

## Virulence genes and antibiotic resistance profile of *Vibrio* species isolated from fish in Egypt

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### Abstract

*Vibrio* species are significant pathogens affecting aquatic species. Around 12 species of *Vibrio* can cause a gastrointestinal illness (gastroenteritis) in humans resulting from eating contaminated food such as raw or undercooked shellfish. The indiscriminate use of antibiotics accelerates the development of resistance representing a severe challenge for controlling *Vibrio* outbreaks. In this study, the antibiotic resistance profile and the prevalence of pathogenic *Vibrio* species of apparently healthy and diseased fishes isolated from different types of fish in Kafr EL-Sheikh Governorate in Egypt during 2018 were determined. Samples obtained from fishes were inoculated onto a *Vibrio*-selective medium (TCBS) and phenotypically identified using the biochemical characteristics and representative cultures were checked by PCR to confirm the identified isolates. In the present study, *V. alginolyticus* (16.00%) was the predominant species followed by *V. cholerae* (7.33%) and *V. parahaemolyticus* (5.33%). The tested isolates were resistant to ampicillin (80.00%) and sensitive to ciprofloxacin and norfloxacin (100%). A total number of 15 *Vibrio* isolates (five *Vibrio parahaemolyticus*, five *V. alginolyticus*, and five *V. cholerae*) were screened for five housekeeping genes and pathogenic virulence markers by PCR. Results showed that 100% of the *V. parahaemolyticus* isolates carried the *tlh* gene and 60.00% carried the *tdh* gene. In *V. alginolyticus*, 100% of the isolates carried the *collagenase* gene 0.00% carry the *tdh* gene; and 80.00% of *V. cholerae* isolates carried the *ctx* gene. The results showed that many isolates in this study had virulence characteristics that might correspond with the potential of infections and diseases.

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### Introduction

Vibriosis is a globally threatening bacterial disease affecting mariculture with high mortalities and severe economic losses.<sup>1</sup> Many species of *Vibrio* are considered pathogens to humans and are known as foodborne disease.<sup>2</sup> Vibriosis is among the most severe infectious diseases affecting fishes, bivalves; and crustaceans. It commonly occurs in salt and brackish water fishes causing a huge economic loss in the mariculture industry.<sup>3</sup> The predominant pathogenic species associated with saltwater are *V. anguillarum*, *V. parahaemolyticus*, and *V. vulnificus*, and in freshwater are *V. mimicus* and *V. cholerae*.<sup>4</sup> *Vibrios* have a highly plastic genome and can transmit the virulence genes horizontally among environmental and

pathogenic *Vibrio* which raises the number of pathogenic *Vibrio* strains in the aquatic environment.<sup>5</sup> A wide range of products including hemolysins, proteases, biofilm formation, phospholipases, cytotoxins, quorum sensing, siderophores and the presence of phage share the virulence of *Vibrio* species.<sup>6-8</sup> The swarming motility of *Vibrio* is implicated in its virulence,<sup>9</sup> while, hemolysin is the most critical virulence factor reported in *Vibrio* and related to both human and fish diseases.<sup>10</sup> Previous studies showed various virulence genes such as *Outer membrane protein (OMP)*, *thermolabile hemolysin (TLH)*, *toxR*, *collagenase* and cholera toxin that contributed to the virulence of the pathogens. *Collagenase* was widely used in molecular identification of *V. alginolyticus*, capable of degrading basal epithelial membrane and; conjunctive

