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FOREWORD

International trade in agricultural products and commodities has been expanding greatly during the last decade with a value estimated at US $583 billion, which represents 42,5% of the world exports of primary products (WTO, 2002). A substantial proportion of international food trade originates in developing countries. The increase in the international market has also enlarged the possibility of introducing new food borne pathogens into the importing countries (e.g. novel *Salmonella* spp. strains) or of spreading pathogens across boundaries from endemic to low incidence areas (e.g. *Vibrio cholerae*). As a result of this, some countries have imposed food safety-based technical barriers to trade including, for instance, restrictions due to scientifically insupportable low tolerance levels for *Salmonella*, Listeria and other pathogens. Such barriers can effectively limit or block international trade in food and can result in substantial economic loss to the exporting country. This situation has generated an urgent need to identify the major food microbial contaminants and their levels in the main foods exported in the international food market, as well as the need to produce specific data to conduct better risk assessments on food in importing and exporting countries.

In August 1997, an FAO/IAEA Consultants Meeting on Microbiological Contamination of Food was held in Vienna with the objectives to 1) evaluate the importance of microbiological contamination of foods in relation to international trade and identify major food borne microbiological contaminants of relevance to international trade, 2) examine the impact of national, regional and international agreements, standards, guidelines/legislation and requirements concerning microbiological contaminants in food traded internationally, 3) advise on areas in food safety microbiology which the FAO/IAEA Training and Reference Centre for Food and Pesticide Control should cover, and 4) prepare a plan of activities for the FAO/IAEA Training and Reference Centre in Food Safety Microbiology for the short and medium terms. As a result of the discussions at the consultants meeting, a plan for four types of activities was approved: i) training of regulators and processors in HACCP-based food production, ii) enhancement of laboratory activities for specific purposes, iii) compilation of microbiological methods, and iv) research elements. In conjunction with the research elements it was recommended that a Coordinated Research Project (CRP) on Determination of Human Pathogens Profiles in Food by Quality Assured Microbial Assays be held.

The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture initiated the CRP in 1998 with 12 research contract holders and three research contract agreements. The first meeting of this CRP was hosted by the International Atomic Energy Agency in Vienna, (2–6 November 1998). The second meeting was hosted by the Department of Food Technology and Human Nutrition, Faculty of Agricultural Engineering and Technology, Institute Petanian, Bogar, Indonesia (16–20 October 2000) and the final meeting was held in Mexico DF, Mexico (22–26 July 2002) at the Faculty of Veterinary Medicine, Universidad Autonoma de Mexico.

This publication presents the research results obtained during the period 1998–2002. The IAEA officers responsible for this CRP and publication were P. Loaharanu and during the last phase T. Rubio of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. The latter was responsible for organizing the final research coordination meeting and finalizing the preparation of its proceeding for publication with the assistance of K. Newton who compiled the first draft of the manuscript.

EDITORIAL NOTE

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SUMMARY OF THE COORDINATED RESEARCH PROJECT

1. INTRODUCTION

In the year 2002, agricultural commodities represented 9,3% (US $583 billion) of world merchandise trade and 42,5% of world exports of primary products (WTO, 2002). During recent years, the exportation of agricultural commodities has increased an average of 8% which has increased the possibility of introducing new pests and/or pathogens or of spreading pests or pathogens across boundaries from endemic to low incidence areas. Some countries have imposed food safety-based technical barriers to trade such as unsupportable low tolerance levels for some microbial pathogens which has produced unnecessary restrictions in the international trade.

At present, international trade is governed by the World Trade Organization (WTO), which was established in 1995 as a successor of the General Agreement on Tariffs and Trade (GATT). The WTO is the only global international organization dealing with the rules of trade between nations. At its heart are the WTO agreements, negotiated and signed by the bulk of the world's trading nations and ratified in their parliaments. There are two agreements of particular relevance to international trade in food and agricultural commodities: Application of Sanitary and Phytosanitary Measures (SPS) and Technical Barriers to Trade (TBT). Regarding food safety matters, relevant provisions of the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) apply. The overall objective of the SPS Agreement is to permit countries to take legitimate measures to protect the life and health of their consumers (in relation to food safety matters), while prohibiting them from using those measures in a way that unjustifiably restricts trade. The TBT seeks to ensure that technical regulations, standards and analytical procedures for assessing conformity with technical regulations and standards do not create unnecessary obstacles to trade.

In order to facilitate trade in food products and to meet the WTO-SPS Agreement of relevance to international trade in food and agricultural commodities, several important activities have been identified:

(1) The collection of data on major incidents of microbiological contamination in foods implicated in the rejection of food shipments in international trade, and the economic impact of these rejections;

(2) Use of analytical methods in food microbiology (conventional and rapid methods) certified by national or international bodies, including an assessment of the possibility for the harmonization of such methods;

(3) Improving the quality of reference laboratories, especially in developing countries; and

(4) Training of reference laboratory personnel, especially in developing countries.

Some of these activities can be supported by the FAO/IAEA Joint Division of Nuclear Techniques in Food and Agriculture and its Training and Reference Centre for Food and Pesticide Control. These activities could be carried out under its mandate "to assist Member States and their institutions to fulfil requirements to support the implementation of international standards/agreements relevant to food safety and control." Therefore, and taking into account the recommendations of the FAO/IAEA consultants meeting on microbiological contamination of food held in Vienna (1997), the Joint FAO/IAEA Division implemented a

Coordinated Research Project on Determination of Profiles of Human Bacterial Pathogens in Foods for Export by Introduction of Quality-Assured Microbiological Assays.

The CRP started in 1998 with 15 participants. During the early years, four laboratories withdrew from the project. Reasons for withdrawal included inability to proceed with the nominated plan (two contracts); relocation of the chief investigator from the government laboratory to private industry and considerable delay in replacement with a suitable scientist (one contract); and closure of the participating laboratory (one contract).

Thousands of samples in a wide variety of foods were selected by the participants to be studied during different points of the food chain: meat (chicken, bovine and pork), seafood (shellfish such as shrimp, prawns, scampi, squid, lobsters and different types of fish like salmon, cuttle fish, rohu, fin herring, catfish, milkfish, tilapia), spices (pepper, paprika), frozen vegetables (asparagus, peas and corn) and others products (coconut and dairy products). The pathogenic bacteria included in the studies were *Salmonella* spp. (several serotypes), *Escherichia coli, E. coli 0157:H7, Staphylococcus aureus, Clostridium perfringens, Bacillus cereus, Vibrio choleare, Vibrio parahaemolitycus* and *Yersinia enterolítica*. Table 1 shows the participants' countries as well as the food and microorganisms studied.

The food/food products studied presented a wide range of microbes on and in them at harvest/slaughter. The numbers and types of microbes that comprise this primary contamination vary from one commodity to another, within geographic regions, and with production and slaughtering or harvesting methods. Some can grow on the food during storage, causing spoilage; others may cause illness in consumers either by infection or by intoxication. Levels of contamination can be influenced by changing technologies (e.g. shipment by air freight), and by further processing to add value. All of these aspects were analysed under the CRP implemented.

2. OBJECTIVES OF THE CRP

The overall objectives of this CRP were to assist national food control authorities and institutions to improve food safety and stimulate international trade. The approach taken to achieve this was by determining profiles of selected human bacterial pathogens of concern to importing countries on raw foods and in food products. Anticipated outcomes included increasing assurance in food control measures in exporting countries and facilitation of international trade in foods. Foods that are microbiologically safe would be identified as well as the areas which require improvement. The development of appropriate methodology and laboratory quality systems could also be addressed during the course of the project.

A second objective of this CRP was to support the activities of the FAO/IAEA Training and Reference Centre for Food and Pesticide Control of the Joint FAO/IAEA Division Nuclear Techniques in Food and Agriculture under its mandate "to assist Member States and their institutions to fulfil requirements to support the implementation of international standards/agreements relevant to food safety and control, the safe use of pesticides and sanitary and phytosanitary measures by providing training, quality assurance services and technology transfer."

Table 1. FOODS AND MICROORGANISMS STUDIED UNDER THE CRP

COUNTRY	PRODUCTS	MICROORGANISMS
Australia	Seafood (prawns, lobsters); spices (pepper, paprika); others (coconuts)	Total bacteria counts (TBC) *Salmonella* spp. and serotypes *Vibrio cholerae, Escherichia coli, Staphylococcus aureus*
Austria	Meat (poultry)	*Salmonella* spp. and serotypes.*
Brazil	Meat (poultry, bovine, pork)	*Salmonella* spp. and serotypes; *E. coli* 0157: H7
Chile	Seafood (Salmon); Frozen vegetables (asparagus, peas, corn)	TBC, *Salmonella* spp., *Escherichia coli*, *Vibrio c*holerae, *Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, Listeria monocytogenes*
India	Seafood (shrimp, prawns, scampi, rohu, squid, cuttlefish, fin herring)	TBC, *Salmonella* spp., *Escherichia coli, Staphylococcus aureus, Vibrio cholerae, Vibrio parahaemolyticus, Listeria monocytogenes, Yersinia euterolitica*
Indonesia	Seafood (shrimp)	TBC, *Salmonella* spp, *Enterobacteriaceae, Coliforus, Escherichia coli, Staphylococcus aureus, Vibrio cholerae, Vibrio parahaemolyticus, Listeria monocytogene.*
the Republic of Korea, Republic of	Meat (beef, pork, poultry), meat products, dairy products, vegetables, Seafoods (salmon, shrimp, octopus, cod, and Pollack fish, jelly fish)	*E. coli, E. coli* 0157:H7 * *Salmonella* spp, *Staphylococcus aureus, Vibrio parahaemolyticus, Listeria monocytogenes.*
Mexico	Seafood (shrimp)	*Salmonella* spp., *Vibrio cholerae* *
Nigeria	Seafood (crabs, clams, cuttle fish, shrimp)	*Vibrio cholerae*, *Vibrio parahaemolyticus, Staphylococcus aureus, Listeria monocytogenes*
Philippines	Seafood (prawns, catfish, milkfish, tilapia)	*Salmonella* spp.*
Thailand	Seafood (shrimp)	*Salmonella* spp, *Vibrio spp, Vibrio parahaemolyticus*

*Studies on methodologies.

3. ACHIEVEMENTS OF THIS CRP

The achievements of the work carried out under the scope of this CRP in the past five years are summarized as follows:

It is important to note that it was not possible for a comparison of results among the different countries because the sampling and analytical methods were not equivalent. On the other hand, there was a limit in terms of the bacteria studied as well as its pathogenicity. Some participants did not identify important and potential pathogenic species (e.g. *Campylobacter)* and/or pathogenic strains (e.g. Pathogenic strains of *V. cholerae*). In spite of these weaknesses, the results are useful because they give a general idea about the microbial contamination of the products, some tendencies and the most contaminated food/food products in the international market.

3.1. Determination of profiles of human bacterial pathogens of concern in a variety of foods marketed in international trade

Salmonella

In terms of bacteria contamination, *Salmonella* spp. remains the main concern. This pathogenic bacteria was found in almost all studies/testing programs and in most of the foods analysed (meat, seafoods, spices and coconut).

The ubiquity of *Salmonella* spp. in the natural environment may contribute significantly to the continued prominence of this bacteria in animal meat and its derivate products. The slaughtering-plant operations generally amplify the level of bacterial contamination associated with live animals. In addition, the contaminated water in ocean and aquaculture has become an important source of *Salmonella* spp. in fish and shellfish.

The high prevalence of *Salmonella* spp. in retail poultry reflects the continued inability of the industry to effectively prevent spread of this microorganism during the production, slaughtering and marketing of raw poultry meat. For instance, in Austria it was noted only a moderate declining trend in the contamination of chicken meat with *Salmonella* spp. since 1993 in spite of several measures taken, especially the continued monitoring in parent poultry flocks. In Austria it has also been established that the contamination of poultry is common, both in domestic and imported poultry, with a prevalence of over 30%, being the main source of human Salmonellosis from poultry meat and eggs which have not been properly heat processed, and *S. enteritidis* the most common serotype founded. No reports of beef or pork as a source of human Salmonellosis were communicated in that country during the period 1998–2001.

The study carried out in Brazil (Sao Paulo) showed an average of 9% of the meat and meat product samples to be positive to *Salmonella* spp, with *Salmonella enteritidis* being the serotype mostly isolated. This study also demonstrated that refrigeration (4ºC) and freezing temperatures (-18ºC) did not affect S. enteritidis but only reduced its number.

Salmonella spp. was also present in raw fish and shellfish samples. Although marine waters generally are considered to be free of *Salmonella* spp., estuaries and coastal waters contaminated with human and agricultural wastes pose a human health hazard. At the same time the development of aquaculture (11% of increase per year during the last decade) has generated another important source of *Salmonella* spp. contamination in fish and fish products.

The study carried out in Indonesia showed that shrimp from ocean and aquaculture environments in west, central and east Java were heavily contaminated (10E5.6-7.1CFU/g). *Salmonella* spp. was present in 6,25% of the ocean samples and 12,5% of the aquaculture samples analysed. The level of contamination of aquaculture shrimp was a reflection of water contamination. Most of the ponds were located near households that may be polluted with household water. It was also noted in this study that during the processing of frozen shrimp, *Salmonella* spp. could survive, so processing controls needed to be improved. It was noted that filth and *Salmonella* spp. accounted for 90% of the Indonesian shrimp consignments rejected in the international market.

The study in India included different seafood products processed in six industries (three European Union approved (EUA) and three EU non-approved (EUN)). It was observed that 16,7% of cuttle fish, 28,5% of the shrimp and 40% of the squid EUN industries were positive to *Salmonella*. However, no positive samples of *Salmonella* spp.were found in the EUA plants. This is a good indicator that when GMP and HACCP are applied, their use results in a good product that can easily fulfil the requirements of importing countries.

The studies conducted in Thailand with samples of aquaculture black tiger shrimp showed that *Salmonella* spp. was present in samples of water supply (13,95%), pond water (1,53%), feed materials (1,14%), fresh shrimp at farm (3,17%), fresh shrimp from wholesale market (30,4%) and frozen shrimp destined to exportation (0,21%). The reason of the high percentage founded in the wholesale market was the poor hygienic conditions. In this study, twenty-four different serotypes of *Salmonella* spp. were identified from positive samples. It is important to note that the same serotypes of *Salmonella* spp. reported in Australia in imported shrimp from Thailand were also reported by the participants of the exporting country. This observation may assist in the identification of the source of these strains and its possible control.

The use of rinse water and packaging ice from contaminated sources, poor personal hygiene among non specialized workers and repeated manual handling of raw products during the harvesting and packaging operations may have contributed to the higher rates of contamination in fish and shellfish from Asia.

In Chile, where the aquaculture of salmon were located far from the coast and into hidden areas, surrounded by leafy vegetation and where the water is very cold and clean, the raw material presented a good microbial quality and *Salmonella* spp. was not found. Likewise, no positive samples of *Salmonella* spp. were found in the study carried out in Mexico using the PCR technique and conventional methods.

It can be concluded that the elimination of *Salmonella* spp. at the harvest/production and processing stages would be one of the major advances in food safety and the facilitation of trade.

Escherichia coli/E. coli 0157:H7

Escherichia coli are found in humans as part of the normal intestinal flora shortly after birth. The contamination of food with this bacteria occurs from food handlers or from contact with water containing human sewage. The importance of *E. coli* as a cause of diarrhoeic disease has been increasingly recognized over the last 30 years. Detailed comparison of properties of diarrhoeagenic strains has resulted in the recognition of four categories of *E. coli* associated with the disease. There are enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) and enterohaemorrhagic (EHEC). *E .coli* 0157:H7, the most representative EHEC, can cause severe infection and death in humans, but produces no signs of illness in non-human

hosts; since the first reports (1982), *E. coli* 0157:H7 outbreaks have been rising steadily worldwide.

The work done under this CRP showed that *Escherichia coli* caused no problem during the period of the testing program done in Australia on different food and food products as well as in the study carried out in Chile with frozen vegetables. However, this bacteria was found in the study done in India (25% of the rohu steak samples had *E. coli* exceeding the limit of 20 CFU/g), Indonesia (shrimp in the area of west and central Java had positive samples in the range of 2.4–3.8 (log10 CFU/g) and in the testing program carried out in the Republic of Korea (27% of raw meat and 20% of vegetable samples from importing countries) were positive to *Escherichia coli.*

For this CRP, *E. coli* 0157:H7 was researched only in Brazil and the Republic of Korea, and in both countries' findings, this bacteria was not detected.

Staphylococcus aureus

Staphylococcus aureus is a ubiquitous organism in warmblooded animals and its presence in a low number of raw foods of animal origin is to be expected. In fact, the study carried out in Nigeria showed that 16,5% of the samples were positive to *S. aureus*, however the numbers were within the permitted levels.

This bacteria was not present in the salmon and vegetable samples analysed in Chile and the SET caused no problem in the testing program carried out in Australia. However, important contamination was found in India, Indonesia and the Republic of Korea.

The study done in India showed that 14,3 % of shrimp and 40 % of the squid samples of EUN plants were positive to *S. aureus*. The Indonesian study on shrimp collected from six sampling points during frozen shrimp production revealed that the processing reduced the number of *S. aureus* but did not eliminate them. The testing program carried out in the Republic of Korea with different imported seafoods showed that among six pathogenic bacteria searched, *S. aureus* presented the highest percentage (12,1%). This bacteria was found in many products including smoked salmon, tuna, frozen shrimp and small octopus. The highest contamination was found in shrimp (29%).

Because one of the main sources of contamination of foods with *S. aureus* is without any doubt the food handler, it can be concluded that the problem can be solved with GMP and HACCP.

Vibrios

There have not been any extensive surveys for the presence of pathogenic vibrios in foods other than seafood. Vibrios are naturally occurring environmental bacteria, present in virtually all coastal waters of tropical and temperate regions of the world.

(a) Vibrio cholerae

Costal areas with brackish waters and estuarine regions are niches for many species, including strains of toxigenic 01 *V. cholerae*. This strain is occasionally encountered in the environment in no endemic areas, but they are normally non toxigenic and considered to be non-pathogenic.

Under this CRP only Indonesia detected this pathogenic bacteria, 18,5% of the shrimp samples obtained from the east and central Java were positive to *V. cholerae*. However, *V. cholerae* caused no problem during the course of the testing program carried out in Australia as well as in the studies carried out in Chile, Mexico, India and Nigeria.

(b) Vibrio parahaemolyticus

The gastroenteritis caused by this species has been linked exclusively to the consumption of contaminated seafood. It has a worldwide distribution in warm climates, estuarine and costal environments and has been isolated form many species of fish, shellfish and crustaceans.

The study carried out in Indonesia showed that *V. parahaemolyticus* was frequently identified in aquaculture shrimp (25 % positive samples) but absent in fresh ocean samples. It was also noted that processing during frozen shrimp production did not effectively reduce the incidence of this pathogenic bacteria. In fact, this bacteria was found in 11% of the samples obtained in the market.

In the study carried out in India, 28% of the shrimp samples from the EUN plants were positive with *V. parahaemolyticus*. However, in Nigeria and in Thailand, its incidence was lower (1,3% and 3,8%, respectively).

The the Republic of Korean testing program carried out on imported foods did not show any positive sample with *V. parahaemolyticus.*

Listeria monocytogenes

Although listeriosis has been described for many years, the demonstration of foodborne transmission of the organism has made it one of the major concerns to the food industry. *L. monocytogenes* can contaminate nearly all kinds of food and can grow at refrigerated temperatures and survive at freezing temperatures.

In the Indonesian study, 70% of shrimp samples were positive with *L. monocytogenes*. This bacteria was present in the whole processing line and it survived at freezing temperatures. The percentage of positive samples in the Nigeria study for the same product was very low (only 1,8%).

L. monocytogenes was also present in 2% of the salmon samples analysed in Chile (one of the 48 salmon samples (final product)) where the processing plant had implemented adequate measures in the whole processing line. Some studies have found a high correlation between the occurrence of *L. monocytogenes* in seawater and raw material. However, up to now a clear contamination route has not been determined. In the Republic of Korea this bacteria was isolated in the 9,3% of the smoked salmon samples.

L. monocytogenes was not detected in any of the seafood samples analysed in the study done in India.

Other pathogenic bacteria

Other bacteria like *Bacillus cereus*, *Clostridium perfringens* and *Yersinia enterolitica*. were also searched by some of the participants, but they were not found in the food samples analysed. Frozen vegetable samples analysed in Chile were negative to the presence of

Bacillus cereus and *Clostridium perfringens*. *Yersinia enterolitica* was also not found in any of the seafood samples analysed in India.

One of the gaps of this CRP was the non inclusion of *Campylobacter* spp. in the studies carried out by the participating countries, taking into account that this bacteria is considered by different sources as one of the most frequently isolated bacterial agents implicated in human gastroenteritis produced by contaminated foods. In fact, animals used for food production are a reservoir for *Campylobacters*, because these bacteria generally are commensal organisms in the intestinal tract of a wide variety of animals. *Campylobacter* may be isolated from different environmental sources such as ponds, lakes and creeks as well as seawater, streams, rivers and estuaries that have been tainted by faecal contamination from wild or domestic animals. So the detection of this bacteria in the products studied under this CRP would be a good contribution to the knowledge of the human pathogenic profile in food.

3.2. Food/food products

It is important to note that most of the participants determined bacteria contamination of poultry meat (chicken) and shrimp, both products of great importance in the international markets in terms of volume. Only a few participants analysed other important products in international trade like other seafood (e.g. bivalves) or meat (e.g. pork and beef meat), vegetables etc. Therefore, as a result of the studies carried out by the CRP's participants, we can conclude that the chicken meat as well as shrimp presented a high microbial contamination, including pathogens, and that practices during the production/harvest, processing and distribution must be improved.

3.3. Methodologies

Most of the participants used conventional microbiological methods but some of them also studied rapid methods.

Conventional methods for the detection, isolation and identification of foodborne bacteria require enrichment culturing of food samples for various lengths of time (depending on the target organism) followed by subculturing on a medium that contains selective agents and/or allows for differentiation of targeted bacterial colonies from among the background microflora. Presumptive colonies are ultimately identified by performing a series of biochemical and serological tests. Additionally, tests for the presence of toxins produced by the target organism, as well as for other virulence characteristics, may also be performed. Although traditional cultural methods can be sensitive, such methods are often laborious and time consuming, requiring four to seven days or longer before definitive results can be obtained. During the past 15 years, great strides have been made in the development of rapid methods for detection, identification and enumeration of foodborne pathogens. In addition to providing results in a shorter length of time than by conventional methods, rapid assays can also be more sensitive, specific and accurate than classical methods.

Taking into account that the development of appropriate technology could also be addressed under this CRP, some of the participants evaluated different methodologies for detecting pathogenic bacteria, including rapid methods for the detection, identification and enumeration of pathogenic bacteria. In fact, the participants from Austria, Mexico, the Republic of Korea and Philippines all faced this task.

For instance, the study carried out in Austria evaluated different methods for detection of *Salmonella* spp. in meat, especially in poultry: a) studies on motility media (Modified-

semisolid Rappaport-Vassiliadis (MSRV) media of different manufacturers, novobiocine supplementation to MSRV and DIASALM media, comparison of DIASALM media vs MSRV, and b) antigen detection by a commercial EIA (Vydas system). The MSRV was found to be a suitable method for detection of *Salmonella* spp. in food; it is cost effective but requires experienced personnel. On the other hand, the Vydas system offered a largely automated and quality-assured diagnosis at a higher price.

The study done in Mexico had the objective of using the polymerase chain reaction (PCR technique) for the simultaneous detection of *Salmonella* spp. and *Vibrio cholerae* in frozen shrimp for exportation and to compare the results with conventional methods. Both methodologies gave identical results (the samples were negative in *Salmonella* spp. and *V. cholerae*). The official microbiological methods, including isolation, biochemical and serology tests, took 15 days when the PCR technique was run in 28 hours.

Another example was the study carried out in the Republic of Korea for the detection of different groups of *Escherichia coli* (enterophatogenic *E. coli* (EPEC); enterohemorragic *E. coli* (EHEC); enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC)). For this purpose, a multiple polymerase chain reaction (multiplex PCR) was developed and compared with conventional methodologies used in the Republic of Korea. It was concluded that more research is needed to determine the usefulness of the multiplex PCR for the detection of different groups of *E. coli*.

In the Philippines the traditional methods BAM/AOAC were compared with eight rapid test kits to detect *Salmonella* spp. The BAM/AOAC method is still the most sensitive and specific recommended detection method for *Salmonella* spp. but the assays take 14 days. The rapid test sandwich type antigen-antibody reaction in microtiter well proved to be the best rapid method for monitoring *Salmonella* spp. in aquaculture products. This rapid test had a 99% agreement with BAM/AOAC method.

3.4. Measures of control

It is important to mention that a sampling of shipments of imported foods conducted by Australia during a period of seven years (1995–2001) showed that after 1995 there was a decrease in the proportion of samples which failed to fulfil the standards (1,7% in 1999 versus <1,0% in 2000–2001) and at the same time there was also an increase in the number of samples with low ranges of counts. This improvement in quality may have been driven by commercial factors such as rejection of products but also for an improvement in the microbiological quality of foods in exporting countries.

It is known that during recent years the international market for foods has been subjected to more stringent quality controls enforced by international, regional and national regulations. This fact was demonstrated by the study carried out in India. Data on the export quality of marine and aquaculture fish and fishery products collected from European Union Approved (EUA) and EU-non-approved (EUN) plants located at the east and west coasts of India were analysed for the presence of human bacterial pathogens using standard bacteriological techniques. A total of 126 samples of different seafood (shrimp, prawns, scampi, squid, rohu, fin herring, cuttle fish) were analysed for different pathogenic bacteria. It was observed that the marine products from EUN plants were of poorer microbiological quality as compared with the products from EUA plants. As part of the results of this study, it was stated that there is the need for implementation of better hygienic practices for the improvement of microbial quality of products from EUN plants as well as the need to adopt stringent hygienic practices for the production of good quality aquaculture fishery items.

Additionally, the study conducted in Nigeria showed that at the beginning of the CRP some pathogens were detected in some of samples analysed (e.g. *S. aureus* was present in shrimp, cuttle fish and crabs; *L. monocytogenes* as well as *V. cholerae* were present in shrimp samples); however, at the end of the project these pathogenic bacteria were eliminated at least in the packaged raw seafood products. The reason for the results obtained is the training and the enforcement of GMP and HACCP protocols applied by the exporters.

As a result of the testing program carried out in the Republic of Korea, it was concluded that the microbiological quality of imported seafood products, especially in their raw state, was poor and it was recommended that the regulatory agencies, such as KFDA (the Republic of Korean Food and Drug Administration) paid more attention to the microbiological quality of the imported foods. Similar conclusions were presented by other participants.

It can be concluded that when GAP, GMP and HACCP were applied, the food produced had a good microbiological quality and could comply with the requirements of the international market.

4. CONCLUSIONS AND RECOMMENDATIONS

Consideration of the results obtained from the individual projects allows the following general conclusions to be drawn. The experiences of the investigators during the course of the project, and interactions and discussions at the research coordination meetings, have produced the recommendations tabulated below.

These conclusions indicate that the objectives of this CRP have substantially been met. Action on the recommendations listed below would complete the achievement of the objectives, and would ensure lasting benefits in food safety and in trade in food.

Conclusions

(1) Profiles of selected human bacterial pathogens in some foods, raw materials and products were established.

(2) *Salmonella* spp. remains a major concern in export quality food and is responsible for rejection of consignments in which it is detected.

(3) *E. coli* O157:H7 is of low incidence in raw meat in Brazil and in the Republic of Korea.

(4) Freshly harvested seafood and aquaculture fishery products were found to often carry contaminating bacterial pathogens.

(5) Environmental contamination by bacterial pathogens of the coastal fishing areas and the aquaculture ponds adversely affects the microbiological quality of the seafood products originating from these environments.

(6) Food products obtained from processing plants which followed GMP and HACCP based quality systems were of good microbiological quality and able to comply with the requirements of importing countries. The results demonstrate that good practices in agriculture, processing and distribution produce improvements in the quality of food in trade. This is also evidenced in the improving quality of the foods observed by importing countries.

It is recognized that in certain food producing areas, environmental problems are difficult to address.

(7) The use of internationally recognized methodology by participating laboratories allows direct comparison of results to give a reliable picture of the prevalence of pathogens in foods.

(8) Good progress has been made in the development of laboratory quality systems by the participating laboratories. Comprehensive evaluations of some rapid methodologies were made by two participants. These developments mean that improved confidence in, and timeliness of results, become possible.

(9) The findings of the CRP have contributed to improved national food safety programmes in several countries. The results of the CRP will be useful to assist national food control authorities and institutions to improve food safety and to facilitate international trade.

Recommendations

(1) In recognition that laboratory analysis is only one part of the quality control of the total food chain, it is recommended that attention be directed by national authorities in the improvement of the microbiological safety of the environment, agricultural practices, processing, storage, transport and distribution of foods.

(2) Implementation of Good Manufacturing Practices and HACCP-based quality systems should be accelerated to assure the production of good quality foods that meet international specification and standards.

(3) International organizations should continue to support national governments to implement HACCP-based food safety systems in order to improve food safety and to facilitate international trade in food commodities.

(4) It is recommended that the incidence of *Campylobacter* and other emerging foodborne pathogens be examined in the near future.

(5) It is recommended that training activities in analytical methodology and quality systems based on ISO 17025 be organized and supported by national and international organizations. Three possible schemes are suggested:

(a) Centralized facility such as Seibersdorf where trainees attend training courses;

(b) Provision of training fellowships for trainees to attend an existing training laboratory; and

(c) Provision of funding for an expert to visit laboratories for training purposes.

Support is essential for the establishment of laboratory proficiency testing programs as an integral quality assurance tool for laboratory quality systems.

(6) Recent advancements in microbiological analysis using molecular and immunological techniques increases the need for training personnel involved in food analysis.

(7) An advisory and information centre should be established to collect, update, and disseminate information on national and international standards. Efforts to harmonize

international standards and testing methodology as undertaken by Codex and ISO should be encouraged and supported.

Central development of protocols and guidelines for the laboratory documentation required for technical and quality systems accreditations is also recommended. Availability of this documentation to National laboratories would greatly facilitate their accreditation process.

COMPILATION OF MICROBIOLOGICAL TESTING RESULTS FOR HUMAN PATHOGENS AND INDICATORS BACTERIA IN FOODS IMPORTED BY AUSTRALIA DURING RECENT YEARS

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Abstract

Australia is a net food exporting country, but also imports a considerable quantity of food from worldwide sources. Monitoring for safety of foods imported into Australia is the responsibility of the Australian Quarantine Inspection Service (AQIS). AQIS carries out the inspection and testing of food imports under the Imported Foods Inspection Program (IFIP). An advisory committee which contains food safety experts, conducts risk assessments on foods and advises AQIS on risk categorisation of the foods, and on the testing to be conducted. The rate of testing of individual food types is determined initially by the risk category, with high risk foods undergoing a higher rate of sampling than do lower risk foods. When a good safety history is established through the testing program for a high risk product from a particular producer, testing frequency is reduced. Sampling rates switch from sampling of all shipments to random sampling. The inspection and testing program is designed to comply with WTO requirements.

1. INTRODUCTION

The Australia New Zealand Food Standards Code (now Food Standards Australia and New Zealand) contains microbiological standards, sampling plans and methods of analysis to be used for a variety of foods. Imported foods must comply with the relevant standard. Where foods are not covered by Standards, the advisory committee sets acceptable levels for appropriate pathogens or indicator organisms. Survey programmes are also carried out under the IFIP as new safety risks are identified. Use of Australian Standard Methods of analysis is required where the relevant method is available. However, alternative methods are permitted provided that they have been validated to give the same results as the AS method. Where the target organism is not covered by an AS method, an internationally recognised method such as ISO, BAM, AOAC is acceptable.

