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Existence and characteristics of *Vibrio* species isolated from fish marketed in Sohag governorate, Egypt and their control by essential oils

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Abstract

The aims of this study were isolation and characterization of *Vibrio* species isolated from fish meat. A total 100 fish samples including chilled *Tilapia nilotica*, chilled cat-fish (*Clarias gariepinus*), frozen mackerel and frozen shrimp were collected from various markets in Sohag governorate, Egypt. *Vibrio* species were isolated from 41% of the examined samples. The prevalence of *Vibrio* was the highest in chilled *Tilapia nilotica* (80%) followed by frozen shrimp (40%) then frozen mackerel (32%) finally chilled cat-fish was (12%). Seven *Vibrio* species were identified using the biochemical tests including *Vibrio cholerae, Vibrio metschnikovii, Vibrio parahaemolyticus, Vibrio carchariae, Vibrio vulnificus, Vibrio damsel* and *Vibrio mimicus*. For further confirmation selected isolates were identified by a multiplex PCR by using species-specific primers to amplify gene regions in three species *sodB* gene for *Vibrio cholerae*, *flaE* gene for *Vibrio parahaemolyticus* and *Hsp60* gene for *Vibrio vulnificus*. The isolated *Vibrio* species was analyzed for their susceptibility to four antibiotics trimethoprim/sulfamethoxazole, amikacin, streptomycin and erythromycin. All isolates were susceptible to trimethoprim/sulfamethoxazole except *Vibrio mimicus* Furthermore, the resistance of *Vibrio cholera* against streptomycin and erythromycin was recorded. Testing the ability of *Vibrio* for biofilm formation on Congo red plates was resulted in some *Vibrio* species including *Vibrio cholera*, *Vibrio metschinkovii* and *Vibrio damsela* had the ability to form biofilms. The impact of some essential oil including oregano, olive and rosemary oils was investigated. As result, rosemary essential oil had a great antibacterial activity against all isolated *Vibrio* species while both oregano and olive oil had no antibacterial activities on *Vibrio* species. The results of the current work concluded the occurrence of *Vibrio* species in some examined fish samples and rosemary essential oil is a promising compound for controlling *Vibrio*.

Keywords: *Vibrio*, PCR, Antibiotic resistance, Essential oils, Biofilm.

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Introduction

Fish is an important source of protein and other nutrients as it contains vitamins, minerals and essential fatty acids, fish is on high demand by most people, not only because of its wide availability, but because it is known as a cheap and safest source of animal protein (FAO, 2012).

Vibrio species are one of the most important groups of bacteria that cause food borne diseases as a result of the consumption of partially cooked fish or shellfish or contaminated fish (Anjay et al., 2014). Diseases caused by *Vibrio* species were reported in aquatic animals such as fish, oysters, shrimp and lobster (Chrisolite et al., 2008). Members of the genus *Vibrio* are gram negative, rods that have a single rigid curve or straight and are motile with a single polar flagellum when grown in liquid medium (Kaysner et al., 2004). The number of *Vibrio* species known as pathogenic strains is at least eleven strains including, *Vibrio* parahaemolyticus as the main cause of foodborne gastroenteritis, *Vibrio* cholerae as the main cause of diarrhea (Holmberg et al., 1992) and *Vibrio* vulnificus that cause 95% of all deaths associated with seafood consumption (Rosche et al., 2006).

Vibrio cholerae, the causing agent of cholera is broadened in marine and fresh water fish. Specified serogroups O1and O139 of this bacteria are responsible for epidemics and pandemics, Non-O1 and non-O139 *Vibrio cholera* serogroups are also related to *Vibrio cholera* gastroenteritis as well as wound infection and bacteremia (Deshayes et al., 2015). *Vibrio parahaemolyticus* is a common cause of foodborne illness that causes diarrhea and gastroenteritis among population all over the world and responsible for food poisoning (Christopher et al., 2011).

