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Prevalence of Antibiotic-Resistant *Vibrio* Isolated from Some Marketed Fish in Egypt with a Decontamination Trial by Lemon juice

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Abstract

Vibrio species are major sea foodborne pathogens that cause gastroenteritis as a serious disease of human public health due to the consumption of undercooked or raw fish. In the current study, a total of 100 fish samples (Nile tilapia, Nile perch, Meagre, and Sea bass) were collected randomly from retail markets in Egypt to investigate the prevalence of *Vibrio* species. The results revealed that *Vibrio* species isolated with an overall percentage of 52% of all examined fish. Bacteriological and chemical examinations revealed 42.3% *V. parahaemolyticus*, 26.92% *V. mimicus*, 19.23% *V. alginolyticus*, 9.62% *V. vulnificus* and 1.92% *V. cholera*. Antibiotic sensitivity declared high resistance of the isolates to different antimicrobial agents used in Egypt including Ampicillin (100%), Nalidixic acid (88.3%), Streptomycin (84.2%), Sulphamethoxazol (70.7%) and Oxytetracycline (64.8%) and it had sensitivity to Amikacin (94%), Ciprofloxacin (70.5%), Gentamicin (58.9%) with an average MAR index of 0.576. By polymerase chain reaction, all examined *Vibrio* isolates were positive for 16SrRNA specific for *Vibrio* spp. and harbored *tox*R gene virulence gene. Finally, dipping of tilapia in lemon juice 5% for 2 h reduced *V. parahaemolyticus* count by 0.42log cfu/g (62.08%). Consequently, hygienic measures should be approved to control the contamination of fish in the markets and the aquatic environment. Regular monitoring of fish for antibiotic resistance by *Vibrio* species, and their molecular characterization is necessary to improve the safety of seafood. Dipping fish in lemon juice is an efficient strategy for reducing *V. parahaemolyticus* load in fish.

KEYWORDS
Antibiotic resistance, Fish, Lemon juice, *Vibrio* spp., Virulence gene

INTRODUCTION

Aquatic species are mostly high in nutrients of global importance, including high-quality animal protein, a major source of essential amino acids, omega-3 fatty acids, its richness in phosphates and calcium, and its supply of many vitamins (Byrd *et al*., 2021). Although seafood is a nutritious element, it is incriminated as a possible cause of many foodborne pathogens. *Vibrio*s are of the most common widespread foodborne pathogens in surface waters associated with food poisonings (Trinanes and Martinez-Urtaza, 2021). They are naturally occurring in the estuarine-marine and freshwater habitats worldwide in association with aquatic animals. The most important *Vibrio* spp. associated with the consumption of raw or undercooked fish are *V. parahaemolyticus* and *V. vulnificus*, however *V. cholera*e is mainly associated with waterborne disease (Baker-Austin *et al*., 2017). The pathogenicity of *V. parahaemolyticus* associated with seafood is confirmed by the presence of the *tox*R gene that is associated with cytotoxic activity and hemolysis of *V. parahaemolyticus* in the host cell (Ahmed *et al*., 2018). Antibiotics are used mainly in fish farms for treating, preventing infections, and promoting growth which causes an increase in *Vibrio* species resistance to several used antibiotics and causes a potential effect on public health due to transmission of resistant bacteria through food or the transmission of resistance genes to consumers (Binh *et al*., 2018; Liu *et al*., 2018). Specific organic acids are used for controlling microbial contamination and deploying foodborne pathogens during food production and processing because of their inhibiting effect against *V. parahaemolyticus*. Citric acid represents the primary organic acid in lemon juice which is used for flavoring and preservation for food (Nawi *et al*., 2017). It is considered a natural preservative for meat due to its antioxidant and antimicrobial activities (Morshdy *et al*., 2021a). Consequently, the current study was planned to evaluate the prevalence of *Vibrio* spp. in marketed fish samples in Sharkia and Cairo Governorates, Egypt. The biochemical identification, virulence gene determination, and antibiotic resistance profile of *Vibrio* isolates were scrutinized. The antibacterial effect of lemon juice on *V. parahaemolyticus* was also investigated.

MATERIALS AND METHODS

Samples collection

One hundred fish samples (Nile tilapia, Nile perch, Meagre, and Sea bass), 25 of each were collected randomly from Sharkia

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and Cairo Governorates, Egypt, and aseptically transferred to the Meat Hygiene Laboratory, Food Control Department, Faculty of Veterinary Medicine, Zagazig university, Egypt.

Isolation and identification of Vibrio spp.