The Australian Government Analytical Laboratories have conducted the testing since the inception of the IFIP in the early 1990s. Thus our results of testing provide a record covering a number of years of the occurrence of bacteria of food safety and hygiene concern in imported foods.

The results of testing for certain commodities have been collated and are reported here as the Australian Government Analytical Laboratories (AGAL) component of this FAO/IAEA cooperative research programme.

2. MATERIALS AND METHODS

Sampling of shipments was conducted by trained quarantine inspectors according to the requirements of the Australia-New Zealand Food Standards Code (ANZFSC). Samples tested consisted of five sub-samples each of 25g of pepper and paprika, and 10 sub-samples each of 25g of coconut from each shipment. Sub-samples were composited for testing, and results

were reported as “Detected” or “Not Detected” per 25g. Five sub-samples of crustaceans were drawn also, with individual packs drawn when possible and sub-samples taken aseptically when necessary. These sub samples were individually tested.

Standard Plate Counts, *E. coli* and *Salmonella* testing were done by the Australian Standard methods (1) with minor, validated deviations. *V. cholerae* testing was done by the FDA, BAM method (2), and SET testing was done by Tecra or bioMerieux Vidas EIA methods. The latter test was conducted by AGAL Melbourne while all other testing was done by AGAL Sydney.

Isolates of *Salmonella* from positive samples were submitted to a reference laboratory for serotyping.

The AGAL Sydney Microbiology laboratory has NATA technical accreditation for SPC, *E. coli* and *Salmonella* testing (as per ISO/IEC 17025 since July 2001). AGAL Melbourne has been accredited for SET testing for two years. Our organization also has Quality Systems-accreditation.

3. RESULTS

Table 1. RESULTS OF MICORBIOLOGICAL TESTING OF SAMPLES OF FROZEN, COOKED PRAWNS AND LOBSTERS IMPORTED INTO AUSTRALIA DURING THE PERIOD JULY 1995 TO DECEMBER 2001

PLATE COUNT 30C/3d (% in range)											
Prawns/ Lobsters	**10-100**	**E2-E3**	**E3-E4**	**E4-E5**	**E5-E6**	**>E6***	**Total No.**	**E.coli Total No.**	**Salmonella# Total No.**	**V. cholerae No.**	**SET No.**
Jul-Dec 95	0	7	37	26	21	8.5	142	142, 1+ve, MPN 4	0 +ve	142, 0+ve	142, 0+ve
Jul-Dec 95	2.3	18	25	38	4.5	2.2	255	253, 4+ve MPN 4, 4, 9, 21	250, 2+ve 1 Thailand, 1 Cuba, *S.thompson S.senftenberg*	255, 0+ve	255, 0+ve
Jan-Jan 97	5	22	29	30	12	2	407	362, 1+ve MPN 7	362, 0 +ve	325, 0+ve	340, 0+ve
Jan-Dec 98	7	28	29	31	4	1	405	362, 4 +ve MPN 4, 4, 7, 210	2 +ve Thailand, Malaysia, *S.houten, S.weltervreden*	206, 0+ve	371, 0+ve
Jan-Dec 99	2.9	23.5	37.3	30.6	4.5	1.2	510	475, 1+ve MPN=4	470, 8+ve, 7x Thailand, Malaysia, *S.houten, 4xS.Stanley, S.newport S.saintpaul, S.bareilly*	120, 0+ve	470, 0+ve
Jan-Dec 00	6.3	23.4	39.2	23.2	6.1	1.8	380	60, 2+ve MPN=4, 23	60, 0+ve	20, 0+ve	120, 0+ve
Jan-Dec 01	8.1	26.5	44.9	17.7	1.6	1.3	385	385 0+ve	375, 1+ve Malaysia, *S.bareilly*	162, 0+ve	365, 0+ve

* *SPC results >E6 exceed the relevant standard.

Total number of samples tested, number positive, source and serotype.

Table 2. RESULTS OF MICROBIOLOGICAL TESTING OF SAMPLES OF PEPPER, PAPRIKA AND COCONUT IMPORTED INTO AUSTRALIA DURING THE PERIOD JULY 1995 TO DECEMBER 2001

PEPPER	**Salmonella***
Jul 95-Dec 95	43, 0 +ve
Jan 96-Dec 96	118, 2+ve, the Republic of Korea, Netherlands, *S. weltervreden, S. saintpaul*
Jan 97-Dec 97	144, 2+ve, Malaysia, the Republic of Korea, *S. give, S. weltervreden*
Jan 98-Dec 98	113, 5+ve, Hong Kong, Malaysia, China, Taiwan, Egypt *2x S. weltervreden, S. paratyphi var Java, S. mbandaka, S. newport*
Jan 99-Dec99	123, 7+ve 3xMalaysia, India, HK, Vietnam, Slovenia *S. mikawasima, S. potsdam, S. weltevreden, S. livingstone, S. anatum, S .thompson, S. javiana*
Jan 00-Dec 00	139, 2+ve, 2xMalaysia, *S. paratyphi B var Java, S.javiani*
Jan 01-Dec 01	172, 2+ve, Malaysia, China, *S. weltevreden*, S.subsp.1ser 9,12:--:1,5
PAPRIKA	
Jun 95-Dec 95	29, 1+ve, Spain, *S. meunchen*
Jan 96-Dec 96	39, 3+ve, USA, Spain*, S. sandiego, S. agona,* 2xS.subsp.
Jan 97-Dec 97	27, 1+ve, Spain, *S. subsp*.
Jan 98-Dec 98	24, 0+ve
Jan 99-Dec 99	48, 1+ve, Spain, *S. typhimurium*
Jan 00-Dec 00	43, 0+ve
Jan 01-Dec 01	33, 0+ve
COCONUT	
Jun 95-Dec 95	30, 2+ve, Philippines, Vietnam, *2x S. weltervreden*
Jan 96-Dec 96	39, 1+ve, Sri Lanka, *S. vvittingfoss*
Jan 97-Dec 97	33, 1+ve, source NA, S.subsp.
Jan 98-Dec 98	34, 0+ve
Jan 00-Dec 00	24, 0+ve
Jan 01-Dec 01	39, 1+ve, Thailand, *S. lexington*

* Total number of samples tested, number positive, source, and serotype

4. CONCLUSIONS

4.1. Crustaceans

A sustained improvement in the SPC is apparent during the testing period. The proportion of samples which failed on SPC decreased after 1995, and the proportions of samples in the lower ranges of counts increased. This improvement in quality (as measured by SPC) may have been driven by commercial factors such as rejection of product by Australia. However, exporting countries have demonstrated that improved quality can be achieved. This improved quality and consumer safety of foods entering world trade will benefit both the exporting and the importing countries.

E. coli, V. cholerae and SET caused no problems during the course of this testing program.

4.2. Crustaceans and spices

Few samples and few positive results make trend analysis unreliable for the incidence of *Salmonella*. But the results indicate that *Salmonella* continues to be an intermittent problem in imported foods. After its detection, importers have the options to destroy, re-export or treat the foods to eliminate the *Salmonella* before release of the food onto the market. All of these options add to the costs of trade and the elimination of *Salmonella* at the production and processing stages would be a major advance in food safety and in facilitation of trade.

The same serotypes of *Salmonella* as are reported in shrimp in the report from Thailand, are commonly found in Thai shrimp imported into Australia. This observation may assist in the identification of the source of these strains and possible control.

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STUDIES ON METHODS FOR DETECTION OF *SALMONELLA* SP. IN MEAT WITH REGARD TO EQUIVALENCY AND COMPATIBILITY

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Abstract

This contribution summarizes research activities on the evaluation of methods for detection of *Salmonella* in meat, especially poultry. The following items were under study: (1) studies on motility media for detection of *Salmonella* spp.: evaluation of MSRV media of different manufacturers; evaluation of novobiocine supplementation to MSRV and DIASALM media; abuse studies of incubation temperature; comparison of DIASALM medium vs. MSRV; (2) *Salmonella* antigen detection by a commercial EIA (Vidas System). The findings and information from other sources (references, technical papers) are to be combined in a database to give a comprehensive overview on the currently applied methodology. Basic considerations on the structure of this database are demonstrated.

1. INTRODUCTION

Salmonella is still the most important infective agent of foodborne disease with known epidemiology in Austria. However, in some provinces the number of reported cases of Campylobacteriosis is going to exceed that of Salmonellosis (FEIERL, 2002) [9], which is in accordance with a European trend (BGVV, 2001) [2].

The number of human *Salmonella* isolates in 2000 was 7439 (with 6526 isolates originating from food poisoning), with a moderately declining trend (-5 to -10% / year) observed since 1993. This was attributed to several measures, especially monitoring of *Salmonella* in parent poultry flocks. The main source for human foodborne Salmonellosis was still poultry and eggs which had not been properly heat processed, and no reports of beef or pork as a source of human Salmonellosis have been communicated in the last four years. Since the remarkable rise in incidence of Salmonellosis in 1990, *S. enteritidis* accounts for the majority of the human *Salmonella* isolates and ca. one third of non- human isolates.

Antibiotic resistance of human *Salmonella* isolates stayed quite constant at a near zero (Ciprofloxacin) to five (Ampicillin, Tetracyclin) percentage level for most of the common antibiotics [1].

Extrapolation of these data on the epidemiological situation on humans and food for human consumption in Austria has to be made with great care (the impact of “under- reporting” is not known). However, these data show that there is still a need for improved surveillance of (food borne) Salmonellosis in Austria. One of the basements of effective surveillance is not only regularly updated information on the epidemiology of a given pathogen in different countries,

but also the quality of the diagnostic methods employed to generate data. This research project aims at compiling currently used procedures for detection of *Salmonella* in meat and, where necessary, to generate data on (promising) procedures.

As an additional benefit, this work also served as part of a postdoctoral training of food hygiene lecturers at universities in Ethiopia and Egypt. Training university teachers should result in a "multiplication effect" and booster knowledge transfer. Trainees were supplied with a basic collection of reagents and chemicals for further work in their home countries.

2. SUBTASK 1: EXPERIMENTAL SECTION

Subtask 1 was devoted to two groups of diagnostic methods:

2.1. Evaluation of motility media for the detection of *Salmonella* spp.

Motility media offer a cheap and rugged diagnostic method; while some authors point out the "selective isolation" character, others accept motility media, as "modified-semisolid Rappaport-Vassiliadis" Agar as a stand-alone technique equal to selective solid agar plates [16, 20]. Unlike selective solid media, enrichment procedures may be confined to primary non-selective enrichment only without compromising sensitivity. A time gain of 24 h compared to ISO standard method [11] is another important benefit.

2.2. Evaluation of a test kit for *Salmonella* (antigens), with a large degree of automation and quality assurance

Such tests may be benefitial as they provide fast results, even with not highly educated laboratory staff [3, 13]. The findings of this work were disseminated as quickly as possible by publishing in journals or presentation on conferences, with acknowledgement to the IAEA.

The impact of inherent drawbacks, as non-motile Salmonellae for group (a) and, for group (b) the significance of sole antigen detection (and not viable bacteria) and financial costs, was well recognized, but not evaluated in this contribution.

3. EVALUATION OF MOTILITY MEDIA FOR THE DETECTION OF *SALMONELLA* SPP.

3.1. Objectives

- Productivity of three brands of MSRV medium (OXOID, MERCK and BIOKAR),
- Salmonella load on chicken cuts,
- Serotypes of *Salmonella* isolated from chicken carcasses and cuts.

3.2. Summary

The study showed that MSRV medium of OXOID and MERCK were more productive than BIOKAR when a mall volume of highly concentrated *Salmonella* suspensions were applied, whereas studies with larger inoculation volume from 0.2 to 0.5 ml (total volume per plate) proved that this effect was negligible in routine use. The *Salmonella* load of chicken carcasses ranged between 32 and 2900 Salmonellae per whole skin. *S. hadar* was the most frequent isolate followed by *S. enteritidis*.

3.3. Materials and Methods

3.3.1. Preparation of the MSRV agar

MSRV agar base (BIOKAR BK 134, MERCK 1.09878, OXOID CM 910) was dissolved in water by heating in a steam pot under frequent agitation. After cooling the agar to 50°C, ca 12 ml of the agar was poured into sterile petri dishes and left aside at room temperature to solidify. Solidified plates were stored in the refrigerator for not more than two days until usage. Novobiocine was not added to either of the media.

3.3.2. Preparation of the chicken samples

Thirteen chicken carcasses and 14 chicken pieces (four wings, five livers and backs, each) bought from different supermarkets and open markets in Vienna, Austria were transported to the laboratory at ambient temperature. The skin of the carcass was removed aseptically piece by piece with sterile forceps and scissors and put into sterile stomacher bags fitted with filter mesh inserts. After recording the weight, a proportional volume of buffered peptone water (bpw, OXOID CM 509) was added to the stomacher bag and the sample was macerated with a Stomacher 400 for two minutes at high speed. The suspension was then transferred to a metal beaker and homogenized for one minute by Sorvall highspeed blender. The chicken pieces were weighed, put in sterile plastic bags and shaken for one minute manually using both hands. This was followed by homogenization as stated above. Suspensions of both types of samples were incubated at 37°C for 18-20 hours.

3.3.3. Inoculation of the msrv agar for chicken samples

Three drops of the incubated samples (about 0.1-0.15 ml per drop) were placed at three different spots on the solidified msrv medium and incubated at 41-42°C for 24 hours.

3.3.4. Quantative and qualitative determination of Salmonella

Salmonella load per 100 g of chicken skin and chicken pieces was determined by the most probable number technique (MPN). The sample was rinsed and the rinse liquid divided into nine fractions with a weight ratio of 30: 30: 30: 3: 3: 3: 0.3: 0.3: 0.3. These fractions were first incubated at 37°C for 18-20 hours for pre enrichment and later on transferred to MSRV medium. Results were read and the index number was formed (DE MAN, 1983). The corresponding MPN number gives the count number per 30% of the whole sample. The MPN was multiplied by 3.33 to get the most probable number per whole sample, see Figure 1.

Figure 1: DETERMINATION OF SALMONELLA LOAD.

1) Aseptically remove skin and macerate in 300 ml buffered peptone water (BPW) with a Stomacher (Stomacher 400, 2 x 2 min. high speed).

(2) Separate rinse liquid from solids.

(3) Record the volume of the liquid, divide in nine fractions according to the following ratio: 30:30:30: 3:3:3: 0,3:0,3:0,3 (weight base).

3 x 90 ml 3 x 9 ml 3 x 0.9 ml*

*To avoid drying, add 2.1 ml BPW to the tubes

(4) Incubate at 37°C for 20 h

(5) Place three drops (ca. 0.4 ml) of the incubated and enriched sample at three different spots on the MSRV medium.

(6) Incubate at 42°C for 20 h, then read plates. No. of pos.** triplicate petridishes = MPN index value. According to MPN table (DE MAN, 1983) [4] the index value of 320 corresponds to a most probable number of 9.1 (95% limit: 1.8-36).

3 x 90ml 3 x 9 ml 3 x 0,9 ml

3 **2** **0**

(7) Multiply MPN with 3.33 to get number of *Salmonella* in the sample.
** Confirm with H- Agglutination (Latex agglutination)
Note: Volumina and results given here serve only as an example.

(8) Multiply MPN with 3.33 to get number of *Salmonella* in the sample.
** Confirm with H- Agglutination (Latex agglutination)
Note: Volumina and results given here serve only as an example.

3.3.5. Reading of MSRV and confirmatory reactions

After incubation at 42°C for 20 hrs, the MSRV plates were read. Homogenous swarming zones of adequate size were considered presumptive positive (SCHALCH and EISGRUBER, 1997) [19]. Samples were confirmed as *Salmonella* positive by latex agglutination (OXOID F.T. 203) of a loopful of agar taken from the periphery of the swarming zone. After subculture on solid selective medium (XLD, OXOID CM 469), the major sero groups B, C, D and E were determined by agglutination of colony material with the corresponding antiserum (BEHRING). Positive samples were transferred to slant agar and transported to the *Salmonella* Reference Central Laboratory at Graz, Austria for further determination of the serotypes.

3.3.6. Evaluation of productivity of MSRV media when small volumes are applied

The productivity of the medium was determined by central application of 10µl of a suspension of *Salmonella* pure cultures in late lag phase with a density of approx. 10^8 cfu/ ml. After incubation the diameter of the opaque swarming zones formed in the medium was measured in cm with a ruler. Maximum value was nine (equating to the petri dish diameter).

3.4. Results and discussion

Table 1 shows the results of the productivity of three brands of MSRV presented as migration zone diameter in cm. The values given are arithmetic means of each four results. *Salmonella* could be detected with all three types of the MSRV agar, however, with different productivity. On the average, the productivity of the OXOID and MERCK products was higher. BIOKAR product revealed the least productivity under the test conditions carried out with pure cultures of *Salmonella* (obtained from the National Reference Center in Graz).

All serotypes of *Salmonella* fully migrated through the OXOID MSRV agar, whereas pure cultures of *S. panama*, *S. agona* and *S. hadar* partially migrated through the MERCK MSRV agar. *S. panama, S. agona, S. virchov, S. hadar, S. senftenberg, and S. heidelberg* partially migrated into the BIOKAR MSRV agar. *S. newport, S. anatum and S. enteritidis* (both phage types 4 and 12) fully migrated in all three brands of the MSRV agar.

Out of the total of 27 chicken carcasses, pieces and livers, 17 (62.96%) were contaminated with *Salmonella* (Table 2). The *Salmonella* load of chicken carcasses ranged between 36 and 2900 per whole skin and that of chicken pieces was between 30 and 1500 per 100 g. One chicken carcass and one liver were highly contaminated with *S. enteritidis* (Table 3). A total of 17 strains of *Salmonella* with six different serotypes (*S. enteritidis, S. hadar, S. indiana, S. typhimurium, S. heidelberg, and S. braenderup*) were isolated from chicken carcasses and chicken pieces. Eight of 17 (47.05%), four of 17 (23.52%) and two of 17 (11.76%) of the isolates were found to be *S. hadar, S. enteritidis and S. braenderup*, respectively (Table 4). *S. hadar* was the major isolate.

A series of studies have been conducted to find out a shortened and a suitable method to detect *Salmonella* in food and the environment. However, most of the proposed methods could not find wide application due to the fact that some of these methods require investment on apparatus and reagents or are not economical for small laboratories dealing with very number of samples. The modified semi solid Rappaport-Vassiliadis agar has been found to be a suitable method for detection of *Salmonella* in food [12]. As opposed to other standard cultural methods, the final result can be obtained within 48 hours and modifications with a total investigation time of 24 hours have been developed [5, 18]. MSRV is cost effective, not sophisticated, but requires experienced personnel.

Table 1. SWARMING ZONE DIAMETER (CM) INDUCED BY PURE CULTURES OF SALMONELLA IN 3 COMMERCIAL BRANDS OF MSRV AGAR (MAXIMUM VALUE = 9)

Sample	Salmonella	OXOID	MERCK	BIOKAR
		Diffusion diameter (cm)	Diffusion diameter (cm)	Diffusion diameter (cm)
1	*S. panama*	9	4	4
2	*S. enteritidis, sublethal*	9	9	6
3	*S. agona*	9	5	7
4	*S. virchow*	9	9	7
5	*S. typhimurium*	9	9	6
6	*S. infantis*	9	9	6
7	*S. newport*	9	9	9
8.	*S. hadar*	9	5	7
9	*S. anatum*	9	9	9
10	*S. senftenberg*	9	9	5
11	*S. heidelberg*	9	9	7
12	*S. enteritidis* pt 12	9	9	9
13	*S. enteritidis* pt 4	9	9	9

Table 2. RATE OF CHICKEN CARCASSES AND CHICKEN PIECES CONTAMINATED WITH SALMONELLA

Sample	No. examined	positive	
		n	%
Carcass	13	8	61.53
Wings	5	4	80
Backs	5	3	60
Livers	4	2	50
Total	27	17	62.96

Table 3. LOAD OF SALMONELLA AND SEROTYPES ISOLATED FROM CHICKEN CARCASS AND CHICKEN PIECES

Sample No:	Sample type	Salmonella load (cfu per sample)	Serotype isolated
1	Carcass	2900	*S. enteritidis*
2	Carcass	ND	-
3	Carcass	92	*S. hadar*
4	Carcass	93	*S. indiana*
5	Carcass	36	*S. typhimurium*
6	Carcass	150	*S. heidelberg*
7	Carcass	92	*S. enteritidis*
8	Carcass	ND	-
9	Carcass	ND	-
10	Carcass	430	*S. hadar*
11	Carcass	ND	-
12	Carcass	ND	-
13	Carcass	92	*S. hadar*
14	Liver	ND	-
15	Liver	150	*S. hadar*
16	Liver	1500	*S. enteritidis*
17	Liver	ND	-
18	Wings	ND	-
19	Wings	74	*S. hadar*
20	Wings	30	*S. enteritidis*
21	Wings	43	*S. hadar*
22	Wings	150	*S. braenderup*
23	Back	30	*S. braenderup*
24	Back	150	*S. hadar*
25	Back	ND	-
26	Back	ND	-
27	Back	30	*S. hadar*

ND = not detected in the whole carcass and chicken pieces

Table 4. SALMONELLA SEROTYPES ISOLATED FROM CHICKEN CARCASSES AND CHICKEN PIECES (%)

No.	Serotypes	n	%
1	*S. enteritidis*	4	23.5
2	*S. hadar*	8	47
3	*S. indiana*	1	5.9
4	*S. typhimurium*	1	5.
5	*S. heidelberg*	1	5.9
6	*S. braenderup*	2	11.8

The principle of the detection, the ability of *Salmonella* to migrate through semisolid agar with higher osmotic pressure, implies the main disadvantage that non-motile *Salmonella* cannot be detected [10]. However, since the proportion of non-motile *Salmonella* is estimated to be less than 1% [6], this should not hinder its routine application. Another drawback of the method is that contaminant microorganisms such as *Citrobacter* and *Proteus* may migrate through the media thus resulting in false positive negative results [17]. However, this problem can be overcome to a certain extent by agglutinating presumptive *Salmonella* positive samples with *Salmonella* H antiserum [7, 21]. Our results indicate that although all three types of the MSRV agar contain the same ingredients, there is variation in the diameter of migration zones when inoculation volumes are small. This may be attributed to the quality of gel incorporated in the medium. But it has to be pointed out that when inoculation volumes are 0.2-0.45 ml, no significant differences in the swarming zones were found in routine application of these three media on the detection of *Salmonella* spp. in 300 chilled and frozen chicken cuts and offals [15].

4. STUDY 2 (presented by Paulsen [14])

4.1. Objectives

- Performance of MSRV agar media of different brands
- Novobiocine supplementation to MSRV agar
- Enrichment at 41°C

MSRV agar media of different manufacturers were used for detection of *Salmonella* in 300 samples of poultry meat (cuts, carcasses). Protocol was as described in (a)-1. All media gave the same performance. Novobiocine supplementation was found not to be essential. Enrichment at 41°C (same temperature as for MSRV agar) instead of 37°C resulted in an increase of doubtful results. Of the 78 positive results, 77% were serogroup D, 17%C and 6% B. One sample yielded a false positive result with all media under study.

5. STUDY 3 (PUBLISHED BY FARGHALY [8])

5.1. Objective

Testing of a semisolid motility agar for detection of *Salmonella* (DIASALM agar).

5.2. Summary

DIASALM agar is a semisolid selective-differential medium for the detection of S*almonella*. The medium consists of a motility feature (similar to MSRV agar) and a differential system of sugar (sucrose and lactose) fermentation and H_2S formation. The usefulness of the medium was compared with MSRV medium in 167 samples of a variety of meat and meat products. By using DIASALM and MSRV agars, *Salmonella* were recovered from 27% of the samples. The agreement between DIASALM and MSRV agars was 100%. The addition of Novobiocine to DIASALM agar (factory recommendation: 10 mg/l) was found useful. Unlike MSRV agar, the H_2S detection system embedded in DIASALM also allows for detecting non-motile *Salmonella*.

5.3. Materials

Samples were obtained from local markets, retail shops and butchers in Vienna. The total of 167 samples consisted of chicken (retail cuts: n=82; chicken viscera: n=43), pork and beef cuts (n=17), fermented sausage (spreadable "Mettwurst", n=8), minced meat (pork and beef, n=7), paté (n=5), luncheon meat (n=5).

5.4. Method

For detection of *Salmonella*, 25 g were taken from the sample and homogenized (Stomacher 400) with 225 g buffered peptone water (MERCK 1.07228) which had been prewarmed to 37°C. This suspension was incubated for 20-24 hrs at 37°C. From this enrichment culture, three drops (total ca. 0.3 ml) were placed on MSRV agar and one drop was placed centrally on DIASALM agar (ca. 0.1 ml). After 20-24 hrs of incubation at 42°C, plates were checked for typical signs of *Salmonella* (for MSRV, see SCHALCH and EISGRUBER, 1997 [19]; for DIASALM primarily the motility zone, and then the purple halo and the blackened inoculation area were considered to be significant). Suspect plates were checked with *Salmonella* latex test (OXOID FT 203) and an agar cube from the periphery of the motility zone was streaked onto XLD selective agar (OXOID CM 469). Typical colonies were checked with API 20E (BIO MERIEUX) and *Salmonella* antiserum (BEHRING). Subcultures were then sent to the National Reference Centre in Graz, Austria for typing.

MRSV agar was prepared without addition of novobiocine (SCHALCH and EISGRUBER, 1997), while DIASALM agar (MERCK 1.09803) was prepared with addition of 10 (recommended concentration) and occasionally also 0,5 and 20 mg novobiocine (SIGMA N 1628) /l. Agar plates were stored in a refrigerator and not older than two days before use. Nine reference strains were obtained from the National Reference Centre in Graz, Austria (*S. enterica* serotypes Typhimurium 2x, Enteritidis 3x, Hadar 1x, Agona 1x, Anatum 1x, Virchow 1x).

5.5. Results

5.5.1. Performance of the diagnostic system

The appearance of the motility zone on DIASALM agar is the result of several reactions listed in Table 2. The potential presence of suspect *Salmonella* is quite easily detected on DIASALM agar due to the occurrence of a distinct opaque violet motility zone. The size of this zone differs clearly from the occasionally occurring purple diffusion zone around the inoculum due to diffusion of metabolites produced by non or marginal motile microorganisms growing at the inoculum centre. Lactose positive *Citrobacter freundii* and *Enterobacter cloacae* can be easily distinguished from *Salmonella* by their utilization of sugar incorporated in DIASALM agar; motility of lactose negative isolates of *Citrobacter freundii* in this study was suppressed by 10 mg/l novobiocine. After an additional 24 hrs of incubation, both species expressed small, non-homogenous spotted motility zones, while those of Salmonellae stayed homogenously with diameters up to 9 cm.

On DIASALM agar inoculated with mixed cultures (data not shown) the blackening due to H_2S formation at the inoculation spot is easily detected on the bottom side of the plate.

As the colours of the DIASALM medium result from various reactions (Table 7), storage of cultured plates may result in colour changes: yellow zones caused by acidification by e.g. lactose fermentation may disappear and be replaced by violet colour (alcalisation by peptone utilisation).

5.5.2. Recovery of Salmonella

A total of 167 samples of meat and meat products were tested of which *Salmonella* were recovered from 45 samples. In detail, 47.5% of chicken retail cuts, 13.9% of chicken viscera and 0% of the other types of meat and meat products were found positive. Agreement of DIASALM and MSRV was 100%. The ranking of the frequency of the isolated serotypes was: *S. enterica* serotype Enteritidis (71.1%) > Hadar (13.3%) > Indiana (9%) > Typhimurium (2.2%) > Heidelberg (2.2%) > Braenderup (2.2%).

Table 5. INDICATORS OF GROWTH ON DIASALM AGAR

Appearance	Explanation	Comment
Opaque – turbid zone	Motility zone	Opaque zone is distinct from the diffusion zone of acid (yellow) or alkali (puple- blue) that can be observed around the opaque motility zone
Green colour of DIASALM turns to whitish or gets transparent	Malachite green bound to amino groups and therefore discoloured	
Yellow motility zone	Metabolism of lactose, or saccharose produces acid resulting in bromocresolpurple shifting the motility zone to yellowish	E.g. novobiocine resistant *E. coli*
Purple motility zone	No lactose or saccharose metabolism. Alkali production due to metabolism of peptones, shifts bromocresolpurple indicator in the motility zone to purple	E.g. *Salmonella*
Violet motility zone	No metabolism of lactose or saccharose. Alkali production due to metabolism of peptones, shifts bromocresolpurple in the motility zone to purple. The absence of discolouration of malachite green produces a final colour of the motility zone of violet (green plus purple)	E.g. *Salmonella*
Black to brown areas*	H_2S formation	E.g. in centre: non motile Salmonellae, *Proteus* sp. or *Citrobacter freundii*

*in centre of inoculation when viewed from bottom of plate or when the plate is placed on a lighted glass surface. When paper discs impregnated in 10% iron ammonium sulphate are placed 3 cm diagonally from the inoculation after incubation a clear black- brown colour occurs in centre and also a black brown "feather" around the paper disc.

6. (B) EVALUATION OF A TEST KIT FOR SALMONELLA:

Study published by PAULSEN [15]

6.1. Objectives

Comparison of a partially-automated *Salmonella* antigen- EIA with cultural detection (motility media)

6.3. Summary

The Vidas ICS/SLM system allows the detection of *Salmonella* sp. as antigens in food in a four-step procedure: cultural enrichment, immunoconcentration (ICS), post enrichment, antigen detection (SLM), see Figure 2. A preliminary result can be obtained at the end of the day following the day of arrival of the sample, i.e. in an investigation time of 24-28 hrs. This method was compared to the widely accepted, rapid, sensitive and low-cost MSRV agar method. Both methods were applied on frozen as well as chilled poultry meat samples (n=122), (see Table 8). After some modification of the Vidas ICS/SLM protocol, no significant differences between MSRV agar and Vidas SLM were found in additional 36 chilled and 58 frozen meat samples. The Vidas ICS/SLM produced no false positive results (Table 9).

