Isolation, Identification and comparative study for detection of *Vibrio parahaemolyticus* in fresh seafood from Damietta Governorate markets, Egypt.

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Abstract— Seafood is one of the most important fishery products of the coastal governorates in Egypt. In recent years, Vibriosis has been an important cause of fish production loss and human health risk. The objective of this study is to detect the prevalence and quantification of *Vibrio parahaemolyticus* (VP) in 100 (25/ species) of seafood samples; collected from coastal regions and fish markets in Damietta; by traditional bacteriological isolation methods and quantitative real time polymerase chain reaction technique (QRT-PCR) as a molecular method for rapid and sensitive detection. It was found that by using traditional bacteriological isolation methods; the incidence of *Vibrio parahaemolyticus* were 7 (28%), 1 (4%), 7 (28%), 6 (24%) in examined Shrimp , Crabs , Bivalve molluscs and brush tooth Lizard fish samples, respectively. While molecular detection system(QRT-PCR) revealed that the incidence of *Vibrio parahaemolyticus* were 20 (80%), 18(72%), 21(84%), 21(84%) in examined Shrimp, Crabs, Bivalve molluscs and brush tooth Lizard fish samples, respectively. In conclusion; these data indicate that QRT-PCR can provide sensitive species-specific detection and quantification of *V. parahaemolyticus* in seafood without prior isolation and characterization of the bacteria by traditional microbiological methods.

Key Words: QRT-PCR, Sea food, , TLH, TDH, TRH, *Vibrio parahaemolyticus.*

1 Introduction

Seafood benefits to human health are noted for multiple bodily organs and physiological functions. Seafood compares favorably with other protein sources as it offers superior macronutrients in the ideal form of lean proteins combined with omega-3, healthy type of fat, and a wide array of highly bioavailable micronutrients. According to the U.S. Food and Drug Administration (FDA), certain varieties of seafood are excellent sources of potassium, vitamin A, vitamin E and vitamin C, vitamin D, calcium. Eating seafood regularly can also potentially lower your risk for developing colon, breast or prostate cancer. Pregnant women can also benefit from the high levels of protein, zinc and iron found in seafood **[1],[2].**

The safety of seafood is increasingly becoming an important public health issue as it an important vehicle for pathogenic microorganisms to humans. This is due to the global rise in the number of people vulnerable to disease coupled with the customary tendency of the world population to consume raw or improperly cooked seafood.

Seafood is prone to contamination by spoilage and pathogenic bacteria due to the ubiquity of many of these microorganisms in marine environments. Another major source in the spread of bacteria is the cross contamination from utensils and surfaces during food processing as well as the water used during processing **[3]**. It is estimated that 76 million people fall ill and 5,000 die annually from food-borne illness, and a large proportion of these cases can be attributed to Salmonella *spp.* and *Vibrio parahaemolyticus*. For example, nearly 28% of *Vibrio* infections occurred; 23% were reported eating a single seafood item and 5% of patients handling seafood **[4],[5],[6]**. Most of the Vibrio species are pathogenic to humans; VP a marine inhabitant food-borne pathogen cause acute gastroenteritis, diarrhea primarily after the consumption of raw or undercooked seafood; usually occurs in summer (from June to October) and may be life threatening for people with weak immune disorders, although the infection is often self-limited**[7].** Because V. parahaemolyticus has the potential to transmit through seafood because its popularity as a source of healthy protein including crab, shrimp, lobster, fine fish, and oysters; it extends throughout the world **[8],[9],[10].**

The pathogenicity of Vibrio species species is due to the massive production of several virulence factors viz. enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/or haemagglutinins **[11].** Both the thermostable direct haemolysine (TDH) and thethermostable direct hemolysin related hemolysin (TRH) genes are correlated with toxigenic VP strains, however, these genes do not completely account for the pathogenicity of *V. parahaemolyticus*. Thermolabile haemolysine (TLH) gene, a species-specific marker targeted during the genetic detection of *V. parahaemolyticus*; exhibits phospholipase activity and the ability to lyse human erythrocytes. Furthermore the expression of tlh was strongly upregulated under conditions simulating the

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human host intestinal environment. Therefore, TLH is assumed to have a role in *V . parahaemolyticus* similar to TDH **[12]**.

