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Prevalence of Antibiotic Resistant *Aeromonas* and Molecular Identification of *Aeromonas hydrophila* Isolated from Some Marketed Fish in Egypt

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Abstract

Aeromonas hydrophila, is an important foodborne bacterial disease in the aquaculture. The present study aimed to investigate the prevalence of *Aeromonas* species, virulence genes associated in *A. hydrophila* and to determine the antimicrobial susceptibility of *Aeromonas* spp. isolated from fish samples (tilapia, mugil, tuna, saurus, pagrus and shrimp) collected from Zagazig city markets, Sharkia Governorate, Egypt. *Aeromonas* spp. was isolated with a percentage of 39.3% of all examined fish samples. Four *Aeromonas* species (*A. hydrophila*, *A. caviae*, *A. fluvialis* and *A. sobria*) were isolated from the tested fish samples (12%, 15.3%, 2.7% and 9.3%, respectively). *A. hydrophila* was only isolated from Tilapia, Saurus and Shrimp samples (16%, 28% and 28%, respectively). Aerolysin (*aerA*) and haemolysin (*ahh1*) were expressed in 100% and 75% of the *A. hydrophila* isolates. Antibiotic susceptibility testing of *Aeromonas* spp. revealed marked resistant for testing antibiotics; Ampicillin (100 %), Erythromycin (100 %), Tetracycline (83.3 %), Sulphamethoxazol (75 %), Cefotaxime (50 %) and Cephalothin (50 %). Dipping of Nile tilapia in lemon juice 5% for 2 h reduced *A. hydrophila* counts by 0.45 log cfu/g (64.44%). In conclusion, the present study confirms contamination of fish by *Aeromonas* spp. Immersion of fish in in lemon juice 5% is an efficient policy for reducing *A. hydrophila* in fish.

KEYWORDS
Fish, *Aeromonas* spp.,Antimicrobial susceptibility, *aerA*, *ahh1*, Lemon juice

INTRODUCTION

Fish and shellfish are considered one of the vital sources of nutrition. It provides human with essential fatty acids especially Omega 3 fatty acids, amino acids, high quality animal protein, vitamins and minerals that required for health and growth. Quality deterioration of fresh fish rapidly occurs during handling and storage fish products leading to limitation of its shelf life (Hafez *et al*., 2018). Some exogenous factors such as polluted waters, post capture contamination (FAO, 2014), improper unhygienic storage and handling of fish (Sarkar *et al*., 2013) could accelerate fish and shellfish spoilage via pathogenic and spoilage bacteria and poses a health risk during processing of food (Al Bulushi *et al*., 2010). *Aeromonas* species are commonly associated with many environmental sources and food (Das *et al*., 2013). *Aeromonas hydrophila*, *A. caviae* and *A. sobria* are the most common *Aeromonas* spp. incriminated in human gastrointestinal disorders (Stratev *et al*., 2012). *A. hydrophila* is considered one of the most important zoonotic foodborne pathogens. It had serious impact on human including gastroenteritis, skin and wound infections, meningitis, septic arthritis, diarrhea (traveler's diarrhea) and fulminating septicemia (Salunke *et al*., 2015). In addition to endocarditis, osteomyelitis and a high mortality rate in immune-compromised persons (Cetin and Sarimehmetoglu, 2009). The pathogenesis of *A. hydrophila* is mainly due to presence of numerous virulence factors including, aerolysin (*aerA*) and haemolysin (*ahh1*) (Martin-Carnahan and Joseph, 2015).

Extensive use of antibiotics in the aquaculture for controlling *A. hydrophila* infection is considered the main cause to increase antibiotic resistance levels and disseminating of multidrug-resistant bacteria (Yildirim-Aksoy and Beck, 2017). Direct spread of antibiotic resistant bacteria to consumers through food or via resistance genes transmission poses a possible human health hazard (Binh *et al*., 2018). With increasing of consumers' demand for natural, non-toxic and safe preservatives, lemon juice could be used in an economic and safe way to decrease the contamination of bacteria in sea food and to limit the illness associated with seafood due to its antioxidant and antimicrobial activities (Fadel and El-Lamie 2019). The objectives of the current study were to determine the prevalence and types of *Aeromonas* spp. in fresh and marine fish in Egypt, detect *aerA* and *ahh1* virulence associated genes in the identified *A. hydrophila* isolates and to investigate antibiotic resistance profiles among isolated *Aeromonas* strains. In addition to evaluate the effect of lemon juice in the reduction of *A. hydrophila* count.

