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Isolation, identification and antibiotic susceptibility of *Vibrio parahaemolyticus* isolated from shrimp aquaculture

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Abstract *Vibrio parahaemolyticus* is a chief foodborne pathogen accountable for main monetary damages in aquaculture and a peril to social wellbeing. *V. parahaemolyticus* was isolated from private aquaculture farms in the delta region in Kafr El-Sheikh Governorate, Egypt. A total of 160 examined *Mugil capito* (*Chelon ramada*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*) and *Penaeus vannamei* (shrimp) samples were collected from the aquaculture arrangement. 25% of samples were positive for *V. parahaemolyticus*. Shrimp agronomy faced grave glitches with diseases caused by *V. parahaemolyticus*, which have been frequently associated with fatalities both, in hatcheries and growout pools. The *Vibrio* species isolates from infected shrimp samples were biochemically and molecularly identified. Results revealed that, only 5 (12.5%) isolates from *Penaeus vannamei* (shrimp) were found positive for the thermostable direct hemolysin (*tdh*) and the the rmost able direct hemolysin-related hemolysin virulence factor genes (*trh*). The isolates were found to be 99% identical to the position strain *V. parahaemolyticus* and were deposited on GenBank under the accession number (OM654368). The antibiotic sensitivity test of the virulent strain of *V. parahaemolyticus* MAA3 showed highly resistance to ampicillin (10 µg) , amikacin (30 µg), cefotoxime (30 µg), ceftazidime (30 µg), and intermediate sensitivity to Erythromycin (15 µg). On the other hand, it was highly sensitive to ceftriaxone (30 µg), chloramphenicol (30 µg), cotrimoxazole (25 µg), trimethoprim (1.25 µg), sulfamethoxazole (23.75 µg), ciprofloxacin (5 µg), doxycycline HCl (30 µg), gentamycin (10 µg), levofloxacin (5 µg), tetracycline (30 µg), and tobramycin (10 µg). This study showed that *Vibro parahaemolyticus* have the ability to resist several antibiotics in addition to being very sensitive to other antibiotics, and the presence of virulence factors in *Vibro parahaemolyticus* represents a major threat to human health and economic resources in the future. To improve seafood safety, it is necessary to continuously evaluate the antibiotic and molecular features of *V. parahaemolyticus*.

Keywords: *Vibrio parahaemolyticus*, aquaculture, , Shrimp, isolation, antibiotic

1.Introduction

Fisheries represent an important sector of the Egyptian national income because about 26.7 % of the total Egyptian fish production comes from natural resources **[1].** Marine fishes represent the major investment choice for the national fishermen and a vital source of income in many developing countries **[2].** However, aquaculture quiet has to face some severe experiments such as derisory fish feed resources, disease occurrences, pathogen microscopic organism infection; insufficient marketing, poor water quality, poor running, a deficiency of fake feeds, effluence, a deficiency of structure and a lack of veterinary service provision [**3**]. Disease occurrences following impurities caused by pathogenic bacteria have been reported among countless aquatic fish. Bacterial diseases are one of the most important

snags facing fish engineering and are responsible for heavy mortality **[4].** The majority of bacterial diseases are produced by some contributing managers which include bacteria from short, Gram-negative rods going to the families *Enterobacteriaceae*, *Pseudomonadaceae (Pseudomonas*) and *Vibrionaceae* (*Vibrios*) **[5]**. *Vibrio parahaemolyticus* is a Gram-negative halophilic bacterium, foodborne pathogen occurs in marine situations [**6**]. Also, contact with *V. parahaemolyticus* on the skin can cause an open wound in the skin and may result in wound infection. But, not all *V. parahaemolyticus* strains are pathogenic and competent of instigating disorder in humans [**7**]. Fish souks, fish collecting areas, sea water and sometimes fresh water are the main causes through which this bacterium may infect humans0.

. Shrimps and fishes scan hoard the bacterium from the aquaculture before garnering method. The growth of *V. parahaemolyticus* is related to water temperature and season as well [**8**].

