L., GALL, D., SMITH, P., BOSSE, J., NICOLETTI, P. AND KELLY, W., Use of divalent cation chelating agents (EDTA/EGTA) for reduction of non-specific serum protein interaction in enzyme immunoassay, Vet. Res. Commun. **18** (1995[a]) 433-437.

[9] NIELSEN, K. H., GALL, D., KELLY, W., HENNING, D., AND GARCIA, M. M., Enzyme immunoassay: application to diagnosis of bovine brucellosis, Monograph (1992) Agriculture Canada.

[10] ABALOS, P., IBARRA, L., PINOCHET, L., NAVIA, F., BOISIER, X., Residual anti-*Brucella abortus* strain 19 antibodies detected in adult cattle by two indirect-ELISA tests, Veterinary Record **138** (1996) 140.

[11] GALL, D. AND NIELSEN, K. H., Improvements to the competitive ELISA for detection of antibody to *Brucella abortus* in cattle sera, J. Immunoassay **15** (1995) 277-291.

[12] NIELSEN, K. H., KELLY, L., GALL, D., BALSEVICIUS, S., NICOLETTI, P. AND KELLY, W., Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis, Vet. Immunol. Immunopathol., **46** (1995b) 285-291.

[13] NIELSEN, K.H., KELLY, L., GALL, D., BALSEVICIUS, S., J. BOSSE, NICOLETTI P., KELLY, W., Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis, Preventive Veterinary Medicine **26** (1996b) 17-32.

[14] UZAL, F.A., CARRASCO, A.E., NIELSEN, K.H., Evaluation of a competitive ELISA for the diagnosis of bovine brucellosis, Vet. Res. Comm. **20** (1996) 421- 426.

COMPARISON OF DIFFERENT SEROLOGICAL ASSAYS FOR THE DIFFERENTIAL DIAGNOSIS OF BRUCELLOSIS

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Abstract

COMPARISON OF DIFFERENT SEROLOGICAL ASSAYS FOR THE DIFFERENTIAL DIAGNOSIS OF BRUCELLOSIS

Two indirect and two competitive Enzyme-linked immunosorbent assays (I-ELISA102, I-ELISA103, C-ELISA1 and C-ELISA2 respectively) have been evaluated in comparison with traditional test such as Radial Immunodiffusion (RID), Complement Fixation (CF), Rose Bengal Agglutination (RB) and Rivanol agglutination (RV) The sera analysed included 1018 sera obtained from non-vaccinated bovines, 848 sera from brucellosis free herds calf vaccinated with Strain-19, 295 sera obtained from brucellosis free herds adult vaccinated with Strain-19 and 665 sera from *Brucella abortus* biotype 1 (field strain) infected herds Cut-off off values calculated by ROC analysis were established for each ELISA Although all ELISAs fulfilled the requirements for sensitivity and specificity, in our hands C-ELISA2 performed slightly better than the other assays for differentiating infected from vaccinated bovines The specificity of this test was similar to that of RID assay which is known to have high specificity for differentiating adult vaccinated from infected bovines The kappa value among the different tests was good and within the limits of reproducibility and performance expected for the different assays

From the different immunoenzymatic assays, the C-ELISA2, which uses LPS as antigen and a monoclonal antibody against the C/Y epitope as competing reagent, seems to be the most promising of the ELISAs and therefor can be recommended for screening a large number of serum samples on a laboratory basis

1. INTRODUCTION

Brucella species (*B. abortus, B. melitensis* and *B. suis*, *B ovis* and *B canis*) are responsible for brucellosis in animals and humans causing severe economic and public health problems [1]. There is clear evidence that *Brucella species* are capable of surviving and multiplying within cells [2,3]. This fact could explain the marked tendency of the disease to result in focal involvement, forms with long evolution, and frequent relapses. There is also indication that LPS and related polysaccharides (NH), which are the most important antigens of *Brucella*, are implicated in the pathogenesis of these bacteria [4,5]. An upgraded model of the outer membrane of smooth *Brucella* displaying the most conspicuous antigens is presented in Figure 1.

The physical and chemical characteristics of *Brucella* LPS and antigenically related polysaccharides (NH) have been extensively documented [4,5,6,7] The antibody response elicited against *Brucella* LPS and the antigenically related native hapten polysaccharide (NH) during infection is by far the strongest when compared to those induced by other molecules of this microorganism [8] Consequently, *Brucella* LPS has been considered the most important antigen during the immune response in brucellosis and the target for many serological and immunological studies [4,5,8,] In contrast to enterobacterial LPS and other polysaccharide molecules, *Brucella* LPS is capable of inducing strong IgG and IgM antibody responses

A total of 11 epitopes (Figure 2) have been recognised in *Brucella* LPS [9,10] Four of them (A, M, C/Y and C) have been identified in the O polysaccharide chain The C and C/Y epitopes are found in all smooth type LPSs The A epitope is characteristic of *B abortus* (biotype 1) whereas the M epitope is found in *B melitensis* (biotype 1) species In some cases both the A and M epitopes can be found in the same bacterial strain as in the case of some strains of *B suis* (biotype 4) Two epitopes are found in the core oligosaccharide (R1 and R2), three in the lipid A (LA1, LA2 and LA3) and two more in the lipid A associated peptide (LAOmp3-1 and LAOmp3-2) Most of the serum antibodies from infected or immunised animals are directed against the C/Y epitope present in the O-antigen and NH polysaccharide Antibody responses against other epitopes located in these polysaccharides, as well as in the core oligosaccharide and lipid A moieties, although perceptible are produced in minor quantities (Figure 3)

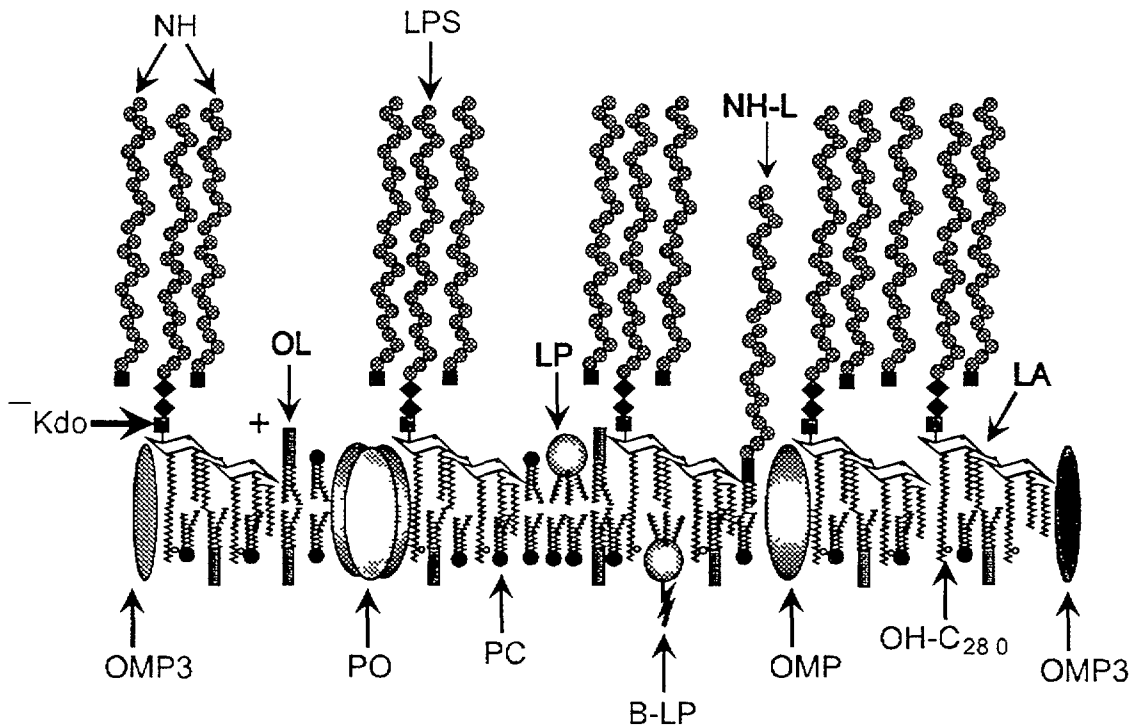

FIG 1 Schematic representation of S Brucella spp OM 2-deoxy, D-manno octulosonic acid (Kdo), lipid A (LA), free lipoprotein (LP), bound lipoprotein (B-LP) lipopolysaccharide (LPS), native hapten polysaccharide (NH), lipid bound NH (NH-L), phosphatidylcholine (PC), hydroxylated $C_{28\,0}$ fatty acid (OH-$C_{28\,0}$), ornithine lipids (OL), OM proteins (OMP) OM proteins group 3 (OMP3), and porin (PO)

A broad spectrum of activities concerning antibodies against *Brucella* LPS and NH have been described For instance, it has been shown that antibodies against LPS and NH epitopes produced during infection are of higher affinity than those produced during vaccination or immunisation with purified molecules [8] Moreover, most of these antibodies correspond to the IgG1 isotype, suggesting a T dependent response Opsonising and complement fixing antibodies facilitate phagocytosis and intracellular destruction of ingested *Brucella* [11] Several experiments have demonstrated that passively transferred polyclonal or monoclonal antibodies in mice can protect against challenges by pathogenic bacteria [12] Antibodies of the IgG class directed against the C/Y epitope seem to be the most protective of all [8,12] Finally it was proved that passively transferred antibodies against O chain and NH from infected animals into mice and rabbits were capable of inducing a strong type I hypersensitivity reaction after injection of minimal quantities of these polysaccharides [13] For all these reasons the detection of IgG1 antibodies against C/Y epitopes located in the O chain polysaccharide of LPS in serological test has been considered a key factor in the diagnosis of brucellosis [8,14]

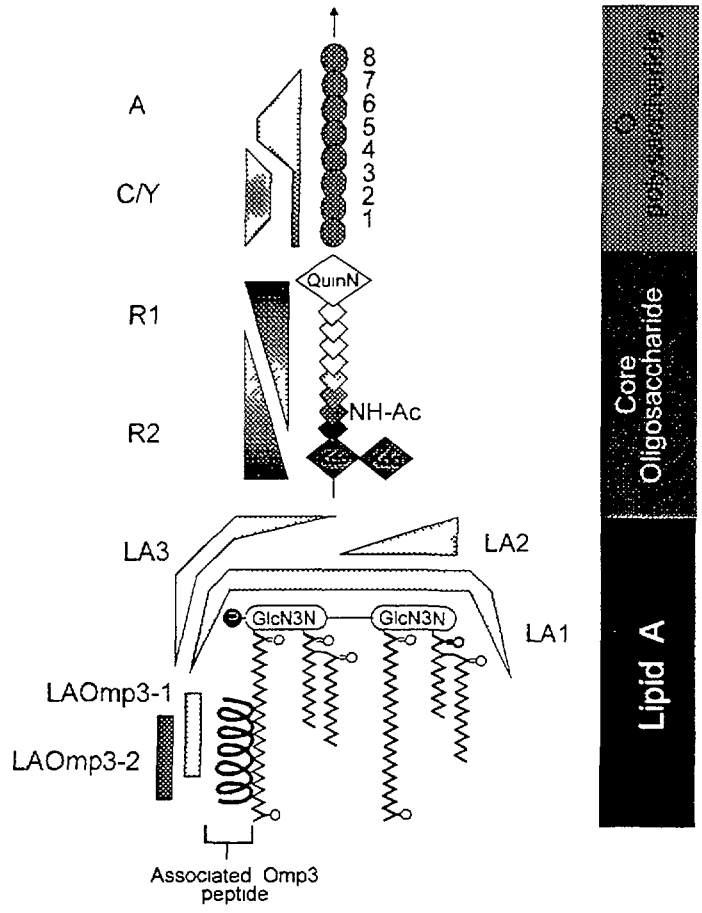

FIG 2 Schematic representation of B abortus (biotype 1) lipopolysaccharide The different epitopes recognised by sera from infected bovines and monoclonal antibodies are indicated The geometric diagrams indicate the degree of reactivity

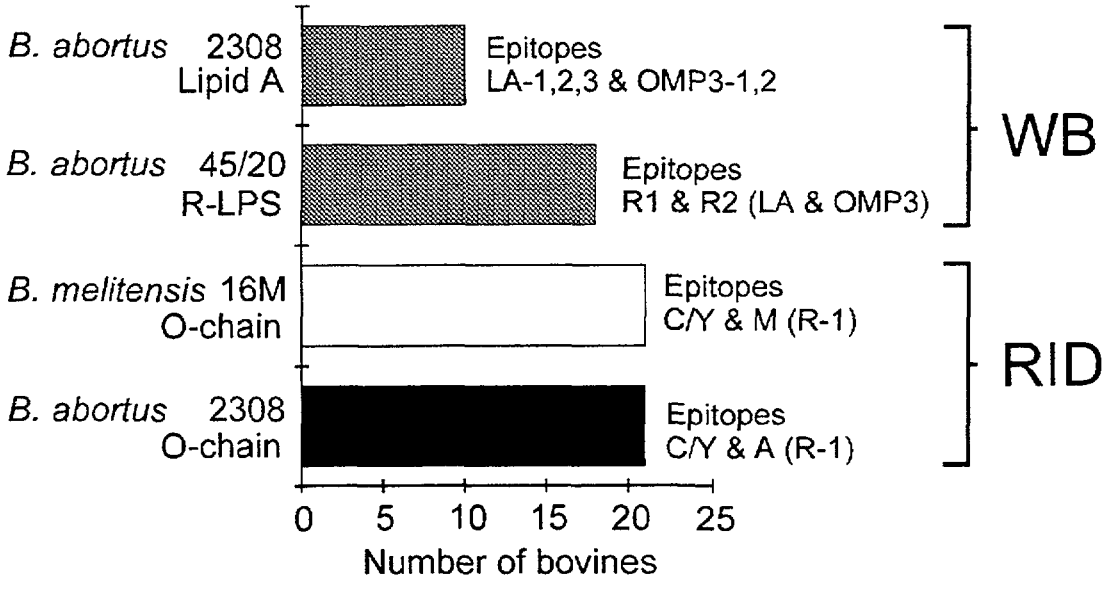

FIG 3 Western blot and Radial immunodiffusion of sera from B abortus (biotype 1) infected bovines against LPS derived antigens

2. MATERIAL AND METHODS

Two indirect and two competitive Enzyme-linked immunosorbent assays (I-ELISA102, I-ELISA103, C-ELISA1 and C-ELISA2 respectively) have been evaluated in comparison with traditional test such as Radial Immunodiffusion (RID), Complement Fixation (CF), Rose Bengal Agglutination (RB) and Rivanol Agglutination (RV). All the ELISA kits and their respective protocols and computation analysis were supplied by the Joint FAO/IAEA Division, Vienna, Austria and carried out as described in previous works [14,15,16]. The traditional serological assays were developed and performed as described elsewhere [17]. The sera analysed included 1018 sera obtained from non-vaccinated bovines, 848 sera from brucellosis free herds calf vaccinated with Strain-19, 295 sera obtained from brucellosis free herds adult vaccinated with Strain-19 and 665 sera from *B. abortus* biotype 1 (field strain) infected herds. The data obtained from the analysis of the samples of vaccinated and infected bovines was plotted in frequency histograms and the diagnostic sensitivity and specificity calculated as described elsewhere [16] using complement fixation (1/40) as standard test. Receiver operator analysis (ROC) for determination of cut-off value was performed with modifications as described elsewhere [18,19].

3. RESULTS

The frequency distribution of infected and vaccinated bovines for the different ELISAs is presented in Figure 4, 5, 6, 7. Even though a moderate overlapping between the infected and vaccinated animals is detected, a clear cut-off off value calculated by ROC analysis was established for each ELISA assay (Figure 8). Although all ELISAs fulfilled the requirements for sensitivity and specificity (Table I and II), in our hands C-ELISA2 performed slightly better than the other assays for differentiating infected from vaccinated bovines. The specificity of this test was similar to that of RID assay which is known to have high specificity for differentiating adult vaccinated from infected bovines. The kappa value among the different tests was good and within the limits of reproducibility and performance expected for the different assays (Table III).

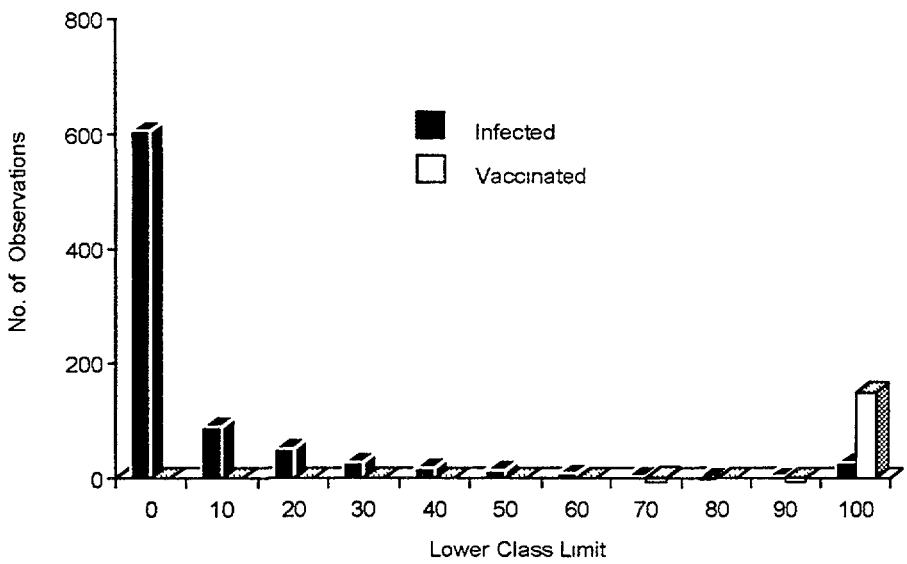

FIG. 4. Frequency distribution of infected and vaccinated bovines in the indirect ELISA (I ELISA102).

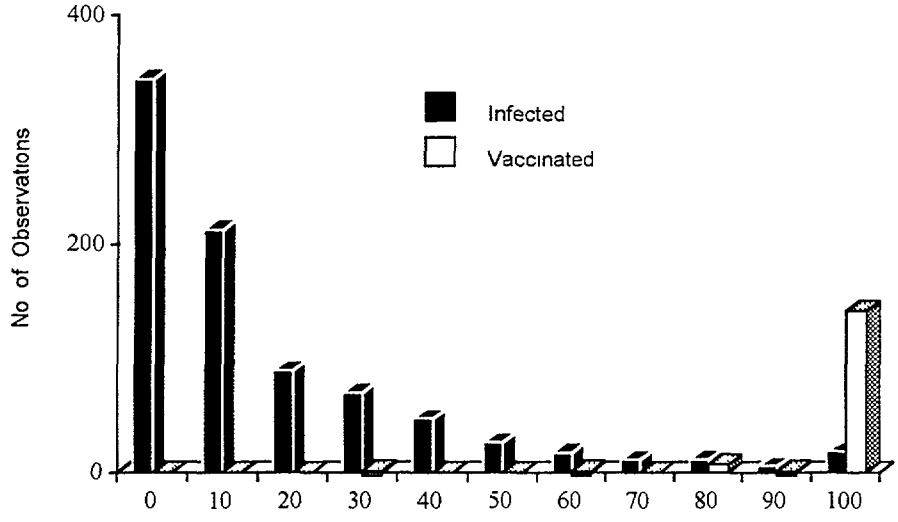

FIG 5 Frequency distribution of infected and vaccinated bovines in the indirect ELISA (I ELISA103)

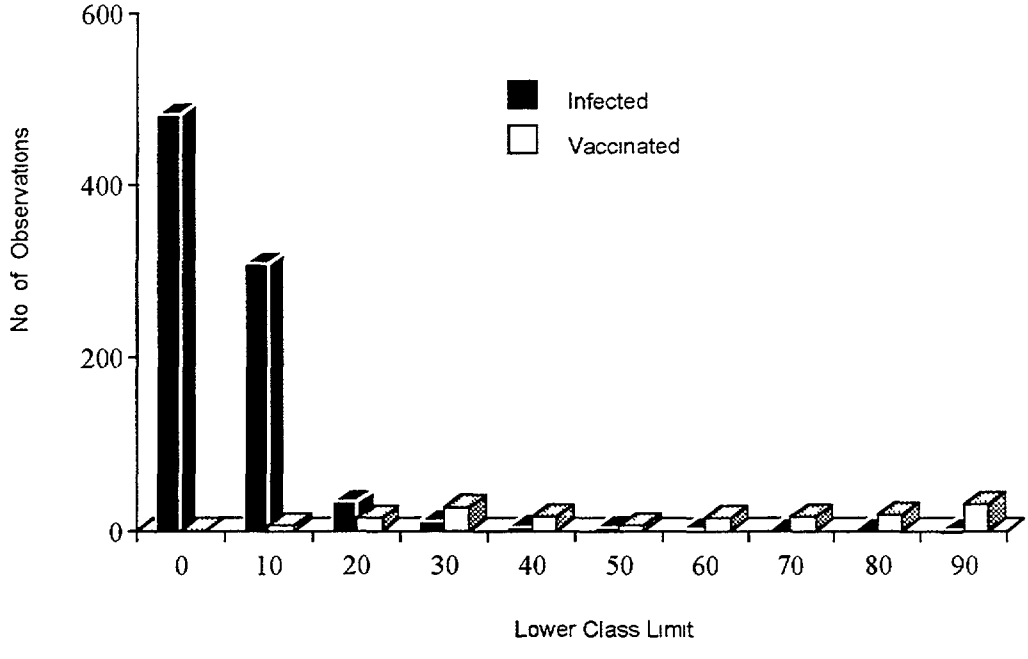

FIG 6 Frequency distribution of infected and vaccinated bovines in the competitive ELISA (C-ELISA1)

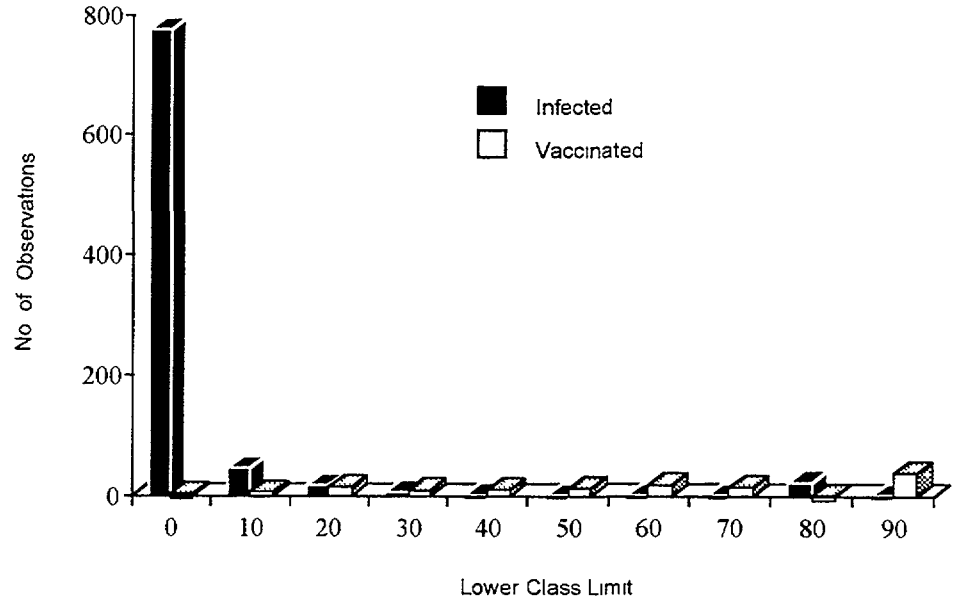

FIG 7 Frequency distribution of infected and vaccinated bovines in the competitive ELISA (C-ELISA2)

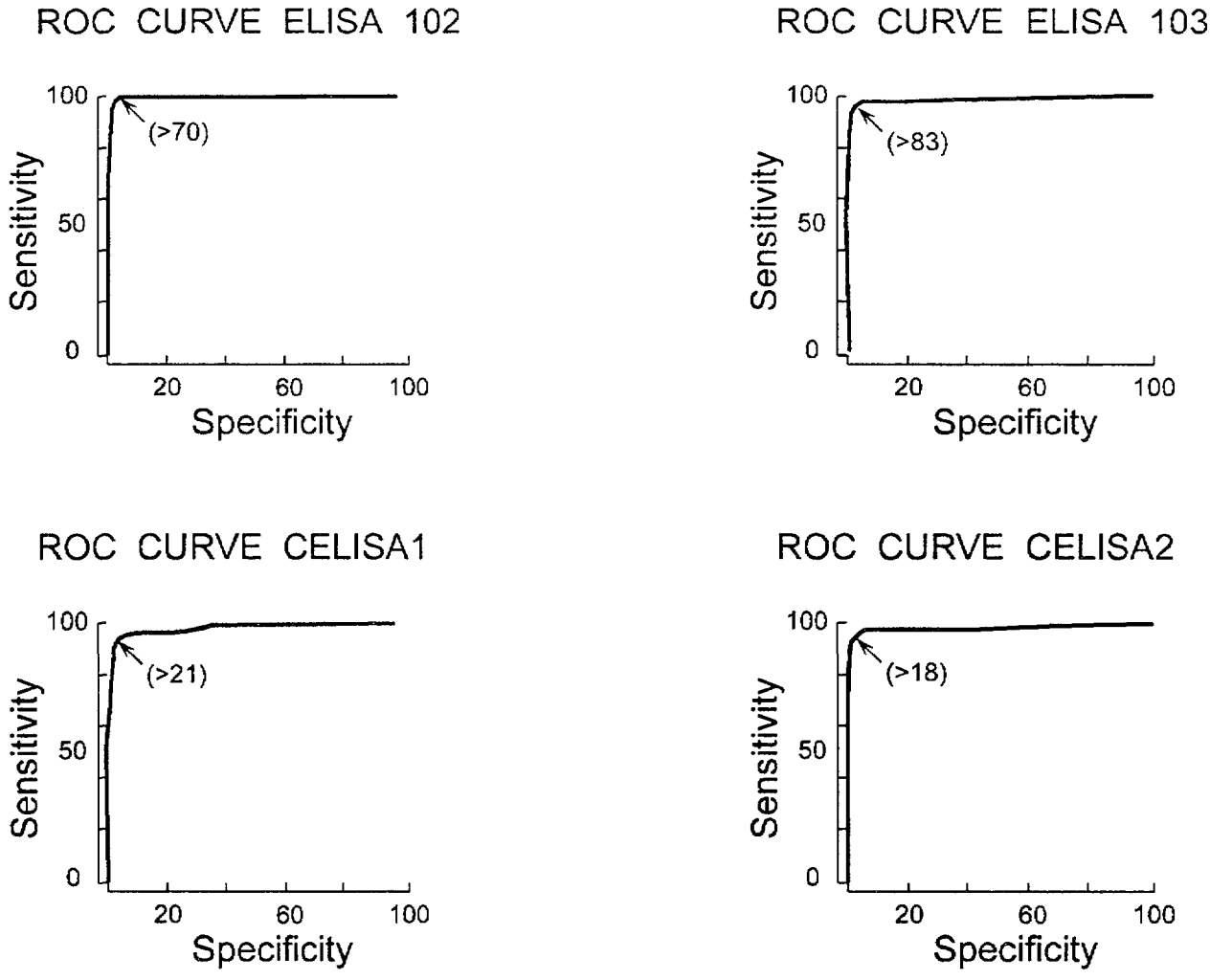

FIG 8 Receiver operator (ROC) curves for each ELISA assay The arrow shows the cut-off point

TABLE I. SENSITIVITY AND SPECIFICITY OF THE DIFFERENT ELISAS

Assay	Sensitivity	Specificity
IELISA102 (70%)	100% (99 44 to 100)	95 65 ± 1 37%
IELISA103 (65%)	99.34% (96 66 to 100)	93 54 ± 1.65%
CELISA-1 (21%)	95.39 ± 3.33%	94 36 ± 1.52%
CELISA-2 (21%)	94 08 ± 3 75%	96 83 ± 1.18%

For sensitivity positives defined RB/CFT[-], N =152
For specificity positives defined RB/CFT[-], N =851

TABLE II SPECIFICITY OF THE DIFFERENT ELISAS IN ADULT VACCINATED AND CALFHOOD VACCINATED BOVINES

Assay	Adult vaccinated	Calfhood vaccinated
IELISA102 (70%)	95 67 ± 2 77%	92 12 ± 1 95%
IELISA103 (65%)	97.11 ± 2 28%	92 80 ± 1 87%
CELISA1(21%)	97 11 ± 2 28%	93 75 ± 1 75%
CELISA2 (21%)	97 60 ± 2 08%	96 74 ± 1 28%

Adult vaccinated, positives defined RB/CFT[-], N =208
Calfhood vaccinated, positives defined RB/CFT[-], N =736

TABLE III KAPPA VALUES FOR THE DIFFERENT ELISAS

	ELISA102	ELISA103	CELISA1	CELISA2
ELISA102	-	-	-	-
ELISA103	0 814 (0 768 - 0 860)	-	-	-
CELISA1	0 774 (0 723 - 0 825)	0 790 (0 742 - 0 839)	-	-
CELISA2	0 826 (0 780 - 0 873)	0 770 (0 717 - 0 822)	0 801 (0 751 - 0 850)	0 836 (0 790 - 0 883)

95 % Confidence Limits in Brackets

4. DISCUSSION

Control and eradication of brucellosis requires at least four different coordinated measures vaccination, diagnosis, removal of reactors and epidemiological surveillance. If one of these actions is absent or is partially accomplished, then the disease remains as a constant or periodically emergent nightmare For the first of the requirements it has been clear for many years that *B abortus* S-19 is an efficient vaccine against bovine brucellosis and for the future there are in process several live experimental vaccines (e.g. non pathogenic rough *B. abortus* RB51 and transposon *B abortus* 2308 derived mutants) For the second of the conditions, we have demonstrated (together with our colleagues from other countries) the usefulness of several serological assays which allow us to distinguish vaccinated from infected bovines with high sensitivity and high specificity. The last two conditions are political and therefore out of the scope of this discussion.

From the different immunoenzymatic assays, the C-ELISA2, which uses LPS as antigen and a monoclonal antibody against the C/Y epitope as competing reagent, seems to be the most promising of

the ELISAs. This is due to several clear cut facts. For instance, the C-ELISA2 has excellent sensitivity and specificity with good reproducibility and possesses a convenient cut-off value for diagnostic purposes. In addition, this test uses purified LPS as antigen which is relatively easy to prepare and standardise (for serological analysis). Finally it is not restricted for bovines and can be adapted for different species of animals such caprines, ovines, suines, dogs, horses and humans (in these species the test has been tested with positive experiences). One of the only restrictions of these assay is that is not suitable for testing samples in the field, since it requires a relatively sophisticated equipment (ELISA reader, computers), suitable laboratory conditions and skilled technicians. However this test could be recommended for screening a large number of serum samples on a laboratory basis. For testing samples in the field it is probably more realistic to use Rose Bengal test (which possesses high sensitivity) in combination with a RID assay (which is known to have high specificity). These two tests are simple, robust, long term tested and do not require sophisticated equipment and combined generate a powerful tool for sera testing in the field. Similar to the CELISA-2 both tests can be used for the diagnosis of *Brucella* infection in other species, including humans [8].

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REFERENCES

[1] CORBEL, M. J. et al., Bergey's Manual of Systematic Bacteriology, Vol 1. Williams and Wilkins Co., Baltimore (1984) p. 377.

[2] CANNING, P., Advances in brucellosis research, L. G. Adams (ed.), Texas A&M University Press, College Station, (1990) p. 151

[3] RILEY, L. AND D. C. ROBERTSON, Brucellacidal activity of human and bovine polymorphonuclear leukocyte granule extracts against smooth and rough strains of *Brucella abortus*. Infect. Immun. **46** (1984) 231-236.

[4] ARAGÓN, V. R et al., Characterization of *Brucella abortus* and *Brucella melitensis* native haptens as outer membrane O-type polysaccharides independent from the smooth lipopolysaccharide, J. Bacteriol. **178** (1996) 1070-1079.

[5] CHERWONOGRODZKY, J. W. et al., Animal Brucellosis, K. Nielsen and B. Duncan (ed.). CRC, Press, Inc. Boca Raton, Florida, (1990) p.19.

[6] FREER, E. et al., Heterogeneity of *Brucella abortus* lipopolysaccharides, Res Microbiol. **146** (1995) 569-578.

[7] MORENO, E. et. al., *Brucella abortus* 16S rRNA and lipid, A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class Proteobacteria, J Bacteriol. **172** (1990) 3569-3576.

[8] DÍAZ-APARICIO, E. V. et al., Comparative analysis of *Brucella* serotype A and M and Yersinia enterocolitica O:9 polysaccharides for serological diagnosis of brucellosis in cattle, sheep, and goats, J Clin Microbiol. **31** (1993) 3136-3141.

[9] BUNDLE, D. R., et al., Structural elucidation of the *Brucella melitensis* M antigen by high-resolution NMR at 500 MHz, Biochemistry **26** (1987) 8717-8726.

[10] ROJAS, N. et al., Immunochemical identification of *Brucella abortus* lipopolysaccharide epitopes, Clin Diagn Lab Immunol. **1** (1994) 206-213.

[11] CANNING, P. C. et al., Opsonin-dependent stimulation of bovine neutrophil oxidative metabolism by *Brucella abortus*, Am J Vet Res. **49** (1988) 160-163.

[12] MONTARAZ, J. A. et al., Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies, Infect Immun. **51** (1986) 961-963.
[13] DÍAZ, R., OYELEDUM, M. A., Studies of some biological activities of "*brucella*" endotoxin in normal and infected animals and the role of the hypersensitivity factor, Ann. Sclavo. **19** (1977) 117-130.
[14] NIELSEN K. et. al., Enzyme immunoassay: application to the diagnosis of bovine brucellosis. Agriculture Canada Monograph (1992).
[15] NIELSEN K. et al., Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis, Vet. Immunol. Immunopathol. **46** (1995) 285-291.
[16] NIELSEN K. et al., Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis, Prev. Vet. Med. 26 (1996) 17-32.
[17] ALTON, G. G. et al., Techniques for the brucellosis laboratory, INRA , Paris (1988) p190.
[18] METZ, C., Basic principles of ROC analysis, Semin. Nucl. Med. **8** (1978) 283-300.
[19] STENSON, H., SIGNAL: a supplementary module for SYSTAT and SYGRAPH. SYSTAT, Inc. Evanson, IL (1988).

FIELD TRIAL OF A BRUCELLOSIS COMPETITIVE ENZYME LINKED IMMUNOABSORBENT ASSAY (ELISA)

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Abstract

FIELD TRIAL OF A BRUCELLOSIS COMPETITIVE ENZYME LINKED IMMUNOABSORBENT ASSAY (ELISA)

The purpose of this study was to evaluate the performance of a competitive ELISA system for the diagnosis of bovine brucellosis in comparison to conventional serological tests routinely used in Argentina A total of 2 500 serum samples, comprising *Brucella*-free herds, vaccinated cattle and naturally infected animals, was tested by the following tests buffered plate agglutination, Rose Bengal, 2-mercaptoethanol, complement fixation, and indirect and competitive ELISAs Specificity and relative sensitivity at each test were determined The competitive ELISA was considered suitable for detection of vaccinated animals and had higher specificity than the other tests The results point to the potential use of the test as a complementary assay in the brucellosis control programme in Argentina

1. INTRODUCTION

Bovine brucellosis has been a major disease for many years in Argentina Although considerable efforts have been undertaken to control it, in some regions of the country, prevalence rates are still high ranging from 30 to 35% [1]. As an action of the National Control and Eradication Program, massive vaccination of female calves, at an age between 3 and 10 months, using the S-19 vaccine strain, has been carried out during the last two years Vaccine coverage raised from 45% in 1994 to 85% in 1996 [2,3].

The Buffer Plate Agglutination Test (BPAT) is considered the screening diagnostic assay in Argentina. Negative animals are not further tested and positive samples are tested by Tube Agglutination (TA), 2-mercaptoethanol (2-ME) and Complement Fixation (CF) as complementary tests [3] However, many animals have vaccine-induced antibodies over a long period, especially those vaccinated around 10 months of age. Therefore, in infected herds it is difficult to distinguish antibody titres generated after vaccination from those produced due to natural field infections. None of the usual serological tests is able to distinguish antibodies produced against S-19 vaccine strain from natural infections [4]. A specific and reliable test will be desirable in any control and eradication program to enable to distinguish vaccinated from infected animals.

The purpose of this study was to evaluate the performance of a Competitive ELISA (C-ELISA) for the diagnosis of bovine brucellosis in the differentiation of *Brucella*-infected from *Brucella*-vaccinated cattle, in comparison to the Indirect ELISA (I-ELISA) and to conventional serological techniques used in Argentina.

2 MATERIALS AND METHODS

2.1. Experimental Design

Sera from 2,500 animals from three different groups were analysed:

Group A: 500 serum samples collected from non-vaccinated cattle from brucellosis-free herds A herd was considered free if no clinical and/or bacteriological evidence of brucellosis was registered and CF or 2-ME or Rivanol tests were negative for at least 2 years prior sampling.

Group B: 1,000 serum samples collected from S-19 calfhood-vaccinated cattle from brucellosis-free herds. Vaccinated herds had no clinical and/or bacteriological evidence of brucellosis for at least 2 years prior sampling, but had low prevalence rates (< 1%) of CF or 2-ME or Rivanol positive results during the last 2 years prior sampling.

Group C: 1,000 serum samples collected from vaccinated cattle from *Brucella*-infected herds, in which field strains of *B. abortus* had been isolated. In infected herds some animals had clinical and/or bacteriological evidence of brucellosis and high prevalence rates (> 5%) of CF or 2-ME or RIV positive results in animals older than 18 months were observed at the time of sampling. Samples were collected in a total of 12 farms, being 4 for each group.

2.2. Serological Tests

The BPA and RB results were expressed as positive or negative. Classification of cattle as seronegative or seropositive was set at a titre of 1:25 for the 2-ME and at a titre of 1:10 for the CF. These conventional tests were performed as described elsewhere [5,6].

Four ELISA systems were used :

a) an indirect ELISA (IE-1) using smooth lipopolysaccharide (SLPS) antigen and a mouse monoclonal anti-bovine IgG1 (MabM23) conjugated to horseradish peroxidase (HRPO) [9];

b) a modification of the IE-1 (IE-2) using SLPS and Mab M23, specific for bovine IgG1, conjugated with HRPO. In this technique, the sera is previously treated with EDTA/EGTA (dilution 1/50) [10];

c) a competitive ELISA (CE-1) using O-polysaccharide of *B. abortus* S1119-3 as antigen and YsT9 monoclonal antibody conjugate for competition;

d) a competitive ELISA (CE-2) using SLPS of *B. abortus* 1119/3 as antigen and a mouse monoclonal antibody (Mab M84) conjugate.

The local cut-off values were set at 40% positivity (%P) for IE and 40% inhibition (%I) for CE. The cut-off were obtained by testing negative serum samples [4]. The results were recorded as the mean of the optical density (O.D.) value of duplicates in all ELISA systems.

The BPA (12% Cel. Vol.) and RB (4,2% Cel. Vol.) antigens and all ELISA reagents and plates were provided by the FAO/IAEA. The antigens for 2-ME and CF tests were produced at INTA following standardised procedures [7].

All ELISAs were performed using polystyrene microplates (NUNC 269620,) and O.D. values were measured in a Multiskan II microplate reader linked to a 486 personal computer, using the FAO/IAEA software program BREIA 1.02.

2.3. Estimation of assay performance [8]

$$\text{Diagnostic Specificity} = \frac{\text{number of test negative cattle}}{\text{number of true negative cattle}} \times 100$$

$$\text{Relative Sensitivity} = \frac{\text{number of comparative test positive}}{\text{number of relative test positive}} \times 100$$

3. RESULTS

Table I shows the diagnostic specificity (D.S.) of each test in relation to negative serum samples (group A). The D.S. was 100% for CF, 100% for 2-ME, 98.6% for I-ELISA, 99.8% for C-ELISA, 99.8% for BPA and 97.7% for RB. Table II shows the D.S. of the sera from vaccinated animals (group B). D.S. for CF was 96.4%, 2-ME 93.6%, I-ELISA 95,8%, C-ELISA 97,5%, BPA 35.4% and for RB 37.6%. Table III shows the relative sensitivity (R.S.) for the CF test considering

sera from infected animals (group C). The R.S. for 2-ME was 99.8%, I-ELISA 98 2%, C-ELISA 97.3%, BPA 99% and RB 96% Tables IV and V show the kappa agreement and the ROC analyses respectively

TABLE I. DIAGNOSTIC SPECIFICITY (DS) OF SEROLOGICAL TESTS FOR BOVINE BRUCELLOSIS CONSIDERING THE NEGATIVE HERDS (Group A, n = 500)

	BPA	RB	CF	2-ME	IE-1	IE-2	CE-1	CE-2
+	1	11	0	0	7	9	4	1
-	499	489	500	500	493	491	496	499
DS%	99.8	97.8	100	100	98.6	98.2	99 2	99 8

2-ME Positive = 1 25 or more CF Positive = 1 10 or more
CE-1 LPS + O-Chain, cut-off 40%
CE-2 LPS + M84 (EDTA/EGTA) cut-off 40%P
IE1 LPS + M23 (EDTA/EGTA) IE-2 LPS + M23, cut-off 40%I

TABLE II. DIAGNOSTIC SPECIFICITY OF SEROLOGICAL TESTS CONSIDERING SERA FROM VACCINATED ANIMALS (Group B, n = 1,000)

	BPA	RB	CF	2-ME	IE-1	IE-2	CE-1	CE-2
+	322	364	28	46	51	50	27	17
-	678	636	972	954	949	950	973	983
DS%	67.8	63.6	97.2	95.4	94 9	95	97.3	98.3

2-ME Positive = 1 25 or more, CF Positive = 1 10 or more
CE-1 LPS +O-Chain, Cut-off 40%
CE-2 LPS + M84 (EDTA/EGTA) Cut-off 40%P
IE-1 LPS + M23 (EDTA/EGTA) IE-2 LPS + M23, Cut-off 40%I

TABLE III. RELATIVE SENSITIVITY (R S.) OF CF TEST CONSIDERING SERA FROM INFECTED ANIMALS (Group C, n = 1,000)

	BPA	RB	2-ME	IE-1	IE-2	CE-1	CE-2
+	999	971	998	982	969	965	975
-	1	29	2	18	31	35	25
RS%	99.9	97.1	99.8	98.2	96.9	96.5	97.5

RS Relative Sensitivity (CF= 1 10 or more)
2-ME Positive = 1 25 or more
CE-1 LPS + O-Chain Cut-off 40%
CE-2 LPS + M84 (EDTA/EGTA) Cut off 40%P
IE-1 LPS + M23 (EDTA/EGTA) IE-2 LPS + M23, Cut-off 40%I

TABLE IV. KAPPA AGREEMENT

	C-ELISA-1-OC	C-ELISA-2-M84	I-ELISA-1-ADRI	I-ELISA-2-IAEA
C-ELISA1	-	-	-	-
C-ELISA2	0.931 (0.903-0.958)	-	-	-
I-ELISA1	0.812 (0.769-0.855)	0.855 (0.817-0.893)	-	-
I-ELISA2	0.910 (0.879-0.941)	0.927 (0.899-0.955)	0.824 (0.782-0 866)	-

95% Confidence Limits in brackets

TABLE V. ROC ANALYSIS (Area under the ROC Curve)

ELISA	Area under ROC curve
IE-1ADRI	0.983 (95% CI = 0.978 to 0.995)
IE-2 (IAEA)	0.983 (95% CI = 0.978 to 0.995)
CI-1 (O-Ch)	0.991 (95% CI = 0.979 to 0.996)
CE-2 (M-84)	0.992 (95% CI = 0.980 to 0.998)

4. DISCUSSION AND CONCLUSIONS

In Argentina the bovine brucellosis control and eradication campaign was initiated through a national resolution in November of 1993 [2] and considerable efforts have been undertaken to achieve its goals. An insufficient vaccination coverage has substantially contributed to the spread of the disease. In Argentina, where the use of the S-19 vaccine is compulsory since 1982, vaccine coverage of female calves has never been more than 50% until recently. Due to an animal health information campaign during the last 3 years vaccination coverage raised to approximately 90% in 1996 [2]. The use of a whole set of diagnostic serological techniques is mandatory. Performance of these tests is expensive and has created a certain reluctance in the farmer community. Thus, a unique and standardised technique could have an important impact in our brucellosis program. Additionally, none of the conventional tests used for diagnosis is able to differentiate *Brucella*-vaccinated from *Brucella*-infected animals

Enzyme immunoassays for diagnosis of brucellosis have been studied for many years. In this study it could be demonstrated that the diagnostic specificity and relative sensitivity of ELISA is comparable to CF and 2-ME, which are the official complementary tests in Argentina. Validation of the I-ELISA some years ago corroborated its high sensitivity but its major drawback was the low specificity and its incapacity to differentiate *Brucella*-vaccinated from infected animals [4,8,9,]. The competitive ELISA proved to be the most suitable test to identify vaccinated animals. The specificity of the test was higher than other tests including CF. Although no significant differences between both competitive ELISAs could be observed, the C-ELISA using SLPS antigen is preferred because the antigen can be easily produced at low costs. Most of the false negative animals were common to all complementary tests. Interestingly, most of them are corresponding to *Brangus* breeds (cross breeding between *Bos angus* and *Bos indicus*).

Application of ELISA is feasible on large scale due to its reproducibility and easy standardisation. Complement fixation has a great performance but its application is cumbersome. The 2-ME test takes 48 hrs. to be completed and the reagent is toxic. Due to our conditions at farm level e.g. brucellosis prevalence and compulsory vaccination of female calves the C-ELISA could be applied, replacing the conventional tests as a complementary test of the official screening test (BPA). This study demonstrates that the ELISA test would be very useful to the Control and Eradication Program of Bovine Brucellosis in Argentina.

