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Article:

Virulence of *Vibrio species* isolated from some types of fish and shellfish

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Abstract

Vibrio species. are microbial foodborne pathogens with high public health hazards. Pathogenicity of *Vibrio spp.* is encoded by a wide spectrum of virulence factors. This work was conducted to isolate and identify *Vibrio spp*. from 150 fish and shellfish samples sold in Sohag markets and to determine the prevalence of *Vibrio spp*. and their virulence genes by molecular techniques. *Vibrio spp.* were isolated from 40% of the examined samples. Bacteriological and biochemical investigations showed that the highest rate of *Vibrio spp.* recovered from fish and shellfish was *Vibrio. mimicus* (43.3%) followed by *V. vulnificus* (16.7%), while the least percentage was *V. anguillarium* (1.7%). Samples were amplified by PCR technique for more accurate identification, all isolates were positive to *16S rRNA* specific for *Vibrio spp*., all (7) isolates of *V. parahemolyticus* were positive for *toxR, trh* , while *tdh* gene was not identified in any of *V. parahemolyticus* isolates. *sodB* gene of *V. cholerae* was found in all four tested isolates while *ctxAB* and *ompW* genes were seen only in three isolates. All the identified isolates of *V. mimicus* encode *sodB* gene. *hsp60* specific for *V. vulnificus* was detected in all isolates. Fish and shellfish in Sohag city were highly contaminated with *Vibrio spp.* causing a potential health hazard. PCR techniques represent a quick, accurate, and reliable method for detection of virulence genes of *Vibrio spp*. Strict infection control and hygienic measures should be applied at fish farms and markets to minimize food contamination with *Vibrio spp.*

Keywords: *Vibrio spp.*, fish, shellfish, virulence genes, PCR.

Introduction

Fish and seafood are the most reliable alternatives for red meat, and represent an excellent source of various nutritional elements **[1]**. They can be infected with many foodborne pathogens. The genus *Vibrio* is gram-negative bacteria that comprises important pathogenic *spp.* for human and aquatic organisms. They are widespread in shellfish, finfish, and marine ecosystems **[2]**. Twelve *Vibrio spp.* are known to be pathogenic to humans such as *V. parahaemolyticus, V. cholerae* and *V. vulnificus* are the *spp.* commonly related to seafood-borne infections all over the world **[3]**. *V. parahaemolyticus* is the major food-borne pathogen worldwide, causing acute dysentery, abdominal pain, diarrhea, nausea, vomiting, fever, chills, water-like stools and shock **[4]**. *V. cholera* is a well-documented human pathogen, causing lethal diarrhea. Cholera epidemics in human are mainly caused by *V. cholerae* serogroups O1 and O139 **[5]**. *V. mimicus* can produce gastroenteritis, otitis, and acute cholera- like diarrhea **[6]**. *V. vulnificus* is an opportunistic pathogen that is intimately linked to *V. cholerae and V. parahaemolyticus*. *V. vulnificus* infections, can lead to septicemia and up to 50% deaths. Exposure of wounds to contaminated fish and shellfish can induce cutaneous infections and necrotizing fasciitis, which may require limb amputation **[7]**. The pathogenicity of *Vibrio spp.* is caused by many virulence factors encoded by virulence genes. Generally, virulence factors allow pathogens to invade and destroy the host by enabling pathogenic adherence and entrance, establishment, multiplication and prevention of host defenses **[8]**. Pathogenic strains of *V. cholerae O1/O139*

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encode 2 principal virulence factors; enterotoxin called "cholera toxin" (CT); a product of the *ctxA,* and *ctxB* genes) and the toxin coregulated pilus (TCP) colonization factor: *tcpA*), together enable bacterial cell adherence and colonization to the host intestine causing severe diarrhea with the risk of dehydration **[9]**. The main contributors to *V. parahaemolyticus* pathogenicity are the thermostable direct hemolysin (*tdh)* and *tdh*-related hemolysin (*trh)*. The biological impacts of these proteins include hemolytic and cytotoxic activity **[10]**.This study was aimed to isolate and identify *Vibrio spp.* from fish and shellfish in Sohag city and to detect their virulence genes with molecular techniques.

Materials and Methods

This work was conducted between November 2022 to November 2023 in Food Hygiene Department, Faculty of Veterinary Medicine, Sohag University and Microbiology Department, Animal health research institute, Sohag branch.

