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Microbiological and molecular characterization of *Vibrio cholera* and *Vibrio parahaemolyticus* isolated from Tilapia fish (*Oreochromis niloticus*)

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Abstract

Vibrio species are zoonotic pathogens that can affect humans by different routes such as ingestion or contact causing gastrointestinal diseases and wound infection. This study aimed to detect *V. cholera* and *V. parahaemolyticus* in Nile Tilapia fish collected from Aswan Governorate, Egypt. A total of 52 muscle samples were collected from Tilapia fish and were subjected to microbiological and molecular characterization. Alkaline peptone water media was used for the enrichment of the samples then followed by inoculation onto thiosulfate citrate bile salt sucrose (TCBS) agar media for the isolation of *Vibrio* species, biochemical tests were performed to identify *V. cholera* and *V. parahaemolyticus*, and then they were confirmed by *Vibrio* genus-specific gene and virulence genes by PCR . Out of 52 fish muscle samples (Nile Tilapia) 32 appeared as yellow colonies, 2 samples showed green colonies and 18 samples showed mixed yellow and green on TCBS agar. Only 40 samples show biochemically positive for *Vibrio* species. 15 random samples were amplified to 16srRNA gene by PCR technique for more accurate identification resulting in 14 isolates being positive to 16srRNA, 8 isolates positive for *sodB* gene-specific of *V. cholera*, and 6 isolates positive for *toxR* gene-specific of *V. parahaemolyticus*. Out of 8 *V. cholera* isolates were found high resistance rate to amoxiclav (87.5%), gentamicin (75%), chloramphenicol (50 %) and ampicillin (50 %). Conversely, (50 %) sensitive to tetracycline, ciprofloxacin and, trimethoprim-sulphamethoxazole, and the average MRA of *V. cholera* was 0.48. On the other hand, all 6 *V. parahaemolyticus* isolates were resistant to amoxiclav (100%), tetracycline (100%), gentamicin (100%) and chloramphenicol (100%) However, all isolates sensitive to trimethoprim-sulphamethazole (100%) but half of them sensitive to ciprofloxacin (50%) and ampicillin (50%), and the average MRA of *V. parahaemolyticus* was 0.59. In conclusion, this study showed that *V. cholera* is the most dominant pathogenic one then followed by *V. parahaemolyticus* in Nile tilapia fish resulting in economic losses and causing public health problems.

Keywords: Vibrio, Tilapia fish, PCR, Resistant

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Competing interest: The authors have declared that no competing interest exists.

Introduction

There is no doubt that human illness as a result of bacterial infection is common, and Vibriosis is a worldwide life-threatening bacterial disease that affects mariculture, resulting in high mortality and severe economic losses. Some *Vibrio* species have been linked to several foodborne disease outbreaks around the world (Gobarah et al., 2021).

The *Vibrio* species is widely distributed in marine and estuarine environments and transmitted to human through eating raw or undercooked seafood can result in gastrointestinal infections (Lee et al, 2018). *Vibrio* is Gram-negative bacteria, halophilic, facultative anaerobic that is associated with high salinity (30–35 ppt), high temperature, parasitic infestation, and mechanical injuries, all of these factors suppress immunity and make fish more susceptible to vibriosis (El-Bouhy et al., 2016).

The pathogenicity of *Vibrio* species is accelerated by a broad range of virulence factors encoded by virulence genes. In general virulence factors allow pathogens to infect and damage the host by enabling pathogenic adherence and entrance, establishment and multiplication and prevention of host defenses (Ruwandeepika et al., 2012).

The major virulence factors of cholera are mainly associated with the *CTX* genetic element which corresponds to *CTX Φ* (prophage), a lysogenic filamentous bacteriophage. The genetic element comprises of two gene clusters, the core and the RS2 regions. The core region contains *ctx* genes encoding the cholera toxin (CT) (Yi Y et al., 2014). Toxigenic *V. cholera* isolates carry the *ctxAB* genes which play a role in the appearance of the effective cholera toxin (Oliver et al., 2013).

In terms of *V. parahaemolyticus* ̕pathogenicity, thermostable direct hemolysin (*tdh*) gene, TDH-related hemolysin (*trh*) gene, T3SS systems (T3SS1 and T3SS2) are the virulence factors own by pathogenic *V. parahaemolyticus* that initiate an infection (Letchumanan et al., 2017*).* The *tdh* and *trh* genes are considered major virulence factors in *V. parahaemolyticus* as they have hemolytic activity in the host cells (Raghunath et al., 2015).

Vibrio can produce adherence factors, which allow it to adhere to surfaces and form biofilms. Biofilm cells are more resistant to disinfectants and antibacterial agents than free-swimming bacteria, so that environmental survival, infectivity, and transmission of this pathogen is enhanced due to its strong biofilm formation ability (Elexson et al., 2014).