ACKNOWLEDGEMENT

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REFERENCES

[1] NAVARRO, F., GREGORET, R., BIDONDE, J., SAMARTINO, L., Prevalence of Bovine Brucellosis in Buenos Aires Province, Rev. Soc. Med. Vet. (1997) in press.

[2] SENASA annual book. National Programs of Animal Health (1995, 1996).

[3] Ministry of Agriculture, National Control and Eradication Program of Bovine Brucellosis. Resolution 1269 November (1993).

[4] NIELSEN, K., A brief review of diagnosis of bovine brucellosis by detection of antibody. Arch. Med. Vet. XXVII (1995).

[5] ALTON, G., JONES, L.M., ANGUS, R.D., VERGER, J.M., Techniques for the brucellosis laboratory. INRA, Paris, (1988).

[6] Pan American Health Organisation, Pan American Zoonosis Centre, The Complement Fixation Test for the diagnosis of bovine brucellosis, Technical note N.° 24. (1981).

[7] Pan American Health Organisation, Pan American Zoonosis Centre, Elaboration and standardisation of antigens for the diagnosis of bovine brucellosis, Technical note N° 3. (1971).

[8] SARAVÍ, M., WRIGHT, P., GREGORET, R., GALL, D., Comparative performance of the enzyme-linked immunosorbent assay (ELISA) and conventional assays in the diagnosis of bovine brucellosis in Argentina, Vet. Imm. Immunop. **47** (1995) 93-99.

[9] Office International des Epizooties (OIE), World organisation for animal health, Manual of Standards for Diagnostic Test and Vaccines, Chapter 3.2.1 Bovine brucellosis, Third Edition (1996).

[10] NIELSEN, K., KELLY, L., GALL, D., SMITH, P., BOSSE, J., NICOLETTI, P., KELLY, W., The use of divalent cation chelating agents (EDTA/EGTA) to reduce non-specific serum protein interaction in enzyme immunoassay. Vet. Res. Comm. **18** (1994) 433-436.

FIELD TRIAL OF BRUCELLOSIS COMPETITIVE ELISA

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Abstract

FIELD TRIAL OF BRUCELOSIS COMPETITIVE ELISA

2990 sera samples from cattle were tested for antibodies to *Brucella abortus* using 8 serological tests for The tests used were Rose Bengal (RBT), Buffer Plate Agglutination Test (BPAT), Complement Fixation (CFT), 2 Indirect and 2 Competitive Enzyme Linked Immunosorbent Assays (ELISA) Bacteriological evaluation from milk was done also All tests were compared with respect to diagnostic specificity in vaccinated herds which were considered to be *Brucella*-free The diagnostic specificity of the Indirect and Competitive ELISA was greater than 99,8% Estimates of relative sensitivity were obtained from infected herds The diagnostic sensitivity of the Indirect ELISA was greater than 95,8% and for the Competitive ELISA between 98,8 and 100 %, the last value refers to the Competitive ELISA Prototype II (SLPS antigen/M84 Mab), which was found highly suitable to differentiate vaccinated from *brucella*-infected cattle The use of C-ELISA II for monitoring bovine populations under an eradication programme is recommended

1. INTRODUCTION

The occurrence and persistence of serum antibodies following *brucella* strain 19 vaccination is the major disadvantage since antibodies may interfere with detection of brucellosis infected cattle The diagnostic problem is related to the type of test used and to the interpretation of results The Competitive ELISA for detection of bovine serum antibodies to *Brucella abortus* field strains generally do not react with sera containing residual antibody from vaccination with *B. abortus* S-19 The Joint FAO/IAEA Division is carrying out a field trial in Latin America to validate the diagnostic performance of competitive brucellosis ELISAs for the differentiation of vaccinated from infected cattle and to compare the diagnostic performance characteristics of the competitive ELISAs to the indirect ELISA and to the standard serological techniques.

2 MATERIAL AND METHODS

2.1. Test groups

2 1.1 Sera from non vaccinated/brucellosis-free herds:

991 sera from non vaccinated cows from a brucellosis-free area in the south of Chile, Punta Arenas, without clinical or bacteriological history of brucellosis for more than 2 years prior to sampling and no CFT or 2ME or RIV reactors for more than 2 years prior to sampling.

2.1 2. Sera from S-19 calfhood vaccinated/brucellosis-free herds:

985 sera from vaccinated cows from brucellosis-free herds in the X region of Chile with calfhood vaccination between 3-8 months of age, in which there has been no clinical and/or bacteriological evidence of brucellosis for at least 2 years prior to sampling.

2 1.3 Sera from vaccinated or non-vaccinated cattle from Brucella-infected herds:

1.004 sera from cows belonging to infected herds with prevalences between 5-15% and bacteriological evidence of brucellosis. Most of these cows were calfhood vaccinated. Some received adult vaccination with a reduced doses of S-19.

2.2. Serological evaluation

Blood samples were taken from each cow for serological evaluation with a careful identification for each animal. Each serum sample was stored in two vials. Data were electronically stored in a spread sheet format (Excel software). Data for analysis included each cow's identification number, age, vaccination status, vaccination age, culture results and serological test data.

2.3. Serological tests

2.3.1. Rose Bengal plate test (RBT:
Antigen lot/Batch 46G774 Rhône Merieux- Francia, supplied by IAEA/OIE protocol.

2.3.2. Buffered plate agglutination test (BPAT):
Buffered plate antigen Lot SR 2-95-01, supplied by IAEA/OIE protocol.

2.3.3. Complement fixation test (CFT):
Based on the 50% haemolytic unit, taking as a model the "Maltener" reaction described by Wadsworld. The complement is tittered by Von Krog formula [2] Reagents belong to the Servicio Agrícola y Ganadero.

2.3.4. Indirect ELISA:
Kit supplied by Joint FAO/IAEA Division and endorsed protocol followed.

2.3.5. Indirect ELISA. ADRI:
Kit supplied by ADRI Nepean.ADRI, Nepean protocol.

2.3.6. Competitive ELISA, Prototype I:
(O-chain antigen, YST9 Mab). Chemicals supplied by the Joint FAO/IAEA Division, biologicals supplied by ADRI, Nepean. IAEA protocol.

2.3.7. Competitive ELISA, Prototype II:
(SLPS antigen, M84 Mab). Chemicals supplied by the Joint FAO/IAEA Division, biologicals supplied by ADRI, Nepean IAEA protocol.

2.4. Bacteriological evaluation

Quarter milk samples were collected under sterile conditions and transported immediately to the laboratory. After 8 to 24 hours gravity cream was transferred to culture plates. Samples from aborted foetuses were taken and cultured in selective, solid media.

3. RESULTS

TABLE I. NUMBER OF SERA TESTED FOR EACH GROUP BY DIFFERENT TESTS

Test	Number of sera from neg. herds	Number of sera from inf. Herds	Number of sera from vacc. herds	Total number of sera tested
RB Chile	991	1.013	985	2.989
RB Canada	991	1.013	985	2.989
BPAT	991	1.014	985	2.990
C.Fixation	991	1.015	985	2.991
I-ELISA ADRI	991	1.012	985	2.988
I-ELISA.IAEA	991	1.013	985	2.989
C-ELISA I O-chain	991	1.015	985	2.991
C-ELISA II LPS.	991	1.014	985	2.990

3.1. Data Analysis

The relative sensitivity and specificity of sera from infected cattle and brucellosis-free animals were calculated using the CF as the gold test (Table II)

TABLE II. SENSITIVITY OF DIFFERENT TESTS

Test	Cut-off	Sensitivity
ADRI-ELISA	(30%)	95.84 +/- 1.51%
IAEA-ELISA	(22%)	96.88 +/- 1.31%
CELISAI	(22%)	98.81 +/- 0.82%
CELISA II	(29%)	100%
CFT	(AC+)	93.23 +/- 1.83%
CFT	(AC-)	93.09 +/- 1.85%

ELISA positives defined (RB/BPAT/CFT(+)),N=674/671
CFT positives defined (RB/BPAT(+)),N=724

The specificity of the tests was calculated using sera from non-vaccinated and brucellosis-free herds (Table III)

TABLE III. SPECIFICITY OF DIFFERENT TESTS

Test	Cut-off	Specificity
ADRI-ELISA	(30%)	100% (99.52-100)
IAEA-ELISA	(22%)	99.80% (99.19-99.96)
CELISAI	(22%)	100% (99,52-100)
CELISA II	(29%)	99.80% (99.19-99.96)
CFT	(AC+)	98.08 +- 0.85%
CFT	(AC-)	99.60 +- 0.39%

N= 991
95% CI in brackets

The relative specificity of the tests were calculated using sera from vaccinated and brucellosis free cattle (Table IV).

TABLE IV. RELATIVE SPECIFICITY OF DIFFERENT TESTS

Test	Cut-off	Specificity
ADRI-ELISA	(30%)	95.74 +/- 1.26%
IAEA-ELISA	(22%)	96.04 +/- 1.22%
CELISAI	(22%)	96.45 +/- 1.15%
CELISA II	(29%)	100%
CFT	(noAC)	97.36 +/- 1.00%

N=985

The kappa agreement of different tests is shown in Table V.

TABLE V. KAPPA AGREEMENT

	ADRI	CELISA I	CELISA II
ELISA ADRI	-	-	-
CELISA I	0.963 (0.94-0.975)	-	-
CELISA II	0.962 (0.94-0.975)	0.987 (0.980-0.995)	-
ELISA IAEA	0.963 (0.94-0.976)	0.968 (0.956-0.981)	0.968 (0.956-0.981)

95% confidence limits in brackets

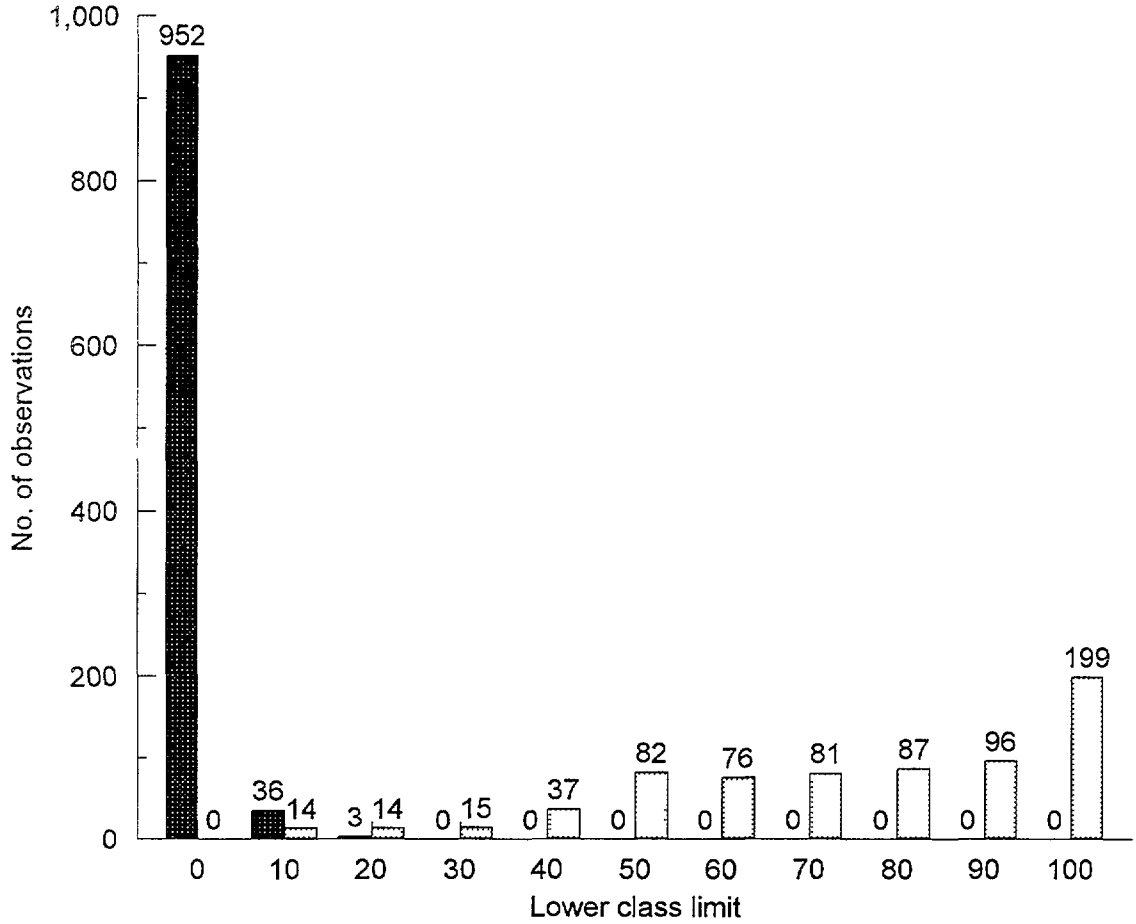

FIG. 1. Frequency distribution ADRI ELISA, Chile.

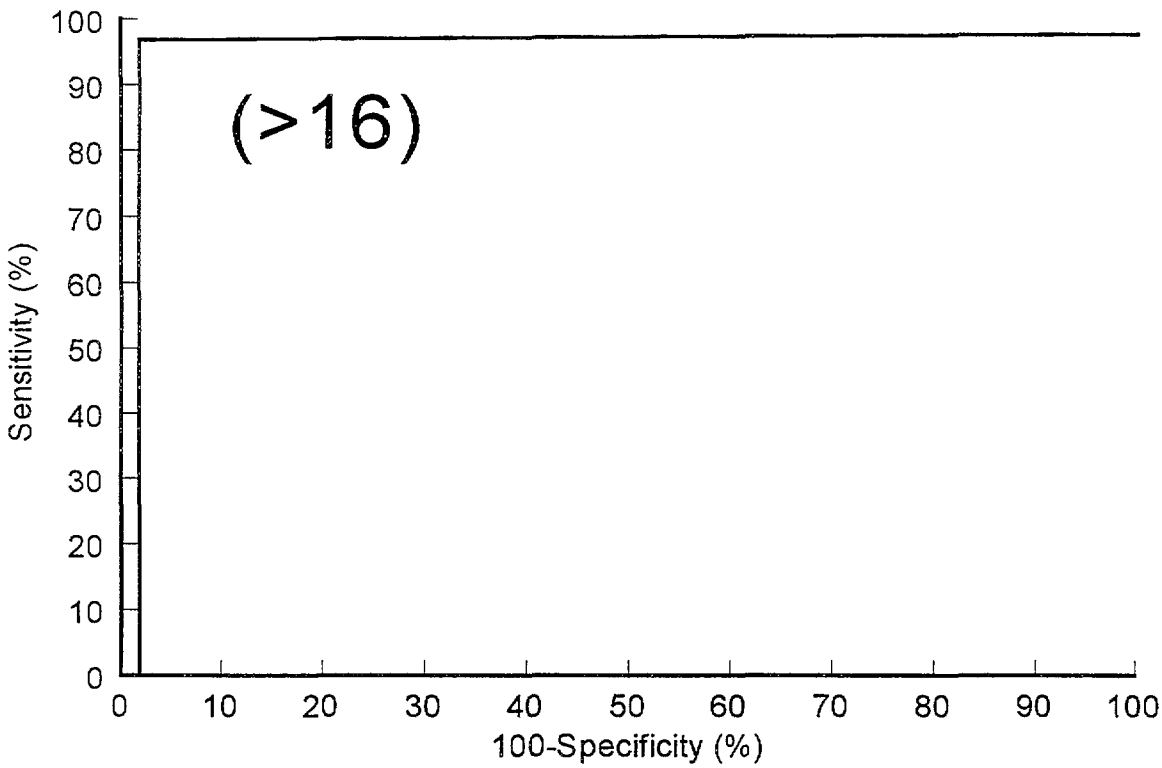

FIG. 2. ROC curve ADRI ELISA.

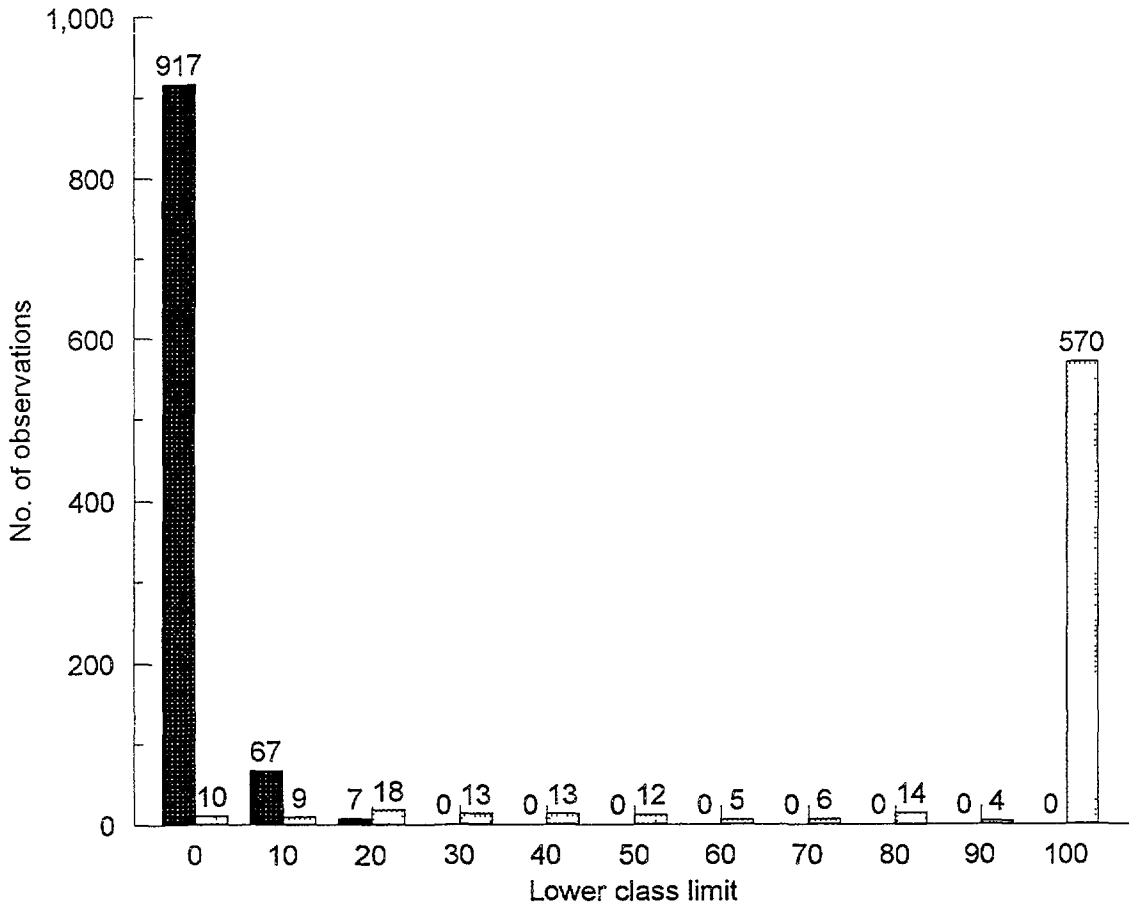

FIG. 3. Frequency distribution IAEA ELISA, Chile.

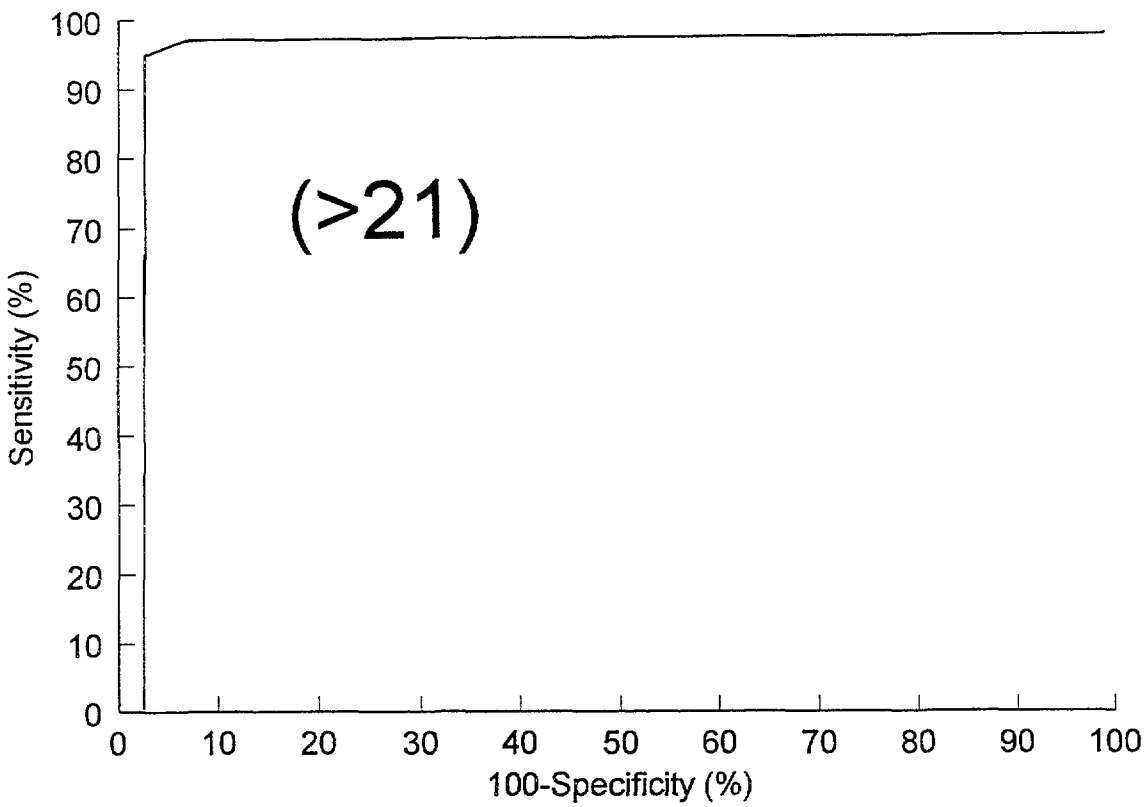

FIG. 4. ROC curve IAEA ELISA.

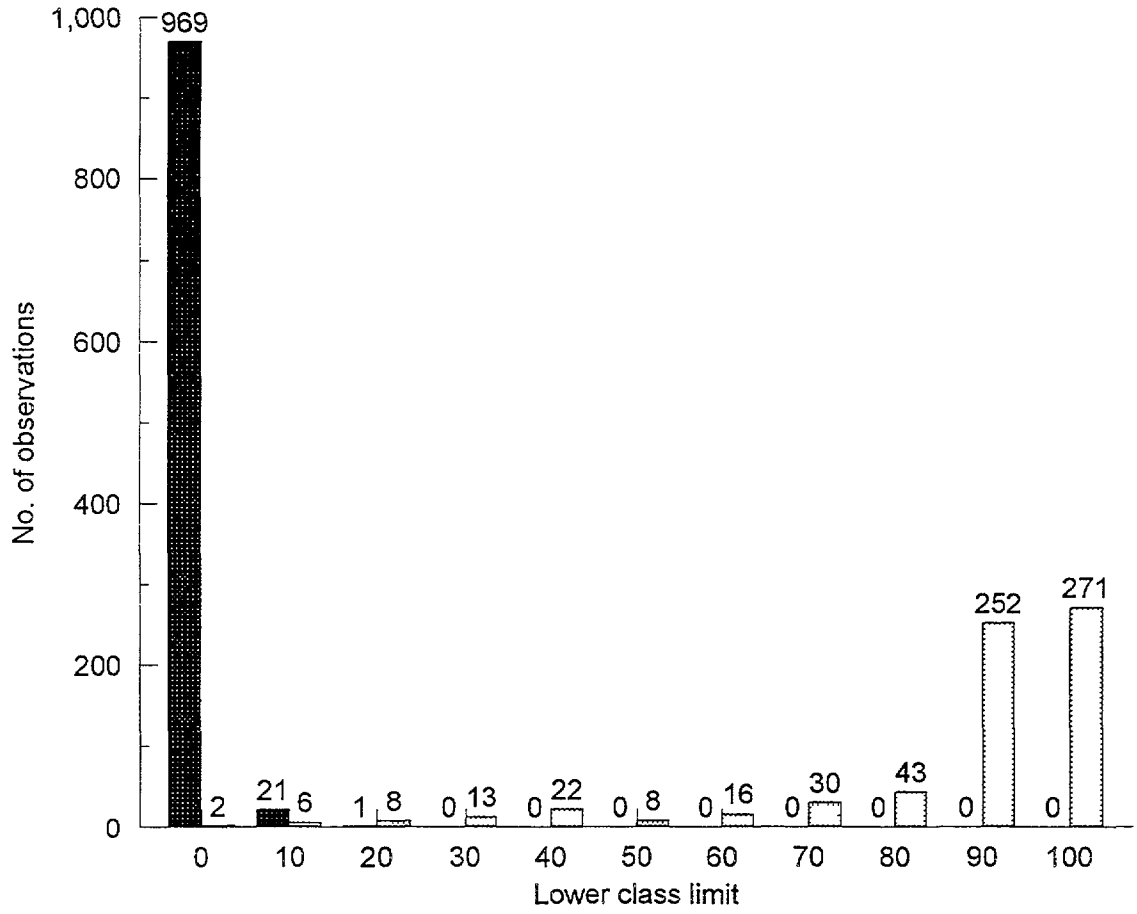

FIG 5. Frequency distribution CELISA1, Chile

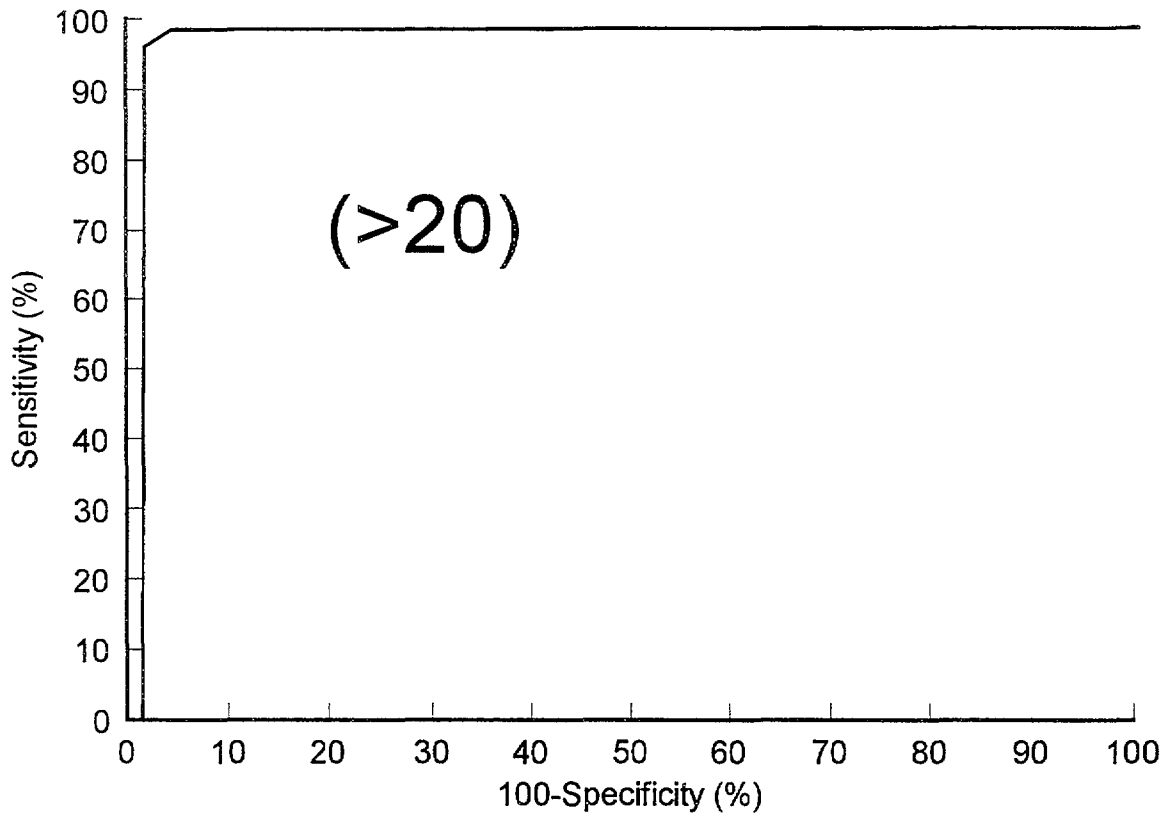

FIG 6 ROC curve CELISA1 OC

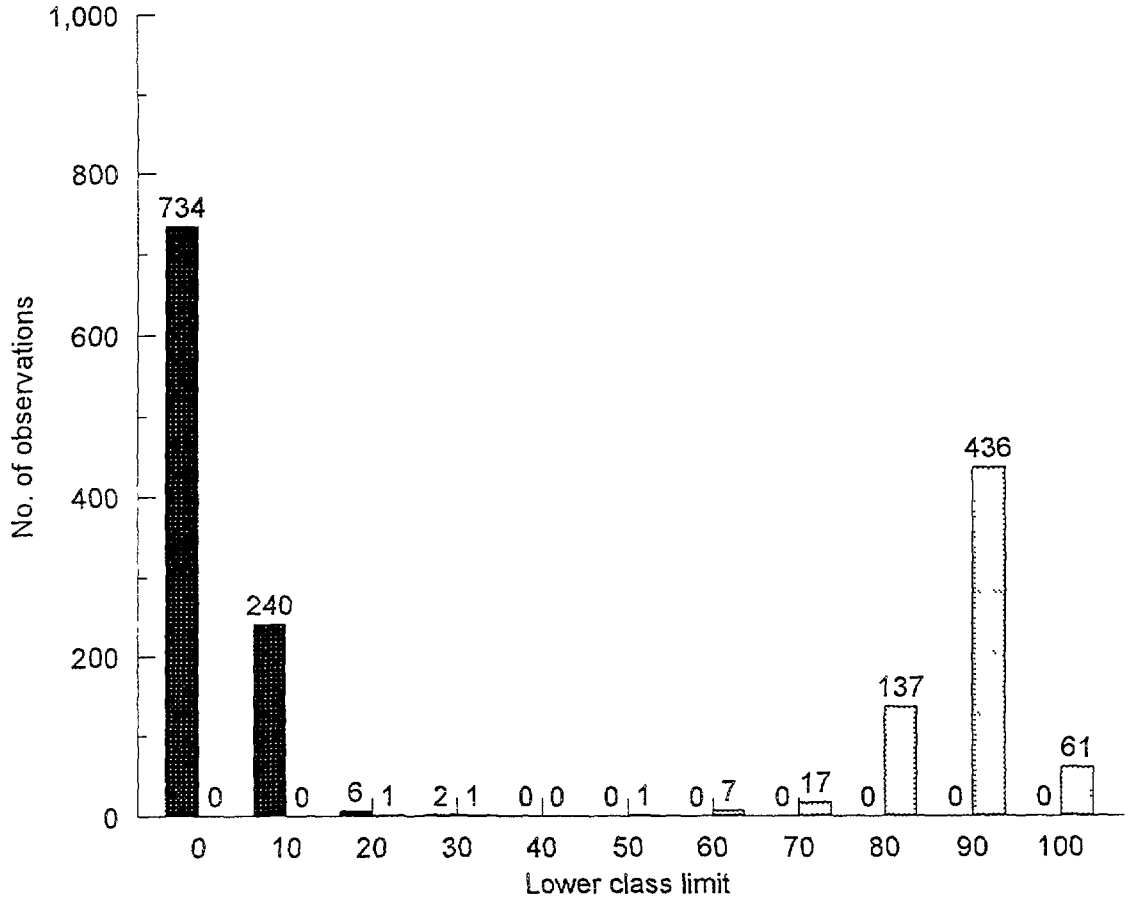

FIG. 7. Frequency distribution CELISA2, Chile.

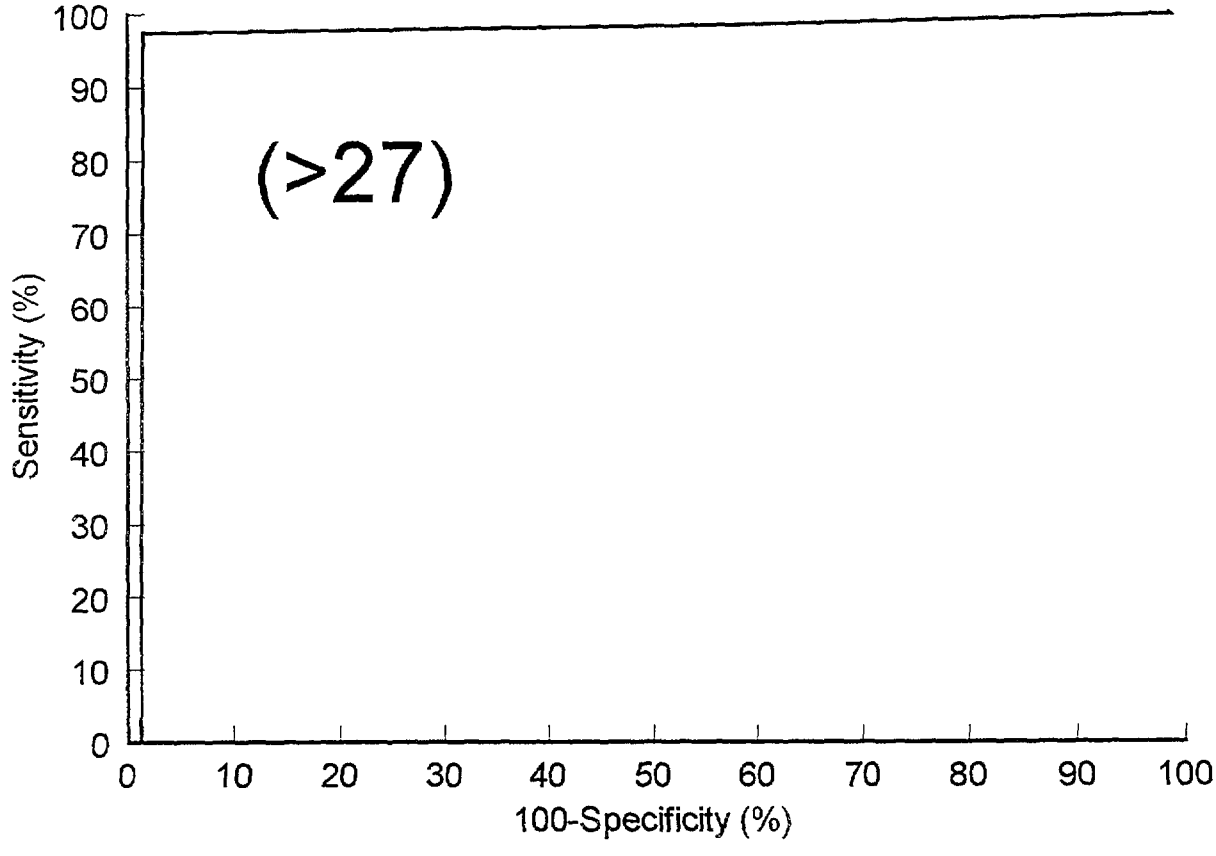

FIG. 8. ROC curve CELISA2 M84.

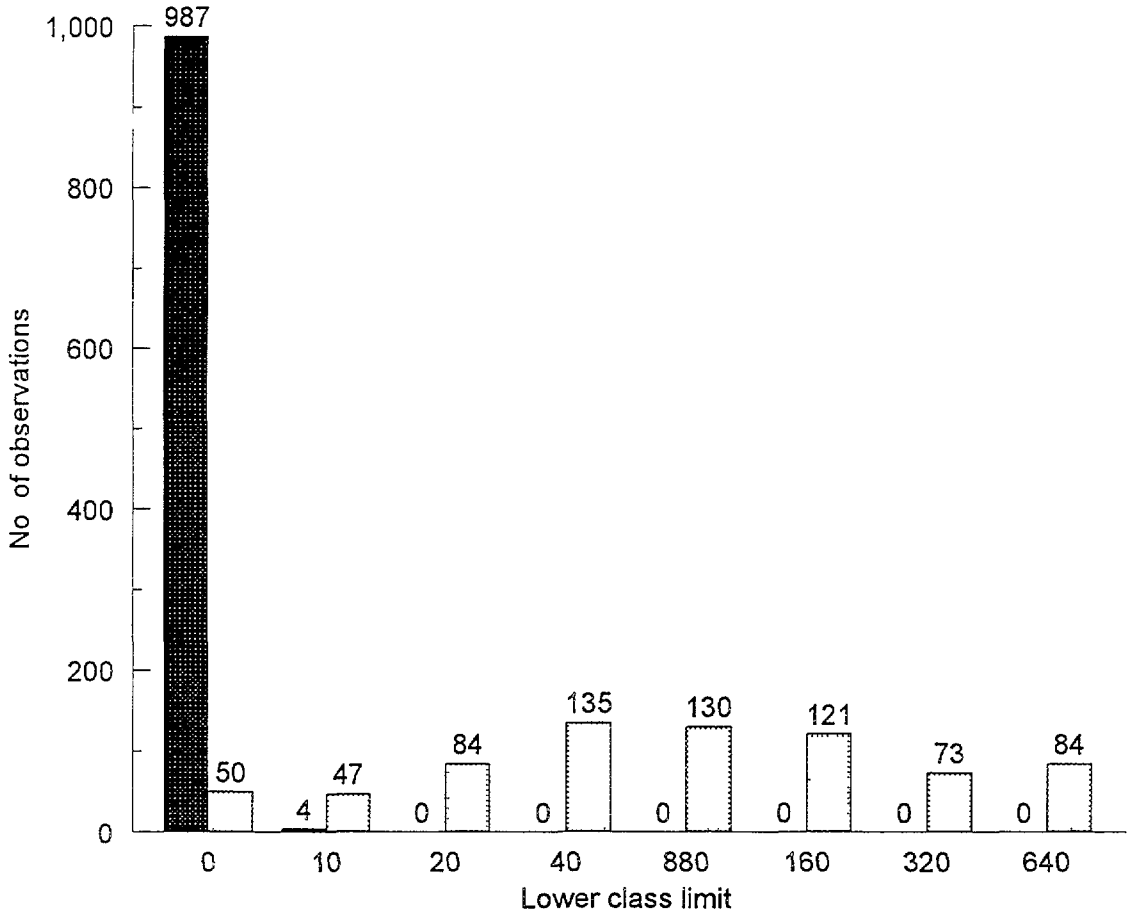

FIG. 9. Frequency distribution CFT, Chile.

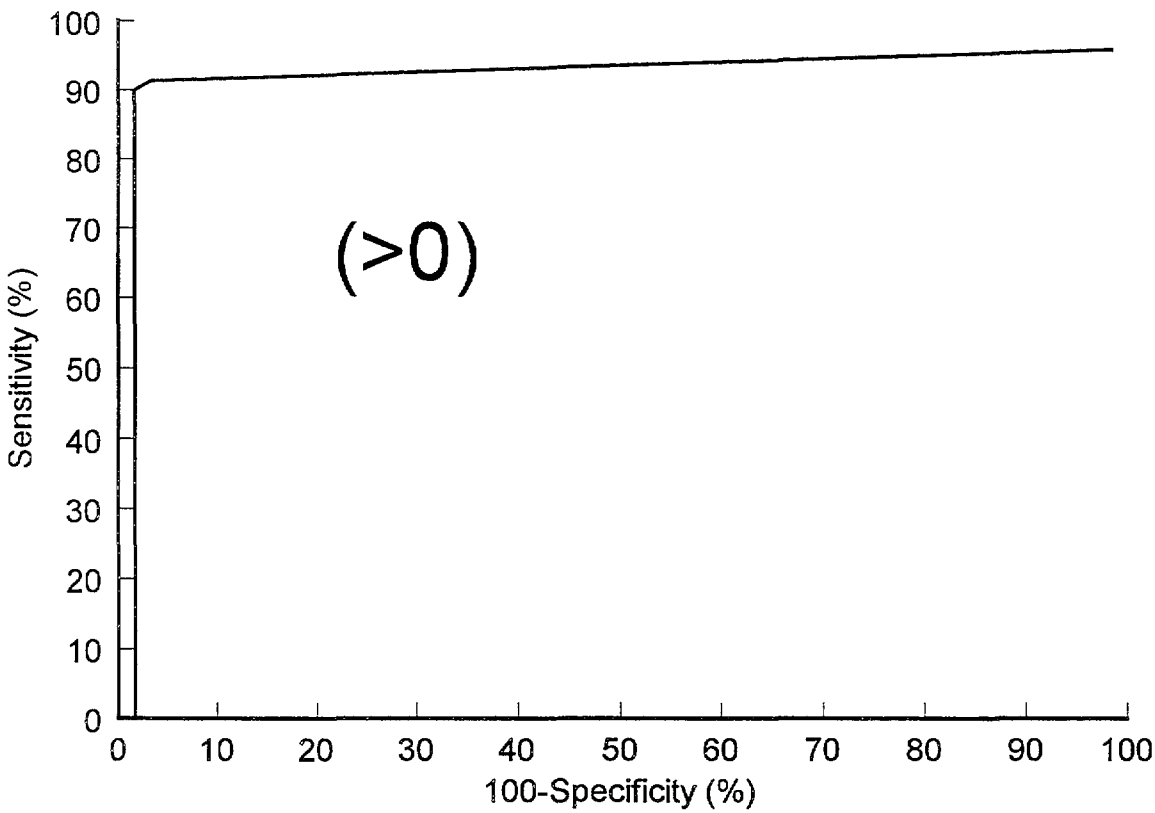

FIG. 10. ROC curve CFT.

4. CONCLUSIONS AND RECOMMENDATIONS

Standardised ELISAs cut-off values were defined under the epidemiological conditions in Chile. From Table II and III it can be seen that ELISAs (Indirect and Competitive) have better sensitivity and specificity than the Complement Fixation thus detecting more infected cattle than the conventional test. This is in agreement with previous findings [7,8,9].

The BPAT, RB, Complement Fixation test and the Indirect ELISA do not distinguish vaccinal antibody from that due to infection. Competitive ELISA II (which uses LPS antigen and monoclonal antibody Mab 84 as competing reagent) showed the best performance in differentiating vaccinated animals with *B.abortus* strain 19 and those who have been exposed to cross-reactive organisms.

The Indirect and Competitive ELISA showed a high reproducibility and were easy to standardise. Results show that the C-ELISA II provides higher accuracy than the conventional tests and that it would be very useful in the National Bovine Brucellosis Eradication Programme in Chile.

ACKNOWLEDGEMENTS

This research was carried out with the support of the Animal Production and Health Section of the FAO/IAEA Joint Division in Austria, the Animal Disease Research Institute (A.D.R.I.), Canada and the Servicio Agrícola y Ganadero, Chile (SAG) as part of a Regional Research Programme. We thank especially Klaus Nielsen, David Gall and Axel Colling for their support and valuable help.

REFERENCES

[1] ALTON, G.G., JONES, L.M., ANGUS, R.D., VERGER, J.M., Technique for the Brucellosis Laboratory, Institut National de la Recherche Agronomique, Paris (1988) .

[2] CEPANZO, Centro Panamericano de Zoonosis, Fijación del complemento para el diagnóstico de la brucelosis, Nota Técnica N **24** (1981) 30p.

[3] MAC MILLAN, A., Conventional serological tests, In: Nielsen, K. and Duncan, J. R., Animal Brucellosis, CRC Press, Inc. Florida, (1990) 153-198.

[4] NICOLETTI, P., Further evaluation of serological test procedures used to diagnose brucellosis, In: Nielsen, K., Gall, D., Kelly, W., Vigliocco, A., Henning, M., García, M., Desarrollo del inmunoensayo. Aplicaciones del enzimo inmunoensayo para el diagnóstico de la brucelosis

[5] NIELSEN, K., DUNCAN, J.R., Antibody isotype response in adult cattle vaccinated with *Brucella abortus* S-19, Vet. Immunol. Immunopathol. **19** (1988) 205-214.

[6] NIELSEN, K., Enzyme immunoassay development, Agriculture Canada, Animal Disease Research Institute, Nepean, Ontario, Canada, (1991) 52p.

[7] NIELSEN, K., GALL, D., KELLY, W., HENNING, D. AND GARCÍA, M., Enzyme immunoassay: Application to diagnosis of bovine brucellosis, Published by Agriculture Canada, Animal Diseases Research Institute, Canada, (1992) 203.

[8] NIELSEN, K., GALL, D., KELLY, W., VIGLIOCCO, A., HENNING, M., GARCÍA, M., Aplicación de los inmunoensayos en la serología de la brucelosis, (1996).

[9] WRIGHT, P.F., NIELSEN, K., Application of enzyme immunoassay in Veterinary Medicine: Serodiagnosis of bovine brucellosis, In: T. T. Ngo. ed. Nonisotopic Immunoassay, Plenum Press, New York, USA, (1988) 129-146.

[10] WRIGHT, P.F., NIELSEN, K., KELLY, W., Primary binding techniques for the serodiagnosis of bovine brucellosis: Enzyme immunoassay, In: Nielsen, K. and Duncan, J.R., Animal Brucellosis, Ed. CRC. Press. Inc. Florida, USA, (1990) 199-235.

[11] WRIGHT, P.F., NILSSON, E., VANROOIJ, E., LELENTA, M., JEGGO, M., Standardisation and validation of enzyme linked immunosorbent assay techniques for the detection of antibody in infectious diseases diagnosis, Rev. Sci. Tech. Off. Int. Epiz. **12** (1993) 435-450.