6.3. Material /samples

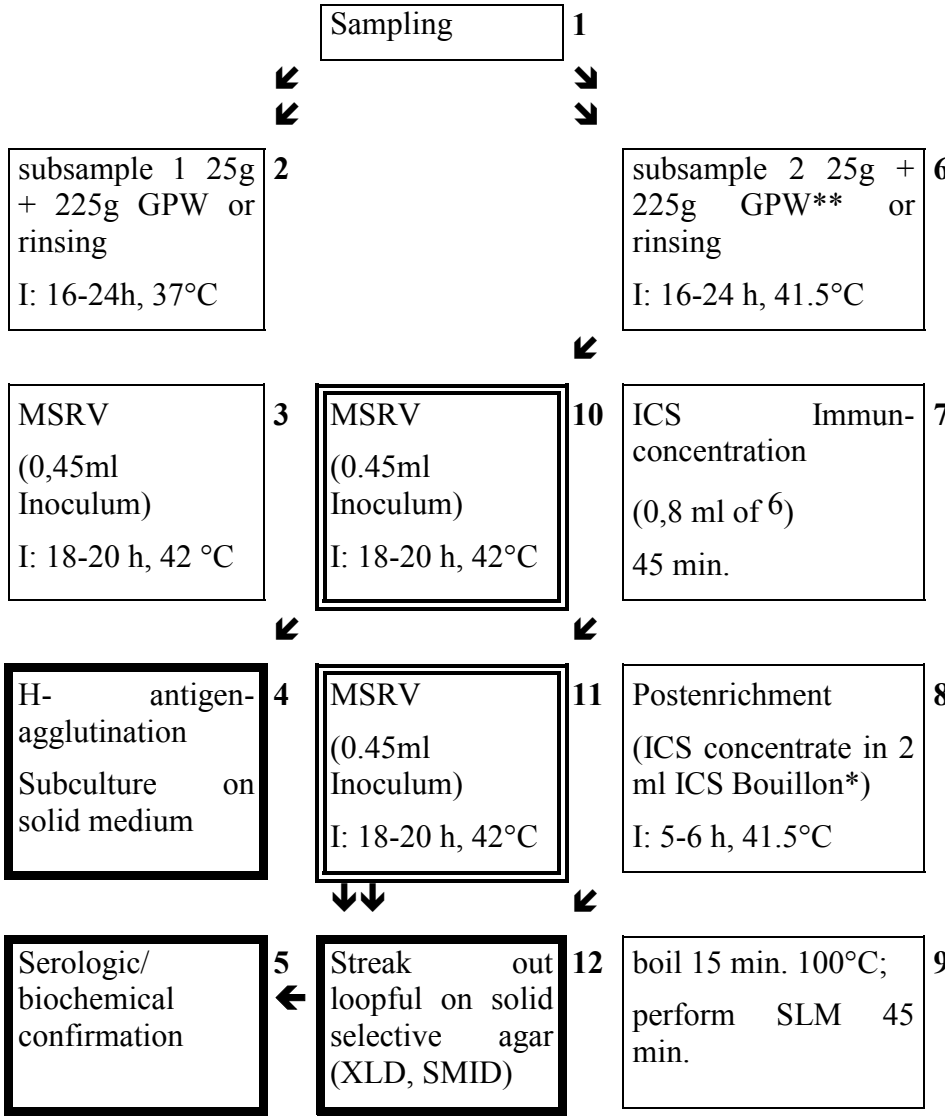
Table 6 summarizes the materials and samples used.

Table 6. SAMPLES AND SAMPLING PROCEDURE FOR EVALUATION OF THE PERFORMANCE OF A PARTIAL AUTOMATED SALMONELLA - EIA

Series	**Type of sample**	**Number of samples**	**Storage conditions**	**Sampling**	**Amount of sample**
1	chicken cuts and organs	122	-18°C, 2 weeks to 6 months	destructive	2 subsamples each 25 g
2	chicken skin, liver, gizzard	36	+ 4°C, < 5 days	rinsing and then division in two parts	total sample (50-250 g)
3	as #2	58	-18°C, 5 weeks to 6 months	destructive	2 subsamples each 25 g

Test protocol

Fig.2: Protocol MSRV agar and Vidas ICS/SLM



Legend:

= confirmatory reaction included in the method

= control test for the comparison of methods

GPW ... buffered peptone water; I ... Incubation.

Differences between **9** and **11** = "A" Differences (more attributable to different sensitivities of the detection methods used); between **3** and **9** = "B" Differences (also influenced by uneven distribution of bacteria on samples).

***** ICS Bouillon prewarmed to 41,5°C prior to use.

****** GPW for frozen samples prewarmed to 41,5°C.

Flow of working steps indicated by arrows.

6.4. Results

The results are summarized in Table 7.

Table 7. RESULTS AND AGREEMENT OF CULTURAL AND EIA TESTING OF POULTRY MEAT AND POULTRY OFFALS

Series	**# pos. total**	**# pos. MSRV 37° C**[3-5]**	**# pos. MSRV 41,5°C**[10-5]**	**# pos. MSRV from ICS Bouillon[11-5]**	**# pos. 10 µl ICS broth streaked on SMID/XLD[12-5]**	**# pos. ICS/ SLM[9]**	**"A"**	**"B"**
1	31	31	24	20	n.u.	18	*6 (ICS)	7 (41,5°C)
2	16	12	16	15	15	15	1 (ICS)	4 (37°C)
3	11	11	11	n.u.	11	11	0	0

n.u. = not investigated.

*Two samples were ICS negative and MSRV with 41,5°C enrichment positive; two samples were ICS/SLM and MSRV (enrichment at 41,5°C) negative; two samples could not be tested further.

**= incubation temperature for enrichment.

"A" = Differences between ICS/SLM and MRSRV from the same enrichment culture;

"B" = Differences between MSRV (enrichment at 37°C) and MSRV (enrichment at 41°C); less productive method in brackets.

Bold numbers in superscript relate to Figure 1.

7. SUBTASK 2: COMPILATION OF METHODS

The second subtask was to compile methods for detection of *Salmonella* in foods (protocols were taken from scientific or technical publications and national or international standards). The minimum set of data on each method is given in Table 8, note that each reference etc. has its own entry line. The next step is to fit the data in a convenient, user-friendly database.

Table 8. MINIMUM INFORMATION ON METHODS FOR DETECTION OF SALMONELLA IN FOOD (EXAMPLE)

#	Short name	Enrichment	Detection **C**ultural / **I**mmuno ?	Details on detection	Sens.	Spec.	**Nature of samples**	**N**ational standard/ **C**ompany protocol/ **S**cientific publ. ?	Reference	Further information file*
04	Vidas	Two Step (BPW and RV+ Muller Kaufmann)	I		0.95	0.91	poultry meat	C	BioMerieux	-
32	DIASALM	One Step (BPW)	C	Motility medium (SIM plus sugars)	0.99	0.98	meat	S	FARGHALY et al., 2001	far.doc

* gives details on the protocol or the principle of detection

8. SUMMARY AND CONCLUSIONS

The results of Subtask 1 show that selective motility agars for detection of *Salmonella* in (poulty) meat allow a quite reliable "presumptive diagnosis". The same holds true for the Vidas system. While Vidas offers a largely automated and quality assured diagnosis at the price of higher financial costs, selective motility agars offer a cheap alternative, provided that they are used by trained staff.

The process of compiling all methods currently used for detection of *Salmonella* in (poulty) meat is not finished yet.

ACKNOWLEDGEMENTS

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PRESENCE OF *SALMONELLA* SPP AND *ESCHERICHIA COLI* O157:H7 IN RAW MEAT, IN SÃO PAULO CITY, BRAZIL AND EVALUATION OF LOW TEMPERATURE (REFRIGERATION AND FREEZING) RESISTANCE OF THESE BACTERIA

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Abstract

A total of 253 samples of raw meat (bovine, swine and poultry) were collected in local supermarkets in Sao Paulo, Brazil, and analysed for *Salmonella* and E. coli 0157:H7, in order to determine their microbiological quality and compliance with the established standards for these two bacteria. Additionally, samples of ground beef were artificially contaminated with *S. enteritidis* and E. coli 0157:H7, in order to determine the effect of refrigeration and freezing temperature during the storage period (4ºC for 5 days, and - 18ºC for 90 days, respectively). Twenty-three samples (9.1%) were positive to *Salmonell*a and seven different serotypes were isolated, *S. enteritidis* being the most common serotype founded (34,8%). The results of the artificially contaminated samples showed that E. coli 0157:H7 was sensitive to refrigeration as well as freezing temperature. *S. enteritidis* was only affected by freezing temperature during the storage time.

1. INTRODUCTION

Salmonellosis is considered the major public health problem in many countries worldwide. Many foods, particularly those of animal origin are important vehicles for *Salmonella* transmission. Animal carriers have an important role in *Salmonella* epidemiology for the microorganism is continually released in their feces.

Conditions of production, transportation to slaughterhouse, and meat processing may favor conditions microorganism dissemination. Contamination inside the processing plants may occur during several operations, and the source of the microorganisms may be the environment, utensils, handlers and cross-contamination between animals (Bryan & Doyle, 1995) [4]. At the site of food preparation, cross-contamination may occur if time and temperature conditions are condusive (Varnam & Evans, 1991) [28].

Nowadays, *Salmonella* is one of the most frequently involved microorganisms in food poisoning (Bean *et al*., 1997; Fantasia & Filetici, 1994; 1999; Peresi, *et al*., 1998) [2, 11,18]. Tavechio *et al* (1996) related that in Sao Paulo from 1991-1995, *S. enteritidis* was predominantly from nonhuman sources [26]. This serotype first appeared in 1993 associated with food borne outbreaks. In 2000, the Sao Paulo city (CVE/SES-SP), notified that 13,7% of the total outbreaks reported were related to *Salmonella* (602 cases) [Online] ftp://ftp.cve.saude.sp.gov.br/doc [7].

E. coli O157:H7 is another important food borne pathogen of public health interest due to the increasing incidence of human disease and the association with various kinds of foods, often those of animal origin. Since 1982, when *E. coli* O157:H7 was first identified as a cause of food borne illness, many outbreaks have been reported; CDC, 1994; Besser *et. al* 1993; Griffin & Tauxe, 1991) [6, 3, 14].

This bacterium is an important pathogen in USA, Europe and Japan and in some countries of the southern hemisphere like Argentine, Australia, Chile and South Africa. In Argentina, *E. coli* O157:H7 has been associated with patients presenting hemolytic uremic syndrome (HUS) and children with bloody diarrhea (López *et. al*. 1997) [16]. In São Paulo.in 1990, one strain of *E. coli* O157:H7 was isolated from a patient with AIDS presenting diarrheal disease. In 2001, two cases of *E. coli* O157:H7 illness were reported, both in Campinas, state of São Paulo, in June and July (IRINO *et al*., 2002) [15].

Studies showed that cattle and sheep carry *E. coli* O157:H7 more frequently in summer (Armstrong *et. al*, 1996; Cassin, *et. al*, 1998) [1, 5]. The presence of the organism in meat from meat exporting countries such as Brazil has become an important trade issue.

The purposes of this study were to verify the incidence of *Salmonella* spp and *E. coli* O 157:H7 in raw meat traded in São Paulo, Brazil and evaluate the survival pattern of *Salmonella* and *E. coli* O157:H7 strains at different times of refrigeration and freezing in ground beef.

2. MATERIALS AND METHODS

2.1. Samples

A total of 253 samples of raw meat (bovine, swine and poultry) collected in local supermarkets of São Paulo, Brazil were analyzed in order to determine their microbiological quality and their compliance with established legal standards. Samples were placed in plastic bags, and transported under refrigeration to the Food Microbiology Section of Instituto Adolfo Lutz, Central Laboratory, São Paulo.

2.2. Strains

Salmonella Enteritidis was isolated at the Food Microbiology Section of Instituto Adolfo Lutz, São Paulo, from food implicated in food borne disease outbreaks. *E. coli* O157:H7 IAL 1848 was obtained from the Bacterial Culture Collection Section at the same institute.

2.3. Isolation of *Salmonella* from raw meat

Conventional modified procedures, recommended by the American Public Health Association were used (Flowers *et. al.* 1992) [12].

Pieces of bovine, swine and poultry meat were analyzed by homogenization and poultry carcasses were analyzed by rinsing technique.

2.4. Isolation and Identification of *Salmonella*

Pre-enrichment was performed by incubating samples in buffered peptone water (BPW) overnight at 35ºC. Selective enrichment was performed in selenite cystine (SC) and modified Rappaport-Vassiliadis (RV) broth, and incubated at 42ºC/24-48h. Each enrichment broth was streaked onto selective plates: Salmonella-Shigella agar (SS), brilliant green agar (BG) and

bismuth sulfite agar (BS), and incubated for 24h at 35ºC (SS and BG) and 48h at 35ºC (BS). Characteristic colonies of each plate were biochemically tested using IAL medium for Enterobacteriaceae characterization (Pessoa et. al., 1972) [21], incubated for 24h at 35ºC and were serologically tested using polyvalent somatic and flagellar antisera produced by Bacteriology Section of Instituto Adolfo Lutz. The positive strains were sent to that section for complete serotyping.

2.5. Isolation and Identification of *E.coli* O157:H7

Meat samples were homogenized with BPW and incubated at 35ºC for six hours. Each sample was plated onto MacConkey-sorbitol agar and incubated at 35ºC for 24h. At least three colonies were submitted to biochemical and serological tests.

2.6. Evaluation of refrigeration and freezing effects on *Salmonella* Enteritidis and *E. coli* O157:H7 inoculated in raw meats

2.6.1. Preparation of meat

Samples of ground beef were obtained at local supermarkets in the city of São Paulo, Brazil.

2.6.2. Preparation of cultures/sample contamination

S. enteritidis strain was cultivated in Brain Heart Infusion broth (BHI) at 35º C/18-24h, after incubation, serial dilutions up to 10^{-4} were performed in BPW and 4,5 ml of this dilution was inoculated in 450g of ground beef.

2.6.3. E. coli O157:H7 strain was cultivated and inoculated in ground beef as described for Salmonella

After homogenization by hand massage in the plastic bags containing the inoculated meat samples, samples were separated into ten portions of 25g each, and placed in sterile plastic bags. One portion was immediately analyzed. The others were analyzed after 24h, 72h, 96h and 120h at 4ºC, and after 24h, seven days, 30 days, 60 days and 90 days at -18ºC.

2.7. Analysis of inoculated meat samples

Each 25g ground beef sample was homogenized with 225 ml of BPW in a stomacher 400 Laboratory Blender (Seward Medical Ltd., London, England) and subsequent decimal dilutions were made using the same diluent. Enumeration of *S. enteritidis* and *E. coli* O 157:H7 was performed using the Most Probable Number (MPN) technique according to the APHA procedure (Peeler et. al., 1992) [19]. Isolation and identification were performed using the procedures described above.

Three separate trials were performed for each strain of *S. enteritidis* and *E. coli* O 157:H7.

3. RESULTS AND DISCUSSION

From 253 raw meats samples, 9.1% (23 samples) were positive for *Salmonella*. Table 1 shows the results.

A total of seven serotypes of *Salmonella* spp. were isolated, as seen in Table 2. The most common isolates were *S. enteritidis* which was isolated from eight samples (three chicken,

three bovine, one swine and one swine sausage). The second most common was *S. typhimurium* and Salmonella (I 4, 5, 12:i:-). Both isolated in three samples.

According to Tavechio *et al*. (1996) the *S. enteritidis* serotype was mostly isolated from human sources from 1991-95 [26]. One strain of *S. typhimurium* and one strain of *S. emek* were lysine decarboxylase negative (LDC). This occurrence is not frequent, but this information is an important consideration in the identification of these strains, since they may be misdiagnosed as *Citrobacter* spp.

No samples were positive for *E. coli* O 157:H7. This result is in accordance with those obtained by Silveira *et. al* (1999) [25]. However, in 2001, two clinical cases occurred in São Paulo. In Brazil, the prevalence of *E. coli* O157:H7 in healthy dairy cattle has also been reported to be very low [8].

Table 1. INCIDENCE OF SALMONELLA SPP. IN RAW MEAT SAMPLES OBTAINED IN THE CITY OF SÃO PAULO, BRAZIL

Type of Sample	No. of samples tested	Positive (%)
Beef	96	7 (7,3)
Chicken	65	3 (4,6)
Swine	23	2 (8,7)
Swine sausage	56	11 (19,6)
Chicken sausage	13	0 (0)
Total	253	23 (9,1)

Several other researchers have investigated the prevalence of *Salmonella* spp. in raw meat. *S. Enteritidis* was the most prevalent serotype [9, 13, 17, 27]. In the city of São Paulo, in 2002, it was reported that 22,5% of raw meat samples were harboring *Salmonella*, with *S. enteritidis* being the most frequent serotype (28,8%), Paula et al, (2000) [18]. This is in agreement with the predominance of *S. enteritidis* serotype in reported food borne outbreaks.

Table 2. SALMONELLA SEROTYPES FOUND IN RAW MEAT SAMPLES OBTAINED IN THE CITY OF SÃO PAULO, BRAZIL

Serotypes	Number of samples (%)
S. enteritidis	8 (34,8)
S. typhimurium	3 (13)
S. I4.5,12:i:-	3 (13)
S. london	2 (8,7)
S. infantis	1 (4,3)
S. agona	1 (4,3)
S. derby	1 (4,3)
S. panama	1 (4,3)
S. senfetenberg	1 (4,3)
S. I13.23:z:-	1 (4,3)
S. emek	1 (4,3)
Total	23 (100)

3.1. Survival trials

Figures 1 and 2 present *S. enteritidis* and *E. coli* O157:H7 population in ground beef (log MPN/g) kept under refrigeration (4ºC) for different times.

Figure 1

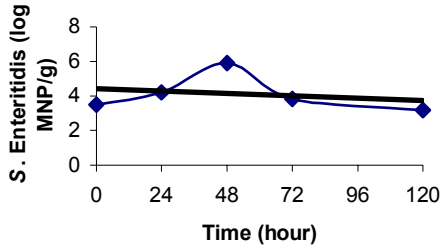
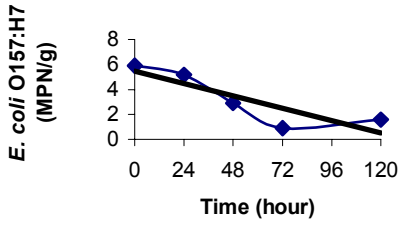


Figure 2



Figures 3 and 4 present *S. enteritidis* and *E. coli* O157:H7 population in ground beef (log MPN/g) kept under freezing (-18ºC) for different times.

Figure 3

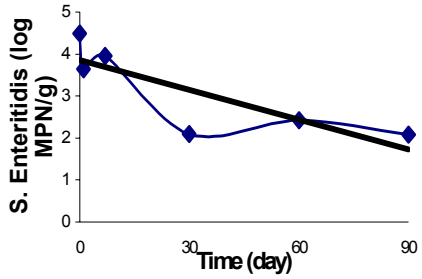
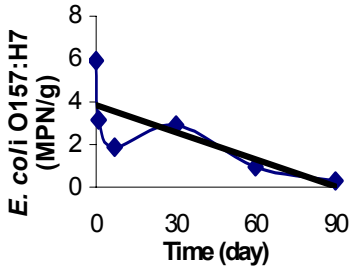


Figure 4



The results of the present trial showed that the strain of *E. coli* O157:H7 used was sensitive to refrigeration and freezing temperatures. In both cases, the initial population was reduced in four and six log, respectively during the storage period. Data from literature also indicates that *E. coli* O 157:H7 is affected by freezing [23, 26]. Those researchers concluded that *E. coli* O157:H7 death in ground beef after freezing and thawing ranged from 0.62 to 2.52 log10 CFU/g. On the other hand, Saad & Franco (1999) observed that *E. coli* O157:H7 counts in inoculated ground beef under refrigeration remained relatively constant throughout the 96 hours of their trial. Doyle & Schoeni (1987) showed that *E. coli* O157:H7 in ground beef may survive up to nine months at -20º C [10]. The freezing sensitivity of different strains from the same genus and species may vary depending on the strain, kind of substrate, freezing procedure (adaption to conditions), and temperature. Among genera, sensitivity may also differ, as shown in the present study in wich *Salmonella* presented higher resistance than *E. coli* O157:H7.

Salmonella e*nteritidis* population submitted to refrigeration (4ºC) was not affected throughout 120 hours of trial; however, under freezing, the initial population was reduced two log. According to Varnam & Evans, 1992, *Salmonella* population is reduced at freezing temperatures, and growth of *Salmonella* strains at temperature below 7ºC depends on the strain and serotype, and population is reduced at freezing temperature. Strains of *Salmonella* are less resistant to freezing than *S. aureus* or vegetative cells of *Clostridium*.

4. CONCLUSIONS

All analyzed samples were negative for *E.coli* O157:H7.

S. enteritidis was the predominant *Salmonella* serotype isolated from raw meat.

One strain of *S.typhimurium* and one strain of *S. emek* were lysine decarboxylase negative (LDC).

Results of the present study suggest that the *S. enteritidis* population in laboratory contaminated ground beef was not influenced by refrigeration, but was reduced by freezing temperature.

E. coli O157:H7 was sensitive to both refrigeration and freezing temperatures.

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DETERMINATION OF BACTERIAL PATHOGEN IN FOODS FOR EXPORT AND THEIR RAW MATERIAL

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Abstract

Chile is a South American country with an important fish and shellfish production. These products are some of the most important items for the economy of the country. From 1998 to 2001, Chile exported $1 137 625 788 of fish and shellfish. Statistics also show that frozen vegetables are fast becoming high on the food export list. During recent years (1998 to 2001) $223 312 248 worth of frozen vegetables were exported to different countries. This study was performed to trace the presence of pathogens in some of these Chilean foods to be exported: 97 samples of salmon and 84 samples of different frozen vegetables (asparagus, peas and corn) were analyzed in order to determine their levels of microbial contamination. Total bacteria counts (mesophilic aerobes bacteria), *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens*, *Salmonella* spp. and *Listeria monocytogenes* were tested. *Vibrio cholerae* was tested only in salmon samples. The analysis of salmon samples showed that the raw material presented a very good quality. However, during the filleting process the fish was contaminated, presenting higher total bacteria counts. Only one of the 48 final product samples presented contamination with a pathogenic bacteria (*Listeria monocytogenes* (<100 cfu/g)). Frozen vegetable samples (raw material and final products) did not present any of the pathogen bacteria studied. The mesophilic aerobes bacteria counts were reduced during processing due to the effectiveness of the good manufacturing practices and the technological process used.

1. INTRODUCTION

There is a wide recognition of the role of foods in spreading diseases and there is a general awareness of the need to set up safety and quality systems in food production. During recent years many reports have been published on food contamination with different types of pathogenic bacteria like *E. coli* 0157 H7, *Listeria monocytogenes,* some *Shigella serotypes.* In the USA, 20 000 persons become sick each year because of the contamination of meat and meat products with *E. coli* O157 H7.

2. MATERIALS AND METHODS

Fish samples were obtained from a fish exporting company and at three different points of the processing line: a) raw material (freshly caught and gutted fish), b) filleting point (after the fish bone is removed), and c) final product (frozen and vacuum packaged salmon fillet). A total of 97 samples were analyzed: 27 samples of raw material, 22 samples from the filleting point and 48 samples of the final product.

Raw material, as well as samples at the filleting point and at the end of the process were obtained from the same production lot.

Vegetable samples were obtained from two companies which produce and process different types of frozen vegetables. The process included a blanching (short heat 95-99ºC for 3-5 minutes) which is necessary to reduce microbial numbers by one or two log cycles, inactivate enzymes and stabilize the products during the frozen storage.

[†] Deceased, February 2001.

A total of 84 vegetable samples (20 asparagus, 30 peas and 34 corn samples) were analyzed: a) 36 samples of raw material (fresh vegetables) and b) 48 of the final products (frozen and packaged vegetables).

The determination of the total bacteria counts (mesophilic aerobic bacteria), and the enumerations and/or isolations of Escherichia coli, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, Salmonella spp., Listeria monocytogenes and Vibrio cholerae were carried out using the methodology described in the 8th Edition of the Bacteriological Analytical Manual published by the US FDA - 1995. Vibrio cholerae was tested in fish samples only**.**

3. RESULTS AND DISCUSSION

3.1. Frozen salmon

The results are presented in Table 1.

The results show that raw material is of good microbiological quality. However, the same samples presented a higher level of contamination after the filleting process.

The reason for the good microbiological quality of the raw material may be due to the location of the aquacultures. These are in the South of the country where the climate is cold. Additionally, the salmon culture lagoons are far from the coast and into a hidden area, surrounded by leafy vegetation where the water is very cold and clean. The fish was maintained at refrigerated temperature immediately after the capture and during transportation. The temperature of the fish was checked at their arrival at the processing plant. If the temperature was over 3.5ºC the fish was rejected.

Half of the samples tested were processed manually and the other half were mechanically processed. The samples processed manually presented higher total bacteria counts than the samples processed mechanically.

Final products presented a low mesophilic aerobes bacteria count due to the smoking process and the vacuum packaging. However, one of the 48 samples of the final product was *Listeria monocytogenes* positive (<100cfu/g).

Although the plant had implemented adequate measures in the processing line to control the mesophilic aerobic counts, these measures did not eliminate *L. monocytogenes*. Two samples were positive for *Listeria monocytogens* at the filleting point, and one at the end of the processing line (final product).

Some studies have found a high correlation between the occurrence of *L. monocytogenes* in the seawater and the raw material. However, up to now a clear contamination route has not been found.

The results obtained indicate that further preventive measures must be considered for control of *L. monocytogenes* contamination.

3.2. Frozen vegetables

The results are presented in Tables 2, 3 and 4.

The raw materials (different types of fresh vegetables) were produced and harvested by the same processing companies. These companies used good agriculture practices (GAP) as well as good manufacturing practices (GMP), including during the transportation of the raw materials to the processing plant immediately after the harvest. This is an important factor contributing to good microbiological quality. Strict control in these early steps in the food chain avoids high contamination and multiplication of microorganisms during this time.

The samples did not present any of the pathogenic microorganisms studied. The total bacteria counts were reduced substantially during the processing steps. The time between the count-reducing blanching step and freezing was very short, in order to prevent bacterial regrowth.

The microbiological quality of the final products was very good.

Table 1. FROZEN SALMON - BACTERIOLOGICAL ANALYSIS TO FROZEN SALMON FOR EXPORT

Analysis	**Raw material**	**Manipulation Point**	**Final product**
Samples	N = 27	N = 22	N = 48
Enumeration mesophilic aerobes cfu/g	< 2.5 x 10^3 100%	< 2.5 x 10^3 50.0% 2.5 x 10^3 - 2.5 x 10^4 36.4% >4.3 x 10^4 - 2.5 x 10^5 9.0 % >2.5 x 10^5 4.6 %	< 2 x 10^3 95.8 % 4.2 x 10^5 4.2 %
MPN *S. aureus*	<3 ger/g	<3 ger/g	<3 ger/g
MPN *E. coli*	<3 ger/g	<3 ger/g	<3 ger/g
Detection *Salmonella*	Negative in 25 g	Negative in 25 g	Negative in 25 g
Enumeration *B. cereus*	<1 x 10^2 cfu/g	<1 x 10^2 cfu/g	<1 x 10^2 cfu/g
Enumeration *C.perfringens*	<1 x 10^2 cfu/g	<1 x 10^2 cfu/g	<1 x 10^2 cfu/g
Detection *L. monocytogenes*	Negative in 25 g	2 positive 20 Negative in 25 g	1 positive* 47 Negative in 25 g

Table 2. ASPARAGUS - BACTERIOLOGICAL ANALYSIS TO FROZEN ASPARAGUS FOR EXPORT

Analysis	**Raw material**	**Final product**
Samples	N =10	N = 10
Enumeration mesophilic aerobes cfu/g	< 6.7 x 10^3 10% > 6.7 x 10^4 – 2.5 x 10^5 80% > 2.2 x 10^5 10%	< 2.5 x 10^3 100 %
Enumeration *S. aureus*	< 10 cfu/g	< 10 cfu/g
MPN E. coli	<3 ger/g	<3 ger/g
Detection *Salmonella*	Negative en 25 g	Negative en 25 g
Enumeration *B. cereus*	< 1x 10^2 cfu/g	< 1x 10^2 cfu/g
Enumeration *C. perfringens*	< 10 cfu/g	< 10 cfu/g
Detection *L. monocytogenes*	Negative en 25 g	Negative en 25 g

Table 3. PEAS - BACTERIOLOGICAL ANALYSIS TO FROZEN PEAS FOR EXPORT

Analysis	**Raw material**	**Final product**
Samples	N =14	N = 16
Enumeration mesophilic aerobes cfu/g	1.5×10^4 -1.5×10^5 21.4% 1.5×10^5 –2.5×10^6 42.8% $>2.5 \times 10^6$ - 2.5×10^7 28.6% $> 2.5 \times 10^7$- 2.5×10^8 7.1%	2.5×10^2 - 2.5×10^3 43.8% $>2.5 \times 10^3$ -2.5×10^4 56.2 %
Enumeration *S. aureus*	< 10 cfu/g	< 10 cfu/g
MPN *E. coli*	<3 ger/g	<3 ger/g
Detection *Salmonella*	Negative in 25 g	Negative in 25 g
Enumeration *B. cereus*	< 1×10^2 cfu/g	< 1×10^2 cfu/g
Enumeration *C. perfringens*	< 10 cfu/g	< 10 cfu/g
Detection *L. monocytogenes*	Negative in 25 g	Negative in 25 g

Table 4. CORN- BACTERIOLOGICAL ANALYSIS TO FROZEN CORN FOR EXPORT

Analysis	**Raw material**	**Final product**
Samples	N =12	N = 22
Enumeration mesophilic aerobes cfu/g	$2.5 \times 10^4 \times 2.5 \times 10^5$ 16.6% $>2.5 \times 10^5$-2.5×10^6 25.0% $>2.5 \times 10^6$ 58.3 %	2.5×10^2 - 1.4×10^3 36.5 % 2.5×10^3 - 2.5×10^4 63.6 %
Enumeration *S. aureus*	< 10 cfu/g	< 10 cfu/g
MPN *E. coli*	<3 ger/g	<3 ger/g
Detection *Salmonella*	Negative in 25 g	Negative in 25 g
Enumeration *B. cereus*	< 1×10^2 cfu/g	< 1×10^2 cfu/g
Enumeration *C. perfringens*	< 10 cfu/g	< 10 cfu/g
Detection *L. monocytogenes*	Negative in 25 g	Negative in 25 g

4. CONCLUSIONS

1. The microbiological quality as measured by the aerobic plate count of the salmon samples was very good. Three of the 97 samples presented contamination with *L. monocytogenes* and further control measures may be required for this organism.

2. The reasons of the good bacteriological quality of the salmon samples were the good sanitary conditions of the aquaculture, adequate temperature and contamination control during the transportation as well as the good manufacturing practices applied in the processing plant.

4. The main reasons for the excellent quality of the different vegetable samples were the agricultural practices applied in the field, as well as the good practices applied during transport and in the processing plants

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MICROBIOLOGICAL QUALITY OF SOME MAJOR FISHERY PRODUCTS EXPORTED FROM INDIA

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Abstract

The export quality marine and aquaculture fish and fishery products were collected from European Union Approved (EUA) and EU-non-approved (EUN) plants located at east and west coast of India and were analysed for the presence of human bacterial pathogens using standard bacteriological techniques. A total of 126 samples comprising of 26 marine shrimp (*Penaeus indicus*), 18 freshwater prawn, scampi (*Macrobrachium rosenbergii)*, 40 squid (*Loligo* sp.), six cuttle fish (*Sepia* sp.), 30 rohu (*Lobia rohita*) and six long fin herring (*Citrocentrus* sp.) were analysed. The samples were screened for aerobic plate count (APC) and pathogens including *Salmonella* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus, Staphylococcus aureus, Escherichia coli*, *Listeria monocytogenes,* and *Yersinia enterocolitica.* It was observed that the marine products from EUN plants were of poorer microbiological quality as compared with products from EUA plants. Samples of squid (40%) and shrimp (28.5%) from EUN plants were found to be contaminated with *Salmonella.* However, *V. cholerae, L. monocytogenes,* and, *Y. enterocolitica* were not detected in any of the samples tested. Shrimp samples (28%) from EUN plants were positive for *Vibrio parahaemolyticus.* Shrimp (14.3%) and squid (40%) were also contaminated with coagulase positive *S. aureus*. *Salmonella* contamination was observed in 16.7% of the cuttle fish samples from EUN plants. Whole herring samples were of acceptable microbiological quality. Of the freshwater items analyzed, whole rohu samples had higher microbial load as compared to processed rohu samples. All the rohu samples were free from the pathogens, however, 25% of the rohu steak samples had *E. coli* exceeding the limit of 20 cfu/g. Both whole as well as headless scampi harboured higher microbial load; whole (50%) and headless (41%) scampi samples were contaminated with *Salmonella* spp. The results suggested a need for implementation of better hygienic practices for the improvement of microbial quality of products from EUN plants. In addition, it is necessary to adopt stringent hygienic practices for production of good quality aquacultured fishery items.