Most pathogenic *V. parahaemolyticus* can produce two chief pollutants, thermos table direct hemolysin (TDH) and TDH-related hemolysin (TRH) [**9, 10**]. The gene *tdh* is a protein that supports in aperture development, which has been associated with the invsion of bacteria, and *trh* plays a foremost role in virulence. Furthermore, both poisons have the features of hemolytic toxicity, enterotoxigenicity, cardiac toxicity, and cytotoxicity that can affect the infected hosts [**11, 12, 13**]. The pathogenesis of *V. parahaemolyticus* is then immobile open to inquiry and has not been entirely explained **[14, 15].**

The objective of the current study is the isolation and documentation of *Vibrio parahaemolyticus* from shrimp samples. Also, offer vital information on the prevalence, antibiotic susceptibilities patterns profiling of *V. parahaemolyticus*

2. Materials and methods

2.1. Collection of samples

160 samples of fish and shrimp were randomly collected from El-Burullus Lake and private aquaculture farms in the delta region in Kafr El-Sheikh Governorate, Egypt. The shrimp (*Penaeus vannamei*). All samples were transported in sterile plastic bags to circumvent adulteration. The samples were deposited in an protected box holding crushed ice. The samples were nearly transferred to the Laboratory (Research & Development Center, Ghalioun, Kafr El-Sheikh Governorate, Egypt).

2.2. Clinical and post-mortem (PM) examination

The collected shrimp were examined clinically for the detection of external abnormalities or lesions, then subjected to PM examination for the detection of any macroscopical lesions in the organs [**16]**. Live samples were immersed in tricaine methanesulfonate (Sigma-Aldrich) for 10 min., following cessation of the opercular movement according to American Veterinary Medical Association guidelines on euthanasia **[17].**

2.3. Samples processing

The samples were splashed thoroughly with sterile distilled water past to check. 25 g from each sample was aseptically relocated into a sterile polyethylene stomacher bag and blended with 225 ml of sterile chalky saline peptone water (ASPW) in a stomacher homogenizer at 230 rpm.

2.4. Isolation and purification of *Vibrio* spp.

Vibrio spp. were isolated by using thiosulfate citrate bile salt agar medium (TCBS). One loopful from the previous homogenate sample was streaked over sterilized TCBS. All plates were examined for the presence of green colonies, round, 2-3 mm diameter colonies (suspect: *V. parahemolyticus* or *V. vulnificus*) or yellow colonies, round, 2-3 mm diameter colonies (suspect: *V. cholera*, *V. fluvialis* or *V. alginolyticus*). Single camp from each grown type of *Vibrio* colonies was lined onto TCBS agar plates for distillation and raised at 30°C for 24 h (18).

Identification of isolated *Vibrio* spp.

Morphological, microscopic and biochemical characterization:

The pure colonies of suspected *Vibrio* spp. were subjected to Gram staining and biochemical tests (oxidase, catalase, Ornithine

decarboxylase, triple sugar iron (TSI), and indole test (**19**).

Assessment of hemolytic activity

The hemolytic activity of isolated *Vibrio* strain was detected according to the method described by Brutscher et al. **(20)**.

Confirmation of species by VITEK2

All positive *V. parahaemolyticus* isolates were extra established by the VITEK 2 system (BioMérieux, Mabrat El Asafra, Egypt) using a VITEK 2 GN ID card. For VITEK 2 assays, pure segregates were speckled on GA plates and gestated at 37 ^{o}C dramatic. 1–3 isolated colonies were nominated from each GA plate and deferred in saline for training of inoculum to find an absorbance of _0.5 McFarland Units before actuality endangered to VITEK 2 analysis (**21**).

Molecular identification of the isolated *Vibrio* strain via PCR

Further identification for the *Vibrio* species was completed by 1 rDNA. The retrieved *Vibrio* species was cultured on tryptic soya agar with 2% NaCl for genomic DNA extraction.