COUNTRY REPORTS

Part B: FOOT-AND-MOUTH DISEASE

FMD antigen ELISA

SEROLOGICAL CHARACTERISATION OF FOOT-AND-MOUTH DISEASE TYPE "O" FIELD ISOLATES FROM PERU: 1992–1994

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Abstract

SEROLOGICAL CHARACTERISATION OF FOOT-AND-MOUTH DISEASE TYPE "O" FIELD ISOLATES FROM PERU 1992-1994

Nineteen field isolates of foot-and-mouth disease Virus (FMDV) recovered from bovine epithelial samples corresponding to outbreaks present in different regions of Peru, between 1992-1994 were studied The relationship of the virus isolates to the O/Urubamba vaccine strain of Peru was determined by the calculation of the "r" values obtained by the liquid-phase blocking ELISA All the isolates showed "r" values higher than 0 66 indicating that the vaccine strain should protect against the field strains Characterization of the field isolates by a trapping ELISA using a panel of monoclonal antibodies against FMDV O/Switzerland and O/Caseros, showed slight differences in the profiles of the field isolates when compared with the O/Urubamba vaccine strain, but no differences were found among all the isolates

1 INTRODUCTION

Foot-and-mouth disease is a very important disease of cattle, sheep, goats and pigs in Peru The first laboratory diagnosis of the disease was reported in 1951. In Peru the FMDV types O, A, and C have been isolated In addition the presence of Vesicular Stomatitis Virus (VSV) serotypes New Jersey and Indiana has been reported. In the last ten years no isolation of the FMDV type C nor VSV serotype Indiana has been reported. The use of improved techniques for the rapid diagnosis and characterization of isolates such as the enzyme linked immunosorbent assay (ELISA) have been extensively described [1 - 5]

The establishment of ELISA as a routine method for the diagnosis and serotyping of FMD and VS, as well as for the detection and titration of antibodies in order to assess the immune status of the cattle population has been an important development. ELISA has also been used for the epidemiological monitoring of new field strains and measuring the protection given by the vaccine strains [6]

During the years 1992 to 1994, several outbreaks of FMDV type "O" were reported from all regions of the country [7,8]. The vaccine being used for the National Control Programme contains the strains O/1, A/24 and C/3 formulated with oil adjuvant.

The objective of this study was the characterization of the new isolates of FMDV present in Peru and in particular their relationship to the reference vaccine strain used in the FMD National Control Programme

2. MATERIAL AND METHODS

2.1. Viruses and cell cultures

Viruses originally isolated from bovine epithelial samples from different regions of Peru, are listed in Table I. The antigens were passaged once in primary bovine thyroid cells (BTY) and used for the serological characterization. The vaccine strain O/Urubamba, grown in BHK/21 cells was obtained from the FMD Vaccine Production Laboratory of the National Institute of Health.

2.2. Polyclonal antisera

Rabbit and guinea pig anti-FMDV polyclonal antisera were provided by the World Reference Laboratory (WRL) Pirbright, U.K. and used as described by Ferris [9].

Bovine post-vaccinal sera from cattle vaccinated during the regular FMD vaccination program in Peru were used as reference sera to calculate the "r" values.

Rabbit anti-mouse and rabbit anti-guinea pig immunoglobulins conjugated to horseradish peroxidase were obtained from a commercial source.

2.3. Monoclonal Antibodies

A panel of eight neutralizing Mabs produced against the FMDV strains O/1/Switzerland/65 (B2, C6, C8, C9 and D9) and O/1/Caseros (OC1, OC2,and OC3) were provided by Dr J. Crowther, WRL, Pirbright, U.K..

2.4. ELISA method

Each isolate and the vaccine strain were titrated by the indirect sandwich ELISA described by Hamblin et al [1]. A fixed concentration of the field virus equivalent to a concentration giving an optical density (OD) of 1.5 was reacted with bovine post-vaccinal sera previously titrated against the O/Urubamba vaccine strain using the liquid-phase blocking ELISA (LPBE). The ELISA employed to obtain the "r" values described by Hamblin et. al. [2]. The "r" value was calculated using the titer of the reference sera against the field virus/the titer of the reference sera against the homologous vaccine strain.

The sandwich ELISA was used to screen the reactivity of Mabs against the field strains captured using rabbit antisera coated plates. The antigens were previously titrated to give an OD values of 1.2. The various Mabs were added as duplicate samples at a concentration that was found to give the plateau maximum absorbance when titrated against O/BFS virus in the sandwich ELISA. The reactions of the Mabs were standardized with reference to the concentrations of the captured viruses; these were determined using a polyclonal guinea pig antibody system.

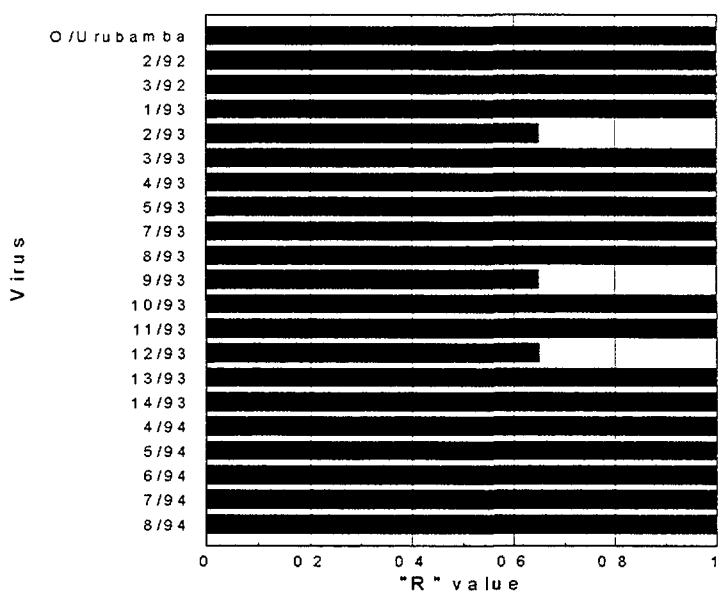

FIG. 1. "R" values of FMDV type "O" field isolates (Peru: 1992-1994).

3. RESULTS

The titers of the field strains and reference vaccine strain giving an OD of 1.5 are shown in Table 1. The reference sera were obtained from a pool of sera giving an end point titration of >128. The selected antigen dilutions were reacted with the reference sera and the titer obtained against each field strain is shown in Table I. The "r" values obtained are presented in Figure 1 and indicate that all the field isolates showed a close relationship to the reference vaccine strain giving values of 0.66-1.00.

The reactivity of the Mabs with the field strains and reference strain was carried out as described by Samuel et. al. [12], and the profiles were obtained using the software developed at the WRL (Figures 2, 3, 4, 5). These results confirmed the conclusions derived from the "r" values.

The profiles showed that the binding of the C6 Mab was greatly reduced in the field isolates when compared with the O/Urubamba vaccine strain, but all the isolates showed similar profiles.

TABLE I. FMDC TYPE "O" FIELD ISOLATES, PERU: 1992-1994

No/Year	Location	Antigen titer	Serum titer
2/92	Lima	1/6	384
3/92	Lambayeque	1/10	512
1/93	Piura	1/8	384
2/93	Huancavelica	1/8	256
3/93	Ayacucho	1/6	384
4/93	Pasco	1/8	384
5/93	Puno	1/6	384
7/93	Arequipa	1/6	384
8/93	Apurimac	1/6	384
9/93	Cusco	1/8	256
10/93	Puno	1/2	384
11/93	Lima	1/6	512
12/93	Tacna	1/8	256
13/93	Cusco	1/8	512
14/93	Cusco	1/4	512
4/94	Huancavelica	1/6	512
6/94	Arequipa	1/6	512
7/94	Arequipa	1/6	512
8/94	Ancash	1/8	384
O/Urubamba		1/6	384

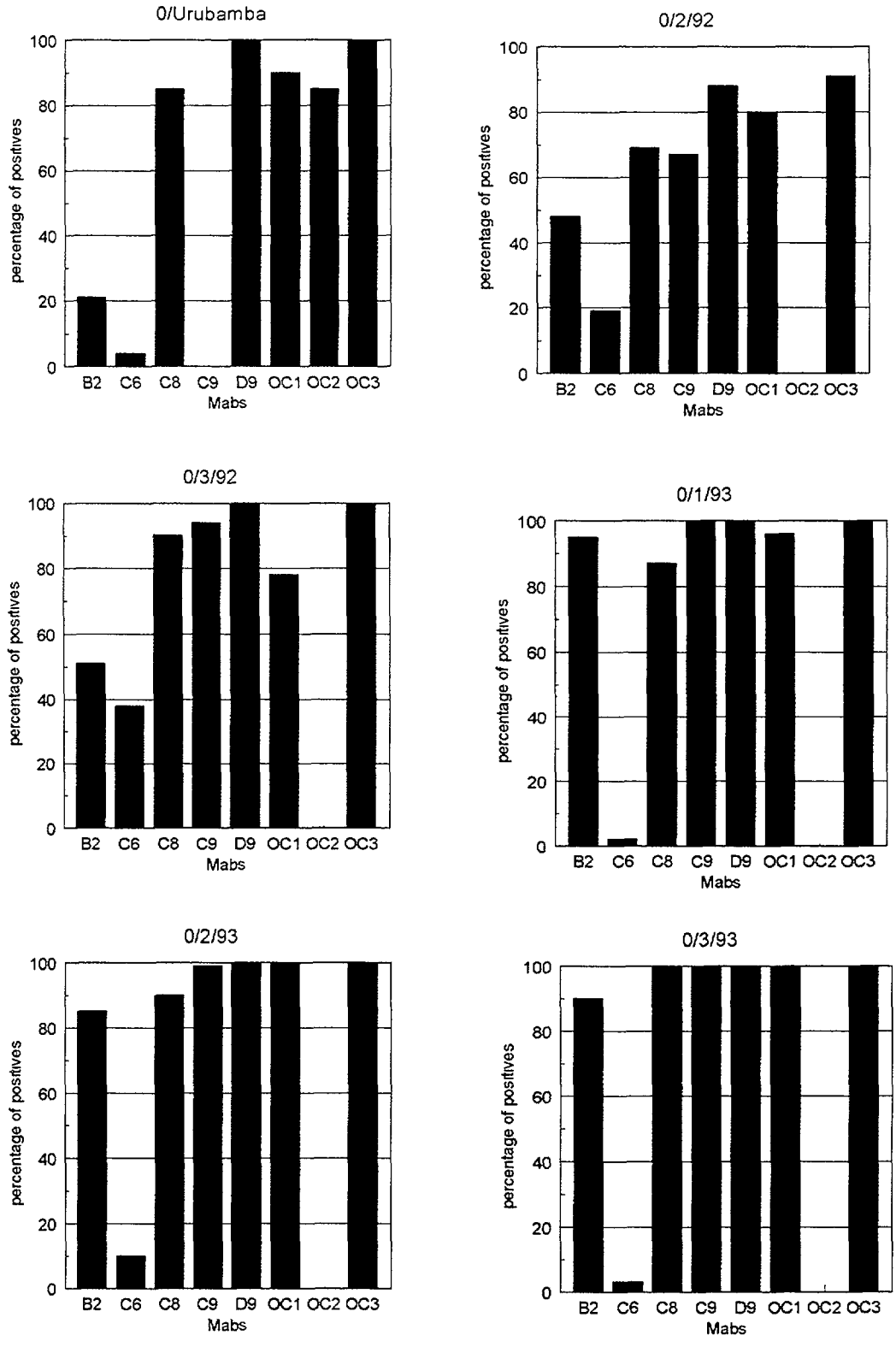

FIG 2 Profile of field isolates of FMDV type "O" by a panel of O/BFS monoclonal Ab

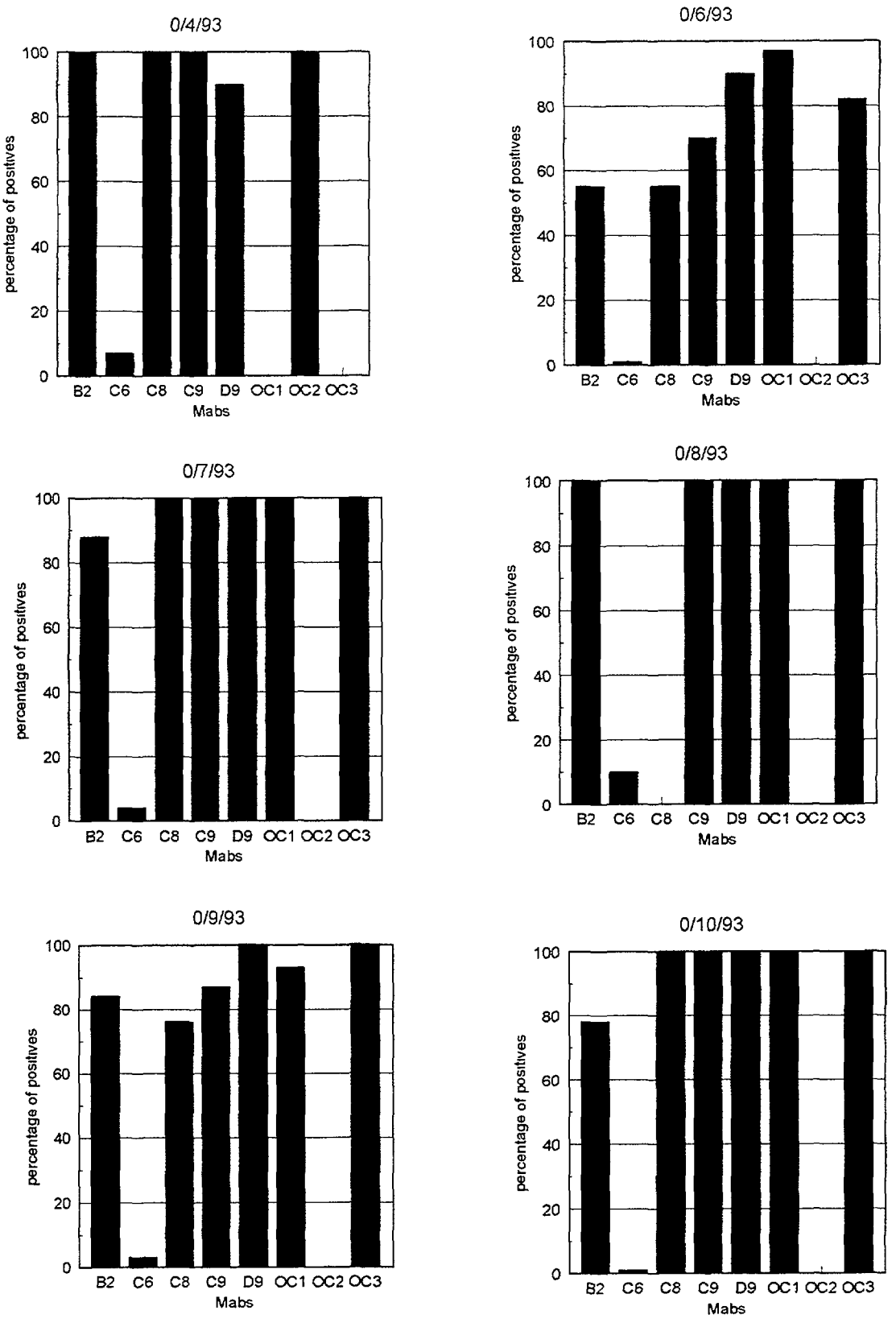

FIG 3 Profile of field isolates of FMDV type "O" by a panel of O/BFS monoclonal Ab

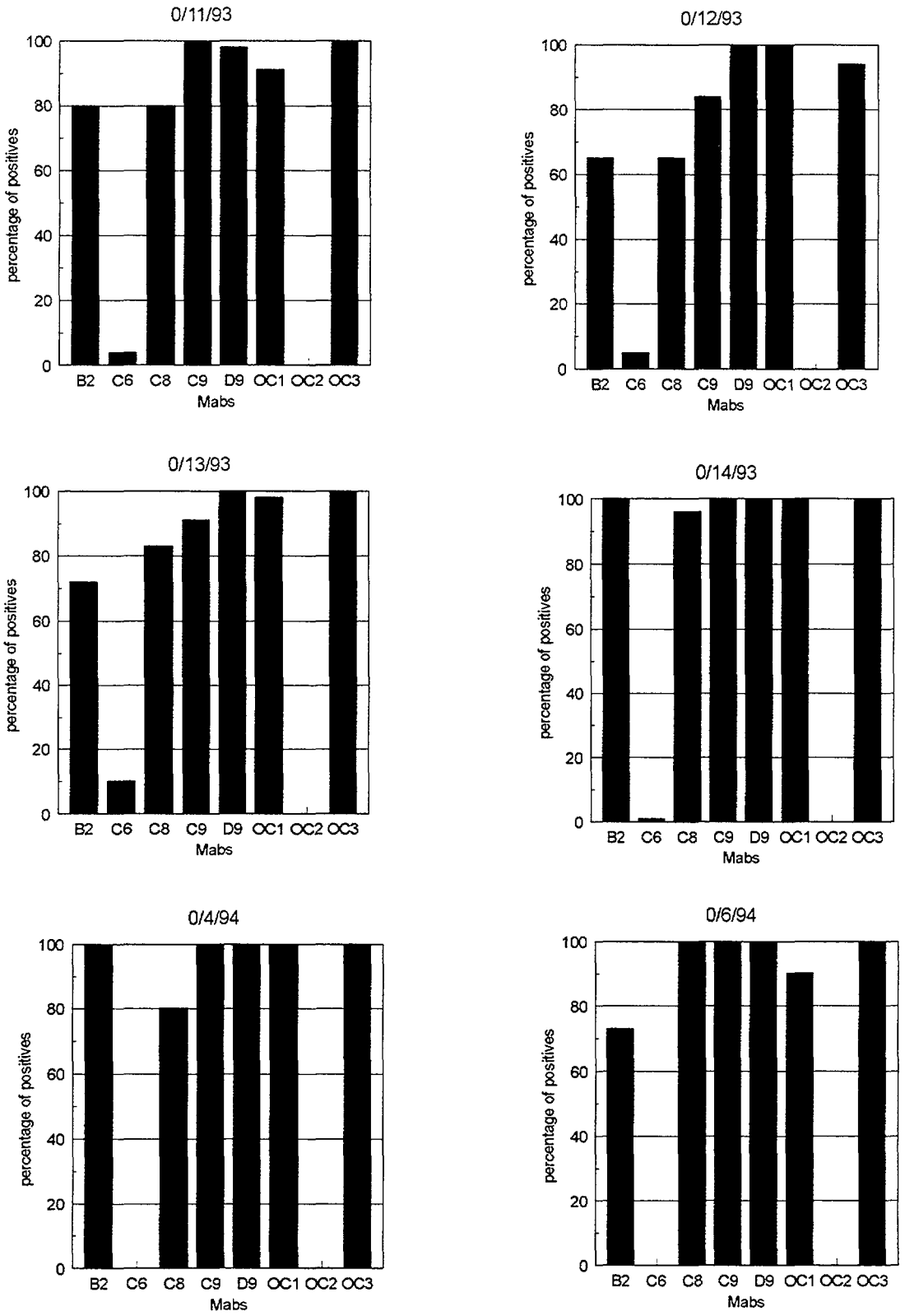

FIG. 4. Profile of field isolates of FMDV type "O" by a panel of O/BFS monoclonal Ab.

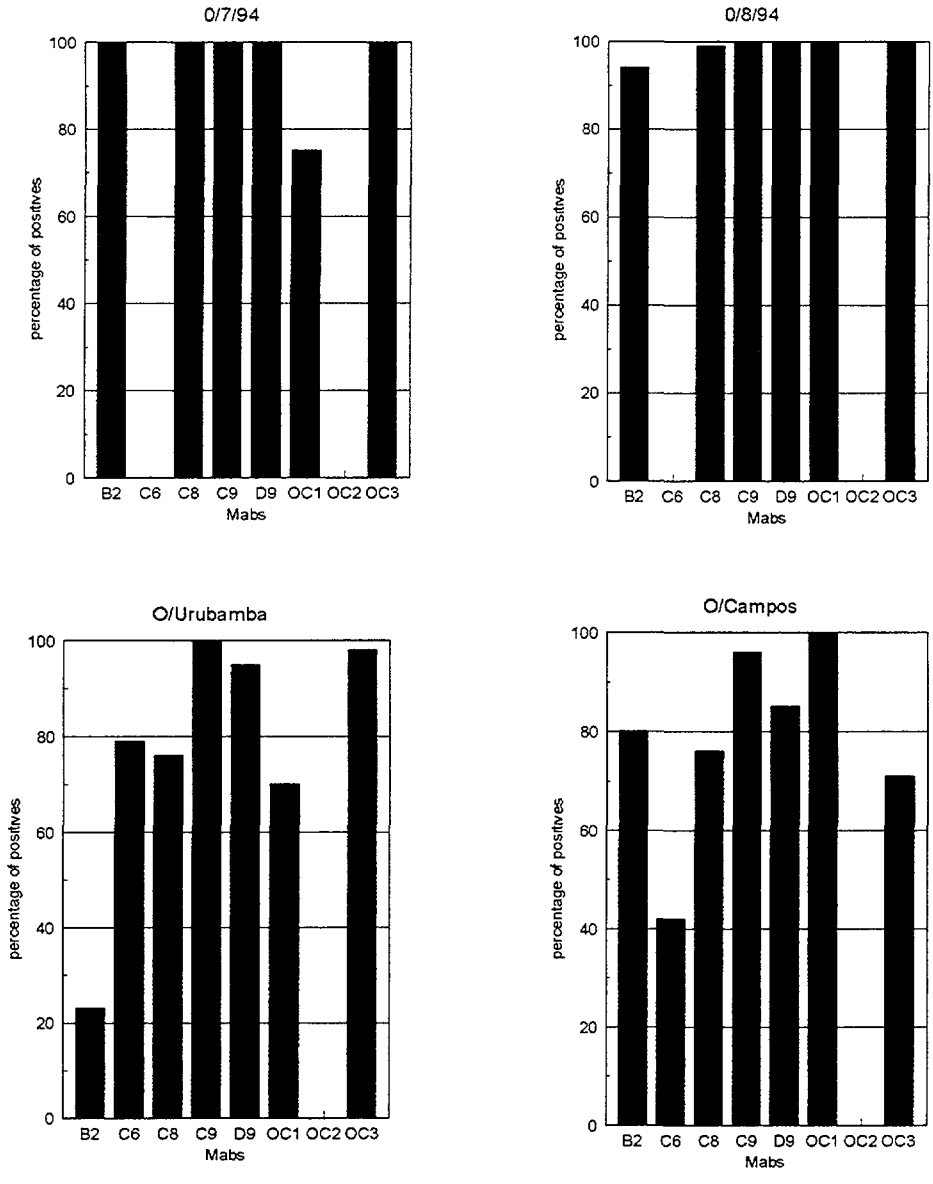

FIG. 5. Profile of field isolates of FMDV type "O" by a panel of O/BFS monoclonal Ab.

4. DISCUSSION

The FMD diagnostic laboratory must identify and characterize FMD virus in samples submitted, and also advise the FMD National Control Programme on the appropriate vaccine strains to be used for control measures. The advantages of ELISA techniques have been emphasized by different authors [4,5,9]. The LPBE was developed to replace the Virus Neutralization Test (VNT) [1] and correlates directly with VNT and protection [10]. LPBE incorporated into a two-stage system provided a practical and rapid method to study the relationship field isolates and vaccine strains [3]. Interpretation of "r" values, according to Ferris and Donaldson [11] indicates that there were not significant differences between the field isolates and the vaccine strain.

Characterization of FMDV using Mabs has been extensively described [12,13]. Recently five antigenic sites for the FMDV type O have been identified by Mabs. [14]. In this study the panel of Mabs employed were raised against O/Switzerland and O/Caseros, and recognize the five immunodominant epitopes. Characterization of the strains under study would be improved if Mabs against O/Urubamba were available. The Mabs profiles of the field isolates showed a similar pattern. All the field isolates showed low binding with the C6 Mab, whereas high reactivity was shown between

the vaccine strain and the Mab. This slight difference suggests that the field strains did not originate from the vaccine or from an FMD vaccine production laboratory.

The consistency of Mab profiles among field isolates reflects that circulating virus in the field is conserved in cattle population with low immune status. Mab profile results provided a more detailed antigenic characterization and agreed with the "r" value findings. This study demonstrated that monitoring of field strains using ELISA provided valuable data for epidemiological surveillance and assessment of suitable vaccine strains.

ACKNOWLEDGEMENTS

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REFERENCES

[1] HAMBLIN, C., BARNETT, I.T.R, AND HEDGER, R.S., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, I.Development and method of ELISA, J. Immun Methods **93** (1986) 115-121.

[2] HAMBLIN, C., BARNETT, I.T.R. AND CROWTHER, J.R., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, II. Application, J. Immun. Methods **93** (1986) 123-129.

[3] FERRIS, N.P., DAWSON, M., Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of FMD and VSV, Vet. Microbiol. **16** (1988) 201-209.

[4] CROWTHER, J.R., ELISA in FMD diagnosis and differentiation and the use of monoclonal antibodies, In: 17th Conference of the Office International des Epizooties Foot-and-Mouth Disease Commission, Paris, OIE (1986) 178-195.

[5] ALONSO, A., MARTINS, M.A., GOMES, M. DA P., ALLENDE, R., SONDAHL, M.S., Foot-and-mouth disease virus typing by Complement fixation and Enzyme linked immunosorbent assay using monovalent and polyvalent antisera, J. of Veterinary Diagnostic Investigation 4 (1992) 249-253.

[6] KITCHING, R.P., RENDLE, R., FERRIS, N.P., Rapid correlation between field isolates and vaccine strains of foot-and-mouth disease virus, Vaccine **6** (1988) 403-408.

[7] Pan American Foot-and-mouth Disease Center, Epidemiological Monthly Report, **26** (1993).

[8] Pan American Foot-and-mouth Disease Center, Epidemiological Monthly Report, **27** (1994).

[9] FERRIS, N.P., Selection of foot-and-mouth disease antisera for diagnosis, Rev. Sci. Tech. Off. Int. Epizoot. **6** (1987) 127-140.

[10] VAN MAANEN, C AND TERPSTRA, C., Comparison of a liquid-phase blocking sandwich ELISA and a serumneutralization test to evaluate immunity in potency tests of foot-and-mouth disease vaccines, J.Immunol.Meth. **124** (1989) 111-119.

[11] FERRIS, N.P., DONALDSON, A.I., The World Reference Laboratory for Foot-and-Mouth Disease: a review of thirty-three years of activity (1958-1991), Rev. sci. tech. Off.int. Epiz. **11** 657-684.

[12] SAMUEL, A.R., KNOWLES, N.J., SAMUEL, G.D. AND CROWTHER, J.R., Evaluation of a trapping ELISA for the differentiation of foot-and-mouth disease virus strains using monoclonal antibodies, Biologicals **19** (1991) 229-310.

[13] CROWTHER, J.R., The use of monoclonal antibodies in the molecular typing of animal viruses. Rev. sci. tech. Off. int. Epiz. **12** (1993) 369-383.

[14] CROWTHER, J.R., FARIAS S., CARPENTER, W.C., SAMUEL, A.R., Identification of a fifth neutralizable site on type O foot-and-mouth disease virus following characterization of single and quintuple monoclonal antibody escape mutants, Journal of General Virology **74** (1993) 1547-1553.

FIELD ASSESSMENT OF THE ENZYME LINKED IMMUNOSORBENT ASSAY FOR FOOT-AND-MOUTH DISEASE VIRUS DIAGNOSIS AND TYPING

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Abstract

FIELD ASSESSMENT OF THE ENZYME LINKED IMMUNOSORBENT ASSAY FOR FOOT-AND-MOUTH DISEASE VIRUS DIAGNOSIS AND TYPING.

The objective of the present study was to evaluate the enzyme linked immunosorbent assay (ELISA) in comparison with the complement fixation test (CFT) for the diagnosis and typing of foot-and-mouth disease (FMD) virus (FMDV). Diagnostic material was epithelium from either suspected cases of FMD or from animals experimentally infected with FMDV. Epithelial suspensions and supernatant fluids from cell culture passage were assayed by CFT and ELISA. The superiority of the ELISA over the CFT was demonstrated:
1) the detection rate was 23% higher than that of CFT on original (epithelial) suspensions (OS) submissions of all sample (positive and negative) and 30% higher on supernatant fluids from cell culture passage, 2) the detection rate of ELISA on OS of confirmed positive samples was 28% higher than that of CFT, 3) no significant differences were observed in the detection and typing rates between the PANAFTOSA and FAO/IAEA ELISA kits ($P<0.05$) and 4) the sensitivity of the ELISA was 16 to 85 times higher than that of CFT when serial dilutions of sample homogenates were examined.

1. INTRODUCTION

Foot-and-mouth disease (FMD) is economically the most important viral disease of domestic animals. The causative agent is a Picornavirus and displays remarkable antigenic variability: seven distinct serological types and more than 60 subtypes and variants have been identified world-wide [1]. Types O, A, and C of foot-and-mouth disease virus (FMDV) are prevalent in South America.

The effectiveness of control measures for FMD relies upon rapid, sensitive and reliable diagnostic procedures. In this regard, the complement fixation test [2] has been used extensively, however, it has disadvantages mainly related to its relative insensitivity and constraints derived from the anticomplementary effects of some samples.

ELISA for antigen detection and typing has been previously described [3] and the procedure with some modifications is being applied as routine in most FMD diagnostic laboratories [4,5]. In this paper, the efficiency of the ELISA and the CFT for FMDV diagnosis has been compared using epithelial samples from animals experimentally and naturally infected with FMDV and antigens derived from their cell culture passage. Two laboratories were involved in the present study: the National Control Laboratory (SENASA) and the Virology Institute of INTA. The former conducted the CFT and cell culture passage and the latter the ELISA determinations.

2. MATERIALS AND METHODS

2.1. Samples

Field samples (N=85) from suspected cases of FMD received for diagnosis at the SENASA during 1991 and 1992 were used in the present study. Upon receipt, each sample was ground in phosphate buffered saline (PBS) using a pestle and mortar with the aid of sterile sand to yield a 20%

(w/v) suspension (original suspension, OS). The suspensions were clarified by centrifugation at 1500 g for 10 min Each OS was divided into 3 fractions. one each for CFT, ELISA and virus isolation determinations OS which were either not typed or which gave anticomplementary results by CFT were passaged in BHK-21 roller bottles as previously described [5] CFT and cell culture passage were carried out at SENASA laboratory OS and supernatants of cell culture passage were submitted to INTA laboratory for antigen detection and typing by ELISA.

2.2. ELISA

Indirect sandwich ELISA kits provided by either FAO/IAEA or PANAFTOSA for FMDV antigen detection and typing were used The FMDV strains used to produce capture (rabbit) and detection antibody (guinea pig) for each kit are listed in Table I

TABLE I FMDV STRAINS USED TO RAISE ANTISERA FOR FAO/IAEA AND PANAFTOSA ELISA KITS

VIRUS	IAEA/FAO kit	PANAFTOSA kit [5]
FMDV-O	O1 BFS	O1Campos-Br/58, O1 Caseros-Arg/67, O2 Brescia-Italy/47, O3 Venezuela/51, O6 UK/24, O8 Bahia-Br/ 60, O Magd-Col/78, O MS-Br/ 80, O RS- Br/80
FMDV-A	A5 Allier A22 IRQ 24/64 A24 Cruz-Br/55	A5 West-Ger/48, A24 Cruz-Br/55, A32 Ven/70, A79 Arg/79, A Est-Ven/80, A 81 Arg/81, A84 S Carl-Br/84, A Col/84, A85 Col/85
FMDV-C	C1 Noville-Swit/65	C1 Noville-Swit/65, C2 Pando-Ur/44, C3 Res-Br/55, C3 Indaial-Br/71, C3 Arg/85, C4 T Fuego-Arg/66, C5 Arg/69
VSV-New Jersey	New Jersey	New Jersey Costa Rica/66
VSV-Indiana	Indiana C	Indiana 1-2-3

Briefly, ELISA plates (Nunc- Maxisorp, Denmark) were coated overnight at 4°C with O, A, and C FMDV rabbit antiserum at the appropriate dilution in 0 05 M carbonate-bicarbonate buffer, pH 9 6 Samples and control antigens were added and plates incubated at 37°C for 1 hour on a rotary shaker Detection of captured virus was performed by adding specific guinea pig antisera to O, A and C FMDV followed by the addition of peroxidase conjugate (rabbit anti guinea pig IgG, Dako Corporation). Guinea pig antisera and conjugate were diluted in PBS containing 0.05% Tween 20 and 5% skimmed milk. Plates were washed with PBS after each incubation step O-phenylenediamine and hydrogen peroxide composed the chromogen/ substrate mixture and plates were incubated for 15 min at room temperature (RT) The reaction was stopped with 1.25M sulphuric acid and read on a Dynatech Multiskan reader at 492 nm. Positive samples to a specific serotype showed a corrected absorbance value of above 0 1

The ELISA using the PANAFTOSA kit was operated as described above but with some modifications: 1) after coating with rabbit antisera, plates were blocked for 1 hour at RT with PBS containing 1% ovalbumin and 2) control antigens, detection antibody and conjugate were diluted in PBS containing 0 05% Tween 20, 1% ovalbumin, 2% non-immune bovine serum and 2% non-immune rabbit serum.

2.3. Complement fixation test

The CF_{50} tube test [6] as used for vesicular stomatitis virus and FMDV typing by diagnostic

laboratories in South America and at the PANAFTOSA was employed and carried out at SENASA laboratory.

2.4. Virus isolation

Field samples giving negative results by CFT were inoculated onto BHK-21 roller bottles at SENASA laboratory as previously described [5].

Cultures which either showed no evidence of cytopathic effect CPE up to 48 hours post inoculation or which were negative by CFT were further passaged in cell culture a maximum of two times. Aliquots from cell culture passage were stored at -20°C for 4-8 weeks before submission to INTA laboratory for ELISA determinations. It should be noted that not all the material passaged in cell culture was available by the time the ELISA was performed.

2.5. Relative sensitivity between the ELISA and CFT

Serial dilutions of several OS were prepared in PBS containing 0.05% Tween 20 for ELISA determinations (double dilution range from 1/2 up to 1/512) and in PBS for CFT determinations (dilutions 1/1.5, 1/2, 1/3 and 1/4) to compare the relative sensitivity between the two assays.

3. RESULTS

3.1. Comparison of FAO/IAEA ELISA with CFT

The numbers of OS and cell culture harvests which were found to be positive by use of the CFT and the FAO/IAEA ELISA are recorded in Table II.

TABLE II. COMPARISON OF ANTIGEN DETECTION AND TYPING BY FAO/IAEA ELISA AND CFT ON ORIGINAL (EPITHELIAL) SUSPENSIONS AND ON SUPERNATANT FLUID DERIVED FROM THEIR CELL CULTURE PASSAGE

Test comparison	Sample	No. samples	No. +ve FAO/IAEA ELISA	No. +ve CFT
(1)	OS	85	65 (76)*	45 (53)
	Cell culture	106	76 (72)	45 (42)
	Total	191	141 (74)	90 (47)
(2)	OS	71	57 (80)	37 (52)

Test comparison of ELISA and CFT performed on: (1) test samples from all submissions and (2) OS from virus-positive submissions
OS, original suspension* : Percentage of total

The results demonstrate that a higher typing rate was achieved by use of the ELISA. The typing rate obtained by ELISA was similar in tests on OS and cell culture antigens from all sample submissions. This was also true for the CFT but the detection rate(s) was/were lower. In test comparisons on confirmed FMDV positive submissions, the ELISA on both OS and cell culture harvests again gave a higher detection rate.

Six out of 192 samples gave cross-reactivity between serotypes, 5 of which typed as O and A (cell culture passage), and the other (OS) typed as O and C.

3.2. Comparison of PANAFTOSA ELISA kit with FAO/IAEA ELISA and with CFT

The results achieved by use of the ELISA kits received from PANFTOSA and FAO/IAEA and CFT are summarised in Table III and again illustrate that a higher typing rate was obtained by the ELISA.

TABLE III. COMPARISON OF ANTIGEN DETECTION AND TYPING BY BOTH FAO/IAEA AND PANAFTOSA ELISA AND CFT ON ORIGINAL (EPITHELIAL) SUSPENSIONS AND ON SUPERNATANT FLUID DERIVED FROM THEIR CELL CULTURE PASSAGE

No. samples	No. +ve	
	PANAFTOSA ELISA	CFT
64	47 (73)*	35 (55)
	No. +ve	
	PANAFTOSA ELISA	FAO/IAEA ELISA
73	53 (73)	58 (79)

*Percentage of total

No significant differences were found between the detection rate of the two ELISA kits ($P<0.05$). One sample was scored positive by the PANAFTOSA ELISA but not by the FAO/IAEA ELISA, but a positive diagnosis was achieved by both assays after sample cell culture passage and retesting by ELISA. Conversely, 6 samples were scored positive by the FAO/IAEA ELISA but not by the PANAFTOSA ELISA. Cell culture passage of these samples led to positive results by ELISA in 5 out of the 6 cases.

One out of 73 samples derived from cell culture passage gave a cross-reaction between types C and O by both ELISA kits.

3.3. Relative sensitivity between the FAO/IAEA ELISA and CFT

ELISA was found to be 16 to 85 times more sensitive than CFT in detecting FMDV (Table IV).

TABLE IV. RELATIVE SENSITIVITY BETWEEN THE FAO/IAEA ELISA AND CFT FOR DETECTION OF FMDV ON SERIALLY DILUTED ORIGINAL (EPITHELIAL) SUSPENSIONS (OS)

Sample	CFT	ELISA
R636	1/2*	1/32
L20	1/3	1/32
227	1/1.5	1/32
228	1/3	1/64
6466	1/1.5	1/16
OC	1/3	1/256

*Final dilution of OS which was found to be positive

4. DISCUSSION

The superior sensitivity of the ELISA over the CFT has been extensively documented [3,4,7]. The ELISA kits provided by FAO/IAEA and PANAFTOSA have been applied as routine diagnostic procedures and typing in many FMD laboratories. The results obtained with the ELISA under our laboratory conditions have confirmed the advantages of this method over the CFT [3,4,5,7].

We detected FMDV antigen by ELISA in 73% of OS samples that gave positive results in OS or cell culture passage by either ELISA or CFT, and in 52% by CFT. This superiority was lower to that reported by Westbury et al. (1988) [7] who found 58% more positives by ELISA than by micro-CFT in confirmed positive samples. The lower figure achieved in this study could be due to a higher sensitivity of the CFT in tubes applied here [5] and the quality of epithelium samples.

ELISA was more efficient than CFT in detecting antigen either in OS or in cell culture passage. Detecting rates were similar in both categories of test material. Previous studies have reported a higher detection rate on cell culture supernatants than on OS [4,5]. The lower detection rate obtained by us on cell culture samples could be due to antigen degradation after storage at -20°C for 4-8 weeks before running the ELISA. Additionally, there were probably supernatant fluids tested which were derived from cell cultures either showing no or low amounts of CPE.

Similar detection and typing rates were achieved by both ELISAs (FAO/IAEA and PANAFTOSA kits) in spite of differences in strain components, suggesting an adequate coverage of the different antiserum reagents to FMDV strains prevalent in Argentina in 1991 and 1992.

The cross-reactivity shown with samples originated from cell culture passage could be due to reactivity of capture and detector antibody to BHK-21 components of cell culture supernatants. Antisera included in the FAO/IAEA kit were prepared against purified, inactivated 146S antigens derived from FMDV propagated in BKK-21 cells. Besides having an anti-FMDV activity and antisera contain antibodies to BHK-21 cells and bovine gamma globulin. Antibodies to the latter component have been "blocked" but not those to BHK cells. The phenomen was also shown by PANAFTOSA kit in 1/64 samples tested. The cross-reactivity between O and C found in this OS sample could be explained by the presence of two serotypes in the one specimen [8] as a consequence of either a natural mixed infection or by contamination of the sample by another serotype. However, virus neutralization analysis using antisera against O and C revealed the presence of one type (C FMDV type) (data not shown).

The sensitivity of the ELISA was 16 to 85 times higher than that of the CFT when serial dilutions of sample homogenates were examined. This estimation is consistent with previous sensitivity assessment: minimum detection levels of 8 ng/ml of 140S can be detected by ELISA [3], whereas a minimum of 500 ng/ml of 146S is needed to achieve a positive result by CFT [4].

A rapid confirmation of the presence and type of FMDV achieved by use of the ELISA has significant impact in initiating rapid emergency activities to restrict an outbreak.

In conclusion, the higher detection rate of the ELISA over the CFT and its easy performance, strongly recommends its use for swift and reliable diagnosis of FMDV.

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REFERENCES

[1] PEREIRA, H.G., Subtyping of foot-and-mouth disease virus, In: "Developments in Biological Standardization" (C. Mackowiak and R.H. Regamey, eds.) Vol.35 (1977) pp.167-174 Karger, Basel.

[2] BUCKLEY, L.S., OSBORNE, R.W. & Pereira, H.G., Laboratory diagnosis of foot-and-mouth disease and swine vesicular disease, Bull. Off. Int. Epizoot. **83** (1975) 123-129.

[3] ROEDER, P.L. & Le Blanc Smith, P.M., Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis, Res. Vet. Sci. **43** (1987) 225-232.

[4] FERRIS, N.P. & DAWSON, M., Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases, Vet. Microbiol. **16** (1988) 201-209.

[5] ALONSO, A., MARTINS, M., GOMES, M.P.D. et al., Foot-and-mouth disease virus typing by complement fixation and enzyme-linked immunosorbent assay using monovalent and polyvalent antisera, J. Vet. Diagn. Invest. **4** (1992) 249-253.

[6] ALONSO, A. MARTINS, M., GOMES, M. P.D., et al. (1991). Development and evaluation of an enzyme-linked immunosorbent assay for detection, typing, and subtyping of vesicular stomatitis virus, J.Vet. Diagn. Invest. **3** (1992) 287-292.

[7] WESTBURY, H.A., DOUGHLY, W.J., FORMAN, A.J. et al., A comparison of enzyme linked immunosorbent assay, complement fixation and virus isolation for foot-and-mouth disease diagnosis, Vet. Microbiol.**17** (1988) 21-28.

[8] FERRIS, N.P., OXTOBY, J.M. & HUGHES, J.F., Multiple-infected diagnostic specimens from foot-and-mouth disease endemic regions, Rev.sci.tech.Off.int.Epiz. **14** (1995) 557-565.

EVALUATION OF AN INDIRECT ELISA FOR DETECTION AND TYPING OF FOOT-AND-MOUTH DISEASE VIRUS

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Abstract

EVALUATION OF AN INDIRECT ELISA FOR DETECTION AND TYPING OF FOOT-AND-MOUTH DISEASE VIRUS

An Indirect enzyme linked immunosorbent assay (ELISA) kit was used for diagnosis of foot-and-mouth disease virus (FMDV) types O1, A24, C3 which occurred in Rio Grande do Sul State, Southern Brazil during 1984-1994 The samples were randomly selected and tested by ELISA, Complement Fixation Test (CFT) and in tissue culture Out of 106 samples 78 (73,5%) were positive by ELISA and 39 (36,8 %) were found positive in CFT, when original suspensions were used Once these samples were inoculated onto tissue culture both tests gave similar results, although ELISA picked up more positive samples during the 1st passage in tissue culture The negative samples (16) included in this study were negative in all tests The ELISA was more sensitive than and as specific as CFT ELISA and tissue culture together were shown to be a better system for detection of foot-and-mouth disease virus antigen than CFT

1. INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals and is one of the most important viral disease affecting livestock Its major effect is as a constraint to international trade (export/import) between FMD-free countries and those in which FMD is endemic (South America, Africa and Asia). As Rio Grande do Sul State, Brazil, is involved in the Programme of Control and Eradication of FMD in the River Plate Basin Area, involving Argentina, Brazil, Paraguay and Uruguay [1] it is very important to have early notification of outbreaks and rapid diagnosis. Any failure in diagnosis will affect disease control and will favor the spread of infection All strategies of control and eradication of FMD in this area rely on effective vaccination of cattle, a network of veterinary officers and efficient diagnostic laboratories. Thus, it is necessary that laboratory tests for FMD should have very good sensitivity, specificity and reliability

In most countries of South America detection and typing of FMDV has been carried out by CFT [2,3], however, CFT has many disadvantages such as: low sensitivity, it is cumbersome, time consuming, and requires a good laboratory structure The advantages of indirect ELISA for typing of FMDV have been described [4,5,6,7,]. Thus, the purpose of this investigation was to evaluate an ELISA kit for FMD antigen detection and compare it with the CFT and tissue culture, using the virus collection of IPVDF's FMD Unit and samples submitted from outbreaks, at IPVDF-Regional Diagnosis Laboratory, Rio Grande do Sul State.

2 MATERIAL AND METHODS

2.1. Field Samples

Epithelial samples collected from 1984 up to 1994 were sent to IPVDF, Regional Diagnosis Laboratory. A total of 90 positive samples (Type O1: 25; type A24: 46; type C3: 19) and 16 known negative samples were stored at -20°C in PBS, pH 7.4 50 % glycerol. All samples were tested by ELISA, CFT and inoculated onto tissue culture (roller bottles) either as original suspensions or as tissue culture supernatants.

2.2. CFT

CFT used was a tube test (CF 50%) standardized by the Panamerican Foot-and-Mouth Disease Center (PAFMDC) for FMDV [2].

2.3. ELISA procedure

An ELISA kit provided by the Joint FAO/IAEA Division , Vienna, Austria was used. It is based on an indirect sandwich ELISA. Briefly, rabbit antisera specific for the different types and subtypes of FMDV and Vesicular Stomatitis Virus (VSV) are adsorbed to polystyrene plates. Following the addition of the test sample, the antigen is trapped by the immobilized antibodies. Specific guinea pig antisera are added to react with the trapped antigen. The reaction is detected by the addition of anti-guinea pig antibody conjugated to horseradish peroxidase (HRP). After the addition of substrate/chromogen a colored reaction develops allowing identification of the antigen [8].

2.4 Virus Isolation

One ml of the original suspensions were inoculated onto cultures of IBRS-2 cells grown in 1 liter disposable plastic bottles. The monolayers were washed with 50 ml of maintenance medium and subsequently 100 ml of the same medium was added. After inoculation these bottles were incubated at 37°C in roller apparatus for 48 hours or harvested earlier if cytopathic effect was observed.