1. INTRODUCTION

India has an 8129 km long coastline, 5.4 million hectare fresh water and 1.2 million brackish water area and is the third largest fish producing nation with an annual production of 5.36 million tonnes. Indian seafood occupies an eminent position in world food trade. During the last decade India has grown as a major exporting country of fishery products consisting of marine, freshwater and aquaculture products to more than 60 countries including the European Union, Japan and USA. Fish export increased from about 140 000 metric tonnes in

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1990 to 440 473 metric tonnes in the year 2001 registering an average annual growth rate of 14.6% in volume and 47.3% in value during the decade [13]. Frozen shrimp formed the major export commodity contributing 70.23% of total value, followed by finfish (12.6%) squid (5.22%) and cuttlefish (4.74%) (Anonymous, 2002).

Production of fish and shellfish by aquaculture is the fastest growing sector of world food economy. World aquaculture output has been growing at 11% per annum during the past decade and has reached 33.3 million tonnes in the year 1999 [8]. In India, fish production by aquaculture has doubled to about 2.0 million tonnes during the last decade [5]. India now ranks second in global aquaculture production and fourth in shrimp production. Farmed shrimp contributes 59% by weight (65 894 metric tonnes) and 86% by value ($806 million) of India's total shrimp exports [5]. At present, 830 000-hectare water areas are under fresh water aquaculture operation in India. The state of Andhra Pradesh in India with 90 638 hectares under aquaculture cultivation tops in production of freshwater prawns. In the year 2000–2001, Andhra Pradesh produced 67 615 metric tonnes of shrimp [13]. Fresh water giant prawn, *Macrobrachium rosenbergii* (scampi) for which there is a huge demand, is one of the major varieties that are grown in the country. In the year 2000-2001, out of the total export of 7500 tonnes of scampi, 7000 tonnes were exported from Andhra Pradesh [18].

Fishery products have been recognized as a major carrier of food-borne pathogens. The contamination can occur prior to harvest, during harvest and processing operations, distribution, storage, and preparation of the product [25]. Some of the common pathogens found in fishery products are *Salmonella* spp*., Staphylococcus aureus, Vibrio cholerae, V. parahaemolyticus, Yersinia enterocolitica, Listeria monocytogenes, Campylobacter jejuni,* and *Escherischia coli* [24]. Presence of these pathogens in processed food products including fishery products has been a major reason for the rejection by importing countries. Analyses of 11 132 samples of fish, shellfish, crustaceans and other aquatic creatures, which were imported in USA during 1990 to 1998, showed that 7.2% of the samples were contaminated with *Salmonella*. On a regional basis, incidence of *Salmonella* was highest in imports from Central Pacific (12.5%) and Africa (11.5%) followed by Southeast Asia (7.8%) [14]. Aquacultured fishery products are more prone to various safety hazards including contamination by pathogenic microorganisms than are ocean fish. The safety issues associated with aquacultured products have been highlighted by a Joint FAO/IAEA/WHO study group (1999). During recent years, fish exports have been subjected to stringent quality evaluation enforced by international regulatory authorities. The main quality problems faced by Indian seafood exports are microbiological. This includes high bacterial load, aerobic plate count (APC) and the presence of pathogens like *Salmonella* spp., fecal coliforms, and *Vibrio cholera* [20]. Failures of Indian seafood consignments to meet the high hygienic standards have resulted in their rejection or detention by the importing countries. During June 1995 to December 1997, 31 fish consignments exported to European Union (EU) countries were found to be of poor hygienic quality. This led to a ban of fish export from India to EU countries (Anonymous, 1998; Anonymous, 1999)b [2, 3]. In addition, during the same period, many consignments exported to USA and Japan was also rejected for the same reason [20].

During the last few years, enforcement of Hazard Analysis Critical Control Point (HACCP) protocol in units processing fishery products for export has been made mandatory by the European Union, USA and other countries [1]. The Marine Products Export Development Authority (MPEDA), the Government agency that coordinates export of fishery products from India, has taken various measures to improve the quality of seafood to meet international standards. Such measures include training programms, supervision and advice on design of processing plants, quality of water, workers' hygiene, handling of the fish, etc. as per the

HACCP protocol [3, 9]. Although these efforts have resulted in improvement of quality, partial lifting of the ban and permitting a few processors to export fish to EU countries, some of the 'EU approved' exporters are finding it difficult to maintain acceptable quality of their products. This has caused loss of their 'EU approved' status and hence license to export to EU countries (MPEDA, personal communication).

The present study was conducted as part of a five-year Coordinated Research Project sponsored by the Joint FAO / IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna. The main objective was to determine the profiles of human bacterial pathogens in some of the major fishery products exported from India. Mumbai is a major seafood-processing centre on the western coast of India and exports about 9% of total processed seafood produced in the country. Kakinada in Andhra Pradesh is one of the major fresh water aquaculture centres on the east coast of India. For the present study, samples from processing plants from Mumbai and Kakinada were analysed. The products selected for analysis include processed marine (*Penaeus indicus*) and processed as well as whole fresh water prawn (scampi - *Macrobrachium rosenbergii*), squid (*Loligo* sp.), cuttle fish (*Sepia* sp.) and marine (herring - *Citrocentrus* sp.) and fresh water finfish (rohu - *Lobia rohita*).

2. MATERIALS AND METHODS

2.1. Chemicals and media

Bacteriological Media were from Hi Media Laboratories Ltd., Mumbai, India. Rabbit plasma for coagulase assay was from Becton Dickinson, France.

2.2. Sampling

Frozen processed rohu samples from a local unit in Mumbai were packed aseptically in sterile polyethylene bags and brought to the laboratory under frozen condition and kept at -20°C till the analysis. The frozen processed (gutted and steaked) as well as whole rohu and scampi samples from a processing plant in Kakinada, Andhra Pradesh were brought to Mumbai by air in frozen condition. A total number of 30 rohu and 18 scampi samples were collected.

Frozen or processed and ready-to-freeze squid, cuttle fish and shrimp were collected from five different processing units in Mumbai. From each plant, six to twelve random samples of the commodity were collected. Each unit sample weighing about 250 to 300 g. was randomly picked up from different locations of the frozen slab. The samples of frozen processed cuttle fish and whole herring were collected from two different processing units in Mumbai.

2.3. Microbiological analysis

The microbiological analysis was performed as per standard methods adopted from Bacteriological Analytical Manual Online, US, FDA for detection, enumeration and identification to species level of individual organisms (BAM, 2001) [5]. Respective reference organisms were used for verification of the identification procedures employed.

2.3.2. Aerobic Plate Count (APC)

Twenty-five g of the sample were homogenized in 225 ml of sterile physiological saline. After serial dilution up to fourth dilution, the samples were pour-plated on Plate Count Agar (PCA). The colonies were counted after 48 hours of incubation of the plates at 37°C.

2.4. Salmonella

Twenty-five grams of the sample was homogenized in 225 ml of lactose broth and incubated overnight at 37°C. After the initial pre-enrichment step, samples were further enriched in Selenite Cystine Broth, Tetrathionate Broth at 37°C and Rappaport Vassiliadis (RV) Medium at 42°C. A loopful of growth from each of these media was streaked on Bismuth Sulphite Agar (BSA), Xylose Lysine Desoxycholate agar (XLDA) and Hektoen Enteric agar (HEA). Both atypical and typical colonies from each of these plates were picked up, purified and identified further by subsequent biochemical tests.

2.4.1. Vibrio cholerae

Twenty-five grams of the sample were homogenized in 225 ml of Alkaline Peptone Water (APW) and incubated overnight at 37°C. After secondary enrichment for six hours in APW, a loopful of the culture from primary and secondary enrichment media was streaked on Thiosulphate Citrate Bile Sucrose Agar (TCBS). Yellow, translucent colonies from each plate were picked up for further biochemical tests.

2.4.2. Vibrio parahaemolyticus

Fifty grams of the sample were homogenized in 450 ml of sterile 3% saline. The homogenate was serially diluted in Glucose Salt Teepol Broth (GSTB) containing 3% NaCl and appropriate dilutions were incubated for 24h at 37^{o}C. Loopfuls of the culture from each of the tubes showing turbidity were plated on to TCBS agar containing 3% NaCl. Greenish and bluish green colonies were selected for further biochemical tests.

2.4.3. Escherichia coli

Enumeration of *E. coli* was performed using a five-tube most probable number (MPN) technique that consisted of pre-enrichment at 37°C for 48 h in Lauryl Sulphate Tryptose Broth (LSTB) followed by selective enrichment at 45.5 ± 0.5°C for 48h in *Escherichia coli* Broth (ECB). The positive tubes were plated on Eosin Methylene Blue (EMB) agar. Purple coloured colonies with a metallic sheen were selected and the presence of *E. coli* was confirmed by Indole, Methyl Red, Voges-Proskauer and Citrate (IMViC) tests. The final enumeration of *E. coli* was done following McCardy's table.

2.4.4. Staphylococcus aureus

Dilutions made for the Aerobic Plate Count were plated on Baird Parker's agar (BPA). After 24-48 h of incubation at 37° C, the characteristic black colonies with a peripheral clearance zone were counted and typical isolates were tested for coagulase activity. Coagulase activity was checked by Tube Coagulase Test as per BAM protocol.

2.4.5. Listeria monocytogenes

Twenty-five grams of the sample was homogenized in 225 ml of the BHI (Brain Heart Infusion) broth and incubated for 48 h at 4°C. Secondary enrichment was done in Listeria Enrichment Broth (LEB) for 48 h at 37°C. Public Health Laboratory Services (PHLS, UK) method comprising of primary enrichment in 1% buffer peptone water (24h at 30°C) followed by secondary enrichment in *Listeria* selective broth (LSB) for 48 h at 30°C was also used for enrichment of *Listeria* sp. Loopful of culture was streaked on to Listeria Selective Agar (LSA). Characteristic positive colonies were picked up for further biochemical confirmation.

2.4.6. Yersinia enterocolitica

Twenty-five grams of the sample was homogenized in 225 ml of the BHI (Brain Heart Infusion) broth and incubated for 48 h at 4°C. Secondary enrichment was done in Sorbitol Bile Broth (SBB) for 48 h at 30°C. Further plating was done in Yersinia Selective Agar (YSA) to isolate the characteristic [bull's eye red] positive colonies. A battery of biochemical tests was employed to confirm the presence of this organism.

3. RESULTS AND DISCUSSION

The samples of marine shrimp, squid and cuttle fish were procured from six processing units in Mumbai, which include three EU approved (EUA) and three non-EU approved (EUN) plants. The EUN plants, since having no EU approval, are exporting to countries other than EU. The results of the microbiological analysis of these samples are shown in Table 1. It can be seen that the microbial quality of shrimp and squid from EUA plants was better than the EUN plants. The aerobic plate counts of both the products from EUA plants were approximately one log cycle lower than their EUN counterparts. Shrimp (28.5%) and squid (40%) samples from EUN plants were contaminated with *Salmonella*; however, the samples obtained from EUA plants were free from this pathogen (Table 1). In addition, 14.3% of shrimp and 40% of squid samples collected from EUN plants were positive for coagulase positive *S. aureus,* the level of incidence being above the prescribed upper limit of 100 cfu per gram as per Specification by Export Inspection Agency (EIA), India (Table 2). All the samples were free from pathogens such as *L. monocytogenes, V. cholerae* and *Y. enterocolitica.* Although some samples were positive for *E. coli,* the counts were below the permissible level of <20 cfu/g. The 28% of the shrimp samples from EUN plants showed the presence of *V. parahaemolyticus.* This pathogen is present in the marine environment and has been reported in shrimp (Bandekar *et al*, 1982) [6]. The cuttle fish analyzed under the study were from EUN plant. A great degree of variation was found in the APC ranging from 10^2 to 10^5 cfu/g (Table 1). Though all the cuttle fish samples were positive for *S. aureus*, the isolates tested were all coagulase negative. All the samples were free from *E. coli, L. monocytogenes and V. cholerae,* but 16.67% of samples were contaminated with *Salmonella.*

The present results suggest that products processed by EUN approved plants were of comparatively poorer microbiological quality than those from EUA units. The EUA plants are operating under the protocols provided by European Union, which are based on GMP (Good Manufacturing Practices) and SSOP (Sanitation Standard Operating Procedures) and the HACCP (Hazard Analysis Critical Control Point) protocols. The superior quality of the products from EUA plants results from strict implementation of the hygiene practices. This may not be the case in EUN plants. This study clearly shows the importance of good handling practices in product quality and the need for improvement of existing sanitary and hygienic practices in the processing of the fishery products in the EUN plants.

The microbiological quality of whole and headless shrimp from Kakinada was poor (Table3). In 80% samples of whole shrimps and 50% samples of headless shrimps the APC was $>10^5$ cfu/g. 33% of samples of whole shrimps and 42% of headless shrimps) were contaminated with *Salmonella*. *E. coli* was present in 50% of the samples of whole shrimp, however, the counts were < 20 cfu/g. In headless shrimp, 25% of the samples were positive for *E. coli* (all<20 cfu/g). All the samples were free from *V. cholerae, S. aureus* and *L. monocytogenes*.

The samples of processed rohu steaks obtained in four different batches (18 samples) were of good microbiological quality. The Aerobic Plate Count (APC) was $\leq 5 \times 10^5$ cfu/g (Table 4). *E. coli* (<20 cfu/g) was detected in 16% of the samples. All the samples were free from

Salmonella, V. cholerae, S. aureus and *L. monocytogenes*. In one batch of six samples from Kakinada, all samples were found to be of poor microbiological quality having high APC (> 5 x 10^5 cfu/g) and high coliform counts. However, no pathogens were detected in any of the samples. The whole rohu samples obtained from Kakinada exhibited poor microbiological quality. APC was ≥ 5 x 10^5 cfu/g in 33.33% of samples and 80% of the samples were contaminated with *E. coli* (<20 cfu/g) (Table 4). However, all of the samples were free from *Salmonella, V. cholerae, S. aureus* and *L. monocytogenes*. The whole samples of rohu had higher microbial load probably due to contamination from the intestine during processing. Earlier studies on the microbiological quality of rohu marketed in India have shown high level of microbiological contamination with 33% to 40% samples positive for *E. coli*; however, no *Salmonella* contamination was reported (Sinha *et al*, 1991, Sapna Kumari *et al*, 2001). Only 17% samples of herring had APC greater than 5 x 10^5 cfu/g. All the samples were free from *E. coli, Salmonella, V. cholerae,* coagulase positive *S. aureus* and *L. monocytogenes*.

Export Inspection Agency of India (EIA) has stipulated bacteriological specifications for frozen marine shrimp (Table 2). However, no specifications are available for the fresh water aquaculture products. The present results suggest that processed rohu samples are of better microbiological quality than the whole fish probably due to the GMP and SSOP followed in the plant during processing. The comparatively poor quality of freshwater prawns reflects the poor quality of the environment, unhygienic handling and the cultivating conditions such as feed quality. It has been recognized that use of wastewater for fish farming or the practice of fertilizing ponds with animal manure may result in transmission of pathogenic bacteria and parasites to fish. Aquatic birds are known to harbour pathogenic strains of *V. cholerae and Salmonella* spp. that can infect the fish farms [11, 19, 7]. The poor hygienic quality of fishmeal used for aquafeed production may be a source of contamination of these pathogens. A two-year survey carried out in one of the major prawn exporting countries in Southeast Asia has shown that brackishwater ponds and cultured prawns were inherently contaminated with *V. cholerae and Salmonella* [21]. Our results indicate that when farming prawns, better care needs to be taken to avoid contamination from the environment. In addition, further processing like radiation treatment of prawns may be required to eliminate the pathogens from the finished product.

Table 5 gives the profiles of presumptive and confirmed positive isolates of major pathogenic organisms in the products. The detection of pathogens like *Salmonella* and *L. monocytogenes,* involves pre-enrichment, selective enrichment and plating on several media. As can be seen from the table a large number of presumptive positive isolates were obtained. The presumptive positive isolates of individual pathogens need to be confirmed by an extensive number of biochemical tests. Our results showed that the entire presumptive positive isolates of *L. monocytogenes, V. cholerae* and *Y. enterocolitica* could not be confirmed by biochemical testing. Very few isolates of *Salmonella* (40/1249) and *V. parahaemolyticus* (3/167) could be confirmed positive. There is an urgent need for adopting rapid reliable method for the analysis of a large number of samples within shorter time.

None of the 126 samples tested in this study were contaminated with *L. monocytogenes*. Most of the *Listeria* isolates were found to be *L. inocua* [17, 12]. However, in a recent study on the occurrence of *Listeria* sp. in processed fishery products obtained from a seafood processing plant, Jeyasekaran *et al* (2002a) observed that the incidence of *L. monocytogenes* was high in marine headless shrimp in comparison to peeled and undeveined shrimps. The studies reported from a fish processing plant in India indicated that about 4% sea food handlers were carriers of *L. monocytogenes* and 5.1% of the samples from floors, drains, tables and equipments of the plant were positive for this pathogen [16]. The present results suggest a

need to improve the sanitary and hygienic practices followed by the seafood processing plants, especially in EUN plants.

4. CONCLUSIONS

The seafood samples analyzed from EUA plants were of good microbiological quality compared to those from EUN plants. Shrimp (28.5%), squid (40%), scampi (50%) and cuttle fish (16.7%) from EUN plants were contaminated with *Salmonella*. Shrimp (14.3%) and squid (40%) were positive for coagulase positive *S. aureus.* Shrimp (28.5%) samples were also positive for *V. parahaemolyticus*. The aquaculture farming practices, quality of water in the ponds, use of fertilizers or manures, quality of feed as well as hygienic conditions in the processing plants need to be improved for obtaining safe products.

The microbiological protocols for the detection of *Salmonella, L. monocytogenes* and other pathogens are time consuming and laborious and reliable, rapid methods need to be adopted.

Table1. MICROBIOLOGICAL PROFILE OF READY TO EXPORT SHRIMP, SQUID AND CUTTLE FISH

Microbiological parameters	Shrimp Cfu/g		Squid Cfu/g		Cuttle fish Cfu/g
	EUA	EUN	EUA	EUN	EUN
Aerobic Plate Count (APC)	$3.5x10^3$-$6.3x10^5$	$1.6x10^4$-$1.6x10^6$	<10-7.0 $x10^5$	$4x10^2$-$1.5x10^5$	$4.3x10^2$-$1.5x10^5$
Escherichia coli	0-9/g	0-8	0-4	0-8	NIL
Staphylococcus aureus (Coagulase positive)	-	$0\text{-}10^3$ [a]	-	0-200 [c]	NIL
Salmonella	-	+[b]	-	+[c]	+[d]
Vibrio cholerae	-	-	-	-	NIL
Vibrio parahaemolyticus	-	<1/g [e]	-	-	ND
Listeria monocytogenes	-	-	-	-	-
Yersinia enterocolitica	-	-	-	-	ND

a: One out of seven samples of shrimp
b: Two out of seven samples of shrimp
c: Two out of five samples of squid
d: One out of six samples of cuttle fish
e: Enumeration was done by MPN method
ND: Not done.

Table 2. BACTERIOLOGICAL SPECIFICATION BY EXPORT INSPECTION AGENCY, INDIA, FOR FROZEN SHRIMP AND FISH

Criteria	Shrimp	Fish
Aerobic Plate Count at 37°C	1 x 10^6 cfu/g	5 x 10^5 cfu/g
Escherichia coli	20 per g	20 per g
Staphylococcus aureus (Coagulase positive)	100 per g	100 per g
Salmonella	Nil in 25 g	Nil in 25 g
Vibrio cholerae	Nil in 25 g	Nil in 25 g

Table 3. MICROBIOLOGICAL PROFILE OF READY TO EXPORT WHOLE & HEADLESS SCAMPI

Microbiological parameters	Whole (cfu/g)	Headless (cfu/g)
Aerobic Plate Count	10^4 - 10^6 [a]	X 10^4 – 5.0 X10^5
Staphylococcus aureus (Coagulase positive)	Nil	Nil
Escherichia coli	0 -1	0 – 11
Salmonella	+[b]	+[c]
Vibrio cholerae	Not detected	Not detected
Vibrio parahaemolyticus	ND	ND
Listeria monocytogenes	Not detected	Not detected
Yersinia enterocolitica	ND	ND

a: 2 out of 6 samples were exceeding 1x10^6 cfu/g
b: 3 out of 6 samples positive
c: 5 out of 12 samples positive
ND: Not done.

Table 4. MICROBIOLOGICAL PROFILE OF READY TO EXPORT ROHU AND LONG FIN HERRING

Microbiological parameters	Rohu		Whole Herrings
	Whole (cfu/g)	Steak (cfu/g)	(cfu/g)
APC	10^3 - 10^6 [a]	10^3 - 10^5 [b]	1.5 X 10^4 - 5.5 X 10^5
Escherichia coli	0 - 6	0 - 55[d]	Nil
S. aureus (Coagulase positive)	Nil	Nil	Nil
Salmonella	Nil	Nil	Nil
Vibrio cholerae	Nil	Nil	Nil
Vibrio parahaemolyticus	ND	ND	ND
Listeria monocytogenes	Nil	Nil	Nil
Yersinia enterocolitica	ND	ND	ND

a: 2 out of 6 samples were exceeding $5x10^5$ cfu/g
b: 3 out of 24 samples exceeding $5x10^5$ cfu/g
c: 1 out of 6 samples exceeding $5x10^5$ cfu/g
d: 4 out of 24 samples exceeding 20cfu/g.
ND: Not done

Table 5. TOTAL NUMBER OF ISOLATES OF PATHOGENS IDENTIFIED UP TO BIOCHEMICAL LEVELS[a]

Pathogen	Presumptive positive isolates	Confirmed positive isolates
Staphylococcus aureus (Coagulase positive)	65	4
Salmonella	1249	40
Vibrio cholerae	240	0
Vibrio parahaemolyticus	167	3
Listeria monocytogenes	859	0
Yersinia enterocolitica	97	0

a: Total number of fishery samples analysed was126.
b: *Salmonella* identified biochemically.

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DETERMINATION OF CONTAMINATION PROFILES OF HUMAN BACTERIAL PATHOGENS IN SHRIMP OBTAINED FROM JAVA, INDONESIA

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Abstract

Shrimp continues to be an important export commodity for Indonesia and contributed significantly to the country's revenue. However, shrimp exports have been frequently rejected by importing countries due to filth, *Salmonella* and insanitary conditions. This study was conducted to evaluate the profiles of bacterial contamination of ocean and aquaculture shrimp obtained from the area of West, Central and East Java; frozen shrimp and shrimp during industry production of frozen shrimp. The study indicated that both ocean and aquaculture shrimp obtained from the study area were heavily contaminated. On the average, shrimp obtained from West Java were more contaminated than those obtained from East and Central Java. The total bacterial counts were generally higher in ocean shrimp than those of aquaculture ones. *Salmonella* was present in two of 32 samples of ocean shrimp and in four of 32 samples of aquaculture shrimp obtained from the study area. *Vibrio cholerae* was not detected in shrimp from West Java, but was found in three out of 16 samples obtained from East and Central Java. *V. parahaemolyticus* was frequently identified in aquaculture shrimp but absent in fresh ocean shrimp. Studies on shrimp collected from six sampling points during frozen shrimp production revealed that processing will reduce the number of total bacterial, *E. coli*, and Staphylococal counts. However, the processing did not effectively reduce the incidence of *Salmonella* or *V. parahaemolyticus* when the raw material has been contaminated with the pathogens. Sizing and grading as well as arrangement of shrimp before freezing were considered as the critical points where bacteria should be controlled to inhibit growth and cross contamination with bacteria such as *Listeria*. Implementation of Good Agricultural Practices in production of raw shrimp as well as Hazard Analysis Critical Control Point at the line processing are expected to improve the quality of fresh and frozen shrimp.

1. INTRODUCTION

Shrimp is an important and expensive commodity in international trade. Indonesia, Mexico, Ecuador, China, Thailand, India, Vietnam, and the Philippines are the largest exporters of shrimp (Nickelson, 1992) [9]. In Indonesia the export volume of shrimp and fish has been decreasing within the past five years; however, they still contributed revenue to the amount of $1 048 423 975 in 2001 (Ministry of Industry and Trade, 2002) [7]. Shrimp from Indonesia is primarily exported as frozen headless shrimp to Japan and USA. However, shrimp from Indonesia is subject to automatic detention due to the risk of bacterial pathogens. The presence of filth and and *Salmonella* accounted for 90% of the rejection. Additionally, other factors such as environmental issues and antibiotic contamination may have caused the export to decrease.

[†] Deceased, January 2000.

Shrimp for export are generally caught from the ocean and aquaculture ponds. Although most of the frozen shrimp processors are big industries using modern facilities and equipment and complying with the Good Manufacturing Practices, the fresh shrimp were generally caught by traditional fishermen with low knowledge in good handling practices and sanitation.

The objectives of the research were to determine (1) the bacterial contamination profiles of fresh shrimp collected from ocean and aquacultures of the northern coast of Java; (2) bacterial contamination profile of the existing frozen shrimp products available in Indonesian markets; and (3) the fate of bacterial contaminants in shrimp during in line processing in the preparation of frozen shrimp.

2. MATERIALS AND METHODS

2.1. Collection of shrimp samples

Samples of fresh ocean and aquaculture shrimp from the provinces of West, Central and East Java were obtained during the rainy season of 1998-1999, 2000, 2001, respectively. Ocean shrimp samples were collected from four catching place in West Java, i.e. Sukabumi (W1), Indramayu (W2), Jakarta (W3); and Tangerang (W4); two catching places in East Java, i.e. Tuban (E1) and Surabaya (E2), and two places in Central Java, i.e., Semarang (C1) and Pekalongan (C2). Aquaculture shrimp cultured in the pond were collected from aquaculture places of the three areae above, i.e. from Karawang (W5), Indramayu (W6), Tangerang (W8) and, Cirebon (W8), Tuban (E3), Surabaya (E4), Semarang (C3) and Pekalongan (C4).

Frozen shrimp were collected from five seafood processing industries, namely industries I1, I2, I3, I4 and I5 and from four different grocery stores, i.e. G1, G2, G3 and G4 located in West Java.

Shrimp samples for line processing study were obtained from an industry located in Java. Samples were collected at six sampling points during frozen shrimp processing. Samples were obtained (1) during receiving, (2) after head removal, (3) after sizing and grading (4) after final rinsing in water containing 30 ppm chlorine (5) after arrangement and water filling and (6) after freezing.

3. MICROBIOLOGICAL ANALYSIS

Black tiger shrimp samples were collected aseptically, heads were removed and the tails were analyzed promptly (frozen shrimp was thawed in refrigerator prior to analysis). Aquaculture water was also evaluated for its bacterial contents as supporting data. Each study was repeated two to five times. A composite sample of 450 g was collected and prepared for microbiological analysis. The analysis included the examination of total plate counts, *Staphylococcus*, coliform, *Enterobactericeae* and *E. coli* counts. Presence of *Salmonella, V. cholerae*, *V. parahamolyticus*, and *Listeria monocytogenes* (only for shrimp of line processing) was also determined.

The methods to quantify total microbes, staphylococci, coliform, *Enterobactericeae* and *E. coli* as well as procedures to detect the presence of bacterial pathogens were in accordance with AOAC (1995) [1]. Total plate counts determined in Plate Count agar (PCA), *Staphylococcus* counts in Baird Parker tellurite egg yolk Agar (BPA), coliform counts in Violet Red Bile Agar (VRBA), *Enterobactericeae* counts in VRBA + 1% glucose, and *E. coli* counts in Eosine Methylene Blue Agar (EMBA).

Procedures for detection of pathogenic bacteria in shrimp consisted of enrichment/selective enrichment, isolation and confirmation steps. The media for enrichment were Selenite Cystine Broth for *Salmonella*, Alkaline Peptone Water (APW) for *V cholerae* or APW + 3% NaCl buffer for *V. parahaemolyticus*, and Half Fraser Broth for *Listeria*. The selective media used to isolate the pathogenic bacteria were Hektoen, Bismuth Sulfite and XLD Agar for *Salmonella*, Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar for *V.cholerae* and *V. parahaemolyticus*, and PALCAM Agar for *Listeria*. Confirmation of *Salmonella* was conducted in Triple Sugar Iron (TSI) Agar and Lysine Iron Agar (LIA) followed by API 20E test kit. Presence of *V. cholerae* and *V. parahaemolysis* was confirmed in TSI and SIM or SIM supplemented with 3% NaCl. Presence of *Listeria* was confirmed by growing the typical isolates from PALCAM in TSAYE Agar and tested for motility, Gram staining and catalase activity.

4. RESULTS AND DISCUSSION

4.1. Bacterial contamination profile of fresh shrimp

The study showed that the level of contamination of fresh shrimp varied between sampling sites. However, in general it was found that on average, shrimp from West Java contained more bacteria and was more frequently contaminated with human pathogens than shrimp from Central and East Java (Table1). The bacterial load ranged from (log_{10}CFU/g) 5.6 to 7.1, indicating a high level of contamination. The counts were similar to those obtained by Cann (1977) who reported a bacterial load between 10^5-10^7 per g of shrimp [3]. Most were spoilage bacteria such as those belonging to *Enterobacteriacea* which ranged from (log_{10}CFU/g) 5.6 to 6.8.

Table 1. BACTERIAL CONTAMINATION PROFILE OF FRESH OCEAN SHRIMP OBTAINED FROM JAVA

Bacteria	Log $_{10}$ CFU/g or + (present) and - (absent)							
	West Java				East Java		Central Java	
	W1	W2	W3	W4	E1	E2	C1	C2
Total Plate Count	7.1	5.9	6.2	6.1	5.7	5.6	5.7	6.3
Staphylococcus	5.3	4.4	4.8	4.5	3.3	3.2	3.1	4.1
Enterobacteriaceae	6.8	5.5	5.3	5.6	<1	4.0	2.5	4.2
Total Coliform	6.1	4.7	4.9	5	<1	3.4	2.1	3.8
E. coli	2.8	3.3	3.3	3.3	<1	3.7	2.4	3.8
Salmonella	+[*]	-	-	-	-	-	-	-
	(2/4)[**]	(0/4)	(0/4)	(0/4)	0/4	(0/4)	(0/4)	(0/4)
V. cholerae	-	-	-	-	-	-	-	+
	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(1/4)
V. parahaemolyticus	-	-	-	-	-	-	-	-
	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)

[*)] *S. paratyphi A*

[**)] Number in parenthesis is the number of positive isolates per number of samples.

The number of *E. coli* was low only in samples obtained from Surabaya, East Java (E1), but ranged from (log_{10}CFU/g) 2.4-3.8 in shrimp from other locations. Presence of these bacteria indicated fecal contamination as a result of poor sanitation. *Salmonella* was found in shrimp obtained from West Java with a frequency of 12.5% or 6.25% of overall samples. This number was lower than that in a report by Heinitz et al. (2000) who stated an isolation frequency of 7.2% of imported seafood during 1990-1998 in the USA and a 12.5% isolation rate of the pathogen from seafood imported from Central Pacific area (Australia, Cocos islands, Fiji, Indonesia, Malaysia, New Zealand, New Guinea and Singapore). *V. cholerae* was detected in one out of 32 samples, while *V. parahaemolyticus* was absent in all raw ocean shrimp samples. This result was different from Nascumento et al. (1998) who reported isolation of *V. parahaemolyticus* of ocean shrimp obtained from the coastal area of Maranhao state, Brazil [8].