Ethical considerations

The current study was approved by the Veterinary Medical Research Ethics Committee, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt, according to the OIE standards for use of animals in research with number **Soh.un.vet /00065R**

Collection and preparation of samples

150 random fish and shellfish samples including *Nile tilapia* and *Lates niloticus* as freshwater fish, mullet and mackerel as marine water fish, shrimps and oyster as shellfish (25 of each), were collected from retail shops and fish markets in Sohag city in clean sterile bags and transported to the laboratory in ice boxes to be prepared and examined as soon as possible. The scales and fins of the fish samples were removed, and the skin was sterilized with alcohol and flamed with a sterile spatula. The muscles above the lateral line were removed, while shellfish (shrimps and oysters) were washed with running water then sterilized by alcohol, and flamed, and then the carapace was removed aseptically to expose the flesh.

Enrichment of the samples

Ten grams of prepared samples were homogenized under aseptic circumstances in a sterile homogenizer containing 90ml of sterile alkaline peptone water (3%Nacl and pH 8) **[11]**.

Isolation of *Vibrio spp.*:

A loopful from incubated broth culture of alkaline peptone water (3%Nacl) was separately streaked onto Thiosulfate citrate bile and sucrose agar (TCBS) and incubated at 37°C for 24 hrs. Typical colonies of *Vibrio spp.* on TCBS appeared as smooth green (sucrose negative) or yellow (sucrose positive) **Figure 1.,** then they were purified onto a Tryptic soya agar and incubated at 37°C for 24 hrs **[12]**.

Microscopic examination:

Films from suspected purified colonies were prepared, fixed and stained by Gram stain. The *Vibrio spp.* were Gram-negative, highly motile, curved rods single or strung together in S-shapes or spirals under microscopic examination. **[12]**.

Biochemical identification of different *Vibrio spp.*:

Purified isolates were examined by biochemical tests which included oxidase test, salt tolerance test to detect the growth of *Vibrio spp.* on 0% and 6% NaCl, O-nitrophenyl-beta D-galactosidase test (ONPG), voges-proskauer test (VP) and lysine decarboxylase test **[13]**.

Molecular identification of *Vibrio spp.* and their virulence genes

1. DNA extraction of *Vibrio* isolates: The DNA was extracted using the QIAamp DNA Mini kit Catalogue No.51304 (Qiagen, Germany, GmbH) following the manufacturer's recommendations.
2. Polymerase chain reaction (PCR) amplification using oligonucleotide primers: The oligonucleotide primers with sequences. **[14-17]** Table 1 (Metabion, Germany) were used for the amplification of the Vibrio spp. to detect 16S rRNA gene specific for Vibrio spp , then their virulence genes as the following for each primer: 12.5 µl of 2X DreamTaq Green buffer (Takara; Code No. RR310A), 1 µl of the forward and reverse primers (20 pmol) for each. 5 µl of template DNA and 5.5 µl nuclease-free water were added and thoroughly vortexed, then PCR tubes were cycled using an applied biosystem 2720 thermal cycler (Life technologies, Germany) with some modifications in cycling conditions as shown in **Table 2**.
3. Analysis of the PCR products: The amplified PCR products were visualized using agarose gel electrophoresis with ethidium bromide (Applichem, Germany) and running buffer (1× TBE). 100 bp DNA Marker (Qiagen, Germany, GmbH) was used to determine the product sizes. The gel was photographed using a gel documentation system (Alpha Innotech, Biometra). **[14]**.

Statistical analysis

Data were analyzed using GraphPad Prism 9.5.1 software (GraphPad Software Inc., San Diego, CA, USA). Where Chi square test was used to assess whether a significant difference between the identified *Vibrio spp.* in fish and shellfish, "P" value of <0.05 was considered statistically to be significant **[18]**

Table 1: Oligonucleotide primers sequences for *Vibrio spp.* and some virulence genes.