V.cholera is a causative agent of cholera. Although cholera is rare in the developed world, it is considered as a major cause of morbidity and mortality worldwide, especially in developing countries in Asia, Africa and Latin America, *V.cholera* is endemic, in places with high density, poor sanitation and access to safe drinking water is scarce (Vezzulli et al., 2013). Cholera is characterized by a secretory acute diarrhea may lead to severe dehydration and death within hours if not promptly treated (Kitaoka et al., 2011*). V.cholera* is transmitted mainly in a faecal–oral mode via contaminated drinking water, although the bacterium can also transmit through person-to-person close contact (Goh et al., 2017).

V. parahaemolyticus causes at least 30000 food borne infections per year so it

causes epidemic and sporadic cases of gastroenteritis after consumption of raw or undercooked seafood (Xie et al., 2015). Gastroenteritis caused by *V. parahaemolyticus* characterized by watery diarrhoea and abdominal cramps in most cases, along with nausea, vomiting, fever and headache. The incubation period is usually between 12 and 24 hours and the disease usually resolves in three days (Rhamtulla et al., 2015*).*

This study aimed to detect the presence of *Vibrio cholera* and *Vibrio parahaemolyticus* in Tilapia fish samples were collected from Aswan Governorate, Egypt which can be achieved by conventional isolation and identification, molecular identification of genus specific primer , species specific primer and some virulence factors by PCR, antibiotic sensitivity test and detection of Biofilm formation.

Materials & Method

Collection and preparation of samples

A total of 52 random samples of apparently healthy Nile Tilapia fish collected from different markets in Aswan governorate, Egypt. All samples were collected in ice box and transferred with a minimum of delay to the microbiology lab, faculty of veterinary medicine, south valley university and were subjected for the bacteriological examination. The fish's scales and fins were removed, and the skin was sterilized by alcohol then flamed by a sterile spatula. After removing the muscles above the lateral line, 5 g were transferred under aseptic conditions to a sterile homogenizer containing 45 ml of sterile alkaline peptone water (3% Nacl and pH 8) (Saad et al, 2015).

Isolation and Identification of *Vibrio* species

A loopfuls from each previous cultured tube were separately streaked onto Thiosulfate citrate bile and sucrose agar (TCBS), then the medium was incubated at 37°C for 24 h (Saad et al., 2015). To purify the *Vibrio* suspect colonies, a single green and yellow colonies from each grown type was streaked onto the other TCBS agar plates and incubated overnight at 37°C. This procedure was repeated until pure consistent colonies were obtained (Azawi et al., 2016). Identification was accomplished based on the results of microscopic observation of stained smears using Gram staining and biochemical examination were carried by using: Indole test, Citrate utilization test, Triple sugar iron test and Salt tolerance test to detect growth of *Vibrio* species on 0%, 3% and 6% Nacl *(*Rahman et al., 2010).

Molecular identification of *Vibrio* species and virulence genes

DNA extraction of *Vibrio* isolates

The boiling lysis technique was used to extract DNA from tryptic soya agar (TSA) colonies. About 2-3 colonies of bacterial cells were suspended in 100 µl of distilled water and mixed gently. The suspension was heated in water bath at 90°C for 10 minutes then centrifuged at 12,000 rpm for 3 min, the supernatant containing the template DNA was stored at −20°C for PCR. (Park et al., 2013).

Detection of *Vibrio 16S rRNA* gene, Species-Specific and Virulence genes

Fifteen random Vibrio species isolates were subjected to molecular confirmation which performed by PCR using 16S rRNA gene to detect *Vibrio* species, *sodB* gene used for detection *V. cholera*, and *toxR* gene used for detection *V. parahaemolyticus*.

ctxAB used to identify cholera toxin and *tdh* gene used to identify thermostable direct hemolysin and *trh* gene used to identify *tdh*-related hemolysin of *V. parahaemolyticus.*

PCR amplification were done by using the published primers, a gene was performed to identify the *Vibrio* genus using a DNA thermal cycler (Thermo cycler, ASTEC, Japan).The list of primers used in our study is shown in the following table (1).

Table (1) Oligonucleotide primers sequences used in this study

Target gene	Sequence	Amplified product	Reference
Vibrio 16S rRNA	CGGTGAAATGCGTAGAGAT TTACTAGCGATTCCGAGTTC	663 bp	*Tarr et al., 2007*
V. parahaemolyticus toxR	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368 bp	*Kim et al., 1999*
V. cholerae sodB	AAG ACC TCA ACT GGC GGT A GAA GTG TTA GTG ATC GCC AGA GT	258 bp	*Tarr et al., 2007*
V. parahaemolyticus trh	GGCTCAAAATGGTTAAGCG CATTTCCGCTCTCATATGC	250 bp	*Mustapha et al., 2013*
V. parahaemolyticus tdh	CCATCTGTCCCTTTTCCTGC CCAAATACATTTTACTTGG	373 bp	
V. cholerae ctxAB	GCCGGGTTGTGGGAATGCTCCAAG GCCATACTAATTGCGGCAATCGCATG	536 bp	*De Menezes et al., 2014*

Under standardized cycling conditions, the PCR assay was optimized in a 25 µl reaction mixture containing 2 µl of DNA template, 12.5 µl of 2x master mix (Go Taq Green Master Mix, Promega), 0.5 µl each of forward and reverse primers (10 pmol/l), and the rest of the volume was made by adding nuclease free water.Cycling conditions of different primers as mentioned in the table (2). PCR products were subjected to gel electrophoresis with 1.5% agarose gel. Then the gel was submerged in ethidium bromide as fluorescent dye and visualized using Gel Documentation unit (BIORAD, USA) (Suresh et al., 2018).

