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Occurrence of *Escherichia coli* O157 in Some Marine Fishes and Shellfish Sold in Isfahan markets, Iran

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***Escherichia coli* O157 is a food-borne pathogen that attributed to the contamination of some sea foods including fish and shellfish. This study was aimed to identification of Escherichia coli in some marine fish and shellfish such as, lobster, crab and shrimp, and to investigate the occurrence of *Escherichia coli* O157 as a food born bacterial pathogen. The study was carried out in Isfahan markets (Iran) during spring and summer 2011. A total of 190 samples (90 fish, 40 lobster, 30 shrimp and 30 crabs) were collected, Samples were randomly obtained from the fish markets and examined based on their growth characteristics on specific culture media and molecular (PCR) tests. Among all samples, 49 cases (25.78%) contaminated by *E.coli*, that among these isolates two cases were distinguished as an *E.coli* O157. In these samples *E.coli* O157:H7 was not identified. The highest and lowest contamination rate was observed among fishes and crab with 34.44 and 10 percents, respectively. Also the contamination rates of *E.coli* in different seasons were 15.78 and 10 percent in spring and summer, respectively. It can be concluded that marine fish and shellfish can carry some pathogenic bacteria as *E.coli* O:157 that have risks for human health.**

Key Words: *Escherichia coli* O157, fish, lobster, crab, shrimp.

Shiga toxin-producing *Escherichia coli* (STEC), also called verotoxin-producing *E. coli* (VTEC), has emerged as a pathogen that can cause food poisoning and severe and potentially fatal illnesses (Paton and Paton, 1998). Fish and shellfish are highly perishable, and prone to vast variations in quality due to differences in species, environmental habitats and feeding habits. In addition, they can also function as carriers of several microbial and other health hazards (Yagoub,2009). Most fish related food borne illness are traced to *Salmonella*, *Staphylococcus spp.* , *Escherichia spp.* , *Vibrio parahemolyticus*, *Clostridium perfringens, Clostridium botulinum E*, and *Enteroviruses* (*CFSAN*,2001). Fishery products are important not only from a nutritional point of view, but also as an item of international trade and foreign exchange earner for a number of countries in the world (Yagoub SO ,2009). The presence of *E. coli* as well as verotoxigenic *E. coli* O157:H7 in fish meal was investigated by Thampuran *et al* .(2005). In the study typical *E. coli* O157 or labile toxin-producing *E. coli* wasn't seen in the fish and fishery environments of Cochin (India). *Aeromonas spp.*, *Plesiomonas shigelloides, Vibrio cholerae* 01, *Vibrio*

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parahaemolyticus, and Vibrio vulnificus were isolated from different organs of fishes (Ristori *et al*., 2007). It was found that the hygienic quality and freshness of fish and shellfish decreased in summer, especially for clam and mussel (Hwang *et al*.,2004). The microbial quality of the tilapia indicated that all tissue samples except muscle tissues were contaminated with fecal coliform were *Escherichia coli* is the most common contaminant and is often encountered in high numbers (Thampuran *et al*.,2005). Bacterial microbiota associated with fresh raw shrimp was *Aeromonas, Pseudomonas, Vibrio, Flavobacterium* and *Serratia* (Jeyasekaran *et al*.,2006). Heinitz *et al* (2000). found that 10% of imported and 2.8% of domestic raw seafood was positive for *Salmonella.* Also *Enterococcus sp, Aeromonas sp,* fecal and total coliform, the presence of *Listeria sp* and *Salmonella spp* from the external surface of tilapias were shown by Morales *et al* (2004). The hazards and challenges associated with handling fish during farming and capture and the environmental contaminants in seafood may pose a risk to human health (Håstein *et al*.,2006) . In the most cases microbiological changes occur when shrimps are insufficiently iced and improperly stored at elevated temperatures (Reilly *et al*.,1986).