Fresh shrimp obtained from ponds also had high bacterial contamination that varied in each location (Table 2). However the total bacterial counts of the aquaculture shrimp were generally lower, with the exception of shrimp from E1. Bacterial contamination of ocean shrimp may have come from poor handling during transport from the ocean. Some of the traditional fishermen do not have appropriate cold storage and may be equipped with insanitary containers. The number of *E. coli* in aquaculture shrimp was generally higher than that of ocean shrimp, although in E1 this was not the case.

Aquaculture shrimp from Central Java contained the lowest bacterial counts. However the *E. coli* contamination was low in shrimp obtained from East Java. *Salmonella* was isolated more frequently in aquaculture shrimp, especially those collected from West Java (12.5%). *V. cholerae* was frequently isolated from shrimp collected from East Java, while shrimp collected from all four locations in West Java contained *V. parahaemolyticus*. V. *cholerae* in crustacean has been reported as the vehicle for some cholera outbreak in the US (CDC, 1986) and Peru (CDC, 1991) [4, 5].

The level of contamination of aquaculture shrimp was a reflection of the contamination of water (Table 3). Most of the ponds were located near households that may be polluted with household waste. The highest level of *E. coli* in aquaculture shrimp obtained from Pekalongan, Central Java (C4) as shown in Table 2 was correlated with the high contamination of the water from the same pond (Table 3). A similar pattern was observed for *Salmonella* and *V. cholerae*. However, *V. parahaemolyticus* was not isolated from the pond water but was isolated from the shrimp obtained from East Java.

4.2. Microbiology of frozen shrimp available in the market

Bacterial contamination profiles of frozen shrimp available in the market are presented in Table 4. The frozen samples contained high bacterial counts, and none of the samples met the microbiological standards for frozen shrimp issued by many agencies. The Indonesian National Standard requires that frozen shrimp should contain less than 5×10^5 CFU/g (raw) or 2×10^5 CFU/g (cooked) for total plate counts, 10 CFU/g (raw) or negative (cooked) of *E. coli*, and no *Salmonella* in 25 g of sample. ICMSF states that frozen shrimp should contain less than 10^6 CFU/g total plate counts, 400 CFU/g coliforms, 25 CFU/g *E. coli*, and 100 CFU/g *Staphylococcus*. The USA states that good frozen shrimp should contain less than 4.0×10^5 CFU/g total plate counts, while those containing 4×10^5 to 1.9×10^6 CFU/g total plate counts are categorized as fair quality.

Table 2. BACTERIAL CONTAMINATION PROFILE OF FRESH AQUACULTURE SHRIMP OBTAINED FROM JAVA

Bacteria	Log $_{10}$ CFU/g or + (present) and - (absent)							
	West Java				East Java		Central Java	
	W1	W2	W3	W4	E1	E2	C1	C2
Total Plate Count	5.3	5.9	5.0	4.8	6.2	4.9	4.5	4.7
Staphylococcus	4.5	5.3	4.6	3.5	<1	2.9	3.3	3.4
*Enterobacteriacea*e	5.3	5.7	4.5	4.4	<1	4.0	3.4	2.6
Total Coliform	4.2	5.2	4.5	4.2	<1	3.2	3.4	2.5
E. coli	2.3	3.4	3.7	2.3	<1	2.7	2.8	4.0
Salmonella	+*	-	+*	+*	-	-	-	-
	(1/4)**	(0/4)	(1/4)	(2/4)	(0/4)	(0/4)	(0/4)	(0/4)
V. cholerae	-	-	-	-	-	+	+	+
	(0/4)	(0/4)	(0/4)	(0/4)	(0/4)	(2/4)	(1/4)	(2/4)
V. parahaemolyticus	+	+	+	+	-	+	-	-
	(2/4)	(2/4)	(1/4)	(2/4)	(0/4)	(1/4)	(0/4)	(0/4)

*) *S. paratyphi A*

**) Number in parenthesis is the number of positive isolates per number of samples

Table 3. MICROBIOLOGICAL QUALITY OF AQUACULTURE WATER*)

Bacteria	Log $_{10}$ CFU/g or + (present) and - (absent)			
	East Java		Central Java	
	E3	E4	C3	C4
Total Plate Count	6.2	4.9	4.5	4.7
Staphylococcus	<1	2.9	3.3	3.4
Enterobacteriaceae	<1	4.0	3.4	2.6
Total Coliform	<1	3.2	3.4	2.5
E. coli	<1	2.7	2.8	4.0
Salmonella	-	-	-	-
	(0/2)	(0/2)	(0/2)	(0/2)
V. cholerae	-	+	+	+
	(0/2)**	(1/2)	(1/2)	(1/2)
V. parahaemolyticus	-	-	-	-
	(0/2)	(0/2)	(0/2)	(0/2)

*) Ponds of West Java were not evaluated

**) Number in parenthesis is the number of positive isolates per number of samples

Analysis of bacterial contamination of frozen shrimp obtained from grocery stores suggested that the number of bacteria in sample G1 to G4 shrimp were similar to those obtained from the industry. The result suggested that frozen distribution/transportation between the industry and grocery stores was well maintained. In general the number of total microorganisms varied in shrimp obtained from the five industries and four grocery stores. *V. parahaemolyticus* was isolated from shrimp from G2 and G3. Frozen shrimp from G2 was also contaminated by *V. cholerae*. None of the samples contained *Salmonella*. This result suggests that frozen shrimp in the market did not contain pathogens as frequently as the fresh ones, because the pathogens may have been reduced by processing and frozen storage.

Table 4. BACTERIAL CONTAMINATION PROFILES OF FROZEN SHRIMP PRODUCTS AVAILABLE IN THE MARKET

	Log $_{10}$ CFU/g or + (present) and - (absent)								
Bacteria	Industries					Groceries			
	I1	I2	I3	I4	I5	G1	G2	G3	G4
Total Plate Count	5.4	5.6	6.0	6.0	5.8	7.0	6.0	6.4	6.4
Staphylococcus	2.8	3.8	4.7	5.0	3.4	4.3	3.8	3.9	3.9
Enterobacteriaceae	3.0	3.0	4.2	5.2	3.5	5.3	4.8	4.4	3.4
Total Coliform	2.4	2.7	3.6	4.0	2.5	4.7	4.7	3.9	3.9
E. coli	<1	1.8	2.2	2.7	1.8	3.5	2.2	2.7	2.7
Salmonella	- (0/4)*	- (0/4)	+ (1/4)	- (0/4)	- (0/4)	- (0/4)	- (0/4)	- (0/4)	- (0/4)
V. cholerae	- (0/4)	- (0/4)	- (0/4)	- (0/4)	- (0/4)	- (0/4)	+ (2/4)	- (0/4)	- (0/4)
V. parahaemolyticus	- (0/4)	- (0/4)	- (0/4)	- (0/4)	+ (0/4)	- (0/4)	+ (1/4)	+ (3/4)	- (0/4)

*) Number in parenthesis is the number of positive isolates per number of samples

4.3. Fate of Bacterial Contaminants in Shrimp during Frozen Shrimp Processing

This study was conducted in the processing industry which processes aquaculture shrimp for frozen shrimp processing. Typical processing line of frozen shrimp in the industry consisted of (1) receiving, (2) first rinse in 100 ppm chlorinated water, (3) head removal, (4) second rinse in 30 ppm chlorinated water, (5) sizing and grading I, (6) sizing and grading II, (7) weighing, (8) final rinse, (9) arrangement and water filling, (10) freezing, (11) glazing, (12) metal detection, (13) packaging, and (14) storage.

Analysis of shrimp at six sampling points suggested that total bacterial contamination of the raw shrimp received by the processor was similar to those found in aquaculture shrimp as shown in Table 2. After second rinse, the bacterial population decreased due to the use of chlorine. After sizing and grading, however, the bacterial counts tended to increase, probably due to the inadequate control of time spent during this manual step. The bacterial counts also

increased during shrimp arrangement and water filling into trays for freezing which may have been caused by inadequate control of water temperature and lengthy time allocated during this process. However, after freezing, the bacterial counts decreased and thus resulted in products complying with the Indonesian standard for frozen shrimp. The average bacterial count of the finished products in this study was $\log_{10}$CFU/g 5.4 or 2.5 x 10^5 which was lower than the standard of 5x10^5. Although the raw material contained *E. coli* at levels up to $\log_{10}$CFU/g 2.9, processing decreased the count significantly.

Most of the products contained *Salmonella* which was present in the receiving materials and survived subsequent processing. Only one out of the five finisheded positive-evaluated was free of *Salmonella*. *V. cholerae* was absent in sampling points. *V. parahaemolyticus* was present in three of the five finished products. This bacteria was present in the raw material and survived freezing. The number of samples positive for *Listeria* increased during processing, suggesting contamination within the industrial facilities. *Listeria* has been reported as psychrotrophic organisms and was reported to be found in the food processing plant [2]. Listeria isolates from the study were not further identified, to determine if they were pathogenic. Contamination from pathogens other than *Listeria* did not occur during processing suggesting adequate hygiene practices. Results of this study are summarized in Table 5.

Table 5. BACTERIAL CONTAMINATION PROFILES DURING FROZEN SHRIMP PROCESSING

	Log CFU/g*			Present (+) or absent (-)*			
Steps during processing	Total Plate Count	*Staphylococcus*	*E. coli*	*Salmonella***	*V.cholerae*	*V.parahaemolyticus*	*Listeria spp.*
Receiving material	5.4	2.7	2.9	+ (5/5)	- (0/4)	+ (4/5)	+ (2/5)
After head removal	5.3	2.0	2.4	+ (5/5)	- (0/4)	+ (4/5)	+ (3/5)
After sizing and grading	6.7	2.1	<2	+ (5/5)	- (0/4)	+ (4/5)	+ (3/5)
After final rinse	5.6	1.3	0-<2	+ (5/5)	- (0/4)	+ (3/5)	+ (5/5)
After arrangement and water filling	5.6	1.4	2.3	+ (5/5)	- (0/4)	+ (3/5)	+ (5/5)
After packaging	5.4	1.3	0-<2	+ (4/5)	- (0/4)	+ (3/5)	+ (3/5)

*) Number in parenthesis is the number of positive isolates per number of samples
**) Confirmation using API 20E are incomplete

5. CONCLUSIONS

Ocean shrimp from Java had a higher bacterial contamination than did aquaculture shrimp. Shrimp samples obtained in the West Java contained more bacteria and were more frequently contaminated with *Salmonella* than those obtained from East and Central Java area. Several frozen shrimp products available in the Indonesian market did not meet the standard required by the Ministry of Industry and Trade for total plate counts, but only one out of 36 samples

tested was contaminated by *Salmonella*. This differed on shrimps sampled from in-line processing in industry. Although the total plate counts, *Staphylococcal* and *E. coli* counts met the standard requirement, *Salmonella* and *V. parahaemolyticus* were detected in the finished frozen product. The pathogens originated from the raw material and steps in the processing were not adequate to reduce the incidence of the pathogens. Control of processing temperature and chlorine concentration in the rinse water needs to be improved. Implementation of Good Agricultural Practices in production of raw shrimp as well as Hazard Analysis Critical Control Point at the line processing are expected to improve the quality of fresh and frozen shrimp.

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DEVELOPMENT OF A RAPID DETECTION METHOD FOR PATHOGENIC *E. COLI* GROUP BY MULTIPLEX PCR AND DETERMINATION OF PROFILES OF FOOD PATHOGENS FROM IMPORTED SEAFOOD IN THE REPUBLIC OF KOREA

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Abstract

A total of 347 samples of imported seafood products were purchased in retail markets, in order to determine their contamination with pathogenic bacteria: *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes*, *Vibrio parahaemoliycus*, Escherichia coli and Escherichia coli 0157:H7. The results showed that 12,1% of the samples was positive to *S. aureus* and 2,1 % of the samples were contaminated with *L. monocytogenes* and E. coli. No *Salmonella* spp*, V. parahaemolyticus* and E. coli 0157:H7 were found. *S. aureus* was present in a wide variety of seafood products, being frozen shrimps the most contaminated one (29% of the frozen shrimps samples). *L. monocytogenes* was isolated only in smoked samples, whereas E. coli was found in smoked salmon as well as in frozen pollack flesh samples.

1. INTRODUCTION

Pathogenic *Escherichia coli* is a group of bacteria, including enteropathogenic *E. coli* (EPEC), enterohemorragic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC). Human gastrointestinal illness caused by EPEC, EIEC and ETEC has been recognized for several decades. Pathogenicity of EHEC was recognized in 1982 when it was associated with two food-related outbreaks of hemorrhagic colitis in Oregon and Michigan in the United States. *E. coli* O157:H7, the most representative EHEC, can cause severe infections and death in humans, but produces no signs of illness in its non-human hosts. Since the first reports, *E. coli* O157:H7 outbreaks have risen steadily worldwide.

E. coli O157:H7 has been isolated from various sources, including feces, raw milk, raw meat and poultry, cheese, dairy products, and other processed foods. It has been known to cause severe diseases, including hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). The virulence factors of *E. coli* O157:H7 are two Shiga-like toxin (SLT, or Verotoxin), SLT I (=VT I) and SLT II. (=VT II). Besides *E. coli* O157:H7, Shiga-like toxin-producing *E. coli* (STEC) strains are an important cause of epidemic and endemic diarrhea, hemorrhagic colitis, and HUS. Serotype O157:H7 is the most typical in EHEC group, however, more than 60 other serotypes of *E. coli* also produce Shiga-like toxins and has reported to be associated with outbreaks and sporadic disease. In fact, it has been reported that non-O157 STEC infection occupied 10-30% of HUS occurrence in German, Italy and the U.S. from the late 1980s.

The conventional method for isolation of *E. coli* O157:H7 includes examining its incapability of hydrolyzing sorbitol on SMA (Sorbitol MacConkey Agar). However, this method is only available for O157:H7, and is time consuming. Other techniques such as PCR, which is rapid and specific, have been developed and applied to detect or differentiate species. Curently, PCR can detect only one species at a time. Therefore, development of a rapid and convenient

PCR technique that is able to detect and differentiate several species, for example between pathogenic *E. coli*, in a single run is in demand.

The purposes of study (Project 1) conducted during 1998-1999 were 1) for the development of a multiplex PCR method for the detection and differentiation of 4 groups of pathogenic *E. coli*, and 2) to examine the incidence of pathogenic *E. coli*, including O157:H7 in domestic and imported foods in the Republic of Korea using conventional and multiplex PCR methods.

Food poisoning outbreaks increase during summer season, between June and October, in the Republic of Korea. According to food poisoning incidence statistics, there were 93 outbreaks and 6400 cases in 2001. The major causative foods were meat and its processed products, seafood and its processed products, and ready-to-eat lunch-box made of rice cake. Seafood and shellfish were responsible for about 25% of total outbreaks. *Salmonella*, *S. aureus* and *V. parahaemolyticus* were the major causative microorganisms.

Worldwide, food poisoning outbreaks have increased continuously. Expansion of international trade, consumer demand for "lightly processed" foods, mass production of foods, and changes in eating habits were the main reasons for this. In the Republic of Korea, the consumption of fresh fruits and vegetables (especially organic) as well as seafood products has greatly increased. The average annual amount of seafood products consumed in the Republic of Korea is 45kg/person (edible portion base) and it is rising continuously. In other words, about 3.4 million tons are required for annual seafood consumption in the Republic of Korea. However, production of domestic seafood products has gradually decreased mainly due to the contamination of adjacent seas as well as UN Ocean Regulation inaugurated from 1997 (e.g., 200 nautical miles National limits). Therefore, import of seafood products is increasing. Importation of other food products, especially agricultural products, has gradually and greatly increased, which means the Republic of Korea is now a major food products importing country.

Coinciding with the increase of imported food products, the food poisoning incidence has increased, particularly that caused by seafood, in spite of strengthened quarantine inspection. One of the reasons may be the eating of raw fish or shellfish such as oysters or abalone. the Republic of Korea and Japan consume raw fish called "sushi" or "sashimi", and certain other types of raw seafood imported for sushi or sashimi. These foods are recognized as good, expensive and healthy products in both countries. However, eating raw seafood in the summer is a food poisoning risk. Many countries monitor imported food products, especially high-risk products such as seafoods. The results of this monitoring could be used in the management of regulation policy or setting of microbial standard. Therefore, it is timely to monitor the profiles of food poisoning bacteria in various imported seafood products to check out their microbial safety and possible role in the increased food poisoning incidence by seafood (Project 2).

2. MATERIAL AND METHODS

Project 1 (1998-1999)

2.1. Food samples

100 domestic and imported food samples were purchased from retail markets in nationwide. These included 30 meat (beef, pork, chicken), 20 meat products, 20 dairy products, and 30 vegetables. All samples were transported under refrigeration to the laboratory.

2.2. Bacterial strains

E. coli ATCC 43894 (serotype O157:H7, VT I+, VT II+), *E.coli* ATCC 12807 (serotype O126: H, EAF+), *E. coli* ATCC 25922 (negative reference) and *Shigella flexneri* ATCC 33938 (*ipa* I+) obtained from National Institute of Health, the Republic of Korea, and *E. coli* H10407 (serotype O78:H11, LT+. ST+) obtained from CDC, U.S.A. were used as reference cultures.

2.3. Isolation and identification of *E. coli.* and *E .coli* O157:H7

The FDA Bacteriological Manual was used for isolate *E. coli* and *E. coli* O157:H7 from food samples. Suspected colonies from selective agar were identified with gram staining, biochemical tests, API 20E (Biomeuriex) and Biolog Microstation System (Biolog Inc.). The confirmed isolates were reserved for further identification of pathogenicity.

2.4. MPCR (Multiple Polymerase Chain Reaction)

Modified method of Strockbine *et al*. was used for MPCR conditions. Template DNA was prepared as: 1) boiling microfuge tube, containing 50µl distilled water and a single colony, for 15min at 100^{o} C, 2) centrifuge for 15min at 10 000 rpm, and 3) take top layer. The DNA sample was amplified in a 30.2ml reaction mixture of the following constitution; 3µlof 10X PCR buffer (100mM Tris-HCl, pH9.0, 50mM KCl, 0.1% Triton X-100, promega), 1.5ml of 25mM MgCl2, 3µl of dNTPs (mixture of 1mM dATP, 1mM dCTP, 1mM dTTP, and 1mM dGTP), 1µl of multiplex primer (Table 1), 0.2ml of *Taq* polymerase (5U/µl), 15.5µl of distilled water. Six microliters of template DNA were added to it. Then the mixture was amplified in a Touch Down Thermal Cycler (Hybaid, UK). Parameters for the amplification cycle were denaturation for 30 sec at 95^{o}C, annealing for one min at 55^{o}C, and extension for 1min at 72^{o}C. After the 35th cycle, the PCR tube was incubated for 10min at 72^{o}C. The amplified DNA was separated by gel electrophoresis using 1.5% agarose.

2.5. Serotyping

The serotypes of isolates were determined by the slide and tube agglutination methods using the O and H antisera (Denka Seiken, Japan), respectively.

2.6. Liquid-liquid hybridization

To develop the liquid-liquid hybridization method, the PCR ELISA (DIG detection, Roche) kit was applied. The two primers used here were VT I and VT II. The procedures for developing this liquid-liquid hybridization method are: 1) make biotin-labeled capture probe with VT I, 2) immobilize the capture probe to streptavidin coated microtiter well, 3) label digoxigenin to VT II primers, 4) hybridize the biotin-labeled capture probe with digoxigenin labeled oligonucleotide, 5) remove unbound DNA by washing, 6) visualize the hybrids with an anti-digoxigenin peroxidase conjugate, and the colorimetric substrate ABTS, and 7) analyze the sample with an ELISA plate reader.

Project 2 (2000-2001)

2.7. Food samples

347 imported seafood products were purchased from retail markets. They were 75 smoked salmon, 42 tuna, 100 frozen shrimp, 28 small octopus, 42 frozen cod flesh, 40 frozen Pollack flesh, and 20 jelly fish. All samples were transported under refrigeration to the laboratory.

2.8. Isolation and quantification procedures

Isolation procedures used were FDA BAM. In the last stage of confirmation, various biochemical or molecular tests were conducted on typical colonies from selective medium. To determine the level of contamination, a quantification test was performed on all positive samples.

2.9. Enterotoxin test

Reversed passive latex agglutination kit (SET-RPLA, Denka Seiken, Japan) was used for staphylococcal enterotoxin test. The enterotoxin type was determined followed by agglutination.

2.10. PCR

For the accurate confirmation of isolates, traditional boiling lysis PCR was conducted. The amplified DNA was separated by gel electrophoresis using 1.2% agarose.

3. RESULTS AND DISCUSSION

Project 1 (1998-1999)

The virulence factors used in the construction of multiplex PCR primer were verotoxin I and II for EHEC, heat labile (LT) and heat stable (ST) toxin for ETEC, *ipa* I for EIEC and EAF for EPEC. The oligonucleotide sequence of individual primers is described in Table 1. The MPCR amplification conditions were established, and sensitivity and accuracy was tested with reference cultures. The results are shown in Figure 1.

The incidence of *E. coli* determined by conventional isolation was eight (27%) and six (20%), 0 in raw meat and vegetable samples respectively, in the 30 samples of each tested. These isolates were tested with MPCR to identify the pathogenicity (virulence factors). Five isolates, all from raw meat, were pathogenic *E. coli* (Table 2). However, no *E. coli* O157 was detected. All isolates from vegetables proved to be non-pathogenic *E. coli*. This vegetable result agrees with those of the monitoring program. In 1999, we examined various vegetables (over 500 samples) for the presence of *E. coli* O157:H7 as well as pathogenic *E. coli* but no positive sample was found. The absence of *E. coli* in meat and dairy products implies that processed products, both domestic and imported, were produced and maintained under good sanitary condition and safe manner.

Table 1. OLIGONUCLEOTIDE SEQUENCE OF PRIMERS FOR MPCR

Type	Toxin	Primer	Amplification size
EHEC	VT	VT I: CAG TTA ATG TGG TGG CGA AG CAC AGA CTG CTG CAG TGA GG	475bp
		VT II: CT TCG GTA TCC TAT TOC CGG CGC TGC AGC TGT ATT ACT TTC	863bp
ETEC	LT	LTL\: TCT CTA TGT GCA TAC GGA GC LTR: OVA TAC TGA TTG COG CAA T	320bp
	ST	ST1-1: TTA ATA GCA COC GGT ACA AGC AGG ST1-2: OCT GAC TCT TCA AAA GAG AAA ATT AC	147bp
EIEC	*ipa*	Shig-1: TGG AAA AAC TCA GTG OCT CT Shig-2: CCA GTC CGT AAA TTC ATT CT	422bp
EPEC	*eae*	EAF 1: CAG GGT AAA AGA AAG ATG ATA A EAF 2: TAT GGG GAC CAT GTA TTA TCA	397bp

EHEC: enterohemorrhagic *E. coli*
ETEC: enterotoxigenic *E. coli*
EIEC: enteroinvasive E. coli
EPEC: enteropathogenic E. coli
VT: verotoxin
LT: heat-labile enterotoxin
ST: heat-stable enterotoxin
ipa I : invasion-plasmid antigen I
EAF: EPEC adherrence factor

Table 2. PATHOGENICITY OF FOOD ISOLATES BY MPCR

Source of isolates	*Virulence factor*	*Serotyping*
Beef	LT+	O159
Beef	EAF+	O8
Beef	VT I+	O55
Pork	LT+	O159
Chicken	LT+	UT

UT: Untyphable

LT: heat-labile enterotoxin

EAF: EPEC adherrence factor

VT I: verotoxin I

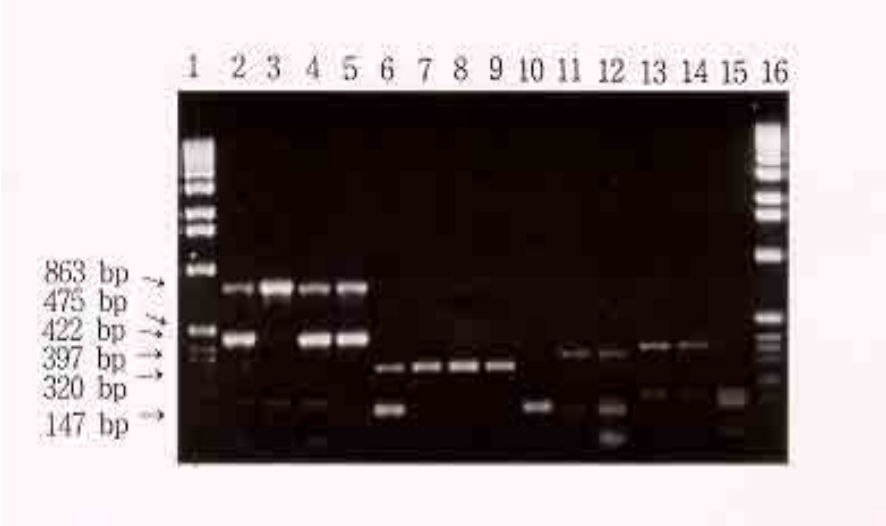


Figure 1: MPCR amplication of VT I, VT II, LT, ST, EAF, *ipa* I gene

PCR products were amplified in the region of 320 bp for LT, 147 bp for ST, 475 bp for VT I, 863 bp for VT II, 422 bp for *ipa* I and 397 bp for EAF gene, respectively.

Lane 1, 16: 1 kb DNA marker, lane 2: *E. coli* ATCC 43894 (VT I+, VT II+), lane 3: F101 (VT II+), lane 4: *E. coli* O157:H7 (isolated from stool of cattle, VT I+, VT II+), lane 5: F66 (VT I+, VT II+), lane 6: *E. coli* ATCC H0407 (LT+, ST+), lane 7: S25 (LT+), lane 8: S26 (LT+), lane 9: F60 (LT+), lane 10: F38(ST+), lane 11 : *E. coli* ATCC 12807 (EAF+), lane 12 : F62 (EAF+), lane 13: *Shigella flexneri* 33938 (*ipa* I+), lane 14: F74 (*ipa* I+), lane 15 : *E. coli* ATCC 25922 (negative control).

Currently available solid-solid liquid hybridization is time consuming and is not suitable for monitoring laboratories such as the Republic of Korea Food and Drug Administration's Regional Branches or Quarantine Stations where testingof large quantities of domestic and imported food products is conducted. Liquid-liquid hybridization is more rapid than solid-solid liquid hybridization, which takes usually overnight. Therefore, we tried to develop

liquid-liquid hybridization using the two primers, VT I and VT II. As described in the method section, we used the PCR ELISA (DIG Detection) kit to develop a liquid-liquid hybridization method.

The PCR ELISA is a ready-to-use kit providing all materials for the experiment. The concepts tried were 1) attach biotin to VT I to make the capture probe and then immobilize the probe to streptavidin coated microtiter wells, 2) attach digoxigenin to VT II primers and hybridize it with the biotin-labeled capture probe, 3) analyze the sample with ELISA pate reader followed by removing unbound DNA by washing, and visualizing the hybrids with an anti-digoxigenin peroxidase conjugate and the colorimetric substrate ABTS. However, the background was too high and the hybridization result could not be determined. It seems possible that the immobilization of the biotin-labeled capture probe was not achieved successfully. Research to resolve the problem is continuing.

Project 2 (2000-2001)

347 imported seafood products were purchased from retail markets and six major foodborne pathogens were monitored. The tested products and their exporting countries are shown in Table 3. A variety of products were imported from China, whereas most smoked salmon and frozen shrimp was imported from Norway and Thailand.

The overall prevalence of pathogens of 16.1% (56 out of 347) was considered fairly high (Table 4). Contamination rate of *S. aureus* was the highest (12.1%), while that of *L. monocytogenes* and *E. coli* was 2.1%. No *Salmonella* spp., *V. parahaemolyticus* or *E. coli* O157:H7 was found. *S. aureus* was found in many products including smoked salmon, tuna, frozen shrimp and small octopus. The most highly contaminated product by *S. aureus* was frozen shrimp (29.0%). *E. coli* was detected in four out of 75 (5.3%) smoked salmon and three out of 40 (7.5%) frozen pollack flesh. *L. monocytogenes* was isolated seven out of 75 (9.3%) smoked salmon. The most frequently contaminated product was frozen shrimp, and smoked salmon was contaminated with the largest range of pathogens. No tested microorganism was found in frozen cod flesh and jellyfish.

The prevalence rate of 16.1% is not such a great concern to public health in samples that are consumed after proper cooking. From the aspect of public health or food hygienic regulation, *S. aureus*, *L. monocytogenes* and *E. coli* in smoked salmon would be a more serious problem because it was a completely processed ready-to-eat product. The contamination rate with those three bacteria was 20% and this might present a food poisoning hazard. The microbial standard for ready-to-eat products in the Republic of Korea Food Sanitation Act is zero tolerance. *V. parahaemolyticus* was not isolated from any tested samples. All products tested in this study were frozen and there may be slight chance of survival of *V. parahaemolyticus*.

In the enterotoxin test for *S. aureus* isolates, 57.1% produced type A. No B, C, or D was detected (Figure 2). The type A staphylococcal enterotoxin was found most in frozen shrimp from China (47%). The quantification test for all positive samples showed the average contamination was less than 100cfu/g. The isolates of *S. aureus* and *L. monocytogenes* were confirmed by PCR (Figure 3 and 4).

Table 3. SEAFOOD PRODUCT SAMPLES BY EXPORTING COUNTRIES

	Total	**N**	**U**	**S**	**I**	**T**	**C**	**A**	**Sp**	**R**	**Uk**
Smoked salmon	75	69	-	-	-	-	-	-	-	-	6
Tuna	42	-	-	29	-	-	-	-	-	-	13
Frozen shrimp	100	-	-	4	8	52	27	8	-	-	1
Small octopus	28	-	-	-	-	-	21	-	7	-	-
Frozen cod flesh	42	-	9	-	-	-	4	-	-	27	2
Frozen pollack flesh	40	-	6	-	-	-	3	-	-	29	2
Jellyfish	20	-	-	-	-	7	9	-	-	-	4
Total	**347**	**69**	**15**	**33**	**8**	**59**	**64**	**8**	**7**	**56**	**28**

N: Norway

U: USA

S: Singapore

I: India

T: Thailand

C: China

A: United Arab Emirates

S: Spain

R: Russia

U: Unknown

Table 4. ISOLATION RATES OF FOOD PATHOGENS FROM IMPORTED SEAFOOD PRODUCTS

Products	**Total positives/ samples (%)**	**No. of isolates/samples(%)**					
		***S au*[a]**	***Sal*[b]**	***L.mo*[c]**	***Vib*[d]**	O157[e]	E.coli
Smoked salmon	15/75 (20.0)	4/75 (5.3)	-	7/75 (9.3)	-	-	4/75 (5.3)
Tuna	7/42 (16.6)	7/42 (16.6)	-	-	-	-	-
Frozen shrimp	29/100 (29.0)	29/100 (29.0)	-	-	-	-	-
Small octopus	2/28 (7.1)	7/28 (7.1)	-	-	-	-	-
Frozen cod flesh	0/42 (0)	-	-	-	-	-	-
Frozen pollack flesh	3/40 (7.5)	-	-	-	-	-	3/40 (7.5)
Jelly fish	0/20 (0)	-	-	-	-	-	-
Isolation rate (%)	**56/347** (16.1)	**42/347** (12.1)	**-**	**7/347** (2.1)	**-**	**-**	**7/347** (2.1)

a: *S. aureus*

b: *Salmonella*

c: *L. monocytogenes*

d: *V. parahaemolyticus*

e: *E. coli* O157:H7

Table 5 shows the isolation rates of tested microorganisms by importing countries. Seafood products imported from China showed most positive results (39.1%) while no bacteria were detected in products from USA and Spain. Major portion of those positives were *S. aureus* indicating poor food handling practices. Seafood products from Thailand were least contaminated.