Bacterial DNA isolation: DNA was extracted from the Cell using Solgent purification bead. The 16S rDNA was amplified by means of polymerase chain reaction (PCR) using the bacterial universal primer 27F **(22).**

Solgent PCR conditions were as follows: 95 ◦C denaturation for 15 min, followed by 95 ^{o}C for 20 s, 30 cycles at 50 ^{o}C for 40 s, 72 ^{o}C for 90 s, and a final step of 72 ^{o}C for 5 min. Reaction mixtures were purified using Solgent PCR purification kit

Sequencing of 16S rDNA of the bacterial isolates were done in SolGent (Solution for Genetic Technologies), The resulting 16S rDNA sequences were compared to GeneBank entries via NCBI BLAST home page (https://blast.ncbi.nlm.nih.gov/Blast/). The sequence was aligned to related genus using Clustal X, version 2.0 and a phylogenetic tree was constructed by the neighbor – joining method using MEGA with maximum composite like hood method **[23].**

2.5. Polymerase Chain Reaction (PCR) Assay

The extraction of chromosomal DNA was through expending the ulcer DNA mining method according to the technique of Mahmud et al. **[24].** Presumptively branded isolates were added reputable by simplex PCR for the gene, a species precise gene for *V. parahaemolyticus* [**25, 26]**, PCR analyzes were agreed upon to the perceive tdh and trh genes labelled by **Bag et al. [26].** The PCR-amplified products were separated in an agarose gel of 1.5% damaged in MIDORI green supper and projected above UV light with a GelDoc Go *imaging* system **[(27, 28].**

2.6. Antibiotic susceptibility test of the *Vibrio* species isolate

The isolated *Vibrio* strain was examined for its antibiotic susceptibility by the disc diffusion method (29). Antibiotics (TM, India) were used as listed in **Table 1.** 100 µl of bacterial suspension (1.5×10^8 CFU/ml) was adjusted to 0.5 MacFarland standards, and inoculated into sterilized Muller-Hinton agar (MHA) (HIMEDIA) with 3% NaCl and poured on the plate and the discs were spread over the agar surface with sterile forceps and after media solidified, every antibiotic disc was placed (6 mm) and incubated for 24 h at 30 ^{o}C±1. **[28**].

Table (1): Different antibiotics used for antibiotic susceptibility test

Antimicrobial discs	**Concentration (µg)µg**
Chloramphenicol	30
Co-trimoxazole	25
Trimethoprim-Sulfamethoxazole	1.25 /23.75
Ceftriaxone	30
Ciprofloxacin	5
Cefotoxime	30
Ceftazidime	30
Tetracycline	30
Doxycycline HCl	30
Levofloxacin	5
Tobramycin	10
Erythromycin	15
Gentamycin	10
Amikacin	30
Ampicillin	10

2.7. Statistical analysis:

Using the SPSS PC Version computer program, a statistical analysis was performed to determine the significance of the incidence of Vibrio parahaemolyticus among examined shrimp and fish.

3. Results

3.1. Clinical and necropsy findings of fish samples:

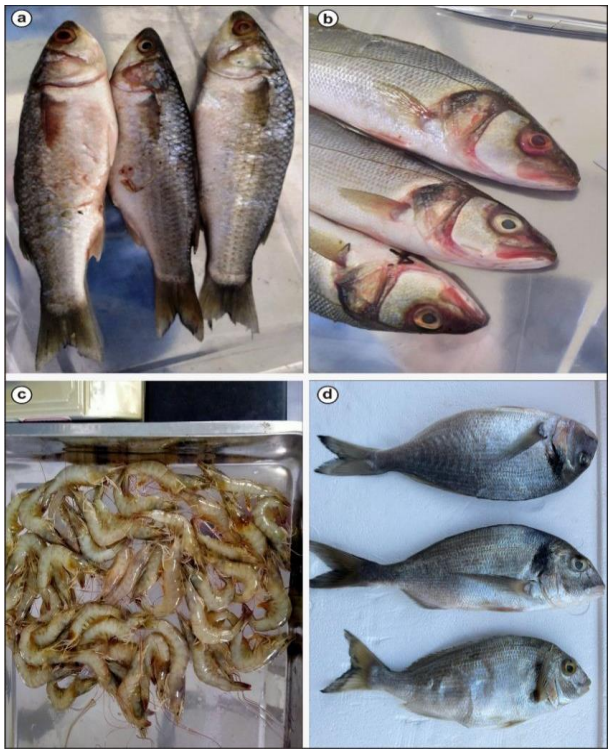

Fig 1: a. *mugil capito (Chelon ramada)* showing hemorrhage in eye and hemorrhagic area around operculum, **b**. *Sea bass (Dicentrarchus labrax*) showing hemorrhage in eye, hemorrhagic areas on the mouth, at the base of fins and around the operculum, **c.** destruction of the antennal flagellum in *Penaeus vannamei shrimp*, uropod and brown to black spots on the its skeleton and reddish correlation of the body and **d**. *Sea bream (Sparus aurata)* showing skin hemorrhage.