3. RESULTS

The results obtained by ELISA and CFT with original suspensions are shown in Table I. ELISA was positive in 73.5% while 36.8% were positive by CFT. All samples(positives and negatives) were inoculated onto tissue culture(three passages) and results are shown in Table II. Both tests successfully detected virus in the tissue culture supernatants but ELISA identified more positive results than CFT at the 1st passage. In these cases the samples were inoculated (2nd and 3rd passages) to increase the virus titer and subsequently CFT gave a positive typing. ELISA was not able to detect FMDV in 28 original suspensions(26.5%) and CFT failed to detect virus in 67 original suspensions(63.2%). The results with negative samples(16), included in this experiment, had complete agreement in all tests. The sensitivity and specificity of ELISA and CFT are shown in Tables III and IV.

TABLE I. TYPING OF FMDV BY ELISA AND CFT USING ORIGINAL SUSPENSIONS OF FIELD SAMPLES

	CFT	ELISA
Positive	39(36.8%)	78(73.5%)
Negative	67(63.2%)	28(26.5%)
Total	106	106

TABLE II. TYPING OF FMDV BY ELISA AND CFT ON CELL CULTURE HARVESTS

		1st passage	2nd passage	3rd passage
CFT	Positive	79(75.5%)	90(85.0%)	
	Negative	27(25.5%)	16(15.5%)	16
ELISA	Positive	90(85.0%)		
	Negative	16(15.0%)	16	16

TABLE III. SENSITIVITY AND SPECIFICITY OF ELISA FOR DETECTING FMDV IN EPITHELIAL SAMPLES (ORIGINAL SUSPENSIONS)

		Positive	Negative	Total
ELISA	Positive	78	0	78
	Negative	12	16	28
	Total	90	16	106

Sensitivity: 86.6% / Specificity: 100%

TABLE IV. SENSITIVITY AND SPECIFICITY OF CFT FOR DETECTING FMDV IN EPITHELIAL SAMPLE S (ORIGINAL SUSPENSIONS)

		Positive	Negative	Total
CFT	Positive	39	0	39
	Negative	51	16	67
	Total	90	16	106

Sensitivity: 43.3% / Specificity: 100%

4. DISCUSSION

CFT has been used in South America as the standard test for diagnosis of FMD and other vesicular diseases since 1960 and recommended by the PAFMDC to be used at diagnostic laboratories in all countries in this continent [2]. Since ELISA has been shown to be a sensitive test for diagnosis of FMD [2,3,4,5,6,7,9,10] it is now in use in a majority of laboratories throughout the world for antigen and antibody detection. In this study it was possible to confirm once more the disadvantages of CFT in relation to ELISA (Tables I - IV) for detection and typing of FMD. One disadvantage of the ELISA Kit was the short shelf life of the reconstituted positive controls. Once diluted they kept acceptable activity for no more than three months as an average between the two batches received for this investigation. It is an aspect that will need additional studies with diluents that may improve antigen stability. Cross reactions were not a problem. When they occurred (four tissue culture samples O/C) they were probably due to high antigen content, since it was not detected when original suspensions were typed. Also in this study it was possible to show (Table II) that ELISA and tissue culture were the best system for the detection of FMDV.

As ELISA proved to be simple to perform, rapid and has high sensitivity it will be very useful for FMD control and diagnosis in countries or areas such as the River Plate Basin Programme where disease needs to be confirmed as soon as possible in order to prevent spread and involvement of new areas.

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REFERENCES

[1] COSALFA- Comisión de la Lucha Contra la Fiebre Aftosa, Informe Final XX Reunión Ordinaria, Montevideo, Março, (1993) p.25-26.

[2] ALONSO, A., Diagnóstico de las Enfermedades Vesiculares, Rio de Janeiro: CPFA, 1986. 40p. (Series de Manuales Técnicos, 15).

[3] ALONSO, A.F., MARTINS, M..A., GOMES, M.P.D., ALLENDE, R., SÖNDAHL, M.S. Foot-and-Mouth Disease virus typing by complement fixation and ELISA tests using monovalent and polyvalent antisera, J. Vet. Diagn. Invest. **3** (1992) 287-292.

[4] HAMBLIN, C., ARMSTRONG, R. M., HEDGER, R.S., A rapid enzyme-linked immunosorbent assay for the detection of foot-and-mouth disease virus in epithelial tissues, Veterinary Microbiology, Netherlands, 9 (1987) 435-443.

[5] FERRIS, N.P., DOWSON, M., Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular disease, Veterinary Microbiology, Netherlands **16** (1988) 201-209.

[6] ROEDER, P. L., Le BLANC SMITH, P. M., Detection and typing of FMD virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis, Research in Veterinary Science, USA, **43** (1987) 225-232.

[7] KITCHING, R.P., Current status for the standardization and supply of reagents for FAO/IAEA FMD ELISA kits. In: IMMUNOASSAY METHODS FOR THE DIAGNOSIS AND EPIDEMIOLOGY OF ANIMAL DISEASES IN LATIN AMERICA, Bogota, Colombia, Proceedings of the 2nd Research Coordination Meeting FAO/IAEA/SIDA 1992, FAO/IAEA, 4-5.

[8] FAO/IAEA, FMD ELISA kit manual, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Austria (1992).

[9] ABU-ELZEIN, E., CROWTHER, J., Enzyme-labeled immunosorbent assay techniques in foot-and-mouth disease virus research, J.Hyg. **80** (1978) 391-400.

[10] CROWTHER, J.R., ABU-EL ZEIN, E.M.E., Detection and quantification of foot-and-mouth disease virus by enzyme-labelled immunosorbent assay techniques, Journal of General Virology, London, **42** (1979) 597-602.

NEW DEVELOPMENTS IN FOOT-AND-MOUTH DISEASE DIAGNOSIS

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Abstract

NEW DEVELOPMENTS IN FOOT-AND-MOUTH DISEASE DIAGNOSIS

A variety of newer diagnostic procedures based around the use of molecular technologies are now being undertaken to further characterise the foot-and-mouth disease (FMD) virus enabling a deeper understanding to be gained of the pathogenesis and epidemiology of this disease Such approaches have categorically identified the carrier state and highlighted the importance of carrier animals in control programmes Use of the polymerase chain reaction provides even further insight into the carrier animal but interpretation of data has to be undertaken with caution The role of non-structural proteins can provide further insight into an animals response to both vaccination and natural infection and could provide a basis for separation of the carrier state Finally the pivotal role of monoclonal antibodies in all aspects of FMD research is now clear and these highly specific reagents are now being used for a variety of research and diagnostic purposes within the FMD field

1 INTRODUCTION

The routine use of molecular biological techniques in the diagnostic laboratory has not only greatly increased the speed, specificity and sensitivity of foot-and-mouth disease (FMD) diagnostic tests, but has also greatly assisted in our understanding of the epidemiology and pathogenesis of the disease Novel, or improved, techniques have been applied both to genomic studies, using nucleotide sequencing and polymerase chain reaction (PCR), and to antigenic studies, using expressed proteins and monoclonal antibodies, to define more precisely the antigenic nature of the virus and the immune response it provokes.

2 FMD CARRIERS

Using these novel techniques [1], it has been possible to show identity between strains of FMD virus found in African buffalo and cattle, and between strains causing outbreaks in cattle over extended periods [2,3]. For the first time there is laboratory confirmation that cattle and buffalo carrying FMD virus can initiate new outbreaks of disease. The carrier animal is defined as one from which live FMD virus can be isolated 28 days after contact with infection This may be a fully susceptible animal which develops clinical disease and in which virus persists following recovery, or a vaccinated animal that has contact with live virus and fails to develop clinical disease, but becomes a carrier In the carrier state, FMD virus persists in the pharyngeal region, in the presence of specific anti-FMD virus antibodies in the circulation and pharyngeal secretions. Sheep and goats are reported to carry FMD virus for up to nine months, cattle and African buffalo for up to, and possibly longer than, three and five years respectively; pigs do not become carriers, and other susceptible wildlife ungulates probably carry virus for only a relatively short period [4]. In spite of considerable research effort, the specific cells in the pharynx in which FMD virus persists, have not been identified.

The importance of the carrier animal in the epidemiology of FMD has long been assumed, and for the purposes of international trade, any animal with FMD virus antibody is considered a potential carrier As the economic consequences of an outbreak of FMD in the European Union (EU) would be considerable, even a very small risk is unacceptable. However, until recently there was only circumstantial evidence to link outbreaks of FMD with carrier animals.

Although the EU ceased prophylactic vaccination against FMD after 1991, the option to vaccinate is retained, should slaughter and other zoosanitary measures not be effective in controlling an outbreak. A vaccine bank of concentrated FMD antigens is currently being set up on four sites within the EU. Should vaccination be used to help control an outbreak of FMD, all vaccinated, sero-positive cattle,

and sheep, would be considered potentially to have had contact with live virus, and therefore, possibly to be carriers. If these animals were not very soon slaughtered after the outbreak had been controlled, they would require constant surveillance to ensure that they did not mix with susceptible animals. It is likely that if vaccination were reintroduced, large numbers of animals would be involved, making permanent supervision impossible. In addition, the EU perceives a risk from FMD carrier animals following the extension of trading agreements to countries outside of the EU and from which live animal imports occur. Already, in March 1993, there has been an example of live cattle being brought into Italy with false certification, causing an outbreak of FMD.

However, there is no reliable technique available to distinguish between the serologically positive carrier and non carrier animal, and because of the costs of an outbreak of FMD only a 100% sensitive test would be acceptable. One hundred percent specificity would not be essential, assuming the number of false positive reactions was within acceptable limits. Because of the requirement to identify the carrier animal, much of the research into new diagnostic tests has been directed towards this aim.

The traditional method used to identify the carrier is the probang cup [5]. A scraping is made of the pharynx and anterior oesophagus and the material collected is used to inoculate susceptible cells in tissue culture. The most sensitive tissue culture system is primary bovine thyroid cells. The sensitivity of the test can be further increased by homogenising the sample with Arcton or Freon before inoculation of tissue culture. However, single sampling, even under the most suitable conditions, including those of collection and transport to the laboratory, results in less than 50% of carriers being identified. Repeat sampling improves the opportunity to isolate virus, but many carriers will still be missed.

3. POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a technique which amplifies a specific genome segment of virus in a diagnostic sample. It relies on prior knowledge of the nucleotide sequences flanking the region to be amplified. For the identification of FMD virus genome, a c-DNA segment is first produced from a short DNA oligonucleotide primer which is complementary to the FMD virus genome in the sample. Following this, and using an additional oligonucleotide primer complementary to the distal end of the c-DNA, a further complementary sequence is produced. These two complementary strands of DNA are then amplified through a series of primer annealing, extension and thermal denaturation in the presence of a mixture of nucleotides and thermostable DNA polymerase. Each cycle doubles the quantity of amplified segment to a level that can be detected on an agarose gel. The specificity of the amplified sequence can be confirmed with a labelled DNA probe, by restriction endonuclease digestion or by nucleotide sequencing a region or all of the product. A range of specific primers is used in the World Reference Laboratory (WRL), Pirbright which can identify all known strains of serotype A, O, C, and Asia 1. They are not yet available for the SAT serotypes.

When applied to probang samples, PCR has increased the sensitivity of the detection of carriers considerably. In one trial reported by Amarel-Doel et al. [6] in which 101 probang samples were examined, 39 were negative by PCR and tissue culture, 19 were positive by both tests and 26 were positive by PCR and negative on tissue culture. However, 17 were positive on tissue culture and negative by PCR. There appears to be non-specific inhibitors of PCR, such as blood, which can cause false negatives. The use of PCR for identifying carriers is therefore limited, although it is a valuable additional test to the traditional tissue culture technique. In addition, it could be argued that the demonstration of FMD virus genome by PCR does not necessarily indicate the presence of live virus in the sample.

Nevertheless, PCR is likely to be used more routinely as more experience with the technique is acquired, and the causes of false negative results identified. It has already been used specifically to identify strains of virus present in FMD vaccines when the live vaccine strains have not been made available to the WRL. It is also now being used to obtain directly the nucleotide sequence of strains of FMD virus present in diagnostic samples. This avoids the requirement to grow isolates on tissue culture which could result in the selection of strains better able to grow in culture or even predispose to mutation of the original strain present.

4. NON-STRUCTURAL PROTEINS

An alternative approach to identifying the carrier animal which, although indirect, would have greater application, is to develop a serological test. Current serological tests are able to distinguish animals which have had previous contact with FMD virus antigen from naive animals which have not. However, they cannot distinguish animals which have been merely vaccinated from those which have been infected, nor between recovered animals which have eliminated the virus and persistently infected carrier animals.

Conventional FMD vaccines consist of inactivated whole virus preparations with a suitable adjuvant. Vaccination therefore results in the production of antibodies to the structural proteins on the surface of the virion, and should the animal subsequently be exposed to infection these antibodies will inactivate live virus and provide protection. In principle, the immune response to infection with FMD virus differs from the response to vaccination. During the process of viral replication FMD virus is initially produced as a single polypeptide chain which is subsequently broken down into a number of structural proteins, which form the virion itself, and a number of non-structural proteins (NSP's), which have biological activity either on the FMD virus polypeptide or on the host cell (Figure 1). A number of these NSP's have been shown to be immunogenic. Theoretically therefore, the detection of antibody to NSP's should indicate infection rather than vaccination. However, in practice, trace amounts of NSP's can be found in commercial vaccines which also induce the production of antibody, particularly following repeated vaccination.

The NSP which provokes the strongest immune response and which has consequently been the best studied is the viral RNA polymerase (protein 3D, also known as the Virus Infection Associated Antigen, VIAA). This protein, like most NSP's, is highly conserved between strains and even between serotypes of FMD virus, holding out the possibility of a single serological test capable of detecting infection with any of the seven serotypes of the virus. Unfortunately a reliable, sensitive and specific assay for antibody to VIAA has proved elusive and despite the fact that the first test for antibody to VIAA was reported over 25 years ago [7], no VIAA test has yet found general acceptance. Conventional assays for antibody to VIAA use a semi-purified antigen prepared from the virus grown in tissue culture. When used for immunodiffusion, the antigen results in a test with poor sensitivity and specificity [8] and, when used in ELISA, there are problems of inadequate reproducibility (WRL, unpublished findings).

Molecular biological techniques have now been applied to clone the genes coding for the NSP's into a number of different vector systems and work is underway both at the WRL and other laboratories in Europe and South America to explore the potential of these expressed products as antigens in ELISA. The potential use of these antigens is considerable as it should be possible to develop a test which can detect infection with any serotype in a single assay and differentiate animals which have been infected from those which have been vaccinated. Furthermore it should be possible to identify the carrier animal, as animals which have been infected and subsequently eliminate the virus produce a different spectrum of response to NSP's from animals which go on to become carriers. An ELISA using an expressed protein has the added advantage that the antigen presents no biological hazard.

Initial work using an expressed VIAA [9] was disappointing as the test had poor specificity and hence could only be used as a screening test to evaluate whether or not a population of animals had been exposed to FMD infection. Subsequently, expressed proteins have been used successfully in radioimmunoprecipitation [10] and immunoblotting studies [11, 12] to differentiate infected from vaccinated animals and also tentatively to differentiate carriers from non-carriers. However, neither of these techniques is suited to screening the large numbers of animals which would require testing should ring vaccination ever be used to contain future outbreaks within the EU. For this purpose it would be essential to use ELISA. At the WRL the major NSP's have been expressed in E coli as fusion proteins attached to glutathione s-transferase (GST). Following purification, and either with or without cleavage from the GST, these recombinant NSP's are recognised by immune sera from animals infected with a range of serotypes of FMD virus. When used in a simple indirect ELISA, recombinant 3D is able to differentiate infected from naive cattle. The sensitivity of the test is only slightly lower than the conventional liquid phase blocking ELISA of Hamblin et al. [13] and the specificity is approximately 95%. Vaccinated animals, especially those which have received more than one dose of vaccine, give a

positive reaction, as can up to 5% of normal bovines. It therefore appears that the recombinant (and possibly the native) 3D protein shares epitopes with other antigens which are encountered by cattle, perhaps the polymerase of other enteroviruses. Further work is required to characterise the recombinant NSP's and the immune response against them. A range of ELISA techniques are currently being evaluated with the intention of producing ELISA's for routine diagnostic use which are capable of detecting antibody to FMD virus NSP's.

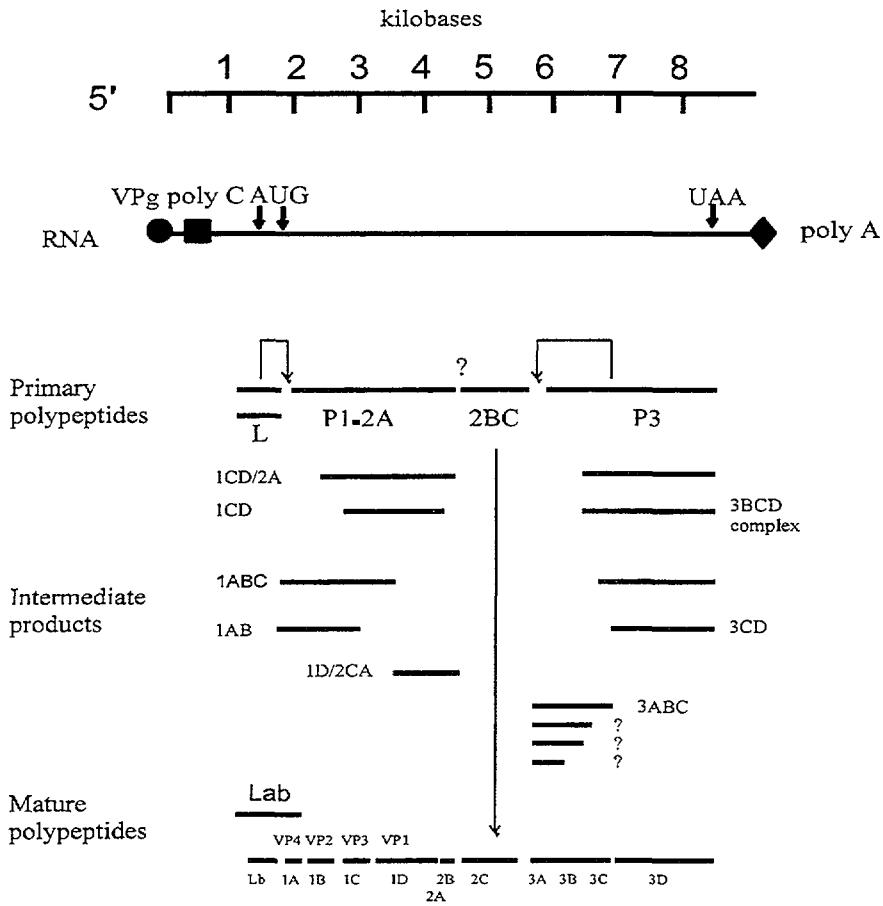

FIG. 1. Genomic organization and proteolytic processing of the FMDV polyprotein.

5. MONOCLONAL ANTIBODIES

Monoclonal antibodies (Mabs) are the products of a clone of identical, immortal antibody-producing cells. They consist of a population of identical antibodies all of which have specificity for one single, definable epitope (antigen binding site). This contrasts with polyclonal antisera, produced by immunisation of animals, which consist of mixed populations of antibodies against a range of different epitopes. Mabs against FMD virus have been produced since the 1980's and have provided useful information about the structure and function of the virus. Only now are they starting to be more widely applied to improve FMD diagnosis.

Mabs can be used in ELISA to detect either antigen or antibody. Mabs have been applied with some success to FMD antigen detection ELISA's with the aim of increasing specificity. A serotype-specific Mab-based antigen detection ELISA currently in use at the Istituto Zooprofilatico Sperimentale, Brescia, Italy [14], relies on a mixture of at least 3 different Mabs against each of the serotypes O, A and C to detect FMD virus in clinical samples. The use of multiple Mabs has the advantage that it is less likely that a field virus will fail to possess at least one of the epitopes recognised by the antibodies. If a Mab can be produced against an epitope shared by all the serotypes of FMD virus it should be possible

to detect virus of any serotype with a single assay. Smitsaart et al. [15] reported an assay using a Mab reactive against an epitope on the 12S sub-unit of FMD virus which was shared between 6 of the 7 serotypes of FMD virus. Work is continuing to produce a Mab reactive against an FMD virus-specific epitope which is sufficiently stable to form the basis of a diagnostic test.

In the FMD virus antibody detection ELISA Mabs can be used either to trap the FMD virus antigen or, more usually, to compete with test sera for binding to antigen. Due to the unique specificity of Mabs, competition assays are able to differentiate serological responses to antigens which are very closely related, such as antibodies to rinderpest and peste des petits ruminants [16]. Competition [14] and complex-trapping-blocking [17] ELISA's using combinations of serotype-specific Mabs have been developed. As described for a non-serotype specific antigen detection ELISA, the identification of an epitope shared between all of the seven serotypes of FMD virus could be the basis of a non-serotype specific competition ELISA able to detect antibody to any strain of FMD virus. Due to their highly conserved nature, epitopes on the NSP's of the virus are the most likely candidates for such a site. However, the search is complicated by the fact that antibody to NSP's is not neutralising. Therefore it is not possible to raise Mab escape mutants and sequence them to identify the sites at which the Mabs bind. Alternative approaches are required such as scanning libraries of overlapping peptides corresponding to the predicted amino acid sequence of the protein to identify the residues important in Mab binding.

Mabs now also play an important role in characterising field, vaccine and reference strains of FMD virus. Panels of Mabs have been produced and characterised against strains of FMD virus types O, A, C & SAT 2. Many Mabs have also been produced against strains of other serotypes and characterised to varying degrees. Attempts have been made over several years to establish a bank of FMD Mabs at the WRL, Pirbright with limited success and a new initiative has recently been launched under the auspices of the Community Reference Laboratory section of the WRL. These panels of Mabs are used to characterise the major epitopes of strains of FMD virus. Comparisons between field and vaccine strains, or between seed strains and final vaccines strains, give precise information about the similarity of the epitopes examined. Once sufficient information is available about which of these epitopes are important for protection and how this relates to Mab binding, it should be possible to predict on the basis of a Mab profile whether a vaccine strain is likely to give protection against a particular field strain. Furthermore our knowledge of the link between the genotype (nucleotide sequence) and phenotype (Mab profile) of viruses increases as more and more strains are examined. This holds the prospect of eventually being able to predict important nucleotide sequence changes on the basis of Mab profiles, which would save the considerable time and expense of sequencing large numbers of isolates.

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REFERENCES

[1] KITCHING, R.P., The application of biotechnology to the control of foot-and-mouth disease virus, Br.Vet. J. **148** (1992) 375-388.

[2] DAWE, P.S., FLANAGAN, F.O., MADEKUROZWA, R.L., SORENSEN, K.J., ANDERSON, E.C., FOGGIN, C.M., FERRIS, N.P., KNOWLES, N.J., Natural transmission of foot-and-mouth disease virus from African buffalo (Syncerus caffer) to cattle in a wildlife area of Zimbabwe, Vet.Rec. **134** (1994) 230-232.

[3] HARGREAVES, S.K., OIE Scientific Conference on the Control of Foot-and-Mouth Disease, African Horse Sickness and Contagious Bovine Pleuropneumonia, OIE, Paris, France, (1994) pp 9-10.

[4] SALT, J.S., The carrier state in foot-and-mouth disease - an immunological review, Br.Vet. J. **149** (1993) 207-223.

[5] SUTMOLLER, P., GAGGERO, A., Foot-and-mouth disease carriers, Vet. Rec. 77 (1965) 968-969

[6] AMAREL-DOEL, C.M.F., OWEN, N.E., FERRIS, N.P., KITCHING, R.P., DOEL, T.R., Detection of foot-and-mouth disease viral sequences in clinical specimens and ethyleneimine-inactivated preparations by the polymerase chain reaction, Vaccine **11** (1993) 415-421.

[7] COWAN, K.M., GRAVES, J.H., A third antigenic component associated with foot-and-mouth disease infection, Virology **30** (1966) 528-540.

[8] McVICAR, J.W., SUTMOLLER, P., FMD: The agar gel diffusion precipitin test for antibody to virus-infection-associated (VIA) antigen as a tool for epizootiologic surveys, American Journal of Epidemiology **92** (1970) 273-278.

[9] VILLINGER, F., MUELLER, H.K., BRUCKNER, L., ACKERMANN, M., KIHM, U., Antibodies to FMD virus infection associated (VIA) antigen: use of a bio-engineered VIA protein as antigen in an ELISA, Veterinary Microbiology **20** (1989) 235-246.

[10] TESAR, M., BERGER, H-G , MARQUARDT, O., Serological probes for some FMD virus non-structural proteins, Virus Genes **3** (1989) 29-44.

[11] NEITZERT, E., BECK, E., DE MELLO, P.A., GOMES, I., BERGMANN, I.E., Expression of the aphthovirus RNA polymerase gene in *Escherichia coli* and its use together with other bioengineered non-structural antigens in detection of late persistent infections, Virology **184** (1991) 799-804.

[12] BERGMANN, I.E., DE MELLO, P.A., NEITZERT, E., BECK, E., GOMES, I., Diagnosis of persistent aphthovirus infection and its differentiation from vaccination response in cattle by use of enzyme linked immunoelectrotransfer blot analysis with bio-engineered non-structural viral antigens, American Journal of Veterinary Research **54** (1993) 825-831.

[13] HAMBLIN, C., BARNETT, I.T.R , HEDGER, R.S., A new enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against FMD virus, 1. Development and method of ELISA, Journal of Immunological Methods **93** (1986) 115-121.

[14] BROCCHI, E., DE SIMONE, F., BUGNETTI, M., GAMBA, D., CAPUCCI, L. Report of the Research Group of the Standing Technical Committee of the European Commission for the Control of FMD, Lindholm, 1990 pp. 83-88 (Appendix 14).

[15] SMITSAART, E.N., SAIZ, J.C., YEDLOUTSCHNIG, R.J., MORGAN, D.O., Detection of foot-and-mouth disease virus by competitive ELISA using a monoclonal antibody specific for the 12S protein subunit from six of the seven serotypes, Veterinary Immunology and Immunopathology **26** (1990) 251-265.

[16] ANDERSON, J., MACKAY, J.A., The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes, Epidemiology and Infection **112** (1994) 225-231.

[17] VAN MAANEN, C., A complex-trapping-blocking ELISA using monoclonal antibodies and detecting specifically antibodies directed against foot-and-mouth disease types A, O and C, I. Method and characteristics, Veterinary Microbiology **24** (1990) 171-178.

COMPARISON OF COMPLEMENT FIXATION AND ELISA FOR DIAGNOSIS OF FOOT-AND-MOUTH DISEASE

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Abstract

COMPARISON OF COMPLEMENT FIXATION AND ELISA FOR DIAGNOSIS OF FOOT-AND-MOUTH DISEASE
Foot-and-mouth disease (FMD) virus is characterised by its rapid transmission and its great antigenic variability which require a rapid and accurate diagnosis in the laboratory, in order to initiate an immediate response for control From these studies it is clear that Enzyme linked immunosorbent assay (ELISA) has the advantage over the Complement fixation test (CFT) of being a test of high sensitivity and specificity Therefore, this technique is now used in our laboratory for diagnosis to detect FMD virus (O-A-C) in epithelia from animals affected by the disease

1. INTRODUCTION

Foot-and-mouth disease has a great capacity for spread due to the movement of infected animals, contaminated animal products and escape of virus from the laboratory. FMD is caused by a virus, classified as an Enterovirus, belonging to the family Picornavirus and genus Aphtovirus. There are 7 serotypes of virus and within each serotype there is a spectrum of antigenic variants

2. MATERIALS AND METHODS

2.1. Field samples

Epithelium from the mouth or feet of affected cattle (38 samples) and from BHK cell culture (4 samples) [1,2]. The epithelia samples had previously been submitted to SENACSA, kept at - 20°C. in glycerine and phosphate buffer. The samples were examined by the ELISA [3] and compared with the Complement fixation test [4,5] for diagnosis and typing.

2.2. ELISA procedure

For the diagnosis and typing of FMD virus by ELISA the following materials were used:

- Rabbit Capture Antisera (O-A-C-NJ-I-and negative sera)
- The samples: Epithelium samples or BHK tissue culture suspension.
- Antigen reference: 0-A-C-NJ-I-and negative
- Plates
- Coating buffer
- PBS
- Tween 20
- Conjugate
- Substrate
- Multichannel and single channel pipettes
- Sulphuric Acid.
- Assay tubes
- Shaker
- Spectrophotometer Multiskan plus (filter: 492 mm.)

TABLE I RESULTS ON FMD ELISA AT SENACSA

No	Date	Plate	Test sample	ELISA	CFT
01	10 01 94	1	Epit Mouth	0	0
02	10 01 94		Epit Mouth	0	0
03	10 01 94		Epit Foot	Neg	Neg
04	14 01 94	2	Epit Foot	0	0
05			Epit Mouth	Neg	Neg
06			Epit Mouth	Neg	Neg
07	31 01 94	3	Epit Mouth	0	0
08			Epit Foot	0	0
09			Epit Foot	0	0
10	22 02 94	4	Epit Mouth	0	0
11			Epit Mouth	0	0
12			Epit Mouth	0	0
13	22 02 94	5	Epit Foot	0	0
14			Epit Foot	0	0
15			Epit Foot	Neg	Neg
16	22 02 94	6	Epit Mouth	0	0
17			Epit Mouth	0	0
18			Epit Mouth	0	0
19	22 02 94	7	Epit Mouth	0	0
20			Epit Mouth	0	0
21			Epit Mouth	0	0
22			Epit Mouth	0	0
23			Epit Mouth	0	0
24	23 02 94		Epit Mouth	0	0
25			Epit Mouth	0	0
26			Epit Mouth	0	0
27	23 02 94	9	Epit Mouth	0	0
28			(BHK3)	C	C
29			Epit Mouth	0	0
30	23 02 94	10	Epit Foot	0	0
31			BHK2	A	A
32			BHK3	C	C
33	23 02 94	11	Epit Mouth	0	0
34	23 02 94		Epit Mouth	0	0
35			Epit Mouth	0	0
36			Epit Mouth	0	0
37			BHK3	A	A
38	23 02 94		Epit Mouth	0	0
39			Epit Mouth	0	0
40			Epit Mouth	0	0
41			Epit Mouth	0	0
42			Epit Mouth	0	0

The assay procedure was as follows:

1- Solid Phase: Antisera Rabbit (O,A,C-NJ-I-and negative sera) dilution 1/100, 18 h, 4°C. Wash 5 times
2- Control Antigen: Dilution used O,A,C, 1/40 - 1/160 with PBS, 0.01 M, pH 7,4, NJ-I 1/50 - 1/200
Test samples: Suspension of epithelium
1 h 37°C on shaker washing 5 times
3- Detector guinea pig antisera: Dilution 1/100 (O,A,C-NJ-I-and negative)
4- Conjugate anti guinea pig: Dilution 1/300
5- Substrate OPD + $H_2 0_2$ 15 minutes
6- $H_2 SO_4$ (0.25 M)
7- Read at 492 nm

3. RESULTS

Virus types O, A and C were identified from the samples prepared from epithelia and suspensions of BHK cell cultures. Results were calculated using the background control adding the optical density (OD) value of the columns five and six divided by two; this result is the mean background OD. The mean OD of the antigen controls of the serotypes O-A-C-NJ-I-Neg were calculated and subtracted from the OD value of the background. This was repeated for every sample. All positive samples by complement fixation were also positive by ELISA (Table I).

4. DISCUSSION

Rapid and specific laboratory diagnosis is required for FMD, in order to differentiate this disease from others caused by vesicular viruses, and to identify the serotype of FMD virus. In the results reported here no difference was found between the ELISA and complement fixation test, however, other workers have reported increased sensitivity of ELISA, and the reagents used in ELISA can more easily be standardised and stored.

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REFERENCES

[1] ABU ELZEIN, E.M.E., CROWTHER, J.R., The specific detection of foot-and-mouth disease virus whole particle antigen (140 S) by enzyme labelled immunosorbent assay, J. Hyg. **83** (1979) 127-134.

[2] HAMBLIN, C., et al., A rapid enzyme linked immunosorbent assay for the detection of foot-and-mouth disease virus in epithelial tissues, Vet. Microbiol. **9** (1984) 435-443.

[3] CROWTHER, J.R., ABU ELZEIN, E.M.E., Detection and quantification of foot-and-mouth disease virus by enzyme labelled immunosorbent assay techniques, J. gen. Virol. **42** (1979) 597-602.

[4] FERRIS, N.P., DAWSON, M., Routine application of enzyme linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases, Vet. Microbiol. **16** (1988) 201-209.

[5] GOMES, M.P.D., Application of enzyme linked immunosorbent assay for the diagnosis of foot-and-mouth disease virus and vesicular stomatitis in comparison with the complement fixation, Bol. Centr. Panam. Fiebre Aftosa **55** (1989) 21-25.

COUNTRY REPORTS

Part B: FOOT-AND-MOUTH DISEASE

FMD antibody ELISA

VALIDATION OF A FOOT-AND-MOUTH DISEASE ANTIBODY ELISA IN FIVE LATIN AMERICAN COUNTRIES

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Abstract

VALIDATION OF A FOOT-AND-MOUTH DISEASE ANTIBODY ELISA IN FIVE LATIN AMERICAN COUNTRIES
The work plan consisted of using a liquid phase blocking ELISA test for the detection of antibodies to foot-and-mouth disease virus (FMDV) using the following categories of sera (A) Spot test 120 non-infected/non-vaccinated bovine sera diluted 1 32, (B) Titration test 120 bovine sera from animals vaccinated with trivalent oil vaccine, bled 30 days after vaccination, (C) Titration test with sera from non-infected/non-vaccinated bovines that presented titers >1 32 in the spot test To detect FMD positive animals in the field, the spot test established with a cut-off of 1 32 demonstrated in this work a good specificity with the non-vaccinated group, where 3 animals out of 120 were considered positive The antibody titration test is an excellent tool to determine the level of antibodies in cattle populations The protocol indicates that positive sera from the spot test should be tested in the titration assay in a starting dilution of 1 32 We suggest to use a lower starting dilution (1 16) in order to start below the discriminative of positive spot test sera 1 32 for the titration assay procedures

1 INTRODUCTION

A liquid phase blocking ELISA for detection of serum antibodies to FMDV [1,2] was used according to the protocol established in the first Research Coordinated Meeting FAO/IAEA/PAHO-PANAFTOSA [3,4] held in September, 1995 in Rio de Janeiro, Brazil The biological reagents [5] were supplied by PANAFTOSA and the chemicals reagents by IAEA. PANAFTOSA made the necessary adjustments in the test and the determination of the upper and lower control limits which then were introduced in the FAO/IAEA ELISA software programme EDI

The test is based upon specific blocking of liquid phase FMDV antigen [6] by antibodies in serum samples

2 MATERIAL AND METHODS

2.1. Serum samples

2 1 1 Sera from vaccinated animals

One hundred and twenty sera from 18-24 months old animals, vaccinated with trivalent oil adjuvant vaccine and bled 30 days after vaccination

2 1 2 Sera from non-infected animals

One hundred and twenty sera from 18-24 months old animals from selected herds without history of FMD, not vaccinated and tested previously to confirm that all were FMD antibody free

Sera were kindly supplied by the Vaccine Control Laboratory of Brazil, Ministry of Agriculture (MAARA-LARA/RS) Rio Grande do Sul State, which carries out the official vaccine potency control for commercial FMD vaccines. Sera were used in the test without treatment or preservation procedures.

2.2. ELISA biologicals

2 2 1 Virus strains

For this work the reference strains O_1 Campos-Br.1/58, A_{24} Cruzeiro-Br 1/55 and C_3 Indaial-Br 1/71 were used Colombia and Venezuela in view of being free of FMD virus strain C, only received O and A virus strains [7]

Viruses were obtained from BHK-21, C-13 (Baby Hamster Kidney) cell cultures, inactivated by Binary Ethylenimine (BEI), treated with 50% v/v with sterile glycerol and stored at -20°C [8,9].

2 2 2 Trapping antibodies

Hyperimmune sera were obtained by inoculations of rabbits with above mentioned virus strains, after cesium chloride gradient purification, and stored at -20°C

2 2 3 Detecting antibodies

Hyperimmune antisera were produced in guinea pig [10] with the strains previously mentioned, adapted in this species and stored at -20°C

2 2 4 Conjugate

The conjugate, IgG-anti-guinea pig labeled peroxidase, was produced at PANAFTOSA and distributed to the five laboratories.

2 2 5 Control sera

Positive control sera: Pool of sera from bovines vaccinated and revaccinated with monovalent oil adjuvant vaccines using each one of the strains mentioned above. Positive control sera were divided in two groups: strong positive (++) and weak positive (+) and stored at -20°C

Negative control: Pool of bovine sera from FMD and Vesicular Stomatitis Virus (VSV) free areas All biological reagents with their respective working dilutions established, were aliquoted, labeled and distributed by PANAFTOSA to the five participating laboratories as shown in Table II and III

2.3. ELISA chemicals, consumables, manual and software programme for data management

Chemicals (Carb Bicarb. tablets, PBS tablets, Tween 20, Phoshp/Citr Tablets, H_2O_2 tablets, OPD tablets, skimmed milk powder, ovalbumen grade II, V, conjugate and normal rabbit serum), consumables (tips, NUNC and polypropyelene plates, minor laboratory equipment, the ELISA software programme EDI and the protocol were supplied by the Joint FAO/IAEA Division, Vienna, Austria.

2.4. Liquid phase blocking ELISA

Values for Upper (UCL) and Lower (LCL) Control Limits for the first level of acceptance of the test were obtained based on the Optical Density (O.D.) of the antigen (1 0-1.5) as recommended by the Joint FAO/IAEA Division Manual Virus concentrations were determined by separate titration of each of the virus strains (O, A and C)

The second level of acceptance of the controls (strong positive (++), weak positive (+), negative (-) and Antigen Control (Ca)) expressed as Percentage of Inhibition (P.I.) were obtained using four replicates of 25 tests ran over a period of several days. Replicates were done for each control and for each virus strain A control chart developed by PANAFTOSA using physical, chemical and biological control parameters and based on O.D. values was applied P I values were calculated using statistical analysis and included in the FAO/IAEA ELISA software programme EDI 2 11 as shown in Table I.

TABLE I UPPER CONTROL LIMITS (UCL) AND LOWER CONTROL LIMITS (LCL) FOR ANTIGEN CONTROL (Ca) AND STRONG POSITIVE (C++), WEAK POSITIVE (C+) AND NEGATIVE (C-) CONTROL SERA FOR SEROTYPE A, O AND C ANTIGENS

Parameter			Value/Virus		
			O	A	C
LCL	OD	Ca	1 06	1 08	1 07
UCL	OD	Ca	1 34	1 30	1 31
LCL	PI	C++	93	89	90
UCL	PI	C++	96	95	95
LCL	PI	C+	63	59	63
UCL	PI	C+	81	75	80
LCL	PI	C-	-06	09	01
UCL	PI	C-	38	40	36
LCL	PI	Ca	-41	-39	40
UCL	PI	Ca	25	23	24

Ca Antigen Control
OD Optical Density
PI Percent Inhibition

TABLE II. BIOLOGICALS SUPPLIED BY PANAFTOSA FOR ARGENTINA, BRAZIL AND PARAGUAY

COORDINATED RESEARCH PROGRAMME FOR THE VALIDATION OF FMD ANTIBODIES ELISA TEST - FAO/IAEA/PAHO-PANAFTOSA - (2nd phase)

A) SERA

1) 120 bovine negative sera

2) 120 bovine sera from vaccinated cattle with oil adjuvant trivalent vaccine, bled 30 days after vaccination.

B) REAGENTS

Trapping Antibodies (Rabbit)	Amount (ml)	Final Dilution
O_1 Campos-Br.1/58	0,250	1/2.000
A_{24} Cruzeiro-Br.1/55	0,300	1/1.500
C_3 Indaial-Br.1/71	0,250	1/2.000
(Storage at - 20°C)		
Detecting Antibodies (Guinea Pig)		
O_1 Campos-Br.1/58	1,00	1/300
A_{24} Cruzeiro-Br.1/55	1,50	1/200
C_3 Indaial-Br.1/71	0,50	1/600
(Storage at - 20°C)		
Inactivated Virus/Glycerinated		
O_1 Campos-Br.1/58	40,00	1/16
A_{24} Cruzeiro-Br,1/55	20,00	1/30
C_3 Indaial-Br.1/71	10,00	1/60
(Storage at - 20°C)		
Conjugate/Peroxidase		
IgG anti Guinea Pig-Lot 83	10,00	1/80
(Storage at + 4°C)		
Positive control serum (++)		
O_1 Campos-Br.1/58	1,50	1/32
A_{24} Cruzeiro-Br.1/55	1,50	1/32
C_3 Indaial-Br.1/71	1,50	1/32
(Storage at - 20°C)		
Positive control serum (+)		
O_1 Campos-Br.1/58	1,50	1/32
A_{24} Cruzeiro-Br.1/55	1,50	1/32
C_3 Indaial-Br.1/71	1,50	1/32
(Storage at - 20°C)		
Negative control serum		
Bovine serum - Lot 02	4,50	1/32
(Storage at - 20°C)		
Blocking serum		
Bovine serum	60,00	-
(Storage at - 20°C)		

TABLE III. BIOLOGICALS SUPPLIED BY PANAFTOSA FOR COLOMBIA AND VENEZUELA

COORDINATED RESEARCH PROGRAMME FOR THE VALIDATION OF FMD ANTIBODIES ELISA TEST - FAO/IAEA/PAHO-PANAFTOSA - (2nd phase)

A) SERA

1) 120 bovine negative sera

2) 120 bovine sera from vaccinated cattle with oil adjuvant trivalent vaccine, bled 30 days after vaccination.

B) REAGENTS

Trapping Antibodies (Rabbit)	Amount (ml)	Final Dilution
O_1 Campos-Br.1/58	0,250	1/2.000
A_{24} Cruzeiro-Br.1/55	0,300	1/1.500
(Storage at - 20°C)		
Detecting Antibodies (Guinea Pig)		
O_1 Campos-Br.1/58	1,00	1/300
A_{24} Cruzeiro-Br.1/55	1,50	1/200
(Storage at - 20°C)		
Inactivated Virus/Glycerinated		
O_1 Campos-Br.1/58	40,00	1/16
A_{24} Cruzeiro-Br,1/55	20,00	1/30
(Storage at - 20°C)		
Conjugate/Peroxidase		
IgG anti Guinea Pig-Lot 83	10,00	1/80
(Storage at + 4°C)		
Positive control serum (++)		
O_1 Campos-Br.1/58	1,50	1/32
A_{24} Cruzeiro-Br.1/55	1,50	1/32
(Storage at - 20°C)		
Positive control serum (+)		
O_1 Campos-Br.1/58	1,50	1/32
A_{24} Cruzeiro-Br.1/55	1,50	1/32
(Storage at - 20°C)		
Negative control serum		
Bovine serum - Lot 02	4,50	1/32
(Storage at - 20°C)		
Blocking serum		
Bovine serum	60,00	-
(Storage at - 20°C)		

The assay was developed following strictly the previously established protocol, published and distributed by the Joint FAO/IAEA Division, Vienna, Austria to assure a maximum of standardization between participating laboratories.

3. RESULTS

A total of 46 ELISA plates were used and the following results obtained:

3.1. Spot test

Performed with 120 non-infected bovine sera diluted 1:32. Out of 9 ELISA plates 4 plates were "outside limits" (2 because of Ca values , 1 because of the C++ P.I. values and 1 because of Ca and P.I. values). Five serum samples were positive for virus A only. Specificities for the different antigens of the spot test are given in Table IV.

TABLE IV. SPECIFICITY OF SCREENING ASSAY (Spot test 1/32) FOR SEROTYPES O, A AND C

Virus	C	O	A
Total neg. sera	120	120	120
Test pos.	0	4	0
Test neg.	120	116	120
Specificity (%)	100	97.5	100

3.2. Antibody titration

Performed with 120 sera from cattle vaccinated with FMD trivalent oil adjuvant vaccine using O_1 Campos-Br.1/58, A_{24} Cruzeiro-Br.1/55 and C_3 Indaial-Br.1/71 virus. Out of 36 ELISA plates 15 were "outside limits" (11 because of OD values for the Ca, 2 because of C+ PI values and 2 because of C++ PI values). Four dilutions were used for this titration: 1:10, 1:50, 1:250 and 1:1250. Results are shown in Tables IV-VII.

3.3. Antibody titration of positive sera in the spot test

Five sera, which were positive in the spot test were further titrated 1:32, 1:64, 1:128 and 1:256 against virus A. Three sera were positive at dilution of 1/32 dilution. The two remaining resulted negative.
Sensitivity of the titration assay according to the dilution is shown in Tables V-VIII.

TABLE V. SENSITIVITY OF TITRATION ASSAY (CUT-OFF > 1:10)

Virus	C	O	A
Total of pos. sera	120	120	120
Test pos.	120	120	120
Test neg.	0	0	0
Sensitivity (%)	100	100	100

TABLE VI. SENSITIVITY OF TITRATION ASSAY (CUT-OFF > 1:50)

Virus	C	O	A
Total of pos. sera	120	120	120
Test pos.	119	93	111
Test neg.	1	24	8
RT	-	3	1
Sensitivity (%)	99.16	77.5	92.5

RT = retest

TABLE VII. SENSITIVITY OF TITRATION ASSAY (CUT-OFF > 1:250)

Virus	C	O	A
Total of pos. sera	120	120	120
Test pos.	48	14	44
Test neg.	60	104	71
RT	12	2	5
Sensitivity (%)	40	11.6	36.6

RT = retest

TABLE VIII. SENSITIVITY OF TITRATION ASSAY (CUT-OFF > 1:1250)

Virus	C	O	A
Total of pos. sera	120	120	120
Test pos.	3	1	3
Test neg.	113	119	114
RT	4	0	3
Sensitivity (%)	2.5	0.83	2.5

RT = retest

4. DISCUSSION

The validation of a liquid phase blocking ELISA for detection of FMD antibodies proposed by FAO/IAEA, together with PAHO-PANAFTOSA and 5 laboratories (Argentina, Colombia, Brazil, Paraguay, and Venezuela) proved to be a valuable exercise.