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tissue that leads to extra-intestinal pathology and dissemination to blood stream.<sup>11</sup> Minimum 12 *Vibrio* spp. have been recognized as pathogenic strains; that induced foodborne diseases. *V. parahaemolyticus*, along with other *Vibrio* spp. causes 25.00% of the entire foodborne diseases.<sup>12</sup> *V. para-haemolyticus* produce hemolysin as an enterotoxin that induces blood cell lysis in the infected organisms. The *tlh* gene encode to thermostable hemolysin as a specific marker to identify *V. parahaemolyticus* species.<sup>13</sup> *V. para-haemolyticus* have many virulence factors including thermostable direct hemolysin (tdh), TDH related hemolysin (trh) and adhesin. Thermostable direct hemolysin encoded by the *tdh* gene has a role among the most important virulence factors in *V. parahaemolyticus*.<sup>14</sup> The cholera pathogenesis is complex and includes several genes such as *ctx*, *zot*, *tcp*, and *rfbO1*. These genes are used as a marker of virulence, although cholera toxin is the most vital epidemic marker.<sup>15</sup> *Vibrio* species identification is based principally on their morphological and, biochemical characteristics, and are confirmed by PCR. The purpose of this research was to detect the antibiotic profile and study some virulence genes (*tdh*, *tlh*, *collagenase*, *ctx*) in *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae* isolated from fishes in Kafr EL-Sheikh Governorate in Egypt.

## Materials and Methods

**Sampling.** A total number of 150 different types of fish samples were collected alive at random samples from markets at Kafr El-Sheikh Governorate. Alive and freshly dead Fish samples were transported to the Department of Microbiology, Animal Health Research Institute in Kafr El-Sheikh Governorate. Fishes were examined clinically for any abnormalities; including hemorrhages, skin ulceration, fin erosion, and abdominal distention. The bacteriological isolation of *Vibrio* species was done from samples of kidney, heart, liver, gills, and skin according to Noga.<sup>16</sup>

**Bacterial isolation.** Under the aseptic condition samples were obtained from liver, kidney, heart, spleen, and gills taken on trypticase soya broth (Oxoid, Basingstoke, Hampshire, United Kingdom) with 3.00% NaCl (Oxoid, Basingstoke, Hampshire, United Kingdom) and put in an incubator at 30.00 °C for 18 - 24 hr then

streaked on TCBS agar (Himedia, Maharashtra, India). Colonies of yellowish and greenish color were examined after incubation at 30.00 °C for 18-24 hr; using the standard method described in the FDA Bacteriological Analytical Manual.<sup>17</sup> All fish handling was conducted under the guidelines for the care and use of animals for scientific purposes established by the Ethics Committee of the Animal Health Research Institute, Giza, Egypt (Approval No. 83429).

**Identification of bacterial strains.** For phenotypic identification pure cultures were subjected to Gram staining and viewed microscopically. Biochemical and molecular methods were done on strains to identify them at the species level. Biochemical tests like oxidase, catalase, citrate utilization, gelatin hydrolysis, TSI, lysine decarboxylase, growth in 2.00 - 10.00% NaCl, indole, Voges-Proskauer, methyl red, string test, urease, H<sub>2</sub>S and ONPG were performed for the identification as well as strain differentiation of bacteria (Table 1).<sup>18</sup> Representative cultures (10.00%) were confirmed using PCR.

**Antibiotic susceptibility testing.** Fifteen of *Vibrio* spp. isolates were tested for susceptibility to different antibiotics (Himedia) including gentamycin (10.00 µg per disk), ampicillin (10.00 µg per disk), streptomycin (10.00 µg per disk), nitrofurantoin (300 µg per disk), ciprofloxacin (5.00 µg per disk), sulpha trimethoprim (25.00 µg per disk), nalidixic acid (30.00 µg per disk), cefotaxime (30.00 µg per disk), erythromycin (15.00 µg per disk), amikacin (30.00 µg per disk) and norfloxacin (10.00 µg per disk) by disk diffusion method as previously described.<sup>19</sup>

**Genetic identification and DNA extraction.** DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) with modifications from the manufacturer's recommendations. Briefly, 200 µL of the sample suspension was incubated with 10.00 µL of proteinase K and 200 µL of lysis buffer at 56.00 °C for 10 min. After incubation, 200 µL of 100% ethanol was added to the lysate. Then, the samples were washed and centrifuged as suggested by the manufacturer. Nucleic acid eluted with 100 µL of elution buffer provided in the kit. The concentration of DNA was measured using Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA).