Table (2) Cycling conditions of the different primers during cPCR

Target	Primary denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No. of cycles	
Vibrio 16S rRNA	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. cholerae sodB	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 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 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.**

Detection of Biofilm formation of *Vibrio* species

By using Congo Red Agar

The capsule production of the *Vibrio* strains was evaluated by culturing them on Congo red agar (CRA) as described by (Freeman et al., 1989). Fifteen isolates were studied by cultivation them on Congo Red Agar (CRA) plates, CRA was prepared by adding 0.4g of Congo red dye and 18g of sucrose to 500 ml of distilled water mixed them gently and then autoclaved. Inoculate suspected colonies on CRA plates then incubated at 37°C for 24 hr. Following incubation, Colonies that absorbed Congo red dye (CR+) appeared red colonies but (CR-) remained translucent or white colonies *(*Payne et al., 1977*)*.

By using Microtiter Plates

Bacteria were grown in Tryptic soy broth for *V. cholera* but in *V. parahaemolyticus* add 3% Nacl to broth overnight at 30°C and diluted to 1:100 w/v (in TSB with 1 % glucose). 200 µl of each isolate dilution were seeded in triplicate wells of sterile 96- well microtiter plate and incubated at 37°C for 24 h. After three washing in phosphate buffer saline (PBS), wells were dried and then fixation by methanol then emptied plate and dried for 20 min. After that adherent biofilms were stained with 200 µl crystal violet for 15 minute, then acetic acid was added for solubilization of dye, the absorbance of the adherent biofilm was measured at 490 nm in microplate reader. The Interpretation of biofilm production if OD ≤ ODc = no biofilm producer; ODc< OD ≤ 2 ✕ ODc= weak biofilm producer; 2 ✕ ODc <OD≤4 ✕ ODc= moderate biofilm producer; 4 ✕ ODc <OD= strong biofilm producer (Stepanovic et al., 2007).

Antibiotic sensitivity test

15 isolates of *Vibrio* species, 9 from *V. cholera* and 6 from *V. parahaemolyticus* were subjected to antibiotic susceptibility against 7 antibacterial drugs; ciprofloxacin, tetracycline, ampicillin, gentamicin, chloramphenicol, amoxiclav and Trimthoprim/ sulfamethoxazole. A single colony of the isolate was picked with a sterile loop and inoculated into 2ml of sterile Tryptone Soy Broth. The broth was incubated at 37° C for 4 hours, to obtain young growth. After that, the plates with Muller Hinton agar were prepared and allowed to dry for 5 minutes. With sterile forceps, antibiotic discs were applied to the surface of the inoculated plates. Within 15 minutes of applying the discs, the plates were inverted and incubated at 37°C. The plates were examined after 18 - 24 hours of incubation, and the diameters of the inhibition zones were measured with a transparent plastic ruler *(Ukaji et al., 2015).* The results were recorded as resistant or sensitive and intermediate by comparing the inhibition zone diameter with the *CLSI (2015).*

Determination of MAR index

Determination of MAR index followed the procedure described by (Osundiya et al. 2013) in which the number of antibiotics an isolate is resistant to (a) is divided by the total number of the antibiotics used in the study (b). The calculating formula is shown below:

MAR Index = a /b

Table (3) showed results of isolation and identification of *Vibrio*

Culture on TCBS agar	No of isolates	Biochemical test	No of isolates
Yellow colonies	50 (71.4%)	Suspected *V.cholera*	33 (66%)
Green colonies	20 (28.6%)	Suspected *V. parahaemolyticus*	7 (35%)

Result

Cultural characterization of *Vibrio* species

On TCBS, 52 samples showed the characteristic colonies; yellow colonies were detected in 50 samples and green colonies were detected in 20 samples (Table 3). Mixed colonies (yellow and green) were detected in 18 samples out of the 52 samples.

Biochemical characterization of *Vibrio* species

By Gram (0%) stain; *Vibrio* species appeared as gram negative, curved rod, non-capsulated, non-spore forming and motile organism. In this study, out of 50 yellow colonies 33 samples were biochemically suspected to *V. cholera* and out of 20 green colonies (7) samples were biochemically suspected to *V.parahaemolyticus*. Out of 33*V.cholera* isolates (32) showed positive growth on Nacl (0%), 16 showed growth on Nacl (3%) and only 2 showed growth on Nacl (6%), However out of 7 *V. parahaemolyticus* isolates all 7 showed growth on Nacl and Nacl (3%), but only 3 showed growth on Nacl (6%) as showed in table (4).