To detect FMD positive animals in the field, the spot test established with a cut-off of 1:32 demonstrated in this work a good specificity with the non-vaccinated group, where 3 animals out of 120 were considered positive.

O.D. values for the antigen control established by PANAFTOSA fell within the predetermined range (1.0-1.5) and were included in the FAO/IAEA ELISA software EDI. A high degree of precision in the results was observed. It is recommended that P.I. values which are established as border values for the acceptance of the plate should be accepted e.g. a test value $\geq$ the Lower Control Limit (LCL) or a test value $\leq$ the Upper Control Limit (UCL) should be taken as accepted by the software programme EDI.

The antibody titration test is an excellent tool to determine the level of antibodies in cattle populations. The protocol indicates that positive sera from the spot test should be tested in the titration assay in a starting dilution of 1:32. We suggest to use a lower starting dilution (1:16) in order to start below the discriminative of positive spot test sera 1:32 for the titration assay procedures.

Looking on the results there was no difference using the PANAFTOSA software or FAO/IAEA software (EDI). The main difference between the two programmes is that PANAFTOSA software expresses results in logarithmical functions and EDI uses values expressed as percentage of inhibition P.I. as recommended by OIE (Office International des Epizooties).

REFERENCES

[1] AVRAMEAS, S., TERNYMCK, T., GUESDON, J.L., Coupling of enzymes to antibodies and antigens, Scand J. Immunol. **8** (1978) 7-23.

[2] SANCHEZ-VIZCAÍNO, J.M., ALVAREZ, M.C. ,Técnicas inmunoenzimáticas, ELISA en patología animal y vegetal, Paris, OIE (Serie Técnica 7) (1987).

[3] HAMBLIN, C., BARNETT, I.T.R., HEDGER, R.S., A new enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, I. Development and method of ELISA, J. Immunol. Methods **93** (1986) 115-121.

[4] HAMBLIN, C., BARNETT, I.T.R., CROWTHER, J.R., A new enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, II. Application. J. Immunol. Meth. **93** (1986) 123 - 129.

[5] GOMES, M.P.D., SONDAHL, M.S., MARTINS, M.A., OLASCOAGA, R.C., ALONSO, A.F., Application of enzyme linked immunosorbent assay for the diagnosis of foot-and-mouth disease virus and vesicular stomatitis in comparison with the complement fixation, Bol. Centr. Panam. [1] Fiebre Aftosa **55** (1989) 21-25.

[6] McCULLOUGH, K.K., CROWTHER, J.R., BUTCHER, R.N., A liquid-phase ELISA and its use in the identification of epitopes on foot-and-mouth disease virus antigen, J. Virol. Methods **11** (1985) 329-338.

[7] ROEDER, P.L., LE BLANC SMITH, P.M., Detection and typing of foot-and-mouth disease virus enzyme-linked immunosorbent: A sensitive, rapid and reliable technique for primary diagnosis, Res. Vet. Sci. **43** (1987) 225-232.

[8] BAHNEMANN, H.G., Binary ethyleneimine as an inactivant for foot-and-mouth disease virus and its application for vaccine production, Arch. Virol. **47** (1975) 45-56.

[9] BAHNEMANN, H.G., Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethyleneimine, Vaccine **8** (1990) 299-303.

[10] ALONSO, A., MARTINS, M.A., GOMES, M.P.D., ALLENDE, R., SONDAHL, M.S., Foot-and-mouth disease virus typing by complement fixation and ELISA tests using monovalent and polyvalent antisera, J. Vet. Diagn. Invest. **4** (3) (1992) 247-253.

VALIDATION OF THE FAO/IAEA/PANAFTOSA ELISA KIT FOR DETERMINATION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS

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Abstract

VALIDATION OF THE FAO/IAEA/PANAFTOSA ELISA KIT FOR DETERMINATION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS

A Liquid phase blocking sandwich ELISA (LPBE) for the detection of foot-and-mouth disease (FMD) antibodies, serotypes O, A and C was validated using sera from bovines free of antibodies and vaccinated bovines This technique proved to be sensitive and specific for the study of these antibodies This kit has been prepared by the Pan American Foot-and-Mouth Disease Center (PAHO/ WHO) in collaboration with the Animal Production and Health Section of the Joint FAO/IAEA Division, Vienna, Austria and the Institute for Animal Health in Pirbright, United Kingdom

1 INTRODUCTION

Foot-and-mouth disease (FMD) is the animal disease that causes the highest economic losses in the livestock industry of Argentina. The FMD eradication plan (1993-1997) is based on mass vaccination of bovines, regionalization, epidemiological surveillance and active participation of all interested sectors

As a consequence of the implementation of this plan, no outbreaks of the disease have been observed since April 1994 and in 1997 the Office International des Epizooties (OIE) officially recognized Argentina as "Freedom from FMD, with vaccination".

The main tasks performed in our laboratory are investigations on suspected samples for the diagnosis of vesicular disease or FMD virus, the control of all the FMD vaccines used and serological assays for epidemiological surveys and vaccine immunity studies.

An antigen detection ELISA is used to serotype virus samples and the liquid phase blocking ELISA (LPBE) is used for the detection of FMD serum antibodies [1-3]. As part of a project to extend the use of the LPBE in South America, we have evaluated an ELISA kit provided by the Joint FAO/IAEA Division and the Pan-American Foot-and-Mouth Disease Center (CPFA-OPS) for serological studies.

2 MATERIALS AND METHODS

Test serum samples and the biological reagents were supplied by CPFA-OPS Chemical reagents, consumables, the protocol and the ELISA software were supplied by the Joint FAO/IAEA Division

2.1. Serum Samples

2 1 1 Sera from non-infected animals

One hundred and twenty sera from 18-24 months old bovines from selected herds without neither history of FMD infection nor vaccination and tested previously by CPFA to confirm that all were FMD antibody free.

All these sera originated from Rio Grande do Sul State- Brazil, utilized by the vaccine control laboratory (MAARA-LARA/RS).

2.1.2. Sera from vaccinated animals:

One hundred and twenty sera from 18-24 months old bovines, vaccinated with trivalent oil adjuvant vaccine and bleed 30 days after vaccination.

2.2. Virus Strains

For this work the strains O1 Campos-Br.1/58, A24 Cruzeiro-Br.1/55 and C3 Indaial-Br.1/71 were used. These viruses were obtained from BHK-21, C-13 cell culture, inactivated by binary ethylenimine (BEI), treated with sterile glycerol (50% v/v) and stored at -20°C.

2.3. Trapping antibodies

Hyperimmune sera to each of the serotypes were obtained by inoculation of rabbits with one of the previously mentioned virus strains and stored at -20°C after cesium chloride gradient purification.

2.4. Detecting Antibodies

Hyperimmune antisera were produced in guinea pigs against the strains previously mentioned using live virus adapted to this species and stored at -20°C.

2.5. Conjugate

The conjugate (Peroxidase labeled goat immunoglobulins to guinea pig immunoglobulins) was produced by CPFA.

2.6. Control sera

Positive control sera: pools of sera from vaccinated and revaccinated bovines with monovalent oil adjuvanted vaccines manufactured with each of the virus strains previously mentioned and divided in two groups strong positive (C++) and weak positive (C+) and stored at -20°C.

Negative control sera: a pool of bovine sera from FMD and Vesicular Stomatitis Virus (VSV) free areas.

2.7. Liquid phase blocking ELISA

The assay was followed strictly in accordance to the established protocol distributed by the Joint FAO/IAEA Division.

2.8. Software

The plates were read in a Multiskan spectrophotometer (MCC 340) and optical density values were interpreted by software supplied by the FAO/IAEA Joint Division (ELISA Data Interchange; EDI 2.1.1) for calculation of percentage inhibition values, control and plate acceptance.

3. RESULTS

The internal upper and lower control values are shown in Table I.

TABLE I. UPPER CONTROL LIMITS (UCL) AND LOWER CONTROL LIMITS (LCL) FOR STRONG POSITIVE (C++), WEAK POSITIVE (C+) AND NEGATIVE (C-) CONTROL SERA FOR A, O AND C ANTIGENS

Parameter			Value/Virus		
			O	A	C
LCL	OD	Ca	0.957	0.967	1.013
UCL	OD	Ca	1.760	1.729	1.444
LCL	PI	C++	86	75	83
UCL	PI	C++	100	99	99
LCL	PI	C+	63	59	63
UCL	PI	C+	81	75	80
LCL	PI	C-	-06	09	-01
UCL	PI	C-	38	40	36
LCL	PI	Ca	-41	-39	-40
UCL	PI	Ca	25	23	24

Ca Antigen Control
OD Optical Density
PI Percent Inhibition

The group of 120 sera from non-infected cattle gave entirely negative results in the screening test (1/ 32 final dilution) for the three serotypes under study (O, A and C).

Ninety sera from the group of vaccinated animals were titrated with a five fold dilutions starting at 1/10 until 1/1250. The titers obtained for the three serotypes under study gave variable results. Figures 1, 2, and 3 show the titer for serotype C, A and O respectively. An antibody titer of ≥ 1/112 indicates that the animal is protected against infection from the homologous antigen.

According to these results the specificity was 100 % for the three serotypes, the sensitivity for virus O was 97,7 %, for virus A 92,2 % and for virus C 93,3 %.

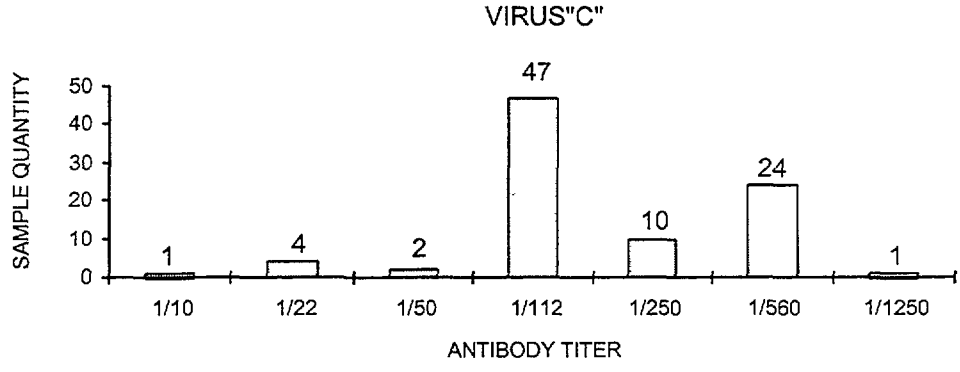

FIG. 1. Antibody titer of 120 sera from cattle vaccinated against serotype "C" (titers of ≥ 1/112 are considered as protective).

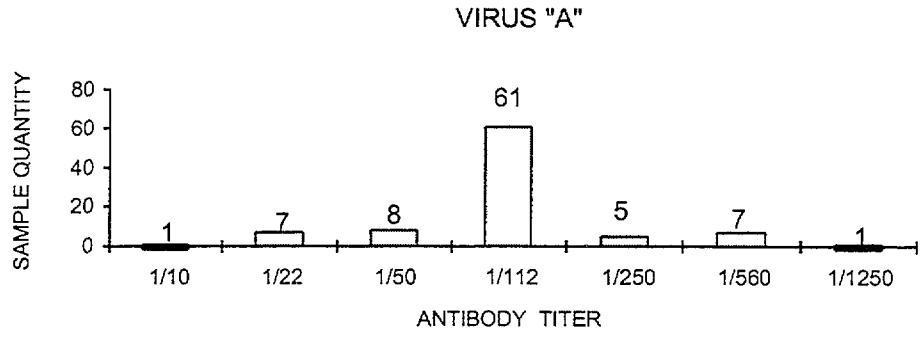

FIG. 2. Antibody titer of 120 sera from cattle vaccinated against serotype "A" (titers of ≥ 1/112 are considered as protective).

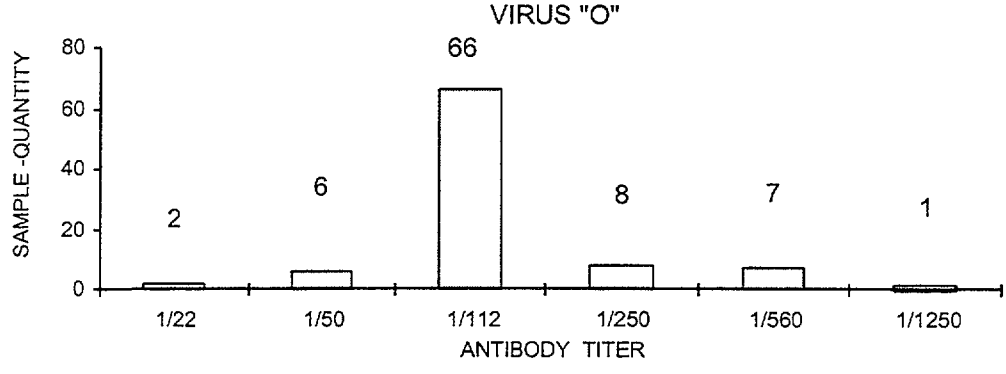

FIG. 3. Antibody titer of 120 sera from cattle vaccinated against serotype "O" (titers of ≥ 1/112 are considered as protective).

4. DISCUSSION

The LPBE proved to be specific and sensitive to be used for FMD antibody detection. In FMD free areas without vaccination it can be applied for import/export testing of animals in order to determine the absence or presence of the disease. In areas where vaccination against FMD is carried out the LPBE is a useful tool to determine the protection level of the vaccinated animals, but since the technique does not discriminate antibodies due to vaccination from antibodies due to infection it can not be used to determine the presence or absence of the disease. During the first run the internal control values were too narrow and a high percentage of plates were rejected due to outside limits status. The values for the upper and lower internal control limits were analyzed and reestablished taking into consideration all data produced by the 5 participating laboratories and by PANAFTOSA. The new values, which are shown in Table I proved to be more suitable.

REFERENCES

[1] HAMBLIN, C., BARNETT, I., HEDGER, R., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, J. Immun. Methods **93** (1986) 115-121.

[2] HAMBLIN, C., BARNETT, I., CROWTHER, J., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, J. Immun. Methods **93** (1986) 123-129.

[3] PERIOLO, O., SEKI, C., GRIGERA, P., ROBIOLO, B., FERNANDEZ, G., MARADEI, E., D'ALOIA, R., LA TORRE, J., Large-scale use of liquid phase blocking sandwich ELISA for the evaluation of protective immunity against aphtovirus in cattle vaccinated with oil adjuvant vaccines in Argentina, Vaccine **11** (1993) 754-760.

VALIDATION OF FAO/IAEA/PANAFTOSA ELISA KIT FOR THE DETECTION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS IN VENEZUELA

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Abstract

VALIDATION OF FAO/IAEA/PANAFTOSA ELISA KIT FOR THE DETECTION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS IN VENEZUELA

A liquid phase blocking ELISA (LPBE) supplied by FAO/IAEA/PANAFTOSA has been evaluated for the qualitative and quantitative detection of specific antibodies to "O" and "A" serotypes of foot-and-mouth disease (FMD) A total of 240 bovine sera were analyzed 120 sera from non-infected and non-vaccinated cattle were tested in a screening test showing a specificity of 99,2% 120 from vaccinated cattle were tested in a titration assay giving a sensitivity of 99,2% For serotype "O" the titration test showed a protection of 80% and for serotype "A" 75,6% Antibody titers fluctuated between >112 and >1250 which indicates protection

1. INTRODUCTION

The liquid phase blocking ELISA [1,2] developed to identify specific antibodies to foot-and-mouth disease (FMD) [3] in serum of cattle in the field is a very useful method It is able to determine the status of immunity in cattle and has been used to monitor the success of vaccination against FMD

The Joint FAO/IAEA Division and the Panamerican Center for Foot-and-Mouth Disease (PANAFTOSA) have agreed on a protocol [4] to validate a LPBE kit Five countries (Argentina, Brazil, Colombia, Paraguay and Venezuela) were selected to standardize a technique that supports the vaccination campaign through seroepidemiological studies and which can be used to control the quality of FMD vaccine.

The national livestock population in Venezuela represents approximately 18 000 000 bovine, 5 000.000 swine (126.512 sows) and a less important number of ovine and caprine species Efforts to eradicate FMD in Latin America have been agreed through the establishment of a hemispheric eradication plan In June 1996 in Brasilia countries have committed themselves to eradicate FMD from the continent by the year 2009. Following this plan, Venezuela implemented a control and eradication programme, which is based on three pillars: 1) mass vaccination of bovine 2) epidemiological surveillance and 3) active participation of all sectors involved Encouraging results have been reported from the south of Venezuela, Bolivar state, where no outbreaks have been observed since June 1994. The reason for this development is the very strict application of a vaccination plan, covering approximately 90% of the cattle and it is estimated that this area will be declared free of FMD in 1998.

In the view of the existing eradication plan, it is necessary to replace the present diagnostic technique, which uses inoculation of life virus in suckling mice with a LPBE, which uses inactivated virus as the assay antigen.

2 MATERIALS AND METHODS

Test serum samples and the biological reagents were supplied by PANAFTOSA. Chemicals, consumables and the ELISA software were supplied by the Joint FAO/IAEA Division.

2.1. Serum Samples

2.1.1. Sera from non-infected, non-vaccinated animals

One hundred and twenty sera from 18-24 months old cattle from selected herds without neither history of FMD infection nor vaccination. Sera were tested previously at PANAFTOSA to confirm freedom of FMD antibody. Sera were supplied from the vaccine control laboratory, MAARA-LARA/RS, Rio Grande do Sul State, Brazil.

2.1.2. Sera from vaccinated animals

One hundred and twenty sera from 18-24 months old cattle, vaccinated with trivalent oil adjuvant vaccine and bled 30 days after vaccination.

2.2. Virus strains

For this work the strains O_1 Campos-Br.1/58 and A_{24} Cruzeiro-Br.1/55 were used. Viruses were obtained from BHK-21, C-13 cell cultures, inactivated with binary ethylenimine (BEI), treated with sterile glycerol (50% v/v) and stored at -20°C.

2.3. Trapping antibodies

Hyperimmune sera to each of the serotypes were obtained by inoculation of rabbits with one of the previously mentioned virus stains, after cesium chloride gradient purification, and stored at -20°C.

2.4. Detecting antibodies

Hyperimmune antisera were produced in guinea pigs against the strains previously mentioned using live virus adapted to this species and stored at -20°C.

2.5. Conjugate

The conjugate (Peroxidase labeled goat anti guinea pig immunoglobulin) was produced at PANAFTOSA.

2.6. Control Sera

2.6.1. Positive control sera

Pools of sera from vaccinated and revaccinated cattle with monovalent oil adjuvanted vaccines manufactured to each of the virus strains previously described and divided in two groups: strong positive (C++) and weak Positive (C+), and stored at -20°C.

2.6.2. Negative control sera

Pool of bovine sera from FMD and Vesicular Stomatitis Virus (VSV)-free areas.

2.7. Liquid Phase Blocking ELISA

The assay was followed strictly in accordance to the established protocol distributed by the Joint FAO/IAEA.

2.8. Software

The plates were read in a Multiskan spectrophotometer (MCC 340) and optical density values were interpreted by software supplied by the Joint FAO/IAEA (ELISA Data Interchange, EDI 2.1.1).

3. RESULTS

The antigen control (Ca) and Percentage of Inhibition (PI) values for the upper and lower control limits for serotype "O" and "A" Antigen were redefined during the final Research Coordination Meeting in Vienna in April, 1997. These values are shown in Table I.

TABLE I. UPPER CONTROL LIMITS (UCL) AND LOWER CONTROL LIMITS (LCL) FOR ANTIGEN CONTROL (Ca) AND STRONG POSITIVE (C++), WEAK POSITIVE (C+) AND NEGATIVE (C-) CONTROL SERA FOR SEROTYPE O AND A ANTIGENS

Parameter			Value/Virus	
			O	A
LCL	OD	Ca	0.96	0.97
UCL	OD	Ca	1.76	1.73
LCL	PI	C++	86	75
UCL	PI	C++	100	99
LCL	PI	C+	63	59
UCL	PI	C+	81	75
LCL	PI	C-	-06	09
UCL	PI	C-	38	40
LCL	PI	Ca	-41	-39
UCL	PI	Ca	25	23

Ca Antigen Control
OD Optical Density
PI Percent Inhibition

The analysis of results obtained from 38 ELISA-plates that were processed in relation to the acceptance levels is shown in Table II.

TABLE II. PERCENTAGE OF ACCEPTED ANTIGEN CONTROL (CA) AND PI CONTROL VALUES (SEROPTYPE O AND A)

Antigen control (Ca) (%)		Accepted PI control values (%)		
Serotype		Controls	O	A
O	68	C++	89	89
A	37	C+	68	58
		C-	84	74
		Ca	100	100

The screening test of 120 sera from non-vaccinated an non-infected bovine gave a specificity of 99,2%. The same percentage was obtained for the positive sera to "O" and "A" serotypes of FMD virus, coming from vaccinated bovines.

One hundred and twenty positive sera from vaccinated cattle were titrated to both "O" and "A" serotypes using five fold dilution series (1/10-1/1250). 95 (79%) out of 120 cattle have antibody titers ≥ 1/112 against serotype O and 91 (76%) out of 120 cattle have antibody titers ≥ 1/112 against serotype A. It is concluded that 79% of the cattle are protected against an infection with serotype O and 76% are protected against serotype A (Table III).

TABLE III. ANTIBODY TITER OF 120 VACCINATED CATTLE IN THE TITRATION ASSAY

	1/112	1/250	1/560	1/1250	>1/1250
Serotype O	59	8	26	1	1
Serotype A	66	8	14	1	2

4. CONCLUSIONS AND DISCUSSION

Results shown in Table II demonstrate that the first level of acceptance based on the average of the control value of the antigen of both serotypes needs to be adjusted in order to reach a significant percentage of "accepted" plates. Since the antigen is dissolved in glycerin the pipetting techniques may also play a significant role for the consistency of the Ca values. Our study revealed that 68% of the plates were accepted for serotype O and 37% for serotype A at the first level of acceptance (antigen control).

Concerning the second level of acceptance e.g. values for upper and lower control sera for C++, C+, C- Ca, Percentage of Inhibition, PI our results showed a high percentage of "within limits" status (> 80 %). Only the values for the weak positive control (C+) need adjustment. In this category only 68% were "within limits" for serotype O and 58% for serotype A respectively.

A certain variation between serotypes O and A was observed. Another source of variation was observed when different microplates were used. The EDI program rejects a plate when the value of a control serum is a border value. EDI should be modified in the sense that border values still are regarded as "within limits". Another solution is to take this particularity into account when control values are established.

In the group of non-vaccinated and non-infected animals one animal out of 120 was classified as positive. We therefore conclude that the screening assay at a standard dilution of 1/32 (cut-off value) gave a specificity of 99,2%.

The titration assay is an excellent tool to determine serotype specific antibody levels in cattle and to assess their immune status. Out of 120 vaccinated cattle, 95 were classified as positive (titer ≥ 1/112) giving a sensitivity of 79% for serotype O. Out of the same group 91 were classified positive(≥ 1/112 for serotype A giving a sensitivity of 76%.

Taking into consideration the adjustments and recommendations made in this paper we conclude that the liquid phase blocking ELISA showed good specificity and sensitivity. Performance, standardization and interpretation of results are easy and the fact that only inactivated virus material is involved makes the test suitable to support an FMD eradication campaign e.g. in FMD free areas. In these aspects the test clearly exceeds other more cumbersome serological tests e.g. the serum protection test or virus neutralization test which at present are still in use. Nevertheless, a new ELISA, which detects antibodies to non-structural FMD virus protein and which differentiates FMD-infected from vaccinated cattle is needed to round up the diagnostic spectrum to effectively support an FMD eradication campaign.

ACKNOWLEDGEMENTS

We thank the Joint FAO/IAEA Division and the Panamerican Foot-and-Mouth Disease Center for (PANAFTOSA) for their support and for giving us the opportunity to carry out this project in Venezuela. We also thank Dr. Sara Papo (IIV- FONAIAP) for her valuable assistance in translating this paper into English.

REFERENCES

[1] HAMBLIN, C., BARNETT, I.T.R. AND CROWTHER, J.R., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus II, Application, J. Immunol. Methods **93** (1986) 123-129.

[2] HAMBLIN, C., BARNETT, I.T.R. AND HEDGET, R.S., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus I. Development and Method of ELISA, J. Immunol. Methods **93** (1986) 115-121.

[3] CROWTHER, J.R., Foot-and-Mouth Disease, Methods of Enzymatic Analysis, Third Edition, Vol. X. Antigens and Antibodies, VCH Verlagsgesellschaft mbH Germany (1986) 433-447.

[4] FMD ELISA KIT, Liquid phase blocking enzyme immunoassay for detection of antibodies to foot-and-mouth disease virus, Bench Protocol Version FMDVABLA 1.0. Joint FAO/IAEA Division, Animal Production and Health Section, February 1996.

INCORPORATION OF THE ELISA TECHNIQUE TO DETERMINE ANTIBODY LEVELS AGAINST FOOT-AND-MOUTH DISEASE

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Abstract

INCORPORATION OF THE ELISA TECHNIQUE TO DETERMINE ANTIBODY LEVELS AGAINST FOOT-AND-MOUTH DISEASE

Two groups of sera were evaluated by a liquid phase blocking ELISA (LPBE) for the detection and quantification of foot-and-mouth disease (FMD) antibodies to serotypes O, A and C to assess the sensitivity and specificity of the assay The first group consisted of 120 sera from non-infected and non-vaccinated cattle, which were tested by a screening assay at a fix dilution of 1/32 The second group consisted of 120 sera from cattle vaccinated with a trivalent (O,A and C) vaccine Sera from this group were titrated in a five fold dilution range 1/10, 1/50, 1/250 and 1/1250

1 INTRODUCTION

Foot-and-mouth disease (FMD) is one of the main causes of enormous economic loss to Latin American countries. Paraguay is now in an advanced stage of the implementation of the control and eradication of the disease through a comprehensive vaccination campaign.

Two areas of the country have never experienced FMD nor has FMD vaccine ever been used. One area, "Nueva Asuncion" is at the border with Bolivia and the other area is an isolation station named "Quyquyho". These two areas have provided animals free of FMD antibodies for use in studies to evaluate the efficacy of FMD vaccines. Adult cattle are vaccinated once and calves twice per year with a trivalent (O,A and C) oil emulsion vaccine. The vaccination programme is linked with other control measures and has been successful in preventing any FMD outbreaks since 1995. The Office International des Epizooties (OIE) has recognized these efforts by designating Paraguay the status "free of FMD with vaccination". Several assays have been used for the detection of the virus and the antigen detection ELISA has replaced the complement fixation assay [1,2] Currently the virus neutralization test (VNT) is employed for serological investigation.

The aim of this project was to evaluate a liquid phase blocking ELISA (LPBE) for the detection and quantification of FMD antibodies [3,4].

2. MATERIALS AND METHODS

Materials and equipment were supplied by the Joint FAO/IAEA Division, PANAFTOSA and Servicio Nacional de Salud Animal:

2.1. Joint FAO/IAEA Division

ELISA chemicals, consumables, manual and software programme for data management:

Chemicals (Carb. Bicarb. tablets, PBS tablets, Tween 20, Phoshp/Citr. Tablets, H_2O_2 tablets, OPD tablets, skimmed milk powder, ovalbumen grade II, V, conjugate and normal rabbit serum), consumables (tips, NUNC and polypropyelene plates) minor laboratory equipment, the ELISA software programme EDI and the protocol.

Equipment·

Photometer Flow Laboratories, Titertek Multiskan Plus MkII Microplate Reader with an interference filter of 492 nm, orbital shaker Flow Laboratories, Titertek Microplate Shaker, Washer Flow Laboratories, Titertek Handiwash Microplate Washer, Finnpipette digital multichannel pipettes

variable ranges from 5-50µl and 50-300µl, quality tips Finnpipette single channel variable ranges from 5-50µl, 50-200µl and 200-1000µl, reagent troughs suitable for simultaneous multichannel pipetting of a single reagent, microplates, Nunc Immuno Maxisorp flat bottom 96 well microplates, U-bottom 96 well microplates, pH meter and pH strips, Vortex mixer.

2.2. PANAFTOSA

Biologicals:
Trapping antibody rabbit anti-FMDV serotypes O, A and C, control antigens FMDV serotypes O, A and C in aliquots in 50% glycerol, control sera anti-FMDV serotypes O, A and C (C++ antibody = strong positive, C+ antibody = moderate positive, C- antibody = negative) for each serotype, detecting antibody guinea pig anti-FMDV serotypes O, A and C, anti-species conjugate (rabbit horseradish peroxidase-conjugate), anti-guinea pig immunoglobulin.

2.3. Servicio Nacional de Salud Animal

Water purification system, refrigerator range of +2°C to +6°C, freezer -20°C, incubator +35°C-+39°C, water bath +35°C - +39°C, balance accuracy of +/- 0.1 gr., timer, glassware/plastic ware, beakers 20-4000 ml, flasks 50-1000 ml, graduated cylinders 10-2000 ml, graduated pipettes 1-20ml, safety bulbs, storage bottles with closures 1-1000 ml dilution tubes 2-5 ml, suitable racks, cryopreservation vials polypropylene with screw cap 1-5 ml.

2.4. Screening assay

The solid phase was prepared using trapping antibodies from rabbit antisera O_1 Campos 1/2000, A_{24} Cruzeiro1/1500 and C_3 Indaial 1/2000, adsorbed to Nunc Immunoplate Maxisorp 100µl /well [5]. The plates were incubated at 4°C for 18 hours. Then they were washed with PBS + tween 20 0,05% with three cycles of washing. Then the plates were blocked with ovalbumen grade V, 1%, 100µl each well and incubated at room temperature. The plates were washed three times and kept at 20°C until use. Control and sample sera were diluted at 1/16 resulting in 40 test sera for every serotype/plate. Then O, A and C antigen was added using dilutions 1/16, 1/30 and 1/60.The plates were incubated at 37°C shaking in an orbital shaker for one hour. Then, the plates were washed in three cycles and the respective antibodies were added 50µl each well per detecting antibody, O_1Campos 1/300, A_{24} Cruzeiro 1/200 and C_3 Indaial 1/600 and incubated at 37°C with shaking for 30 minutes. Plates were washed and horseradish peroxidase conjugate was added at 1/100 and incubated at 37°C with shaking for 30 minutes, 50µl each well. Plates were washed and the substrate/chromogen (OPD+H_2O_2) were added 50µl, serum samples and a blanking plate a column. The optical density values were read in a plate reader (Multiskan Plus II) at 492 nm wavelength.

2.5. Titration Assay

Solid phase the same as screening assay. U-bottom, 96 well microplates, polypropylene plate: Control sera (C++,C+ and C-) were diluted at 1/16 (60µl in every 4 well). Serum samples were diluted at five fold dilution range 1/5,1/25, 1/125, and 1/625 respectively each well 60µl, 10 sera for every plate. After this step, the respective antigen diluted (O 1/8, A 1/15, C 1/30) was added 60µ l/well. Plates were incubated at 37°C with shaking for one hour. Then, 50µl from each well was transferred to a previously washed solid phase and incubated at 37°C, 30 minutes with shaking. Plates washed were added with detecting antibody, O_1 Campos 1/300 , A_{24} Cruzeiro 1/200, C_3 Indaial 1/600 and incubated 37°C, 30 minutes. Plates were washed and conjugate at dilution 1/100 was added. The plates were incubated 30 minutes at 37°C with shaking. The plates were washed three times with wash buffer, and the substrate /chromogen OPD was added 50µl in all the plate (every plate) plus a clean plate used as "blanking plate" was added with OPD 50µl in a column and all the plates were incubated for 15 minutes at a room temperature, in a dark place. The reaction was stopped by the addition of a stop solution, sulfuric acid 50µl each well in the plates tested and the blanking plate. The reaction(optical density values) was read in a spectrophotometer at 492 nm wavelength.

3. RESULTS

TABLE I. FIRST LEVEL OF MICROPLATE ACCEPTANCE ANTIGEN CONTROL (Ca)*

Virus strain	N° of plates tested	Within control limits (no)	Within control limits (%)	Outside control limits (no)	Outside control limits (%)
O_1 Campos	15	2	13	13	87
A_{24} Cruz	15	9	60	6	40
C_3 Ind	15	3	20	12	80
Total	45	14	31	31	69

* Optical density(OD)values

Table I shows that for the virus O, of 15 plates tested, 2 (13%) plates were within control limits (Antigen Control Ca). For the virus A, of 15 plates tested, 9 (60%) plates were within control limits(Ca). For the virus C, of 15 plates rested, 3(20%) plates were within control limits(Ca).

TABLE II. SECOND LEVEL OF MICROPLATE ACCEPTANCE CONTROL SERA (STRONG POSITIVE (C++), MODERATE POSITIVE (C+), NEGATIVE (C-) AND ANTIGEN CONTROL (Ca)

Virus strain	No. of plates tested	C ++	C +	C -	Ca
O_1 Campos	15	5/15	3/15	14/15	15/15
A_{24} Cruz	15	0/15	3/15	13/15	15/15
C_3 Ind	15	3/15	2/15	9/15	15/15

Table II shows that for serotype O, of 15 plates tested, 5 plates were within control limits for strong positive (C++), 3 plates for moderate positive(C+). For serotype A, no plate was within control limits for strong positive(C++), 3 plates were within control limits for moderate positive (C+). For serotype C, only 3 plates were within control limits for strong positive (C++) and 2 plates for the moderate positive (C+).

For the second level of microplate acceptance results from the second run were less acceptable than from the first run.

TABLE III. SCREENING METHOD (SERUM SAMPLES OF NON-VACCINATED ANIMALS)

Virus strain	No. of tested sera	No. of negative	No. of positive	No. of sera to be retested	% of negative sera
O_1 Campos	120	119	1	0	99
A_{24} Cruz	120	73	17	30	61
C_3 Ind	120	120	0	0	100

Table III shows that for serotype O of 120 negative samples, 119 (99%) samples were negative. For serotype A, 73(30%) samples were negative, 17 were positive and 30 needed to be retested. For serotype C, 120 samples were negative, as expected.

TABLE IV. TITRATION METHOD (VACCINATED ANIMALS)

Virus strain	No. of tested sera	No. of positive	No. of negative	No. of sera to be retested	% of negative sera
O_1 Campos	120	84	26	10	70
A_{24} Cruz	120	26	49	45	22
C_3 Ind	120	79	23	18	66

Table IV shows that for serotype O out of 120 positive samples 84 (70%) samples were positive, 26 samples were negative, and 10 samples had to be retested. For serotype A out of 120 positive samples, 26 (22 %) samples were positive, 49 samples were negative, and 45 samples had to be retested. For serotype C, 79 (66%) samples were positive, 23 samples were negative, and 18 had to be retested.

CONCLUSIONS AND DISCUSSION

The method, which was performed was the same as in the first phase, only the peroxidase/conjugate was diluted 1/100 according to the batch for the second phase. Upper and lower control limits the strong positive (C++) were increased for three strains of virus (O_1 Campos, A_{24} Cruzeiro and C_3 Indaial). The results obtained in this work show that for serotype A_{24} Cruzeiro, the values did not fall, as we expected, within the control limits for the strong positive (C++). A high percentage of the results were lower than in the first phase and it is concluded that further work might be necessary to overcome these difficulties.

REFERENCES

[1] ABU ELZEIN, E.M.E., CROWTHER, J.R., The specific detection of foot-and-mouth disease virus whole particle antigen (140 S) by enzyme labelled immunosorbent assay, J. Hyg. **83** (1979) 127-134.

[2] HAMBLIN, C., et al., A rapid enzyme linked immunosorbent assay for the detection of foot-and-mouth disease virus in epithelial tissues, Vet. Microbiol. **9** (1984) 435-443.

[3] CROWTHER, J.R., ABU ELZEIN, E.M.E., Detection and quantification of foot-and-mouth disease virus by enzyme labelled immunosorbent assay techniques, J. gen. Virol. **42** (1979) 597-602.

[4] FERRIS, N.P., DAWSON, M., Routine application of enzyme linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases, Vet. Microbiol. **16** (1988) 201-209.

[5] GOMES, M.P.D., Application of enzyme linked immunosorbent assay for the diagnosis of foot-and-mouth disease virus and vesicular stomatitis in comparison with the complement fixation, Bol. Centr. Panam. Fiebre Aftosa **55** (1989) 21-25.

THE USE OF A LIQUID PHASE BLOCKING ELISA KIT FOR DETECTION OF ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS IN COLOMBIA

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Abstract

THE USE OF A LIQUID PHASE BLOCKING ELISA KIT FOR DETECTION OF ANTIBODIES AGAINST FOOT-AND MOUTH DISEASE VIRUS IN COLOMBIA

The objective of this study was to undertake an interlaboratory comparison of a liquid phase blocking ELISA for detection of antibodies to FMD virus For that purpose sera from 120 vaccinated, 120 infected and 120 FMD negative cattle were tested All sera were tested in a screening assay at a dilution of 1/32 Positive sera were tested in a titration assay (1/10, 1/50, 1/250, 1/1250) For serotype O_1 Cruzeiro 108 sera from the FMD-free group were classified as negatives giving a specificity of 90% For the same serotype the group of infected/ vaccinated cattle gave 114/115 positive results showing a sensitivity of 95% respectively 96% For serotype A_{24} Cruzeiro from the FMD-free group 85 sera were classified as negatives giving a specificity 71% For the same serotype the group of infected/ vaccinated cattle gave 90/99 positive results showing a sensitivity of 75% respectively 82% The predictive value of the assay was good as results expected for the different serum categories were mainly confirmed in the test Nevertheless a high number of plates were rejected due to "outside limits" and further adjustments are necessary to obtain more reliable results

1 INTRODCUTION

In some regions of Colombia foot-and-mouth disease virus serotypes O_1 Campos and A_{24} Cruzeiro exist endemically At present the country is involved in the hemispheric foot-and-mouth disease eradication plan. To achieve this objective it is necessary to use techniques with a higher sensitivity and specificity than the traditional diagnostic serological tests [1,2,3,4,5] The use of a liquid phase blocking ELISA, LPBE is of great benefit in areas, where FMD prevention, control and eradication programs are carried out. The LPBE provides more reliable results because it is very sensitive and specific. Other advantages are the fast delivery of results - usually within the same day - and the fact that the technique is easy to perform and does not require special laboratory conditions e.g cell culture or CO_2 environment

2 MATERIAL AND METHODS

The assay is based on specific blocking of a defined amount of FMDV antigen by antibodies in the test sample during the liquid phase [6,7]. After the test serum is allowed to react with specific FMDV antigen, the test serum/antigen mixture is transferred to an ELISA plate coated with FMDV serotype specific trapping antibodies The presence of antibodies to FMDV in the serum sample will result in the formation of immune complexes and consequently reduce the amount of free antigen trapped by the immobilized rabbit antisera. In turn, less amount of guinea pig anti-FMDV detecting antibodies will react in the next incubation step. After the addition of enzyme labeled (horseradish peroxidase, HRP) anti-guinea pig immunoglobulin and substrate/chromogen solution a reduction of color development will be observed when compared to control containing free antigens only The bench protocol of the Joint FAO/IAEA Division was followed [8].

A total of 360 sera from cattle were tested from 3 different categories as shown below·

- 120 bovine sera from free areas of FMD (provided by CPFA)
- 120 bovine sera from vaccinated cattle with trivalent vaccine (provided by CPFA)
- 120 bovine sera from FMD infected animals obtained from outbreaks which naturally occurred in different regions of Colombia.

3. RESULTS

For serotype O_1 Cruzeiro 108 sera from the FMD-free group were classified as negatives giving a specificity of 90%. For the same serotype the group of infected/ vaccinated cattle gave 114/115 positive results showing a sensitivity of 95% respectively 96%. For serotype A_{24} Cruzeiro from the FMD-free group 85 sera were classified as negatives giving a specificity 71%. For the same serotype the group of infected/ vaccinated cattle gave 90/99 positive results showing a sensitivity of 75% respectively 82% (Table I).

TABLE I. RESULTS ACCORDING TO GROUP OF SERA AND SEROTYPE

Bovines	Samples	O Virus			A Virus		
		P	N	R	P	N	R
Free	120	5	108	7	21	85	14
Infected	120	114	4	2	90	15	15
Vaccinated	120	115	0	5	99	10	11

P = positive
N = negative
R = retest

A high number of plates was classified "outside limits" because the positive serum controls were out of the upper and lower limits, although the negative serum control and the antigen control were within limits.

4. CONCLUSIONS AND DISCUSSION

Although most of the plates were rejected due to "outside limits" the predictive value of the assay remained good as results expected for the different serum categories were confirmed in the test as shown in Table I.

Further adjustment for the upper and lower control limits is necessary to obtain reliable results. It could be observed that the problem was more noticeable for the disease free sera, where a small percentage of the samples were positives. In the case of sera from infected animals, the highest percentage was positive. A similar result was observed in the group of sera from vaccinated animals.

In the group of sera from infected animals positive results were obtained for both serotypes. The reason for this is that these animals live in FMD endemic areas where additionally vaccination is carried out.

Once having standardized this technique it will be used as a routine test all over the country to monitor the success of the vaccination programme, which is being applied systematically every six months.

ACKNOWLEDGEMENTS

We like to thank PANAFTOSA and the Joint FAO/IAEA Division for their support to carry out this project.

REFERENCES

[1] CROWTHER, J.R., Foot-and-Mouth Disease, Methods of Enzymatic Analysis, Third Edition, Vol. X. Antigens and Antibodies, VCH Verlagsgesellschaft mbH Germany (1986) 433-447.

[2] CROWTHER, J.R., ABU ELZEIN, E.M.E., Detection and quantification of foot-and-mouth disease virus by enzyme labelled immunosorbent assay techniques, J. gen. Virol. **42** (1979) 597-602.

[3] FERRIS, N.P., DAWSON, M., Routine application of enzyme linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases, Vet. Microbiol. **16** (1988) 201-209.

[4] ALONSO, A., MARTINS, M.A., GOMES, M.P.D., ALLENDE, R., SONDAHL, M.S., Foot-and-mouth disease virus typing by complement fixation and ELISA tests using monovalent and polivalent antisera, J. Vet. Diagn. Invest. **4** (3) (1992) 247-253.

[5] GOMES, M.P.D., SONDAHL, M.S., MARTINS, M.A., OLASCOAGA, R.C., ALONSO, A.F., Application of enzyme linked immunosorbent assay for the diagnosis of foot-and-mouth disease virus and vesicular stomatitis in comparison with the complement fixation, Bol. Centr. Panam. [1]Fiebre Aftosa **55** (1989) 21-25.

[6] HAMBLIN, C., BARNETT, I.T.R., HEDGER, R.S., A new enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, I. Development and method of ELISA, J. Immunol. Methods **93** (1986) 115-121.

[7] HAMBLIN, C., BARNETT, I.T.R., CROWTHER, J.R., A new enzyme linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, II. Application. J. Immunol. Meth. **93** (1986) 123 - 129.

[8] FMD ELISA KIT, Liquid phase blocking enzyme immunoassay for detection of antibodies to foot-and-mouth disease virus, Bench Protocol Version FMPVABLA 1.0. Joint FAO/IAEA Division, Animal Production and Health Section, February 1996.

STUDY OF FAO/IAEA/PANAFTOSA ELISA KIT FOR THE DETECTION OF ANTIBODIES AGAINST FMD

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Abstract

STUDY OF FAO/IAEA/PANAFTOSA ELISA KIT FOR THE DETECTION OF ANTIBODY AGAINST FMD

Two groups of sera were used to evaluate a liquid phase blocking ELISA (LPBE) for the detection of antibody against foot-and-mouth disease virus One hundred and twenty sera, from animals with no previous history of FMD infection or vaccination, were analyzed by screening assay at a final dilution of 1 32 A second group of 120 sera, from animals vaccinated with an oil trivalent vaccine (O, A,C) were tested by titration in the LPBE All the sera were tested against virus of three FMD serotypes, using O_1 Campos, A_{24} Cruzeiro, C_3 Indaial virus strains

1 INTRODUCTION

The production of beef and dairy cattle depends greatly upon the health status of the animal Foot-and- mouth disease (FMD) is the most important disease of livestock [1] in Brazil. The disease is seldom fatal in adults (e.g 5% mortality rate) Once the animal has recovered it reaches the productivity status of milk and beef yield as an uninfected animal Myocarditis is often a consequence of FMD in young animals and mortality rates of 50% are common in this group.

Until 1994, the virus neutralization test (VNT) [2] and the complement fixation test (CFT) [3,4] were used in our laboratory for FMD diagnosis. Disadvantages of the CFT are a lack of sensitivity and its complexity of performance together with the need to use live FMD virus, which presents a disease security hazard. This last point is also a disadvantage of the VNT together with the need to use tissue culture cells and the concurrent disadvantages that their use entails in terms of variable cell growth and sensitivity, cell contamination and protracted time for test completion.

An eradication programme has been implemented to control FMD in the country The milestone of FMD control in Brazil has been, and remains, FMD vaccination together with official control of livestock movement. As a consequence of this programme, there are now areas in the south of the country, without FMD outbreaks for four years. These areas are anticipated to gain recognition by the Office International des Epizooties (OIE) as "FMD-free area with vaccination"

Continuation of the vaccination programme entails that the quality of each batch of vaccine must be stringently assessed. Groups of cattle are immunized with vaccine. Formerly these vaccinated animals were then challenged with live virus at 28 days post-vaccination to determine the efficacy of the vaccine. However in the field of FMD serology, the liquid phase blocking ELISA (LPBE) [5,6] has been increasingly employed in many FMD laboratories around the world. This assay is now being used in our vaccine control tests, to evaluate the immunity of vaccinated animals and has totally replaced the need for cattle challenge by virus.

As part of an intention to extend the use of this assay in other South American countries we have further evaluated its applicability for general FMD serology through the use of an FAO/IAEA/PANAFTOSA ELISA kit for the detection of antibody against FMD.