Vibrio spp. isolation was performed according to FDA (2004). About 10 grams of fish flesh were homogenized aseptically in 90 mL of sterile alkaline peptone water (APW, Micro Master - India) and incubated for 24–48 h at 35±2°C (ISO-TS-21872-1, 2007). A loopful of each inoculated APW broth was streaked onto Thiosulfate citrate bile salts sucrose agar plates (TCBS, Hi-Media - India) and the plates were incubated at 37°C for 24 h. The presumptive *Vibrio* colonies (yellow-green or greenish blue) were picked up, purified, and then biochemically identified according to ISO/ TS 21872-1 (2007) and ISO/ TS 21872-2 (2007).

Molecular identification

Biochemically identified colonies were genetically verified using 16SrRNA primers specific for *Vibrio* species then confirmed by the presence of specific primers targeting the *tox*R virulence gene to identify *V. parahaemolyticus*. Extraction of bacterial DNA was performed according to QIAamp DNA Mini kit manufacturer's guidelines (QIAGEN GmbH, Hilden, Germany, Catalogue no.51304). The amplification of the 16SrRNA gene and *tox*R gene was performed using primers from Metabion (Germany). The 16SrRNA primers were sense 5'CGGTGAAATGCGTAGAGAT'3 and antisense 5'TTACTAGCGATTCCGAGTTC'3 with 663 bp. product size (Tarr *et al*., 2007). The primers used for detection of *tox*R gene were sense 5'GTCTTCTGACGCAATCGTTG'3 and antisense 5' ATACGAGTGGTTGCTGTCATG '3 with 368 bp. product size (Kim *et al*., 1999; Kim and Lee, 2017).

Antibiotic susceptibility

The antibiotic susceptibility test of 34 *Vibrio* isolates from 5 different *Vibrio* genera was performed against 14 different antibiotics by Kirby–Bauer disc diffusion method. The single-disk diffusion method was performed as motioned by Amalina *et al*. (2019) and the zones of inhibition were estimated according to NCCLS (2001). The antibiotics used (Oxoid Limited, Basingstoke, Hampshire, UK). The Multiple antibiotic resistance index (MAR) for each isolate was determined as MAR index = a (the number of antibiotics that the isolate is resistant to) / b (total number of antibiotics tested).

Using lemon extract as natural potential decontaminant

Two concentrations (3% and 5%) of lemon extract were prepared as dipping solutions for the inoculated tilapia fish. For each experiment, a total of 12 tilapia fish samples (200 grams, each) were aseptically divided into four groups (3 fish, each). The experimental trials were repeated in triplicates. One ml of the *V. parahaemolyticus* broth that was adjusted to 0.5 McFarland was inoculated by pipetting over each fish. The inoculated fish were remaining at room temperature (25°C) for 30 minutes. Then fish has divided into four groups; the 1st group was positive control and was dipped in sterile distilled water; the 2nd group was dipped in lemon extract solution of 3%; the 3rd group was dipped in lemon extract solution of 5%, and the 4th group was negative control (without the microorganism inoculation). All treated and control groups were examined to estimate the antimicrobial effect of lemon extract against *V. parahaemolyticus* after 0.5, 1 and 2 h.

Statistical analysis

All bacteriological values have existed as means ± standard error (S.E). All statistics were evaluated at a 95% level of confidence by SPSS and One-way analysis of variance (ANOVA). Significant differences between the means were performed by the DUNCAN test. P-values less than 0.05 were considered statistically significant.

RESULTS

Bacteriological examination of fish samples revealed that *Vibrio* species present with an overall percentage of 52%. The highest isolation percentage was in Nile perch (80%) but the lowest isolation percentage was in Nile tilapia (16%) (Table 1). The most predominant isolated *Vibrio* species were *V. parahaemolyticus* (42.3%), followed by *V. mimicus* (26.92%), and the lowest % was *V. cholera* (1.92%) (Table 1).

It was found that all examined *V. parahaemolyticus* isolates were positive for 16S RNA and *tox*R gene (Figure 1).

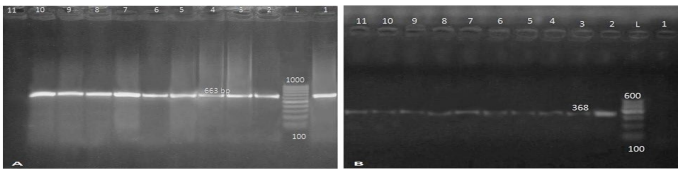

Fig. 1. Exemplar for 1.5% agarose gel. L:100- bp Ladder. (A) Amplification of 16SrRNA specific for *Vibrio* spp., (11: negative control, 1: positive control 2: 10 positive *Vibrio* isolates). (B) *V. parahaemolyticus tox*R gene (1: negative control, 2: positive control, 3:11 *V. parahaemolyticus* isolates harbored *tox*R gene).