MATERIALS AND METHODS

Collection of samples

A total of 150 fish samples (tilapia, mugel, tuna, saurus,

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pagrus and shrimp), 25 of each were randomly collected from different localities and fish markets at different sanitation levels in Zagazig city, Sharkia Governorate, Egypt.

Isolation of Aeromonas species

Ten gram of fish flesh were aseptically homogenized in 90 mL of alkaline peptone water (APW, Micro Master - India) and incubated for 18 h at 30˚C (Villari *et al*., 2000). A loopful of each turbid inoculated APW broth was streaked onto *Aeromonas* Agar media (Lab M, UK) and incubated at 37°C for 18-24 h. Suspected colonies (translucent, pale green colonies) were purified and biochemically identified (Carnahan *et al*., 1991).

Molecular identification

DNA extraction was performed according to the manufacturer's instructions of QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany). *A. hydrophila* isolates that were biochemically identified in the current study were subjected to molecular identification (Wang *et al*., 2003) of *aerolysin* (*aerA*) and *haemolysin* (*ahh1*) virulence associated genes. The primers used for detection of *aerA* gene were sense 5′ CAAGAACAAGTTCAAGTGGCCA '3 and antisense 5′ ACGAAGGTGTGGTTCCAGT '3 with a product size of 309 bp. The primers for *ahh1* gene were sense 5′ GCCGAGCGCCCAGAAGGTGAGTT'3 and antisense 5′ GAGCGGCTGGATGCGGTTGT '3 with 130 bp. product size (Stratev *et al*., 2016). Amplified DNA fragments were analyzed by 2% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1X TAE buffer (0.04 M Tris, 0.02 M Acetic acid, 0.002 M Na_2 EDTA) at 100 V for 45 min with 8 µl PCR product. The gel was stained with ethidium bromide, captured and visualized on UV transilluminator.

Antibiotic susceptibility

Antimicrobial susceptibility for *Aeromonas* spp. was tested by the single diffusion method according to Stratev *et al*. (2015). Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated bacterial strains (Oxoid Limited, Basingstoke, Hampshire, UK). The antimicrobial susceptibility testing was applied according to the guidelines of NCCLS (2001). The Multiple Antibiotic Resistance (MAR) index for each strain was determined according to the formula stipulated by Singh *et al*. (2010) as follow: MAR index = No. of resistance (Isolates classified as intermediate were considered sensitive for MAR index) / Total No. of tested antibiotics.

Lemon juice solution as natural potential decontaminants

The isolated *A. hydrophila* that was positive for *aerolysin* and *haemolysin* virulence genes together in the current study was used for the experimental trail as previously describe by Morshdy *et al*. (2022). About 12 freshly caught Nile tilapia fish for each experiment (200 grams, each) were divided into four groups as 3 fish in each group. Nile tilapia fish were disinfected by dipping in ethyl alcohol (70%) for 5 min and they were allowed to dry. About one ml of *A. hydrophila* broth (adjusted to 0.5 McFarland) was inoculated via pipetting above each fish. Then the inoculated fish were maintained at 25°C for 30 minutes. Lemon juice solutions 3% and 5% were prepared for immersion of the Nile tilapia fish that were inoculated with *A. hydrophila*. After that, fish samples were divided in to four groups; the 1st group without the microorganism inoculation (negative control); the 2nd group, was immersed in sterile distilled water (positive control); the 3rd group, was immersed in 3% lemon juice solution; the 4th group was immersed in 5% lemon juice solution. Bacterial count was measured after 0.5, 1 and 2 h in triplicate to determine the antimicrobial effect of lemon juice against *A. hydrophila* and the results reported as mean values and standard error.

Statistical analysis

All statistics were assessed by One-way analysis of variance (ANOVA) and SPSS at 95% level of confidence. All values are present as means ± S.E. Significantly difference is considered when $P < 0.05$. Significant differences were determined by DUNCAN test.