Target gene	Sequence	Amplified product	Reference
Vibrio 16S rRNA	CGGTGAAATGCGTAGAGAT	663 bp	**Tarr et al. [14]**
	TTACTAGCGATTCCGAGTTC		
V. parahaemolyticus toxR	GTC TTCTGACGCAATCG TTG	368 bp	**Kim et al. [15]**
	ATACGAGTGGTTGCTGTCATG		
V. parahaemolyticus Trh	GGCTCAAAATGGTTAAGCG	250 bp	**Mustapha et al. [16]**
	CA TTTCCGCTCTCATATG C		
V. parahaemolyticus tdh	CCATCTGTCCCTTTTCCTGC	373 bp	
	CCA AATACA TTTTACTTGG		
V. mimicus sodB	CAT TCG GTT CTT TCG CTG AT	121 bp	
	GAAGTGTTAGTGATTGCTAGAGAT		
V. vulnificus hsp60	GTC TTA AAG CGG TTG CTG C	410 bp	**Tarr et al. [14]**
	CGCTTCAAGTGCTGGTAGAAG		
V. cholerae sodB	AAG ACC TCA ACT GGC GGT A	248 bp	
	GAAGTGTTAGTGATCGCCAGAGT		
V. cholerae ctxAB	GCCGGG TTG TGG GAA TGC TCCAAG	536 bp	**De Menezes et al. [17]**
	GCC ATA CTA ATTGCGGCA ATCGCATG		
V. cholerae ompW	CACCAAGAAGGTGACTTTATTGTG	304 bp	
	GGT TTG TCG AAT TAG CTT CACC		

Table 2: Cycling conditions of PCR primers of Vibrio spp. and some virulence genes.

Target	Primary denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No. of cycles	
Vibrio 16SrRNA	94˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
V. parahaemolyticus toxR	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.
V. parahaemolyticus Trh	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
V. parahaemolyticus Tdh	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.
V. mimicus sodB	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
V. vulnificus hsp60	94˚C 5 min.	94˚C 30 sec.	57˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.
V. cholerae sodB	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
V. cholerae ctxAB	94˚C 5 min.	94˚C 30 sec.	59˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
V. cholerae ompW	94˚C 5 min.	94˚C 30 sec.	59˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

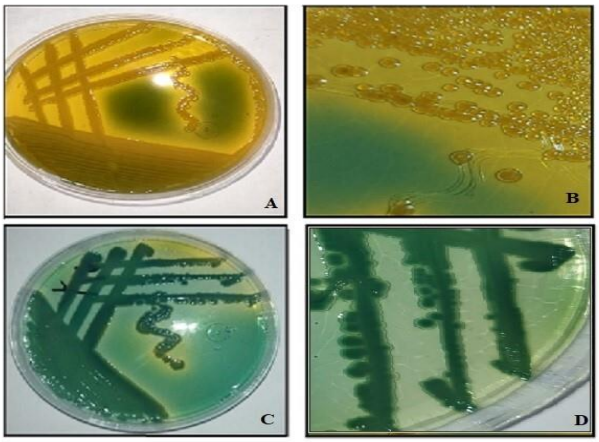

Figure 1.: Colonies of Vibrio spp. on TCBS agar
A. Yellow colonies of Vibrio spp. on TCBS agar.
B. Colonies of Vibrio spp. are large, smooth and slightly flattened with opaque centers and translucent peripheries as in V. cholera, metshinkovii, and V. anguillarium.
C. Green colonies of Vibrio spp. on TCBS agar.
D. Colonies of Vibrio spp. are round, opaque, green or bluish, 2 to 3 mm in diameter as in V. parahaemolyticus, V. mimicus, V. vulnificus, and V. damsela.

Results

Out of 150 examined samples there were 60 samples positive for *Vibrio spp.* with a percentage of 40%. The highest incidence of *Vibrio spp*. was in **freshwater fish** (44%) followed by **shellfish** (40%) while the least incidence was in **marine fish** (36%) **Table 3**. *Nile tilapia* had the highest rate of Vibrio *spp.* 13 (52%), followed by shrimp 12 (48%), mackerel 11 (44%), *Lates niloticus* 9 (36%), oyster 8 (32%) while the mullet had the least incidence (28%) **Table 4**. Chi-square, df = 4.667, 5, p value = 0.4579. No significant statistical difference was found. Bacteriological and biochemical examinations revealed 7 vibrio *spp.* recovered from fish and shellfish. The highest incidence of *Vibrio spp.* was *V. mimicus* (43.3%) followed by *V. vulnificus* (16.7%), *V. Parahaemolyticus* and *V. metshinkovii* were detected in 11.7% each, *V. damsela* in 8.3%, and *V. cholerae* in 6.7%, while the least percentage was *V. anguillarium* (1.7%) **Table 5.** There was a high significant statistical difference among different *Vibrio* spp. in fish and shellfish samples ($p< 0.01$, $\chi 2$ 49.7) **Table 5.**

