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RESEARCH ARTICLE

Detection of *tdh* and *trh* genes from Vibrio parahaemolyticus Isolated from Gastroenteritis Cases and Shrimp Samples

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The present research was interested with the extraction of DNA from 30 *Vibrio parahaemolyticus*strains which isolated from gastroenteritis and 25 from shrimp samples by using the boiling method after confirmation of its identification by *toxR* based PCR, .then polymerase chain reaction was performed to screen on the two genes *tdh and trh* which encodes for TDH and TRH toxins by using specific primers and amplification program. The results showed that 50% and 66.6% of*V.parahaemolyticus* isolated from gastroenteritis have the *tdh and trh* genes respectively, while those isolated from fresh and frozen shrimp samples have the two genes with lower percentages 12%,40% and 4%,12% also respectively.

INTRODUCTION

*Vibrio parahaemolyticus*is a gram-negative halophilic bacterium distributed in temperate and tropical coastal waters throughout the world (Prescott *et al.,* 2012) , It is a food borne-pathogen and a major cause of gastroenteritis particularly from traditional consumption of raw or uncooked seafood, however, not all the environmental strains are considered pathogenic , the clinical isolates of *V. parahaemolyticus*most often produce either the thermostable direct hemolysin (TDH) or TDH related hemolysin (TRH) encoded by *tdh*and *trh*genes respectively (Zhang and Austin, 2005),therefore, for disease prevention, after isolation of *V. parahaemolyticus*from seafood , the isolated strains were characterized to fully evaluate the risk posed by the implicated food (Cheng *et al*., 2008).

*V. parahaemolyticus*is is a major cause of food-borne illness in the world, In Asia, approximately half of the food poisoning outbreaks in Taiwan, Japan, and several Southeast Asian countries are due to this bacterium (Tepliski*et al*., 2009). Raw or undercooked fish and seafoods have been implicated as a major vehicles of *V. parahaemolyticus* infection to humans (Hui-Min.,2014). The presence of thermostable direct hemolysin (TDH) is a proven virulence factor which can cause gastroenteritis, a proposed virulence factor, the TDH-related hemolysin (TRH) encoded by *trh*gene, has been discovered in clinical stains of *V. parahaemolyticus*lacking *tdh*(Gomathie et al.,2013).

European Commission concluded that the practice of judging seafood exclusively based on the total *V.parahemolyticus*counts without consideration of the virulent factors such as *tdh*and *trh*is not appropriate and the total counts are not indicative enough for the presence of pathogenic Vibriosas non pathogenicVibriosthat can be present in seafood or environmental waters, therefore the presence of hemolysin genes is always considered as markers of its pathogenicity (EC, 2001). More than 90% of

clinical *V. parahaemolyticus*strains possess *tdh*(DePaola*et al*., 2000; Wong *et al*., 2000b). In contrast, the *tdh*and *trh*genes were rarely detected in the environmental strains of *V. parahaemolyticus*(Kadhim,2012).The incidence of pathogenic *V. parahaemolyticus*has been reported to be less than 1-2% among environmental strains (Sujeewa*et al*.,2009), but studies using molecular techniques indicate higher prevalence of pathogenic strains.The presence of TDH was also demonstrated on Wagatsuma agar which was referred as Kanagawa phenomenon positive (beta hemolysis) on Wagatsuma blood agar (Konema*et al.*, 2006).

It has been demonstrated that the PCR technique can detect a low number of specific bacteria against a large background of other prokaryotic and eukaryotic cells and organic materials which may present in the samples , those properties make PCR a suitable method for analyzing environmental samples (Kadhim,2012).

The objective of this study was to determine the prevalence of virulent strains of *V. parahaemolyticus*isolated from gastroenteritis patients and from fresh and frozen shrimp which was ready for human consumption in Mosul city in Iraq.

Materials and Methods :

Strains of*V. parahaemolyticus*:

*V. parahaemolyticus*strains which isolated from gastroenteritis cases and from shrimp samples in a previous study were maintained on Tryptone Soya agar (TSA) slants containing 3 % (w/v) Nacl . Biochemical tests and growth characteristics on several media were performed to confirm identity and purity of the cultures , then confirmation of the identification was doen by using *toxR* based PCR.

.**Buffers and Solutions:**

The following buffers and solutions were prepared according to(Tada *et al*.,1992).

Loading buffer, Tris EDTA, Tris-Borate-EDTA (1X), DNA Ladder(100 bp), Agarose gel (1%, 1.3%), Go Taq Green master mix, Tryptone Soy Agar with3% Nacl.