The present purpose is to investigate the occurrence of any human bacterial pathogens in the marine fishes and shellfish taken for examination and also exploring the possible route of entry into food chain through the sea foods. Thus this study was designed to detect *E. coli* O157 in fish and shellfish, and to characterize these isolates by means of bacteriology, biochemical and PCR tests.

MATERIAL AND METHODS

Study area

Fish and shellfish were collected randomly from Isfahan fish markets during the spring and summer 2011. A total of 190 samples (90 fishes, 30 shrimps, 40 lobsters and 30 crabs) were collected. The species of the fish and shellfish included Tigertooth croake (*Otolithes ruber*), mackerel (*Scomberomorus commerson*),white shrimp (*Pnaeus indicus*) , lobster (*Panulirus pencillatus*) , Black pomfret (*Parastromateus niger*) and crab (*Nursia plicata*) . Specimens placed on sterilized bags and, under standard condition, transported to the laboratory on ice within one hour.

Sampling and Isolation of *E. coli*

To examine the bacterial organisms in the samples, the methods of culture and plating as described by Venkataraman and Sreenivasan (1952) were followed. The muscles of fish and shellfish were dissected and processed as described by Hossain (2008). The respective portions were cut, homogenized and after serial dilution using sterile saline, they were subjected in the *Violet Red Bile Agar* (VRBA) medium , and incubated at 37°C for 24 h. Culture technique procedures recommended by Bergey's Manual (1948) were followed. Five colonies that are grown in VRBA medium inoculated in *Brilliant Green Lactose Bile (BGLB)* Broth, and incubated at 44.5°C for 24 h, then *Eosin Methylene Blue (*EMB) medium (Oxoid Ltd., Hampshire, England) was used for isolation of Escherichia.coli colonies. To identify and characterize the isolated pathogens, biochemical parameters such as indole, methyl red, Voges-Proskauer, and citrate tests were carried out. Identification of *Escherichia coli* O157 was performed with sorbitol MacConkey (sMAC) agar and incubation in 37°C for 24 h. Nonsorbitol fermenting and sorbitol fermenting colonies on sMAC were identified as *E. coli* using standard methods.

PCR for detection of *E.Coli* O157:H7

DNA extraction

For each sample, DNA was extracted from enrichment broths before or after an 8-0.22 µm filtration step. One milliliter of the 20-24 h-sample broth was centrifuged at 12000 g for 3 min. The pellet was washed twice with PBS with Tween 20 (2%) and three times in PBS alone. After centrifugation, 100 µl or 400 µl of InstaGene Matrix (Bio-Rad, Hercules, USA) were added to the pellet from filtered broths or not, respectively. The mixture was incubated at 56°C for 30 min, vortexed for 15s and then incubated at 100°C for 15 min. After incubation, these suspensions were vortexed for 15s, centrifuged at 12 000 g for 10 min and the supernatant was conserved at –80°C for PCR analysis.

Primers

The oligonucleotide primers that specifically amplified 259bp fragments (for O157) and 625-bp fragments (for O157:H7) were used for

PCR amplification, based on method of Gannon *et al* (1997) ; Paton and Paton (1998).Target primers for amplifying segments of genes are listed in Table 1.

PCR amplification test

The amplification reactions were performed in 50 µl reaction mixtures containing 0.1 mM of each dexoynucteotide, 15 pmol of each primer, 50 mM KCl, 10 mM Tris-Hcl (pH = 9), 2 mM MgCl2,10%dimethyl sulfoxide (DMSO, Sigma), 1.5 U of Taq DNA polymerase (Sigma) and 40 ng of template DNA. The PCR reaction was carried out in a PCR programmed thermocycler (Eppendrof, Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany Co.) using the thermal profiles: initial cycle 94°C for 5 min, followed by a further 35 cycles: denaturation at 94°C for 60s; annealing at 58°C for 60s , and extension by polymerase at 72°C for 60 s. a final extension step of 5 min at 72°C was performed. The negative control (no template DNA) was distilled water. The PCR products were detected by electrophoresis of 20 µL of each amplification mixture in 2% agarose gel in 1% Tris-acetate-EDTA buffer, after which the gel was stained with ethidium bromide (0.5µg. ml^{-1}).