2. MATERIALS AND METHODS

Test serum samples and biological reagents were supplied by the CPFA-OPS. Chemicals were distributed by the Joint FAO/IAEA Division.

2.1. Serum Samples

Serum from non-infected animals one hundred and twenty sera from 18-24 months old cattle from selected herds without neither history of FMD-infection nor vaccination and tested previously by CPFA to confirm freedom of FMD antibody

All sera originated from Rio Grande do Sul state, Brazil, utilized by the vaccine control laboratory (MAARA-LAR/RS)

Sera from vaccinated animals one hundred and twenty sera from 18-24 months old cattle, vaccinated with trivalent oil adjuvant vaccine and bled 30 days after vaccination

2.2. Virus strains

For this work the strains O_1 Campos-Br,1/58, A_{24} Cruzeiro-Br 1/55 and C_3 Indaial-Br 1/71 were used These viruses were obtained from BHK-21, C-13 cell culture, inactivated by binary ethylenimine (BEI), treated with sterile glycerol (50% v/v) and stored at -20C

2.3. Trapping antibodies

Hyperimmune sera to each of the serotypes were obtained by inoculation of rabbits with one of the previously mentioned virus strains, after cesium chloride gradient purification, and stored at -20 C

2.4. Detecting antibodies

Hyperimmune antisera were produced in guinea pigs against the strains previously mentioned using live virus adapted to this species and stored at -20 C

2.5. Conjugate

The conjugate (Peroxidase labelled goat immunoglobulins to guinea pig immunoglobulins) was produced by CPFA

2.6. Control sera

Positive control sera pools of sera from bovines vaccinated and revaccinated with monovalent oil adjuvant vaccines manufactured to each of the virus strains previously described were divided in two groups strong positive (C++) and weak positive (C+) and stored at -20 C

Negative control sera a pool of bovine sera from FMD and Vesicular Stomatitis Virus (VSV) free areas

2.7. Liquid Phase Blocking ELISA

The assay was followed strictly in accordance to the established protocol, published and distributed by the Joint FAO/IAEA Division, designed to produce a standard level of results

2.8. Software

The plates were read in a Multiskan spectrophotometer (MCC 340) and optical density values were interpreted by software supplied by the Joint FAO/IAEA Division (ELISA Data Interchange, EDI 2 11) for calculation of percentage inhibition values and control and plate acceptance

3 RESULTS

TABLE I NUMBER OF PLATES DONE

Serotype	Plates accepted	Plates rejected
O	0	19
A	4	15
C	4	15
Total	8	49

The 120 sera from non-infected cattle were tested against O, A and C serotype

TABLE II SCREENING TEST

Results	O	A	C
Negative	120	117	120
Positive	-	2	-
Retest	-	1	-
Total	120	120	120

One hundred and twenty sera were titrated against the three virus serotypes

TABLE III TITRATION ASSAY

Serotype	Protected	Non-protected	Retest	Total
O	-	-	-	-
A	27	11	2	40
C	33	2	5	40
Total	60	13	7	80

4 CONCLUSION AND DISCUSSION

A high percentage of plates were considered "outside limits" Eighty six percent of plates were rejected at first level of acceptance, which is based on the average of the control value of the antigen The values for the three serotypes still need further adjustment in order to get a higher percentage of plates accepted or "within limits"

The values for the C++ PI were too narrow and because of this a lot of plates were rejected or "outside limits"

The results of the screening test could not be considered conclusive because all the plates were rejected at first level of acceptance Since all plates were "outside limits" we think the negative results on O_1 Campos serotype are not reliable as well as to the other serotypes (A_{24} Cruzeiro, C_3 Indaial)

Table III shows the results from the titration assay, where exclusively results of accepted plates were considered Out of 120 sera 40 sera were analyzed to the A_{24} Cruzeiro and to C_3 Indaial serotypes For the O_1 Campos serotype it was impossible to obtain results Of those sera, 67,5 % were identified as protected (>1/112) against serotype A_{24} Cruzeiro and 82,5% were identified as protected (≥ 1/112) against serotype C_3 Indaial

The above mentioned problems indicate that the assay needs further adjustment and recalculation of the upper and lower contrl values of all controls Ca, C++, C+, C-, in order to get plates

accepted. The software program EDI should be modified in a way that border values are accepted.

Once these limitations are solved, the LPBE proved to be an easy to perform, rapid and safe assay. Using inactivated virus this assay is a very useful tool for an FMD eradication programme. We think that final, conclusive results about specificity and sensitivity of the assay can be obtained once the described limitations have been overcome.

ACKOWLEDGEMENTS

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REFERENCES

[1] BACHARACH, H.L., Fiebre Aftosa, Boletim do Centro Panamericano de Febre Aftosa **7** (1972) 1-33.

[2] MACKOWIAK, C. & LANG, R. Emploi des cultures de tissue dans le titrage du virus aphteux et la recherche des anticorps, Bull.Off.int.Epizoot. **49** (1958) 99-106.

[3] MARUCCI, A.A., Amer. J. Vet. Res., **18** (1957) 785-791.

[4] MARUCCI, A.A., Amer. J. Vet. Res.**19** (1958) 979-984.

[5] HAMBLIN, C. et al., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, I. Development and method of ELISA, J Immunol Methods, **93** (1986) 115-121.

[6] HAMBLIN, C. et al., A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus, II. Application, J Immunol Methods **93** (1986) 123-129.

COUNTRY REPORTS

Part C: BABESIOSIS

Babesiosis antibody ELISA

SERO-EPIDEMIOLOGICAL SURVEY ON BOVINE TICK-BORNE DISEASES IN THE LESSER ANTILLES

XA9848670

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Abstract

SERO-EPIDEMIOLOGICAL SURVEY ON BOVINE TICK-BORNE DISEASES IN THE LESSER ANTILLES

As part of a tick-borne disease control programme in the Lesser Antilles, studies were undertaken to determine the prevalence of cowdriosis, babesiosis and anaplasmosis in an effort to determine what the impact of tick eradication would be The epidemiological situation for bovine babesiosis and anaplasmosis is unstable in all the islands of the Lesser Antilles, but the clinical cases are only recorded in imported breeds, which represent less than 5% of the cattle population The native cattle population react as if naturally resistant When the *A variegatum* tick eradication campaign begins, it will be necessary, by the end of the acaricide treatment regime, to immunize all the imported cattle born during that period, and possibly all of the seronegative imported cattle already on the islands Both Antigua and Guadeloupe have a long history of infestation with the tick and both have experienced clinical cases of cowdriosis On the other islands, less than 6% of the sera were positive and this correlates well also with an apparent absence of clinical cases of cowdriosis

1 INTRODUCTION

The zoo-sanitary situation of livestock in the Caribbean region is poorly known As part of a joint project funded by the French Government (FIC), a regional survey began in 1992, mainly for determining the prevalence of ticks and tick-borne diseases.

Two tick species, *Amblyomma variegatum* and *Boophilus microplus*, are involved in the transmission of cowdriosis (heartwater) for the first one, of babesiosis and probably also anaplasmosis for the second one.

This study focuses on the presence of cowdriosis in the Lesser Antilles and on the epidemiological situation of babesiosis (*Babesia bovis & B. bigemina*) and anaplasmosis (*Anaplasma marginale*). The beginning of an eradication campaign against *A variegatum* this year, requires particular attention with regard to the impact on the other tick species *B microplus* and on the incidence of transmitted diseases.

2 MATERIAL AND METHODS

2.1. Sampling strategy

A cluster sampling was applied. Each island was divided into districts or municipalities; in each district/municipality, one per cent of the herds were randomly selected and all the animals sampled in each herd For each herd a file was completed, including data on the farming and the tick control For each sampled animal, the breed, sex, age and presence of ticks were noted

2.2. Serological tests

For cowdriosis an indirect ELISA test was used [1]. For *Babesia bovis* an indirect ELISA test was provided by the Animal Production and Health Section of the Joint FAO/IAEA Division, Vienna, Austria. For *B bigemina* and *Anaplasma marginale* a DOT-ELISA test was used [2].

3. RESULTS

4.

A total of 1795 sera were collected from 547 herds randomly distributed on the 11 islands. The serological results are presented in the Table I. In 8 islands, the age of the sampled animals was obtained. Five hundred and seventy six 12 month old calves were examined.

For the 3 islands of St-Kitts & Nevis, Barbados and Grenada, the age of the animals was not determined. To calculate the inoculation rates *h*, it was assumed that the mean age was the same as the mean for other 8 islands. The inoculation rates are presented in Figure 1 for each island and each disease, and the risk areas determined according to Mahoney, 1972 [3].

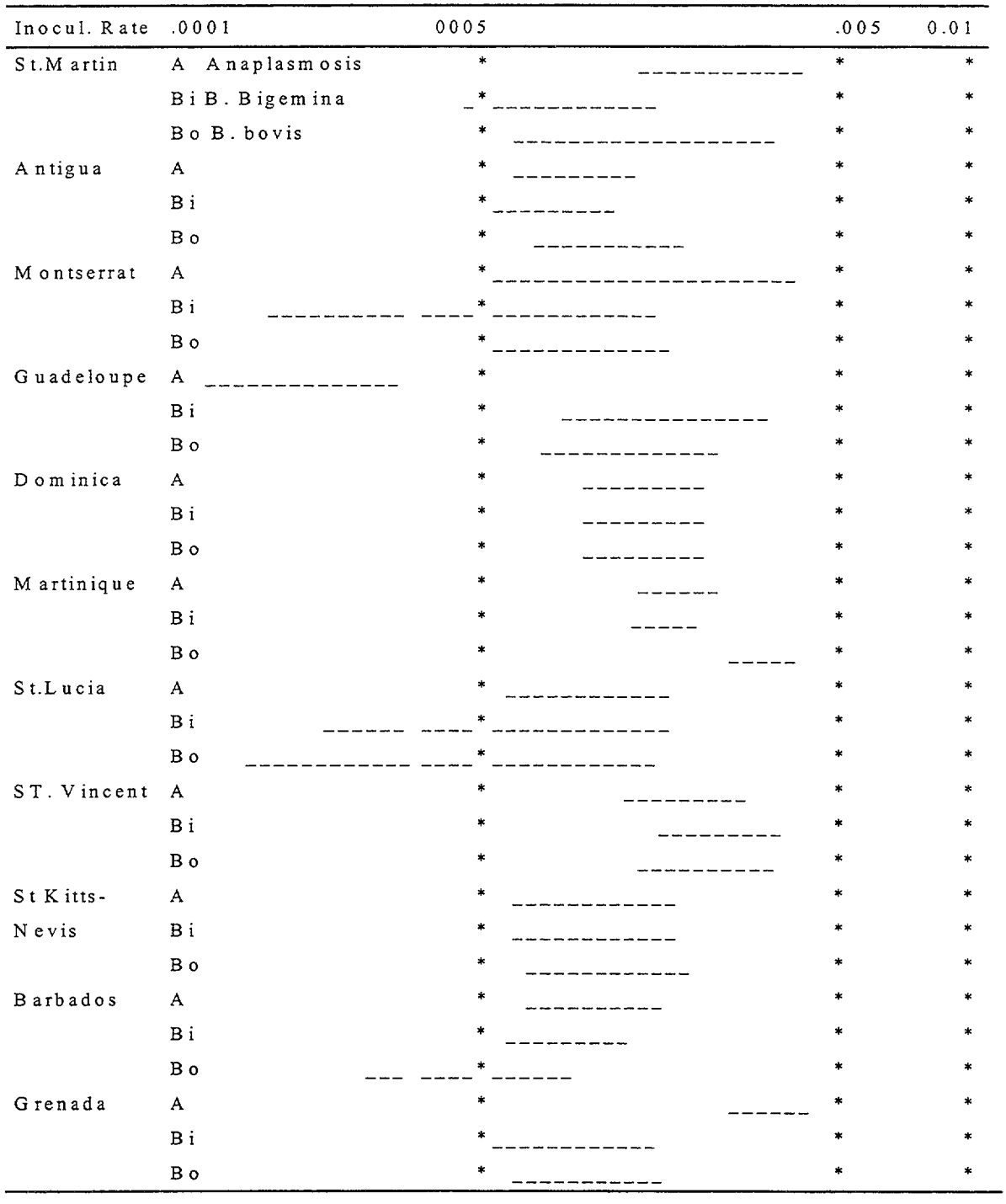

FIG. 1. Inoculation rate for anaplasmosis and babesiosis and critical level for enzootic stability (inoculation rate > 0.005)/instability (low risk for inoculation rate (<0.0005, maximum risk for inoculation rate between 0.0005 and 0.005).

TABLE I. SEROPREVALENCES & INOCULATION RATES OF TICK-BORNE DISEASES IN THE LESSER ANTILLES

Island	ANA		BBI		BBO		CW
	%	H	%	H	%	H	%
St Martin	68	0.0034	32	0.0011	44	0.0017	0
Antigua	29	0.0012	22	0.0009	36	0.0016	13
Montserrat	45	0.0017	18	0.0006	27	0.0009	0
Guadeloupe	1	0.0004	58	0.0034	38	0.0019	19
Dominica	47	0.0023	46	0.0022	47	0.0023	3
Martinique	52	0.0027	47	0.0023	69	0.0042	3
St. Lucia	36	0.0014	28	0.0010	24	0.0010	3
St Vincent	51	0.0023	58	0.0028	55	0.0026	2
St Kitts-Nevis	34	0.0014	34	0.0014	38	0.0016	0
Barbados	45	0.0020	30	0.0012	22	0.0008	6
Grenada	71	0.0041	25	0.0010	33	0.0013	2

ANA=Anaplasmosis, BBI=Babesia bigemina, BBO=B. bovis, CW=Cowdriosis, H=Inoculation rate, %=Seroprevalence

4. DISCUSSION

4.1. Discussion on anaplasmosis and babesiosis

Except for anaplasmosis in Guadeloupe (probably absent), the seroprevalence of the 3 tick-borne diseases for 6-12 month old calves ranged from 18% to 71% on the different islands. Even when the inoculation rates were calculated from the confidence intervals of the seroprevalence rates, the great majority of *h* values fell within the area of instability with maximum risk. No value reached the typical value of enzootic stability. In isolated cases, disease existed in an unstable situation with a low risk of clinical cases e.g. *B. bigemina* in Montserrat and St Lucia and *B. bovis* in St Lucia.

The unstable situation with maximum risk would be manifested by numerous outbreaks of anaplasmosis and babesiosis. In Guadeloupe (E. Camus, personal communication) between 1988 and 1993, 19 clinical cases of *B. bovis* and 1 clinical case of *B. bigemina* were diagnosed in Holstein, Limousin and Charolais but not a single case in Creole Zebu cattle which represents 95 % of the cattle population. In Martinique, between 1983 and 1992, 9 cases of *B. bovis* in Holstein and 4 cases of anaplasmosis in Holstein and Brahman were reported [4]. In Nevis not a single case of babesiosis during a 4 year observation period was noted [5]. In St. Lucia an outbreak of anaplasmosis and babesiosis was observed in imported Holstein but not in native cattle [6].

Only rare cases were recorded in imported animals and mainly in dairy cattle. In Guadeloupe, the local Creole cattle are naturally resistant to cowdriosis, babesiosis and anaplasmosis [7]. In Martinique, there is a majority of Brahman Zebu, and the *Bos indicus* are known to be more resistant to babesiosis.

Is there a possibility that "the Australian model" is not applicable to other countries? The model was used in Brazil and Uruguay for babesiosis, and in Australia and Indonesia for anaplasmosis [8,9]. What would be the impact of a 3 year *A. variegatum* eradication campaign on *B. microplus* and the transmitted diseases? Considering the 20 year eradication campaign of *B. microplus* in Puerto Rico when the foci of *A. variegatum* were rapidly controlled, the eradication campaign will probably reduce the population of *B. microplus* and the seroprevalence rates of babesiosis and anaplasmosis, but is unlikely to eradicate *B. microplus* [10]. In Martinique, a strong *A. variegatum* tick control program resulted in a decrease of the seroprevalence rates from 71% to 50 % for anaplasmosis, from 83% to 60 % for *B. bovis* and 59% to 44 % for *B. bigemina*[11]. In the Lesser Antilles, the eradication campaign will reduce the percentage of immune animals and, after the 3 year acaricide treatment, the non-immune animals will

face contacts with a growing number of infected *B. microplus*. This phenomenon will be particularly critical for imported cattle.

The epidemiological situation for bovine babesiosis and anaplasmosis is unstable in all the islands of the Lesser Antilles, but the clinical cases are only recorded in imported breeds, which represent less than 5% of the cattle population. The native cattle population react as if naturally resistant. When the *A. variegatum* tick eradication campaign begins, it will be necessary, by the end of the acaricide treatment regime, to immunize all the imported cattle borne during that period, and possibly all of the seronegative imported cattle already on the islands.

4.2. Discussion on cowdriosis

Antigua and Guadeloupe were found to have a high percentage of seropositive cattle; 13% and 19% respectively. Both islands have a long history of infestation with the tick and both have experienced clinical cases of cowdriosis [12]. On the other islands, less than 6% of the sera were positive and this correlates well also with an apparent absence of clinical cases of cowdriosis. The 6% observed on Barbados probably indicates the limit of specificity of the ELISA test. Unfortunately, when sheep are considered, higher seroprevalences are observed on Martinique (15%) and Montserrat (11%) where cowdriosis is certainly absent [13]. Antibodies to *Cowdria ruminantium* are known to cross-react with *Ehrlichia ovina* and this *Ehrlichia* (and other species) are actively investigated on both islands of Martinique and Montserrat [14].

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REFERENCES

[1] MARTINEZ, D., COISNE, S., SHEIKBOUDOU, C., JONGEJAN, F., Detection of antibodies to *Cowdria ruminantium* in the serum of domestic ruminants by indirect ELISA, Revue, Elev. Méd. Vét. Pays. Trop. **46** (1993) 115-120.

[2] MONTENEGRO-JAMES, S., GUILLEN, T., TORO, M., Dot Elisa para el diagnostico serologico de la anaplasmosis y babesiosis bovina, Rev. Cientifica, FCV de Luz II (1992) 23-29.

[3] MAHONEY, D.F., ROSS, D.R., Epizootiological factors in the control of bovine babesiosis, Aust. Vet. J. **48** (1972) 292-298.

[4] ALONSO, M., CAMUS, E., RODRIGUEZ DIEGO, J., BERTAUDIERE, L., TATAREAU, J.C., LIABEUF, J.M., Current status of bovine haemoparasitic diseases in Martinique, Rev. Med. Vét. Pays. Trop. **45** (1992) 9-14.

[5] HADRILL, D.J., BOID, R., JONES, T.W., BELL-SAKUY, L., Bovine babesiosis on Nevis Implications for tick control, Vet. Rec. **126** (1990) 403-404.

[6] KNOWLES, R.T., MONTROSE, M., CRAIG, T.M., WAGNER, G.G., LONG, R.F., Clinical and serological evidence of bovine babesiosis and anaplasmosis in St Lucia, Vet. Parasitol., **10** (1982) 307-311.

[7] CAMUS, E., Etude épidémiologique de la cowdriose à *Cowdria ruminantium* en Guadeloupe, Etudes et synthèses de l'Institut d'Elevage et de Médecine Vétérinaire des Pays Tropicaux, Maisons-Alfort, (1989) 152 p.

[8] SMITH, R.D., MADRUGA, C.R., KESSLER, R.H. et al., Field validation of computer simulation model of bovine babesiosis, In: Proc. 2nd Symp. Computer Applc. Vet. Med. Am. Vet. Comparative Soc. College Vet. Med., Mississipi State Univ. (1984) 341-345.

[9] WILSON, A.J., RONOHARDJO, P., Some factors affecting the control of bovine anaplasmosis with special reference to Australia and Indonesia, Prev. Vet. Med. **2** (1984) 121-134.

[10] CROM, R.L, Heartwater in Guadeloupe and in the Lesser Caribbean, Ann NY Acad Sci. (1992) 653-655.

[11] CAMUS, E., BARRÉ, N., Vector situation of tick-borne diseases in the Caribbean, Vet. Parasitol. **57** (1995) 167-176.

[12] PERREAU, P., MOREL, P.C., BARRÉ, N., DURAND, P., Existence de la cowdriose (Heartwater) à *Cowdria ruminantium* chez les petits ruminants des Antilles françaises (La Guadeloupe) et les Mascareignes (La Réunion et Ile Maurice), Rev. Elev. Méd. Vét. Pays. Trop. **33** (1980) 21-22.

[13] CAMUS, E., MARTINEZ, D., BEAUPERTHUY, L., BENDERDOUCHE, A., COISNE, C., CORBETTE, C., DENORMANDIE, N., GARRIS, G., HARRIS, E., KING, T., LANNOY, L., LOUVET, A., LUNEL, E., MONTROSE, M., NISBETT, B., NYACK, B., ROBINSON, J., ROUCHOSSE, P., SWANSTON, G.B., THIÉBOT, B., THYE, G.D., Heartwater in Guadeloupe and in the Lesser Caribbean, Rev. Elev. Med. Pays. Trop. **46** (1993) 109-114.

[14] DU PLESSIS, J.L., CAMUS, E., OBEREM, P.T., MALAN, L.T., Heartwater serology: some problems with the interpretation of results, Onderstepoort, J. Vet. Res. **545** (1987) 327-329.

VALIDATION AND USE OF AN ELISA KIT FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN CUBA

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Abstract

VALIDATION AND USE OF AN ELISA KIT FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN CUBA.

Babesia bovis, the most important etiological agent causing bovine babesiosis, is widely distributed in Cuba and affects mainly adult cattle. A survey of the prevalence of the disease in cattle using an ELISA kit (FAO/IAEA) revealed that 34.2% of the animals between 6 and 18 months of age were positive to *Babesia bovis*, whereas 69.9% of the cattle older than 18 months were positive. Antibodies to *Babesia bovis* were detected in 96.9% of calves vaccinated with an attenuated *Babesia bovis* vaccine. A good correlation was found between the results of ELISA kit with those from indirect immunofluorescence and immunoperoxidase tests developed in Cuba.

1. INTRODUCTION

Babesia bovis is the principal atiological agent of bovine babesiosis which is responsible for high morbidity and mortality of cattle in tropical areas. In Cuba this parasite is widely distributed and is responsible for a disease that is of considerable economic importance.

In order to detect the immune response of cattle to *B. bovis* infection, several tests, e.g. agglutination [1], fluorescence antibodies [2] and immunoenzymatic assays [3,4,5] have been developed.

The enzyme linked immunosorbent assay (ELISA) has been described as a very sensitive assay [6]. In this paper, an ELISA kit for detection of antibodies to *B. bovis* was used for a survey and a control programme in Havana province, and it was compared with other serological tests developed in our laboratory for the diagnosis of this disease.

2. MATERIAL AND METHODS

2.1. ELISA kit

An indirect ELISA test kit for detection of antibodies to *B. bovis* was provided by the Animal Production and Health Section of the Joint FAO/IAEA Division for evaluation in our country. The assay procedure was used as described in the FAO/IAEA manual.

Optical density (OD) readings, representing color development in ELISA, were expressed as a percent of the OD of the positive serum control (PP). The cut-off point, separating positive from negative animals, was determined using serum samples that were collected from 15 splenectomized calves negative to hemoparasites. The cut-off was calculated as twice the mean of the PP value of these sera.

One hundred thirty-three samples collected from calves 1 month after vaccination with an attenuated *B. bovis* vaccine, and sera negative to haemoparasites were used to calculate the sensitivity and specificity of ELISA test. For each plate the results were considered valid when the results of positive (C++), moderate positive (C+) and negative (C-) sera were between the control limits recommended in the manual of the ELISA kit.

2.2. Serological survey

Nine hundred and ninety-seven blood samples were collected from cattle from 4 farms in Havana Province. Three of the four farms investigated were randomly sampled. Approximately 20% of

cattle on each farm were sampled and the cattle placed into two groups according to age (6 - 18 months and > 18 months) All cattle on the fourth farm (> 18 months) were sampled These cattle were breeding bulls known to have a very low prevalence of *B bovis* by the indirect immunofluorescence test All sera were analyzed using the ELISA kit to determine the seroprevalence of *B bovis*

2.3. Comparison of ELISA with indirect immunofluorescence test (IFAT) and immuno-peroxidase test (IPT)

The ELISA was compared with IFAT and IPT using 445 serum samples that included 60 sera from known infected animals, 15 sera from known uninfected animals, and 370 sera from animals of unknown infection status that were collected from the farms

2.4. Immunofluorescence test

Slides coated with antigen and stored frozen were allowed to thaw for 10 minutes at 37°C Each slide was divided into twelve rectangular compartments with a marker pen Test sera were diluted 1 100 in phosphate buffered saline pH 7 2 (PBS), added to the slide, and incubated for 30 min at 37°C in a moist chamber The slides were washed three times with PBS and then allowed to react for 45 min at 37°C with a rabbit anti-bovine IgG fluorescein conjugate diluted 1 400 The slides were washed three times with PBS, glycerol-PBS was added to the surface, and the slides were examined using an oil immersion × 100 objective of a fluorescence microscope

2.5. Immunoperoxidase test

This test was performed as described for IFAT except the use of an horseradish peroxidase conjugate diluted 1 200 in 1% egg albumin in PBS The slides were washed three times and the substrate solution (H_2O_2 and 4-chloro-1- naphtol) diluted in TRIS buffered saline (pH 7 4) was added Finally the slides were examined with an oil immersion × 100 objective of a light microscope

2.6. Control of *B. bovis* vaccine application

For two of the farms investigated, the efficacy of the control program was calculated using 1,335 calves vaccinated with an attenuated *B bovis* vaccine One month after vaccination, 10% of the animals were investigated for the presence of antibodies to *B bovis*

3 RESULTS

The cut-off point in the ELISA, based on 15 splenectomized calves, was determined to be 14 5% of the positive control serum (14 5% PP) The frequency distribution of PP values was obtained by the analysis of all samples (Figure 1) A distinct bimodal distribution was not observed but two peaks could be distinguished The cut-off determined from the graph was estimated to be between 13 and 23% Using the cutoff of 14 5% PP, the sensitivity of the ELISA for vaccinated calves was 96 9% at a specificity of 100%

The results of the survey using the ELISA kit are summarized in Table I These results show that for the 3 farms taken together, antibodies to *B bovis* were detected in 32 4% of cattle 6 to 18-months of age, and in 69 9% of adult cattle For the remaining farm (breeding bulls) the sero-prevalence was only 3 1% A 92 8% correlation was found between the results obtained using the ELISA kit and IFAT (Table II) For the samples that were not in agreement between the two tests, 3 8% were positive by ELISA and negative using IFAT whereas 3 4% were IFAT positive but ELISA negative

The comparison between the ELISA kit and the IPT is summarized in Table III A 94% correlation was observed between the tests Of the 27 serum samples that did not agree between the

tests. 3.3% were positive by ELISA but negative using IPT whereas 2.7% were IPT positive but ELISA negative.

TABLE I. RESULTS OF A SEROLOGICAL SURVEY OF *B.BOVIS* USING THE FAO/IAEA ELISA KIT

Farm	No. of samples	Age (months)	Positive sera	Percentage
1	263	6 - 18	98	37.2
	206	> 18	142	68.9
2	237	6 - 18	66	27.8
3*	129	> 18	4	3.1
4	102	6 - 18	42	41.1
	60	> 18	44	73.3

* breeding bull

TABLE II. CORRELATION OF ELISA AND IFAT FOR THE DETECTION OF *B. BOVIS* ANTIBODIES

Correlation between tests	Fraction from total	Percentage
Agreement		
188 positives 225 negatives	413/445	92.8%
Disagreement		
17 positives by ELISA and negatives by IFAT	17/445	3.8%
15 positives by IFAT and negatives by ELISA	15/445	3.5%

TABLE III. CORRELATION OF ELISA AND IPT FOR THE DETECTION OF *B.BOVIS* ANTIBODIES

Correction between tests	Fraction from total	Percentage
Agreement:		
190 positives 220 negatives	418/445	94%
Disagreement:		
15 positives by ELISA and negatives by IFAT	15/445	3.3%
15 positives by IPT and negatives by ELISA	12/445	2.7%

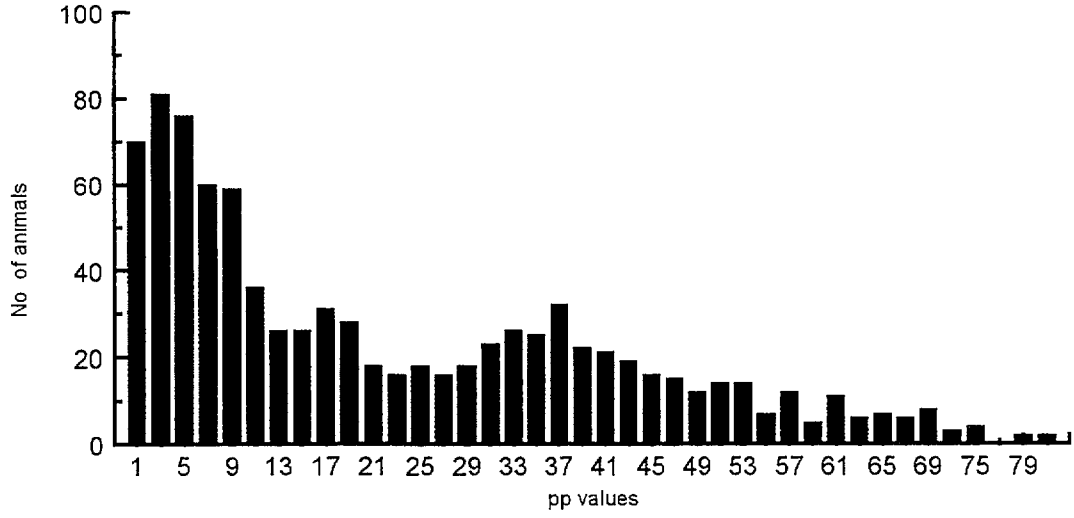

FIG 1 Frequency distribution of pp-values

4 CONCLUSIONS

The difference in the determination of cut-off point using negative sera from splenectomized calves and the frequency distribution method was probably due to the fact that a portion of the investigated population used to determine the frequency distribution was older than 18 months The cut-off for Cuba is probably between 13% and 23 % PP

The specificity of ELISA determined in this work was very high (100%) However, because a limited number of samples were used for making this determination, evaluation of more sera is needed to confirm this result

The ELISA was successfully used in the survey for the detection of specific antibodies to *B bovis* and in a babesiosis control program Three of the four farms investigated would appear to be at risk from babesiosis because the percentage of seropositive cattle was lower than necessary to obtain endemic stability of the infection in the population In the other farm the seroprevalence to *B bovis* was very low and the situation is potentially unstable, but the risk of clinical babesiosis is minimal

The high percentage vaccinated calves with antibodies to *B bovis* indicated that the vaccine was successful in stimulating a response to *B bovis*, the ELISA kit was useful in confirming this observation The 3 1% of vaccinated calves that were negative by ELISA could be attributed to the interval of only one month between vaccination and collection of the serum samples It is possible that some calves had not yet produced detectable antibodies A longer time interval for the collection of sera after vaccination (2 months) could give a better estimation of an immune response to *B bovis* vaccination

The ELISA kit (FAO/IAEA) and IFAT had a good correlation for the detection of antibodies to *B bovis* The IFAT has probably been the most widely used test for the detection of antibodies to *Babesia sp* [7] It has been standardized in Cuba and is still being used routinely for this purpose However, the problem with IFAT is that the test is subjective and vulnerable to technician bias in reading of the slides microscopically

The immunoperoxidase test also had a good agreement with the ELISA kit Blandino et al [8] reported a relative sensitivity of 97% when this assay was compared to IFAT, this is similar to the results obtained by Kung [5] with the S-ELISA This technique is simple to perform and did not require special equipment

ELISA allows a far better standardization than other assays, its results are read and computed automatically For this reason it is a very useful technique for mass screening

ACKNOWLEDGEMENTS

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REFERENCES

[1] MONTENEGRO, S., JAMES, M. A., LEVY, M. G., PRESTON, W.D., ESPERANZA, J., RISTIC, M., Utilization of culture derived soluble antigen in the latex agglutination test for bovine babesiosis and anaplasmosis, Rev. Parasit. **8** (1981) 291.

[2] ALONSO, M., BLANDINO, T., LARRAMENDI, R., JIMENEZ, T., MESA, J., Inmunofluorescencia indirecta en el diagnóstico de la babesiosis bovina en Cuba, Rev. Salud Anim. **10** (1988) 197.

[3] ROSE, R., JACOBSON, R. H., GALE, K. R., WALTISBUHL, D. J., WRIGHT, I.G., An improved ELISA for the detection of antibodies against *B. bovis* using either a native or a recombinant *B. bovis* antigen, Parasitol. Res. **76** (1990) 648.

[4] MONTENEGRO, S. Dot ELISA para el diagnóstico serológico de la babesiosis y anaplasmosis, XII Congreso Panamericano de Ciencias Veterinarias, La Habana, Cuba, 1990.

[5] KUNG, U. M. W., GOODGER, B. V., A slide enzyme-linked immuno-absorbent assay (SELISA) for the diagnosis of *Babesia bovis* infections and for screening of *Babesia*-specific monoclonal antibodies, Int. Jour. Parasit. **20** (1990) 341-345.

[6] WALTISBUHL, D.J., GOODGER, B.V., WRIGHT, I.G., COMMINS, M. A., MAHONEY, D.F., An enzyme linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle. Parasitol. Res. **73** (1987) 126-131.

[7] PAYNE, R. C., OSORIO, O., Tick-borne diseases of cattle in Paraguay. I Seroepidemiological study on anaplasmosis and babesiosis. Trop. Anim. Hlth. Prod. **22** (1990) 53-60.

[8] BLANDINO, T., BARRERA, M., ALONSO, M., Prueba de inmunoperoxidasa para el diagnóstico serológico de *Babesia bovis*, Progreso de la Medicina Veterinaria **2** (1992) 11.

SEROEPIDEMIOLOGICAL STUDY OF *BABESIA BOVIS* IN SUPPORT OF THE URUGUAYAN *BOOPHILUS MICROPLUS* CONTROL PROGRAMME

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Abstract

SEROEPIDEMIOLOGICAL STUDY OF *BABESIA BOVIS* IN SUPPORT OF THE URUGUAYAN *BOOPHILUS MICROPLUS* CONTROL PROGRAMME

Bovine blood samples were collected from a region endemic for *Boophilus microplus* and consisting of 125 ranches with a cattle population of 76,918 A total of 1,728 cattle were bled (1,485 adults and 243 calves less from 1 year of age) from 27 ranches This sample size was determined to provide incidence and prevalence values with a precision of ±10% at a confidence level of 95% The FAO/IAEA ELISA kit was used to detect antibody to *Babesia bovis* Dispersion (proportion of ranches with babesia infection) was estimated to be 70 5% ± 8 8 (SD) A positive ranch was defined as having one or more test-positive animals Apparent prevalence (proportion of cattle with a positive test result) within the region was estimated to be 3 5% ± 0 3, with the range from 0 to 18 5% Incidence based on apparent prevalence in calves less than 1 year of age was estimated to be 2 8% The dispersion, apparent prevalence, and incidence data for this region of Uruguay will be compared through a repeated sampling of cattle in this area during a three-year period to assess the effectiveness of the eradication/control campaign

1 INTRODUCTION

Since 1941, the Uruguayan Government have mandated a campaign against the tick, *Boophilus microplus* Historically there have been advances and reverses in this campaign Recently greater financial support has resulted in an intensified program [1] Uruguay is situated between the 30° and 35° parallels, south latitude, which represents a marginal area for *B microplus* development Since the climate influences the tick population, the enzootic status of babesiosis caused by B*abesia bovis* and *Babesia bigemina* may change as a function of climate in different parts of the country [2,3,4].

Haemoparasites are responsible for significant losses to the cattle industry of Uruguay [5] Their distribution is similar throughout the country Prevalence studies performed with the indirect fluorescence antibody (IFA) technique for *Babesia* spp., and the card agglutination test for *Anaplasma* spp , have identified ranches having enzootic instability for *B bovis* and *Anaplasma marginale,* and other ranches with enzootic stability for *B bigemina and A marginale* [6,7,8] The stability was found to be greater north of the Rio Negro (32° South Latitude) than it was south of the river The Ministry of Agriculture and Fisheries is seriously considering the eradication of ticks south of the Rio Negro and plan to develop effective control of the disease in the northern region of the country [1].

The implementation of a program to control *B microplus* requires a thorough knowledge of the haemoparasite status in the areas where the campaign is going to be enforced Accordingly, it is necessary to·

- evaluate the campaign by monitoring disease prevalence at the beginning and at its different stages as it progresses
- identify areas of high tick prevalence that are enzootically stable. Acaricide treatment used in stable areas reduces the tick population resulting in cattle not becoming exposed or immune to the organisms, this makes them susceptible to haemoparasite infection, morbidity, and mortality
- identify areas of low tick prevalence that are enzootically stable. As tick populations increase, cattle are at greater risk of haemoparasitic infections and their consequences.

The ELISA technique is an established diagnostic procedure and has been used in Uruguay for detection of antibody to *B bovis* [9]. The objective of the present work was to carry out a seroepidemiological study using the ELISA technique in an area where the campaign against *B microplus* is going to be introduced Dispersion, prevalence, and incidence of *B bovis* was determined through a statistically determined sample size of adults and calves which were representative of a population of 76,000 cattle in the region.

2 MATERIALS AND METHODS

2.1. Experimental area

The 8th Police Section in the Department of Lavalleja (34° south latitude) was selected This region consists of 125 ranches with 76,918 cattle The geographic characteristics of the zone are rocky fields with mountain bush vegetation, which makes tick control difficult Official data indicate that a high percentage of ranches in this area are infested with *B microplus*

2.2. The study population

The population for study was selected randomly in two steps First, the ranches were stratified according to the size of their bovine population as shown in Table I Ranches were selected at random from throughout the area The number of ranches selected was based on the dispersion of ticks in the area (about 60% of the farms were tick-infested) The statistical model for selecting ranches indicated that 27 ranches would need to be sampled This represented 21 6% of the total number of herds in the area

TABLE I CHARACTERISTICS OF THE STUDY POPULATION OF ANIMALS

Stratum	Number of cattle	Ranches (n)	Number of adult sera per ranch	Number of calf sera per ranch
I	100 - 500	23	55	9
II	501 - 1000	3	55	9
III	1001 - <	1	55	9
Total		27	1485	243

Previous studies in a similar geographic area suggested that an incidence of 2% and prevalence of 5% of babesiosis was to be expected in the study area The sample size for each ranch was determined to be nine calves less than one year of age and 55 adults (greater than two years of age) This sample size was estimated to provide incidence and prevalence values with a precision of ±10% at a confidence level of 95% The 1,728 cattle sampled represented 2 2% of the 76,918 total animals in the area

After venopuncture, blood samples were placed in a refrigerated box, transported to the laboratory, maintained in a refrigerator until processing, and serum was harvested within 3 days The serum tubes were labeled and stored at - 20°C until used Additional information was obtained from each farm relative to type of management, class of activity, number of cattle, presence of ticks, and previous experience with haemoparasitic diseases

2.3. Serological testing

An ELISA kit from the Joint FAO/IAEA Division, Austria, was used for serological diagnosis as described previously [10] The assay was validated for Uruguayan conditions by testing 200 known uninfected and 80 known infected cattle

2.4. Analysis of results

Dispersion, apparent prevalence, and incidence of haemoparasites was calculated with a 95% confidence interval [11,12] Dispersion was defined as the proportion of ranches with a *Babesia sp* infection A positive ranch was defined as having one or more test-positive animals Dispersion was

estimated based on stratified sampling. Apparent prevalence was defined as the proportion of cattle with a positive test result and was calculated in accordance with the sampling ratio Incidence was defined as the apparent prevalence in calves less than one year of age and also was calculated in accordance with the sampling ratio.

3. RESULTS

3.1. Assay validation

The calculated sensitivity and specificity of the ELISA test was 99% and 98%, respectively (Figure 1). The assay performance was essentially the same as previously published [10].

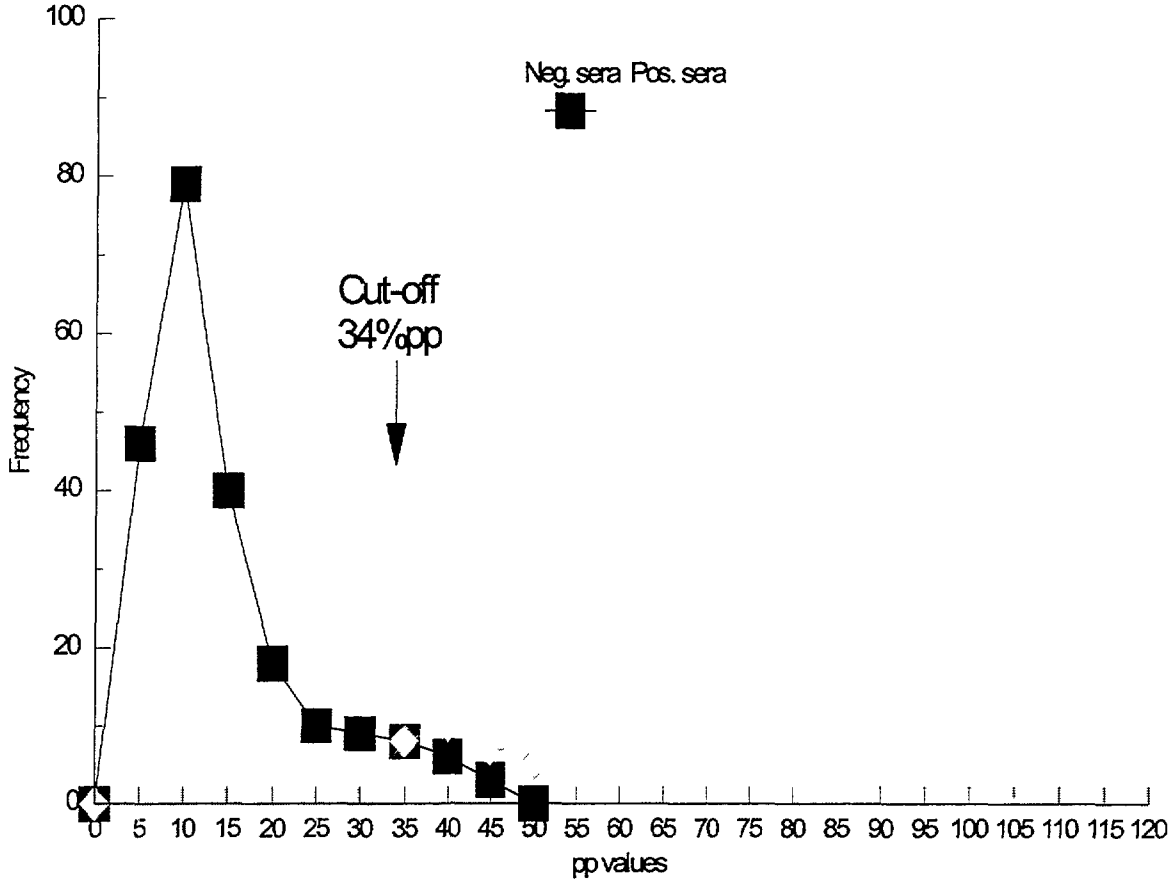

FIG. 1. Representation of the cut-off in pp units from which was established the estimates for sensitivity and specificity of the ELISA assay for antibody to B. bovis.

3.2. Ranch sampling profile

The 27 ranches included in the survey are profiled in Table II. The tick infestation status *(Boophilus microplus)* for each ranch was confirmed by producer responses to survey questions. Sixteen of the 27 ranches (59.3%) had tick infestations.

TABLE II. PROFILE OF THE RANCHES SAMPLED

Stratum	Ranch identity	Presence of ticks	Number of cattle	Number of calves	Percent of calves positive	Percent of adults positive	Overall prevalence
1	820676	+	120	4	-	0	0
1	816529	+	154	32	11	5	6
1	818246	-	156	28	0	9	7
1	820976	-	160	44	11	0	2
1	801033	+	171	58	21	3	7
1	805527	+	180	70	0	6	5
1	805855	-	180	60	0	0	0
1	821204	-	180	11	0	7	6
1	804032	+	200	100	0	0	0
1	802102	-	200	43	0	16	14
1	801948	+	221	63	11	15	14
1	800819	+	250	40	0	0	0
1	805578	+	270	16	11	3	5
1	803346	-	280	50	0	9	8
1	801181	+	310	50	0	2	2
1	S/N [1]	+	320	57	1	4	3
1	806361	+	380	50	21	6	8
1	S/N [2]	+	400	90	0	2	2
1	815506	+	400	200	0	2	2
1	804394	+	420	110	0	22	18
1	803576	-	450	100	11	2	3
1	802552	-	550	140	0	4	4
2	801697	+	582	110	0	7	6
2	S/N [3]	-	660	130	0	2	2
2	815352	-	800	32	0	0	0
2	801972	+	950	100	0	0	0
3	810245	-	1480	4	0	0	0

Dispersion of *B. bovis* in the study area was estimated to be 70.5% ± 8.8% (95% confidence interval). Prevalence as a function of ranch size and age is summarized in Table III. Ranches having 100-500 animals (Stratum I) had 19 times greater prevalence than did ranches having more than 500 cattle (Strata II and III combined). Prior estimates of adult and calf prevalence for the strata were 5% and 2%, respectively. These were similar to the observed prevalences in the study (3.4% and 2.8%, respectively, for adults and calves).