RESULTS

Bacteriological examination of fish samples revealed that *Aeromonas* spp. isolated with a percentage of 39.3% from all examined samples. The highest isolation percentage was in shrimp (60%) and the lowest percentage was in saurus (28%) (Table 1). The most predominant isolated *Aeromonas* spp. were *A. caviae* (15.3%), followed by *A. hydrophila* (12%), *A. sobria* (9.3%) and the lowest percentage was for *A. fluvialis* (2.7%) (Table 1). The highest isolation percentage of *A. hydrophila* was from saurus, shrimp (28% for each) followed by Nile tilapia samples (16%) (Table 1). It was found that all examined *A. hydrophila* isolates were positive for *ahh1* gene. However, 75% was positive for *aerA* virulence gene (Figure 1).

Table 2 shows 100% resistance of the tested *Aeromonas* isolates for both Ampicillin and Erythromycin, 83.3% and 75% resistance to Tetracycline and Sulphamethoxazol, respectively. Meanwhile, *Aeromonas* spp. were more sensitive to Imipenem (83.3 %), Gentamicin (75 %), Ciprofloxacin (75 %). Resistance profile for MRI of *A. hydrophila* isolates ranged from 0.928 to 0.214 with an average of 0.571 (Table 3).

The decontamination of *A. hydrophila* after immersion in lemon juice solution 3% and 5% was evaluated. Table 4 illustrates the mean counts of *A. hydrophila* in the examined samples for

Table 1. Prevalence of *Aeromonas* species isolated from fish samples (n. = 25, each).

Source	Fish type	Number positive (%)	*A. hydrophila*	*A. caviae*	*A. fluvialis*	*A. sobria*
Fresh water	Tilapia	9 (36)	4 (16)	5 (20)	-	-
	Mugil	8 (32)	-	4 (16)	4 (16)	
Marine water fish	Tuna	12 (48)	-	6 (24)		6 (24)
	Saurus	7 (28)	7(28)	-	-	-
	Pagrus	8 (32)	-	8(32)	-	-
	Shrimp	15 (60)	7 (28)	-	-	8 (32)
Total (150)		59 (39.3)	18 (12)	23 (15.3)	4 (2.7)	14 (9.3)

Table 2. Antimicrobial susceptibility of *Aeromonas* species (n. =48).

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	NO.	%	NO.	%	NO.	%
Ampicillin (AM)	0	0	0	0	48	100
Erythromycin (E)	0	0	0	0	48	100
Tetracycline (T)	0	0	8	16.7	40	83.3
Sulphamethoxazol (SXT)	8	16.7	4	8.3	36	75
Cefotaxime (CF)	8	16.7	16	33.3	24	50
Cephalothin (CN)	12	25	12	25	24	50
Amikacin (AK)	24	50	4	8.3	20	41.7
Ceftazidime (CZ)	28	58.3	4	8.3	16	33.3
Amoxicillin (AMX)	32	66.7	0	0	16	33.3
Levofloxacin (L)	28	58.3	16	33.3	12	25
Meropenem (M)	32	66.7	4	8.3	12	25
Ciprofloxacin (CP)	36	75	0	0	12	25
Gentamicin (G)	36	75	4	8.3	8	16.7
Imipenem (IPM)	40	83.3	4	8.3	4	8.3

Table 3. Antimicrobial resistance profile of *Aeromonas* species (n. =48).

Aeromonas spp.	NO. of isolates	Antimicrobial resistance profile	No. of antibiotic	MAR index
A. hydrophila	5	AM, E, T, SXT, CF, CN, AK, CZ, AMX, L, M, CP, G	13	0.928
A. hydrophila	5	AM, E, T, SXT, CF, CN, AK, CZ, AMX, L, M, CP	12	0.857
A. hydrophila	4	AM, E, T, SXT	4	0.286
A. hydrophila	4	AM, E, T	3	0.214
			Average	0.571
A. caviae	6	AM, E, T, SXT, CF, CN, AK, CZ, AMX, L, M, CP, G, IPM	14	1
A. caviae	5	AM, E, T, SXT, CF, CN, AK, CZ, AMX	9	0.643
A. caviae	3	AM, E, T, SXT, CF, CN	6	0.428
A. caviae	4	AM, E, T, SXT	4	0.286
A. caviae	2	AM, E	2	0.143
			Average	0.5
A. sobria	4	AM, E, T, SXT, CF, CN, AK	7	0.5
A. sobria	4	AM, E, T, SXT	4	0.286
			Average	0.393
A. fluvialis	2	AM, E	2	0.143
			Average	0.143