Preparation 0f primers:

The primers were obtained from New England Biolabs Inc. in Gold Oligo

Grade correlated to *V. parahaemolyticustdh*and *trh*genes with 50% GC contents The primers were prepared according to(Tada *et al*., 1992).

*tdh*gene, R: 5'- CCACTACCACTCTCATATGC -3'

*tdh*gene (F): 5' – GGTCTAAATGGCTGACATC– 3'

*trh*gene, (R): 5' GGCTCAAAATGGTTAAGCG-3'

*trh*gene (F): 5'– CATTTCCGCTCTCATATGC – 3'

Extraction of DNA:

Colonies of bacterial strains from TSA+3% Nacl were mixed with 500 µl of sterile deionized water inside eppendorf tubes. It was mixed well by using vortex mixer. The suspension was heated for 10 min in a water bath and then cooled immediately. Cell debris of these cell lysates was pelleted by centrifugation (at 13000 rpm for 2 min) and the supernatants were used as DNA templates in PCR assay.

Reaction mixture for *tdh and trh* genes:

The reaction mixture was prepared at 25 volume master mix as follows:

12 .5 µ	Go taq green Master mix
1 µ	Fwdtdh F or Fwdtrh F
1 µ	Rev tdh R or Rev trh R
4 µ	Template
6 .5 µ	Free nuclease water

PCR-Protocol:

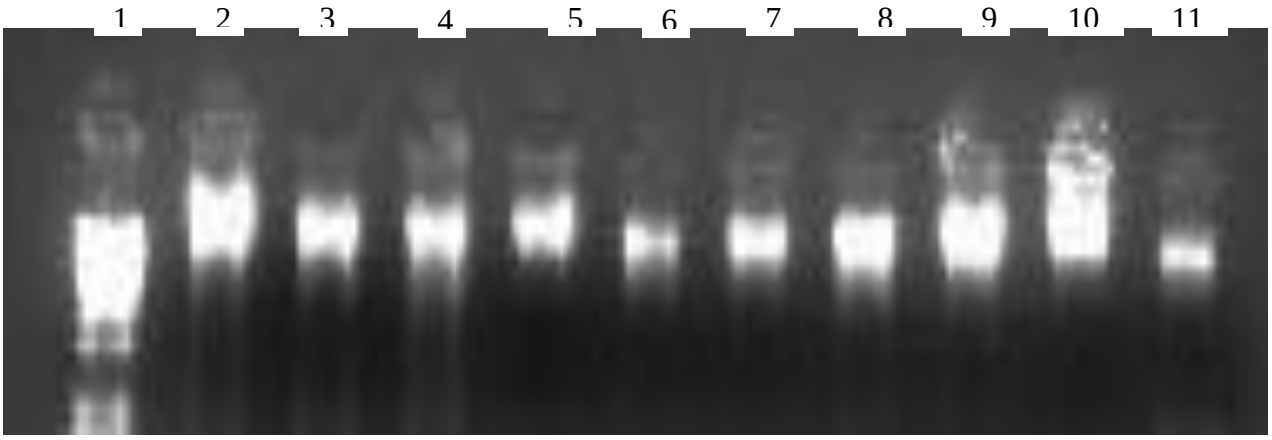
PCR was carried out using 0.5 ml microfuge tubes. The cycling conditions were as follows: predenaturation at 96°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1min, with a final extension at 72°C for 7 min at the end of 35 cycles.

Agarose gel electrophoresis:

The PCR products were run on 1.3% agarose gel (Sigma) in 1x Tris-Borate- EDTA (TBE). 10µl PCR products were loaded into sample wells and the voltage used was 100 volt for 1 h. The gel was then stained by ethidium bromide (0.5µg/ml) solution for 1 min and destained with distilled water for 30 min. Then the gel was visualized and photographed under UVtransilluminator.

Results and Dicussion :