Table 2 illustrates 100% resistance of the tested *Vibrio* isolates to Ampicillin, 88.3% and 84.2% resistance to Nalidixic acid and Streptomycin, respectively. Resistance profile for MRI of *Vibrio* isolates ranged from 0.071 to 1 with an average of 0.576 (Table 3).

Results in Table 4 revealed that dipping tilapia fish in lem-

Table 1. Prevalence and species of *Vibrio* isolates in raw marketed fish samples (No = 25 of each)

Fish	Number (%) Positive	*Vibrio* species									
		V. alginolyticus		*V. parahaemolyticus*		*V. cholera*		*V. vulnificus*		*V. mimicus*	
		No.	%	No.	%	No.	%	No.	%	No.	%
Nile tilapia	4(16%)	2	3.85	2	3.85	-	-	-	-	-	-
Nile perch	20(80%)	-	-	10	19.23	-	-	2	3.85	8	15.38
Meagre	15(60%)	8	15.38	2	3.85	-	-	3	5.77	2	3.85
Sea bass	13(52%)	-	-	8	15.38	1	1.92	-	-	4	7.69
Total	52(52%)	10	19.23	22	42.3	1	1.92	5	9.62	14	26.92

The percentage of positive samples were calculated from total examined. The percentage of *Vibrio* spp. was calculated from positive isolates.

on juice solution of 5% for 30 min reduced *V. parahaemolyticus* count by 0.31log cfu/g (45.88%).

Table 2. Antimicrobial susceptibility and Resistance profile of *Vibrio* species (No = 34)

Antibiotic	Pattern of Resistance					
	Sensitive		Intermediate		Resistant	
	NO	%	NO	%	NO	%
Ampicillin (AM)	-	-	-	-	34	100
Nalidixic acid (NA)	-	-	4	11.7	30	88.3
Streptomycin (S)	4	11.7	2	6	28	84.2
Sulfamethoxazole (SXT)	4	11.7	6	17.6	24	70.7
Oxytetracycline (T)	8	23.5	4	11.7	22	64.8
Cephalothin (CN)	10	29.2	2	6	22	64.8
Chloramphenicol (C)	6	17.6	8	23.5	20	58.9
Kanamycin (K)	6	17.6	12	35.3	16	47.1
Erythromycin (E)	12	35.3	6	17.6	16	47.1
Norfloxacin (NOR)	16	47.1	4	11.7	14	41.2
Cefotaxime (CF)	14	41.2	8	23.5	12	35.3
Gentamicin (G)	20	58.9	8	23.5	6	17.6
Ciprofloxacin (CP)	24	70.5	2	6	8	23.5
Amikacin (AK)	32	94	-	-	2	6

Table 3. Antimicrobial resistance profile of *Vibrio* strains isolated from examined raw marketed fish samples (No = 34)

Strain	Pattern	Antimicrobial resistance profile	No. of isolates	No. of antibiotic	MAR index
V. alginolyticus	I	AM, NA, S, SXT, T, CN, C, K, E, NOR, CF, G, CP, AK	2	14	1
	II	AM, NA, S, SXT, T, CN, C, K, E, NOR	2	10	0.714
	III	AM, NA, S, SXT	2	4	0.286
V. parahaemolyticus	I	AM, NA, S, SXT, T, CN, C, K, E, NOR, CF, G	3	12	0.857
	II	AM, NA, S, SXT, T, CN, C, K, E, NOR, CF	2	11	0.786
	III	AM, NA, S, SXT, T, CN, C, K	2	8	0.571
	IV	AM, NA, S	2	3	0.214
V. cholera	I	AM, NA, S, SXT, T, CN, C, K, E, NOR, CF, G	1	12	0.857
V. vulnificus	I	AM, NA, S, SXT, T, CN, C, K, E, NOR, CF, G	2	12	0.857
	II	AM, NA, S, SXT, T, CN, C	2	7	0.5
V. mimicus	I	AM, NA, S, SXT, T, CN, C, K, E, NOR, CF	2	11	0.786
	II	AM, NA, S, SXT, T, CN, C, K, E, NOR	2	10	0.714
	III	AM, NA, S, SXT, T, CN, C, K, E, NOR	2	10	0.714
	IV	AM, NA, S, SXT, T, CN, C	2	7	0.5
	V	AM, NA, S	2	3	0.214
	VI	AM, NA	2	2	0.143
	VII	AM	2	1	0.071
				Average	0.576

