Prevalence of *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Salmonella* spp. in Seafood Products Using Multiplex Polymerase Chain Reaction

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Although several etiological agents can be transmitted through seafood consumption, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Salmonella* spp. are considered among the most important pathogens in terms of public health and disease. In this study, multiplex polymerase chain reaction (PCR), as a rapid and cost-effective method, was used to determine the prevalence of these pathogens in 245 samples of raw/fresh, frozen, and ready-to-eat (RTE) seafood products marketed in Iran. The prevalence of *L. monocytogenes* in raw/fresh fish and shrimp samples was 1.4%, whereas 2.9% of the raw/fresh fish and 7.1% of the shrimp samples were contaminated with *V. parahaemolyticus*. No contamination with *L. monocytogenes* and *V. parahaemolyticus* was found in frozen and RTE seafood products. The prevalence of *S. aureus* was found to be higher than other investigated pathogens. *S. aureus* was detected in 5% of the raw/fresh samples of fish and shrimp, 17.5% of the frozen, and 12.3% of the RTE samples. Further, our findings indicate that 2.9% of the fish samples, 4.3% of the shrimp samples, and 1.5% of the RTE samples were contaminated with *Salmonella* spp. Owing to the potential hazard of these pathogenic bacteria, multiplex PCR can provide a rapid and cost-effective method for the surveillance of these pathogens in seafood products.

Introduction

The microbiological safety of seafood is a significant concern of consumers, industries, and regulatory agencies all over the world. The rapid and accurate identification of bacterial pathogens is important, both for quality assurance and to trace bacterial pathogens within the food supply (Bhagwat, 2003; Germini et al., 2009). During the last few years, international standards have agreed on the use of polymerase chain reaction (PCR) for the detection of foodborne pathogens and legislations are implementing new types of analyses as the accepted official methods. For example, European regulation EC 2073/2005 allows for the use of alternative detection methods if based on certified analyses according to international standards (EU, 2005). Multiplex PCR is a relatively new method that can simultaneously amplify template mixture and decrease the detection cost, conquering the weakness of single PCR detecting only one target (Kim et al., 2007; Germini et al., 2009).

In studies of seafoodborne pathogens, four major pathogens have emerged as being of significant importance in terms of human health and disease. These include *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Salmonella* spp. (*Feldhusen*, 2000).

*L. monocytogenes* has been isolated from fish and seafood products all over the world. The contamination rate of seafood products with *L. monocytogenes* can vary from 0% to more than 50% (Ben Embarek, 1994; Jinneman et al., 1999). Contamination of seafood products with *L. monocytogenes* depends on many factors such as cleaning and processing procedures, working habits, and the existence of surface-persistent *L. monocytogenes* types in processing facilities. Moreover, raw materials contaminated with *Listeria* may also be a reason for the contamination of the final product (Rørvik et al., 1997; Miettinen and Wirtanen, 2005).

*V. parahaemolyticus* is a human pathogen that occurs naturally in the marine environments and is frequently isolated from a variety of seafood including fish, shrimp, crab, lobster, scallop, and oyster (Austin, 2010). This pathogen is a common cause of foodborne illnesses in many Asian countries, including Taiwan, China, and Japan, and is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States (Jaksic et al., 2002; Su and Liu, 2007).

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S. aureus is one of the most prevalent causes of gastroenteritis worldwide, which is caused by the ingestion of food that contains preformed toxins. Humans are common carriers of S. aureus in the nose, throat, and skin infections. Therefore, the organism can be easily transferred to seafood during handling (Jablonski and Bohach, 2001). The contamination of seafood could be the result of a combination of improper and unsanitary handling, improper storage, and cross contamination (Huang et al., 2001; Jablonski and Bohach, 2001).

Salmonella can occur both in water, especially of contaminated coastal regions or ponds, and in the fresh fish from these areas. The incidence of salmonellosis caused by the consumption of contaminated seafood, such as raw shellfish, is a primary concern of public health agencies in many countries (Feldhusen, 2000).