Various methods have been developed to detect *V. parahaemolyticus*, including conventional culture based, immunology based, and molecular methods. Conventional culture based methods, conducted by selective isolation of bacteria and biochemical identification, are safe but laborious requiring numerous analytical steps, all of these methods take up to a few days to provide a confirmed result. Apart from being laborious, the sensitivity of these methods needs to be improved as interference from other bacteria in the seafood samples, especially other Vibrio *spp.* can sometimes lead to a false result **[13]**. Furthermore, the colony counting method requires a pathogen to be *culturable***,** which may not always be the case given stringent environmental or nutritional requirements. *V. parahaemolyticus* has been shown to easily enter VNBC state as a response to adversity. The VBNC cell loses the ability to divide but maintains metabolic, gene expression, antibiotic resistance, and pathogenic properties. Nevertheless, this cell can regain its normal culturable state given the right circumstances. Thus, there is a real need to develop rapid methods and strategies for *V. parahaemolyticus* monitoring **[14], [15].**

Rapid detection of food-borne pathogens is important in the food industry, to monitor and prevent the spread of these pathogens through contaminated food products. With the advance of biotechnology, a variety of analytical assays have been reported for the detection of *V. parahaemolyticus*. Hence, establishment of real-time PCR systems is necessarily required for rapid and sensitive detection *of Vibrio parahaemolyticus* to prevent food poisoning and ensure the safety of seafood consumption **[16].** Detection method RTPCR can detect a single copy of a target DNA sequence with respect to single pathogen in food. It is permissible because it is amplifies the target organism sequence use of fluorogenic probes rather than nor serotyping method to detect the presence of specific antigens So, this amplify 1-million-fold of target DNA in less than an hour, with efficiency to the target pathogen producing less false-positives eliminating detection of products that could result from non-specific amplification **[17].**

2 Material and Methods

A total of 100 fresh seafood samples included 25 each of shrimp, crab, bivalve mollusks and brush tooth lizardfish were purchased from different localities of Damietta fish markets. Each sample were packed individually in labeled clean polyethylene bag and transferred in icebox with a minimum of delay to the laboratory of Food Hygiene Department, Animal Health Research Institute (AHRI), Giza, Egypt. Where in the bacteriological and biochemical analyses were done within 24 hours.

- **Sample preparation**.

Finfish and crustaceans were washed thoroughly with sterile distilled water prior to bacteriological examination. The skin was sterilized by alcohol and flamed by sterile spatula. The heads, tails, scales guts and the muscles above the lateral line were removed. The fishes were cut into small pieces using sterile scissors.

- **Sample processing**

A total of 25 g of each 100 samples were homogenized with a conventional blender for 90 s in 225 mL Alkaline Saline Peptone Water (APW) (3%Nacl and pH 8). The pooled sample is then homogenized using a sterile high-speed blender (homogenizer) stomacher.

- **Bacterial reference strain.**

V. parahaemolyticus reference strain (NCTC 10885) supplied by (TCS Biosciences Ltd, Buckingham, United Kingdom) pure strain was obtained from the Food Microbiology Laboratory, Animal Health Research Institute, Dokki, Egypt.