**Table 1.** Primers used in this study.

Target gene	Sequence	Amplified product (bp)	Reference
<i>Collagenase (V. alginolyticus)</i>	CGAGTACAGTCACTTGAAAGCC CACAAACAGAACTCGCGTTACC	737	20
<i>tlh (V. parahaemolyticus)</i>	AAAGCGGATTATGCAGAAGCACTG GCTACTTTCTAGCATTTCCTCTGC	449	21
<i>ompW (V. cholera)</i>	CAC CAA GAA GGT GAC TTT ATT GTG GGT TTG TCG AAT TAG CTT CAC C	304	15
<i>ctx (V. cholera)</i>	ATTTGTTAGGCACGATGATG ATCGATGATCTTGGAGCATTTC	432	22
<i>tdh (V. parahaemolyticus, V. alginolyticus)</i>	CCATCTGTCCCTTTTCCTGTC CCAAATACATTTTACTTGG	373	23

**Target genes.** The *V. parahaemolyticus* was tested for the presence of *tlh* (thermolabile hemolysin) and *tdh* (thermostable direct hemolysin) virulence genes. All *Vibrio parahaemolyticus* strain are known to have *thermolabile hemolysin* encoded by *tlh* gene as species marker. The identification of *V. cholerae* was confirmed with the primer for the gene *ompW*, and virulence was evaluated with the primers for the genes *ctx* (cholera toxin). In the case of *V. alginolyticus* it was tested for *collagenase* and *tdh* virulence genes. *Collagenase* gene was used as a species-specific marker besides its role as a virulent gene. Details of primers used are listed in Table 1.<sup>15,20-23</sup> Initial optimization experiments for each primer were conducted to ascertain optimal PCR conditions for MgCl<sub>2</sub> and annealing temperatures. The PCR was carried out using 25.00 µL reaction volume containing 12.50 µL Emerald Amp GT PCR master mix (2x premix), 6.00 µL of DNA template, 1.00 µL of each forward and reverse primers, and 4.50 µL of sterile distilled water.

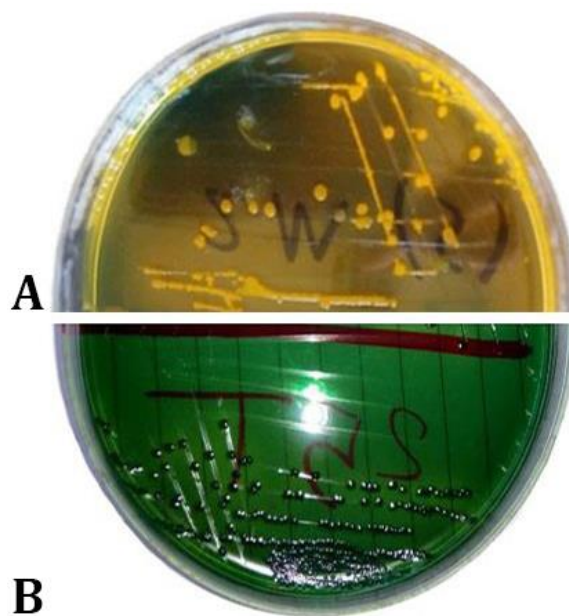
**PCR amplification.** Primers were used in a 25.00 µL reaction containing 12.50 µL of EmeraldAmp Max PCR Master Mix (Takara, Kusatsu, Japan), 1.00 µL of each primer of 20.00 pmol concentration, 4.50 µL of water; and 6.00 µL of DNA template. The reaction was carried out in a thermal cycler (model 2720; Applied Biosystems, Foster City, USA). The thermal cycle process is shown in Table 2. The obtained PCR products were kept at a temperature 20.00 °C for further use. Electrophoresis was used to make qualitative analyses for DNA products from PCR.