Table (4) Growth response of *Vibrio* species to various concentrations of NaCl

Concentration of Nacl %	No. Positive growth response of 33 suspected *V.cholera* isolates	No. Positive growth response of 7 suspected *V. parahaemolyticus* isolates
0 %	32 (96.9%)	7 (100%)
3 %	16 (48.5%)	7 (100%)
6 %	2 (6.25%)	3 (42.8%)

Molecular detection of *Vibrio* species by PCR

Fifteen random suspected isolates amplified for vibrio 16S rRNA, showed 14 isolates positive to 16S rRNA produced clear lighted bands at 663-bp as (fig.1). 9 suspected *V. cholera* isolates amplified for *sodB* showed 8 isolates positive to *sodB* produced light band at 258 bp (fig 2). 6 suspected *V. parahaemolyticus* amplified for *toxR* gene showed all 6 isolates positive to *toxR* produced light band at 368 bp as

(fig.3), then detection of virulence gene (*ctxAB*) of *V.cholera* showed only 3 isolates positive to *ctxAB* (fig.4) and virulence genes of *V. parahaemolyticus* (*tdh* & *trh*) showed 2 isolates postive to *tdh* & *trh* and 1 isolate positive to *tdh* only (fig 5).

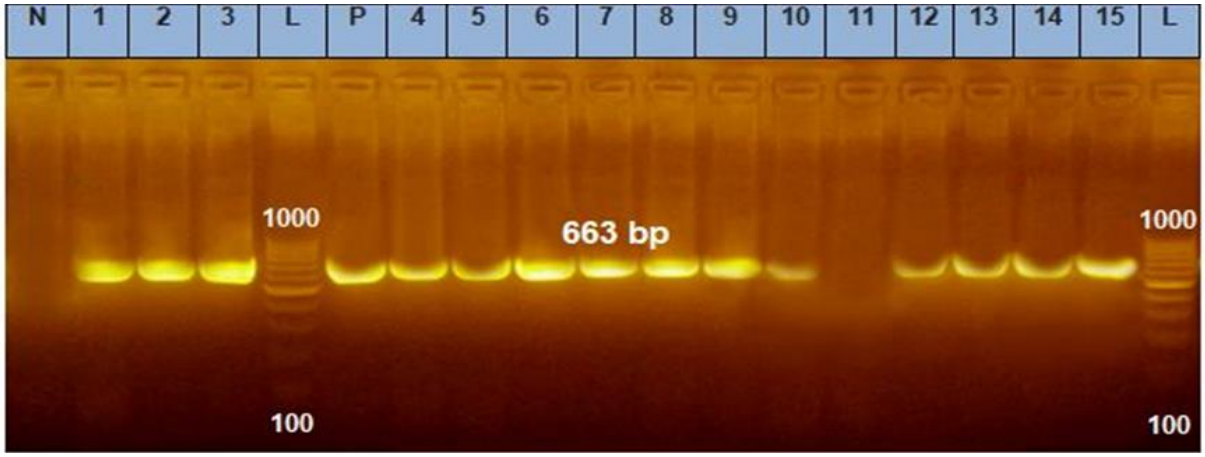

Figure (1) Agarose gel electrophoresis of PCR of 16S rRNA (663bp) for detection of *Vibrio* species, Ladder: 100 bp, N: control negative, P: control positive, Lanes: 1-10 positive for *Vibrio* species, Lane: 11 was negative and Lane 12-15 were positive.

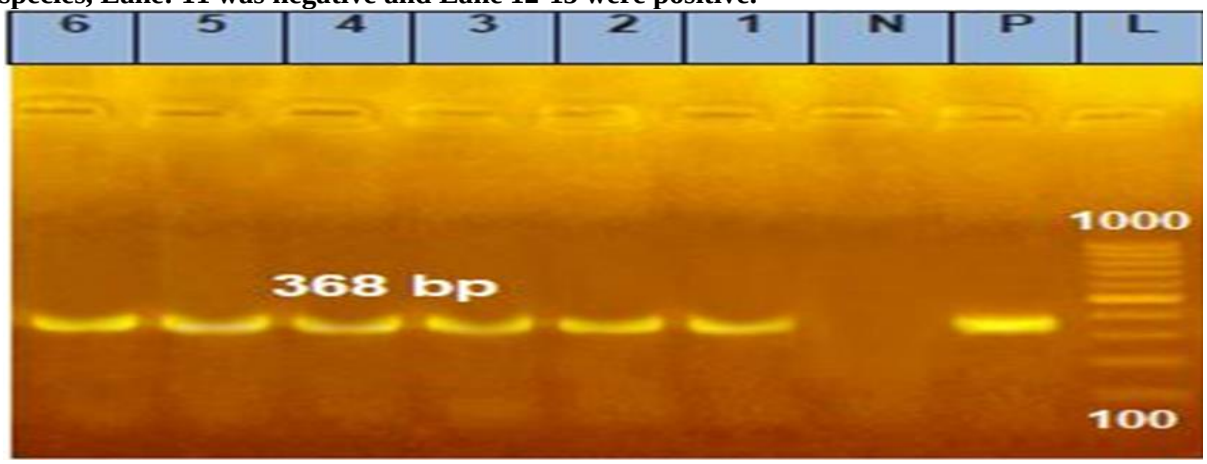

Fig (2) Agarose gel electrophoresis of PCR of *toxR* gene (368bp) for characterization of *V. parahaemolyticus* Ladder: 100bp, P control: positive, N: control negative, Lane: 1-6 were positive for *V. parahaemolyticus.*