From the results of this study, it can be concluded that the sanitation quality of imported seafood products, especially in their raw state, is quite poor. The regulatory agencies such as KFDA (the Republic of Korea Food and Drug Administration) should put more attention to the sanitation quality of imported seafood products and try to find a means to improve it. Also, a public education program should advise that raw seafood be consumed only after proper cooking, especially in summer.

Table 5. ISOLATION RATES OF FOOD PATHOGENS BY IMPORTING COUNTRIES

Country	**Total positives/** samples (%)	**No. of isolates/samples (%)**					
		***S au*[a]**	***Sal*[b]**	***L.mo*[c]**	***Vib*[d]**	**O157[e]**	**E.coli**
China	25/64 (39.1)	24/64 (37.5)	-	-	-	-	1/64 (1.6)
Norway	8/69 (11.6)	-	-	7/69 (10.1)	-	-	1/69 (1.4)
India	2/8 (25.0)	2/8 (25.0)	-	-	-	-	-
Thailand	4/59 (6.8)	4/59 (6.8)	-	-	-	-	-
Arab Emirates	2/8 (25.0)	2/8 (25.0)	-	-	-	-	-
Singapore	4/33 (12.1)	4/33 (12.1)	-	-	-	-	-
Russia	9/56 (16.1)	4/56(7.1)	-	-	-	-	5/59 (8.9)
Unites States	0/15 (0)	-	-	-	-	-	-
Spain	0/7 (0)	-	-	-	-	-	-
Unknown	2/28 (7.1)	2/28 (7.1)	-	-	-	-	-
Isolation rate (%)	**56/347** (16.1)	**42/347** (12.1)	-	**7/347** (2.1)	-	-	**7/347** (2.1)

a: *S. aureus*

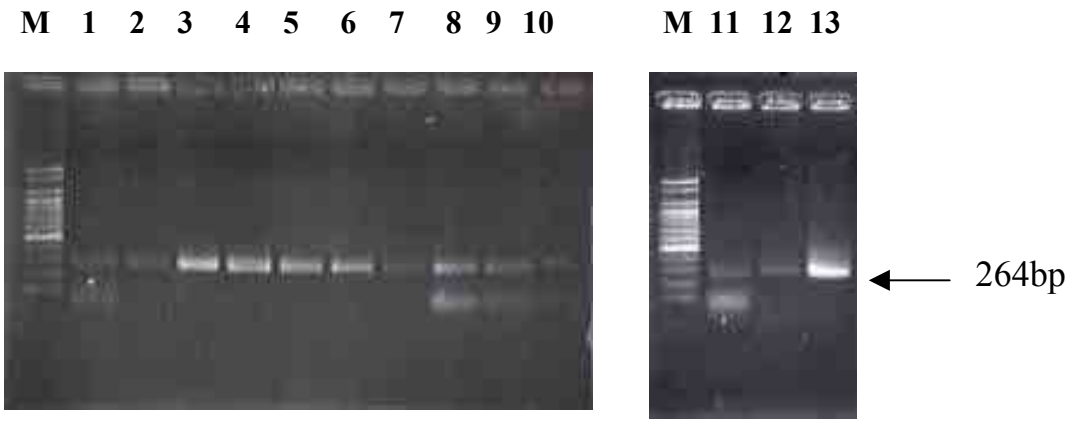
b: *Salmonella*

c: *L. monocytogenes*

d: *V. parahaemolyticus*

e: *E. coli* O157:H7

Fig 3. Confirmation of *S.aureus* isolates by PCR

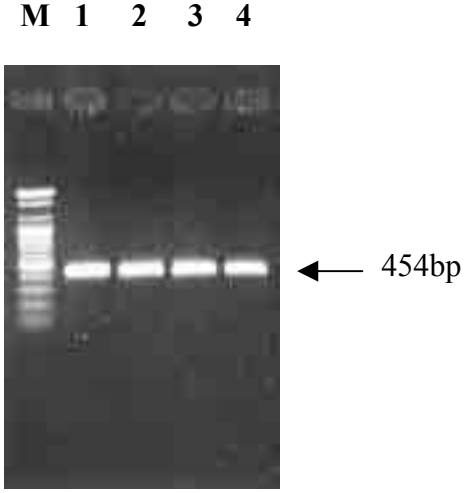


M: 100bp ladder

Lane 1: *S. aureus* ATCC 25923 (reference strain)

Lane 2~13: *S. aureus* isolates

Fig 4. Confirmation of *L. monocytogenes* isolates by PCR



M: 100bp ladder

Lane 1: *L. monocytogenes* ATCC 15313 (reference strain)

Lane 2~4: *L. monocytogenes* isolates

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DETECTION OF *SALMONELLA* SPP., SHIGELLA (FLEXNERI AND SONNEI) AND *VIBRIO CHOLERAE* O1 BY POLYMERASE CHAIN REACTION (PCR) IN EXPORTED SHRIMP FROM THE MEXICAN NORTHEAST COAST

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Abstract

The objective of the present work was to use the PCR technique for the simultaneous detection of *Salmonella* spp and *Vibrio cholerae* O1 in frozen shrimp for export. The DNA segments located in the gene *A* [284 pairs of bases (pb)] from *Salmonella* spp. *locus ial* (217 and 320 pb) from *Shigella flexneri* and *Shigella sonnei* and the gene *ctxA* and *ctxB* (777 pb) from *Vibrio cholerae* O1 were amplified. The different primers that amplify these segments were assayed in a PCR reaction for the simultaneous detection of DNA from the microorganisms. It was not possible to amplify the gene of *Shigella sonnei* and *Shigella flexneri* under the assay's conditions, whilst those of *Salmonella* spp. *and Vibrio cholerae* O1 were successfully amplified. The amplification conditions for the PCR were: 94° C, 58° C and 72° C during 30 cycles, allowing a reduction from 15 days test time with the official microbiological methods to 28 hours (24 for the pre-enrichment and four for the PCR). Samples of raw-frozen-headless shrimps were taken from production plants located in the State of Sinaloa, Mexico. A random sampling procedure was used, according to the guidelines described by the International Commission of Microbiological Specifications for Foods (ICMSF, 1999). Five packages per lot per production plant were obtained. From each individual package (5 pounds 80 OZ ≈ 2.27 kg) three samples were taken for the bacteriological assays to search for *Salmonella* spp. and *Vibrio cholerae* O1, respectively. The samples were also analyzed by PCR. Results showed that none of the samples were positive by PCR to any of the studied bacteria. *Salmonella* spp. and *Vibrio cholerae* O1 were not detected in these samples by the official methods. However, the latter were able to identify other *Vibrio* species and enterobacteria like *Proteus* and *Acromobacter*. These results confirmed PCR's rapidity, sensitivity and specificity.

1. INTRODUCTION

Food safety has received major attention in recent years due to the increasing concern on this issue in the frame of a global market. Microbiological food contaminants may cause serious disease and even death. They originate high costs for health care and a decrease in productivity. Inadequate food safety can also impose adverse economic consequences in developing countries by resulting in the rejection of imported food by their customer countries (OPS/OMS, 1991).

In Mexico, the national shrimp harvest has reached 95 611 tons. This is the third largest national production and the first place in the total value worldwide, worth $4 523 834.00 Mexican Pesos. A total of 38 365 tons with a value of $453 545 is exported annually (SEMARNAP, 1997). The consumption of fish products in Mexico in 1997 was 1 227 919 tons, of which 36 857 tons (5.66%) were shrimp (SEMARNAP, 1997) [17].

During 1995 the Regional Information System for the Epidemiological Surveillance of Food Born Disease (RISES-FBD) reported outbreaks of FBD in different countries. A total of 620 FBD outbreaks were reported and 21 755 persons were affected. This is an average of 35 persons involved in each outbreak and resulted in 49 casualties. The analysis of the total number of outbreaks showed that the most common etiologic agent affecting the largest number of persons was *Shigella* spp.

Rapid testing and confirmation methods, and better precision in recognition of the species or serotypes involved in foodborne outbreaks are required in order to allow early corrective measures to be taken. The detection of microorganisms by the traditional methods of sampling and culturing can take several days and the cost is relatively high. Obviously, this means a delay in time, as well as an important economic loss for enterprises, especially for those that export foods. Therefore, this study was designed to use the Polymerase Chain Reaction (PCR) technique as a simple and fast method with 45a high sensitivity and specificity for the simultaneous detection of the pathogens *Salmonella* spp., *Shigella* and *Vibrio cholerae* O1 in raw frozen shrimp for export.

2. MATERIALS AND METHODS

Samples of raw-frozen-headless shrimp for export were taken from production plants located in the State of Sinaloa, Mexico. The molecular biology analyses were run at the Center of Animal Biotechnology in Guadalajara, Jalisco, Mexico. The official bacteriological methods were performed at the Department of Preventive Medicine and Public Health in the Faculty of Veterinary Medicine, National Autonomic University of Mexico. The study consisted of two phases: I, Experimental Phase and II, Field Study.

2.1. Phase I

During the first phase, the amplification cycle conditions were determined in the laboratory (time and temperature for the PCR technique) using purified DNA for the simultaneous replication of *Vibrio cholerae* O1, *Salmonella* spp., *Shigella flexneri,* and *Shigella sonnei*. Secondly, the effectiveness of the method was confirmed by the use of field samples, intentionally contaminated with known inoculates of the pathogens strains and negative controls (Figure 1, columns 2, 3, and 4). The results were further confirmed with the official microbiological methods. The extraction of the genomic DNA (in raw) was performed, following the procedure described by Sánchez D. (2000) which took four hours.

Figure 1. DNA integrity on an agarosa gel (1%). It can be observed a sole and clear band of high molecular weight. Column 1, Molecular weight marker of 50 pb of GibcoBRL. Column 2 and 3, *Vibrio cholerae* O1 DNA. Column 4, *Salmonella spp.* DNA.

2.1.1. Extraction of the genomic DNA in raw materials

Plates of pure bacterial strains were prepared: *Vibrio cholerae* O1 in agar T1N1, and *Salmonella* spp. and *Shigella flexneri* and *sonnei* in blood agar and *Salmonella* spp. and *Shigella* spp. in Agar. After 24 hours incubation the colonies obtained were used for PCR. One colony was taken and placed in an eppendorf tube of 1.5 ml with 100 µl of buffer D, containing 50 mM KCl, 10 mM Tris HCl, 2.5 mM $MgCl_2$, 0.45% NP 40 and 0.45% Tween with 8 mg/ml Proteinase K. The sample was incubated for one hour at 50 ^{o}C and the enzyme was inactivated by holding the sample at 90°C for 10 min. Finally, 1.5µl was taken for the PCR technique [14].

The sequence of the primers of the gene *inv* A from *Salmonella* spp. which were used for its amplification are shown in Table 1 [12, 15].

Table 1. SEQUENCE OF THE GENE INV A FOR SALMONELLA SPP. PRIMERS

Amplified gene	Size of the PCR product (pb)	Selected primer	Sequence of the primer
Inv A	285	INVA1	5'-gTg AAA TTA TCg CCA CgT TCg ggC AA - 3'
		INVA2	5'-TCA TCg CAC CgT CAA Agg AAC C - 3'

This gene is specific for the *Salmonella* genus and allows the bacteria to penetrate the epithelial cells of the host's intestines. The amplification conditions in the thermocycler are (a) denaturation at 94°C for five min; then (b) 30 cycles at 94°C for 30 s, 53°C for one min and 72°C for 30 s, and (c) a final extension of one cycle at 72°C for five min Figure 2 [15, 4, 9].

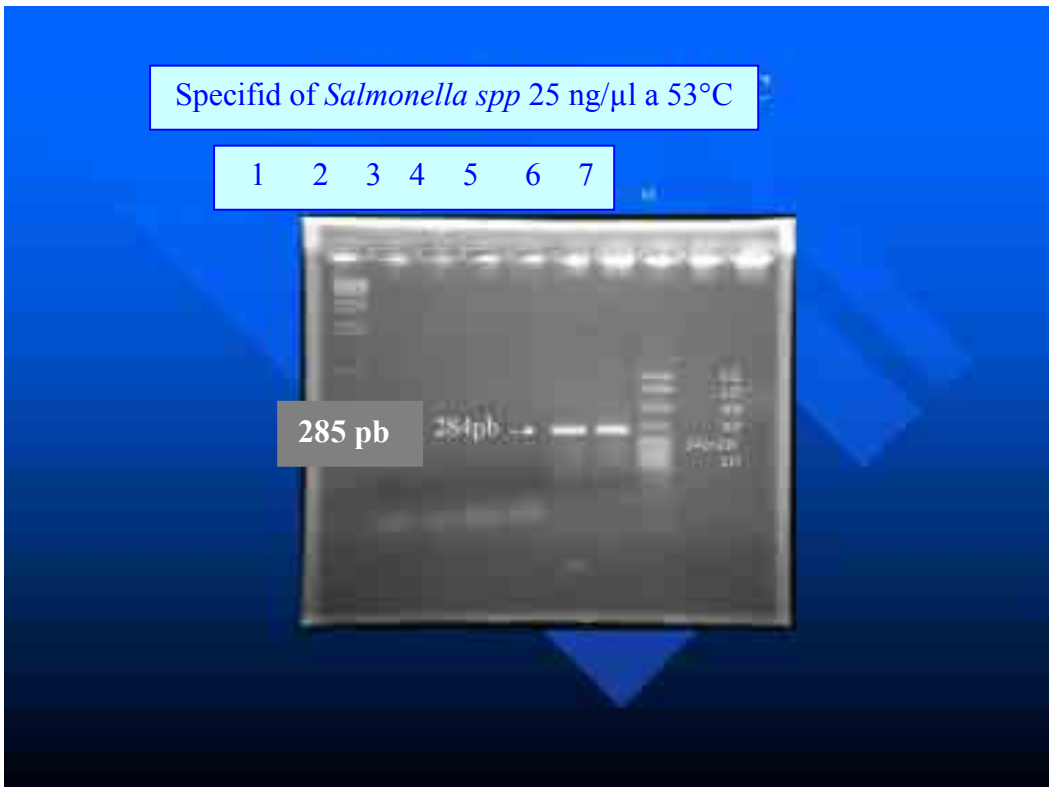

Figure 2. Negative results with DNA from various microorganisms. Column 1 and 8, molecular weight marker of 50 pb of GibcoBRL. Column 2, *E. coli* DNA. Column 3, *Vibrio cholerae* O1 DNA. Column 4, *Yersinia sp* DNA. Column 5, *Shigella spp.* DNA. Column 6 and 7, *Salmonella spp.* DNA.

For *Vibrio cholerae* O1, the primers of the gene *ctxAB*, previously used by Koch *et al.* (1993), were utilized (Table 2).

Table 2. SEQUENCE OF THE GEN CTX AB FOR VIBRIO CHOLERAE 01 PRIMERS

Amplified gene	Size of the PCR product (pb).	Selected primer	Sequence of the primer
Ctx AB	777	VIBRIO P1	5' - TgA AAT AAA gCA gTC Agg Tg - 3'
		VIBRIO P3	5' - ggT ATT CTg CAC ACA AAT CAg - 3'

This gene promotes the production of the cholera toxin that acts on the small intestine's mucosa, causing the typical diarrhea of the disease. The toxin is a protein, which consists of two sub-units, A and B. These are responsible for the attachment of the toxin to the receptor. The conditions of temperature and cycles for the reaction in the thermocycler were: one cycle for the denaturaation at 94°C for five min; followed by 30 cycles at 94°C for 30s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for five min (Figure 3) [4, 7, 8].

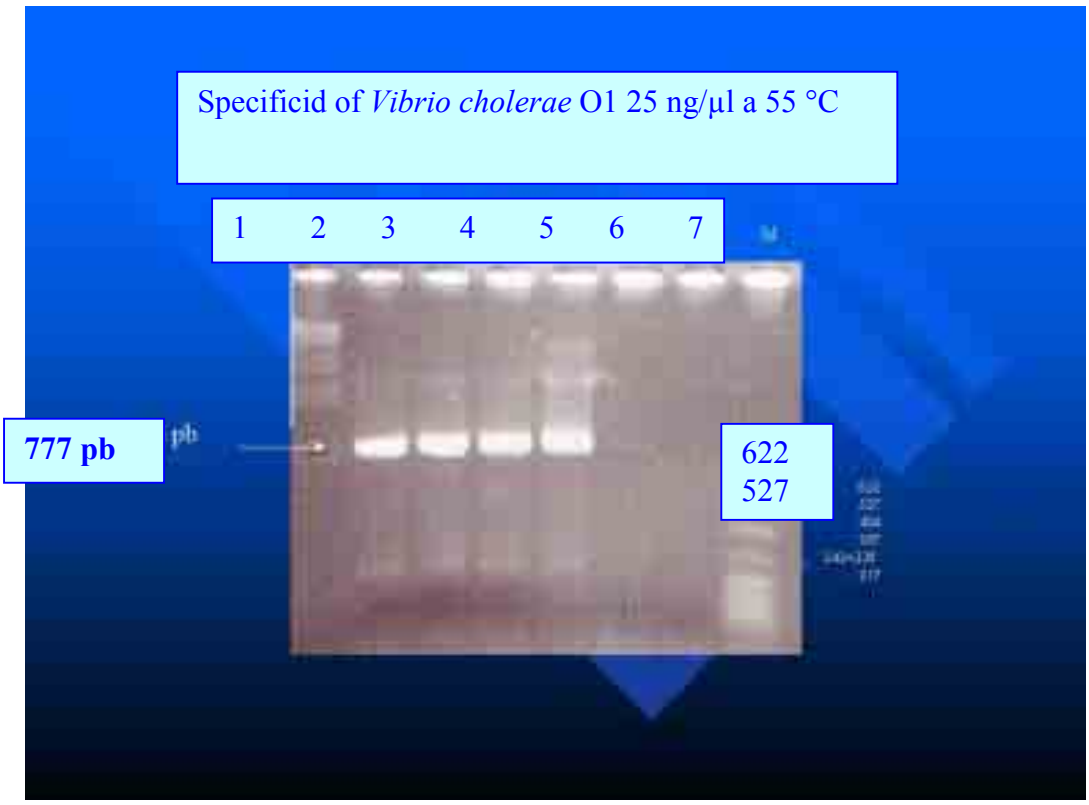

Figure 3. Negative results with DNA from various microorganisms. Column 1 and 8, molecular weight marker of 50 pb GibcoBRL. Column 2, 3, 4 and 5, *Vibrio cholerae* O1 DNA. Column 6, *Yersinia ssp* DNA. Column 7, *Salmonella spp* DNA.

For *Shigella flexneri* and *Shigella sonnei,* primers of the plasmid *ial*, reported by Lindqvist *et al.* (1999) and Frankel *et al*. (1990) were used. They are shown here (Table 3):

Table 3. SEQUENCE OF THE PRIMERS FOR SHIGELLA FLEXNERI AND SHIGELLA SONNEI

Amplified gene	Size of the PCR product (pb).	Selected primer	Sequence of the primer
Plasmid *ial*	217	SHIGELLA P3	5'-TTT TTA ATT AAG AgT ggg gTT-3'
		SHIGELLA P4	5'-gAA CCT Atg TCT ACC TTA CCA g -3'

The dysentery is caused by the penetration of the bacteria into the epithelial tissue of the colon and the consequent destruction of its cells. This penetration ability is due to the plasmid, which has specific receptors for the colon cells. The conditions of cycles and temperature for the reaction in the thermocycler were: one cycle for the denaturaation at 94°C for five min; followed by 30 cycles at 94°C for 30 s, 60° C for one min, 72° C for 30 s, and a final extension at 72° C for 10 min [9, 8, 3].

These sets of primers were tested to achieve a combined or multiplex reaction, with the objective of concurrently detecting DNA of all of the microorganisms using a unique PCR reaction (Figure 4).

This can be achieved because the different PCR products are of different molecular weights (pb). The number of cycles, temperatures and times for the reaction were adjusted in a manner that the primers for *Salmonella* spp. and *Vibrio cholerae* 01 were functional in the same reaction. However, the gene for *Shigella sonnei y Shigella flexneri,* could not be amplified using the above-mentioned conditions. Therefore, further studies are needed for this gene to be integrated in the unique reaction. The reaction used a final volume of 18.25 µl, with a buffer solution (1.0 mM Tris-Cl, 1.5 mM $MgCl_2$ and 50 mM KCl, pH 8.3), 100 µM of each dNTP; 1.4 pM of each primer, 2 U of Taq-polymerase (Boheringer Mannheim), 1 µ of ADN and sterile water.

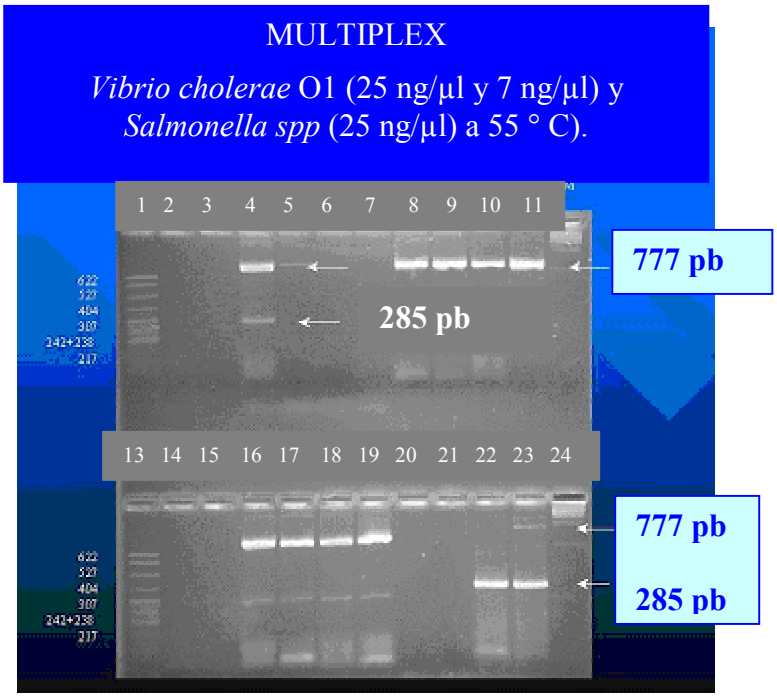

Figure 4. Simultaneous amplification of DNA from *Vibrio cholerae* O1 and *Salmonella spp*. Column 1, 12, 13 and 24, molecular weight marker. Column 4, 16, 17, 18 and 19, simultaneous amplification of DNA from *Salmonella spp.* y *Vibrio cholerae* O1. Column 2 and 3, *E. coli* DNA. Column 5 and 14, negative control. Column 6 and 7, *Yersinia sp* DNA. Column 15 and 20, *Shigella spp* DNA. Column 8, 9, 10 and 11, *Vibrio cholerae* O1 DNA. Column 22 and 23, *Salmonella spp.*. DNA.

2.1.2. Polymorphism of restriction fragments (RFLP)

In order to confirm the identity of the amplification product, it was digested with a specific enzyme that recognizes a specific sequence of nucleotides within the amplified gene. For *Salmonella* spp., the amplification product had a size of 285 pb of the gene *invA*. After being digested by the enzyme Hha 1, it showed two bands of 223 pb and 62 pb, respectively, as shown below:

For *Shigella flexneriy* and *Shigella sonnei*, the amplification product of the *ial* gene had a size of 217 pb. After being digested by the enzyme BST4CI, it showed two bands of 85 and 132 pb, respectively.

For *Vibrio cholerae* O1, the amplification product of the gene ctx AB had a size of 777 pb. After it was digested by the enzyme Cla 1, two bands of 240 and 537 pb, respectively, were observed.

2.2. Phase II

The second phase corresponds to the field study. Samples of raw-frozen-headless shrimp for export were taken from production plants located in the State of Sinaloa, Mexico, close to the Northeast Pacific coast in October 1999. The criteria for the selection of the production plants were: a) exporting plants; b) production capacity between two and 20 tons per week; c) final product presented as "raw-frozen-headless shrimp"; d) technological process subjected to a regular sanitary control; and e) shrimp caught far from industrial zones (bay or high sea) in the Mexican Northeast Pacific coast.

The working frame and the population under study were the shrimp processing plants that were invited to participate. Their production lots were considered as the sample size. Each lot consisted of "master" boxes as the experimental units containing 10 shrimp packages, each of them with five pounds and 80 oz (2.27 kg) of shrimp, which were considered as sub-sample units.

In order to ensure that a microbiologically representative sample of the production lot in each processing plant was obtained, the sanitary conditions and the handling practices of the personnel during product processing were evaluated. This information showed that the process was subjected to a regular sanitary control and it allowed taking a minimal and maximum sample size of five and ten packages per production lot, per plant [6].

When the production lot to be studied was identified, the boxes were numbered by means of a random number table. After the sample units were selected, one package was taken from each box, to complete a total of five packages per production lot per plant.

The procedures followed for sampling, handling and transport of the samples for the microbiological analyses were performed according to the guidelines described in the Manual for Foods, edited by the National Laboratory for Public Health, Ministry of Health, Mexico. From each shrimp package, three samples were taken for the bacteriological assays for *Salmonella* spp. and *Vibrio cholerae* O1. The samples were also analyzed by the PCR technique.

3. RESULTS AND DISCUSSION

CULIACÁN, SINALOA

Results from the isolation of *Salmonella* and *Shigella* sp are shown on Table 4. This table shows that from the isolates, three of them were suspected of containing *Salmonella* spp. (17%) and 15 (83%) to have *Shigella* sp. These 18 colonies were submitted to biochemical tests for their identification.

Table 4. PRESUMPTIVE ISOLATES OF SALMONELLA SPP AND SHIGELLA SP IN FROZEN RAW SHRIMP (from Culiacan, Sinaloa, México, 1999)

	ENRICHMENT BROTH					
N° Package	Kauffman			Selenite		
	SELECTIVE MEANS					
(Analytical samples)	XLD	HEK	SB	XLD	HEK	SB
Package 1 (A-B-C)	1	3	-	-	-	-
Package 2 (A-B-C)	-	2	-	-	-	-
Package 3 (A-B-C)	2	2	-	1	2	-
Package 4 (A-B-C)	2	2	-	-	1	-
Package 5 (A-B-C)	-	1	-	-	-	-

(-) Negative results

XLD: Xilose Lisine Desoxicolate Agar

HEK: Hektoen

SB: Sulfite Bimute Agar

Biochemical tests to identify *Salmonella* spp. and *Shigella* spp. showed that the suspicious colonies were negative to the microorganisms of interest, but were positive to other coliforms, like *Proteus* sp.

Table 5 summarizes the results from the *Vibrio* testing. Presumptive colonies on TCBS were submitted to biochemical tests (T1N1, Tripton, Oxidase, String) in order to confirm *Vibrio* spp. and to serotyping to confirm *V. cholerae* O1. Nine of the 22 (41%) presumptive colonies were *Vibrio* spp. but none confirmed as *V. cholerae* O1.

Table 5. ISOLATION AND IDENTIFICATION OF *VIBIO SPP* AND *VIBRIO CHOLERAE* O1 IN FROZEN RAW SHRIMPS (from Culiacan, Sinaloa, Mexico, 1999)

	MEDIUM		BIOCHEMICAL TEST			
N° Package	TCBS	T1N1	Triptona	Oxidasa	String	Antiserum
(Analytical Samples)				*V. Cholerae O1*		
Package 1 (A-B-C)	-					
Package 2 (A-B-C)	4	4	4	4	2	-
Package 3 (A-B-C)	3	3	3	3	3	-
Package 4 (A-B-C)	11	11	11	-	-	
Package 5 (A-B-C)	4	4	4	4	4	-

TCBS Medium Tiosulfate Citrate Biliae Sacaros

T1N1 Agar Triptone 1% and NaCl 1%.

String (Formación de un hilo mucoide)

(-) Negative Results

The presence of non-cholera *Vibrio* spp. may indicate a possible risk to human health by other pathogenic, marine species.

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Results from the official microbiological methods are shown in Table 6. Seven (7) presumptive colonies were obtained. Four (4) (57%) were presumptive *Salmonella* spp. and three (43%) were presumptive *Shigella* sp.

Table 6. ISOLATIONS OF SAMONELLA SPP. AND SHIGELLA SP. IN FROZEN RAW SHRIMP (from Mazatlan, Sinaloa, Mexico, 1999)

	ENRICHMENT BROTH					
N° Package	Kauffman			Selenite		
	SELECTIVE MEANS					
(Analytical samples)	XLD	HEKTOEN	SB	XLD	HEKTOEN	SB
Package 1 (A-B-C)	-	-	-	-	-	-
Package 2 (A-B-C)	-	-	-	-	1	-
Package 3 (A-B-C)	-	1	-	-	1	-
Package 4 (A-B-C)	1	2	-	-	1	-
Package 5 (A-B-C)	-	-	-	-	-	-

XLD Agar Xilose Lisine Desoxicolate

SB Agar Sulfite Bismute

(-) Negative Results

These presumptive isolates were subjected to biochemical confirmation testing, and none confirmed as pathogens.

Table 7 shows that for *Vibrio* testing, results for presumptive and confirmed tests are as described for Table 5 previously. The possible indicated risk to human health by marine pathogenic *Vibrio* species was also mentioned earlier.

Table 7. ISOLATION AND IDENTIFICATION OF *VIBRIO* SPP. IN FROZEN RAW SHRIMP (Mazatlan, Sinaloa, Mexico, 1999)

	BIOCHEMICAL TESTS					
N° Package	TCBS	T1N1	Trypton broth	Oxidasa	String	Serology
(Analytical Samples)				*V. Cholerae O1*		
Package 1 (A-B-C)	3	3	3	3	3	-
Package 2 (A-B-C)	5	5	4	2	2	-
Package 3 (A-B-C)	4	4	4	4	4	-
Package 4 (A-B-C)	6	6	5	5	5	-
Package 5 (A-B-C)	6	6;	5	5	5	-

TCBS Medium Tiosulfate Citrate Biliae Sacarose

T1N1 Agar Triptone 1% and NaCl 1%

String (Formación de un hilo mucoide)

(-) Negative Result

3.1. PCR Technique

From the sixty samples analyzed, 30 for *Salmonella* spp. and 30 for *Vibrio cholerae* O1, none tested positive. Results are shown in Tables 8 and 9.

Table 8. EVALUATION OF SALMONELLA SPP. AND VIBRIO CHOLERAE O1 USING MULTIPLEX PCR TECHNIQUE, IN FROZEN RAW SHRIMP (from Culiacan, Sinaloa, Mexico, 1999)

N° Package	*Salmonella spp.*	*Vibrio cholerae 01*
Package 1 (A-B-C)	-	-
Package 2 (A-B-C)	-	-
Package 3 (A-B-C)	-	-
Package 4 (A-B-C)	-	-
Package 5 (A-B-C)	-	-

(-) No presence of *Salmonella* spp. and *Vibrio cholerae* O1

Table 9. EVALUATION OF SALMONELLA SPP AND VIBRIO CHOLERAE O1 USING MULTIPLEX PCR TECHNIQUE IN FROZEN RAW SHRIMP (from Mazatlan, Sinaloa, Mexico, 1999)

N° Package	*Salmonella spp.*	*Vibrio cholerae 01*
Package 1 (A-B-C)	-	-
Package 2 (A-B-C)	-	-
Package 3 (A-B-C)	-	-
Package 4 (A-B-C)	-	-
Package 5 (A-B-C)	-	-

(-) No presence of *Salmonella* spp. and *Vibrio cholerae O1*

4. CONCLUSION

Results from both methodologies gave identical results for negative samples. No positive samples were found to *Salmonella* spp., *Shigella* spp. and *Vibrio cholearae* 01. Official microbiological methods including isolation, biochemical and serology tests took 15 days when PCR technique was run in 28 hours. The PCR procedure described shows promise as a rapid and specific technique to detect pathogens in food. Testing to establish the sensitivity of the method will occur in the next phase of the research.