**Analysis of the PCR products.** The PCR products were separated by electrophoresis on 1.00% agarose gel (Appllichem GmbH, Darmstadt, Germany) in 1x Tris-borate-EDTA (TBE) buffer (Thermo Fisher Scientific, Schwerte, Germany) at room temperature using gradients of 5.00 V per cm. For gel analysis, 40.00 µL of the products were loaded in each gel slot. A gene ruler 100 bp ladder (Thermo Fisher Scientific) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech; Biometra, Göttingen, Germany); and the data were analyzed through computer software using gel documentation system (Biometra).

## Results

**Clinical signs of infected fishes.** The clinical examination of diseased fishes showed redness at the base of the anal fin and erosion of the caudal fin, ulcers, detached scales, fin erosion, hemorrhagic areas around the mouth and corneal opacity. The post mortem examination of infected fishes showed congestion and swelling of the spleen, liver, kidney and hemorrhage in the abdominal cavity.

**Identification of *Vibrio* species.** On TCBS agar plates, the bacterial colonies of *V. alginolyticus* were large (2.00 - 4.00 mm) and mucoid yellow, the colonies of *V. cholerae* were large (2.00 - 3.00 mm) and smooth yellow; and the colonies of *V. parahaemolyticus* were large (2.00 - 5.00 mm) and green (Fig. 1). All the *Vibrio* spp. were Gram-negative, straight or curved short rods, non-sporulating and non-capsulated, arranged singly or in chains motile. The biochemical characteristics of *Vibrio* species are shown in Table 3.



**Fig. 1.** Colonial morphology of isolated *Vibrio* spp. on TCBS media. **A)** Yellow colonies on TCBS, and **B)** Green colonies on TCBS.

**Table 2.** Cycling conditions of PCR.

Target	Amplification				No. of cycles	Final extension	Reference
	Primary denaturation	Secondary denaturation	Annealing step	Extension step			
<i>Collagenase (V. alginolyticus)</i>	94.00 °C 5 min	94.00 °C 30 sec	57.00 °C 1 min	72.00 °C 1 min	35	72.00 °C 10 min	20
<i>tlh (V. parahaemolyticus)</i>	94.00 °C 5 min	94.00 °C 30 sec	55.00 °C 40 sec	72.00 °C 45 sec	35	72.00 °C 7 min	21
<i>ompW (V. cholera)</i>	94.00 °C 5 min	94.00 °C 30 sec	57.00 °C 1 min	72.00 °C 1 min	35	72.00 °C 7 min	15
<i>ctx V. (cholera)</i>	94.00 °C 5 min	94.00 °C 30 sec	60.00 °C 40 sec	72.00 °C 45 sec	35	72.00 °C 10 min	22
<i>tdh (V. parahaemolyticus, V. alginolyticus)</i>	94.00 °C 5 min	94.00 °C 30 sec	54.00 °C 40 sec	72.00 °C 40 sec	35	72.00 °C 10 min	23

**Prevalence of *Vibrio* species.** Out of 150 fishes, 24 isolates of *V. alginolyticus*, 11 isolates of *V. cholerae* and eight isolates of *V. parahaemolyticus* were isolated. In the present study, *V. alginolyticus* (16.00%) was the predominant species followed by *V. cholerae* (7.33%) and *V. parahaemolyticus* (5.33%).

**Antibiotic susceptibility.** The tested *Vibrio* spp. isolates showed resistance to ampicillin, erythromycin, streptomycin, and cefotaxime with 80.00%, 40.00%, 33.30% and 26.60%, respectively, however, all the isolates showed sensitivity to ciprofloxacin and norfloxacin with 100% followed by nalidixic acid, sulphatrimethoprim 93.30%, amikacin 86.60%, gentamicin and nitrofurantoin 80.00%.