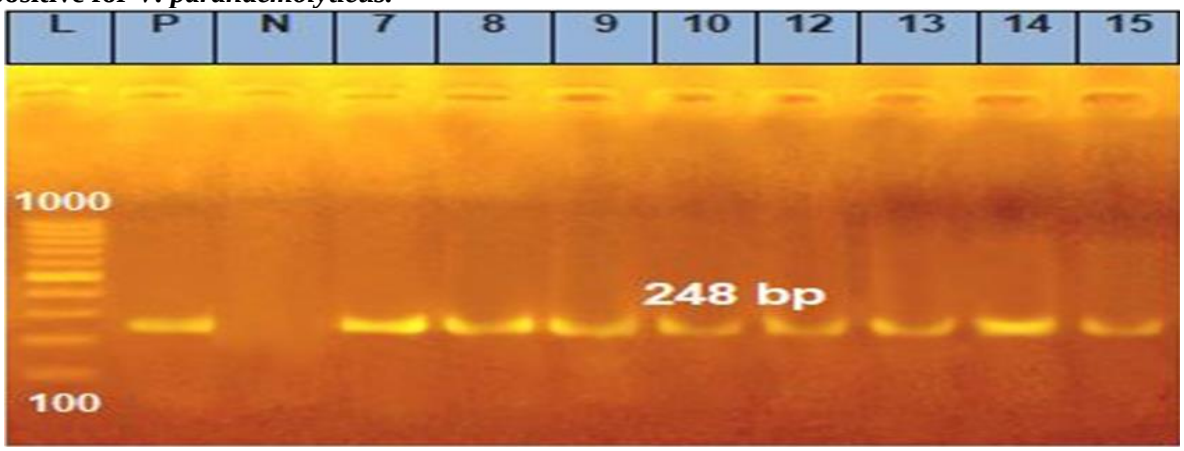

Fig (3) Agarose gel electrophoresis of PCR of sodB gene (248bp) for characterization V. cholera Ladder: 100bp, P: control positive N: control negative and Lane: 7 - 15 were positive for V. cholera.

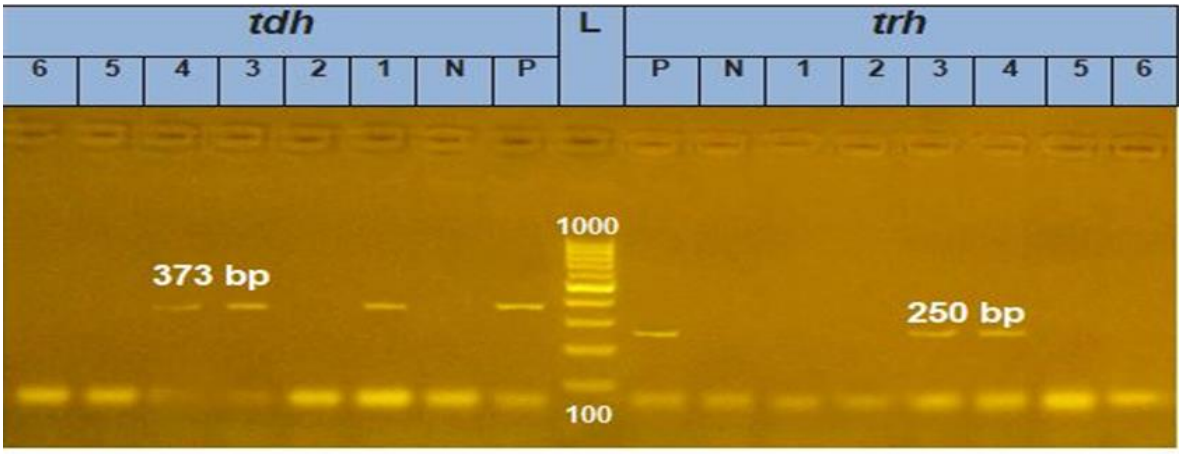

Fig (4) Agarose gel electrophoresis of PCR of *tdh* (373bp) and *trh* (250bp) for of virulence genes of *V. parahaemolyticus* detection Ladder: 100bp, P: control positive, N: control negative, Lane of *tdh* 1, 3& 4 were positive 2, 5& 6 were negative and Lane of *trh* 3& 4 were positive and 1, 2, 5& 6 were negative