TABLE III PREVALENCES AS A FUNCTION OF STRATUM AND AGE

Stratum	Totals (%)	Adults (%)	Calves (%)
I	5,4 ± 0,8	5,6 ± 1,3	3,3 ± 1 3
II and III	0,3 ± 0,3	0,3 ± 0,3	0 0 ± 0,0
Police section	3,5 ± 0,3	3,4 ± 1,1	2,8 ± 1,2

Seventy four percent (20/27) of the ranches surveyed had animals that were positive for *B bovis* Of the 20 infected ranches, only one ranch had a prevalence that exceeded 15% The maximum prevalence for any ranch was 7 0% Using the criterion that calfhood infections are an indication of the incidence of infection, the maximum incidence was 5 8% The ranch that had the maximum prevalence did not have any infected calves whereas the ranch with the maximum incidence had an intermediate prevalence among all ranches (Table II)

4 DISCUSSION

Because the sampling strategy employed gave incidence and prevalence data of babesiosis that were consistent with anticipated results, the data obtained in this survey may be extrapolated to the remainder of the population within the 8th police section in the Department of Lavalleja Among the 70 5% of the ranches that were infected with *B bovis* within the Department, there was no apparent clustering of infected farms (data not shown), rather, infected farms were evenly distributed throughout the region This data would suggest that the tick is also widely distributed within the area, an observation in agreement with prior tick surveys which indicated that 59 3% of all ranches in the area were tick-infested

Although 11 ranches indicated that they had not observed ticks on their ranch eight of the 11 farms had animals that were positive for *B bovis* Because the mean prevalence of seropositive animals on these farms was relatively high (4 2%), it is unlikely that the test was miss-classifying these animals considering that the test sensitivity exceeded 98% A more likely explanation is that ticks are present on these ranches but are not being observed

The low prevalence of babesiosis in the region (3 5% ± 0 3%) indicates that the disease is relatively enzootically stable This is supported by a similar prevalence in adults (3 4%) and incidence in calves (2 8%) On two ranches, the incidence in calves was 21% while the prevalence in adult cattle was only 3% and 6%, respectively This may indicate that the infection is less stable on these farms than on other farms, which is consistent with previous observations [13,14] Both of these farms were known to be infested with ticks Only two of the 11 presumably tick-negative farms had sero-positive calves while six of the 11 had adult sero-positive cattle and no sero-positive calves, these ranches tend to confirm the enzootic stability theory

The relationship between the wide dispersion of vector/haemoparasites and the low overall and local prevalences, depend on several factors Firstly, the study area is located in South Latitude 34° where weather conditions allow only 2 5 to 3 tick generations to develop per annum so that high prevalences of infection are more difficult to obtain [2] Secondly, local variations in prevalence may be due to the interspersed presence of rocky fields covered with brush that protect ticks from cold and heat during certain periods of the year Thirdly, the low infestation levels may be due to the fact that treatments against ticks are applied to cattle each time that tick infestations are detected by producers Because these variables may change over time, enzootic stability may also change Changes in these variables may have resulted in infected herds where they were not expected [15] In our experience, this usually occurs when tick control is lax and the tick population increases favoring transmission to susceptible cattle

Four percent of ranches had greater than 15% prevalence This may have been due to failures in the control or in an improvement of the transmission conditions, placing these ranches at an increased risk of outbreaks One explanation may be an increased resistance to some acaricides that are being used in Uruguay

Although other haemoparasitic infections occur in cattle in Uruguay, infections with *B bovis* are the most common [8] A similar situation is present in Rio Grand region of Brazil [16] Although Argentina has carried out a successful campaign and eradicated the tick below the 30° south latitude, areas enzootic for *B microplus* with high prevalence of haemoparasites still remain above this latitude [17]

Our studies have established a baseline of babesiosis for the 8th Police Department of Uruguay A similar study after 3 years of the campaign will be used to evaluate the effectiveness of the eradication campaign in Uruguay

REFERENCES

[1] MGAP/Sanidad Animal/BID, Proyecto 840, Uruguay (1989)

[2] CARDOZO, H , Estudios sobre la ecologia del *Boophilus microplus* en tres areas enzooticas del Uruguay, Veterinaria, Montevideo, **20** (1984) 4-10

[3] NARI, A , SOLARI, M A , Epidemiologia y control de *Boophilus microplus* en Uruguay, Su relacion con *Babesia* spp XII Congreso Panamericano de Ciencias Veterinarias, LaHabana, Cuba, (1990)

[4] SOLARI, M A et al , Aspectos de la dinamica integral del *Boophilus microplus* y *Babesia spp* en Uruguay X Congreso Latinoamericano de Parasitologia, I Congreso Uruguayo de Parasitologia, Montevideo, Uruguay, (1991)

[5] SOLARI, M A et al , Impact of *Babesia bovis* and *Babesia bigemina* on the production of the beef cattle in Uruguay, Memorias do Instituto Oswaldo Cruz, Rio de Janeiro, **87** (1992)143-149

[6] BERDIE, J , Prevalencia de *Anaplasma marginale* en un area del departamento de Rivera mediante la prueba de aglutinación en placa, Veterinaria, Montevideo **15** (1979) 55

[7] CARDOZO, H , et al , Estudio epizootiológico de los hematozoarios transmitidos por *Boophilus microplus* en un area enzootica del Uruguay, IX Jornadas Uruguayas de Buiatría,Paysandú, (1981)

[8] SOLARI, M A , Aspectos epidemiologicos de babesiosis en Uruguay, Consulta sobre la erradicacion de las garrapatas con referencia especial a las Americas, FAO-AGA TIER/87/27, Mexico, (1987)

[9] CARDOZO, H , SOLARI, M A , ETCHEBARNE, J , Evaluation of an indirect ELISA for the diagnosis of *Babesia* bovis and its use in a Sanitary campaign against the *Boophilus microplus* in Uruguay, Proceedings of a Final Research Coordination Meeting of an FAO/IAEA/SIDA, Costa Rica, (1992) 185-191

[10] [10]CARDOZO, H , SOLARI, M A y ETCHEBARNE, J , *Babesia bovis* ELISA kit validation In Third research coordination meeting of the FAO/IAEA Coordinated research program on immunoassay methods for the diagnosis and epidemiology of animal diseases in Latin America -Guadeloupe, (in press)

[11] COCHRAN, W G , Técnicas de muestreo, John Wiley and sons, London, (1977)

[12] ORGANIZACIÓN PANAMERICANA DE LA SALUD, Procedimientos para estudios de prevalencia por muestreo, Nota técnica **18** (1979)

[13] FRANCHI, M , Datos preliminares sobre incidencia de hemoparasitos en region norte del Uruguay, X Congreso Latinoamericano de Parasitologia, I Congreso Uruguayo de Parasitologia, Montevideo, (1992)

[14] QUINTANA, S , LIUZZI, B , & FABREGAS, B , Babesiosis y Anaplasmosis en el area de acción del Laboratorio Regional Noroeste Paysandú, Período 1980-1990, Veterinaria, Montevideo, **28** (1992) 5

[15] [15]PAIVA, N., Datos preliminares sobre incidencia de hemoparasitos en region este del Uruguay,X Congreso Latinoamericano de Parasitología, I Congreso Uruguayo de Parasitología, Montevideo, (1992).

[16] MANIERO O., LEITE, A., Prevalencia sorológica de *Babesia bigemina* e de *Babesia bovis* em 33 propiedades na zona sul do Rio Grande do Sul, Ministerio da Educacao, Universidade Federal de Pelotas, Curso de Pos-Graduacao em Sanidade Animal, Rio Grande do Sul, Brasil,(1988).

[17] GUGLIELMONE, A., Epidemiologia de los principales hemoparasitos transmitidos por el *Boophilus microplus*. X Congreso Latinoamericano de Parasitología, I Congreso Uruguayo de Parasitologia, Montevideo, (1991).

VALIDATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN CATTLE IN YUCATAN, MEXICO

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Abstract

VALIDATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN CATTLE IN YUCATAN, MEXICO

The ELISA kit provided by the FAO/IAEA for the diagnosis of *Babesia bovis* was validated In order to determine the appropriate ELISA cut-off point that would serve as the threshold between positive and negative samples, 119 serum samples from a Mexican *Babesia*-free zone were analyzed The optimal cut-off point chosen was at 12% of the reactivity of the high positive control serum sample (PP) which resulted in a specificity of 97% One hundred and ninety-six cattle from Wisconsin, USA, were introduced into Yucatan, Mexico, of which 181 were vaccinated with an attenuated live *Babesia bovis* vaccine, 15 animals remained as unvaccinated controls Before and after vaccination all animals were bled and tested by enzyme linked immunosorbent assay (ELISA) and indirect fluorescence antibody test (IFAT) Both tests showed a high degree of correlation in their results To evaluate an immune response to vaccination the optimal cut-off point chosen was 12% PP resulting in a sensitivity 99% and a specificity 95% We concluded that the ELISA test has proved to be useful in Yucatan, Mexico for serological surveys and monitoring the efficiency of vaccination programmes

1 INTRODUCTION

More than one billion cattle in the world are estimated to be at risk of acquiring the tick-borne hemoprotozoan disease babesiosis [1]. Bovine babesiosis, caused by the protozoan parasite *Babesia* spp., continues as a major threat to livestock industries throughout the world *Babesia bovis* and *Babesia bigemina* infect the red blood cells of cattle and area transmitted by the one host tick, *Boophilus microplus*.

The diagnosis of acute babesiosis in cattle is relatively straightforward when clinical signs are evident and supported by microscopic examination of stained blood films [2] On the other hand, mild and subclinical infections are more difficult to recognize since peripheral blood parasitaemias fluctuate and frequently do not rise to levels detectable by microscopy. Although classical serological techniques of babesiosis such as the indirect fluorescence antibody test (IFAT) and complement fixation (CF), have proved useful, they suffer from a number of drawbacks. Generally, these drawbacks relate to a combination of inadequate diagnostic performance, lack of standardization, and/or poor cost efficiency The enzyme-linked immunosorbent assay (ELISA) potentially could resolve these problems In this survey we validated an ELISA kit provided by FAO/IAEA and used it in a serological survey in Yucatan, Mexico

2 MATERIALS AND METHOD

2.1. ELISA Procedure

The ELISA kit used in this study was similar to that described by Waltisbuhl *et al* [3] and was provided by FAO/IAEA [4]. The test was carried out using flat-bottomed 96-well micro-ELISA plates (Nunc-Immunoplate, Denmark). Briefly, the stock antigen was diluted 1:200 in carbonate-bicarbonate buffer pH 9.6 (coating buffer). One hundred microlitres of diluted antigen was added to each well of a micro-ELISA plate and incubated over night at 4°C After incubation the antigen solution was discarded and 100 µl of a blocking solution (5% skimmed milk powder in coating buffer - w/v) was added to each well and incubated for 1 h at 37°C. After blocking, the plates were washed three times in phosphate buffered saline (PBS) pH 7.2 containing 0.001% Tween 20 (PBS-T). The positive reference sera (moderate and strong antibody activity to *B*

bovis) and a negative serum were diluted 1:200 in PBS-T and added to the antigen coated wells in quadruplicate. Test sera at the same dilution were run in duplicate. The plates were incubated for 1 h at 37°C and then washed three times in PBS-T. Rabbit anti-bovine IgG horseradish peroxidase conjugate, diluted 1:11,000 in PBS-T, was then added and a further incubation for 1 hour carried out at 37°C. The plates were again washed three times, a substrate-chromogen solution of H_2O_2 containing O-phenylenediamine was added, and after ten minutes, the reaction was stopped by adding 100µl of 2M sulfuric acid. The intensity of color development was determined by measuring absorbance using a micro-ELISA reader equipped with a 492 nm filter. The results of were expressed as a percent of the high positive control serum sample (PP) [5].

2.2. Cut-off determination

The cut-off value separating positive from negative sera was calculated as 3 standard deviations above the mean of the PP values obtained from 119 adult cattle raised in Toluca in a high altitude tick free zone in central Mexico and 196 cattle imported from Wisconsin, USA, to Yucatan.

2.3. Serum sample

To compare the ELISA with IFAT, 196 cattle (*Bos taurus*) imported from Wisconsin, USA, were to Yucatan were used. The cattle were sent to two farms: 98 animals were placed on ranch 1 and the other 98 on ranch 2. All animals were bled 3 days after arriving in Yucatan. One week later, 181 of the animals received a dose (1ml) of 1 $x10^7$ of *Babesia bovis* vaccine (prepared by 54 rapid cell culture passages) intramuscularly in the neck and 15 control animals received vaccine diluent [6]. Sixty days after vaccination all animals were bled and tested by ELISA and IFAT as described by Todorovic and Long [7]. The pre-vaccination and post-vaccination sera served as known negative and known positive animals, respectively. The specificity and sensitivity of each test were calculated and compared using vaccination status as the gold standard.

3. RESULTS

3.1. ELISA results

The ELISA cut-off point for local conditions and indigenous cattle was determined from 119 negative samples from Toluca in a high altitude *Babesia*-free zone in central Mexico. Three cut-off points of 10%, 12% and 15% PP were calculated (Table I).

TABLE I. CALCULATION OF THE CUT-OFF SEPARATING NEGATIVE FROM POSITIVE SAMPLES BASED ON SERA FROM CATTLE IN THE TOLUCA REGION OF YUCATAN THAT ARE KNOWN TO NOT BE INFECTED WITH *B. BOVIS*

Cut-off (%)	Positives	Negatives	Retest	Spec. (%)
10	6	113	1	95
12	3	116	1	97
15	2	117	1	98

ELSIA results for the pre- and post-vaccinal samples taken from the cattle that were vaccinated at both ranches were used to determine specificity and sensitivity of the ELISA for detection of vaccinal antibody (Table II). All the post-vaccination samples from ranch 1 were

high, indicating an excellent antibody response to the vaccine, whereas, some of the cattle did not respond well on ranch 2. The calculated specificity and sensitivity of the ELISA test using the pre-vaccination samples from both ranches and the post-vaccination samples from only ranch 1 (controls removed), were then used to determine the three cut-off points 10%, 12% and 15% of the PP (Table III). This improved the efficiency of the test.

TABLE II. CALCULATION OF SENSITIVITY AND SPECIFICITY OF ELISA BASED UPON SERUM SAMPLES DERIVED FROM ALL ANIMALS THAT WERE VACCINATED AGAINST *B. BOVIS* ON RANCHES 1 AND 2

Cut-off (%)	Pre-vaccination samples	Post-vaccination samples	Sens. (%)	Spec. (%)
10	179 (-) 15 (+) 2 (r)	6 (1) 172 (+) 3 (r)	97	92
12	183 (-) 9 (+) 4 (r)	13 (-) 160 (+) 8 (r)	92	95
15	189 (-) 3 (+) 4 (r)	26 (-) 150 (+) 5 (r)	85	98

(-) = Test Negative, (+) = Test Positive, (r) = Retest

TABLE III. CALCULATION OF SENSITIVITY AND SPECIFICITY OF ELISA FOR DETECTION OF ANTIBODY TO *B. BOVIS* USING ALL PRE-VACCINATION SERUM SAMPLES FROM RANCHES 1 AND 2 (BUT ONLY POST-VACCINATION SAMPLES FROM RANCH 1)

Cut-off (%)	Pre-vaccination samples	Post-vaccination samples	Sens. (%)	Spec. (%)
10	179 (-) 15 (+) 2 (r)	0 (1) 81 (+) 1 (r)	100	92
12	183 (-) 9 (+) 4 (r)	1 (-) 79 (+) 2 (r)	99	95
15	189 (-) 3 (+) 4 (r)	6 (-) 72 (+) 4 (r)	92	98

The retest values were found to be very similar to the initial results suggesting that the antibody levels were in the equivocal range. The PP value used was an average of the two tests on repeated samples for calculation of the measurement of specificity and sensitivity.

3.2. IFAT results

All the pre and post-vaccination samples were run with the IFAT (Tables IV and V). A few samples from the pre-vaccination population gave a weak fluorescence which was deemed positive. Five samples collected post-vaccination were found to be negative for both ELISA and IFAT.

TABLE IV. SENSITIVITY AND SPECIFICITY OF IFAT BASED UPON ALL CATTLE FROM RANCHES 1 AND 2 THAT WERE VACCINATED WITH AN ATTENUATED *B. BOVIS* VACCINE

Pre-vaccination samples	192 (-) 4 (+)	
		Sensitivity 98% Specificity 98%
Post-vaccination samples	177 (+) 4 (-)	

TABLE V. CALCULATION OF SENSITIVITY AND SPECIFICITY OF IFAT FOR DETECTION OF ANTIBODY TO *B. BOVIS* USING ALL PRE-VACCINATION SERUM SAMPLES FROM RANCHES 1 AND 2 BUT ONLY POST-VACCINATION SAMPLES FROM RANCH 1

Pre-vaccination samples	192 (-) 4 (+)	
		Sensitivity 100% Specificity 98%
Post-vaccination samples	82 (+) 4 (-)	

4. CONCLUSIONS

The cut-off point of 12%, determined from the "local" Mexican population of cattle from Toluca, appeared to provide a satisfactory estimate of sensitivity and specificity for an ELISA that would be used for prevalence studies. Using 196 pre-vaccination samples from the herd that originated in the USA as a gold standard negative population, and 82 (ranch 1) post-vaccination samples as the gold standard positive population, a 12% PP cut-off for the ELISA kit provided a 99% sensitivity and a 95% specificity. A cut-off point of 10% PP resulted in a sensitivity of 100% but a commensurate drop in specificity to 92%, while a cut-off point of 15% gave a sensitivity of 92% and an increase in specificity to 98%. These cut-off points were calculated to illustrate how the selection of an appropriate cut-off point could be driven by the nature of projected studies. For example, if the ELISA were used to confirm results of a highly sensitive but non-specific screening test, a higher cut-off point would be appropriate to enhance specificity in the ELISA.

Running the same 196 pre-vaccination samples and 82 post-vaccination samples in the IFAT test resulted in a specificity of 98% at a sensitivity of 100%. Both the ELISA and IFAT test showed a high degree of correlation in results. This confirmed the observations of Ramirez [8] in a recent study in Yucatan. The 5 post-vaccination samples that were negative in both the ELISA

and IFAT suggests that either both tests were insufficiently sensitive to detect *babesia* antibodies or the vaccine did not induce detectable levels of antibodies. Alternatively these cattle may have been in the early stages of a developing antibody response to vaccination and could become sero-positive over time. This does not however prove that the vaccine failed to protect these animals since cell mediated immunity can play an important role in protection [9].

Our results demonstrate that the indirect ELISA can be used, not only for serological studies, but also to evaluate the ability of vaccinations to induce antibody. Thirty days after vaccination, ELISA was able to detect IgG-antibodies. Since the microtiter ELISA tests are easy to perform and are ideally suited to the processing of large number of test samples [10], the ELISA is an excellent candidate for assessment of antibody responses to *babesia* antigens or infections.

In conclusion the ELISA kit for the detection of *Babesia bovis* worked very well under Yucatan conditions. The kit methodology produced consistently acceptable plates with control values that fell well within the specified acceptable ranges. It is suitable for use in serological studies and vaccination programs.

REFERENCES

[1] KÖNIGSHOFER, H.O. In: PAO-WHO-OIE, Ed. Animal Health Yearbook (Food and Agriculture Organization), Rome, 1977.

[2] ROSS, J.PJ., LOHR, K.F., Serological diagnosis of Babesia bigemina infection in cattle by the indirect fluorescent antibody test, Res Vet Sci. **9** (1968) 557-562.

[3] WALTISBUHL, D.J., GOODGER, B.V., WRIGHT, I.G., COMMINS, M.A., MAHONEY, D.F., An enzyme linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle, Parasitol. Res. **73** (1987) 126-131.

[4] GOODGER, B.V., WRIGHT, I.G., WALTISBUHL, D.J., The lysate from bovine erythrocytes infected with Babesia bovis. Analysis of antigens and a report on their immunogenicity when polymerized with glutaraldehyde, Z Parasitenkd. **69** (1983) 473-482.

[5] WRIGHT, P.F, NILSSON, E., VAN ROOIJ, E.M.A., LELENTA, M., JEGGO, M.H., Standardization and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis, Rev. Sci. Tech. Off. Int. Epiz. **12** (1993) 435-450.

[6] WAGNER, G., HOLMAN, P., CRUZ, D., WAGHELA, S., SAHAGUN, A., SNYDELAAR, A. MEDELLIN, J., DOMINGUEZ, J., RODRIGUEZ, I., Proceedings of the Ninth International Veterinary Haemoparasite Disease Conference, Merida, Yucatan, Mexico, 1993.

[7] TODOROVIC, R.A., LONG, R.F., Comparison of indirect fluorescence antibody (IFA) with complement fixation (CF) test for diagnosis of *Babesia spp.* infections in Colombian cattle, Tropenmed. Parasitol. **27** (1976) 169-181.

[8] RAMIREZ, C.G., Epidemiology of bovine babesiosis in the state of Yucatan, Mexico, Central for Tropical Veterinary Medicine, Mphil. Thesis, Scotland, U.K., 1993.

[9] VALENTIN, A., PRECIGOUT, E., L'HOSTIS, M. CARCY, B., GORENFLOT, A., SCHREVEL, J., Cellular and humoral immune response induced in cattle by vaccination with *Babesia divergens* cultured derived exoantigens correlate with protection, Infec. Immunol **61** (1993) 734-741.

[10] O'DONOGHUE, P.J., FRIEDHOFF, K.T., VIZCAINO, O.G., WEYRETER, H., The detection of IgM and IgG antibodies *Babesia bigemina* in bovine sera using semi-defined antigens in enzyme immunoassays, Vet. Parasitol. **18** (1985) 1-12.

EVALUATION OF AN ENZYME LINKED IMMUNOSORBENT ASSAY KIT FOR THE DETECTION OF *BABESIA BOVIS* ANTIBODIES IN CATTLE IN ARGENTINA

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Abstract

EVALUATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF *BABESIA BOVIS*-ANTIBODIES IN CATTLE IN ARGENTINA.

An enzyme linked immunosorbent assay (ELISA) for the detection of antibodies to *Babesia bovis* was evaluated by using sera of 874 cattle carrying *B. bovis* antibodies, 700 sera of uninfected cattle, and 357 sera from calves from 16 herds subjected to different *B. bovis* inoculation rates. The seropositive/ seronegative cut-off point set as double the mean percent positivity of negative cattle sera (= 16%). The sensitivity of the ELISA (four trials) ranged from 97.1% to 100% and the specificity (three trials) varied from 92.0% to 97.0%. The agreement between ELISA and immunofluorescent antibody test was ≥ 90.0% in 18 of 23 evaluations and it ranged from 86.0% to 88.0% in the remainder. The correlation coefficient between percentage of sera positive to ELISA and IFA test in 16 herds was 0.9958 ($P < 0.001$). The ELISA has the advantages of a high sensitivity, objectivity and capacity to test large number of samples in short period of time and could replace the IFA test specially for epidemiological studies.

1. INTRODUCTION

Babesiosis caused by *Babesia bovis* is the most economically important tick-borne disease of cattle in the Southern Cone of America where it is transmitted only by larvae of *Boophilus microplus* ticks [1]. Clinical cases rarely occur in cattle younger than seven months [2,3]. Infection during this period induces a long-lasting immunity [4], while primary infection later in life can produce severe illness [5]. Therefore, the probability of the occurrence of babesiosis outbreaks in a cohort-herd can be estimated by detecting the proportion of infected cattle, 6-12 months-old using techniques suitable for the diagnosis of babesial antibodies.

The indirect immunofluorescent antibody (IFA) test is widely used to detect *B. bovis* antibodies for epidemiological or experimental studies. However this is a subjective test in which background fluorescence can cause difficulty to arrive at an endpoint serum titration. Microfluorometry can be used to partly solve this problem as demonstrated for a related cattle disease, anaplasmosis [6]. Nevertheless the major problem with the IFA test is the difficulty to process daily a large number of sera as is frequently required in surveys or to use the test to make decisions affecting the economics of vaccination or management of cattle on a regional basis.

Enzyme linked immunosorbent assays (ELISA) have been applied increasingly to detect antibodies against agents of many animal and human diseases. Moreover the ability to couple the detection system to computerized automatic readers makes ELISA a practical and powerful tool for experimental and epidemiological studies, particularly for those diseases in which high quality antigens for ELISA have been developed. The current study was designed to validate an indirect ELISA-kit for detection of antibodies to *B. bovis* under local conditions in Argentina, and to compare the results of ELISA with those of an IFA test. The ELISA data were then used to estimate the endemic stability of *B. bovis* in regions of Argentina.

2. MATERIALS AND METHODS

2.1. ELISA procedure

The antigen was an oxyhemoglobin-free distilled water lysate of *B. bovis* infected erythrocytes preserved in a freeze-dried state [7].

The guidelines provided by the manufacturer of the kit [8] were followed to perform the test. Briefly 100µl of antigen diluted 1:200 in carbonate/bicarbonate buffer pH 9.6 (coating buffer) was adsorbed onto the wells of 96-well microplates (Nunc) by overnight incubation at 4°C. After incubation, excess antigen was removed and 150 µl of 5% skimmed milk in coating buffer were added as blocking solution and incubated for 1 h at 37°C with continuous shaking (Micro Shaker II, Dynatech). Thereafter the plates were washed three times in phosphate buffer saline 0.002M, pH 7.2, containing 0.05% Tween 20 (PBS-T; wash buffer). Sera were added at the dilution 1:200 in 0.01M PBS, pH 7.2, containing 0.05%Tween 20 (diluent buffer). A weak positive (C+), a strong positive (C++), and a negative (C-) control serum, as well as a conjugate control were run in quadruplicate, while test sera were run in duplicate and incubated for 1 h at 37°C with shaking. After five washings, 100 µl of conjugate (rabbit anti-bovine IgG antibody conjugated to horseradish peroxidase), diluted 1:10000 in diluent buffer, was added and incubated for 1 h at 37°C with shaking. The plates were washed and 100 µl of substrate solution (H_2O_2 containing O-phenylenediamine as chromogen) was added and incubated 10 min at room temperature. The reaction was stopped with 100 µl of sulfuric acid 2N. The absorbance values were determined at 492 nm. A Multiskan Plus (type 314) ELISA reader linked to a personal computer (AT 386 IBM clone) and the BAEIA version 1.01 software programme (copyright Walter Kelly, Agriculture Canada 1991-1992) were used throughout. The results for each sample were calculated as a percent of the mean of the quadruplicated C++ sample for each plate and expressed as percent positive (PP) values. Quality control was maintained by assuring that the optical density (OD) readings for each control fell within limits set by the kit manufacturer.

2.2. IFA test procedure

The IFA test was performed as described by Ríos et al. [9] using an antigen prepared from *B. bovis* grown *in vitro*. The antigen was harvested from the cultures when infection of erythrocytes reached 8%. The antigen was kept at -20°C until use. Sera were diluted 1:60 in PBS solution for screening or for the first dilution when endpoint titration of the serum was done. A positive and a negative control serum were added on each smear. A conjugate (fluorescein-labeled rabbit anti-bovine IgG) was used at a 1:80 dilution. The fluorescent reactions were observed with a microscope (Leitz) equipped for epi-illumination using 50-W mercury vapor lamp. A 50X water immersion objective was used.

2.3. Determination of seropositive/seronegative threshold PP value for ELISA (cut-off point)

Five hundred sera from cattle negative to *B. bovis* (bornE and raised in an area of Argentina free of *B. microplus* ticks) were analyzed by ELISA. The threshold was set as double of the mean PP value of these sera from uninfected cattle. To determine the frequency distribution of PP values among uninfected and infected animals, 500 sera from cattle known to be infected with *B. bovis* were also analyzed.

2.4. Determination of the sensitivity and the specificity

The diagnostic sensitivity of the assay was expressed as the number of positive sera/number of sera tested from cattle inoculated with *B. bovis* antigens. The diagnostic specificity of the assay was defined as the number of negative sera/number of sera tested from cattle from a tick free-zone. In all cases a comparison with the IFA test was done, and the degree of agreement between the results of both techniques obtained.

The sensitivity of the ELISA for *B. bovis* antibodies was determined using 374 cattle sera in four trials as follows: Trial 1: Sera from 76 cattle experimentally infected with a pathogenic *B. bovis*

strain; Trial 2: Sera from 201 cattle inoculated with a commercial live immunogen containing vaccinal *B. bovis, B. bigemina* and *Anaplasma centrale* strains; Trial 3: Sera from 70 cattle inoculated with soluble *B. bovis* antigens derived from an *in vitro* culture of this protozoan; Trial 4: Sera from 26 cattle naturally infected with *B. bovis* detected by inspecting thick smears from peripheral blood under oil immersion microscopy.

The specificity was evaluated in three trials involving sera of cattle from an Argentinean region free of *B. microplus* as follows: Trial 1: Analysis of sera from 50 cattle experimentally infected with *B. bigemina*; Trial 2: Analysis of 50 sera from cattle naturally or experimentally infected with *Anaplasma marginale*; Trial 3: Analysis of 100 sera of cattle from the *B. microplus*-free zone with unknown history of haemoparasite infection.

2.5. Cross-sectional studies of cattle herds from areas with different prevalences of *B. bovis* infection

Sera from calves (9-12 months-old) from 16 herds subjected to different *B. bovis* inoculation rates were evaluated by using ELISA and IFA tests to detect antibodies against this protozoan. The degree of agreement between results of both techniques was also determined. The percentage of positive results of each herd tested was used to determine the correlation (r) between IFA and ELISA. Arbitrarily, IFA values were judged as the dependant variable.

3. RESULTS

No major problems were encountered in conducting the ELISA and the OD values were consistently within the acceptance ranges. The mean of the PP values of the negative sera was 8%. Therefore the cut-off point to define the seropositive/seronegative threshold was set at 16%, using published criteria for the ELISA kit. A small overlap in the PP values of positive and negative sera to *B. bovis* antibodies was found (Figure 1). Ten sera from cattle infected with *B. bovis* antigens (1% of all sera analyzed) showed PP values below the cut-off point, while 25 sera (5%) of the total analyzed from cattle not infected with *B. bovis* showed false positive reactions.

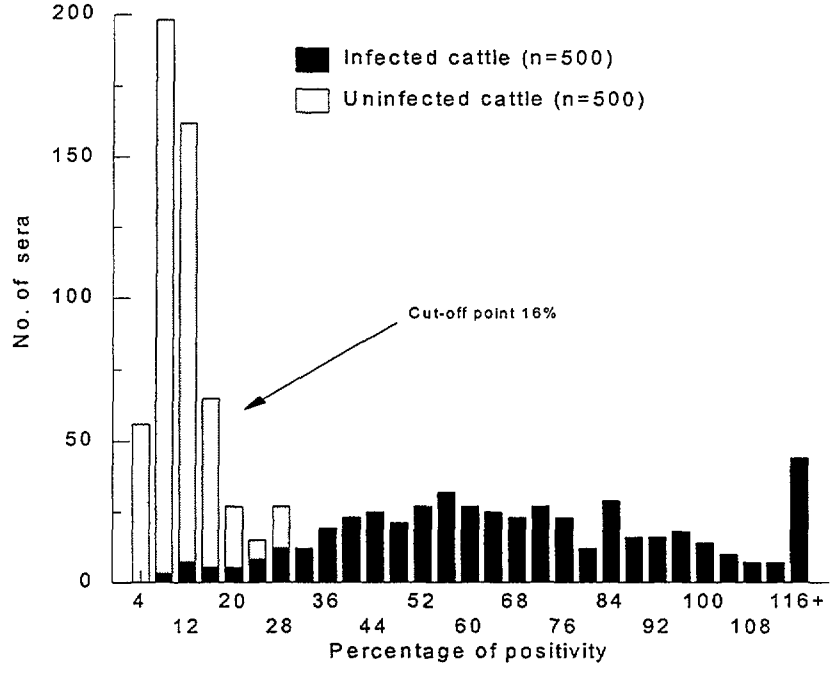

FIG. 1. Frequency distribution of percentage of positivity of sera from cattle infected or uninfected with Babesia Bovis analyzed to determine ELISA seropositive/seronegative cut-off point (twice the mean percentage positivity value of negative sera in relation to a strong positive control).

The sensitivity and specificity of IFA and ELISA along with the degree of agreement between the techniques are presented in Table I. Three false positive reactions to ELISA were found among 50 sera from cattle experimentally infected with *B. bigemina* (PP values 19%, 39% and 44%), four were found among 50 sera of cattle infected with *A. marginale* (26%, 21%, 18% and 16% of PP values) and three false positive reactions were detected in 100 sera of cattle from an area known to be free of *B. bovis* but of unknown history of other haemoparasite infection (22%, 17% and 44% PP values).

The results of the cross-sectional studies of herds are presented in Table II. The concordance value of ELISA and IFA was ≥ 90% in 18 of 23 estimations (results from Table I + Table II) which is consistent with the data presented by Barry et al (10). This resulted in a strong association between the percent of cattle positive to *B. bovis* antibodies using both techniques as shown by the r coefficient of 0.9958 ($P < 0.001$).

TABLE I. SENSITIVITY AND THE SPECIFICITY OF ELISA, ITS COMPARISON WITH AN IFA TEST, AND THE AGREEMENT BETWEEN BOTH TECHNIQUES

Trial	Origin of cattle sera	n	Percentage of positive results		Agreement (%)
			ELISA	IFA	
		SENSITIVITY			
1	Pathogenic B. bovis	77	100	96.1	96.1
2	Vaccinal B. bovis	201	99.5	99.5	98.5
3	Soluble antigens	70	97.1	92.8	91.4
4	B. bovis field strains	26	100.0	100.0	100.0
		SPECIFICITY*			
1	B. bigemina	50	94.0	96.0	90.0
2	A. marginale	50	92.0	96.0	88.0
3	Status unknown	100	97.0	97.0	94.0

* All sera used to test specificity were from cattle borne and raised in the *B. microplus* free area of Argentina

TABLE II. COMPARATIVE PREVALENCE OF ANTIBODIES TO *B. BOVIS* IN CATTLE HERDS NATURALLY INFEECTED USING ELISA AND IFA TEST, AND THE AGREEMENT BETWEEN BOTH TECHNIQUES

Herd	n	ELISA (%)	IFA(%)	Agreement (%)
1	25	4	0	96
2	21	14	24	86
3	15	20	20	87
4	25	100	100	100
5	25	8	0	92
6	25	4	4	100
7	25	24	17	88
8	16	100	100	100
9	25	80	80	92
10	25	68	72	88
11	20	100	100	100
12	20	100	100	100
13	17	100	100	100
14	25	8	4	96
15	23	9	9	100
16	25	4	4	100
TOTAL	418			

4. CONCLUSIONS

As expected, the sensitivity of the assay for detection of *B. bovis* antibodies was high. This is in concordance with the results presented by Barry et al [10] and Waltisbuhl et al. [7] testing cattle infected with vaccinal or pathogenic *B. bovis* strains using other ELISA systems. The assay showed a higher sensitivity than IFA in Trial 1 and 3 but no difference was found in Trials 2 and 4.

Cattle vaccinated with *B. bovis* soluble culture antigens (Trial 3), may be true negatives because they might not have been infected with *B. bovis* strains. Different antigens would be present in the immunizing culture extract vs the antigen used in ELISA thus accounting for the inability of ELISA to detect the antibodies to the immunizing antigens.

The specificity of the ELISA and IFA did not reach the same high level as the sensitivity estimates. The false positive reactions with sera of cattle infected with *A. marginale* or *B. bigemina* is disturbing since the geographical distributions of these haemoparasites match with the distribution of *B. bovis* in several American countries [1]. Waltisbuhl et al. [7] concluded that false positive reactions with an ELISA for *B. bovis* in cattle infected with *A. marginale* might be due to common changes in membrane isoantigens of infected red cells. They proposed further purification of the antigen or absorption of sera with a pool of lysate of normal erythrocytes to improve the specificity of the ELISA. The first option appears to be the best since the absorption of sera will add a time consuming step to the assay procedure. Böse et al. [11] dealing with sera of cattle free of *B. bovis* also found nonspecific reactions using an ELISA to diagnose antibodies to this protozoan. These reactions were probably due to contamination of the IgG conjugate with IgM. More research is needed to further improve the specificity of the assay. Alternatives like increasing the cut-off point are not proper because they will cause a commensurate drop in the sensitivity of the ELISA.

The agreement between both techniques (Table II) was good, ranging between 86 and 100%. Fifty percent of the herds showed 100% of agreement. In cases where the agreement between ELISA and IFA was the lowest (86-92%), the prevalence data indicated the same epidemiological status and obviously the same strategic measures were recommended.

Although the specificity of the ELISA needs to be improved this assay can replace the IFA test to detect *B. bovis* antibodies in cattle. The high degree of sensitivity, objectivity, and its capacity to be adapted to test large number of sera in short period of time are advantages not provided by the IFA test. This ELISA will permit an increase of epidemiological studies of babesiosis. This is especially important in developing countries where areas of enzootic instability to babesiosis are ill-defined, precluding the implementation of preventive measures with economical rationality unless the true infection status of the cattle is known.

ACKNOWLEDGEMENTS

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REFERENCES

[1] GUGLIELMONE, A.A., Epidemiology of babesiosis and anaplasmosis in South and Central America, Vet. Parasitol. **57** (1995) 109-119.

[2] DE VOS, A.J., DALGLIESH, R.J., CALLOW, L.L., Soulsby, E.L. (Editor) Immune Response in Parasitic Infections: Immunology, Immunopathology, and Immunoprophylaxis Protozoa, CRC Press, Boca Raton, FL **3** (1987) 183-222.

[3] GUGLIELMONE, A.A., AGUIRRE, D.H., SPÄTH, E.J.A., GAIDO, A.B., MANGOLD, A.J., DE RÍOS, L.G., Long-term study of incidence and financial loss due to cattle babesiosis in an Argentinean dairy farm, Prev. Vet. Med. **12** (1991) 307-312.

[4] MAHONEY, D.F., WRIGHT, I.G., GOODGER, B.V., A note on the transmission of Babesia bovis (Syn. Babesia Argentina) by the one-host tick, Boophilus microplus, Aust. Vet. J. **55** (1979) 10-12.

[5] SMITH, R.D., OSORNO, B.M., BRENER, J., DE LA ROSA, R., RISTIC, M., Bovine babesiosis: severity and reproducibility of *Babesia bovis* infections induced by Boophilus microplus under laboratory conditions, Res. Vet. Sci., **24** (1978) 287-292.

[6] GOFF, W.L., JOHNSON, W.C., KUTTLER, K.L., Development of an indirect fluorescent antibody test, using microfluorometry as a diagnostic test for bovine anaplasmosis, Am. J. Vet. Res. **46** (1985) 1080 -1084.

[7] WALTISBUHL, D.J, GOODGER, B.V., WRIGHT, I.G., COMMINS, M.A., MAHONEY, D.F., An enzyme linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle, Parasitol. Res. **73** (1987) 126-131.

[8] FAO-IAEA, Babesiosis indirect ELISA kit, Bench protocol version, BBO-1.2 (1987) pp. 37.

[9] DE RÍOS, L.G., AGUIRRE, D.H., GAIDO, A.B., Evaluacíon de la dinamica de la infeccíon por *Babesia bovis* y *Babesia bigemina* en terneros, Diagnóstico por microscopía directa y prueba de inmunofluorescencia indirecta, Rev. Med. Vet. (Buenos Aires) **69** (1989) 254-260.

[10] BARRY, D.N., RODWELL, B.J., TIMMS, P., McGREGOR, W., A microplate enzyme immunoassay for detecting and measuring antibodies to *Babesia bovis* in cattle serum, Aust. Vet. J., **59** (1982) 136-140.

[11] BÖSE, R., JACOBSON, R.H., GALE, K.R., WALTISBUHL, D.J., WRIGHT, I.G., An improved ELISA for the detection of antibodies against *Babesia bovis* using either a native or a recombinant *B. bovis* antigen, Parasitol.Res., **76** (1990) 648-652.

A COMPARATIVE STUDY OF AN ELISA TEST AND AN INDIRECT IMMUNOFLUORESCENCE TEST FOR SEROLOGICAL DIAGNOSIS OF *BABESIA BOVIS* INFECTION

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Abstract

A COMPARATIVE STUDY OF AN ELISA TEST AND AN INDIRECT IMMUNOFLUORESCENCE TEST FOR SEROLOGICAL DIAGNOSIS OF *BABESIA BOVIS* INFECTION.

Detection of antibodies to *Babesia bovis* in cattle is essential for the understanding of the epidemiology of babesiosis and this study was concerned with comparing the indirect fluorescent antibody with the ELISA. Both assays gave rise to 100% sensitivity whilst the ELISA was shown to be marginally more specific at 98%. The ease of use and low cost of the ELISA would make it the more obvious choice in conducting future serological surveys for this parasite.

1. INTRODUCTION

Detection of antibodies to *Babesia bovis* in cattle is essential for the understanding of the epidemiology of babesiosis: Serological tests which have been commonly used include complement-fixation (CF) [1], indirect fluorescent antibody (IFA) [2] and inhibition of hemaglutination (IHA) [3]. More recently, enzyme linked immunoassays (ELISA) have been applied to the serological diagnosis of a number of haemoparasite infections of medical and veterinary importance throughout the world [4]. These tests have been shown to be more specific than those used previously [5]. Barry et al. [6] described the comparison of ELISA and IFA tests with known positive sera where they found more than 95% agreement between the two methods with the ELISA displaying a slightly better sensitivity.

Waltisbuhl et el. [7] reported an improved sensitivity in the ELISA by using horseradish peroxidase instead of alkaline phosphatase in the test. Johnston et al. [8] described an IFA technique which with some modifications, is being used routinely in our laboratory at the Instituto de Pesquisas Veterinárias Desidério Finamor, Porto Alegre, RS, Brazil.

The advantages of the ELISA over IFA are: a) it is more quantitative and not subjective, b) more tests can be performed in a given time, c) it is more sensitive. All these are important considerations when an epidemiological study of an infected region such as the state of Rio Grande do Sul is contemplated.

In this report we describe the comparison of an ELISA for detection of antibodies to *B. bovis* and an IFA test. The viability of the ELISA system for use as an epidemiological tool is also evaluated.

2. MATERIALS AND METHODS

2.1. Field serum samples

One thousand five hundred sixty bovine sera from two regions (Livramento and Bage) in the State of Rio Grande do Sul, southern Brazil, where babesiosis is considered to be enzootic, were collected and stored at -20 °C prior to testing.

2.2. Reference sera

In order to check the specificity of the ELISA, serum samples were collected from 97 cattle, living in one area free of the cattle tick vector, *Boophilus microplus*, and found previously negative on IFA tests, considered at the time as standard. In addition light microscopic examinations of blood smears were also performed which demonstrated the absence of circulating parasites. Sera from 22 known positive animals (as demonstrated by the presence of parasites in blood smears) were obtained from calves experimentally infected with a local strain of *B. bovis* (strain IPV1).

2.3. IFA test: (Preparation of the IFA antigen)

A splenectomized calf was inoculated with *B bovis* parasites At the peak of parasitemia (0 5% of parasitized erythrocytes at day 5), 100 ml of blood was withdrawn and immediately mixed with anticoagulant (4 5% sodium citrate) This was washed twice in PBS and centrifuged at 1,000 g with intervals of 10 minutes between washing Sediment was passed through a fibrous cellulose powder (CF 11, Whatman) and inoculated intravenously into a second splenectomized calf When the parasitaemia reached 5-7%, 500 ml of blood was collected and mixed with anticoagulant at a ratio of 7 1 (v/v) The blood so obtained was then washed three times in PBS with intermittent centrifuge spins at 1,000 g for 15 minutes The washed erythrocytes were finally resuspended in PBS to give a packed cell volume of about 50% Thin unfixed films were made from that suspension on cleaned microscope slides, dried at room temperature, wrapped in tissue paper, and covered with aluminium foil before being stored at -20 °C

2.4. IFA test procedures

The IFA tests were performed as previously described [8] Previous to testing, all sera were diluted 1 40 in PBS The conjugate was anti-bovine IgG fluorescein isothiocyanate (produced in rabbit, by Sera-lab, UK) diluted 1 60 in PBS

2.5. ELISA test

The ELISA reagents were those supplied by the Joint FAO/IAEA Division of the International Atomic Energy Agency (IAEA), Vienna, Austria They were used as specified in the ELISA kit for detection of *B bovis* and all the tests procedures were according to the recommendations by the Joint FAO/IAEA Division, included in the reference kit Basically in this test, the antigen dilution used was 1 600 in carbonate bicarbonate buffer, pH 9 6, and the sera to be tested was diluted 1 200 in PBS with 0 05% tween-20 (PBS-T) with 5% of powder milk Rabbit anti-bovine IgG conjugated with horse radish peroxidase (provided by the IAEA) was used diluted in PBS-T plus 5% of powder milk The substrate used was orthophenyldiamine (OPD) and as stopper, a solution of $1NH_2SO_4$ was used Flat-bottomed microplates (Linbro, Flow laboratories, USA) were used throughout and read at optical density of 492 nm in a microplate reader (Titertek Multiskan, Flow laboratories) Sensitivity and specificity was calculated using the formula presented in Table II

3 RESULTS

3.1. ELISA/IFA comparison

The results obtained with the ELISA and IFA techniques on the 1560 field samples examined are shown in Table I and Figure 1 There was a 90 1% (1406/1560) agreement between positive and negative results in both tests The remainder 9 9% were represented by samples which were 4 2% (66) positive for IFA and negative for ELISA and samples which were 5 6% (88) negative for IFA and positive for ELISA

TABLE I COMPARATIVE RESULTS BETWEEN IFA AND ELISA ON FIELD SAMPLES TESTED FOR DETECTION OF ANTIBODIES AGAINST *BABESIA BOVIS*

IFA +/-	ELISA +/-	IFA/ELISA +/-	IFA+ ELISA-	IFA- ELISA+	Total
1094/466	1116/444	1028/378	66	88	1560

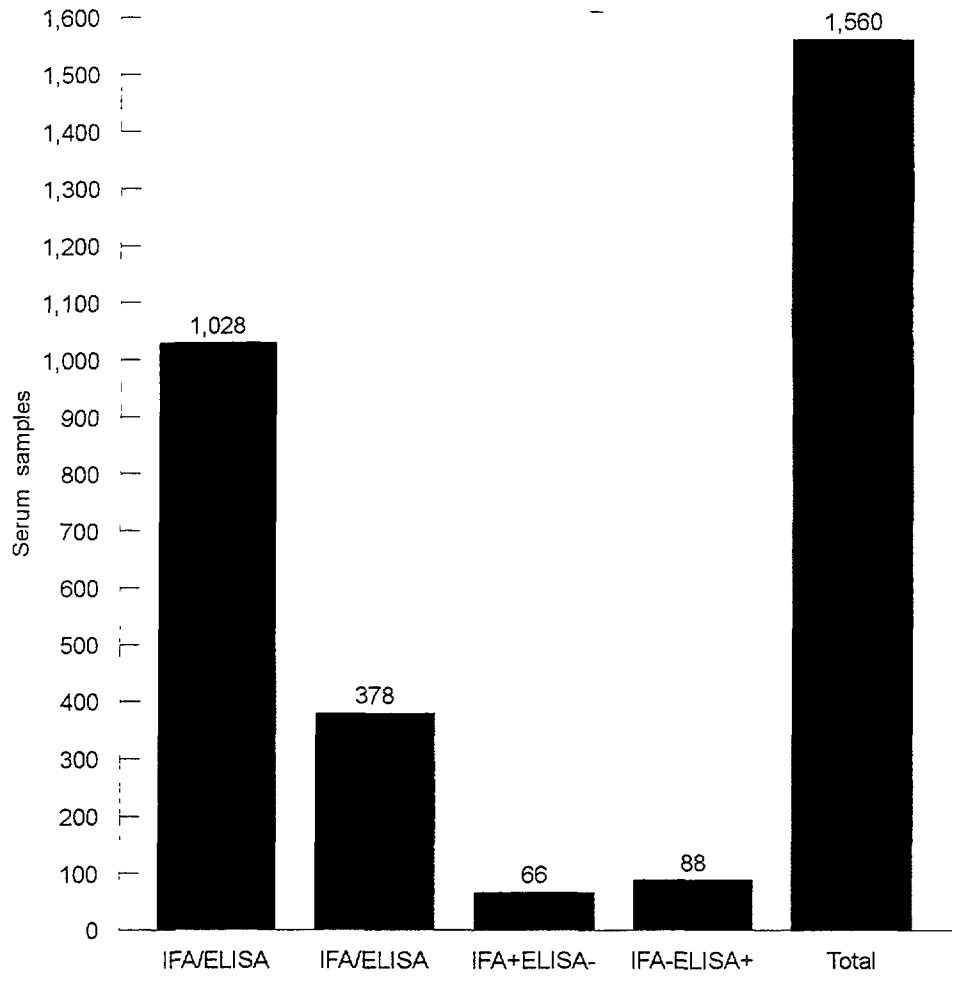

FIG. 1. Comparative results between IFA and ELISA for Babesia bovis of field samples.