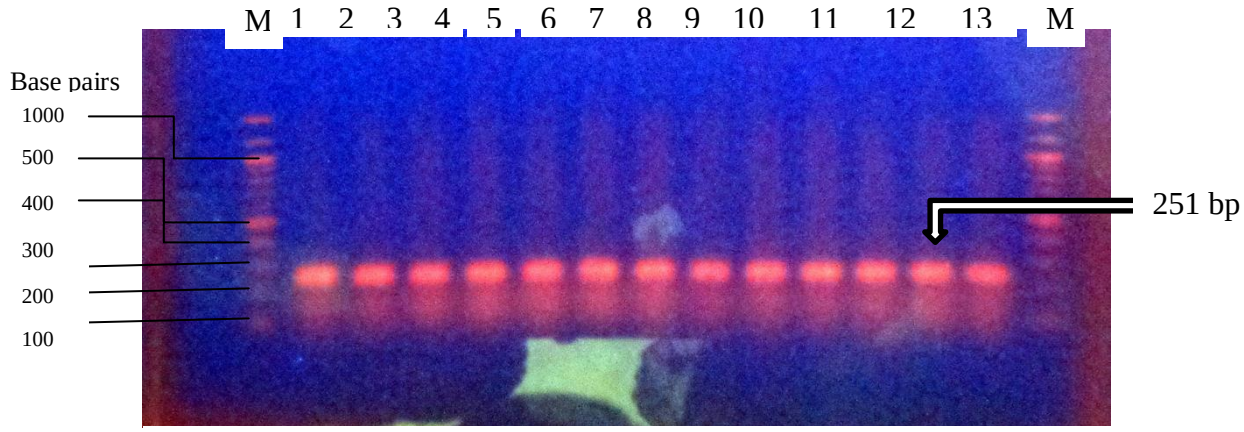
In this research the whole genome of *V. parahaemolyticus* strains isolated from gastroenteritis and shrimp samples was extracted by using boiling method, those strains were identified by routine biochemical and molecular methods. The results showed efficiency of boiling method, It was more faster than other methods used for DNA extraction such as chemical methods in addition it gave reliable results ,this result is agree with (Zulkifli*et al* ., 2009;Elamparithi&Ramanathan ,2011). Figure (1) showed the bands of genomic DNA which extracted by boiling method.



Figure(1) Bands of genomic DNA of *V.parahaemolyticus*

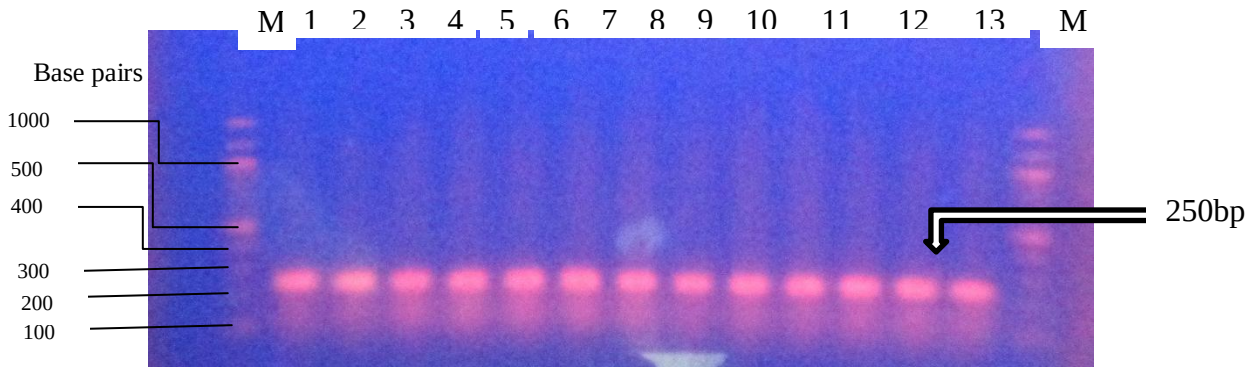
Most virulent strains of *V. parahaemolyticus*were reported to carry the *tdh*gene that encodes TDH toxin or *trh*gene that codes for the TRH toxin while some strains carry both genes (Ohnishi *et al.*, 2011).

The two genes (*tdh*and *trh*) are only present in virulent strains but not in non-virulent *V. parahaemolyticus*. It is not worthy to mention that *tdh*and *trh*like genes have been found in some strains of other *Vibrio* species such as *V. mimicus*,*V. cholera* and *V. hollisae*(Nishibuchi and Kaper,1995). Thus their presence may explain the high level of pathogenic *V. parahaemolyticus*than actual .For this reason in our study *tdh*and *trh*genes were detected only in the isolates that have previously identified as *V. parahaemolyticus*by detecting *tox*R. The polymerase chain reaction was performed to screen or detect the two virulence genes *tdh and trh* by using the primers and amplification program indicated by (Tada *et al*.,1992),All 55 strains which gave positive results for toxR were examined for *tdh*and *trh*genes. Figures (2) and (3) showed the specific *tdh* and *trh* bands genes targeting chromosomal locus at 251 and 250 bp respectively.