MAR: Multiple Antibiotic Resistant: MAR index = a (the number of isolate resistant antibiotics) / b (total tested antibiotics number).
E: Erythromycin; AM: Ampicillin; CN: Cephalothin; SXT: Sulphamethoxazol; AK: Amikacin; G: Gentamicin; T: Tetracycline; AMX: Amoxicillin; CP: Ciprofloxacin; CF: Cefotaxime; L: Levofloxacin; CZ: Ceftazidime; IMP: Imipenem; M: Meropenem.

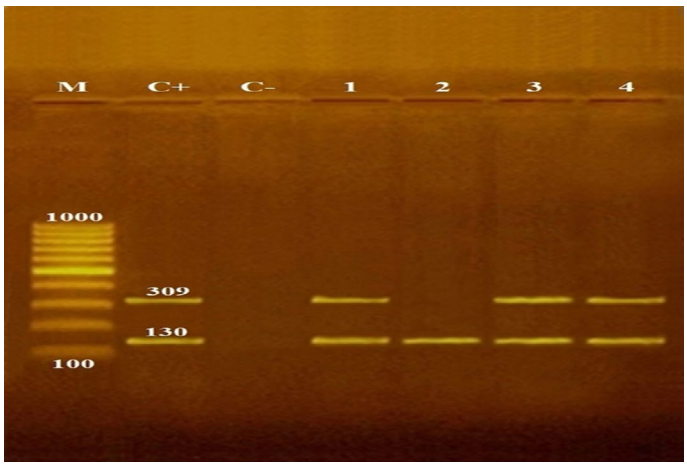

Figure 1. A multiplex PCR exemplar for 2% agarose gel. L:100- bp Ladder. (A) Amplification of *ahh1* gene and *aerA* gene specific for *A. hydrophila* (C-: negative control, C+: positive control 1: 4 positive *A. hydrophila* for *ahh1* gene ;1.3.4 positive *A. hydrophila* for *aerA* gene).

0.5, 1, 2 h. After 2 h immersion in lemon juice 5% a significant decline in *A. hydrophila* count from 7.37±0.47 to 6.92±0.4 log10 cfu /g ($P < 0.05$) with a reduction count (%) of 0.45 (64.44%).

DISCUSSION

The microbial quality investigation of fish is an important issue for public health. Raw fish may act as a vehicle for several microbes' transmission including *Aeromonas* spp. In the present study, *Aeromonas* spp. were identified in 39.3% of the examined fish samples. The obtained results showed that the prevalence of *Aeromonas* spp. in Nile tilapia and *Mugil cephalus* were 9 (36%) and 8 (32%), respectively and the most prevalent *Aeromonas* spp. isolated from Nile tilapia were *A. hydrophila* 4 (16) and *A. caviae* 5 (20), while from fresh *Mugil cephalus* were *A. caviae* and *A. fluvialis* (16%, each). *Aeromonas* spp in the current study were lower than 34 (68%) and 31 (62%) (Ebeed *et al*., 2017); 68 % and 62 % (Saleh *et al*., 2017) and 32 (64%) and 25 (50%) (Kishk *et al*., 2020)

Table 4. Effect of Lemon juice on *A. hydrophilla* count log_{10} cfu/g after different marinating time.