As an important human pathogenic bacterium, *Vibrio* vulnificus has been associated with a small but increasing number of serious life-threatening conditions such as gastro-enteritis and wound infections which might become septicaemic (Mouzopoulos et al., 2008). Indeed, a regular source of infection with this pathogen is the consumption of contaminated raw or undercooked seafood (Drake et al., 2007).

Its importance is considered as a contaminant of raw or undercooked seafood and may lead to acute gastroenteritis including headache, diarrhea, nausea, vomiting and fever (Yang et al., 2008).

The long-dated use of chemical preservatives in large quantities may cause various troubles. Hence, utilization of natural preservatives has extended as an alternative for harmful chemical preservatives. Essential oils and herbal extracts are considered to be excellent natural antimicrobial compound for insurance of food safety (Alizadeh et al., 2017).

Antimicrobial resistance recognized as a significant global threat issue to food security and global public health (FAO, 2016). Hence, the aims of the current works were investigation the occurrence of *Vibrio* species in various types of fish, characterization, analysis antibiotic susceptibility and the ability to form biofilm of *Vibrio* bacterium. Moreover, study the antibacterial activity of some essential oils against *Vibrio* species.

Materials and methods

Sampling method

A total of 100 samples including chilled *Tilapia nilotica*, cat-fish, mackerel

and shrimps (25 each) were collected randomly from the local fish market at Sohag governorate, Egypt. The samples were transferred into cool ice boxes with an internal temperature of +2 to +4°C after collection and were processed within a short time after arrival in Food Hygiene and Control Laboratory at the Faculty of Veterinary Medicine, University of south valley.

Enumeration and identification of *Vibrio* species

Muscles (flesh) of each sample were used for analysis, a 10.0g of muscle sample was enriched in 90 mL alkaline peptone water (APW) by incubation at 37˚C for 18 – 24 h. Two loop full of the culture broth taken from the layer of the APW and undergone a series of tenfold dilutions. From each dilution 1mL was plated on thiosulfate – citrate – bile salt – sucrose agar (TCBS Oxoid Ltd., Basingstoke, England) by the pour plate method and incubated at 37˚C for 18 – 24 h. (FDA, 1992). The green and yellow isolates (Fig. 1) were enumerated and used for further tests according to the biochemical key reported by Jayasinghe et al. (2008) that was designed by using Bergey's manual and FDA manual.

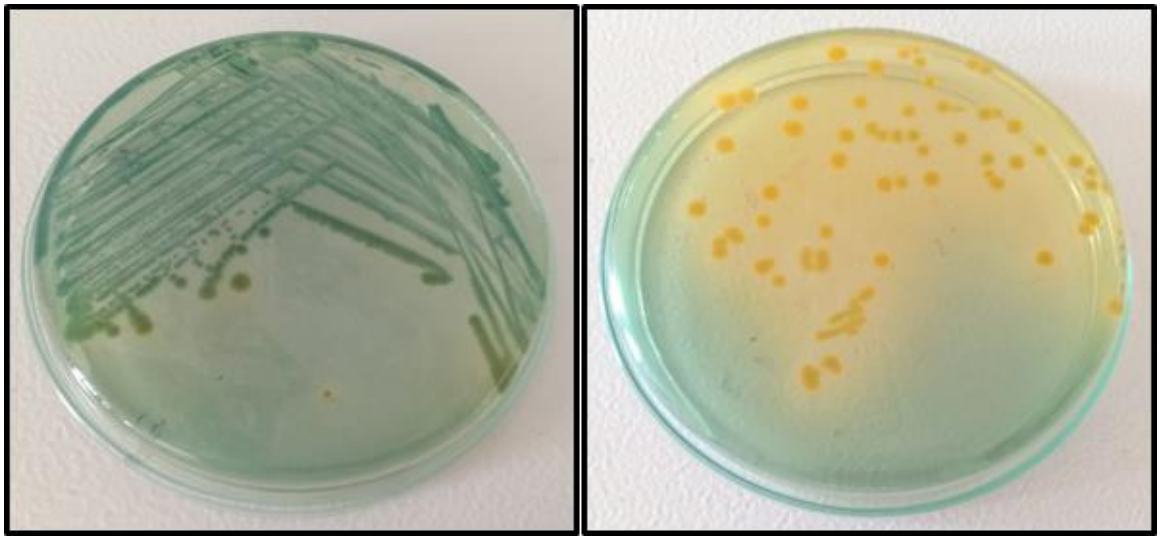

Fig. 1: *Vibrio growth* on TCBS agar: (a) green colonies produced by *vibrio parahaemolyticus, (b)* yellow colonies produced by *vibrio cholera*

