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RESEARCH ARTICLE

Detection and Molecular Characterization of *Vibrio Parahaemolyticus* in Shrimp Samples

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Abstract:

Background:

Food safety has emerged as an important global issue with international trade and public health implications. Bacterial pathogens as*Vibrio parahaemolyticus* recognized as an important cause of foodborne diseases related to the consumption of raw, undercooked or mishandled seafood worldwide.

Methods:

A total of 70 individual wild shrimp samples were collected from shrimp retail outlets in Zanjan, Iran and investigated for the presence of potentially pathogenic strains of *V. parahaemolyticus.*The shrimp samples were immediately homogenized and cultured on TCBS agarand subjected to confirmatory biochemical tests. Polymerase Chain Reaction (PCR) was performed for detection of total and pathogenic *V. parahaemolyticus* by amplification of *vp–toxR,tdh* and *trh* genes.

Results:

The conventional method indicated that 16 (22.8%) of samples were positive for *V. parahaemolyticus*. However, PCR verified that only 12 (17.1%) shrimp samples were positive for *V. parahaemolyticus.* Of the 70 shrimp samples in our study, only 2 (2.8%) *tdh* and 1 (1.4%) *trh* positive strains were identified.

Conclusion:

Detection of *tdh* and/ or *trh* positive *V. parahaemolyticus* in shrimp marketed in Zanjan, Iran shows a probable risk for public health. Therefore, the reliable molecular methods for monitoring of potentially pathogenic *V. parahaemolyticus* are strongly recommended for the routine seafood examination.

Keywords: *Vibrio parahaemolyticus*, Shrimp, PCR, Molecular characterization, *vp–toxR*, *tdh*, *trh* genes.

1. INTRODUCTION

Vibrio parahaemolyticus is a halophilic marine bacterium and some strains can cause gastroenteritis in humans through the consumption of raw, undercooked or mishandled contaminated seafood [1, 2]. Although the gastroenteritis caused by *V. parahaemolyticus* is often self–limited and characterized by diarrhea, headache, vomiting, nausea, abdominal cramps and low fever, the infection may cause septicemia, a life-threatening infection, in immunocompromized patients [3 - 5]. The pathogenic strains of *V. parahaemolyticus* are characterized by the production of Thermo stable Direct Hemolysin (TDH) and/or TDH–Related Hemolys in (TRH) that can lyse the red blood cells on Wagatsuma blood agar (referred to as the Kanagawa phenomenon) and encoded by *tdh* and *trh* genes,

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respectively [3, 6]. The ubiquitous nature of *Vibrio* spp. in marine and estuarine environments makes it impossible to obtain seafood completely free of these species. It has been implicated in several outbreaks of seafood poisoning worldwide [6, 7]. Previous epide miological studies showed that *V. parahaemolyticus* is an important cause of foodborne disease in Asia, South America and the United States. The *V. parahaemolyticus* is frequently isolated from shellfish including oysters, clams, mussels, lobsters, crabs, shrimps and cockles, which provide an excellent substrate for the growth of these micro organisms in the aquatic habitats [8 - 13]. Many studies have been carried out on shellfish and findings concerning the distribution of *V. parahaemolyticus* in oysters and mussels are well documented. However, few data are available forcrustaceans, despite the popularity of crabs and shrimps and their rising consumption worldwide [14]. Shrimp is one of the most important fishery products, and shrimp farming is an important economy characteristic of Iran [13].The frequency of pathogenic *V. parahaemolyticus* infrozen ready–to–eat shrimps for human consumption was recently studied, and 7 to 8% of samples tested positive for *tdh* or *trh* virulence genes in countries such as Malaysia. Therefore, these shrimps might have the potential to cause *V. parahaemolyticus*–associated infections if consumed without further processing [14].

In recent years, *V. parahaemolyticus* has been recognized as the causative agent of 50–70% of all cases of gastroenteritis associated with consumption of seafood [5].

There are different methods for detection of *V. parahaemolyticus* in seafood samples. The most–probable–number (MPN) method is used for enumeration of *V. parahaemolyticus* from food and water, but this method is cumbersome and the recovery of the organism is low [15].The culture-based approaches and PCR technique which is faster, easier and more sensitive can be used for identification of *V. parahaemolyticus* in seafood samples [10]. The *V. parahaemolyticus* strains possess a regulatory gene, *toxR*, which is present in all strains irrespective of their ability to produce *tdh* and/or *trh*.Therefore, the PCR targeted to the *toxR* gene can be used as a method for identification at the species level [16].