AM: Ampicillin, A: Nalidixic acid, S: Streptomycin, SXT: Sulphamethoxazol,T: Oxytetracycline, CN: Cephalothin, C: Chloramphenicol ,K: Kanamycin, E: Erythromycin, NOR: Norfloxacin, CF: Cefotaxime, G: Gentamicin, CP: Ciprofloxacin, AK: Amikacin

Table 4. Effect of Lemon juice on *V. parahaemolyticus* count log_{10}cfu/g in fish after different exposure time

	½ hour			1 hour			2 hours		
	Control	Lemon juice		Control	Lemon juice		Control	Lemon juice	
		L. (3%)	L. (5%)		L. (3%)	L. (5%)		L. (3%)	L. (5%)
Minimum	7.45	7.33	7.19	7.37	7.25	7.03	7.31	7.06	6.81
Maximum	7.79	7.65	7.53	7.65	7.45	7.37	7.55	7.4	7.21
Mean±S.E.	7.62±0.04^{a}	7.49±0.07^{ab}	7.36±0.10^{ab}	7.51±0.06^{ab}	7.35±0.05^{ab}	7.20±0.09^{bc}	7.43±0.08^{ab}	7.23±0.09^{bc}	7.01±0.16^{c}
R. count		0.13	0.26		0.16	0.31		0.2	0.42
R. %		25.9	45.08		30.86	51.23		36.8	62.08

Values within the same raw carrying different superscript letters are significantly different. L.: Lemon extract.

DISCUSSION

Vibrio spp. are microbial foodborne pathogens of water sources that are mainly present in many kinds of seafood, which increase the susceptibility of humans to public health risks (Semenza and Paz, 2021). In the current study, 52% of *Vibrio* species were identified in fish. *V. parahaemolyticus* were identified in 42.3% while *V. cholera*e was found in 1.92%. A higher isolation rate of 82.85% (87 from 105) in freshwater fish was reported in India from which 6 (6.9%) were *V. parahaemolyticus*, 2 (2.3%) *V. vulnificus*, 4 (4.6%) *V. alginolyticus* and 3 (3.45%) *V. cholera* (Suresh *et al*., 2018). In Egypt, *Vibrio* species were isolated from 39% (78/200) of freshwater fishes, from which 23 (29.48%) were *V. harveyi*, 22 (28.20%) *V. anguillarum*, 13(16.67%) *V. vulnificus*, 12(15.38%) *V. alginolyticus* and 8 (10.25%) *V. fluvialis* (El-Sharaby *et al*., 2018). However, a low *Vibrio* isolation percentage of 16% in crustaceans, *V. parahaemolyticus*, and *V. cholera*e were identified at 15.1% and 0.9% (Ahmed *et al*., 2018). *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*, were the most isolated *Vibrio* species from 100 marine fishes (Abdelaziz *et al*., 2017). In China, *Vibrio* species were isolated from freshwater fish as 10.33% (*V. cholera*e), 3.89% (*V. parahaemolyticus*), and 1.24% (*V. alginolyticus*), and 0.76% (*V. vulnificus*) (Yan *et al*., 2019). The variation in *V. parahaemolyticus* incidences may be attributed to improper handling, deficiency of hygiene, variation in storage temperature, and cross-contamination (Letchumanan *et al*., 2015).

The PCR-based assay targeting the *tox*R gene, which is well conserved between *V. parahaemolyticus*, became a popular molecular technique for the detection and identification of *V. parahaemolyticus* in seafood samples (Kim *et al*., 1999; Kim and Lee, 2017; Ahmed *et al*. 2018; Yen *et al*. 2021; Zaafrane *et al*. 2022). The results in the current study indicated the presence of 16Sr-RNA and a regulator toxin (*tox*R) in all examined isolates. All *V. parahaemolyticus* isolates harbored the *tox*R genes were reported by Yen *et al*. (2021) and Zhang *et al*.(2018). However, a lower percentage was reported by Almejhim *et al*. (2021) who found that 26 out of 120 isolates (21.7%) of *V. parahaemolyticus* were positive for the *tox*R gene. Meanwhile, Narayanan *et al* (2020) confirmed 648 *V. parahaemolyticus* by *tox*R gene out of the 721 presumptive isolates.

The presence of antimicrobial residues in aquaculture products represents serious human public health hazards. This health risk occurs through the direct spread of bacterial acquired resistance or indirect spread of resistance genes by horizontal gene transfer from aquatic environments to humans (Sun *et al*., 2015).