**Molecular characterization of some virulence genes in *Vibrio* species.** Fifteen isolates of *Vibrio* spp. (five isolates out of *V. parahaemolyticus*, five isolates out of *V. cholerae*; and five isolates out of *V. alginolyticus*) were tested for the presence of some virulence genes (*tdh*, *tlh*, *collagenase*, *ctx*). The PCR analysis in this study showed that in *V. parahaemolyticus* isolates, *tlh* gene was found in all the five isolates with an incidence of 100% of the strains and *tdh* gene was found in three out of five isolates with an incidence of 60.00% of the strains. In case of *V. cholerae* isolates, *ctx* gene was present in four out of five isolates of *V. cholerae* with an incidence of 80.00% of the strain which indicated that most tested *V. cholerae* species were pathogenic. On the other hand, the *collagenase* gene in *V. alginolyticus* isolates was present in all five isolates of *V. alginolyticus* with an incidence of 100%. None of the five isolates of *V. alginolyticus* harbored *tdh* virulence gene (0.00%), (Fig. 2).

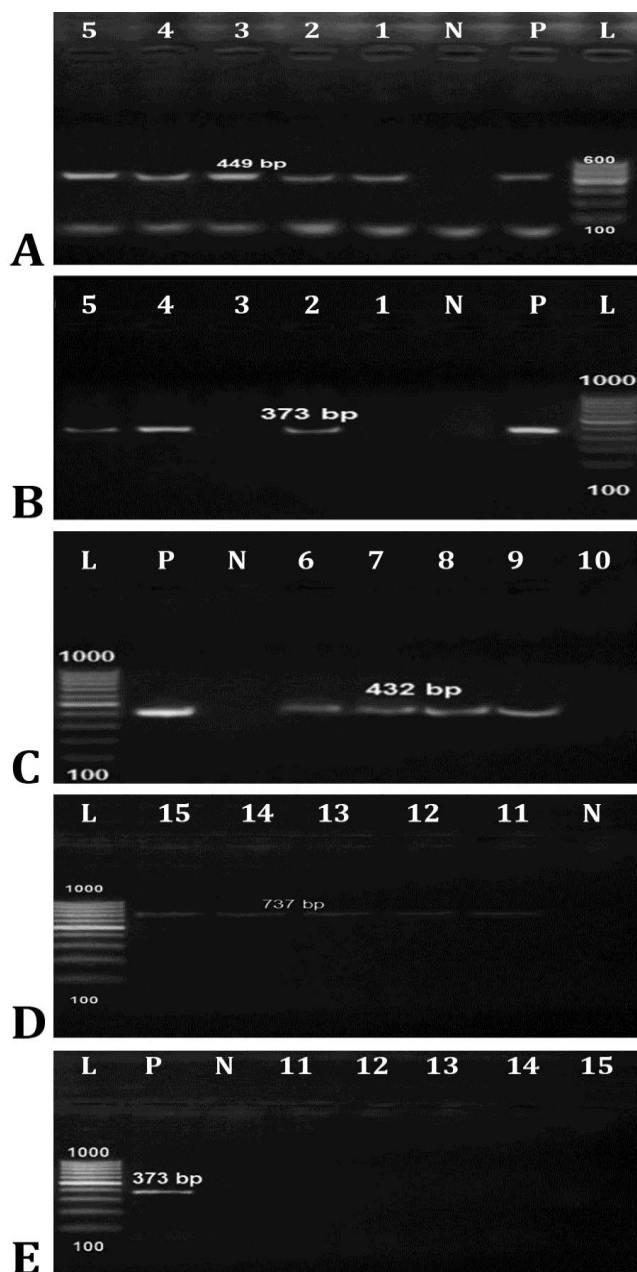
## Discussion

Vibriosis is a universal threatening bacterial disease that affects mariculture with a high percent of mortalities and many economic losses.<sup>24</sup> The rapid spread of resistance among pathogenic bacteria, including *Vibrio* spp, is now an important issue for public health and the production of antimicrobials. *Vibrio* species are inhabitants of healthy fish and can become pathogenic and cause mortality under stress.<sup>1</sup>