Molecular detection of *Vibrio spp.* by PCR techniques Table 6. :

Molecular identification of *Vibrio spp.* revealed that *16SrRNA* gene was detected in all *Vibrio* isolates. **Figure 2.** Concerning to virulence genes to *Vibrio spp.* All 7 isolates of *V. Parahaemolyticus* were positive for *toxR* and *trh,* while *tdh* was not found in any *V. Parahaemolyticus* isolates **Figure 3**. All identified isolates of *V. mimicus* encode *sodB* gene **Figure 4.** The *sodB* gene of *V. cholera* was detected in all isolates, while *ctxAB* and *ompW* genes were detected in three isolates only **Figure 5.** The *hsp60* gene of *V. vulnificus* was identified in all its isolates **Figure 6.**

Discussion

This study showed that the incidence of *Vibrio spp.* among the examined fish and shellfish samples was 40% (60/150). Similar finding was detected in Sohag City by Yousef et al. **[19]** who isolated *Vibrio spp.* in 41% of freshwater, marine fish and shellfish samples. This can be attributed to the same geographic origin, sample source and the method used for detection. Lower results were obtained by Lopatek et al. **[20]** and Elbashir et al. **[21]** who detected *Vibrio spp.* in 19% and 4.5% of fish and shellfish samples. Higher results were reported by Abdalla et al. **[22]** in fish and shellfish sold in United Arab Emirates 64.5% and 92%, respectively. In the current study, freshwater fish represented the highest incidence of *Vibrio spp*. (44%) followed by shellfish (40%) and the marine fish showed the least incidence (36%). Different results were reported by Ibrahim et al. **[11]** who found that incidence of *Vibrio spp*. in the examined samples of fish were 32% ,40%, 52% for freshwater, marine water fish, and shellfish respectively . Also, Nilavan et al. **[6]** found that the incidence of *Vibrio spp.* in freshwater fish was 26%, while in marine fish, it was 48%. Although it was expected that the incidence of *Vibrio spp,* would be higher in marine water fish than in freshwater fish because *Vibrio spp,* is halophilic organism, the current study revealed high incidence of *Vibrio spp*. in freshwater samples. This may be attributed to the fact that freshwater fish were subjected to bad management practices during post-harvest, resulting in poor and unpredictable fish quality in the market.

Table 3. : Incidence of *Vibrio spp.* in relation to type of fish.

Samples type	Number of samples	Positive sample No.	Positive sample %
Freshwater fish	50	22	44%
Shellfish	50	20	40%
Marine water fish	50	18	36%
Total	150	60	40%

Table 4. : Incidence of *Vibrio spp.* in fish and shellfish samples

Source of the isolates	No. of examined samples	*Positive*	
		No.	%
Nile tilapia	25	13	52%
Lates niloticus	25	9	36%
Mullet	25	7	28%
Mackerel	25	11	44%
Shrimp	25	12	48%
Oyster	25	8	32%
Total	150	60	40%

Chi-square, df = 4.667, 5, p value = 0.4579. No significant statistical difference was found.

Table 5. : Prevalence of *Vibrio* serovars in fish and shellfish samples

***Vibrio* spp.**	**Fresh water fish**		**Marine water fish**		**Shellfish**		**Total (n=60)**
	***Nile tilapia* (n=13)**	***Lates niloticus* (n=9)**	**Mullet (n=7)**	**Mackerel (n=11)**	**Shrimp (n=12)**	**Oyster (n=8)**	
V. mimicus	5 (38%)	4 (44.4%)	3 (43%)	4 (36.4%)	6 (50%)	4 (50%)	**26 (43.3%)**
V. vulnificus	2 (15.4%)	3 (33.3%)	0 (0%)	2 (18.2%)	1 (8.3%)	2 (25%)	**10 (16.7%)**
V. Parahaemolyticus	2 (15.4%)	2 (22.2%)	1 (14.3%)	0 (0%)	1 (8.3%)	1 (12.5%)	**7 (11.7%)**
V. metshinkovii	2 (15.4%)	0 (0%)	2 (28.6%)	0 (0%)	3 (25%)	0 (0%)	**7 (11.7%)**
V. damsela	1 (7.7%)	0 (0%)	1 (14.3%)	2 (18.2%)	0 (0%)	1 (12.5%)	**5 (8.3%)**
V. cholerae	1 (7.7%)	0 (0%)	0 (0%)	2 (18.2%)	1 (8.3%)	0 (0%)	**4 (6.7%)**
V. anguillarium	0 (0%)	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	**1 (1.7%)**

High significant statistical difference among different Vibrio spp. in fish and shellfish samples ($p < 0.01$, $\chi 2$ 49.7).