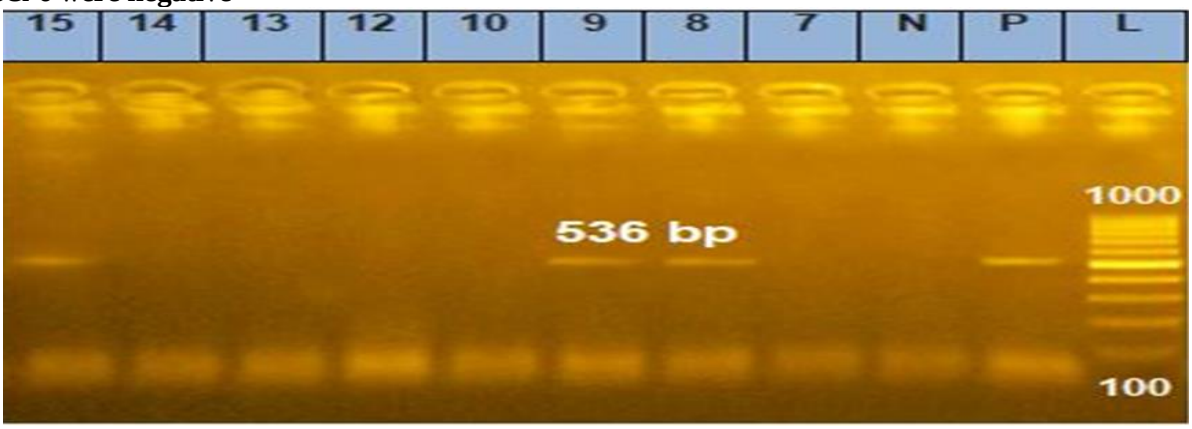

Fig (5) Agarose gel electrophoresis of PCR of *ctxAB* (536bp) for characterization the virulence gene of *V.cholera* Ladder: 100bp P: control positive, N: control negative Lane: 8, 9& 15 were positive and 7, 10, 12, 13& 14 were negative.

Biofilm formation

On Congo red agar out of 8 V. *cholera* isolates 6 (75 %) samples showed red colonies and 2 (25 %) samples showed white colonies. Out of 6 *V. parahaemolyticus* isolates, 5 (83.3%) samples showed red colonies and 1 (16.6%) samples showed white colonies. By using microtiter plate out of 8 *V. cholera* isolates, 3 (37.5%) samples were strong, 3 (37.5%) samples were moderate and 2 (25 %) samples were weak. Out of 6 samples of *V. parahaemolyticus* 4 (66.7%) were strong and 2 (33.3%) were moderate as showed in table (5).

Table (5) Results of Biofilm formation

Number of Isolates	Congo red		Microtiter plate		
	Red	White	Strong	Moderate	Weak
Yellow (8)	6 (75%)	2 (25 %)	3 (37.5%)	3 (37.5%)	2 (25%)
Green (6)	5 (83.3%)	1 (16.6%)	4 (66.7%)	2 (33.3%)	–

Antibiotic sensitivity test

Out of 8 *V. cholera* isolates were found to have a high resistance rate to amoxiclav (87.5%), Gentamicin (75%) and ampicillin (50 %). Conversely, (50 %) sensitive to tetracycline, ciprofloxacin and trimethoprim-sulphamethazole see details in table (6). On the other hand, all *V. parahaemolyticus* isolates (n= 6) revealed that resistant to Amoxiclav, tetracycline, gentamicin and chloramphenicol However, all isolates sensitive to trimethoprim-sulphamethazole and (50%) sensitive to ciprofloxacin see details in table (7).

Table (6) Result of antibiotic sensitivity test for *V. cholera*

Antimicrobial Class	Antimicrobial Agent	Disk diffusion	Number of isolates (%)		
			S	I	R
Penicillins	Ampicillin	10 µg	3 (37.5%)	1 (12.5%)	4 (50%)
	Amoxiclav	30 µg	1 (12.5%)	–	7 (87.5%)
Aminoglycosides	Gentamicin	10 µg	1 (12.5%)	1 (12.5%)	6 (75%)
Tetracyclines	Tetracycline	30 µg	4 (50 %)	1 (12.5%)	3 (37.5%)
Phenolics	Chloramphenicol	30 µg	3 (37.5%)	1 (12.5%)	4 (50%)
Fluoroquinolones	Ciprofloxacin	5 µg	4 (50 %)	1 (12.5%)	3 (37.5%)
Folate Pathway inhibitors	Trimethoprim sulfamethoxazole	1.25/23.75 µg	4 (50%)	3 (37.5%)	1 (12.5%)

Table (7) Result of antibiotic sensitivity test for *V. parahaemolyticus*

Antimicrobial Class	Antimicrobial agent	Disk diffusion	Number of isolates (%)		
			S	I	R
Penicillin	Ampicillin	10 µg	3 (50%)	2 (33.3%)	1 (16.7%)
	Amoxiclav	30 µg	–	–	6 (100%)
Aminoglycosides	Gentamicin	10 µg	–	–	6 (100%)
Tetracyclines	Tetracycline	30 µg	–	–	6 (100%)
Phenolics	Chloramphenicol	30 µg	–	–	6 (100%)
Fluoroquinolones	Ciprofloxacin	5 µg	3 (50%)	3 (50%)	–
Folate pathway inhibitors	Trimethoprim sulfamethoxazole	1.25/23.75 µg	6 (100%)	–	–