The clinical examination of collected *Penaeus vannamei* shrimp revealed the presence of black spots on the gills, pleopods and uropods as shown in **Figure 2.**

Fig 2: *Penaeus vannamei* shrimp with black spots on gills, black spots on pleopods and black spots uropods**.**

Incidence of *Vibrio parahaemolyticus* infection among examined fish and shrimp:

Table (2) showed that, there were significant differences of in the incidences of *Vibrio parahaemolyticus* infection ($P < 0.01$) among the examined fish and shrimp samples. The higher infection observed in *Penaeus vannamei* (shrimp) at 10 (25 %), *Sea bass (Dicentrarchus labrax*) at 8 (20 %), and in *Sea bream (Sparus aurata)* at 6 (15 %) and the lower incidences observed in *Mugil capito* (Chelon ramada) at 2 *(5 %).*

Table 2: Infected and non-infected Fish and shrimp samples

Sample	No. of sample	Numberof the infected Samples	Percentagefinfection(%)
Mugil capito (Chelon ramada)	40	2	5
Seabass (Dicentrarchus labrax)	40	8	20
Sea bream (Sparus aurata)	40	6	15
Penaeus vannamei (shrimp)	40	10	25

$Chi^2 = 7.25^{**}$ ** = Significant at ($P < 0.01$)

3.2.Isolation of *Vibrio* spp. from different fish and shrimp samples.

Twenty bacterial strains were isolated from kidney, spleen, heart and liver under aseptic conditions, plated on sterilized TCBS agar media. Green colonies with black center on TCBS agar media were observed (**Figure 3**). The bacterial isolates were Gram-negative; short comma shaped curved rods.

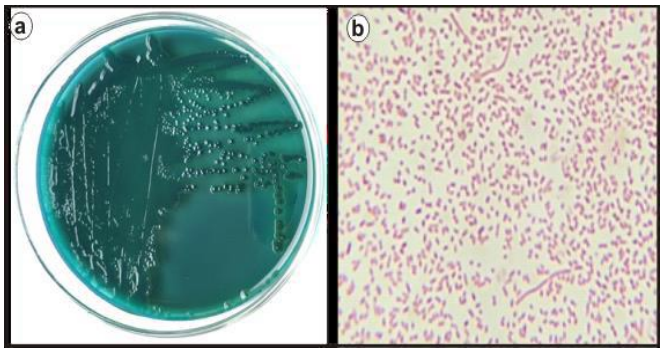

Fig 3: Green colonies with black center of *V. parahemolyticus* colonies on TCBS medium and short- rods gram negative bacteria under microscope.

3.3. Biochemical identification

The identification of bacterial isolates revealed the isolated *Vibrio* species; *is* presumptive *V. parahaemolyticus* and these results were confirmed by a series of biochemical tests as shown in **Table (3)**

Table (3): Identification of presumptive *V. parahemolyticus* by biochemical test

Test	**Reaction**
Oxidase test	Positive
Catalase test	Positive
Indole test	Positive
Ornithine decarboxylase	Negative
Triple sugar iron agar 3% NaCl	
Slant	Red
Bottom	Yellow
Hydrogen sulphide	Negative
Bubbles (gas)	Negative

3.3.1. Biochemical identification by Biomérieux Vitek

The biochemical characterization of *Vibrio* isolates was analyzed using Biomérieux Vitek® 2 GN **(Table 4)**.The GN card contains 18 fermentation tests (adonitol, l-arabinose, d-cellobiose, d-galacturonate, d-glucose, glucose-1-phosphate, d-glucuronate, inositol, and 5-keto-gluconate.

3.3.2. Blood hemolysis of isolated *Vibrio* strain

The strain was beta-hemolytic (complete hemolysis, which is referred to as a clear and colorless medium after growth around the bacterial colonies, (**Figure 4**). So, the *Vibrio* strain was selected as a pathogenic strain.