Biochemical Tests

Oxidase test was conducted by using oxidase test discs (Mast ID oxidase, UK). Furthermore, *Vibrio* species were tested for their ability to grow in 0 and 6% to determine their requirement for Na+ (Choopun et al., 2002). Voges-Proskauer (VP) was performed as reported by Twedt et al., (1984). O-nitrophenyl-beta- D-galactosidase (ONPG) test was carried out by using the ONPG disks (Liofilchem, Italy).

Molecular identification of the isolated *Vibrio* species

Some isolates of *Vibrio* (*Vibrio* cholera, *Vibrio* parahaemolyticus and *Vibrio* vulnificus) were selected for further confirmation. The three sets of oligonucleotide primers used in PCR for detection of sodB, flaE and Hsp60 genes (Table 1). Extraction of DNA according to QIAamp DNA mini kit instructions.The PCR conditions were illustrated in Table 2.

Table1: primers used in PCR for detection of sodB, flaE and Hsp60 genes

Target	Gene	Sequence	Amplified product	Reference
Vibrio cholera	*sodB*	AAG ACC TCA ACT GGC GGT A	248 bp	Tarr et al., 2007
		GAA GTG TTA GTG ATC GCC AGA GT		
Vibrio parahaemolyticus	*flaE*	GCA GCT GAT CAA AAC GTT GAG T	897 bp	
		ATT ATC GAT CGT GCC ACT CAC		
Vibrio vulnificus	*Hsp60*	GTC TTA AAG CGG TTG CTG C	410 bp	
		CGC TTC AAG TGC TGG TAG AAG		

Table 2: PCR conditions

Target gene	Primary denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No. of cycles	
sodB	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
flaE	94˚C 5 min.	94˚C 30 sec.	57˚C 1 min.	72˚C 1 min.	35	72˚C 10 min.
Hsp60	94˚C 5 min.	94˚C 30 sec.	57˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

Antibiotic sensitivity test

Preparation of inoculated test plates and discs implementation Muller Hinton agar plates were all set aseptically for antibiotic sensitivity (Bauer et al., 1966). Colonies were selected and transferred to 5.0 ml of Soyabean Casein Digest Medium (Tryptone Soya Broth) (Micromaster, India). The inoculum was incubated at 35ºC for 2-8 h for the development of moderate turbidity. *Vibrio* species were tested against erythromycin (15 µg), amikacin (30µg), trimethoprim/ sulphamethaxole (1,25/23,75µg), and streptomycin (10µg) (Bioanalyse ASD /TURKEY). Results were interpreted as sensitive, moderate sensitive and resistant using the Clinical and Laboratory Standards Institute (CLSI 2015 and 2018).

Phenotypic characterization of slime-producing *Vibrio*

Qualitative detection of biofilm formation was studied by culturing the obtained *Vibrio* strains on Congo red agar (CRA) (Freeman et al., 1989) .*Vibrio* strains were inoculated into Soyabean Casein Digest Medium (Tryptone Soya Broth) (Micromaster, India) incubated for 2-8 h at 35º to form a moderate turbidity, furthermore on the surface of CRA plates were incubated for 24 h at 30ºC under aerobic conditions and followed overnight at room temperature, slime producing bacteria appeared as blackish colonies, while non-slime producers remained non pigmented (Sechi et al., 2002).