3.2. Specificity and sensitivity

Of 97 examined known negative sera, specificity was shown to be 97.9% for IFA and 98.9% for ELISA. The 22 sera known to contain antibodies to *B. bovis* were all positive in both ELISA and IFA.

TABLE II. CALCULATION OF SPECIFICITY AND SENSITIVITY

Test status	True status		Totals	
	Diseased	Not diseased		
Diseased	a	b	a	b
Not diseased	c	d	c	d
Totals	a + c	b + d	a + b + c + d	

Sensitivity = a/ (a + c) Specificity = d/ (b + d)

4 DISCUSSION

The introduction of washing erythrocytes before a second inoculation into a splenectomized calve, seems to abbreviate timing of *B bovis* parasitaemia The inoculation of a number of leucocytes from the first calve into a second one is markedly reduced, decreasing possibilities for immediate antibody production which probably interferes in the quality of the produced antigen

An explanation for the 9 1% of different results between IFA and ELISA might be the possibility of cross-reaction with other haemoprotozoa (*B bigemina* or *Anaplasma marginale*) previously reported by Waltisbuhl et al [7] In our study concurrent infection may have accounted to such discrepancies Also false positives reactions to *B bovis* were observed in sera from cattle infected with *A marginale* ELISA has been reported as more sensitive than IFA [6] and this can explain the few more positives samples detected by this technique (88 positive by ELISA and negative by IFA) The use of a more sensitive technique is usually desirable This seems to be the case of the ELISA kit provided by Joint FAO/IAEA Division

REFERENCES

[1] MAHONEY, D F , Bovine babesiosis diagnosis of infection by a Complement Fixation Test, Aust Vet J , **38** (1962) 48-52

[2] ROSS, J P J , LOHR, K F , Serologic diagnosis of *Babesia bigemina* infection in cattle by the Indirect Fluorescent Antibody Test, Res Vet Sci , **9** (968) 557-562

[3] GOODGER, B V , Preparation and preliminary assessment of purified antigens in the passive hemagglutination test for bovine babesiosis Aust Vet J , **47** (1971) 251-256

[4] VOLLER, A , BIDWELL, D E , BARTELETT, A , The enzyme linked immunosorbent assay (ELISA) and its applications, Dynatech Europe, Guernsey, GB, (1977)

[5] TODOROVIC, R A , CARSON, C A , Methods for measuring the immunological response to *Babesia* Topics in babesiosis, **12** (1981) 381-408

[6] BARRY, D N , RODWELL, B J , TIMMS, P , McGREGOR, W A , A microplate enzyme immunoassay for detecting and measuring antibodies to *Babesia bovis* in cattle serum, Austr Vet J , **59** (1982) 136-140

[7] WALTISBUHL, D J ,GOODGER, B V , WRIGHT, I G , COMMINS, M A , MAHONEY, D F , An enzyme linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle, Paras Res , **73** (1987) 126-131

[8] JONHSTON, L A Y , PEARSON, R D , LEATCH, G , Evaluation of an indirect fluorescent antibody test for detecting *Babesia argentina* infection in cattle, Aust Vet J , **49** (1973) 373-377

EVALUATION OF AN ELISA KIT IN THE SEROLOGICAL DIAGNOSIS OF *BABESIA BOVIS* FOR EPIDEMIOLOGICAL STUDIES

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Abstract

EVALUATION OF AN ELISA KIT IN THE SEROLOGICAL DIAGNOSIS OF *BABESIA BOVIS* FOR EPIDEMIOLOGICAL STUDIES

An enzyme linked immunosorbent assay (ELISA) kit for detect antibodies to *Babesia bovis*, an intraerytrocitic bovine parasite was evaluated using known negative and positive samples and the results were compared with an indirect immunofluorescent antibody test (IFAT) Results obtained with field samples were used to estimate seroprevalence of *B bovis* in an endemic area to the cattle tick (*Boophilus microplus*) vector of bovine babesiosis Percentage of positivity (PP) values (optical density of tested serum/mean optical density of positive control) on 274 negative samples. had major values ranged in the frequency of 4 0 to 7 0 PP Comparison between ELISA and IFAT showed an agreement of 93 3% on field sera samples, collected in areas of low, good and high soil fertility in the region of Bage (31^0 20'13''S, 54^0 06' 21''W), RS, Brazil From 5,082 tested sera, 3,751 (73%) were positive for *B bovis* antibodies No significant difference ($p>0$ 05) was observed between results from calves living in areas of low and good soil fertility (80 and 82% of seroprevalence, respectively) However, calves living in soil of high fertility showed a minor inoculation rate for *B bovis*, (63% of seroprevalence) indicating needs of measures to prevent losses due to babesiosis

1 INTRODUCTION

The use of the enzyme linked immunosorbent assay (ELISA) techniques since its first description in 1971 [1] is widely distributed for detect antigens and antibodies Briefly, the assay involves 1) the adsorption of antigen or antibody to a solid phase, 2) the addition of sample, 3) incubation and washing steps, 4) addition of enzyme labelled antigen or antibody, and 5) the addition of the enzyme substrate The first ELISA technique for detecting antibodies against a bovine haemoparasite was described in 1976 [2] for *Babesia divergens* Barry et al [3] described the first ELISA for detecting antibodies to *B bovis* showing agreement of more than 95% with the Indirect Fluorescent Antibody Test (IFAT) in a comparative study Also the assay was able to detect *B bovis* antibody 14 days after experimental infection showing more sensitivity than the IFAT Waltisbuhl et al [4] described an ELISA for *B bovis* claiming more sensitivity than the IFAT and using horseradish peroxidase rather than alkaline phosphatase as labelled enzyme

In order to introduce this technique and make it accessible for several laboratories in the world, the Joint FAO/IAEA Division, through its Animal Production and Health Section standardized an ELISA kit and distributed it to laboratories in Latin American countries

This report refers to the results obtained with the ELISA kit for *B bovis* under an FAO/IAEA Research Contract (No 6522) tested at the "Instituto de Pesquisas Desidério Finamor"

2 MATERIALS AND METHODS

2.1. Reference sera samples

Aiming to establish a catalogue of serum bank, 274 samples from a herd of a tick-free area (Santa Vitória do Palmar, extreme south of the State of Rio Grande do Sul) were collected Also blood

samples were obtained and stained by Giemsa for direct observation of haemoparasite These sera being negative on IFAT and negative by light microscopic examination, were taken as negative reference samples A total of 97 sera samples from *babesia*-vaccinated cattle with circulating *B bovis* hemoparasites seen by light microscopic were collected and stored at -20°C prior testing The specificity and sensitivity of the test was based in the results obtained with these sera samples

2.2. Field samples

A survey for *B bovis* antibodies was carried out in the region of Bagé, State of Rio Grande do Sul (31° 20' 13" S, 54° 06' 21" W, 216 m) where 5082 sera samples from calves age between 10 and 14 months-old were collected The farms (68) were selected from three different zones according to type of soil (low, good and high fertility) and 20 calves selected at each farm

2.3. Serological assay

The ELISA kit for *B bovis* antibody was used as specified in the FAO/IAEA manual In order to compare some serological results, an immunofluorescent antibody test (IFAT) for *B bovis* locally produced and used routinely in the parasitology section at IPVDF was taken as reference Herds with a prevalence rate between 15% and 80% were considered to be at risk from babesiosis outbreaks due to *B bovis*

3 RESULTS

Figure 1 shows the distribution of percentage of positive values (PP) obtained with the negative reference samples for *B bovis* Major of these values ranged in the frequency of 4 0 to 7 0 PP being that an average of 4 96 PP was observed on 274 examined sera from negative cattle

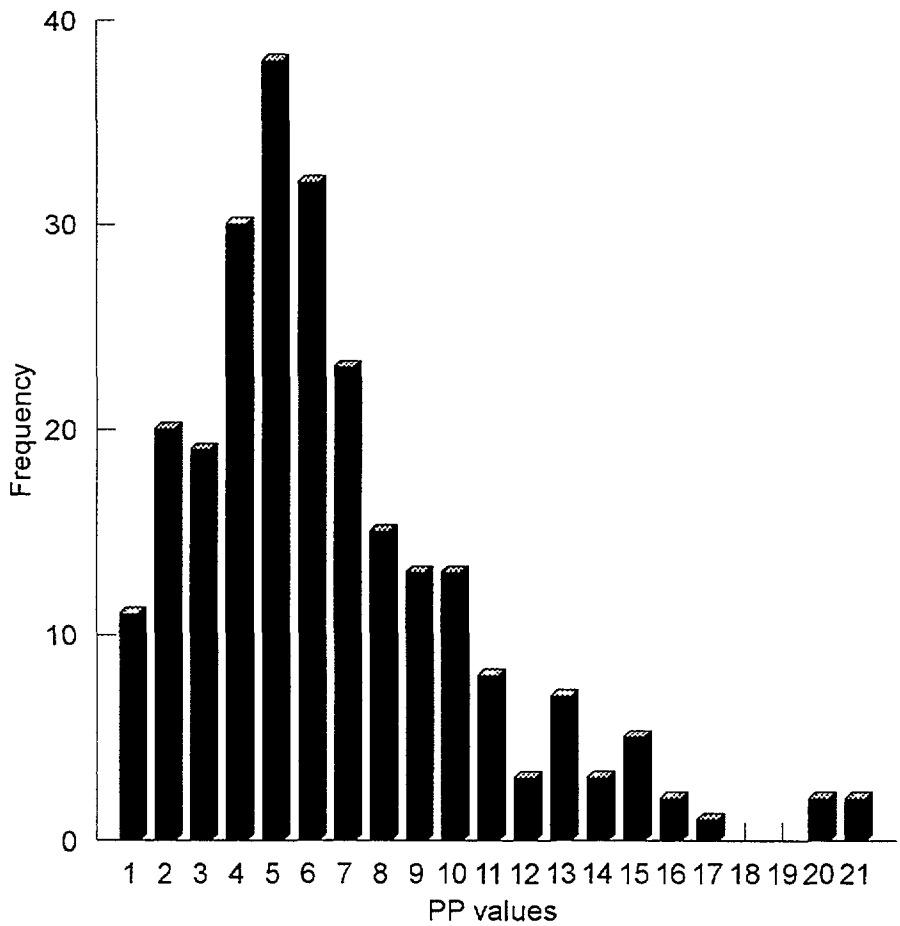

FIG 1 Distribution of PP values for an ELISA kit of B. bovis (values for known negative samples)

A comparative picture (Figure 2) between IFAT and the ELISA kit on field samples showed an agreement of results in 93 3% of the tested samples Nevertheless 3 9% were positive by ELISA and negative by IFAT and 2 7% were positive by IFAT and negative by ELISA

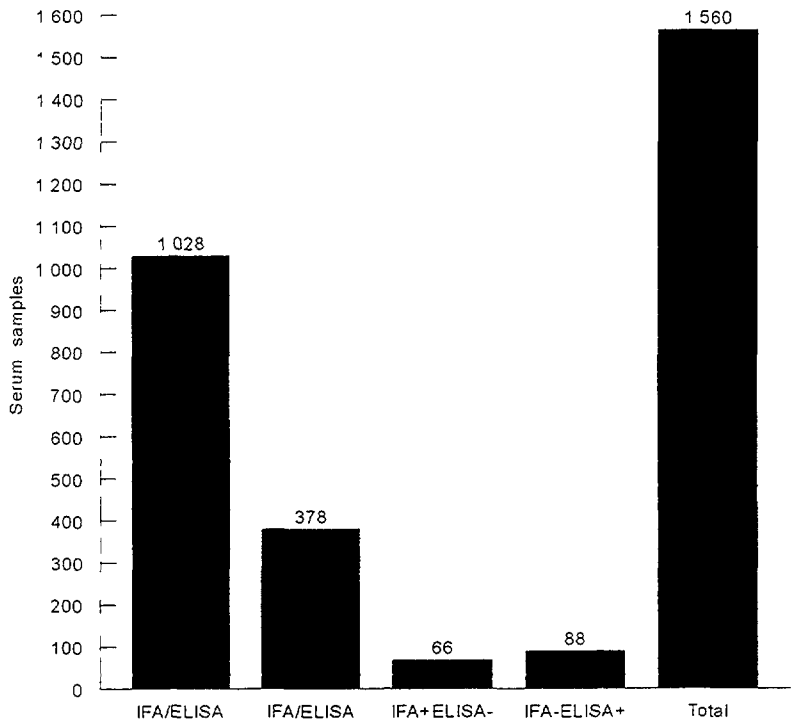

FIG 2 Comparative results between IFA and ELISA for Babesia bovis on field samples

The results with 5082 field samples (Figure 3) showed that 3.751 (73 8%) were positive for antibody against *B bovis* From the three zones, percentages of 80%, 82% and 63% of prevalence were observed respectively for soils with low, good and high fertility

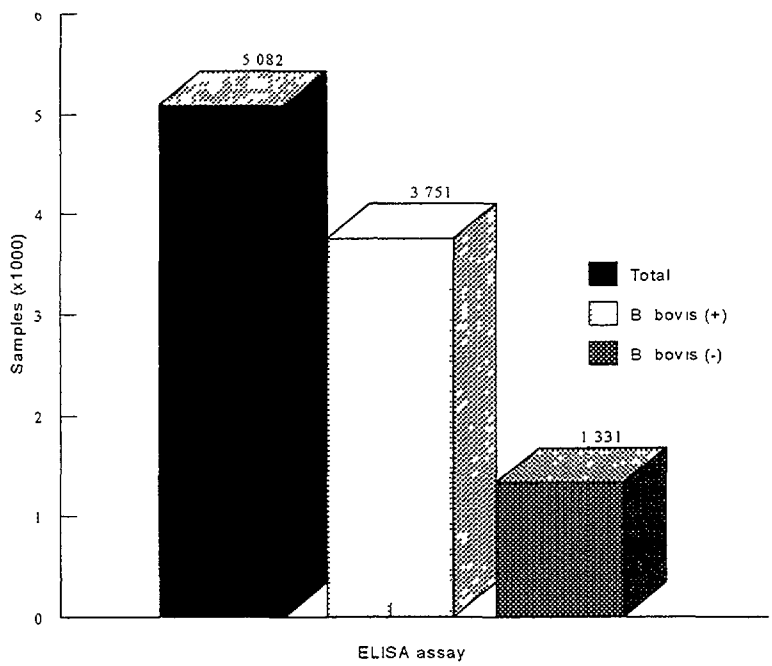

FIG 3 Epidemiology of TBD in RGS state Brazil, Survey in Bagé, RGS

No significant difference ($p>0.05$) was observed between results from calves living in areas of low and good soil fertility. Calves in both these areas were in an area of enzootic stability for babesiosis, since the cattle tick vector *(Boophilus microplus)* is endemic. However, when improving soil fertility and the changes in the pastures management, a lower number of cases was seen in calves indicating a difference in the inoculation rate for *B bovis*.

4 DISCUSSION

The comparison between ELISA and IFAT demonstrated an agreement of 93.3% and revealed the value of the ELISA system which is especially suitable for screening large numbers of samples. Results are expressed in optical density (OD). Thus defined positive and negative values can be expressed and used for further comparisons. For epidemiological surveys this method is very useful and highly applicable. The known negatives and positives samples tested showed the high sensitivity and specificity of the technique, although cross reactions with *B bigemina* and *Anaplasma marginale* were not tested. Sera from animals after 14 days after inoculation with *B bovis* attenuated strain gave positive results for antibody. The antigen dilution recommended (1/200) and the sera dilution (1/200) worked well in our conditions.

Nevertheless a few differences were observed with regard to conjugate titration (variations from 1/9000 to 1/13000 were detected). As described by Mahoney et al. [5] it is possible to predict areas where low and good soil fertility are found, and where a situation of enzootic stability for babesiosis occurs. However, in the area where the soil is very fertil, an enzootic instability was found, indicating that measures to prevent losses due to *B bovis* should be adopted.

REFERENCES

[1] ENGVALL, E., PERLMANN, P., Enzyme linked immunosorbent assay, ELISA III, Quantification of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes, J. Immunol., **109** (1972) 129-135.

[2] PURNELL, R.E., HENDRY, D.J., BIDWELL, TURP, P., Microplate enzyme linked immunosorbent assay for antibody to *Babesia divergens* in cattle, Vet. Rec., **99** (1976) 102.

[3] BARRY, D.N., RODWELL, B.J., TIMMS, P., McGREGOR, W., A microplate enzyme immunoassay for detecting and measuring antibodies to *Babesia bovis* in cattle serum. Aust. Vet. Jou., **59** (1982) 136-140.

[4] WALTISBUHL, D.J., GOODGER, B.V., WRIGHT, I.G., COMMINS, M.A., MAHONEY, D.F., An enzyme linked immunosorbent assay to diagnose *Babesia bovis* infection in cattle, Parasitol. Res., **73** (1987) 126-131.

[5] MAHONEY, D.F., MIRRE, G.B., Bovine babesiosis estimation of rates in the tick vector *Boophilus microplus (Canestrini)*, Ann. Trop. Med. Parasitol. **65** (1971) 309-317.

SOME ASPECTS OF THE EPIDEMIOLOGY OF *BABESIA BOVIS* IN SANTANA DO LIVRAMENTO, SOUTHERN BRAZIL

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Abstract

SOME ASPECTS OF THE EPIDEMIOLOGY OF *BABESIA BOVIS* IN SANTANA DO LIVRAMENTO, SOUTHERN BRAZIL

Some aspects of the epidemiology of *Babesia bovis* were studied in Santana do Livramento, Rio Grande do Sul, Brazil by analysing cattle raising practices applied to 101 herds and by diagnosing *B bovis* antibodies in cattle of about 11 months old using an enzyme linked immunosorbent assay Herds with prevalence of antibodies ranging between 15% to 80 % were considered at risk of babesiosis outbreaks of economic importance (enzootic instability) Fifty three per cent of herds were found in enzootic instability to *B bovis* The proportion of *Bos taurus and B indicus x B taurus herds* in instability were similar (P=0 771, chi square) and the number of acaricides treatments applied yearly had no influence in the instability to *B bovis (P*= 0 866, chi square) Herds maintained along with sheep in a ratio < 1 5 had greater chances to be in enzootic stability due to high antibody prevalence than herds kept under ovine/bovine ratio > 1 5 (P= 0 012, chi square), this probability was further increased in herds maintained on properties greater than 500 ha (P= 0 057, chi square) High *B bovis* antibody prevalence was found in *B taurus x B indicus* herds subjected to an average of 5 8 tick treatments yearly with long residual period acaricides, indicating misuse of the chemicals or tick resistance to them The epidemiological situation to *B bovis* seems to justify vaccination to avoid economic losses in herds in enzootic instability and those in enzootic stability due to low antibody prevalence

1 INTRODUCTION

Babesiosis *(Babesia bovis* and *Babesia bigemina)* is the most important cattle disease transmitted by *Boophilus microplus* ticks in tropical and subtropical areas in South America [1] Calves are protected by non-specific immunity until about seven months of age [2,3] Infection during this period induces a long-lasting immunity, whereas primary infection later in life can produce severe illness [4,5] Therefore the likelihood of babesiosis outbreaks can be indirectly measured by detecting the proportion of infected calves via diagnosing babesial antibody prevalence [6]

The cattle industry is economically important in southern Brazil, where this activity is generally not integrated with agriculture *Bos indicus* x *Bos taurus* cattle, which are generally more resistant to *B microplus* infestations than *B taurus* breeds [7,8,9] are commonly grazed in this region Moreover, cattle and sheep are usually raised together This is relevant in that most of *B microplus* larvae picked up by sheep will not complete their life cycle Therefore, the use of sheep has been suggested as an aid to tick biological control, thereby diminishing the use of acaricides [10]

Farmers and veterinary practitioners claim that under the conditions above, "tristeza parasitária" (a regional term used to describe cattle babesiosis and anaplasmosis) is a serious economic problem, but epidemiological information on this disease complex is scarce In order to broaden our knowledge about this disease, cattle raising practice were obtained through a questionnaire, together with cattle sera that were processed by an enzyme linked immunosorbent assay (ELISA) to determine antibodies to *B bovis*

2. MATERIALS AND METHODS

A questionnaire was sent to 101 livestock owners to determine their opinion on the importance of tick-borne diseases in their herds, and to relate *B. bovis* seroprevalence to: 1) cattle biotypes (*B. taurus, B. taurus* x *B. indicus* or *B. indicus);* 2) property size, discriminated as larger or smaller than 500 ha; 3) ovine/bovine ratio > or < 1.5; 4) number and type of annual acaricide treatments. The herds were located to the north-east of the town of Santana do Livramento forming a circle to the south-west. The maximum distance from the town to a herd was 100 km.

For *B. bovis* serology, a minimum of 20 blood samples from each herd was obtained from cattle with an age of approximately 11 months. Serological tests were performed as described elsewhere [11] with an enzyme linked immunosorbent assay (ELISA) provided by the Joint FAO/IAEA Division. This ELISA was used after confirming an agreement of > 90% with the traditional immunofluorescent antibody test. Herds with prevalence of *B. bovis* between 15 % to 80 % were considered in a high risk area in relation to the possibility of suffering babesiosis outbreaks of economic importance [6]. Chi square test was used for statistical analysis.

3. RESULTS

Not all questionnaires were completely filled out. Therefore the total number of answers for each question varied. Ninety percent of farmers said that "tristeza parasitária" was a problem in their herds, mainly in cattle older than 12 months. Fifty four herds (53 % of the total) were within the area of enzootic instability to *B. bovis*. Of those 47 herds (47% of the total) in enzootic stability, 41 were in this conditions because of *B. bovis* antibodies prevalence > 80 %, and the remainder due to antibody prevalence < 15 %.

The majority of herds were *B. taurus x B. indicus* (n=82) or *B. taurus* (n= 16), 53 % and 51 % of them were in enzootic instability, respectively (P= 0.777, chi square). All herds were treated against ticks using plunge dips; 81% of the grazers used pyrethroids and 19 % formamidinic compounds. The majority of the herds were treated 4-6 times/year but this had no influence on their epidemiological status.

The size of the farms did not affect the epidemiological situation per se. Twenty two farms (26% of the total) had less than 500 ha and 64 (74 %) had over 500 ha. In both cases 55 % of the herds were in enzootic instability.

The analysis of herds grazed along with sheep at a sheep:cattle ratio > or < 1.5 showed that 17 herds (59 %) of the total of 29 herds kept < 1.5 ratio were in a stable situation due to high antibodies prevalence to *B. bovis*. The same situation was found in 30% (17 herds from a total of 56) for those herds with a ratio > 1.5 (p=0.012); five herds in this ratio were in enzootic stability due to low antibody prevalence.

In the strata of 29 herds maintained under the ovine/bovine ratio < 1.5 showed that 28 of them were treated for ticks at least four times yearly using pyrethroids in 26 cases. Thirteen herds (76%) of the 17 herds in enzootic stability were kept in farms bigger than 500 ha, whereas 5 herds (42 %) of the 12 in enzootic instability were from properties of this size (P = 0.057). Again, the number of treatments had no influence on the epidemiological status to *B. bovis*. The 17 herds in enzootic stability due to high antibody prevalence received a mean number of 5.8 +/- 1.03 acaricide treatments per year, while the 12 herds in enzootic instability were treated 5.0 +/- 1.22 times yearly.

4. DISCUSSION

The epidemiology of *B. bovis* appears to follow a classical model [6] in Santana do Livramento, according to the serological data obtained and the opinion of farmers that "tristeza parasitária" usually affects cattle older than one year. This differs from the situation in another region of Brazil (Mato Grosso do Sul), where babesiosis is mainly a problem in cattle younger than seven months [12].

The proportion of herds in enzootic instability to *B. bovis* was high showing regions under a risk of widespread outbreaks of babesiosis. Although the percentage of herds in instability was lower than the figure obtained from the questionnaires in relation to "tristeza parasitária" this may be a result of an overestimation of the problem or that the combination of the outbreaks of *B. bovis, B. bigemina* and *Anaplasma marginale* could result in a problem of the magnitude indicated in the farmers questionnaire. The last situation is the most probable as was shown by Spath [13] in Argentina, where 45 % of the outbreaks were due to anaplasmosis, 8 % to *B. bigemina,* 34 % to *B. bovis* and 13 % due to combinations of both species of *Babesia.*

Nevertheless, the reasons leading to the enzootic instability were not obvious. In the most favourable area for *B. microplus* development in Argentina (border with south Brazil), enzootic instability to *B. bovis in B. taurus x B. indicus* cattle was related to the use of long residual effect acaricides such as pyrethroids [14] that severely depressed tick populations [1]. Therefore it was expected that under high acaricidal pressure with pyrethroids, *B. taurus* x *B. indicus* herds maintained with sheep would usually be in enzootic instability to *B. bovis* or in enzootic stability due to low antibody prevalence.

A superficial analysis appears to support this prediction since 59 % of the herds were in enzootic instability or having antibody prevalences lower than 15 %. Nevertheless the number of tick did not influence the epidemiological situation, and 13 herds maintained in properties larger than 500 ha and under a sheep:cattle ratio < 1.5, but treated almost 6 times/year (most of them with pyrethroids) showed antibody prevalence higher than 80%. These facts show that the assumption was erroneous for a large number of herds. Probably the acaricides were misused or tick resistance is starting to be a problem, as pointed out by Evans [10]. An alternative explanation could be that a high proportion of *B. microplus* larvae are infected with *B. bovis* in southern Brazil. However, there is no local information to support this statement while studies carried out in Australia showed that this rate is extremely low [15].

The sheep:cattle ratio was the parameter most closely related to the epidemiological situation to *B. bovis.* Herds maintained under a ratio < 1.5 had greater chances to be in enzootic instability, especially in properties larger than 500 ha. This may result from a combination of the lower cattle ratio of < 1.5 on tick populations in comparison with higher ratios, difficulties to gather all cattle for tick treatment coupled with bad management of the acaricides and/or tick resistance.

Although further studies are needed to understand the epidemiology of *B. bovis* it appears that the problem justifies vaccination to prevent losses [3]. If this is implemented, herds in enzootic instability due to low antibody prevalence will have to be included in vaccination programmes because any increase in the *B. bovis* inoculation rate may result in devastating outbreaks [16]. Similar studies on the epidemiology of *B. bigemina* and *Anaplasma marginale* are needed to cover all the range of *B. microplus*-transmitted diseases in the region.

REFERENCES

[1] GUGLIELMONE, A.A., Epidemiology of babesiosis and anaplasmosis in South and Central America, Vet. Parasitolog. **57** (1995) 109-119.

[2] DE VOS, A.J., DALGLIESH, R.J., CALLOW, L.L., *Babesia.*In: Immune Response in Parasitic Infections: Immununology, Immunopathology and Immunoprophylaxis, E.J.L. Soulsby (Editor), Vol. 3. Protozoa. CRC Press, Boca Raton, Fl, USA. (1987) pp. 183-222.

[3] GUGLIELMONE, A.A., AGUIRRE, D.H., SPATH, E.J.A., GAIDO, A.B., MANGOLD, A.J., DE RIOS, L.G., Long term study of incidence and financial loss due to cattle babesiosis in an Argentinean dairy farm, Prev. Vet. Med. **12** (1992) 307-312.

[4] MAHONEY, D.F., WRIGHT, I.G., MIRRE, G.B., Bovine babesiosis: The persistence of immunity to *Babesia argentina* and *B. bigemina* in calves *(Bos taurus)* after naturally acquired infection, Ann. Trop. Med. Parasitol. **67** (1973) 197-203.

[5] MAHONEY, D. F., WRIGHT, I.G., GOODGER, B. V., Immunity in cattle to *Babesia bovis* after single infections with parasites of various origin, Aust. Vet. J. **55** (1979) 10-12.

[6] FAO, Ticks and tick-borne diseases control. A practical field manual Vol. II. Tick- borne disease control. FAO, Rome, (1984) pp. 301-621.

[7] LEMOS, A.M., TEODORO, R.L., OLIVEIRA, G.P., MADALENA, E.F., Comparative performance of six Holstein-Friesian x Guzera grades in Brazil, 3. Burdens of *Boophilus microplus* under field conditions, Anim. Prod. **41** (1985) 185-191.

[8] SUTHERST, R.W., MAYWALD, G.F., BOURNE, A.S., SUTHERLAND, J.D., STEGEMAN, D.A., Ecology of the cattle tick *(Boophilus microplus)* in Australia. II. Resistance of different breeds of cattle, Aust. J. Agric. Res. **39** (1988) 299-308.

[9] GUGLIELMONE, A.A., MANGOLD, A.J., GAIDO, A.B., AGUIRRE, D.H., Natural parasitism with *Boophilus microplus* (Acari: lxodidae) in cattle biotypes with different proportions of *Bos taurus* (Spanish and British) and *Bos taurus* genes, Res. Rev. Parasitol. **53** (1992) 77-82.

[10] EVANS, D.E., Tick infestation of livestock and tick control methods in Brazil: A situation report, Insect. Sci. Applic. **13** (1992) 629-643.

[11] FAO/IAEA., Babesiosis ELISA kit manual. Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Austria (1992).

[12] MADRUGA, C.R., BERNE, M.E.A., KESSLER, R.H., GOMES, R.F.C., LIMA, J.G., SCHENK, M.A.M., Diagn6stico da tristeza parasitária bovina no estado de Mato Grosso do Sul: inquérito de opinião, Min. Agric., Emp. Bras. Pesq. Agropec., Cent. Nac. Gado Corte, Circ. Tec. **18** (1986) 32 pp.

[13] SPATH, E.J.A., Un estudio epidemiologico de babesiosis y anaplasmosis en el Valle de Lerma, provincia de Salta. Rev. Med. Vet. (Buenos Aires) **67** (1986) 274-276, 278-281.

[14] MANGOLD, A.J., MARTINO, J.C., VIÑABAL, A.E., GAIDO, A.B., AGUIRRE, D.H. GUGLIELMONE, A.A., Efecto de la flumetrina aplicada por derrame dorsal para el control de infestaciones naturales de *Boophilus microplus* (Can.1988), Vet. Arg. **5** (1988) 108-110-115.

[15] MAHONEY, D.F., MIRRE, G.B., Bovine babesiosis: estimation of rates in the tick vector *Boophilus microplus* (Canestrini), Ann. Trop. Med. Parasitol. **65** (1971) 309-317.

[16] GUGLIELMONE, A.A., Epidemiologia y prevenci6n de los hemoparasitos *(Babesia y Anaplasma)* en la Argentina, In: "Enfermedades Parasitárias de Importancia Economica en Bovinos, Hemisferio Sur, Montevideo, A. Nari y C. Fiel (Editors) pp461-479.

VALIDATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN EL SALVADOR

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Abstract

VALIDATION OF AN INDIRECT ELISA FOR THE DIAGNOSIS OF *BABESIA BOVIS* IN EL SALVADOR

Validation and a preliminary serological study of *Babesia bovis* was made in El Salvador, using the indirect ELISA kit provided by the Joint FAO/IAEA Division of the International Atomic Energy Agency Sera were collected from 545 cattle involving 10 regions of the country and various ages of cattle between 8 and 16 months These were tested from May 1993 to February 1994 A 79 5% prevalence was found, but with a wide range from (5 8-100%), explained by different farm managing systems and different breeds

1 INTRODUCTION

Babesiosis is a tick-borne transmitted disease of cattle The two major tropical species affecting cattle are *B bovis* and *B bigemina.* The infection is characterised by high fever, anaemia, icteria, haemoglobinuria and death in susceptible animals The importance of babesiosis in cattle is the economic loss, especially in tropical and subtropical countries Because of favourable climatic conditions, *Boophilus microplus,* the vector for these parasites is widely found in El Salvador [1,2]

Madrugan et al. [3] found that babesiosis can cause death in calves, but Smith et al [4] considered the disease to be more dangerous to cattle older than 9 months In research studies conducted in Australia, Bartholomew and Callow [5] showed that 76 % of mortality occurs in bovines older than one year In Argentina, Spath [6] stated that 84% of the *B bovis* and *B bigemina* cases were found in bovines of the same age In endemic areas with a high prevalence, cattle are usually protected by antibodies developed through former exposure to babesia at a young age, whereas in areas with a low prevalence outbreaks occur frequently in animals that had not been exposed to the causative protozoan

The only survey in El Salvador took place in 1979 and was limited [2] The objectives of the present work are to validate the Joint FAO/IAEA *B bovis* ELISA kit and to assess the risk of bovine babesiosis in El Salvador.

2 MATERIAL AND METHODS

2.1. Serum Samples

Field blood samples from 545 non-vaccinated young animals between 8 and 16 months of age were collected between May 1993 and February 1994. The samples were collected from farms that received technical assistance from the Animal Health Department of Ministry of Agriculture and Livestock All 8-16 month old cattle were sampled in the selected farms

Due to the wide infestation by *B microplus*, it was impossible to get local negative samples to determine a local cut-off.

2.2. ELISA procedure

The indirect ELISA technique was applied using the kit and methodology as recommended by the Joint FAO/IAEA [7]. The cut-off to determine positive and negative sera was initially set at twice the mean of negative control serum.

3. RESULTS

3.1. Assay standardisation

With all the OD results obtained (positives and negatives) and with the FAO/IAEA established parameters a frequency distribution graph is shown in Figure 1. The cut-off point, calculated from the negative control sera, was 11% PP.

3.2. Serological study

After determining the cut-off, all the data of sera were analysed by origin of the animals, and other parameters such as altitude and breed. Table I represents the prevalence of 545 animals. Most of the regions showed high prevalence (75-100%) although three were below 50%. The sero prevalence distribution according to the altitude is represented in Figure 2. Table II shows that the sero prevalence was also closely linked to the breed of the animals being tested.

The highest (92.3%) and lowest (33.3%) percentages belong to Brahman-cross cattle and Jersey, respectively although it should be noted that the latter breed is highly under represented in the sample.

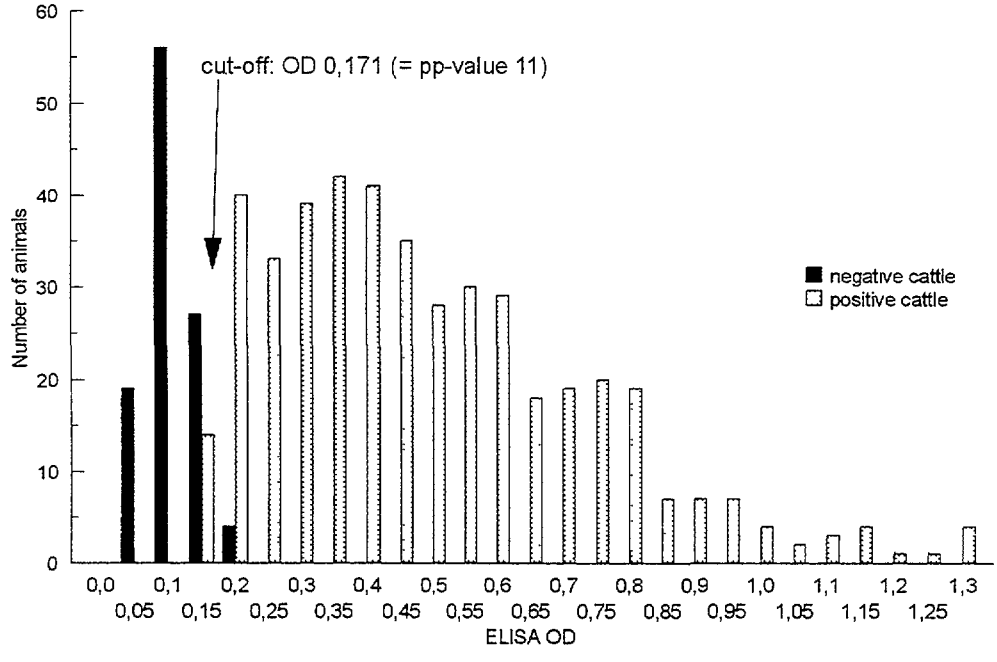

FIG. 1 Frequency distribution graphic of OD values for the indirect babesiosis ELISA (n=536).

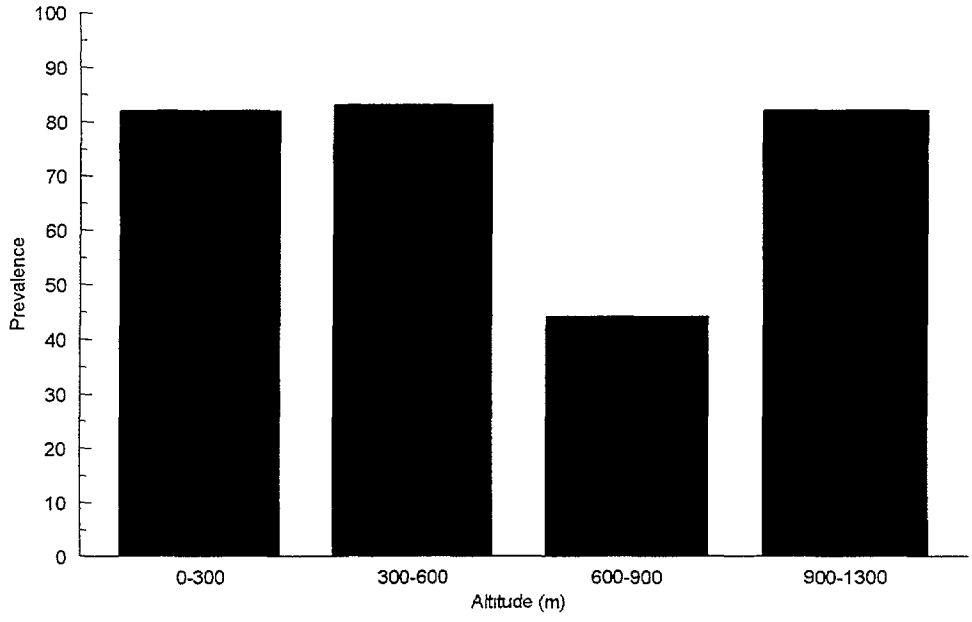

FIG 2 Distribution of sero prevalence to babesia bovis according to altitude

TABLE I BABESIOSIS PREVALENCE DETERMINATION BY STATES AND COUNTIES

State	County	Altitude (meters over sea level)	Total No	Posit	Prev
San Salvador	Ilopango	682	29	8	27 6
Santa Ana	Metapan	475	33	32	97
La Libertad	Ciudad Arce	575	35	27	77
	La Libertad	10	28	25	89 3
Sonsonate	Sonsonate	220	20	20	100
La Paz	Zacatecoluca	210	104	80	76 9
	Santiago Nco	160	72	65	90 3
	Rosario	105	21	18	85 7
	San Juan Talpa	200	17	1	5 8
Chalatenago	La Palma	1000	5	5	100
	San Ignacio	1010	4	4	100
Cuscatlan	Suchitoto	390	8	6	75
Ahuachapan	Ataco	1275	18	6	33 3
San Vicente	San Vicente	390	20	11	55
	Tecoluca	270	53	51	96 2
	San Sebastian	660	7	7	100
	Apastepeque	590	4	4	100
	Verapaz	610	4	3	75
Cabañas	San Isidro	370	39	38	97 4
	Villa Dolores	110	24	22	91 7
TOTAL			545	433	79 45

TABLE II DISTRIBUTION OF SERO PREVALENCE-TO *BABESIA BOVIS* ACCORDING TO BREED

Breed	# Total of animals	Positives	Prevalence
Jersey	18	6	33 3
Holstein	109	85	80 0
Brown Swiss	120	84	70 0
Brahman	57	44	77 2
Cross-Breed	207	191	92 3

4 DISCUSSION

The cut-off was determined from negative sera supplied with the kit, because it was impossible to get known local negative sera Therefore the determination of negative and positive sera needs further investigation However, the test is clearly suitable for detecting herds with babesiosis stability or instability

The average seroprevalence was 79 5% and it is concluded that babesiosis is an endemic disease in El Salvador But there are low prevalence areas, with an unstable situation, where not all the animals had been exposed These cattle are in danger of clinical disease if they are introduced to high prevalence areas or if the infested vector is introduced in these areas For instance the seroprevalence was 5 8% in San Juan Talpa and 85 7% in the neighbouring Rosario (Table I) Of the 20 areas sampled only 3 were in an unstable situation Note however that the sampling strategy was biased because the sampled farms were selected by the Ministry of Agriculture Their management is probably better than in the other ones, due to regular use of acaricides for tick control This could mean that the tick control is possibly less strict in other farms and that the proportion of farms with high seroprevalence and stable situation could be larger than shown by this study

Figure 2 shows 80% of seroprevalence in altitudes below 600 meters Above this (600-900 meters over sea level) low prevalences were found Based on the results in Table II, the Brahman-cross cattle are more resistant to tick infestation and consequently to haemoparasite disease outbreaks than other breeds Sero prevalence levels were low for Jersey cattle, due to good tick control However there is a potential unstable situation with high risk of clinical cases because of the low percentage of immunised cattle This particular herd should be carefully monitored due to the high susceptibility of Jersey to babesiosis and animals should be vaccinated to avoid any clinical case

A practical recommendation could be to carry out a similar survey in all herds with susceptible cattle If the seroprevalence in young animals is high, then the epidemiological stable situation does not require any particular means of control, as demonstrated by the Holstein and Brown Swiss breeds surveyed (Table II) However if the seroprevalence is low and the situation unstable, then cattle should be vaccinated as young animals

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REFERENCES

[1] GUGLIELMONE, A , hemoparasites Diseases Epizootiology in Cattle, FAO (1991)

[2] PAYNE, R C , SCOTT, J M , Anaplasmosis and Babesiosis in El Salvador, Tropical Animal Health and production, **14** (1982) 75-80

[3] MADRUGA, C.R., AYCARDI, E., KESSLER, R.H., SCHENK, M.A.M., DE FIGUEREDO, G.R., CURVO, J.B.E., DE FIGUEREDO, G.R., Levels of anti-*babesia bigemina* and *babesia bovis* antibodies in calves and Ibage breeds and Nelore crosses, ORIGINAL TITLE: Niveis de anticorpos anti-babesia bigemina e babesia bovis, em bezerros da raca Nelore, Ibage e cruzamentos de Nelore, Pesquisa Agropecuaria Brasilera, 1984, **19** (1984) 1163-1168.

[4] SMITH, M.W., A procedure for the infection of susceptible imported cattle with field strains of *Babesia bigemina* and *Anaplasma marginale* in Trinidad, PANS (Pest Articles & News Summaries), **23** (1977) 27-32.

[5] BARTHOLOMEW, R.B., CALLOW, L.L., A benefit-cost study (ex-post) of the development and introduction of a new vaccine against bovine babesiosis, Proceedings, 2nd International Symposium on veterinary epidemiology and economics, 7-11 May 1979, Camberra, Australia, Edited by W.A. Geering, R. T. Roe and L. A. Chapman, Canberra: Aust. Govt. Publ. Serv. English (1980) 448-457.

[6] SPATH, E.J.A., GUGLIELMONE, A.A., DE RIOS, L.E.G., GONZALEZ DE RIOS, L., Evaluation of the epidemiological situation of bovine babesiosis with a simple mathematical model, ORIGINAL TITLE: Utilización de un modelo matemático simple para evaluar la situación epizootiológica de la babesiosis bovina, Revista de Medicina Veterinaria, Argentina, (1987) 126-128, 130-131.

[7] FAO/IAEA., Babesiosis ELISA kit manual. Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Austria (1993).

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