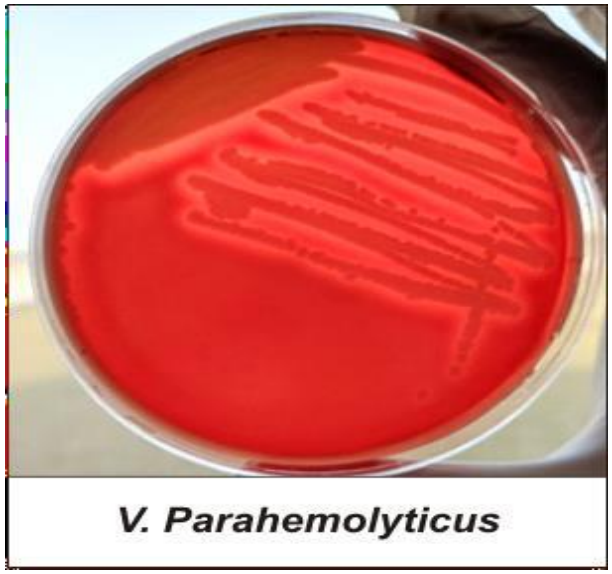

Fig 4: Complete hemolysis of *V. parahemolyticus* (β -hemolysis) on blood agar media.

Table (4): Biochemical tests of suspected *V. parahemolyticus* according to Biomérieux Vitek.

Well	**Test**	**Mnemonc**	**Result**
2	Ala-Phe-Pro-ARYLAMIDASE	APPA	+
3	ADONITOL	ADO	-
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	+
5	L-ARABITOL	IARL	-
7	D-CELLOBIOS	dCEL	-
9	BETA-GALACTOSIDASE	BGAL	-
10	H_2S PRODUCTION	H2S	-
11	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	+
12	Glutamyl Arylamidase pNA	AGL Tp	-
13	D-GLUCOSE	dGLU	+
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	-
15	FERMENTATION/ GLUCOSE	OFF	+
17	BETA-GLUCOSIDASE	BGLU	-
18	D-MALTOSE	dMAL	+
19	D-MANNITOL	dMAN	+
20	D-MANNOSE	dMNE	+
21	BETA-XYLOSIDASE	BXYL	-
22	BETA-Alanine arylamidase pNA	BAlap	-
23	L-Proline ARYLAMIDASE	ProA	+
26	LIPASE	LIP	-
27	PALATINOSE	PLE	-
29	Tyrosine ARYLAMIDASE	TyrA	-
31	UREASE	URE	-
32	D-SORBITOL	dSOR	-
33	SACCHAROSE/SUCROS	SAC	-
34	D-TAGATOSE	dTAG	-
35	D-TREHALOSE	dTRE	+
36	CITRATE (SODIUM)	CIT	-
37	MALONATE	MNT	-
39	5-KETO-D-GLUCONATE	5KG	-
40	L-LACTATE alkalinization	ILATK	+
41	ALPHA-GLUCOSIDASE	AGLU	-
42	SUCCINATE alkalinization	SUCT	+
43	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	-
44	ALPHA-GALACTOSIDASE	AGAL	-
45	PHOSPHATASE	PHOS	-
46	Glycine ARYLAMIDASE	GlyA	-
47	ORNITHINE DECARBOXYLASE	ODC	-
48	LYSINE DECARBOXYLASE	LDC	-
53	L-HISTIDINE assimilation	IHISa	-
56	COUMARATE	CMT	+
57	BETA-GLUCURONIDASE	BGUR	-
58	O/129 RESISTANCE (comp.vibrio.)	O129R	-
59	Glu-Gly-Arg-ARYLAMIDASE	GGAA	+
61	L-MALATE assimilation	IMLTa	-
62	ELLMAN	ELLM	-
64	L-LACTATE assimilation	ILATa	+

+ Positive - Negative

3.3.3. 16S rRNA gene sequence analysis

The nucleotide sequence of the 16S rRNA gene of an isolated suspected *V. parahaemolyticus* showed an identity of 99 to

100 % with sequences of the present *Vibrio* isolates stored in GenBank databases. The nucleotide sequence of PCR yields on behalf of the amplified fragment of 16S rRNA gene of isolated strain was blasted with the extremely parallel sequences in GenBank, NCBI as *Vibrio parahaemolyticus* MAA3. The 16S rRNA partial sequence data of strain *Vibrio parahaemolyticus* MAA3 was deposited in the NCBI database under accession number OM654368. A phylogenetic tree (Figure 5) was also constructed using 16S rRNA gene sequences from the GenBank database.