Table 6. : Incidence of virulence genes of *Vibrio spp.* isolates

Source of isolates	***16Sr RNA***	***V. Parahaemolyticus* (n=7)**			***V. mimicus* (n=26)**	***V. cholera* (n=4)**			***V. vulnificus* (n=10)**
		toxR	***trh***	***tdh***	***sodB***	***sodB***	***ctxAB***	***ompW***	***hsp60***
Nile tilapia	13	2	2	0	5	1	1	1	2
Lates niloticus	9	2	2	0	4	0	0	0	3
Mullet	7	1	1	0	3	0	0	0	0
Mackerel	11	0	0	0	4	2	2	2	2
Shrimp	12	1	1	0	6	1	0	0	1
Oyster	8	1	1	0	4	0	0	0	2
Total	60	7	7	0	26	4	3	3	10

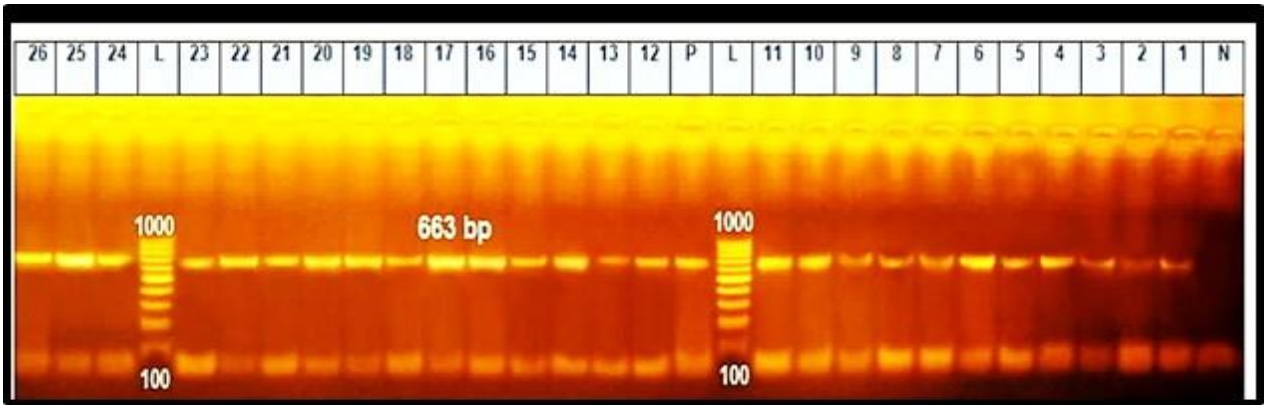

Figure 2: Agarose gel electrophoresis of the PCR product of 16S rRNA genes of Vibrio spp. (663 bp). L: DNA ladder, N: Negative control, P: Positive control. Lanes 1-26: positive samples.

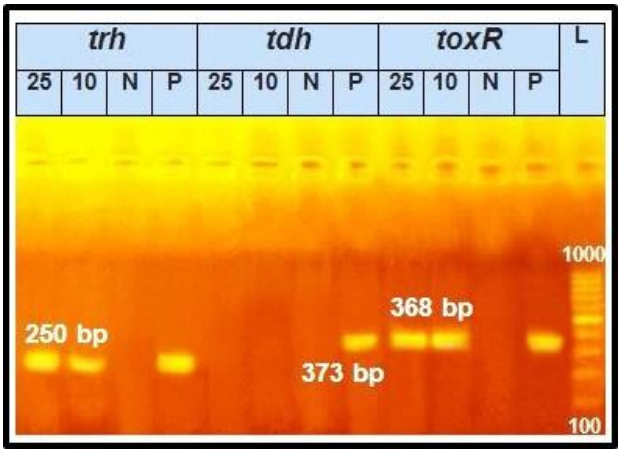

Figure 3: Agarose gel electrophoresis of the PCR product of V. parahemolyticus toxR (368 bp). L: DNA ladder, N: Negative, P: Positive. Lanes 10 and 25: positive samples, Vibrio tdh (373 bp). Lanes 10 and 25: negative samples, Vibrio trh (250 bp). Lanes 10 and 25: positive samples.