	½ h			1 h			2 h		
	Control	Lemon juice		Control	Lemon juice		Control	Lemon juice	
		L. (3%)	L. (5%)		L. 3%	L. (5%)		L. (3%)	L. (5%)
Minimum	7.2	7.06	7	7.21	6.98	6.96	7.24	6.92	6.87
Maximum	7.5	7.3	7.07	7.34	7.1	7.01	7.36	7	7.02
Mean ± S.E.	7.47 ± 0.5^{a}	7.19 ± 0.9^{ab}	7.07 ± 0.4^{b}	7.41 ± 0.65^{ab}	7.05 ± 0.49^{b}	6.98 ± 0.94^{c}	7.37 ± 0.47^{ab}	6.96 ± 0.9^{c}	6.92 ± 0.4^{c}
R. count		0.28	0.4		0.36	0.43		0.41	0.45
R. %		47.46	60.34		56.42	62.84		61.02	64.44

In each row, data followed by different superscript letter is significant, $P<0.05$.

for Nile tilapia and *Mugil cephalus*, respectively. The most frequently identified *Aeromonas* spp in Nile tilapia were *A. hydrophila* (14 %) that was similar to our result, A. carviae (36 %), A. sabria (28 %), *A. veronii* (16 %) and *A. fluvialis* (6 %), While in *Mugil cephalus* it was *A. caviae* (34%), *A. sobria* (26%), *A. veronii* (18 %), *A. hydrophila* (12%) and A. schubertii (10%) (Saleh *et al*., 2017). The most prevalent identified *Aeromonas* species isolated from Nile tilapia was *A. caviae* (36%), *A. sobria* (28%) and from *Mugil cephalus*. *A. caviae* (34%), *A. sobria* (26%), respectively (Ebeed *et al*., 2017). In addition, Ahmed *et al*. (2018) identified *A. hydrophila* with percentage of 4.67 % from Nile tilapia fish and 6.25 % from *Mugil cephalus* fish in Egypt. However, Kishk *et al*., (2020) reported that the most abundant *Aeromonas* spp. from Nile tilapia were A caviae (40.6%), *A. hydrophila* (25%), A sobria (21.9%), *A. veronii* (9.4%), and *A. fluvialis* (3.1%), while from *Mugil cephalus* were A. sobria (44%), *A. caviae* (28%), *A. hydrophila* (20%), and *A. veronii* (8%). In addition, Dhanapala *et al*. (2021) identify eight different *Aeromonas* spp. from freshwater fish as *A. veronii* (75.8%), *A. hydrophila* (9.3%), *A. caviae* (5%), *A. jandaei* (4.3%), A. dhakensis (3.7%),0.6% for each of *A. sobria*, *A. media* and *A. popoffii*.

A. hydrophila harboured *aerA* and *ahh1* genes have the ability to produce cytotoxic and haemolytic actions (Yousr *et al*., 2007). *A. hydrophila* has negatively impact on erythrocytes in form of haemolysis and may lead to diarrhea outbreaks (Rahim *et al*., 1984). *A. hydrophila* virulence associated genes (*ahh1* and *aerA*) were detected in 100% and 75% of the examined *A. hydrophila* isolates. The obtained result was in agreement with Hoel *et al*. (2017) who found that all isolates had high prevalence of hemolysin encoding genes (*hlyA* and *aerA*) with percentages of 99% and 98%, respectively and considered potentially pathogenic, and agree with Yousr *et al*. (2007) who recognized *hlyA* and *aerA* genes in *A. hydrophila* from food origin. Results from this study disagree with the finding of Ahmed *et al*. (2018) who reported that all *A. hydrophila* strains detected were negative for *ahh1* gene, while only three isolates (42.86 %) from Nile tilapia fish and 5 isolates (55.56 %) from *Mugil cephalus* were *aerA* positive. However, Hafez *et al*. (2018) reported 6 *A. hydrophila* isolates (60 %) harbored both *aerA* and *hlyA* genes, 2 (20 %) were positive for *aerA* gene only and 2 (20 %) were positive only for *hlyA* gene.