2.1. Bacteriological Isolation and identification.

Isolation and presumptive confirmation test ***fig.1***. were applied according to the traditional method **ISO/TS 21872/2007[18].**

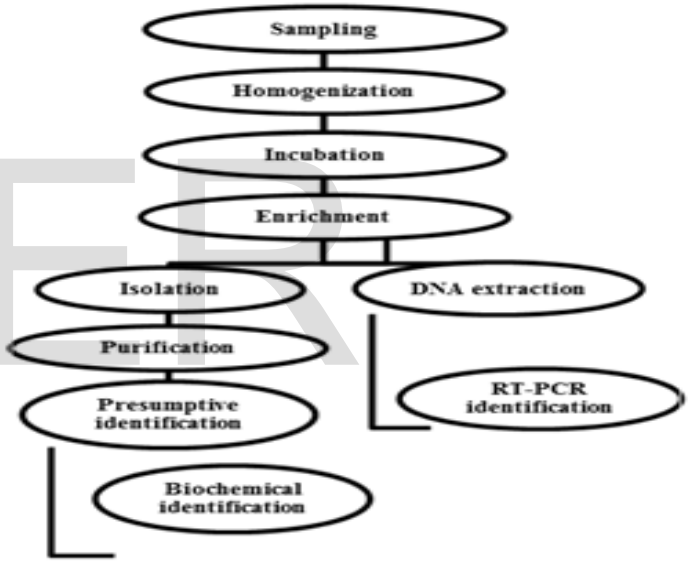

Fig.1. Flow diagrams of the ISO/TS 21872–1 method and PCR method for detection of total and toxigenic Vibrio parahaemolyticus.

2.2.Molecular identification (QRT-PCR system)

- **DNA extraction**

Total cellular DNA was isolated from all samples using QIAamp DNA extraction mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions (QIAamp DNA Extraction kit Mini Handbook, 2016).

Preparation of the QRT-PCR reagent and the DNA template.

All primers and probes were synthesized by (Biosearch Technologies, Denmark). TaqMan primers and probes used in this study for detection and quantification of total and toxigenic V. parahaemolyticus were listed in **Table 1.**

The method was carried out in a final volume of 20 µL of 10 × PCR Buffer using Sato RT q PCR 5 µL master Mix (AMDiagnostics, United Kingdom), 0.8 of 10 pmole forward primer, 0.8

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of 10 pmole reverse primer, and 0.4 µL of 5 pmole probe were used; 2 µL of DNA were used as template. *Stratagene 3005P MXpro Real-Time PCR System (Stratagene, USA)* is used.The cycling parameters were an initial DNA denaturation step at 95°C for 2 min followed by 40 cycles of PCR with DNA denaturation at 95°C for 10 sec and primer annealing at 55°C for 45 Sec.

Table 1. Target genes and oligonucleotide primers and probes used for QRT-PCR detection of total and toxigenic *V. parahaemolyticus*[19].

Target gene	Primer	Oligonucleotide sequence[a] (5′ →3′)	Length (bp)	G-C content (%)	Melting temp. (°C)	Amplicon size (bp)
TLH	F-tlh[b]	5′ AAA GCG GAT TAT GCA GAA GCA CTG 3′	24	45.8	70	450
	R-tlh[c]	5′ GCT ACT TTC TAG CAT TTT CTC TGC 3′	24	41.7	68	
	P-tlh52[d]	5′ TEXR-AAG AAC TTC ATG TTG ATG ACA CT-BHQ2 3′[e]	23	34.8	62	
TDH	F-tdh 170DG[b]	5′ GTA RAG GTC TCT GAC TTT TGG AC 3′	23	43.5	66	229
	R-tdh403[c]	5′ CTA CAG AAT YAT AGG AAT GTT GAA G 3′	25	32.0	66	
	P-tdh-341R[d]	5′ Fam-ATT TTA CGA ACA CAG CAG AAT GA-Tamra 3′[g]	23	34.8	62	
TRH	F-trh82[b]	5′ CCA TCM ATA CCT TTT CCT TCT CC 3′	23	43.5	66	207
	R-trh287[c]	5′ ACY GTC ATA TAG GCG CTT AAC 3′	21	42.9	60	
	P-trh275[d]	5′ Fam-TAT TTG TYG TTA GAA ATA CAA CAA Tamra 3′[h]	25	20.0	60	

[a] Y = C or T; M = A or C; R = G or A. [b] Forward primer. [c] Reverse primer. [d] Oligonucleotide probe. [e] TexR, sulforhodamine (Texas Red) fluorescent dye; BHQ2, Black Hole-2 quencher dye. [f] 6-FAM, 6-fluorescein fluorescent dye; [g] Cy5, carbocyanine fluorescent dye; [h] TET, tetrachloro-6-carboxyfluorescein fluorescent dye.
TAMRA, 5-Carboxytetramethylrhodamine.