Statistical analysis

Data were analyzed to determine if there was a relationship between type of specimen and presence of *E. coli* and *E. coli* O157.

RESULTS

In the present study 190 samples of fish and shellfish were evaluated for detection o *E.coli* O157H:7 .Contamination rates of *E.coli* and *E.coli* O157 in the evaluated specimens are shown in table 2. Our results indicated that 49 samples (25.78%) and 2 samples (4.08%) contaminated to *E.coli* and *E.coli* O157, respectively. From bacteriological, biochemical and molecular tests that were performed on the all samples, two cases were identified as *E.coli* O157. Out of 190 samples no

Table 1. Oligonucleotide primers sequences used for PCR amplification

Gene	Primer	Oligonucleotide sequence (5-3)	Fragment size	Reference
fliCh7	H7-F	GCGCCTGTCGAGTTCTATCGAGC	625	Gannon et al (1997)
	H7-R	CAACGGTGACTTTATCGCCATTCC		
O157	*rfb O157* – F	CGGACATCCATGTGATATGG	259	Paton and Paton (1998)
	rfb O157 - R	TTGCCTATGTACAGCTAATCC		

Table 2. Occurrence of *E. coli* and *E. coli* O157 in the different specimens

Sample type	Sample number	*E coli*		*E coli* O157
		Contaminated	Non contaminated	
Fish	90	31 (34.44%)	57 (63.33%)	2
Lobster	40	7(17.50%)	33(82.50%)	-
Shrimp	30	8(26.66%)	22(73.33%)	-
Crab	30	3(10.00%)	27(90.00%)	-
Total	190	49(25.78%)	139(74.21%)	2(4.08%)

Table 3. Distribution of *E.coli* in the different seasons

Season	Sample				
	Fish(90)	Lobster(40)	Shrimp(30)	Crab(30)	Total(190)
Spring	20(22.22%)	4(10.00%)	5(16.66%)	1(3.33%)	30(15.78%)
Summer	11(12.22%)	3(7.50%)	3(10.00%)	2(6.66%)	19(10.00%)
Total	31(34.44%)	7(17.50%)	8(26.66%)	3(10.00%)	49(25.78%)

E.coli O157:H7 were detected. The highest and lowest contamination of *E.coli* were seen in fish and crab with 34.44 and 10.00 percent, respectively. The contamination rates of *E.coli* in the different seasons were shown in table 3. There were the highest and lowest contamination rates in spring and summer with 15.78 and 10.00 percent, respectively.