Frozen, raw shrimp for export investigated in this study were found to be free of pathogens. Nevertheless, these products are not necessarily free of being a potential risk to human health because many other microorganisms were found. Therefore, the control of the process, the good handling guidelines and the implementation of the HACCP (Hazard Analysis Critical Control Point) system are tools to be used to obtain a safe product.

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DETERMINATION OF PROFILES OF HUMAN BACTERIA PATHOGENS IN NIGERIAN FISH AND SEAFOOD FOR EXPORT

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Abstract

It may be concluded from this project that *L. monocytogenes* and *V. cholerae* are part of the normal flora of the tropical marine and fishing boat environment, and can be controlled through the implementation of safety assurance schemes such as Good Hygiene Practices (GHP) and Hazard Analysis Critical Control Point (HACCP) systems. It is noteworthy that pathogens were detected in seafood at the inception of the project but after the workshop training for the exporters on safe handling practices, these pathogens have been eliminated in packaged raw seafood products. This improvement was also reflected in the low levels of *S. aureus* and *V. parahaemolyticus* obtained in the samples that were analysed. It is expected that with maintenance of, and strict adherence to the GHP and HACCP systems, Nigerian seafood products would be readily acceptable in the EU and the world market.

1. INTRODUCTION

Seafood is an important part of diet in many parts of the world, constituting the main supply of animal protein in some countries. As a result of the low fat content of seafood, health conscious people are turning to seafood consumption as a low fat alternative to red meat. Seafood is known to be more perishable than other high protein foods. Changes in flavour, odour, texture and colour reflect the level of freshness. These changes are caused primarily by microbial activity. The initial number and types of bacteria and storage conditions influence the rate of decomposition.

Safety in seafood products with reference to bacterial contamination is usually concerned with the potential for poisoning. In a simplified overview, poor quality products, spoiled or decomposed, are rarely responsible for food poisoning because they are usually discarded before consumption. Food poisoning in seafood products, as with other foods, except in instances such as scombroid or histamine poisoning, normally is the result of mishandling during or after preparation. The microbial flora of seafood is related to the environment from which the fish are harvested.

For the purpose of this research work, the exported seafood refers to crab claws, cuttle fish fillet and shrimp harvested from the high seas off the coast of Nigeria and neighboring countries.

Seafood borne pathogenic bacteria may be conveniently divided into two groups: the indigenous bacteria and non-indigenous bacteria. The indigenous bacteria consist of *Clostridium botulinum*, *Vibrio cholerae*, *Aeromonas hydrophila,* and *Plesiomonas shigelloides*. These bacteria are widely distributed in the aquatic environment in various parts of the world. The psychrotrophic strains of *C. botulinum* are common in Arctic and colder climates while the Mesophileic *Vibrio* species are normal flora of seafood from a coastal and estuarine environment of temperate or warm tropical zones. The non-indigenous bacteria consist of *Listeria monocytogenes, Salmonella, Shigella, E. coli* and *Staphylococcus aureus*.

The pathogens of concern for this project are *L. monocytogenes, V. cholerae, V. parahaemolyticus* and *S. aureus.*

2. METHODS OF ANALYSIS

The methods employed are as stated in the Bacteriological Analytical Methods (BAM 8th ed. FDA, 1995).

2.1. Enumeration of S.aureus by selective enrichment procedure using the Most Probable Number (MPN)

Stomach 25g of sample in 225ml Buffered Peptone water for 30secs. Make serial dilutions and inoculate 3 tube MPN determinations in Trypticase Soy broth containing 10% NaCl and 1% Sodium Pyruvate. Incubate at 37°C for 48hrs.

From the positive tubes, transfer 3mm loopful to dried Baird-Parker Agar plates. From each plate showing growth, perform the coagulase test. Read the MPN table.

Coagulase test: Transfer suspect colonies into small tubes containing 0.2-0.3ml BHI broths. Incubate at 35°C for 18-24hrs. Add 0.5ml reconstituted Coagulase plasma with EDTA mix thoroughly. Incubate at 35°C and examine periodically over a 6hr period for clot formation. Only firm and complete clots that do not segregate when the tube is tilted are considered positive.

2.2. Procedures for detection of *L. monocytogenes* in Seafoods

Weigh 25g sample into a stomacher blender. Add 225ml of Half Fraser broth (Primary enrichment). Stomach the contents for 1min. Incubate at 37°C for 24hrs. Transfer 0.1ml into 10ml Fraser broth (Secondary enrichment), incubate at 37°C for 48hrs. From the Primary enrichment steak onto duplicate plates of Oxford and Palcam Agar. Streak also from the Secondary enrichment onto Oxford and Palcam agar plates. Incubate plates at 37°C for 48hrs. Check for typical colonies. On Palcam agar, typical colonies are grey-green with a black depressed center and a black halo. While on Oxford agar colonies appear brown black or greenish black with a depressed center and a surrounding black halo.

2.3. Identification

Five typical colonies are streaked onto Trypticase Soy agar with 0.6% yeast extract and incubated at 30°C for 24hrs followed by Biochemical identification.

2.4. Enumeration of V.parahaemolyticus:

Weigh 50g of seafood into a stomacher bag. Add 450ml Alkaline Peptone water and stomach for 30secs. Set up three tubes MPN in 10ml APW using 1ml of the homogenate and dilutions. Incubate at 37°C for three hrs.

From the broth, streak a loopful of surface growth on TCBS. Incubate the plates at 37°C for 18hrs. Examine the plates for typical colonies (green blue colonies). Read the MPN table.

2.5. Detection of *V. cholerae*:

Weigh 25g of sample into a stomacher bag, add 225ml Alkaline Peptone water, stomach for 30secs. Streak TCBS plates at 6 hrs and 24 hrs. Check for typical colonies, which are large, yellow with opaque center and translucent periphery.

2.6. Confirmation of *Vibrio*

Perform oxidase and salt tolerance test.

3. RESULTS AND DISCUSSION

Results are tabulated below:

The need to control and guarantee the safety of exported foods is one of the mandates of the National Agency for Food and Drug Administration and Control (NAFDAC). This was later modified with the addition of the Federal Department of Fisheries as joint Inspector following the harmonization of the Nigerian Fishing Company by the European Union in the late 1990's. Fish and seafood export has since increased to about ten-fold between 1994-2001.

For the first year of this project, 266 samples of seafood for export were analysed. Forty-four (44) samples (16.54%) were found to contain some colonies of *S. aureus*, four samples (1.50%) were positive for *L. monocytogenes* while three samples were positive for *V. cholerae* (Table 1).

The numbers of *S. aureus* were within the permitted level. Their presence in raw food is common. However, the isolation of *V. cholerae* in the August samples was of serious public health concern. The samples in question were pronounced unfit for human consumption.

In the second year, 405 samples were analysed. Four (4) cuttle-fish (0.98%), two (2) crab and crab claws (0.49%), five (5) fillets (1.23%), and forty (40) shrimps (9.80%) were found to have a range of 16-42 colonies of *S. aureus* which is far below the maximum limit of 10^4cfu/g stipulated by the FAO for frozen raw crustaceans (Table 2).

L. monocytogenes was not detected in cuttle fish and crab and crab claws. The detection rate of *L. monocytogenes* in fillet and whole shrimps was very low. This organism was recovered mainly in samples harvested between the months of April and November (Fig. 2).

Eight hundred and thirty-eight (838) seafood samples were analysed from September 2000 to June 2002. None of the samples was found unsatisfactory for counts of *S. aureus.* This finding is expected because the samples were harvested and packaged on board therefore less handling was involved. This is in line with the findings of Nickelson and Fine (1992) that *S. aureus* is seldom isolated from freshly harvested seafood products which had not been subjected to extensive human handling.

Listeria monocytogenes was not detected in any of the samples analyzed during this period. Huss (1994) reported this organism as being normal flora of the aquatic environment, but the FAO expert consultation meeting (1999) reported that freshly harvested seafood from the tropics is generally free of this pathogen.

V. cholera was also not detected in any of the samples. The reason being that training on enforcement and importance of Good Hygienic Practices and implementation of Hazard Critical Analysis Procedures was conducted for the exporters. Vibrios are known to be normal flora of the coastal region in the tropics, therefore the exporters were discouraged from fishing near the coastal areas to prevent their products from being contaminated and rejected and the company being blacklisted.

The range of counts of *V. parahaemolyticus* in the samples was <3 and 43. Kaysner et. al. (1992) reported that isolation of *V. parahaemolyticus* from seafood is not unusual because this organism is a normal saprophytic inhabitant of the coastal marine environment, and is capable of multiplying in the tropical waters. Twedt (1989) found that *V. parahaemolyticus* is present in seafood in low numbers, approximately 10^2/g, and counts increase ten fold during marketing. This however does not constitute an infectious dose; further bacterial multiplication during subsequent food handling is required for problems to occur.

Table 1. SEAFOOD EXPORT SAMPLES ANALYSED FROM JANUARY- AUGUST 1999

Product	Number of Samples Analysed	Number of Positive Samples		
		S. aureus	*L. monocytogenes*	*V. cholerae*
Cuttle -fish	16	2	0	0
Crab and crab claws	22	2	0	0
Shrimps	218	40	4	3

Table 2. SEAFOOD EXPORT SAMPLES ANALYSED FROM SEPTEMBER - AUGUST 2000

Product	Number of Samples Analysed	Number of Positive Samples:		
		S. aureus	*L. monocytogenes*	*V. cholerae*
Cuttle- fish	14	4	0	0
Crab and crab claws	13	2	0	0
Shrimps	354	40	3	0
Fillet	24	5	1	0

Table 3. SEAFOOD EXPORT SAMPLES ANALYSED FROM SEPTEMBER 2000-JUNE 2002

Product	No of Samples Analyzed	Range of Colonies	Range of Colonies	Range of Colonies	Range of Colonies
		L. monocytogenes	*S. aureus*	*V. cholerae*	*V. Parahaemolyticus*
Cuttle fish	49	ND	11-75	ND	<3-15
Crab and crab claws	55	ND	9-43	ND	<3-23
Fillet	31	ND	<3-21	ND	<3-9
Shrimps	703	ND	<3-39	ND	<3-43

Figure 1:

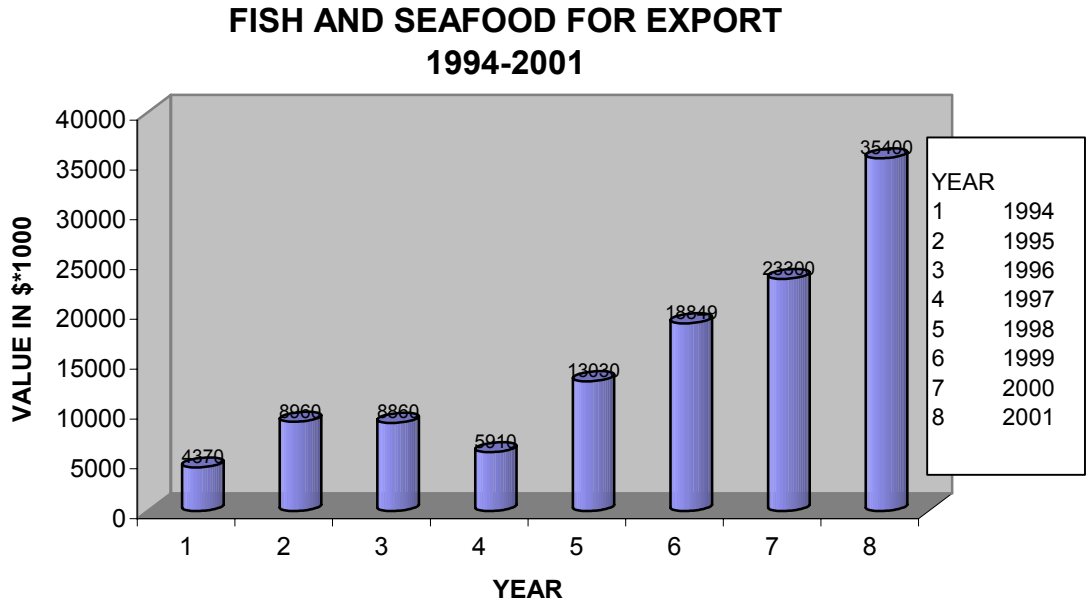
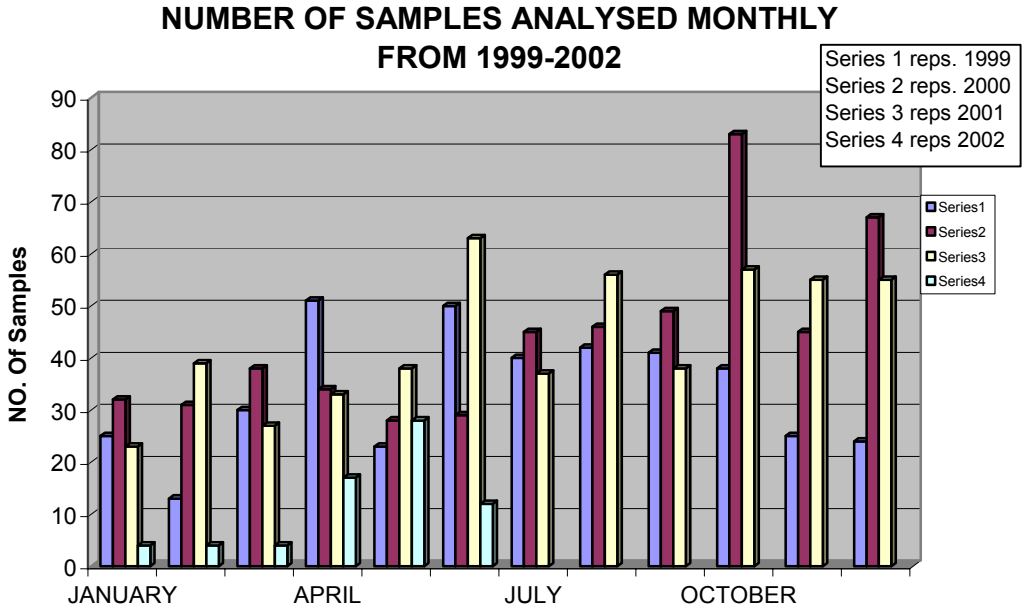


Figure 2:



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HUMAN BACTERIAL PATHOGENS IN EXPORTED AND IMPORTED FOODS AND EVALUATION OF METHODS OF ANALYSIS

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Abstract

Phase I consisted of comparison of recovery of *Salmonella* in inoculated and uninoculated pond prawns, frozen prawns, and imported frozen fish fillet using six available rapid methods vs. conventional method. The best rapid method is S2 which is based on lateral immunoprecipitation. In Phase II, *Salmonella* was monitored in 120 samples of field prawns, catfish, milkfish and tilapia from ponds. S_8 or Tecra Visual Immuno Assay (VIA) for *Salmonella* had 99.2 % agreement with BAM/AOAC method. During Phase III, *Salmonella* was monitored in a further 83 samples of aquaculture prawns and milkfish. Tecra VIA for *Salmonella* remained number 1 in accuracy with 99% agreement with BAM/AOAC method.

1. INTRODUCTION

Salmonellosis has long been considered a classic foodborne infection. Its importance continues to grow despite decreasing reports of outbreaks due to better food handling methods in growing areas, processing plants and in cafeteria and home kitchens as well as better consumer education. The seriousness of the illness and consequences involved even in advanced countries brought about by consumption of contaminated domestic and internationally traded food products is documented.

Recommended standard procedure for the isolation of *Salmonella* by regulatory agencies takes up to four days to provide a negative or a presumptive positive result. It may take another ten days to confirm positive isolates biochemically and serologically. This seriously delays corrective action, when *Salmonella* is found in foods, for example in restaurants, airlines and other commercial food services.

A microbiological rapid test kit is a commercially packaged system containing all supplies, reagents and directions for use for an analytical method. Rationale for the use of rapid tests includes new food safety approaches (e.g., HACCP) and the demands by the client to move food products more quickly in an increasingly competitive global trade. These new rapid tests include the use of newly formulated selective enrichment media, specific enzyme immunoreaction, use of chromogenic media, immunodiffusion or lateral flow immunoprecipitation. While the arsenal for microbiological testing has multiplied and become more diverse, there is reluctance to apply these in developing countries for lack of experience in their use, skepticism in their value, and cost considerations. Moreover, while evaluations of these tests in food have been reported in developed countries, their value for detecting food pathogens in developing countries has not been well established.

This study aimed to evaluate some commercially available rapid tests in their usefulness for detecting *Salmonella* in export and domestically consumed local aquaculture products including pond prawns (*Penaeus monodon*), milkfish (*Chanos chanos*), tilapia (*Tilapia mossambica*) and catfish (*Clarias batrachus*), and imported fish fillets. Important test considerations for acceptability include rapidity, accuracy, sensitivity, and specificity in relation to the USFDA Bacteriological Analytical Manual method for *Salmonella*, and for relative cost and ease of use.

Evaluation guidelines for microbiological rapid test kits were described by Coates (1994) and Agriculture and Agri-Food Canada [1]. Evaluation criteria should include the following:

- Use and recovery of at least three different levels of inoculum - 1-10, 10-100 cells of the target bacteria, and uninoculated samples;

- Matrix - use of at least five different food samples;

- Accuracy - agreement in comparison to a validated or collaboratively studied method;
- Cross-reactivity may be determined by recovery of the pathogen in the presence of competitive organisms; and

- Ruggedness testing.

As a result of the evaluation, data were gathered on potential risks from *Salmonella* in export and domestic market aquaculture products.

2. MATERIALS AND METHODS

This investigation consists of three phases (one year per phase).

2.1. Phase 1-General evaluation of rapid methods

- Preliminary screening of rapid kits in comparison with BAM/AOAC methods based on accuracy and cross reactivity;

- Evaluation of performance of rapid kits in testing of ten inoculated samples each of pond prawns, frozen prawns and imported frozen fish fillets; and

- Evaluation of performance of five rapid kits (S_2-S_6) in monitoring Salmonella in ten samples each of uninoculated pond prawns, frozen prawns and imported frozen fish fillets.

2.2. Phase 2

Monitoring *Salmonella* by three selected rapid kits (S_2, S_6, S_8) in 240 samples collected from 121 ponds of field prawns (86), catfish (8), milkfish (82) and tilapia (64) vs. conventional BAM/AOAC method.

2.3. Phase 3

Monitoring *Salmonella* by two selected rapid kits (S_6 and $S_{8)}$ in 98 samples of field prawns (63) and milkfish (35) collected from ponds, fish ports and markets all over the Philippines vs. conventional BAM/AOAC method.

3. GENERAL EVALUATION OF RAPID METHODS

3.1. Preliminary screening of rapid kits in comparison with BAM/AOAC method based on detection sensitivity with pure cultures of Salmonella

3.1.1. Preparation of the inoculum of Salmonella organisms

Stock cultures of *Salmonella typhimurium, Salmonella enteritidis* or *Salmonella paratyphi*, were subcultured daily for at least three consecutive transfers into nutrient agar slant and incubated at 35°C for 20-24h. Nutrient agar plates were then inoculated with the slant growth and incubated at 35°C for 18-24h. Cell suspensions were prepared by removing growth from the plates with Butterfield's phosphate buffered dilution water (0.0003M pH 7.2). The cell suspension was standardized by adjusting the turbidity to Mcfarland 5 (Ref: USFDA/BAM, 1995. pp. 3.73). An average count of $1x10^9$ organisms/mL was attained by transferring 0.2 mL of the suspension into a 9 mL sterile phosphate buffer. Ten-fold serial dilutions were made and aliquots of the 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilutions plated onto duplicate plates of plate count agar. The plates were incubated at 35°C for 48h before colony enumeration.

3.1.2. Inoculation of Salmonella (target organism) to sterile canned tuna samples

Canned tuna samples were aseptically transferred to 1 kg plastic bags and kept in the freezer. From each sample, 25 grams were transferred to the enrichment broth of the specific rapid test kit. Lactose broth and Tetrahionate broth were used for BAM/AOAC method and test kits without their own enrichment broth. One mL of the 10^{-6}, 10^{-7} and 10^{-8} dilutions of the *Salmonella* suspension was added to the enrichment broths containing the tuna representing three levels of inoculum: 1-10, 10-100 and 100-1000 cells to the tuna samples.

3.1.3. Recovery and Identification

The USFDA/BAM for the conventional method of recovering *Salmonella* was used as the reference method. This involves the use of non-selective lactose broth at 35° C for 24 + 2h followed by selective enrichment in Tetrathionate broth at 42+0.2° C for 24+2h. Differential and selective agar media were inoculated after enrichment. Isolated suspect colonies were then subjected to various biochemical and serological tests for confirmation. Six commercial rapid test kits for *Salmonella* detection, singly or in combination, were obtained for this preliminary evaluation study. They are briefly described and coded as follows:

S-1: This is actually a combination of new methods which this laboratory has decided to explore. The method involves a new selective enrichment medium consisting of peptone water and capsules with time-release enrichment components. After incubation at 35°C for 18-24h, the enrichment broth is streaked onto a non-selective differential chromogenic medium. Suspect *Salmonella* colonies are confirmed using a rapid serological latex agglutination test.

S-2: This rapid test method uses a specially formulated enrichment medium in which the inoculated sample is incubated (for 2h at 35°C and 18h at 43°C). The presence of *Salmonella* was assayed by adding an aliquot of the inoculated enrichment medium to the plastic encased test strip. The assay is based on a lateral immunoprecipitation of *Salmonella* antigen with an immunogold labeled monoclonal antibody.

S-3: This test uses the same non-selective and selective enrichment procedures as the conventional method. The enriched cultures are added to an opening of a bottle with

agar. Results are obtained after incubation at 35^{o} C for 24h. The reaction is based on selective motility and the formation of immunodiffusion band as a result of a positive reaction between the *Salmonella* flagellar antigens and the specific anti-flagellar antibodies added to the reaction tube.

S-4: From Lactose broth, pre-packaged vessels containing media for selective enrichment and biochemical reactions for presumptive identification of motile *Salmonella* are used. Suspect *Salmonella* reactions are subsequently confirmed by serological reactions.

S-5: From the selective enrichment medium (Tetrathionate broth), 100ul aliquots are transferred into each well of anti-*Salmonella* coated microtiter plate wells. The captured *Salmonella* antigens are detected by a conjugated anti-*Salmonella* antibody. Detection is based on a heterogeneous sandwich enzyme-linked immunosorbent assay (ELISA) format. With the use of a sensitive chromogen, the more intense the color which can be read in an ELISA reader (spectrophotometer), the more positive the reaction. This test is amenable for automation.

S-6: This consists of a one-day enrichment and then plating in a differential chromogenic agar. The enrichment broth and differential agar are from the same manufacturer. Confirmation of typical colonies is done using agglutination with latex or polyvalent sera.

S-7: This consists of 24 hours non-selective enrichment with Brain Heart Infusion (BHI) or Peptone water and 24 hours selective enrichment broth in Rappaport–Vassiliadis (RV) broth, six hours post-enrichment in BH1 and ELISA in microtiter wells. The results are read by an ELISA reader.

S-8: This includes pre-enrichment in Lactose broth for 18-22 hours, selective enrichment in Tetrathionate broth and Rappaport–Vassiliadis broth for 16-20 hours at 42^{o}C, 6 hours post-enrichment in M-broth, ELISA and reading results visually using the Color Card.

Results are reported as positive (+) or negative (-) for the presence of *Salmonella*. Duplicate samples were used to spike three levels of *S. typhimurium* and one sample to spike three levels of *S. enteritidis* or a total of nine tests per kit were done.

3.2. Effect of non-target contaminants on the recovery and detection of *Salmonella*

A culture suspension of *S. typhimurium* at McFarland 5 (Ref: USFDA/BAM, 1995, App. 373) was prepared using the same procedure as 1.1.1. Serial dilutions using the same procedure as above were made. For spiking, 1mL of 10^{-8} dilution was added to give 1-10 cells in the canned tuna. Culture suspensions of non-target enterobacteriaceae *E. coli, Klebsiella pneumoniae* and *Yersinia pseudotuberculosis* were prepared as in 1.1.1. For each non-target organism, 1 mL of 10^{-3}, 10^{-5}, 10^{-7} and 10^{-8} serial dilutions were added to the enrichment broth containing the tuna sample with 1-10 cells of *S. typhimurium*. The resulting ratios of target to non-target cells were approximately 1:1, 1:10^{2}, 1:10^{4}, and 1:10^{6}. Duplicate food samples were inoculated with these mixtures for each test kit. Methods for recovery and identification were done as per directions for use in the test kit packages listed in 1.1.3.

3.3. Detection of *Salmonella* in different food matrices

Thirty-one food samples which included 10 pond prawns, 11 frozen prawns, and 10 imported fish fillets were used in the evaluation of rapid test kits versus BAM/AOAC method. Three 25-gram portions were taken from each food sample. Each 25-gram portion of the 31 food samples was evaluated according to three different evaluation tests described below.

3.3.1. Evaluation of detection sensitivity of different methods in spiked samples

The twenty-five gram samples were inoculated with 1-10 cells or 100-1000 cells of various *Salmonella* species (equivalent to 0.04-40 cells per gram). *Salmonella* species used include *S. agona, S. anatum, S. cholerasuis, S. derby, S. potsden, S. paratyphi A and B, S. rostok, S. seftenberg, S. stanley, S. aluhua, S. bareilly, S. cambridge, S. weltevreden, S. moscow, S. newington, S. newport, S. oranienberg, S. poona, S. thompson, S. st. paul, S. dublin, S. eimbuettel, S. enteritidis, S. essen, S. heidelberg, S. infanti, S. kentucky, S. london and S. potsdam*. The isolates were obtained from patients's stool at the Department of Health, Manila. Preparation of inoculum and addition of inoculum are described in 1.1.1 and 1.1.2. The inoculated and uninoculated samples were tested as per direction on use in the test kit package as described briefly in 1.1.3 and confirmed by the USFDA BAM/AOAC conventional method for agreement.

3.3.2. Evaluation of test specificity in foods spiked with different Salmonella *species*

Each 25-gram portion of the 31 samples was inoculated with a loopful of pure cultures of a range of representative strains of *Salmonella*, e.g. serogroups a, b, c, d, e, f as named in 1.3.1 in the various enrichment broths for the rapid kits and conventional method for *Salmonella*. The operating technique for *Salmonella* recovery and identification were those indicated in the instruction sheet for use contained in the kit package and as described briefly in 1.1.3.

3.3.3. Detection of Salmonella *in different field food samples*

Each 25 gram portion of the food samples itemized for Phases 1, 2, 3 above was examined for *Salmonella* after specific enrichment as recommended for the rapid kits and BAM/AOAC method. The operating technique for *Salmonella* recovery and identification were those indicated in the instruction sheet for use contained in the kit package and as described briefly in 1.1.3.

4. RESULTS

4.1. Salmonella

Evaluation data on the different *Salmonella* tests are summarized in Tables 1-4.

4.1.1. Sensitivity of Salmonella *detection test kits in spiked canned tuna samples*

S. typhimurium and *S. entiritidis* were recovered in all tests at all concentration levels from spiked canned tuna samples. Positive detection at the lowest inoculum cell concentration level (1-10 cells /25 grams) indicates that the enrichment methods employed in all tests were able to increase the numbers of *Salmonella* sufficient for detection by the rapid tests kits (usually 10^5-10^7/mL).

4.1.2. Effect of non-target organism on the detection of Salmonella

Regardless of the numbers of contaminating non-target organisms added, none of the tests including the gold standard (BAM/AOAC), were able to detect *Salmonella* when the target cell inoculum level was 0.08 per gram. In comparison to the results for samples spiked with pure cultures of *Salmonella,* the presence of competing flora appears to have a suppressive effect on *Salmonella* multiplication despite the use of selective enrichment. However, when the inoculum level was raised to 0.16 per gram, all rapid tests except S-1 showed positive *Salmonella* detection in practically all levels of contamination with non-target organisms and correlated well with BAM/AOAC. Only S-4 gave a negative result at the highest ratio of *Salmonella* to non-target organism ($1:10^6$). Combined results of 1.1 and 1.2 are shown in Table 1.

4.1.2.1. Calculation of % Sensitivity of different Rapid Tests

Except for S-1, the different commercial rapid tests had high sensitivity agreement with the BAM/AOAC for the detection of *Salmonella* (Table 1). In the presence of contaminating organisms at a Salmonella: non-target cell ratio of $1:10^6$, sensitivity was 100% for S_2, S_3 and S_5, S_8; 95% for S_4, 88% for S_7 and 84% for S_6. These results suggest that all kits except S_1 are suitable as screening methods for sterile samples spiked with pure cultures of *Salmonella*. S_7 did not continue for further evaluation for accuracy. All positive *Salmonella* results in the rapid test screening were confirmed by conventional methods.

4.1.3. Sensitivity and Specificity of tests for Salmonella *detection in spiked pond prawn, frozen prawns and fish fillets*

4.1.3.1. Sensitivity of Salmonella detection tests

Spiked samples with approximately 126 or less *Salmonella* cells per gram were recovered by the conventional BAM/AOAC method except for *S. cambridge* and S. *heidelberg*. Neither of these were detected by the different rapid tests except for one replicate (*S. cambridge)* with S-2 and one replicate (*S. heidelberg*) with S-6. Both of these kit tests detect *Salmonella* antigens so viable cells may not have been present. At levels above 100 cells per gram, the five screening tests showed mostly positive reactions. Considering all cell inoculum levels and species of *Salmonella*, agreement of the rapid kits results with those of the BAM/AOAC were BAM/AOAC 51.5%-91%. Provided that the initial *Salmonella* contamination level of the food matrices was approximately 100-200 cells per gram, S-2 to S-6 would be able to detect consistently the target organism. It should be noted that there was general variation in the detection limit in cells per gram of each test for specific strains of *Salmonella.* This is to be expected since the different tests rely on specific components of the pathogen which may vary between species or strains.

4.1.3.2. Specificity of rapid tests in field samples spiked with various *Salmonella* species

Detection of various *Salmonella* species in pond prawns, frozen prawns and imported fish fillet yielded 100% with BAM/AOAC method, 90% with S-2, 89% with S-5, 85% with S-4, 78% with S_3. S_6 while showing a high % positive on initial screening (96%) showed the lowest specificity (74%) on the confirmation tests.

4.1.3.3. Effect of food matrix on detection of Salmonella in uninoculated samples

These are summarized in Tables 2 and 3.

Pond prawns, frozen prawns and imported fish fillets were found to have *Salmonella* contamination rates of 80%, 64% and 40%, respectively, in the small number of samples tested. Among the rapid test kits for *Salmonella*, only S-2 test showed comparable sensitivity with BAM/AOAC method at 97% (Table 3). The other kits showed poor agreement with BAM/AOAC method, ranging from 58-77%. For all tests done, S-5 had an average of 83.25%.