- **Standard Curve**

Vibrio parahaemolyticus (NCTC 10885) was maintained on trypticase soy agar slants (containing 3% Nacl) at 37 °C. A loopful *V. parahaemolyticus* was transferred aseptically into 10 ml sterile Alkaline Peptone Water plus 3% Nacl and followed by cultivating separately at 37 °C. After incubation, *V. parahaemolyticus* was counted by using spread plate method **[20].** Then adjusted to 10^8 cfu/ml with tube dilution methods; this step will be used in serial dilution used in slandered curve establishment.

3 Results

Table 2. Incidence of *V. parahaemolyticus* based on Cultural Identification methods.

Samples screened	No. of samples	*Vibrio parahaemolyticus* occurrence	Percent/ Type
Shrimp	25	7	28%
Crabs	25	1	4%
Bivalve molluscs	25	7	28%
Lizard fish	25	6	24%
Total	100	21	21%

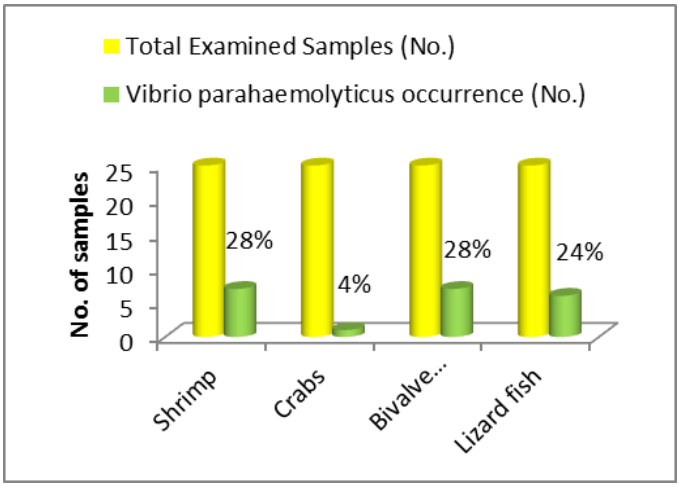

Fig. 2. Column chart representing number of positive VP samples based on Cultural Identification.

As regarded to cultural identification **fig.2.** highest incidence of VP is isolated from 7 (28%) of shrimp samples equally with bivalve molluscs samples followed by bivalve molluscs with incidence 6 (24%), the lowest incidence takes place in crabs with incidence 1(4%); with an overall incidence of 21% (21/100) of the all examined shellfish samples.

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Table 3. Incidence of presumptive *V. parahaemolyticus* isolates based on biochemical confirmation.

Seafood	Colonial morphology Isolation (No.)	Biochemical confirmation	
		(No.)	%
Shrimp	26	11	42.30%
Crabs	30	1	3.33%
Bivalve molluscs	47	12	25.53%
Lizard fish	18	10	55.55%
Total	121	34	28.09%

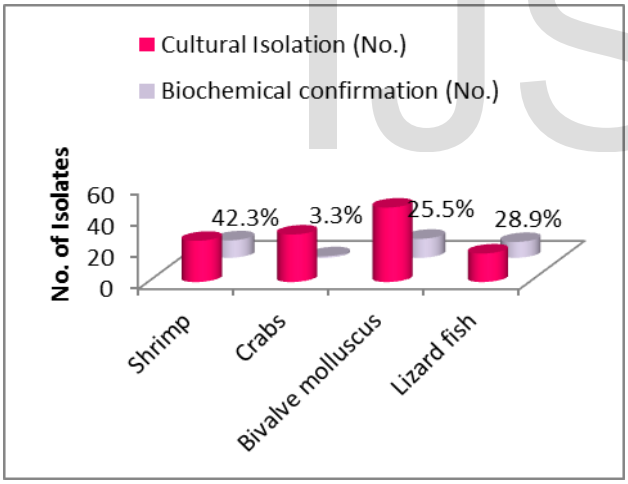

Fig. 3. Column chart representing number of confirmed isolates based on biochemical identification vs colonial morphology isolation.