Discussion

Fish is a low-cost, delicious animal protein with high nutritional value; it is an excellent substitute for red meat, particularly in developing countries. Expanding global aquaculture helps to meet the increased human population's demand. Egypt ranked eighth in aquaculture production in 2014 (FAO 2016). Pathogens such as *Campylobacter*, *Salmonella*, *Vibrio*, *Listeria monocytogenes*, and *Escherichia coli O157:H7* have been linked to major food-borne outbreaks around the world. (Velusamy et al., 2010).

Vibriosis is a worldwide, life-threatening bacterial disease that affects mariculture, resulting in high mortality and

severe economic losses. Some *Vibrio* species have been implicated in numerous outbreaks of foodborne diseases around the world. The genus *Vibrio* contains over eighty-five species that live in marine and natural habitats of seawater and are found all over the world (Gobarah et al., 2021). Human infection sources either through contact with infected fish or orally by consumption of infected fish (Asran et al., 2020).

In our study, the major of cultured isolates showed yellow colonies which were suspected to be *V.cholera*, the minor of cultured isolates showed green colonies which were suspected to be *V. parahaemolyticus* and the rest of cultured isolates showed mixed yellow and green colonies on TCBS similar to colonies detected by (Azwai et al., 2016) and (Suresh et al., 2015).

By Grams stain, the suspected *Vibrio* species showed gram negative, curved rod, motile, non-capsulated and non-spore forming bacteria according to Asran et al., 2020 and Gobarah et al., 2021. The Biochemical suspected isolates were 40 isolates showed typically result to *Vibrio* species 33 samples suspected to be *V. cholera* and 7 samples suspected to be *V. parahaemolyticus*.

In indole test, the positive *Vibrio* species showed red ring at the top of medium like Passalacqua et al., 2016, this result indicated that *Vibrio* spp. had to release tryptophanase enzyme to convert the tryptophan into indole, ammonia and pyruvic acid Csuros et al., 1999. In citrate utilization test, the color of slant agar of positive *Vibrio* species changes from green to blue similar result to Chanderan et al., 2019. The color changes occurred due to the ability of *Vibrio* to break the ammonium phosphate to ammonia by growing on top of agar slant similar result to Goldman et al., 2009. In triple sugar iron test, *Vibrio* species showed either yellow (acidic) or pink (alkaline) color in slant and yellow (acidic) color in butt of medium similar to Dahanayake et al., 2018.

According to salt tolerance test, *V. cholera* isolates showed positive growth on 0% Nacl and 3% Nacl, but showed negative growth on 6% Nacl. These results agree with *(*Elliot et al., 1995) who detected growth of *V.cholera* on 0% Nacl and 3% Nacl but no growth on 6% Nacl, and slightly disagree with (Eashman et al., 2021) who reported growth of *V.cholera* on 0% Nacl and 6% Nacl but no growth on 8% Nacl. However, *V. parahaemolyticus* isolates showed positive growth on 0% Nacl and 3% Nacl but half isolates were positive and the rest were negative growth on 6% Nacl, these results slightly disagree with *(*Elliot et al., 1995*)* who reported growth on 3% Nacl and 6% Nacl, but no growth on 0% Nacl.

16s rRNA gene is used to confirm biochemically identified *Vibrio* and must produce clear light band at 663 bp as mentioned by Tarr et al., 2007. Species specific genes used for confirmation and differentiation among *Vibrio* species. According to Tarr et al., 2007, *V. cholera* was detected by *sodB* gene that show light band at 248 bp and *V. parahaemolyticus was* identified by *toxR* gene that produced light band at 368 bp as mentioned by Kim et al., 1999.

In our study, the occurrence of *Vibrio* species was found 93.3% compared to 98.67%, 82.87% and 27.7% as reported by Noorlis et al., 2011, Suresh et al., 2018 and El-Hady et al., 2015 respectively, the occurrence of *V. cholera* was found 57.1% this result is higher than the result obtained by Asran et al., 2020 and Arunagiri et al.,

2016 who recorded it with percentage 3.45% and 22.4% respectively and the occurrence of *V. parahaemolyticus* was found `42.9% compared to 75.9%, 50% and 17.24% reported by Anjay et al., 2014, Asran et al., 2020 and Arunagiri et al., 2016.

This difference in prevalence percentages may be related to difference in area, fish species, change in the fish immune system and time and methods of sampling and water quality characters (Abdelaziz et al., 2017). The detection of virulence genes in each species is so important to detect the ability of the bacteria to produce the disease. *Vibrio* species are important pathogen affect fish and fish production (Asran et al., 2020*)*.