Study the impact essential oils on isolated *Vibrio* species

The antibacterial activities of the selected essential oils including rosemary oil, olive oil and oregano oil on some isolated *Vibrio* species were studied by agar well diffusion assay techniques (Reeves 1989). In this method, 100 µL of standardized inoculum of each test bacterium (*Vibrio* cholera, *Vibrio* parahaemolyticus, *Vibrio damselae*, *Vibrio vulnificus*, *Vibrio metschnikovii* and *Vibrio mimicus*) were spread onto sterile Muller–Hinton Agar. 8 mm diameter well was cut from the agar using a sterile cork-borer; then each well was filled with 100 µL of the essential oil. The plates were reversed at room temperature for 1 h to allow proper diffusion of the oil into agar and then incubated at 37 °C for 24 h. The clear inhibition zones were recorded in millimeters (Raid et al. 2014).

Results

Bacteriological assay

The results revealed that 41 samples (41%) were contaminated with *Vibrio* species and the mean values were ranged from to 1.2x10 ±1.8 x 10 to $1.4x10^4$ ± 7.3 $x10^3$. chilled Tilapia nilotica showed a high contamination level (80%) followed by shrimp (40%), then mackerel (32%) while cat-fish demonstrated a low contamination level (12%).The chilled Tilapia nilotica show obvious high contamination level with mean value$1.4x10^4$+ 7.3 $x10^3$ CFU/g (Table 3).

Table 3: Statically analytical results of total *Vibrio* count (CFU/g) of the examined fish samples (n=25)

Samples	Positive Samples		Count CFU/g		
	Number	%	Minimum	Maximum	Mean ± SE
Chilled *Tilapia nilotica*	**20**	**80%**	**$1 x 10^2$**	**$9.1 x10^4$**	**$1.4x10^4$ ± 7.3 $x10^{3a}$**
Chilled Cat Fish	**3**	**12%**	**$1 x 10^2$**	**$1.3 x 10^2$**	**$1.1 x10^2$ ± $1.1x10^a$**
Frozen Mackerel	**8**	**32%**	**$1 x 10^1$**	**2.0 x10**	**1.2x10 ±$1.8x10^a$**
Frozen Shrimp	**10**	**40%**	**$1 x 10^2$**	**$1.1x10^4$**	**$2.9x10^3$ ±$1.7 x10^{3a}$**

Letters indicated there were no statistically significant difference between the means at $p < 0.05$

The isolated *Vibrio* species were identified into *Vibrio* cholerae, *Vibrio* metschnikovii, *Vibrio* parahaemolyticus, *Vibrio* carchariae or *Vibrio* harveyi, *Vibrio* vulnificus, *Vibrio* damsela, *Vibrio* mimicus. The results of our study revealed that *Vibrio* cholerae was isolated from chilled Tilapia nilotica followed by shrimp. *Vibrio* damsela was isolated only from chilled Tilapia nilotica. *Vibrio* harveyi was isolated only from cat-fish. On the other hand, *Vibrio* mimicus was highly isolated from chilled Tilapia nilotica followed by mackerel. Other *Vibrio* species were isolated with various percentages (Table 4).

Table 4: Prevalence of various *Vibrio* species in examined fish products samples collected from Sohag Governorate Markets.

Samples	No.	*Vibrio cholerae*		*Vibrio parahaemolyticus*		*Vibrio vulnificus*		*Vibrio damsela*		*Vibrio harveyi*		*Vibrio metschnikovii*		*Vibrio mimicus*	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Chilled Tilapia	25	3	12%	1	4%	3	12%	3	12 %	0	0%	4	16%	6	24 %
Chilled Cat Fish	25	0	0%	1	4%	0	0%	0	0%	1	4%	1	4%	0	0%
Frozen Mackerel	25	0	0%	2	8%	1	4%	0	0%	0	0%	4	16%	1	4%
Frozen Shrimp	25	1	4%	2	8%	1	4%	0	0%	0	0%	6	24%	0	0%
Total	100	4	4%	6	6%	5	5%	3	3%	1	1%	15	15%	7	7%