In the present study, *V. alginolyticus* (16.00%) was the predominant species followed by *V. cholerae* (7.33%) and *V. parahaemolyticus* (5.33%). These results agreed with Abdel-Aziz *et al.*,<sup>25</sup> who revealed that *V. alginolyticus* was the most prevalent followed by *V. parahemolyticus*, *V. cholerae*, and *V. vulnificus* in *P. monodon* farms, also, similar to the result obtained by Al-Taei *et al.*,<sup>26</sup> who found that *V. alginolyticus* was the predominant species followed by *V. cholerae*, *V. furnisii*, *V. diazotrophicus*, *V. gazogenes*, and *V. costicola*. Likewise, Deng *et al.*,<sup>27</sup> isolated *V. alginolyticus*, and *V. parahaemolyticus* from diseased marine fish in South China with a percent of 14.29 and 4.29, respectively. On the other hand, Ahmed *et al.*,<sup>28</sup> recorded that *V. parahaemolyticus* and *V. cholerae* were isolated from crustaceans with 15.10% and 0.90%, respectively, and El-Bouhy *et al.*,<sup>29</sup> found that the total prevalence of *Vibrio alginolyticus* and *Vibrio para-hemolyticus* in *Mugil capito* was 69.76% and 30.24%, respectively. The differences in *Vibrio* spp. prevalence among various species may be attributable to the number of investigated samples, differences in the host susceptibility, degree of water salinity and other different environmental conditions.

**Table 3.** Biochemical characters of isolated *Vibrio* spp. (Elliot *et al.*<sup>18</sup>).

Parameters	<i>V. cholerae</i>	<i>V. alginolyticus</i>	<i>V. parahaemolyticus</i>
Gram stain	-	-	-
Motility	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
Citrate	+	+	+
Indole	+	+	+
Urease	-	-	-
Gelatine liquifaction	+	+	+
Suscebtability to O/129 (150 mg)	+	+	+
<b>Growth in</b>			
0.00% NaCl	+	-	-
3.00% NaCl	+	+	+
6.00% NaCl	-	+	+
8.00% NaCl	-	+	+
10.00% NaCl	-	+	-
Arginine dihydrolase	-	-	-
Ornithine decarboxylase	+	+	+
Lysine decarboxylase	+	+	+
Methyl red	+	-	+
Voges-Proskauer	variable	+	-
ONPG	+	-	-



**Fig. 2.** Agarose gel electrophoresis of PCR products. **A)** Amplification of *tlh* gene at 449 bp. MWM-molecular weight marker (100 - 600 bp DNA ladder), + control (P: Positive, N: Negative) + lanes (1, 2, 3, 4, 5) examined *V. parahaemolyticus*. **B)** Amplification of *tdh* gene at 373 bp. MWM-molecular weight marker (100 - 1,000 bp DNA ladder), + control (P: Positive, N: Negative) + lanes (1, 2, 3, 4, 5) examined *V. parahaemolyticus*. **C)** Amplification of *ctx* gene at 432 bp. MWM-molecular weight marker (100 - 1,000 bp DNA ladder), + control (P: Positive, N: Negative), and lanes (6, 7, 8, 9, 10) examined *V. cholerae*. **D)** Amplification of *collagenase* gene at 737 bp. MWM-molecular weight marker (100 - 1,000 bp DNA ladder), + control (N: Negative), and lanes (11, 12, 13, 14, 15) examined *V. alginolyticus*. **E)** Amplification of *tdh* gene at 373 bp. MWM-molecular weight marker (100 - 1,000 bp DNA ladder), + control (P: Positive, N: Negative), and lanes (11, 12, 13, 14, 15) examined *V. alginolyticus*.

Antibiotic resistance study revealed that the tested *Vibrio* spp. isolates showed resistance to ampicillin (80.00%). This result was similar to the previous studies obtained by Okaoh and Igbino, <sup>30</sup> and Scarano *et al.*, <sup>31</sup> who found that ampicillin showed low efficacy against *Vibrio* spp. In the present study, all the isolates were sensitive to ciprofloxacin and norfloxacin (100%). This indicated that ciprofloxacin and norfloxacin could be used as a drug of choice for the treatment of *Vibrio* infection.