DISCUSSION

Fishery products, which are of great importance for human nutrition worldwide and provide clear health benefits can act as a source of food borne pathogens and may be a potential source of infection (Kromhout *et al*.,1985). Bacterial flora of marine fish, sediments and sea water has been studied world over with a view to explain the spoilage of fish (Yagoub,2009). Among all samples taken from Isfahan markets, 49(27.2%) was contaminated to *E.coli* which is important because of role of its pathogenesis in human. But in the positive samples of *E.coli* only two cases were detected as *E.coli* O157. With considering to table -2 the highest prevalence of *E.coli* with 31(34.4 %) were seen in fish and the lowest in crab with 3(10%). It should be noted that because of low catch rates of the samples especially lobster and crab, the sample size in this study wasn't the same. In the present study *E.Coli* O157 wasn't isolated from crab , but Matulkova *et al* (2012) reported crab meat as a new possible vehicle of *E.Coli* O157 infection. Guyon *et al* (2000) from 150 oysters that tested for faecal coliform bacteria, *Salmonella, E. coli* and *E. coli* O157, recovered one strain of *E. coli* O157 from one sample, which in this strain's *stx* genes were detected. Fish and shellfish can function as carriers of several microbial and other health hazards. Therefore maintenance of quality is of utmost importance in production and trade of fishery products. The first isolation of Shiga toxin-producing *E. coli* (STEC) stx1d strains in shellfish were collected from French coastal environments (Gourmelon *et al*.,2006). This study showed that raw fish and shellfish sold in fish market in Isfahan state could be a source of food- borne bacterial pathogens. Improvements in handling and processing are needed to minimize the prevalence of the pathogenic bacteria. Presence of *E.coli* in these seafood strongly suggests the urgent need to improve the quality control systems. The results may be considered as additional knowledge to enhance proper controlling of the storage life of fish and shellfish, and its products. The potential of seafood to harbor microbial pathogens and causing subsequent illness is well documented for both developed and developing countries (Wright *et al*.,2004). *E.coli* levels correlated very strongly with fecal coliform levels in both fresh and stored oysters and clams, suggesting that there is no advantage in replacing fecal coliforms with E. coli as an indicator of shellfish quality. In comparison between different food borne pathogens include *Vibrio cholerae, Salmonella* , Ecoli and *Listeria monocytogenes* , Significant difference ($p < 0.05$) was observed in *E. coli* of the shrimps collected from different seafood processing plants at different months (Antony *et al*.,2012).

Prevalence of *E.Coli* during the summer is lower than spring (table 3). These results are different from other studies, but they can vary depending on the season. This is agree with Iyer and Joseph (1995), they have also stated that seasons play a role in controlling the bacterial quality of fresh shrimps and observed that the bacterial counts were higher in certain specific seasons. They have recorded a high incidence of *E. coli* in raw shrimps during rainy seasons, which is probably because of the high degree of fecal pollution of water during that period.

Culture of shrimp is one of the fastest growing industries in Iran in the recent years, and the majority of water resources in southern region of Iran have been allocated to shrimp farming. Therefore we should pay much attention to its health management. Immediately after the procurement, there is a significant amount of data on the microbiology of sea food produced or imported in different countries, Therefore maintenance of quality is of utmost importance in production and trade of fishery products. Most of current quality control techniques are time consuming and cumbersome (Yagoub,2009). Both bacteria and fungi are common flora of frozen fish and fish related products during packaging. The frozen fish samples were heavily contaminated which may be as a result of poor sanitary practices employed by the vendors (Adebayo *et al*.,2012). Recovery of typical E. coli O157:H7 was done from fish or shellfish in India, it was proved that strict

adherence to hygienic handling methods and proper cooking or processing is needed before consumption of these products. Human infections caused by pathogens transmitted from fish are quite common. The isolation of enteric pathogenic bacteria from fish that might be transmitted to humans after the handling or consumption of fish was studied in Nile tilapia and 39.5% were *Shigella* sp.; 11.1% were *Salmonella typhi*; 25.4% were *Escherichia coli*. Ten fishes collected from open-air markets revealed *E. coli* (50%) and *S. typhi* (20%) (Onyango *et al*.,2009). The multiplex PCR was compared for simultaneous detection of E.coliO157:H7 *Salmonella* spp and *Listeria monocytogenes* in some seafood from enrichment cultures of various types of artificially inoculated and naturally contaminated foods. This assay was a valuable method for simultaneous rapid screening for the three pathogens in food, even after frozen storage. Although only a few infectious agents in fish are able to infect humans, some exceptions exist that may result in fatalities. However, the greatest risk to human health is due to the consumption of raw or insufficiently processed fish and fish products (Yagoub ,2009)

Results of the present study showed that marine aquatic animal, including fish and shellfish can carry bacteria which are dangerous for human health. According to the molecular detection of E. coli O157 by PCR in *E.coli* isolates, paying much attention to manufacturing, distribution and packaging of these raw seafood is of great importance.

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