Molecular confirmation of the isolated *Vibrio* species

The isolated *Vibrio* cholera, *Vibrio* parahaemolyticus and *Vibrio* vulnificus were confirmed by detection of specie specific genes sodB, flaE and Hsp60, respectively. The results showed the compatibility with biochemical tests results. All the examined isolates for sodB, flaE and Hsp60 were positive as showed in Fig. 2

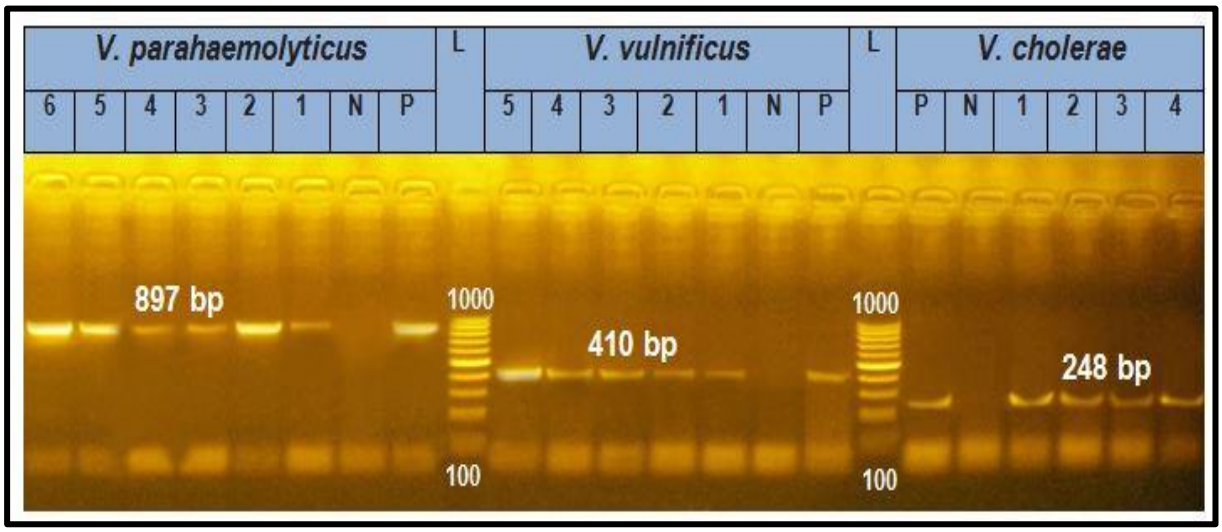

Fig. 2: DNA products from PCR reaction of amplification of *sodB, flaE and Hsp60* genes from isolated *Vibrio* species

Antibiotics susctibility

Four antibacterial compounds were used in disc diffusion tests. The interpretation as sensitive, intermediate and resistant was obtained from the Clinical and Laboratory Standards Institute breakpoints specific for *Vibrio* species (CLSI 2015 and 2018). Breakpoints not available from CLSI for erythromycin were derived from a similar study (Baron et al. 2016). All 12 selected isolates were susceptible to trimethoprim/ sulfamethoxazole except *Vibrio mimicus*. The highest resistance rates were observed for streptomycin (5 isolates) and erythromycin (4 isolates) as shown in Fig.3 (Table 5).

Table 5: Antibiotic susceptibility of *Vibrio* species with antibiotic discs

species	Isolate	Resistance	Intermediate	Sensitive
Vibrio cholera	VC1	S, E	AK	SXT
	VC2	S, E	-	AK, SXT
Vibrio parahaemolyticus	VP1	-	-	S, E, AK, SXT
	VP2	E	-	S, AK, SXT
	VP3	-	-	S, E, AK, SXT
Vibrio damselae	VD	S, E, AK	-	SXT
Vibrio mimicus	VMI	S, SXT	-	E, AK
Vibrio metschnikovii	VME1	S, AK	-	E, SXT
	VME2	-	-	S, E, AK, SXT
	VME3	-	-	S, E, AK, SXT
Vibrio hyrvie	VH	-	-	S, E, AK, SXT
Vibrio vulnificus	VV	-	-	S, E, AK, SXT