The antibiotics misuse in aquaculture results in appearance of multidrug resistance that demonstrates a serious human health risk. In the current study, *Aeromonas* isolates were extremely resistant to Tetracycline and Sulphamethoxazol and recorded 100% resistance to Ampicillin and Erythromycin. Higher resistance for *A. hydrophila* was recorded against Streptomycin, Cloxacillin and Erythromycin (100%, each); Cefotaxime and Sulphamethoxazol (80%, each); Chloramphenicol, Oxytetracycline, Amikacin and Cephalothin (60%, each) (Hafez *et al*., 2018). Meanwhile, Ahmed *et al*. (2018) reported a sensitivity to Imipenem (100%); Gentamycin and Trimethoprim/sulphamethoxazole (56%, each); and a reported a resistance for Cefotaxime (72%); Amoxicillin, Tetracycline and Nalidixic acid (76%, each); Ceftazidime (80%); Amoxicillin/clavulanic acid (84%) and Cefixime (88%). The antimicrobial resistance for *Aeromonas* spp. were 92.5% (amoxicillin), 67.1% (enrofloxacin), 63.4% (nalidixic acid), 26.1% (erythromycin), 23.6% (tetracycline), 18% (imipenem), 16.8% (trimethoprim-sulfamethoxazole), and 16.8% (gentamicin) (Dhanapala *et al*. 2021).

In the present study, most *Aeromonas* spp.and all *A. hydrophila* isolates were resistant to more than three antibiotics and *A. hydrophila* result revealed multiple MAR index ranged from 0.928 to 0.214 with an average of 0.571 that is more than 0.2, which express contamination of fish samples from high-danger sources leading to human public dangers (Letchumanan *et al*., 2015). The MAR indices > 0.2 for *A. hydrophila* isolates have been reported by Dhanapala *et al*. (2021), Ahmed *et al*. (2018) who found that MAR was ranged from 0.11 to 0.88 with an average of 0.489 and Hafez *et al*. (2018) who reported that MAR was varied from 0.214 to 1.00 with the average of 0.614 demonstrating that they coming from hazard source of contamination. The variance in MAR index may be owing to the difference in sample sources, geographic distribution, and used methodology.

Organic acids as citric acid is used to reduce foodborne pathogens and limitation of microbial contamination, thus extending shelf life of food (Lingham *et al*., 2012). Lemon juice shows in vitro antibacterial activity on *A. hydrophila* (Jafarpour *et al*., 2016). In the present study, citric acid (lemon juice) was found to be effective in reducing *A. hydrophila* counts after 2 h marination in lemon juice 5%. Lemon juice showed an ascending increase in the reduction percentages of *A. hydrophila* from 60.34 and 62.84 after 0.5 and 1 h, respectively to reach 64.44% reduction after 2 h dipping at 5% lemon juice concentration. Our result is in agreement with Fadel and El-Lamie (2019) who found that lemon juice is effective in inactivation of *A. hydrophila*. They also found a reduc¬tion of 20.67, 19.61, 20.4, and 42% after decontamination for 0.25, 1, 1.5, and 24 h, respectively. The high-level content of bioflavonoids, pectin, limonene, citric acid, magnesium, calcium, and vitamins in lemon juice potentiate its antibacterial, antiviral, and immune-building effects against disease and infection (Alsaraf *et al*., 2016). The antimicrobial effect of lemon essential oils on gram-negative foodborne bacteria as *A. hydrophila* was reported by Ozogul *et al*. (2015). In Egypt, treatment of fish samples by lemon extract increased the shelf-life, tenderness, enhancing the sensory evaluation and expressing decontaminating effect against *A. hydrophila* count in treated fish samples (Fadel and El-Lamie, 2019). The antimicrobial properties of lemon oil 1% and lemon juice 5% was previously evaluated by Morshdy *et al*. (2021) and Morshdy *et al*. (2022) and they reported that lemon has high acceptable antimicrobial properties and sensory score.

CONCLUSION

The present study concluded that most fish samples under investigation are contaminated with antibiotic resistant *Aeromonas* specially *A. hydrophila* creating a potential foodborne pathogen and may lead to its spread. Higher percentage of isolated *A. hydrophila* harbored haemolytic genes that constitute a human public health risk. It is recommended that hygienic measures during handling, preparation, processing and storage should be implemented to minimize the prevalence of pathogens and the antibiotics miss used in aquaculture should be reduced. In addition, improving the quality control systems for monitoring fish quality. Therefore, contamination with *A. hydrophila* must be reduced by dipping of fish in lemon juice solution which decrease

its microbial load and improving the fish quality.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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