Vibrio isolates in the current study were extremely resistant to Nalidixic acid, Streptomycin, Sulfamethoxazole, Oxytetracycline, and Cephalothin and recorded 100% Ampicillin resistance. This agreed with other studies (Liu, 2017; Ahmed *et al*., 2018; Sony *et al*., 2021). This resistance shows public health alarms since these drugs are used for human disease treatment (Xie *et al*., 2017). In Nigeria, the highest resistance was recorded against Amoxicillin, Augmentin, Gentamicin, Erythromycin, Tetracycline, and Streptomycin (Sony *et al*., 2021). In china, *V. parahaemolyticus* from aquatic isolates were resistant to Streptomycin (90.53%), Ampicillin (33.68%), and Cephalothin (30.53%) with no resistance to Nalidixic acid, Ciprofloxacin or Azitromycin (Xie *et al*., 2017). In India, *Vibrio* species were resistant to Ampicillin (93.38%), Gentamicin and Ceftazidime (80%, for each), Amikacin (66.66), Penicillin (60%), Tetracycline (33.33%), and Streptomycin (6.66%), respectively (Suresh *et al*., 2018). In Malaysia, *V. parahaemolyticus* from fish express resistance to Kanamycin (50%), Amikacin (64%), and Ampicillin (88%) with MRI less than 0.2 for 70% of the isolates (Lee *et al*., 2018). Nearly lower Ampicillin resistance (90%) and Amikacin (60%) in Brazil for *V. parahaemolyticus* and the MAR index was above 0.2 (Adinortey *et al*., 2020)

In the current study, more than 50% of *Vibrio* isolates were resistant to more than five antibiotics and most isolates declare multiple MAR index ranging from 0.071 to 1 with an average of 0.576 that are > 0.2, which point to contamination from hazard sources and show acquired a genetic resistance leading to public hazards to consumers (Tambekar *et al*., 2006; Letchumanan *et al*., 2015). The MAR indices > 0.2 for *V. parahaemolyticus* isolates have been reported by Xie *et al*. (2017) and Ahmed *et al*. (2018) indicating that antibiotics are used widely and randomly. In Malaysia, the isolated *V. parahaemolyticus* express a higher MAR index of 0.36 (Letchumanan *et al*., 2015). The MAR index in Tunisian coast seawater samples ranged from 0.4 to 0.5 (Zaafrane *et al*., 2022). The difference in the MAR index may be due to variation in the geographic distribution, sample sources, and methodology used. Higher MAR in the existing study revealed that the *Vibrio* isolates are from samples of high-danger sources; consequently, surveillance for antimicrobial resistance is necessary.

Treatment with organic acids is one of the modern food processing technologies which depend on non-thermal processing for supplying microbiologically safe and healthy food products. Organic acids are commonly utilized as preservatives and food additives for their antimicrobial activities to extend the shelf life of consumable food (Mathur and Schaffner, 2013; Wang *et al*., 2015). The application of acetic acid can greatly reduce the microbial population on meat surfaces.

In the present study, the antimicrobial activity of lemon juice extract with concentrations of 3% and 5% as a potential decontaminant to raw fish was inoculated with *V. parahaemolyticus*. The results show that the reduction percentage of *V. parahaemolyticus* was 45.08%, 51.23%, and 62.08% after dipping in fresh lemon juice extract of 5% for 0.5, 1, and 2 h, respectively. The antimicrobial effect of lemon juice on *V. parahaemolyticus* as a natural decontamination for seafood was determined by Nawi *et al*. (2017) and the treatment of *V. cholera*e was proved by Sushmita (2022) and Tsai *et al*. (2021). Moreover, a significant reduction counts of *V. parahaemolyticus* in Tilapia fillet pieces, after marinating in freshly squeezed lime juice at 25°C for 30 and 120 min was determined by Ibrahim *et al*. (2018) and Kato *et al*. (2018). The effect of lemon essential oil (1%) was investigated by Morshdy *et al*. (2021b) and they found that it had the greatest acceptable sensory score and antimicrobial properties. Lemon juice is mainly used due to its availability, low price, and minimum or no side effects. The high-level content of citric acid, bioflavonoids, limonene, pectin, calcium, magnesium, and vitamins in lemon juice potentiate its antiviral, antibacterial, and immune building against disease and infection (Alsaraf *et al*., 2016).

CONCLUSION

It was concluded that a higher prevalence level of antibiotic-resistant *Vibrio* species, in local marketed fish harboring *tox*R gene that poses a human public health hazard. It is recommended to apply suitable food safety measures for monitoring fish quality along the food production chain and consumption, the misuse of antibiotics in aquaculture should be authorized under veterinary inspection. The obtained results suggest using natural organic acids like lemon juice as a potential decontaminant for decreasing the microbial load of *V. parahaemolyticus* and improving the fish quality and safety for human consumption.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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