This column chart revealed that only 34 (28.09%) out of the 121 isolates; of colonial morphology (green or bluish green colonies cultivated on TCBS agar) and microscopic examination (Gram-negative, curved rod shape, non-spore forming bacteria); are biochemically identified, distributed as 11(42.30%), 1(3.33%), 12(25.53 %) and 10 (55.55%) from the examined shrimp, crab and bivalve molluscs and lizard fish samples, respectively.

Table 4. Incidence of presumptive *V. parahaemolyticus* isolates based on bacteriological versus molecular identification.

Samples screened	No of samples	Incidence of Positive bacteriological VP isolates confirmed by biochemical tests		Incidence of Positive VP isolates confirmed by molecular assay (RT-PCR)	
		No.	%	No.	%
Shrimp	25	7	28%	20	80%
Crabs	25	1	4%	18	72%
Bivalve molluscs	25	7	28%	21	84%
Lizard fish	25	6	24%	21	84%
Total	100	21	21%	80	80%

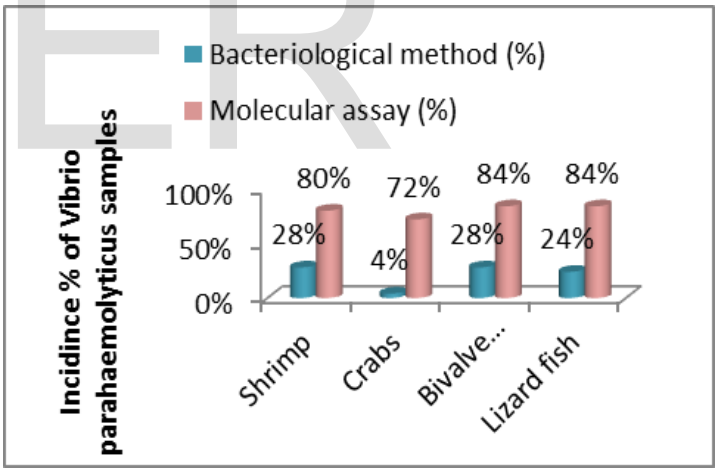

Fig. 4. Incidence (%) of *V. parahaemolyticus* in the examined shellfish samples based on Bacteriological and Molecular identification.

In the present study, bacteriological method based on cultural identification of presumptive Vp is 21%(21/100). However, molecularly identified samples by QRTPCR found to be positive for VP in 20(80%), 18(72%), 21(84%) , 21(84%) in examined Shrimp, Crabs, Bivalve molluscs and Lizard fish samples, respectively. With overall incidence of 80% (80/100) of the all 100 examined shellfish samples.

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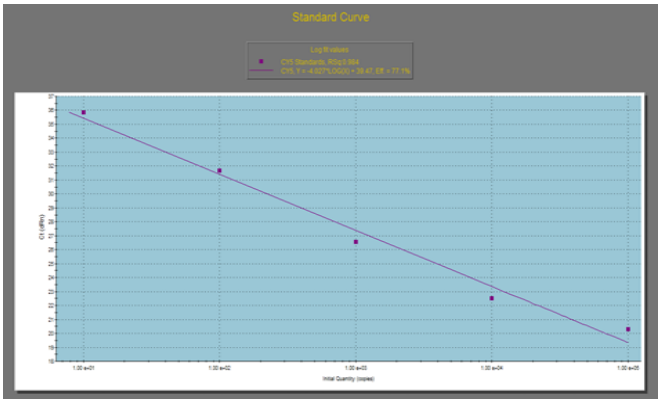

Fig. 5. Standard curve.