The objective of this study was to determine the frequency of pathogenic *V. parahaemolyticus* in wild shrimp samples using the culture and PCR methods based on detection of *tdh* and *trh* virulence genes in Zanan, Iran.

2. MATERIALS AND METHODS

2.1. Sampling

From March to June 2015, a total of 70 individual wild shrimp samples were collected from shrimp retail outlets in Zanjan, Iran. Shrimp samples were packed into a clean polyethylene bag then marked and transported to the laboratory of food microbiology in a cooler with ice packs for analysis within 1 h.

2.2. Reference Strain

The *V. parahaemolyticus* ATCC 17802 waskindly obtained from the Pasteur Institute of Iran and was grown on Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agar (MERCK, Darmstadt, Germany).

2.3. Isolation and Identification of *V. Parahaemolyticus*

The isolation and biochemical identification of *V. parahaemolyticus* were carried out as recommended in the FDA's Bacteriological Analytical Manual. Twenty five gram of the samples were homogenized for 60 s in a stomacher (Heidolph, Schwabach, Germany) with 225 mL of alkaline peptone water (APW) containing 3% NaCl and then incubated for enrichment at 37ºC for 24 h. After primary enrichment, a loopful (without shaking the flask) from each of the enriched homogenate was streaked onto the surface of Thiosulfate Citrate Bile Sucrose (TCBS) agar plates (MERCK, Darmstadt, Germany)and incubated at 37°C for 24 h.On TCBS plates, sucrose negative colonies (green or blue-greencolonies with 2–3 mm in diameter), were picked up and inoculated into tryptonesoya broth with 3% NaCl, incubated at 37°C for 24 h, then purified onto nutrient agar slants with 3% NaCl and subjected for confirmatory biochemical tests using different media contained 2.5% NaCl. Every single colony was screened for Gram staining, motility, oxidase and urease activity, NaCl requirement, citrate utilization, triple sugar iron agar, arginine dehydrolase, lysine and ornithine decarboxylase, O/129 sensitivity, vogese proskauer, indole and acid production from lactose, arabinose, cellobiose, mannitol and mannose.

2.4. Genomic DNA Extraction

A colony of *V. parahaemolyticus* (one colony per sample) was picked from nutrient agar and inoculated into 5 ml of

LB (Luria Bertani Broth, Merck) until the exponential phase with 2 McFarland turbidity with shaking at 120 rpm at 37°C. One ml from an overnight culture in LB was spun at 8,000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 200µL sterile deionized water and boiled at 100ºC for 10 min. The tube was immediately placed in ice for 5 min; then the cell lysate was centrifuged at 13000 r.p.m. for 3 min to pellet the cell debris and the clear supernatant was transferred to a new tube. A 5 µl aliquot of supernatant was used for PCR.

2.5. Molecular Confirmation of *V. Parahaemolyticus* Isolates

Confirmation of presumptive *V. parahaemolyticus* isolates was performed using PCR targeting the *vp–toxR* gene with the following primers and amplicon size 368bp: *toxR–F*:5'–GTCTTCTGACGCAATCGTTG–3' and *toxR–R*:5'–ATACGAGTGGTTGCTGTCATG–3' [2]. A specific primer pairs were also used for detection of *tdh* and *trh* virulence genes in *toxR* positive strains with the following sequences: *tdh–F*:5'–CCACTACCACTCTCATATGC–3' , *tdh–R:*5'–GGTACTAAATGGCTGACATC–3' with amplicon size 251 bp and *trh–F:*5'GGCTCAAAATGGTTAAGCG –3' and *trh–R:*5'–CATTTCCGCTCTCATATGC–3' with amplicon size 250 bp [10]. Single PCR was performed using Dream Taq PCR Master Mix (Thermo Fisher Scientific), which contains Taq polymerase, dNTPs, $MgCl_2$ and the appropriate buffer. Each PCR tube contained 25 µl reaction mixture composed of 12.5 µl of the master mix, 2.5 µl of each forward and reverse primer solution (in a final concentration of 200 nM), 5 µl of DNA and nuclease–free water to complete the final volume. PCR was performed using the Gene Atlas 322 system (ASTEC) with the same cycling conditions for *toxR, tdh* and *trh* genes. Amplification involved an initial Denaturation at 94°C, 5 min followed by 30 cycles of denaturation (94°C, 1 min), annealing (57°C, 1.5 min) and extension (72°C, 1.5 min), with a final extension step (72°C, 8 min). The amplified DNA was separated by submarine gel electrophoresis on 1.5% agarose, stained with ethidium bromide and visualized under UV trans illumination.