The present study was, therefore, carried out to determine the prevalence of L. monocytogenes, V. parahaemolyticus, S. aureus, and Salmonella spp. in raw/fresh, frozen, and ready-to-eat (RTE) seafood products marketed in Iran using multiplex PCR technique.

Materials and Methods
Sampling, preenrichment, and enrichment procedure

During a 10-month period, a total of 245 samples were taken from various seafood products such as raw/fresh fish and shrimp, frozen fish fillets, frozen peeled and deveined (PUD) shrimp, fish nuggets, breaded shrimp, shrimp burgers, and Kentucky shrimp. All samples were placed in cold portable insulated boxes, transported to the laboratory in ice, and processed within 1 h of collection. Twenty-five grams each of the seafood samples were aseptically taken and blended for 60 sec in a stomacher (Bagmixer 400W; Interscience) with 225 mL of Universal preenrichment broth (Atlas, 2006) and incubated at 35°C for 6 h. For enrichment of each pathogen, 10 mL of preenrichment broth was added to an equal volume of double-strength enrichment broths; Listeria enrichment broth, Staphylococcus enrichment broth, alkaline peptone water, and Rappaport-Vassiliadis enrichment broth for L. monocytogenes, S. aureus, V. parahaemolyticus, and Salmonella, respectively (Atlas, 2006). Enrichment broths were incubated for 18–20 h at 35°C, except for Salmonella, which incubated at 42°C.

DNA extraction and multiplex PCR

DNA extraction was performed through boiling. One milliliter of sample of the enrichment broths was centrifuged at 14,000 rpm for 3 min. Bacterial pellets were resuspended in 1 mL of sterile saline solution (0.85% NaCl). Following centrifugation in the same way, the supernatants were replaced with 50 μL of sterile distilled water and incubated at 100°C for 10 min to release the bacterial DNA. After boiling, the clear supernatants obtained after 5 min centrifugation at 14,000 rpm were collected and stored at ~20°C until use. DNA extracted from each specific enrichment broth, assuming equal amounts of DNA, was mixed together and used in multiplex PCRs. The oligonucleotide primers used in this study and multiplex PCR were carried out according to Kim et al. (2007) (Fig. 1). PCRs were performed in a thermal cycler (Eppendorf, Mastercycler Gradient) and PCR products were visualized following gel electrophoresis on 2% agarose gels using ethidium bromide staining.

Limits of detection of multiplex PCR reactions

The sensitivity of multiplex PCRs was assessed on artificially contaminated seafood samples. From the three main categories of seafood products used in this study; raw/fresh, frozen PUD, and breaded shrimp were selected. Twenty-five grams of samples was homogenized in 225 mL of UPB. Subsequently, the homogenates were spiked simultaneously with 1 mL of serial decimal dilutions of the four pathogens. The bacterial concentration of the contaminating cultures was determined by plating on TSA. For each bacterium and product used, three levels of contamination were tested (Table 1). A nonspiked seafood sample was used as negative control. Seafood homogenates were incubated at 35°C for 6 h and then enriched as described earlier. DNA extraction and multiplex PCR were performed in the same way as the seafood samples.