Thermolabile haemolysin (*tlh*) gene was treated with series of 10-fold bacteria dilutions (10^2–10^8 CFU/g) were different concentrations of *Vibrio parahaemolyticus* reference strain (NCTC 10885) are used for future standard curve. The constructed standard curves of tlh is shown in **Fig.5**. Results showed that R2-value was 0.984 for tlh and the slope of the standard curve is −4.027.

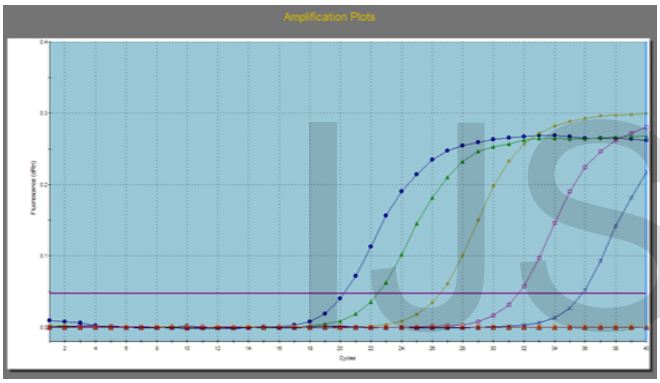

Fig. 6. Amplification plot and cycle threshold (Ct) of standard curve. Showes Ct values range from 20.2 to 36.

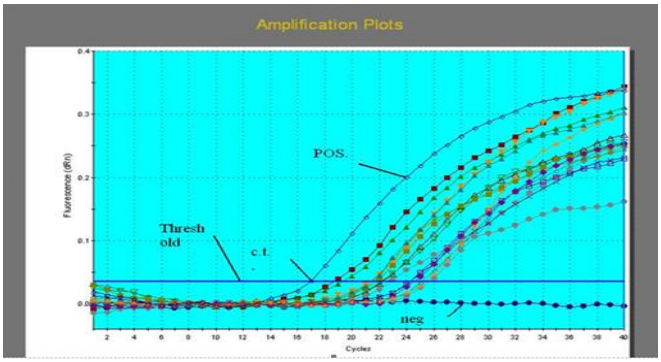

Fig. 7. Results of QRT-PCR for detection of TLH gene in some seafood examined samples.

This figure showing the Amplification Plot with Ct values of some seafood samples; QRT-PCT reagent with Ct values from 19.28 to 37.99; where Ct value >36 considered negative for TLH gene.

There was no expression found for both TDH and TRH genes.

4 Discussion

Vibrio species are Gram-negative, facultative anaerobic, non-spore-forming bacilli which are oxidase positive and as halophilic bacteria widely spread in the sea and brackish water environments worldwide. To ensure its survival in varying environments, *V. parahaemolyticus* has two different types of flagella systems, allowing it to adapt to constantly changing environments. The polar flagellum is responsible for swimming whereas the lateral flagella are closely associated with this warmer cell type transformation and biofilm formation. During infection, V. parahaemolyticus uses the adhesion factors to bind to the fibronectin and phosphatidic acid on the host cell, thus releasing different effectors and toxins into the cytoplasm, causing cytotoxicity and serious diseases **[21].**

To date, there is no information regarding the occurrence of non-pathogenic and pathogenic strains of V. parahaemolyticus in raw fresh seafood from the coasts of Damietta. Egypt; despite the Damietta coasts being recognized as not only one of the most attractive recreation destinations but also a large commercial site, harbor for seafood cultivation and have the most important estuarine location in this region of Egypt.