3. RESULTS AND DISCUSSION

The *V. parahaemolyticus* is an enteric human pathogen that occurs naturally in the marine and estuarine environments worldwide. Several outbreaks of seafood poisoning were caused by *V. parahaemolyticus* in many countries and regions of the world including USA, Japan, India and Taiwan [10]. In this study, a total of 70 individual wild shrimp samples were studied for the presence of pathogenic *V. parahaemolyticus*. A conventional cultural method based on the appearance of green or blue– green colonies on TCBS agar and microscopic examination was detected presumptive *V. parahaemolyticus* in 30 (42.8%) out of the 70 shrimp samples. However, the biochemical tests of the presumptive *V. parahaemolyticus* strains indicated that 22.8% (16/70) of shrimp samples were positive for *V. parahaemolyticus.* Variable incidences of *V. parahaemolyticus* in seafood had been demonstrated using conventional methods. The frequency of *V. parahaemolyticus* in our study was lower than some previous studies. According to Abd–Elghany & Sallam [10] and Quintoil *et al* [17], the frequency of *V. parahaemolyticus* in shellfish samples was 33.3%, and 36.8%, respectively. However, lower incidence of *V. parahaemolyticus* in seafood samples was reported from Italy and Netherlands with 6.2%, 24.3% and 8%, respectively [1, 18, 19].

This variation in *V. parahaemolyticus* frequency among seafood samples may be due to the difference of the geographical region, type of shellfish sample, watersalinity, seasons of sampling, post-harvest practices and hygienic standards applied during the handling, transport and storage of seafood products, as well as the methods used for isolation and identification of the organism [10].

Fast and accurate diagnosis of food–borne pathogens is very important for a positive outcome of eradication programs. PCR based methods which target the conserved region of *V. parahaemolyticus* such as *toxR* gene is more efficient, reliable and faster compare to the conventional techniques [20]. In our study, the biochemically identified isolates were further verified using PCR targeting the *vp–toxR* gene. It has been indicated that 12 (17.1%) samples out of a total 70 shrimp samples were positive for *vp–toxR* gene. In the present study, the frequency of *V. parahaemolyticus* positive samples based on *vp–toxR* gene, was approximately similar to those reported by Abd–Elghany & Sallam in Egypt [10], who found that 16.7% of shellfish samples were positive for *vp–toxR* gene and also by Hassan *et al.* [18] in the Netherlands, who detected that 19% (38/200) of retailed shellfish samples were positive for *toxR* gene. Only a few reports on the frequency of *V. parahaemolyticus* in seafood samples from Iran have been previously published. According to the previous reports from Iran, 9.3% and 11% of the shrimp samples [13, 21] and 21.4% of the fish samples [22] were positive for the presence of this pathogen.

As the presence of *V. Parahaemolyticus* strains carrying *tdh* and/or *trh* genes in seafood represents a public health risk, their detection would be of paramount importance. It is well known that only 1–2% of the environmental strains

possess the *tdh* gene. Of the 70 shrimp samples in our study, only 2 (2.8%) *tdh* and 1 (1.4%) *trh* positive strains were identified. In the previous report from Iran, the prevalence of *tdh*–positive and *trh*–positive *V. parahaemolyticus* was 1.7% and 0.7%, respectively [21]. Similar to our results, in a study conducted in Malaysia, 5 (3.9%) and 1 (0.78%) strains isolated from live and frozen shrimp, respectively were positive for *tdh* gene, whilst 2 (1.56%) and 1 (0.78%) strains were positive for *trh* gene [23]. However, higher incidence of virulence *V. parahaemolyticus* was identified in several studies such as the study conducted in Turkey by Terzi *et al.* [24] who found that 24 (75%) out of the 32 strains isolated from mussel were potentially pathogenic depending on *tdh* and *trh* genes.

CONCLUSION

In conclusion, the detection of *tdh* and/or *trh* positive *V. parahaemolyticus* in shrimp marketed in Zanjan, Iran shows a probable risk for public health. Therefore, intensive and continuous monitoring of potentially pathogenic *V. parahaemolyticus* are strongly recommended in order to evaluate the human health risk arising from seafood consumption.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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