M: 100-bp molecular size marker
Lanes 1-2: isolates from the frozen product
Lanes 3-5: isolates from thefreshshrimp
Lanes 6-13: isolates from gasteroenteritis cases

Figure 2. . The product of amplification of *tdh-ge*ne



M: 100-bp molecular size marker
Lanes 1-2: isolates from the frozen product
Lanes 3-5: isolates from the fresh shrimp
Lanes 6-13: isolates from gasteroenteritis cases

Figure 3. The product of amplification of *trh-ge*ne

The results showed that 50% and 66.6% of *V.parahaemolyticus* isolated from gastroenteritis have the *tdh and trh* genes respectively and these results were in agreement with (Shirai*et al* .,1990; Kaysner*et al.*,1994) who reported that the *tdh* gene was present in 52.3% and 50% of *V. parahaemolyticus* isolated form gastroenteritis respectively, while our results disagree with(Lozano-Lwon*et al.*, 2003; Martinez-Urtaza*et al* .,2004) who reported that the *tdh* gene present in 100% of *V.parahaemolyticus* isolated form gastroenteritis .In respect to the presence of *trh* gene in those strains., our results disagree with (Shirai*et al* .,1990; Martinez-Urtaza*et al* .,2004) who reported that the *trh* gene was present in a percent of 34.3% and 10% respectively. Many studies refered that more than 90% of clinical *V. parahaemolyticus*strains possess *tdh*(Kaysner*et al*., 1990; DePaola*et al*., 2000; Wong *et al*., 2000b).

Our results also revealed that low percentages of shrimp isolates of *V. parahaemolyticus*werecontained *tdh*(12%) and this result was agreed with other studies (Hara-Kudo*et al* .,2003; Rojas *et al* ., 2011) who reported that the *tdh* gene was present in 12% and 10.5% respectively, while was disagree with other studies (Pal &Das ,2010 ; Fuenzalida*et al.,* 2007) who revealed that this gene found in 30% and 50% of total isolates respectively.

Mohammad *et al*. (2005) reported the prevalence of virulent genes in shrimp Malaysia culture environment were 8%, 11% and 17% of frozen shrimp, raw shrimp and pond water respectively. Many reports also showed an increase of the environmental strains carrying the *tdh*and/or *trh*genes (Wong *et al*., 1993; Hervio-Heath *et al*., 2002). DePaola*et al*.(2003) also reported that 12.8% of Alabama oysters were positive for *tdh*positive *V. parahaemolyticus*. It has also been reported that 1-5% of environmental *Vibrio* isolates possess the *tdh*or the *trh*gene (Hervio-Heath *et al*., 2002). The presence of *V. parahaemolyticus*carrying *tdh*and/or *trh*pathogenicity genes in seafood has been considered as a public health risk (Zarei*et al*., 2012).

Detection of *trh*and *tdh*genes are important to study the distribution of pathogenic strains especially in seafood. Most of the seafood from tropical region especially Southeast Asia is known to have high risk of the *V. parahaemolyticus*presence with percentages between 20 - 70% (Wong *et al.*, 2000a). The hot marine water is a major contribution factor to the occurrence of high percentage *V. parahaemolyticus*in the seafood samples. Some researchers reported that the number of total *V. parahaemolyticus*cells did not reflect that of pathogenic strain in seafood. They mostly detected *tdh*positive *V. parahaemolyticus*when the levels of total *V. parahaemolyticus*wereless than the hazardous limit of 10^4 bacteria per gram indicated by USFDA (Raghunath*et al.*, 2008). Thereby, occurrence of pathogenic strain did not correlate with the higher number of total *V. parahaemolyticus*, this suggests that seafood with total *V. parahaemolyticus*count less than hazardous limit are also not completely safe and total count is not a reliable indicator for pathogenic *V. parahaemolyticus*.

Although *V. parahaemolyticus*is widely distributed in the environments and seafood all over the world and most of them are not pathogenic to humans, consumers still need to increase their awareness and ensure that their seafood was cooked properly. The prevention of infection depends on handling of raw seafoods and preparation of finished foods in order to reduce or eliminate foodborne hazards.

The present research considered the first locally study performed in Mosul city/Iraq in order to determine the pathogenic genes *tdh*and *trh*of*V. parahaemolyticus*strains isolated from gastroenteritis patients and from shrimp samples and this study suggest to assess the microbial quality of frozen fish fillet and seafood sold in our country to use the microbiological methods to estimate bacterial numbers, in order to determine fish freshness, hygiene and or evaluate the possible presence of bacteria or organisms of public health importance especially vibrio spp.

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