The increase in seafood consumption and the global warming, resulting in increased ocean surface temperatures, may cause higher prevalence of Vibrio spp., and enhance the risk of Vibrio foodborne infections Fish and fish products are one of the sources of pathogenic bacteria infection in human that could be transmitted to human during processing under poor hygienic conditions **[22].**

Vibrio parahaemolyticus is widely distributed in marine environment and has been recognized as a major cause of foodborne illness associated with the consumption of raw, undercooked or contaminated shellfish. It can cause mild to moderate gastrointestinal infections, which are usually self limiting and rarely fatal **[23].**

The first outbreak of seafood borne disease due to consumption of V. parahaemolyticus contaminated sardine was reported in Japan in 1950. In this outbreak, 20 people were reported dead while over 270 people were likewise hospitalized. More outbreaks involving consumption of contaminated raw or undercooked seafood like oyster has been reported in United States **[24], [25], [26]**. Taiwan **[27], [28]** China **[29]**, Italy **[30]**, Chile **[31]**, Peru **[32]**, Spain **[33]** and Canda **[34].**

Seafood considered in this study are bivalve molluscs (oysters, clams, and mussels), finfish (brushtooth lizard fish) and crustaceans (shrimp and crab). These bivalve molluscs, finfish and crustaceans have a concentration of V. *parahaemolyticus* up to 100-fold higher than surrounding waters due to filter feeding particularly in the summer season which increases the chances of infection and so of a potential public health hazard **[22].**

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particularly in the summer season which increases the chances of infection and so of a potential public health hazard **[22]**.

In the present study, conventional cultural method based on the colonial appearance (green or bluish green colonies cultivated on Thiosulfate citrate bile and sucrose agar **(**TCBS agar) and microscopic examination (Gram-negative, curved rod shape, non-spore forming bacteria) and biochemical identification could detect presumptive *V. parahaemolyticus* in 21 (100%) out of the 100 shellfish and fish samples examined. These 21 samples consisted of 7 (28%),1 (4%), 7 (28%) and 6 (24%) shrimp, crab , bivalve molluscs samples, respectively as declared in **table 2.**
A lower overall (1.71%) incidence of VP takes place in Shanghai **[35].** Another study undergoes in shrimp and crabs in Egypt; the prevalence of VP in the examined shrimp and crab samples was found to be lower in shrimp 20% and higher in crabs 30%, respectively **[36].**The VP incidence in shrimp findings of the present study were in approximation to 22.5 percent reported by **[37]** in Egypt. However, lower incidence of 5.5%; 1.8 % and 5% was recorded by **[38], [39], [40]** respectively. In contrast to the findings of present work, earlier studies conducted by **[41], [42]** reported higher incidence of 80.80% and 83.40 %, respectively.
By contrast, very few data are available for crustaceans and fish, despite the popularity of crabs and shrimps and their rising consumption worldwide. However, in contrast to our prevalence of 4% in crabs, a higher VP incidence of 100% reported by **[43]** in England. Another studies reported a higher VP incidence of 93.3%, 40% in Vietnam and China **[44], [45]** respectively.
Compared to other studies undergoes in bivalve molluscus, based on bacteriological method; the highest VP incidence of 100% in Brazil reports by **[46].** Where a study in United States reported incidence of 77% **[47].**Another studies takes place in Italy was in approximation to our finding by VP incidence 24% **[48].** A lower incidence takes place in Tunisia with VP incidence of 10% **[11].**
However, in contrast to our prevalence of 24% in brush tooth Lizard fish **[44] ,[49]** recorded higher levels in finfish (100%; 66.6%;); China, Malaysia,. Nearly similar results were detected in India by **[50]** where VP occurrence was 22.2%, the occurrence of VP was 29.7%. A lower VP incidence of 13.3% takes place in Japan **[51].**