(AK-30 µg) Amikacin, (S-30 µg) Streptomycin, (E-15 µg) Erythromycin and (SXT 25/23,75µg) Trimethoprim/sulphamethoxazole

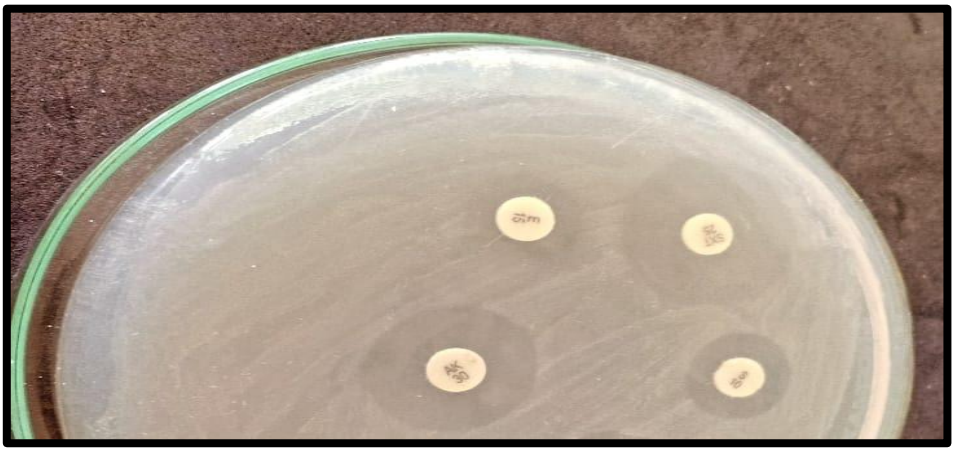

Fig. 3: Antibiotic sensitivity test of *Vibrio* isolate

Slime production on CRA plates

The isolated *Vibrio* cholerae, *Vibrio* metschnikovii and *Vibrio* damsela gave blackish colonies on Congo red media that means they can form biofilm while other species were characterized by pinkish red colonies as shown in Fig.4, and recorded in Table 6.

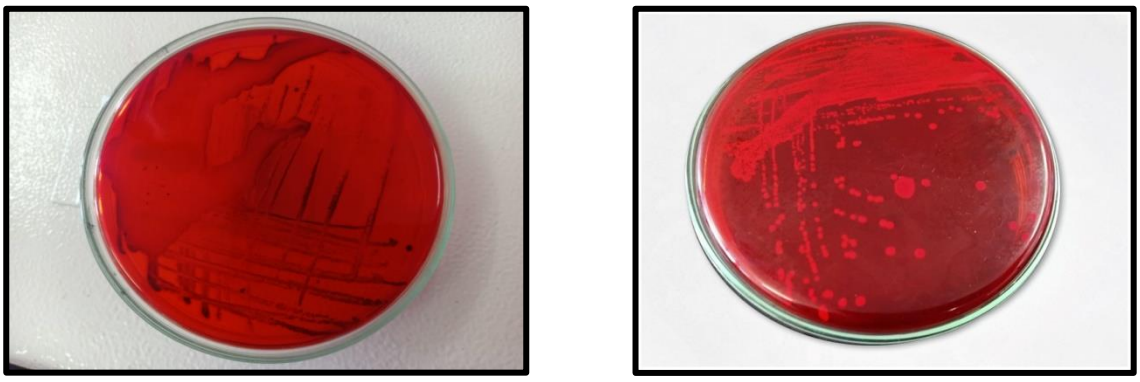

Fig. 4: Testing the ability of *Vibrio* species for producing biofilm on Congo red agar: (a) blackish biofilm producer, (b) pinkish red non-biofilm producer