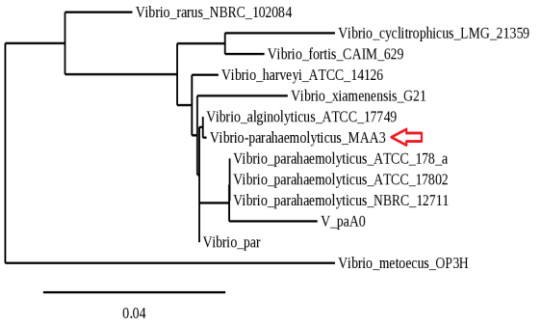

Fig 5: Phylogenetic tree showing evolutionary relationship of *V. parahaemolyticus* MAA3 with the other *Vibrio* species on basis of 16S rRNA gene sequences evolutionary distance.

3.4. Molecular identification of virulent genes of *V.parahaemolyticus* MAA3.

PCR amplification of *tdh, tld, recA, trh,* and *toxR* virulent genes of *V. parahaemolyticus* MAA3 was determined. The strain produced positive amplicon for *tdh* , *tlh* and *toxR* genes that were detected at 373 bp , 449 bp and 368 pb respectively in *V. parahaemolyticus* MAA3 (**Table 4 and Figure 6**). On the other hand, it has negative for *trh* gene.

Table 4: Virulence genes responsible for virulence of *V.parahaemolyticus* .

V. parahaemolyticus	tdh	trh	Tlh	toxR	recA
1	+	-	+	+	+

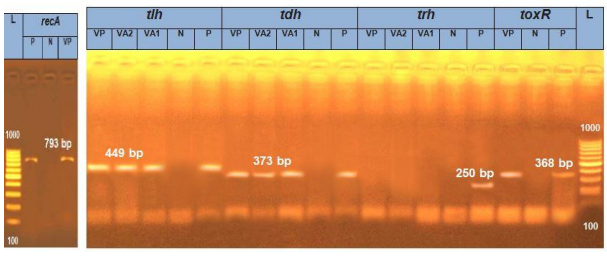

Figure 6: Amplification of 373bp , 449bp and 368pb gene of *tdh* , *trh* and *toxR* gene of *V. parahaemolticus;* Lane L: 100bp marker Sample . N, control negative reaction; P, control positive reaction; PV, 449bp= positive control for *tlh*; gene; PV, 373bp=positive control for *tdh* gene; PV, 368bp positive control for *toxR* , P, control positive reaction for *recA* and control negative reaction for *trh* gene.

3.5. Antimicrobial sensitivity test

The antibiotic sensitivity test of virulent strain of *V. parahaemolyticus* MAA3 showed that, the strain was highly resistant to ampicillin (10 µg), amikacin (30 µg), cefotoxime (30 µg) ,ceftazidime (30 µg), and intermediate sensitivity to erythromycin (15 µg) . On the other hands, it showed highly sensitivity to ceftriaxone (30 µg) ,chloramphenicol (30 µg) , cotrimoxazole (25 µg), trimethoprim (1.25 µg), sulfamethoxazole (23.75 µg), ciprofloxacin (5 µg), doxycycline HCl (30 µg), gentamycin (10 µg) , Levofloxacin(5 µg) , Tetracycline (30 µg), and Tobramycin (10 µg) (**Table 5)**.

Table 5: Antibiotic susceptibility test of virulent strain of *V. parahaemolyticus.*

Antimicrobial Agents	Symbol	Diameter of inhibition zone(mm)			Result
		(R)mm or less	(M) mm range	(S) mm or more	
Ampicillin	AMP	≤13	14-16	≥17	9
Amikacin	AK	≤14	15-16	≥17	14
Cefotoxime	CTX	≤ 22	23-25	≥26	21
Ceftazidime	CAZ	≤17	18-20	≥21	12
Ceftriaxone	CTR	≤19	20-22	≥23	26
Chloramphenicol	C	≤12	13-17	≥18	31
Cotrimoxazole	COT	≤10	11-15	≥16	21
Ciprofloxacin	CIP	≤15	16-20	≥21	25
Doxycycline HCl	DO	≤10	11-13	≥14	25
Erythromycin	E	≤13	14-22	≥23	15
Gentamycin	GEN	≤12	13-14	≥15	15
Levofloxacin	LE	≤13	14-16	≥17	26
Tetracycline	TE	≤11	12-14	≥15	25
Tobramycin	TOB	≤12	13-14	≥15	18