Detection of virulence genes is essential to study the distribution of pathogenic strains especially in seafood. Most types of *Vibrio* species have an extensive reservoir of virulence genes, although they are typically non-pathogenic. The transfer of the nonpathogenic strain to pathogenic strain could be attributed to successful transfer and the mobility of the virulence genes.<sup>32</sup> It may be the aquatic environment harbors different virulence genes distributed among environmental *Vibrios*. Some *Vibrio* species were screened by PCR for the presence of virulence-associated genes to determine the real risk proposed to public health by the existence of this micro-organism in seafood. The presence of *tdh* or *trh*-positive isolates of *V. parahaemolyticus* in freshwater and marine fish indicates a high risk to human health through the consumption of seafood contaminated with toxigenic *V. parahaemolyticus* strains.<sup>33</sup> The prevalence of *tdh* gene in *V. parahaemolyticus* in the current study was high. Three isolates of *V. parahaemolyticus* (60.00%) possessed the *tdh* gene. The *tdh* gene is responsible for the Kanagawa phenomenon which is hemolytic activity caused by lysis of red blood cells on Wagatsuma blood agar.<sup>34</sup> The *tlh* gene was found in all *V. parahaemolyticus* isolates. According to Nordstorm,<sup>35</sup> *tlh* gene was a specific marker for *Vibrio parahaemolyticus* species, and the *tdh* gene regulated virulence factor in *V. parahaemolyticus*. Gutierrez West *et al.*,<sup>21</sup> detected *tlh*, *tdh* genes in 79% and 48% of the isolates, respectively, and confirmed that *tdh* gene was strongly related to the virulence of the human pathogen, while Rojas *et al.*,<sup>36</sup> detected *tlh* gene in 100% of isolates, however *tdh* gene was identified in only 10.50% of the isolates. As the cholera toxin was considered as the most essential epidemic marker. The *ctx* gene from the phage genome encode the cholera toxin and is primarily responsible for the severe watery diarrhea.<sup>13</sup> Therefore, we investigated, in this study, the presence of *ctx* virulence gene of *V. cholerae* which was present in 80.00% of the isolates. This was similar to the result obtained by Hounmanou *et al.*,<sup>37</sup> that found *ctx* gene in 100% of isolates of *V. cholerae* tested, while Awasthi *et al.*,<sup>38</sup> detected *ctx* gene in 20.83% of *V. cholerae* isolates.

*Collagenase* has been widely utilized as a biomarker in molecular identification of *V. alginolyticus* and capable of degrading conjunctive tissue, the basal epithelial membrane that leads to extra-intestinal pathology and dissemination to blood stream.<sup>11</sup> Thermostable direct

hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) genes are among the critical virulence factors in *V. alginolyticus*.<sup>39</sup>

In *V. alginolyticus* we detected *collagenase* gene in all the five isolates (100%) and none of the isolates harbored *tdh* gene. This result was similar to the result obtained by Avsever,<sup>40</sup> who also detected *tdh* gene in 0.00% of the *V. alginolyticus* isolates, however, Nor Najwa *et al.*,<sup>11</sup> detected *collagenase* gene in 67.00% of the isolates, and Gargouti *et al.*,<sup>41</sup> detected *tdh* gene in 2 of 16 (12.50%) isolates of *V. alginolyticus*.

In conclusion, the wide distribution of virulence genes among the tested strains indicated a potential risk for humans so that consumers need to increase their awareness, ensure the proper cooking of seafood and avoid undercooked or cross-contaminated fish. In addition, the collected data indicated that the PCR systems could be useful for rapid detection and differentiation of *Vibrio* spp. in different food matrices as basis for a preventive consumer protection policy.

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### Conflict of interest

The authors declare no financial or conflict of interest regarding this study that could inappropriately influence the work.

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