The cholera toxin gene is one of the most important virulence genes expressed by *V. cholera*. The CTX element contains the *ctx* genes, which encode the cholera toxin CT. This toxin is primarily responsible for profuse secretory diarrhea of infected people with cholera (shrestha et al., 2015).

The 8 *V. cholera* strains amplified to (*ctxAB*) virulence gene which must produce light band 536 bp according to De Menezes et al., 2014. In our study, only 3 strains produced light band at 536 bp. This result disagrees with (Xu et al., 2019) who reported that all isolates were negative for the toxin genes *ctxAB*.

Both *tdh* and *trh* share several biological properties, such as haemolytic activity, enterotoxicity and cytotoxicity (Park et al., 2004). The 6 *V. parahaemolyticus* strains amplified to 2 virulence genes (*tdh* & *trh*) that must produce light band at 273 & 250 bp respectively according to Mustapha et al., 2013. In this study, only two strains produced light band at 273 &250 bp for (*tdh* & *trh*) respectively, one strain produced only light band at 273 bp for *tdh* but negative for *trh* and 3 strains are negative results to both virulence genes.

These results agree with (Ahmed et al., 2018) who reported that 22.6% of seafood samples harbored *tdh* and/or *trh* genes. Additionally, (Xie et al., 2015) reported that 45.9% isolates were positive for only the *trh* gene.

As it is important to detect biofilm formation ability of *Vibrio* species. (Lamari et al., 2018) recommended the Congo Red Agar plate method and polystyrene microtiter plates tests as useful tools for the detection of biofilm formation by *Vibrio* isolates. In our study, the assays performed with the CRA plate method showed the mechanism behind the adhesion of *Vibrio* strains on abiotic surfaces (microtiter polystyrene plate). As by using the CRA plate method, we showed the 11 isolates (6 *V. cholera* and 5 *V. parahaemolyticus*) appeared as red colonies on the Congo red agar which indicate the ability of these strains to produce capsule and plays an important role in biofilm formation. Also, by microtiter plate seven isolates of vibrio (3 *V. cholera* and 4 *V. parahaemolyticus* were formed strong biofilm. their biofilm-forming ability presents a significant public health concern because the biofilms are thought to conserve and absorb nutrients, to be resistant to antibiotics, and to form promising associations with other bacteria or hosts. They are known to revert to an active vegetative state for development and proliferation in a favorable environment (Hall-Stoodley et al., 2004).

Antimicrobial resistant bacteria are becoming prevalent as a result of the widespread use of antibiotics. This is a

major source of concern in both human and animal health because it reflects the drug usage pattern (Xie et al., 2015). In our study, *V. cholera* isolates found resistance rate to ampicillin (50%) and amoxiclav (87.5%). However, (50%) susceptible to ciprofloxacin, tetracycline and trimethoprim-sulfamethoxazole. A similar result found by Shrestha et al., 2015 who reported all clinical *V. cholera* strains were resistance to ampicillin (100%) and susceptible to tetracycline (100%) and (90.9%) was susceptibility to both ciprofloxacin and chloramphenicol. Dengo-Baloi et al., 2017 reported that *V. cholera* from 2012 to 2015 outbreaks had (100%) resistance to ampicillin, (89%) to chloramphenicol, (75%) to trimethoprim /sulphamethazole, (50%) to tetracycline and (0%) to ciprofloxacin.

According to *V. parahaemolyticus* all isolates showed resistant to amoxiclav, gentamicin, tetracycline and chloramphenicol. In contrast, high susceptibility rate to trimethoprim /sulphamethazole (100%) and ampicillin (50%). Lee et al., 2018 reported that the resistance rate of the 165 *V. parahaemolyticus* isolates to ampicillin was (88%). In contrast, high susceptibility rate was seen to chloramphenicol (93%), tetracycline (90%), gentamicin (84%), sulfamethoxazole/trimethoprim (80%) as similar results found by Letchumanan et al., 2019 a large number of isolates showed resistance to ampicillin (85%) and high susceptibility rate was seen to chloramphenicol (92.5%), tetracycline (83.1%), trimethoprim-sulfamethoxazole (75.8%) and gentamicin (70.6%). The difference in antibiotic resistance rate is mainly related to continuous and extensive use of these antibiotics and then it reflects the pattern of drug use (Letchumanan et al., 2015).

In our study, we found the value of MAR index of *V. cholera* range from 0.28 to 0.71 and the average MAR index of *V. cholera* was 0.48 but value of MAR index of *V. parahaemolyticus* ranged from 0.57 to 0.71 and the average MAR index of *V. parahaemolyticus* was 0.59. These results slightly agree with Ahmed et al., 2018 who reported the average MAR index of both *V. cholera* and *V. parahaemolyticus* was 0.678, Xu et al., 2019 reported the values of MAR of the 400 *V. cholera* isolates ranged from 0.00 to 0.70, and Deng et al., 2020 reported the value of MAR of *Vibrio* isolates ranged from 0.00 to 0.60. The difference in the MAR indices might be contributed to samples' origin and source as well as the testing methods (Lesley et al., 2011).

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