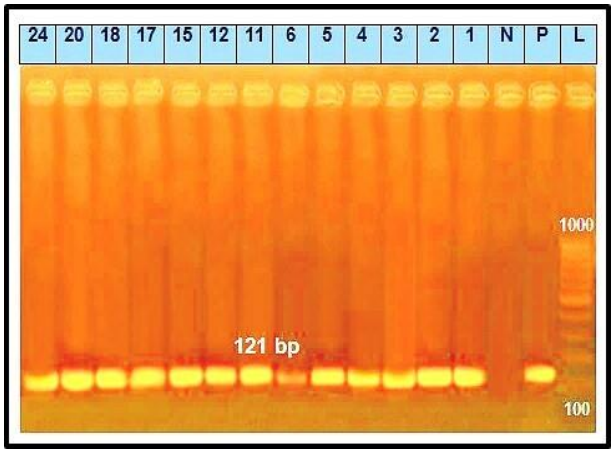

Figure 4: Agarose gel electrophoresis of the PCR product of V.mimicus sodB (121 bp). L: DNA Ladder, N: Negative control, P: Positive control. Lanes 1-13: Positive samples for the sodB gene of V. mimicus.

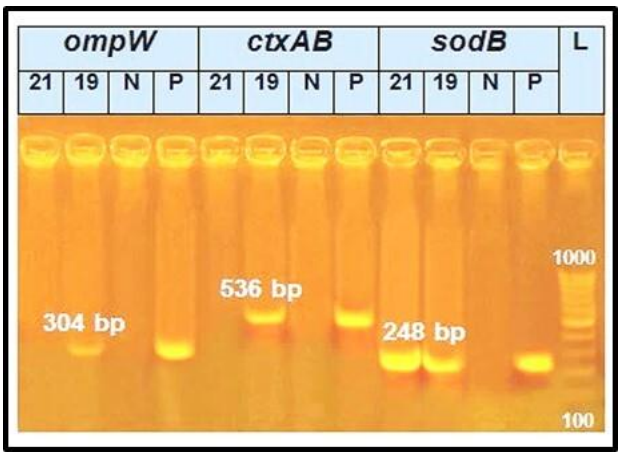

Figure 5: Agarose gel electrophoresis of the PCR product of V.cholerae sodB (248 bp). L: DNA ladder, N: Negative control, P: Positive control, lane 19 and lane 21: positive samples, ctxAB (536 bp). Lane 19: positive, and lane 21: negative samples, ompW (304 bp). Lane 19: positive and lane 21: negative samples.

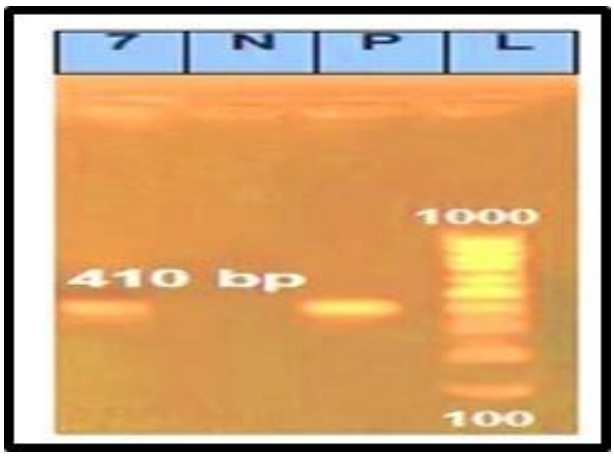

Figure 6: Agarose gel electrophoresis of the PCR product for the hsp60 gene of V. vulnificus. (410 bp). L: DNA ladder, N: Negative control, P: Positive control, lane 7: positive sample for the hsp60 gene of V. vulnificus.