Results and Discussion

In this study, multiplex PCR-based method was used for detection of organisms after enrichment of bacterial cultures. The sensitivity and specificity of this multiplex PCR assay were explained by Kim et al. (2007). They also explained the validity of the multiplex PCR assay for samples of ham, milk, and water. In the present study, sensitivity of multiplex PCR was assessed on artificially contaminated seafood samples (Table 1). As shown, after enrichment, for L. monocytogenes, V. parahaemolyticus, and Salmonella spp., there were no differences in the detection limit between raw/fresh, frozen PUD, and breaded shrimp samples, whereas for S. aureus in raw/fresh shrimp it was higher than in frozen PUD and breaded samples. It is possible that DNA from normal microbiota of raw/fresh samples may decrease the sensitivity of multiplex PCR assay for detecting S. aureus and cause underestimation of prevalence. Overall, the sensitivity of this method together with its ability to give rapid and cost-effective results make it a suitable method to be implemented in control laboratories for the detection of these pathogens in seafood samples.
In the present study, *L. monocytogenes* was detected in 1.4% of the raw/fresh fish and shrimp samples. However, contamination with this pathogen was not observed in frozen and RTE seafood samples at the retail level (Table 2). Only a few reports on the prevalence of *L. monocytogenes* in seafood products in Iran have been previously published. According to Basti et al. (2006), the prevalence of *L. monocytogenes* in fresh fish samples was 2.6%. However, *L. monocytogenes* was not found in 85 samples of fresh and frozen fish and shrimp analyzed by Jalali and Abedi (2008). *L. monocytogenes* contamination of seafood varies with product category. Jorgensen and Huss (1998) demonstrated that the highest prevalence of *L. monocytogenes* is in cold-smoked fish (34%–60%), whereas the lowest is in heat-treated and cured seafood (4%–12%). In general, *L. monocytogenes* is not usually found on fish captured from open waters. However, contamination may take place long before the fish raw material reaches retail trade or processing factories. Potential sources of *L. monocytogenes* on fishing vessels include contamination from water and ice, soiled surfaces, and boxes as well as from human and avian sources. As *L. monocytogenes* is commonly found in coastal waters and in surface waters of lakes, fish captured or cultivated in these waters may possibly carry this microorganism (FAO, 1999).

Our findings indicate that 2.9% of the raw/fresh fish and 7.1% of the shrimp samples were contaminated with *V. parahaemolyticus*. However, no contamination was found in 105 samples of frozen and RTE seafood products (Table 2). This pathogen has been isolated and detected from seafood, particularly shellfish or bivalve mollusks all over the world. In Iran, *V. parahaemolyticus* was detected in 9.3% of the shrimp samples analyzed by Rahimi et al. (2010). In Croatia, during the summer of year 2000, 117 samples of sea fish, shrimp, and bivalve mollusks were sampled and *V. parahaemolyticus* was found in 9.4% of the total samples (Jaksic et al., 2002). A very high level of contamination with *V. parahaemolyticus* has been reported in cockle (*Anadara granosa*) in Malaysia. According to Bilung et al. (2005), of the 100 cockle samples tested, 62 were positive for the presence of *V. parahaemolyticus*. Considering the widespread distribution in marine environments, short generation times, and low infectious doses of pathogenic strains (Kaysner and De Paola, 2000), intensive and continuous monitoring of potentially pathogenic *V. parahaemolyticus* is strongly recommended to evaluate human health risk arising from seafood consumption.

The prevalence of *S. aureus* was found to be higher than other investigated pathogens. *S. aureus* was detected in 5% of the raw/fresh samples of fish and shrimp, whereas the prevalence of this bacterium was 15% in frozen and RTE samples (Table 2). Some reports have presented the incidence of *S. aureus* in seafood: 7% in shrimp, 4% in frozen cuttlefish, 4% in fish (Rodma et al., 1991), 68% in frozen fishery products

<table>
<thead>
<tr>
<th>Pathogen used</th>
<th>Fishery product</th>
<th>Level of contamination (CFU/25 g)</th>
<th>PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Raw/fresh shrimp</td>
<td>0/33/79/112</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td></td>
<td>Frozen PUD shrimp</td>
<td>0/33/79/112</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td></td>
<td>Breaded shrimp</td>
<td>0/33/79/112</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>Raw/fresh shrimp</td>
<td>0/21/56/97</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td></td>
<td>Frozen PUD shrimp</td>
<td>0/21/56/97</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td></td>
<td>Breaded shrimp</td>
<td>0/21/56/97</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Raw/fresh shrimp</td>
<td>0/42/91/172</td>
<td>– / (+) / + / +</td>
</tr>
<tr>
<td></td>
<td>Frozen PUD shrimp</td>
<td>0/42/91/172</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td></td>
<td>Breaded shrimp</td>
<td>0/42/91/172</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>Raw/fresh shrimp</td>
<td>0/18/73/88</td>
<td>– / (+) / + / +</td>
</tr>
<tr>
<td></td>
<td>Frozen PUD shrimp</td>
<td>0/18/73/88</td>
<td>– / + / + / +</td>
</tr>
<tr>
<td></td>
<td>Breaded shrimp</td>
<td>0/18/73/88</td>
<td>– / + / + / +</td>
</tr>
</tbody>
</table>

*a TriPLICATE experiments.
+ : Presence of PCR product; – : Absence of PCR product; (+): weak PCR product.
PUD, peeled and undeveined; PCR, polymerase chain reaction.