Table 6: Ability of *Vibrio* species to form Biofilm

Species	Isolate	Biofilm production	Phenotype of strain on CRA
Vibrio cholera	VC1	+	Blackish
	VC2	+	Blackish
Vibrio parahaemolyticus	VP1	-	Pinkish red
	VP2	-	Pinkish red
	VP3	-	Pinkish red
Vibrio metschnikovii	VME1	+	Blackish
	VME2	-	Pinkish red
	VME3	+	Blackish
Vibrio damselae	VD	+	Blackish
Vibrio mimicus	VMI	-	Pinkish red
Vibrio vulnificus	VV	-	Pinkish red
Vibrio hyrvie	VH	-	Pinkish red

Data are offered as: positive (+), negative (-) for biofilm production

Study the impact essential oils on isolated *Vibrio* species

Rosemary essential oil had a fabulous antibacterial activity against all selected *Vibrio* species, on the other side oregano and olive oil couldn't inhibit any *Vibrio* isolate as shown in Fig.5 and recorded in Table 7.

Table 7: Study the effect of various essential oil on the isolated *Vibrio* species (Diameter of inhibition zone in mm)

Species	Isolate	Oregano oil	Olive oil	Rosemary oil
Vibrio cholera	VC2	-	-	40 mm
Vibrio parahaemolyticus	VP2	-	-	50 mm
	VP3	-	-	55 mm
Vibrio damselae	VD	-	-	20 mm
Vibrio vulnificus	VV	-	-	55 mm
Vibrio metschnikovii	VME1	-	-	50 mm
	VME2	-	-	70 mm
	VME3	-	-	45 mm
Vibrio mimicus	VMI	-	-	30 mm

(-): no inhibition zone was detected.

Fig. 5: Essential oils effect on *vibrio* growth
1. Oregano oil 2. Olive oil 3. Rosemary oil: clear inhibition zone

Discussion

Bacteriological evaluation of fish for the occurrence of *Vibrio* species is important as they are designations of meat quality as well, they may induce food borne illness. The results showed that 41% (41/100) samples were contaminated with *Vibrio* species. The obtained findings were higher than those obtained by Scharer et al. (2011) who proved that *Vibrio* species were found in 45 samples out of 138 ones and Raissy et al. (2013) who revealed that 29.3 % out of the examined fish samples were *Vibrio* positive. These disparate results may be imputed to species differences, in addition to low salinity in River Nile, as Vibriosis is more prevalent in brackish and marine water (Noga, 2010).

The count of *Vibrio* obtained from fish was in range from 1.2 x 10 $\pm$ 1.8x10 to $1.4x10^4 \pm 7.3\ x10^3$ CFU/g. Higher count was obtained by Ebob et al. (2022) who reported that the mean count of *Vibrio* obtained from fish in Nigeria was 11.37±4.82 CFU/g. The obtained data in our study revealed that chilled *Tilapia nilotica* showed a high Vibriosis contamination level (80%), this result was higher than those obtained by Anwar et al. (2010) who reported that the Vibriosis incidence of infection varied among fish type with lowest one in Nile tilapia (12.8%).

The contamination level with *Vibrio* in shrimp was 40% this result is lower than those obtained by Amin et al. (2011) who demonstrated that isolated the percentage of Vibrio species from shrimp fish was 57.3% and Merwad et al. (2011) who reported that the prevalence of Vibriosis was 57.3% in examined white shrimp fish. However, the obtained result was higher than Bakr-Wafaa et al. (2011) who detected *Vibrio* species in 32 % of the total examined shrimp fish.

Cat-fish had the lowest contamination level in current study. The ability, survival and persistence of *Vibrio* to cause infection was attributed to several factors such as sunlight, water temperature and salinity (Lipp et al. 2002).

Notably 6 % of fish samples contain *Vibrio paraheamolyticus* this result was

lower than results were obtained by Yang et al. (2008) who reported that 14.9% of iced and frozen seafood samples in two coastal areas of eastern China were contaminated with *Vibrio parahaemolyticus*. In our study the incidence of *Vibrio* mimicus was 6%, higher results were obtained by Adebayo-Tayo et al. (2011) who reported that the incidence of *Vibrio* mimicus in Nigeria was 15% in fresh seafood samples. The incidence of *Vibrio* parahaemolyticus in cat-fish in our study was 4%, higher results obtained by Noorlis et al. (2011) who isolated *Vibrio parahaemolyticus* from 25% of the cat-fish samples.