R = Resistant, M = Intermediate, S = Sensitive

Discussion

Recently, fisheries represent an important sector of the Egyptian national income because about 26.7 % of the total Egyptian fish production comes from natural resources [**1]**. Bacterial diseases are highly accountable for the heavy death of rough and farm-cultured fish and grounds simple monetary sufferers to fish farmsteads **[4]**. Vibriosis is measured as an

important pathogenic cause of epidemics in Egyptian aquaculture commerce [**29]**. Vibriosis is a human illness caused by pathogenic species of the family Vibrionaceae [**30]**.

Vibrio parahaemolyticus is a marine bacterium that occurs logically in shellfish, oyster, prawn, crab, raw fish, blue mussels, salted herring, roe, fresh water fish, sea fish, etc. Shrimps have been a source of protein for human consumptions from very early times and the most common *Vibrio* species found in shrimp farming were *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus*. The incidence of *V. parahaemolyticus* in shrimp, and fish, was investigated using cultural and molecular methods (**31)**.

In the present work, the results of certainly infested samples signifying disease and the proven signs of varicolored from dark coloring of casing with separate gages, and hemorrhage at the base of some destruction were consistent with the results of El-Bouhy *et al*. [32] and Al-Taee *et al*. **[33]** in collected samples. Post mortem examination revealed clear internal typical lesions. Internally there was congested friable enlarged liver and congested kidney **.** These findings were agreed with those reported by many authors **[34, 35, 36]**.

Bacteriological examination of isolated *Vibrio* species depends mainly on using TCBS agar as a selective media to differentiate between sucrose and non-sucrose fermenter colonies. The results of identification revealed that isolated *Vibrio* strains are sucrose fermenter and regarded as presumptive *V. parahaemolyticus*. This finding was in lines with results obtained by Shionda [37, 38] and Abdellrazeq and khaliel **(39).** Regular bacteriological scrutiny of total 40 shrimp samples resulted in the recovery of only 5 (12.5%) *V. parahemolyticus* isolates.

The findings of the present study were higher than those reported by Khamesipour [40].

The results of biochemical identification showed that isolated *Vibrio* strain*s* were oxidase, catalase, indole, methyl red and citrate positive [**41, 19].** The results of biochemical identification of the isolated strain revealed about a 99 % probability with *V.parahaemolyticus*. As both pathogenic and nonpathogenic strains of *V. parahaemolyticus* exist in fish and shrimp samples, PCR specific for virulence genes (tdh, trh) will help in the detection of the pathogenic strains [**42].** In the current study, out of 5 presumptive *V parahaemolyticus* isolates were positive for virulence genes (tdh or tlh). Also, the results showed that the strain of *V parahaemolyticus* produce a helpful tdh gene with a positive amplicon at 373bp and this result is in agreement with Mustapha *et al.* [43] and Hernández - Robles *et al*. **[44].**

Several structure placement of the 16S rRNA partial gene sequence of presumptive V. parahaemolyticus showed 99% identity with other *Vibrio* species recorded on GenBank with accession number (OM654368).The molecular identification of the *Vibrio* isolate by using 16SrRNA result in a PCR product with positive amplicons at 663bp and these results are in a good agreement with You *et al*., [45] and Abdelaziz *et al*.**, [34].** 16S rRNA gene was used for approval of biochemically well-known *Vibrio* species **[34].**

Results of antibiotic sensitivity toward a virulent strain of *V. parahaemolyticus* showed that the bacterium was highly resistant to ampicillin, amikacin, cefotoxime, ceftazidime, and intermediate sensitivity to erythromycin. On the other hands, it showed highly sensitive to ceftriaxone, chloramphenicol, cotrimoxazole, trimethoprim, sulfamethoxazole, ciprofloxacin, doxycycline HCl, gentamycin, levofloxacin, tetracycline, and tobramycin [**46**].

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