The high incidence of *Vibrio spp.* in shellfish, is based on that shellfish represent an excellent substrate for the microorganisms due to lose texture of their flesh. When the aquatic system was contaminated with pathogenic *Vibrio,* these bacteria become part of shellfish microflora. Studies reported that Free-living vibrios are up taken by filter-feeding shellfish resulting in a higher *Vibrio* spp. incidence **[13].** Incidence of *Vibrio spp.* in marine fish probably reflects the nature of *Vibrio spp*. which is known as a halophilic waterborne bacterium that commonly inhabits environmental water sources worldwide **[11].** Our results demonstrated that the highest incidence of *Vibrio spp.* was detected in *Nile tilapia* (52%) followed by shrimp (48%), mackerel (44%), *Lates niloticus* (36%), oyster (32%) then mullet (28%). Similarly, Yousef et al. **[19]** reported highest incidence of *Vibrio spp.* from *Nile tilapia* followed by shrimp then mackerel with percentage of (80%),(40%) and (32%) respectively. In the present study seven *Vibrio spp*. biochemically identified from fish and shellfish samples. The highest incidence of *Vibrio spp.* was *V. mimicus* (43.3%) then *V. vulnificus* (16.7%), *V. Parahaemolyticus* and *V. metshinkovii* were detected in 11.7% each, *V. damsela* in 8.3%, and *V. cholerae* in 6.7%, while *V. anguillarium* had the least incidence (1,7%). This data was lower than those obtained by Sadat et al. **[23],** and Vu et al. **[24]** who found that the prevalence of *V. parahaemolyticus* were 14%, and 58.3% in fish and shellfish samples respectively. *V. cholerae* was detected in 45% of finfish and crustacean seafood, and in 8.8% of oyster samples **[25-26]**. Also, higher prevalence was obtained by Yousef et al. **[19]** who found *V. metashinkovii* in 15% and *V. damsela* in 12% in all examined fish and shrimp samples.While, *V. anguillarium* was detected in 4% of marine fish samples **[27].** Lower results of *V. vulnificus* were detected with a percentage of 8%**[27]**. Also, Abdellrazeq and Khaliel **[28]** and Nilavan et al. **[6]** reported that 17% and 18.2% of fish samples were contaminated with *V. mimicus*, respectively. While *V. damsela* was in 4% of fish samples **[27]**. In contrast, *V. anguillarium* could not be detected in any fish and shellfish samples from Sohag city **[19].** In this study, the *16Sr RNA* gene was identified in all *Vibrio spp.* Some studies reported the presence of this gene in 82.87%, and 93.3%, respectively**[29,19]**. All the examined isolates of *V. parahaemolyticus* were positive to *toxR* and *trh* genes.Lower results of *toxR*-positive *V. parahaemolyticus* was detected in 35% of oyster samples, while it could not be detected in any of shrimp samples **[30]**. Sadat et al.**[23]**, and Vu et al. **[24]** reported the presence of *trh* in 7·9%, and 8.3% of *V. parahaemolyticus* isolate, respectively. In our study, none of the isolated strains were encoding the *tdh* gene of *V. parahaemolyticus*. Similarly, the hemolysin *tdh* virulence gene did not detected in any of the *V. parahaemolyticus* isolates **[26, 30, 31].** In contrast to this study, *tdh* gene was identified in 0.72% and 2.9% of isolates, respectively **[32,20].** Owing to *sodB* gene of *V. cholerae* it was detected in all four tested isolates while the *ctxAB* and *ompW* genes were detected only in three isolates. Other study repoted that none of the *V. cholera* isolates of fresh water fish carried the *ctxAB* **[33]**, while *V. cholerae ompWp* gene was found with a rate of 8.8%**[34].** Also in 8 out of 14 (57%) of shrimp samples and in 1 out of 10 (10%) of shellfish samples, but none of these samples carried the *ctxAB* gene**[35].** All identified isolates of *V. mimicus* were positive for *sodB* gene. Abdellrazeq and Khaliel **[28]** revealed the *sodB* gene of *V. mimicus* in 2 (11.1%), and 3 (16.6%) of 18 isolates from *Nile tilapia*, and mullet, respectively. This research showed the presence of *hsp60* gene in all isolates of *V. vulnificus. Similarly*, Yousef et al. detected the *hsp60* gene in all *V. vulnificus* isolates**[19].**

Conclution

Vibrios are the most common genera associated with fish and shellfish often causing significant economic losses. Many *Vibrio spp*. are pathogenic to humans and have been implicated in food borne diseases. PCR techniques has become an important diagnostic tool in the detection of foodborne pathogens and determine their virulence genes. Strict preventative measures should be applied at fish markets to prevent food contamination with *Vibrio spp.*

Conflict of interest:

No conflict of interests.

Authors Contributions

All authors had materially participated in the research and article preparation. All authors have approved the final article.

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