In our study, *Vibrio cholerae, Vibrio metschnikovii, Vibrio parahaemolyticus, Vibrio harveyi, Vibrio vulnificus, Vibrio damsela, Vibrio mimicus* were detected *but Vibrio alginolyticus , Vibrio furnissi* and *Vibrio fluvialis* failed to be detected, this result differs from that obtained by Saad et al. (2015) who isolated *Vibrio alginolyticus*, *Vibrio fluvialis, Vibrio damsela, Vibrio furnissi* and *Vibrio mimicus* while, *Vibrio cholerae* and *Vibrio parahaemolyticus* not detected biochemically.

In current study one isolate of *Vibrio harveyi* was identified in cat-fish, this agreed with Austin and Zhang (2006) who reported that *Vibrio harveyi* is a well-known pathogen of both vertebrates and invertebrates and fish infections by this bacterium have been reported from both cultured and wild species. *Vibrio harveyi* was identified as yellow colored colony on TCBS agar as Turgay and Karataş (2016) who proved that all isolates of *Vibrio harveyi* visually appearing as white shiny colonies on Marine agar and yellow-colored colonies on TCBS agar.

The isolates were confirmed by using species specific genes via the multiplex PCR. The isolates were identified as *Vibrio* species by conventional biochemical tests were tested for the *sodB*, *flaE*, *Hsp60* genes-based multiplex PCR to confirm the identification as Raissy et al. (2015) did.

Our study proved that *Vibrio cholera*e can form slime this agreed with Ryjenkov et al (2005) who mentioned that the *Vibrio cholerae* possesses a dual mode of survival, it has the ability to survive as a surface biofilm in aquatic bodies, where it can thrive for years in between cholera epidemics. These surface biofilms are resistant to external stress like predators, antibiotics, chlorine and other factors.

Our study showed that all selected *Vibrio* species were sensitive to trimethoprim/sulfamethoxazole except *Vibrio mimicus*. *Vibrio cholera* showed resistance against streptomycin, erythromycin and was intermediate sensitivity to amikacin that agrreded with that reported by Morshdy et al. (2022). *Vibrio parahaemolyticus* and *Vibrio vulnificus* were sensitive to streptomycin unlike previous studies have suggested that resistance to this antibiotic is common in *Vibrio parahaemolyticus* isolates (Elexson et al., 2014 and Shaw et al., 2014). *Vibrio parahaemolyticus* exhibited both resistant and susceptible characters against streptomycin as reported by Abdul wahab khan et al. (2007). The difference in immune response of fish makes difference in resistance to antibiotics, the type those strains isolated from fish with higher immune responses may have developed mechanisms for enhanced survival compared to fish with lower immunity (Smith et al., 2019). We obtained that *Vibrio parahaemolyticus* also sensitive to erythromycin, amikacin and

trimethoprim/sulfamethoxazole except one isolate resist erythromycin, *Vibrio vulnificus* showed sensitivity to the four selected antibiotics. *Vibrio hyrvie* was sensitive to the four selected antibiotics not in line with Scarano et al. (2014) who reported that over 80% of *Vibrio hyrvie* from fish in Italy showed resistant to ampicillin, amoxicillin and erythromycin.

The effects of essential oils as antibacterial compound were studied in accordance to Harris (2003) who demonstrated that the active constituents in essential oils, influence lots of biochemical processes in the pathogenic bacterial strains, exhibiting interactive cumulative antibacterial effects, we found that rosemary oil showed a great antibacterial activity against all seven different *Vibrio* species, this result agreed with Edris (2007) who reported that essential oils had antimicrobial properties.

The results of this study indicated that *Vibrio* species was a potential pathogen that might be found in fish and shrimps purchased at Sohag governorate and might affect badly on human health. Rosemary essential has a great antibacterial effects on *Vibrio* species and can be considered as a promising anti-*Vibrio* compound.

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