4.1.4. Comparative test times and costs of rapid tests

Test S-2 took the shortest time to complete (20 hours) and needed approximately 40 minutes of analyst's time at about three times material cost compared with BAM/AOAC method (Table 4). S-3 took 72 hours to complete. On a cost per test basis, S_5, S_7 and S_8 were the cheapest. However, there is a need to invest in an ELISA reader for automated quantitation of results for S-5 and S-7. The conventional BAM/AOAC method up to the confirmed test required 14 days, 360 minutes of analyst time and a lot of time and effort for media preparation for a total cost of US $35.29. This includes US $4.98 for material and the rest for labor and other indirect costs. However, considering that most rapid test are presumptive, the BAM/AOAC presumptive tests reduces the time and cost to 150 minutes of technical time and US $1.49 for material cost. Conventional BAM/AOAC method can be reduced to seven days with the use of miniaturized biochemical test kits.

S_8 is the most accurate among the rapid kits in detecting *Salmonella* in aquaculture products. It is also cost effective in needing only 56 hours to complete, 60 minutes of analyst's time and costing US $7.04 per test. Investment in a manual washer is required. Results can be read visually by a color chart.

S_6 requires 48 hours to finish, 50 minutes of analyst's time and costs US $17.17 per test due to high cost of latex agglutination which is imperative due to the high rate of false positive on the diagnostic agar.

5. CONCLUSIONS

The BAM/AOAC method for *Salmonella* is still the most sensitive and specific recommended for detection of this foodborne pathogens but the assay takes 14 days to finish. Among eight tested rapid test kits, S_8, a sandwich type antigen - antibody reaction in microtiter wells proved to be the best rapid method for monitoring *Salmonella* in aquaculture products. It had 99% agreement with BAM/AOAC method.

For rapid test kits, the enrichment procedure, the presence of competing flora, the specific strain of *Salmonella* were the important factors affecting the sensitivity and specificity of the tests.

As to *Salmonella* incidence, average percentage in pond raw prawns (27%), frozen processed prawns (64%), imported fish fillet (40%), milkfish (5.1%) catfish (0%) and tilapia (15.6%).

(Based on Results of Methods done on 1.1 and 1.2)

Table 1. SENSITIVITY OF DIFFERENT TESTS FOR SALMONELLA WITH PURE CULTURES OF SALMONELLA STRAINS AND NON-TARGET ORGANISMS IN STERILE CANNED TUNA

Methods used*	No. of Samples Analyzed	% Agreement with BAM/AOAC	% False Negative (BAM)	% False Negative (rapid kit)	% Sensitivity
BAM / AOAC	39	-	-	-	100%
S_1	19	79%	0	21%	69%
S_2	19	100%	0	0	100%
S_3	19	100%	0	0	100%
S_4	19	95%	0	5%	92%
S_5	10	100%	0	0	100%
S_6	26	84.6%	0	15.4	84%
S_7	26	88.5%	0	11.5	88%
S_8	26	100%	0	0	100%

Computation for Sensitivity and Specificity

$$\% \text{ Agreement} = \frac{\text{Number of samples in Agreement with BAM/AOAC}}{\text{Total number of samples}}$$

$$\% \text{ False negative (BAM)} = \frac{\text{Negative with BAM/AOAC vs. positive with Rapid Test}}{\text{Total number of samples}}$$

$$\% \text{ False negative (Rapid Method)} = \frac{\text{Negative with Rapid Test vs. Positive with BAM/ AOAC}}{}$$

$$\% \text{ Sensitivity} = \frac{\text{Number of samples tested positive}}{\text{Number of samples inoculated with } \textit{Salmonella}} \times \text{Total number of samples}$$

$$\% \text{ Specificity} = \frac{\text{Number of samples tested negative}}{\text{Number of samples not inoculated with } \textit{Salmonella}}$$

NOTE (all samples were inoculated)

(Reference: Flowers et al: Journal Association of Official Analytical Chemists, Vol. 70, No. 3, 1987)

Table 2. DETECTION OF SALMONELLA IN FIELD SAMPLES: FRESH POND PRAWNS, FROZEN PRAWNS IN BLOCKS FOR EXPORT, IMPORTED FISH FILLET, MILKFISH, TILAPIA AND CATFISH USING CONVENTIONAL BAM/AOAC METHOD

Samples	BAM / AOAC Method
Fresh Pond Prawns (159)	
% Positive Samples	27%
Frozen Prawn (11)	
% Positive Samples	64%
Frozen Fish Fillet (10)	
% Positive Samples	40%
Pond Tilapia (64)	
% Positive Samples	15.6%
Pond Catfish (8)	
% Positive Samples	0%
Pond Milkfish (117)	
% Positive Samples	5.1%

Table 3. DETECTION OF SALMONELLA AT VARIOUS INOCULATION LEVELS IN FRESH POND PRAWNS, FROZEN POND PRAWNS IN BLOCKS FOR EXPORT, AND IMPORTED FISH FILLET

Sample No.	*Salmonella spp*.	Inoculation level cells per gram	BAM/ AOAC	S_2	S_3	S_4	S_5	S_6
1	*S. agona*	0.04	+	-/+	+	+/-	-	
2	*S. agona*	0.48	+	-/+	-	-	-	-
3	*S. anatum*	0.16	+	+/+	+	+/+	+	+/+
4	*S. anatum*	3.60	+	+/+	+	+/+	+	+/+
5	*S. cholerasuis*	13.20	+	+/-	-	-	+	+/+
6	*S. derby*	24.00	+	+/+	-	+/+	-	-
7	*S. potsden*	156.00	+	-/+	-	-	-	-
8	*S. paratyphi A*	0.80	+	+/+	+	+/+	+	+/-
9	*S. paratyphi B*	68.00	+	+/+	+	+/+	+	+/+
10	*S. rostock*	16.00	+	+/+	+	+/+	+	+/-
11	*S. seftenberg*	9.60	+	+/+	-	+/+	+	+/-
12	*S. stanley B*	128.00	+	+/+	+	+/+	+	+/+
13	*S. alachua*	0.04	+	-/+	+	-	-	+/-
14	*S. alachua*	0.48	+	-/+	+	-	-	-
15	*S. bareily*	0.32	+	-/+	+	-	-	+/+
16	*S. bareily*	2.76	+	-/+	+	-	-	+/+
17	*S. cambridge*	12.80	-	+/-	-	-	-	-
18	*S. weltevreden*	156.00	+	+/+	+	+/+	+	+/+
19	*S. moscow*	152.00	+	+/+	+	+/+	+	+/-
20	*S. newington*	35.20	+	+/+	-	+/+	-	+/-
21	*S. newport*	224.00	+	+/+	+	+/-	-	+/+
22	*S. oranienberg*	216.00	+	-/-	+	+/+	+	+/+
23	*S. poona*	11.60	+	+/+	-	+/+	+	+/-
24	*S. thompson*	228.00	+	+/+	+	+/+	+	+/+
25	*S. st. paul*	240.00	+	+/+	+	+/+	+	+/+
26	*S. dublin*	136.00	+	+/+	+	+/+	+	+/+
27	*S. eimbuettel*	288.00	+	+/+	+	+/+	+	+/+
28	*S. enteritidis*	120.00	+	+/+	-	+/+	+	+/+
29	*S. essen*	312.00	+	+/+	-	-	+	+/+

Table 3. (Continued)

Sample No.	Sample Code	*Salmonella spp*.	Inoculation Level cells per gram	BAM / AOAC	S_3	S_4	S_5	S_5	S_6
30	F_5	*S. heidelberg*	128.00	-	-/-	-	-	-	+/-
31	F_6	*S. infanti*	296.00	+	+/+	+	+/+	+	+/+
32	F_7	*S. kentucky*	16.40	+	-/-	+	-	+	+/-
33	F_8	*S. london*	148.00	+	+/+	+	+/+	+	+/+
34	F_9	*S. paratyphi C*	52.00	+	+/+	-	+/+	+	+/-
35	F_{10}	*S. potsdam*	184.00	+	+/+	+	+/+	+	+/-
		Total % Agreement with BAM/AOAC			76% / 91%	70%	73%/ 67%	67%	89%/ 1.5%
		Total % Agreement with BAM/ AOAC			76%	70%	67%	67%	51.5%

Table 4. SPECIFICITY OF DIFFERENT DETECTION METHODS FOR SALMONELLA IN FRESH POND PRAWNS, FROZEN PRAWNS IN BLOCKS FOR EXPORTS AND IMPORTED FISH FILLET

Sample No.	*Salmonella spp.*	BAM/ AOAC	S_2	S_3	S_4	S_5	S_6
1	*S. cholerasius*	+	+/+	+	-	+	+/+
2	*S. derby*	+	+/+	+	+/+	+	+/+
3	*S. potsden*	+	+/+	-	+/+	-	-
4	*S. paratyphi A*	+	+/+	+	+/+	+	+/+
5	*S. paratyphi B*	+	+/+	+	+/+	+	+/+
8	*S. rostok*	+	+/+	-	+/+	+	+/-
6	*S. seftenberg*	+	+/+	-	+/+	+	+/+
7	*S. stanley B*	+	+/+	-	+/+	+	+/+
9	*S. cambridge*	+	+/-	-	-	+	-
10	*S. weltevreden*	+	+/+	+	+/+	+	+/+
11	*S. moscow*	+	+/+	+	+/+	+	+/+
12	*S. newington*	+	+/+	+	+/+	+	+/+
13	*S. newport*	+	+/+	+	+/+	-	+/+
14	*S. oranienberg*	+	+/+	+	+/+	+	+/+
15	*S. poona*	+	+/+	+	+/+	+	+/-
16	*S. thompson*	+	+/+	-	+/+	-	+/+
17	*S. st. paul*	+	+/+	+	+/+	+	+/+
18	*S. dublin*	+	+/+	+	+/+	+	+/+
19	*S. eimbuettel*	+	+/+	+	+/+	+	+/+
20	*S. enteritidis*	+	+/+	+	+/+	+	+/+
21	*S. essen*	+	+/+	+	-	+	+/+
22	*S. heidelberg*	+	-/-	+	-	-	-
23	*S. infantis*	+	+/+	+	+/+	+	+/+
24	*S. kentucky*	+	+/-	+	+/+	+	+/+
25	*S. london*	+	+/+	+	+/+	+	+/+
26	*S. paratyphi C*	+	+/+	+	+/+	+	+/-
27	*S. potsdam*	+	+/+	+	+/-	+	+/-
			90%/ 89%	78%	**85%/ 85%**	**89%**	**93/74%**

Table 5.

A. PHASE 1:

DETECTION OF SALMONELLA IN FIELD SAMPLES: FRESH POND PRAWNS, FROZEN PRAWNS IN BLOCKS FOR EXPORT AND IMPORTED FISH FILLETS

	BAM/ AOAC	S_2	S_3	S_4	S_5	S_6
% Agreement with BAM/AOAC		97% /81%	58%	93.5/ 71%	77%	84%/ 45%
% Agreement with BAM/AOAC		97%	58%	71%	77%	45%
Total No. of fresh prawns (10)						
% Positive fresh frozen samples	80%	80%	20%	40%	50%	0%
Total No. of frozen prawns (11)						
% Positive frozen prawns	64%	64%	27%	27%	36%	18%
Total No. of frozen fish fillet samples (10)	40%	30%	10%	40%	30%	0%
% Positive frozen fish fillet sample						
Total % positive	61%	58%	19%	36%	39%	6%

B. PHASE 2:

DETECTION OF SALMONELLA IN FIELD SAMPLES: RAW PRAWNS, MILKFISH, TILAPIA, AND CATFISH

	BAM/ AOAC	S_2	S_6	S_6
% Agreement with BAM/AOAC		60/121 = 49.6%	46.3% /86%	119 /121 = 99.2%
% False Negative		1.6%	0.0% /9.0%	0.8%
% False Positive		48.8%	53.7% /5.0%	0.0%
% Positive Tilapia (T)	15.6%			
% Positive Catfish (H)	0.0%			
% Positive Prawns (P)	23.0%			
% Positive Milkfish (B)	3.3%			

C. PHASE 3:

DETECTION OF SALMONELLA IN FIELD SAMPLES: RAW PRAWNS AND MILKFISH

	BAM / AOAC	S_6	S_8
% Agreement with BAM/AOAC		84 /98 = 85.7%	97/98 = 99%
% Sensitivity		12/26 = 46.0%	26/26 = 100%
% False Positive		12/98 = 12.2%	1/98 = 1%
% False Negative		14/98 = 14.3%	0%
% Specificity		60/76 = 79%	76/76 = 100%
% Positive Prawns (P)	23.3%		
% Positive Milkfish (B)	8.8%		

Table 6. ECONOMIC INDICES OF METHODS FOR SALMONELLA DETECTION

PRODUCT	TIME TO COMPLETE TEST	ANALYST TIME USED	MATERIAL COST	SPECIAL EQUIPMENT
S_1	48 hrs.	50 mins.	893.00	Incubator
S_2	20 hrs.	40 mins.	744.80	Incubator
S_3	72 hrs.	60 mins.	714.52	Incubator
S_4	43 hrs.	50 mins.	722.60	Incubator
S_5	56 hrs.	90 mins.	361.88	Incubator, Elisa Reader
S_6	48 hrs.	50 mins.	893.00	Incubator
S_7	56 hrs.	60 mins.	358.00	Incubator, Washer Elisa Reader
S_8	56 hrs.	60 mins.	366.00	Incubator
BAM/AOAC (Presumptive)	6 days	150 mins.	77.47	Incubator
BAM/AOAC (Confirmed)	14 days	360 mins.	258.72	Incubator

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DETERMINATION OF PROFILES OF *SALMONELLA* AND PATHOGENIC *VIBRIO* SPP. IN BLACK TIGER SHRIMP FOR EXPORT BY INTRODUCTION OF QUALITY ASSURED MICROBIOLOGICAL ASSAYS

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Abstract

Studies were conducted on contamination by *Salmonella* and pathogenic *Vibrio* spp. in samples of aquaculture black tiger shrimp, the water supply (canal water which is supplied as raw pond water before treatment), pond water, feed materials, and fresh and frozen shrimp. *Salmonella* was detected in samples of the water supply, pond water, feed materials, fresh shrimp at farm, fresh shrimp from wholesale market and frozen shrimp destined for export at levels of 13.95%, 1.53%, 1.14%, 3.17%, 30.4% and 0.21% respectively. *V. cholerae* non 01 was found in one sample of water from a culture pond of 131 tested (0.8%). *V. parahaemolyticus* was found in samples of canal water, pond water, fresh black tiger shrimp collected at farms, fresh black tiger shrimp collected at wholesale shrimp markets and frozen black tiger shrimp destined for export at levels of 2.3%, 5.3%, 14.3%, 48 % and 0.2% respectively. The strains identified as *V. parahaemolyticus* were examined for the presence or absence of the TDH and TRH. The incidence of TDH (KP+) was 2.67% (seven of 262 strains) and of TRH (urease reaction) was 1.15 % (three of 262 strains). *Salmonella* and *V. parahaemolyticus* were found in a high percentage in fresh black tiger shrimp collected from wholesale shrimp markets. These shrimp are used as raw material for domestic consumption and for processing for exported shrimp products. Therefore GMP and/or Hazard Analysis Critical Control Point (HACCP) system for shrimp distributors/producers should be applied.

1. INTRODUCTION

Shrimp aquaculture in Thailand has developed since mid 1980s. Shrimp has become a major export commodity. The export of frozen shrimp has risen from 20 000 metric tons in 1983 to 121 000 metric tons in 1991 and became 144 338 metric tons in 2000 at an export value of 60 270 million baht [3, 4]. The problems of export are the quality and safety of food products. Trade restrictions have been enforced concerning the quality of frozen marine products exported from Thailand to some European countries, Japan and USA. The specific problems which affect the exporting of Thai frozen shrimp are contamination by pathogenic microorganisms, especially *Salmonella* and pathogenic *Vibrio* spp.

Salmonella is widespread in animals. Environmental sources of the organism include water, soil, insects, animal feces, raw meats, raw poultry, and raw seafoods. *S. typhi* and the paratyphoid bacteria cause acute disease, are normally septicemic and produce typhoid or typhoid-like fever in humans. Other forms of salmonellosis generally produce milder, gastrointestinal symptoms and have led to public health problems in various countries.

Several investigations on the incidence of *Salmonell*a in fishery products have been conducted. Sajjapala *et al*. (1987) reported that its incidence in frozen shrimp for export from Thailand was 1.2 %. Suwanrangsi *et al*. (1999) also reported that contamination of frozen raw shrimp and frozen processed shrimp were 1.6 % and 1.9 % respectively. However Sajjapala and Phunbua (1994) found *Salmonella* in cooked frozen seafood produced for export to be only 0.33%.

Many *Vibrio* spp. are pathogenic to humans and have been implicated in foodborne disease eg. *V. cholerae, V. parahaemolyticus* and *V. vulnificus etc* [3].

Vibrio cholerae is a waterborne pathogen that causes gastrointestinal disorders with a wide range of clinical manifestations, including vomiting and rice-like diarrhea (Madden *et al*, 1989). The association of human illness with consumption of *V. cholerae*-contaminated oysters, seawater, and other shellfish is well documented [9]. Pathogenic *V. cholerae* produces a heat-sensitive enterotoxin that causes the characteristic cholera symptoms. The species is classified into two major serogroups: O-group-1 and non-01 *V. cholerae.* The O serogroup may have several serotypes, including Inaba, Ogawa, and Hikojima. *V. cholerae* non-O1 also can cause gastrointestinal disease, though typically less severe than that caused by *V. cholerae* O1 [19]. Serotype O139 is an exception, and produces classic cholera symptoms [3].

V. parahaemolyticus is a halophilic bacterium found naturally in estuarine waters and fish. It has a worldwide distribution in warm climate estuarine and coastal environments and has been isolated from many species of fish, shellfish, and crustaceans ([9]. *V. parahaemolyticus* has been implicated in numerous outbreaks of seafood-borne gastroenteritis. However, not all strains are considered pathogenic. Both pathogenic and non-pathogenic forms of the organism can be isolated from marine and estuarine environments and from fish and shellfish dwelling in these environments. Almost all clinical strains and rare environmental strains were shown to exhibit the Kanagawa phenomenon (KP) in early epidemiological studies [7, 14]. KP is a beta-type hemolysis induced by thermostable direct hemolysin (TDH) in a special blood agar medium, Wagatsuma agar. Strains recovered from seafood are usually Kanagawa-negative. Some clinical isolates of *V. parahaemolyticus* produce related hemolysins (TRH) but not TDH. Two other hemolysins, having sequence homology with TDH but exhibiting no hemolysis on Wagatsuma agar, were recently identified and purified [8]. Shirai *et al*. (1990) reported the epidemiologic evidence for the association of TDH and TDH-related hemolysin (TRH) of *V. parahaemolyticus* with gastroenteritis. Urease production correlated with possession of the TRH gene in *V. parahaemolyticus* strains [16].

V. alginolyticus inhabits, often at high numbers, seawater and seafood from throughout the world. It is easily isolated from fish, clams, crabs, oysters, mussels, and shrimp, as well as seawater. Results of many surveys have revealed that this species of *Vibrio* is one of the most commonly isolated vibrios. There are a few published reports describing the symptoms of gastroenteric disease by *V. alginolyticus* [9].

The objective of this study was to investigate the contamination rate of Salmonella and pathogenic *Vibrio* spp. in samples of aquaculture black tiger shrimp, the water supply (canal water which supplied as raw material for pond water before treatment), pond water, feed materials, and fresh and frozen black tiger shrimp destined for export. These will demonstrate the effectiveness of the hygienic practices of Thai shrimp industry and the incidence of these two species of microorganisms in Thailand.

2. MATERIALS AND METHODS

2.1. Sample collection

Samples consisting of canal water which supplied culture pond (86), water from culture pond (131), shrimp feed at shrimp farm (88) and fresh black tiger shrimp (126) were collected from shrimp farm at Samutsakorn during June 1998-June 2002. Fresh black tiger shrimp (125) were obtained from the shrimp market (Bangkok and Samutsakorn wholesale market). All

samples were transported to the laboratory in an icebox and analysed within 3h of collection for microbiological analysis.

Frozen black tiger shrimp (468), eg. uncooked whole frozen shrimp, bread-butter frozen shrimp and cooked-peeled frozen shrimp, etc., which consisted of 269 samples of uncooked frozen shrimp products and 199 samples of cooked frozen shrimp products destined for export were collected from processors during June 1998-June 2002. Contamination of *Salmonella* and pathogenic *Vibrio* spp. in exported frozen shrimp products were determined.

2.2. Microbiological methods for *Salmonella* detection

For isolation of *Salmonella*, the methodology outlined in FDA Manual [1] was used. Samples of 25 g of were homogenized for 2 min with 225 ml of Lactose broth (LB) then incubated 24 ± 2h at 35°C. The incubated sample was shaken well and 1mL was transferred to 10 ml of tetrathionate broth (TTB), 1 ml to 10 ml of Rappaport-Vassiliadis (RV) medium and another 1 ml to 10 ml of selenite cystine broth (SC). The RV medium and TTB were incubated at 42°C and the SC was incubated at 35° C for 24 ± 2h. All samples in TTB, RV and SC broths were streaked on the selective agar xylose lysine desoxycholate agar (XLD), Brilliant green agar (BG) and Bismuth sulfite agar (BS) and incubated for 24 ± 2h at 35° C. After incubation, typical colonies were examined. Suspect *Salmonella* were inoculated onto triple sugar iron agar (TSI), lysine iron agar (LIA), and urea agar slant, then confirmed by serological tests. Positive cultures were serotyped at WHO National *Salmonella* and *Shigella* Center, Division of Clinical Pathology, Department of Medical Sciences, Ministry of Public Health.

2.2.1. Methods for determining the presence of V. cholerae, V. parahaemolyticus and V. alginolyticus

For isolation of *Vibrio* spp., the methodology outlined in FDA's Bacteriological Analytical Manual [2] was used. Samples of 25 g were homogenized for two min with 225 ml alkaline peptone water (APW). Tenfold dilutions of the blended sample were also prepared in 9 ml APW (1:100 and 1:1000 dilutions). Dilutions are made to decrease competition from other vibrios. These enrichment broths were incubated for 6-8h at 35-37°C. The broths were then inoculated to TCBS agar, and the broths were reincubated for a total time of 18-24h. The broths were then plated again on TCBS agar. TheTCBS agar was incubated for 18-24h at 35-37°C**.** Plates were examined for typical colonies.

On TCBS agar, *V. cholerae* form large, smooth, yellow (sucrose-positive), and slightly flattened colonies with opaque centers and translucent peripheries. Confirmation: Carefully pick three or more suspect colonies from each plate, streak each for isolation on tryptic soy agar (TSA, 2% total NaCl concentration), and incubate for 18-24 h at 35-37°C to ensure colonial purity before biochemical testing. *V. cholerae* were examined for aggutination with *V. cholerae* 01, and 0139 antisera.

Sucrose positive yellow and large colonies on TCBS were also purified on TSA and tested for biochemical reactions typical of *V. alginolyticus.*

Sucrose negative bluish green colonies on TCBS were purified on TSA and tested for biochemical reactions typical of *V. parahaemolyticus (*Gram strain, oxidase, salt tolerance, TSI and API 20 E)**.** The strains identified as *V. parahaemolyticus* were examined for the presence or absence of the TDH and TRH genes.

2.2.2. Microbiological Methods MPN for Vibrio parahaemolyticus

The level of *V. parahaemolyticus* in samples was assessed using the most probable number (MPN) method. The methodology outlined in FDA's Bacteriological Analytical Manual for *V. parahaemolyticus* [2] was used. Samples of 50 g were homogenized for two min with 450ml alkaline peptone water (APW). Three-tube MPN tests were set up in APW and incubated for 16-18h at 35-37°C. After incubation, all dilutions containing a turbid tube, and at least one dilution higher, were streaked on TCBS agar with one loopful of inoculum taken from the top 1cm of each enrichment broth. Plates were incubated and examined for typical colonies. Three or more suspect colonies were picked from each plate and streaked on tryptic soy agar (2% total NaCl concentration), and incubated for 18-24 h at 35-37° C to ensure colonial purity. Colonies are identified biochemically. Counts were obtained using MPN tables**.**

3. RESULTS AND DISCUSSION

In this study on the investigation of *Salmonella* and pathogenic *Vibrio* spp. in samples of aquaculture black tiger shrimp, the water supply (canal water which supplied as raw material for pond water before treatment), pond water, feed materials, fresh and frozen shrimp were surveyed.

Results in Table 1 show the incidence of *Salmonella* from several sources. *Salmonella* was found in 12 samples of canal water of 86 tested (14%), but only two samples in pond water of 131 tested (1.5%). In 88 shrimp feed samples collected at farms, *Salmonella* was detected in one sample (1.1%). In samples of fresh black tiger shrimp collected at farms, *Salmonella* was found in four samples (3.2%). *Salmonella* was also found in 38 of 125 samples of fresh black tiger shrimp collected at wholesale shrimp markets (30.4%). In frozen black tiger shrimp destined for export (sampled from processors), *Salmonella* was found in one of 468 samples (0.2%).

Table 1. INCIDENCE OF SALMONELLA IN VARIOUS SAMPLES

Sample	No. of samples	Salm. detected	%
Canal water which supplied culture pond	86	12	14.0
Water from culture pond	131	2	1.5
Shrimp feed	88	1	1.1
Fresh black tiger shrimp from culture pond	126	4	3.2
Fresh black tiger shrimp from wholesale shrimp market	125	38	30.4
Frozen black tiger shrimp	468	1	0.2

Results in Table 2 show that 24 serovars of *Salmonella* were identified from 58 positive samples. Only six serovars were identified from fresh black tiger shrimp harvested from culture pond and the serovars were increased in the handling system particularly at wholesale market. *S. weltevreden* was also found in shrimp on the farm and at the market. This finding is similar to a review of *Salmonella* incidence in aquaculture shrimp by Reilly *et al.* [10]. From this study it was found that *Salmonella* were of very high percentage distributed in fresh black

tiger shrimp collected from wholesale shrimp markets which supplied as raw material for domestic consumption and processing plants for exported shrimp products. Most of wholesale shrimp markets still have very poor hygiene. Therefore the hygiene of the shrimp market is very important for the system of black tiger shrimp production.

It was also found that *Salmonella* were distributed through canal water which supplied as raw material for pond water before treatment. Therefore, the treatment of raw material of water supply is very importance for the system of black tiger shrimp production.

Table 2. SEROTYPES OF SALMONELLA ISOLATED FROM 58 POSITIVE SAMPLES

Sources	Serovars isolated
Canal water which supplied culture pond	*Salmonella bareilly* *Salmonella derby* *Salmonella newport* *Salmonella senftenberg* *Salmonella stanley* *Salmonella thompson*
Water from culture pond	*Salmonella virchow* *Salmonella I 9,12:-:1,5*
Shrimp feed	*Salmonella bareilly*
Fresh black tiger shrimp from culture pond	*Salmonella heidelberg* *Salmonella hvittingfoss* *Salmonella infantis* *Salmonella stanley* *Salmonella virchow* *Salmonella weltevreden*
Fresh black tiger shrimp from wholesale shrimp market	*Salmonella aberdeen* *Salmonella amsterdam* *Salmonella anatum* *Salmonella blockley* *Salmonella derby* *Salmonella give* *Salmonella javiana* *Salmonella kedougou* *Salmonella newport* *Salmonella orion* *Salmonella panama* *Salmonella rissen* *Salmonella schwarzengrund* *Salmonella senftenberg* *Salmonella weltevreden*
Frozen black tiger shrimp	*Salmonella chester*

Table 3 shows that *V. cholerae* was not detected in canal water samples, shrimp feed samples, fresh black tiger shrimp collected at farms, fresh black tiger shrimp collected at shrimp markets and in samples of frozen black tiger shrimp destined for export. *V.cholerae* non 01 was found in one sample of water of 131 tested from the culture pond (0.8%). These findings agree with those of Sajjapala and Phunbua (1994) who reported that *V. cholerae* was not detected in cooked frozen seafood produced for export. Vuttigornphan and Sornphrom (1994) also reported that *V. cholerae* not detected in seafood raw material for export.

V. parahaemolyticus was found in samples of canal water, pond water, fresh black tiger shrimp collected at farms, fresh black tiger shrimp collected at wholesale shrimp markets and frozen black tiger shrimp destined for export at a level of 2.3%, 5.3%, 14.3%, 48% and 0.2%, respectively (Table 3). However nonhuman pathogenic Vibrio*, V. harveyii* was found predominant in frozen black tiger shrimp samples (34.2%).

V. alginolyticus was found in samples of canal water, pond water, fresh black tiger shrimp collected at farms, fresh black tiger shrimp collected at wholesale shrimp markets and frozen black tiger shrimp destined for export at a level of 9.3%, 9.9%, 9.5%, 18.4 % and 3.4%, respectively (Table 3).

Table 3. INCIDENCE OF PATHOGENIC *VIBRIO* SPP. IN VARIOUS SAMPLES

SAMPLE	Positive for 25 g/ml sample No. of samples	*V. cholerae*	*V. parahaemolyticus*	*V. alginolyticus*
Canal water which supplied culture pond	86	Nil	2 (2.3%)	8 (9.3%)
Water from culture pond	131	1(0.8%)	7 (5.3%)	13 (9.9%)
Shrimp feed	88	Nil	Nil	Nil
Fresh black tiger shrimp from culture pond	126	Nil	18 (14.3%)	12 (9.5%)
Fresh black tiger shrimp from wholesale shrimp market	125	Nil	60 (48%)	23 (18.4%)
Frozen black tiger shrimp	468	Nil	1 (0.2%)*	16 (3.4%)

Note: * *V. harveyi* 160 (34.2%)

Table 4 shows the MPN of *V. parahaemolyticus* in various samples. Most of samples contained a very low level of *V. parahaemolyticus*. Fresh black tiger shrimp from the wholesale shrimp market contained the highest level. *V. parahaemolyticus* is potentially present in the raw seafoods in tropical countries, as the environment may promote the growth of this bacteria. Therefore good handling practice of shrimp should be applied, eg appropriate temperature control during handling and transportation. Shrimp should be maintained not higher than 5°C. Improper refrigeration of shrimp contaminated with this organism will allow its proliferation, which increases the possibility of infection in consumers.

Table 3. MPN OF *V. PARAHAEMOLYTICUS* IN VARIOUS SAMPLES

SAMPLE	No. of samples	No. of samples with MPN/g (%) in the range						
		0.3	0.3-<3	<3	3-10	10-100	100-10000	>10000
Canal water which supplied culture pond	81	79	1 (1.2)	-	1 (1.2)	-	-	-
Water from culture pond	119	14 (95.8)	4 (3.4)	-	-	1 (0.8)	-	-
Fresh black tiger shrimp from culture pond	115	-	-	99 (86.1)	4 (3.5)	8 (6.9)	4 (3.5)	
Fresh black tiger shrimp from wholesale shrimp market	122	-	-	76 (62.3)	21 (17.2)	14 (11.5)	10 (8.2)	1 (0.8)

The strains identified as *V. parahaemolyticus* were examined for the presence of the TDH and TRH. TDH (KP+) was found in 2.7% (7 of 262 strains) and TRH (urease reaction) was found in 1.15% (3 of 262 strains). *Sakasaki et al*. [14] also reported that 1% (7 of 650) of environment isolates studied were Kanagawa positive.

4. CONCLUSIONS

A high percentage of fresh black tiger shrimp, collected from wholesale shrimp markets which are used for domestic consumption and for processing for exported shrimp products, was found to be contaminated with *Salmonella* and *V. parahaemolyticus*. Good handling practices and HACCP system for shrimp distributors/producers should be applied in order to improve the safety of this food. Shrimp should be maintained at a temperature not higher than 5°C during handling and transportation. Improper refrigeration of shrimp will allow proliferation of the microorganisms which increases the possibility of infection in consumers.

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