A total of 121 results presumptive *V. parahaemolyticus* colonies were selected based on their colonial morphology from the positive plates of the examined shellfish samples the achieved in **table 3**, revealed that only 34 (28.09%) out of the 121 biochemically identified isolates, distributed as 11 (42.30%), 1 (3.33%), 12 (25.53%) and 10 (18%) from Shrimp, Crab, Bivalve mulluscus and Lizard fish samples, respectively were confirmed biochemically as *V. parahaemolyticus*. Not all the selected 121 isolates recovered by colonial identification were confirmed by biochemical methods to be VP, because some green or blue-green colonies on selective media were not *V. parahaemolyticus* . Higher results obtained by another study; where total biochemically confirmed isolates were 89 (62.24%), distributed as 32 (36%), 35 (39.3%), and 22 (24.7%) from shrimp, crab and cockle samples, respectively **[37]**. Also a study takes place in Turkey reported a higher result; as from total of 47 VP presumptive bluish-green colonies were isolated on TCBS; biochemical tests revealed that 32 of 47 isolates (68%) were identified as *V. parahaemolyticus*; distributed as 8 isolates (25%) salted and fresh fish, and 24 isolates (75%) bivalve molluscs **[52].** The results highlight the added value of using a chromogenic medium for research and isolation of pathogenic Vibrio in seafood, more specific and accurate than TCBS **[53]**.
In this study, molecular identification is established versus bacteriological method; molecularly identified samples by QRTPCR found to be positive for VP in 20(80%), 18(72%), 21(84%), 21(84%) in examined Shrimp, Crabs, Bivalve molluscs and Lizard fish samples, respectively. With overall incidence of 80% (80/100) of the all 100 examined shellfish samples. However overall incidence of VP using bacteriological method is 21(21%) **table 4.**Using QRTPCR; a study in France; reported a slightly higher result of ours with overall incidence of 89.3%; distributed as crustaceans (shrimp and crabs) 79.3%; shellfish (bivalve molluscs) 1.7%. and fish 8.7% **[54].**

In comparing result of both used isolation methods **table 4.;** results is (21%, 80%) using bacteriological, molecular identification; respectively. Our findings are in close agreement with those reported by a study undergone in raw shrimp and crab that reported the occurrence of VP in the examined sea food (20%, 70%) bacteriological, molecular (m-PCR) identification; respectively **[36].** Our study is higher than a study in Netherland where the detection of VP in (8%, 19%) of sea food samples using bacteriological, molecular (PCR) identification; respectively **[55].** Another lower result revealed that VP isolation was (19%, 30%) using bacteriological, molecular (PCR) identification; respectively **[56].**
Furthermore, as observed in this study; molecular method has the ability in detecting more *V. parahaemolyticus* contaminated samples that were negative using the cultural method **[57].** This method allows also the detection of species which may enter in a viable but non-culturable (VBNC) state in response to adverse environmental conditions. VBNC cells no longer grow on conventional media, but may retain their pathogenicity **[58].**

Molecular techniques are also very useful for the identification of the genes associated with species virulence. No gene encoding VP toxin was detected by QRTPCR. These results are consistent with a 2 previous studies carried on seafood samples, oysters, mussels and prawns, collected randomly from markets in Casablanca, Netherland and Morocco **[59], [55],[53]**. A study in Egypt found a very low incidence of toxigenic VP 3 strains; one of these 3 strains that derived from shrimp was positive for both tdh and trh. The second strain (derived from bivalve molluscus) was positive for tdh only, while the third one (derived from shrimp) was positive for trh only **[37].** A higher incidence of virulence genes (tdh and trh sequences) were present in 25% of the *V. parahaemolyticus* positive samples **[54].**

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5 Conclusion

The application of QRTPCR assay allows for a rapid detection of V. parahaemolyticus (took only 3 hours) compared to cultural method and biochemical tests which took 7 days before the results were obtained. It is found that direct PCR method was more sensitive, compared to cultural method

-Although the PCR-based method following the two-stage enrichment was found to be the most rapid, reliable and sensitive method for detection of V. parahaemolyticus, for further examination it is required to keep and to store the bacterial specimens, which can only be obtained by a culture method. Hence, these two methods of detection and identification are complementary to each other, in detecting *V. parahaemolyticus.*

The improvement of the detection methods is required as well; the use of the advanced molecular techniques (RT-PCR), ensures not only a better specify than classical bacteriological techniques for the determination of species, but also allows to focus on the genus of pathogen bacteria.

6 Declaration of interest

Authors declare that there is no conflict of Interest.

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