### Table 2. The Prevalence of *Listeria monocytogenes, Vibrio parahaemolyticus, Staphylococcus aureus,* and *Salmonella spp.* in Seafood Samples Tested

<table>
<thead>
<tr>
<th>Product category</th>
<th>Fishery products</th>
<th>Samples tested</th>
<th>L. monocytogenes</th>
<th>V. parahaemolyticus</th>
<th>S. aureus</th>
<th>Salmonella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw/fresh</td>
<td>Salt water fish</td>
<td>70</td>
<td>1 (1.4)</td>
<td>2 (2.9)</td>
<td>3 (4.3)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td></td>
<td>Shrimp</td>
<td>70</td>
<td>1 (1.4)</td>
<td>5 (7.1)</td>
<td>4 (5.7)</td>
<td>3 (4.3)</td>
</tr>
<tr>
<td>Frozen</td>
<td>Fish fillet</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>3 (15)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PUD shrimp</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>4 (20)</td>
<td>0</td>
</tr>
<tr>
<td>Ready-to-eat</td>
<td>Fish nugget</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1 (10)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Shrimp burger</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (10)</td>
</tr>
<tr>
<td></td>
<td>Breaded shrimp</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>5 (16.7)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Kentucky shrimp</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>2 (13.3)</td>
<td>0</td>
</tr>
</tbody>
</table>
(Sanjeev et al., 1986), and 8% in fish and shellfish (Ayulo et al., 1994). It is well documented that freshly caught seafood are free from S. aureus, and contamination takes place upon handling (Shewan, 1962; Bryan, 1980). Several conditions, such as delay in processing, inadequate refrigeration, poor personal hygiene, and postprocess contamination, are associated with staphylococcal contamination, especially in frozen and RTE samples. Therefore, it is recommended to use sanitary gloves for handling RTE seafood to reduce the problem of S. aureus contamination.

As shown in Table 2, of the 245 samples investigated in this study, Salmonella was found in 6 samples. About 2.9% of the fish samples, 4.3% of the shrimp samples, and 0.95% of the frozen and RTE samples were contaminated with Salmonella spp. The overall prevalence of Salmonella spp. in seafood samples tested was lower than that reported elsewhere. The occurrence of Salmonella in brackish water ponds was monitored over a 2-year period in one of the major shrimp-exporting countries in Southeast Asia. Salmonella were present in 16% of shrimp and 22.1% of mud/water samples (Reilly and Twiddy, 1992). In addition, Salmonella species were present in 21% of eel culture ponds in Japan (Saheki et al., 1989). The presence of Salmonella in water depends on some factors, for example, the pollution from a nearby poultry farm or animal manures. Considering the disease surveillance reports from public health authorities in some countries, human salmonellosis associated with the consumption of seafood occurs very rarely compared with those associated with other foods, for example, poultry products (Reilly, 1998).

In conclusion, although classical microbiological methods offer reliable and standardized procedures for the detection of foodborne pathogens (e.g., ISO standards), they often result in time-consuming analyses not always compatible with the need for rapid results. This study shows that multiplex PCR is a very useful tool for the detection of seafoodborne pathogens, namely, L. monocytogenes, V. parahaemolyticus, S. aureus, and Salmonella spp. The method represents a rapid and cost-effective method for the detection of these pathogens in seafood products. Moreover, the results of the present study can provide a significant contribution to both regulatory agencies and seafood industries. The surveillance of these pathogenic bacteria in harvested seafood and seafood products marketed is crucial to safeguarding public health.

Acknowledgment

This study was supported by a research grant provided by the Shahid Chamran University of Ahvaz.

Disclosure Statement

No competing financial interests exist.

References


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