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Antimicrobial resistance genes of *Vibrio parahaemolyticus* and *Aeromonas hydrophila* isolated from Nile tilapia and Mugil fish farms in Kafr-Elsheikh governorate, Egypt.

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ABSTRACT

The progressive expansion of aquaculture practices led to the development of bacterial disease outbreaks, otherwise, the continuous and extensive use of antibiotics to overcome these diseases. The objective of our study was to investigate the antibiotic susceptibility and antibiotic resistance genes of *Vibrio parahemolyticus* (*V. parahemolyticus*) and *Aeromonas hydrophila* (*A. hydrophila*) species isolated from Nile tilapia and Mugil fish farms in Kafr El-Sheikh province, Egypt. A total of 100 clinically diseased fish were bacteriologically examined. The result recorded65 isolates of *Vibrio* species and *V. parahemolyticus* was isolated with an incidence of 55.4%. Out of 100 examined fish samples 72 *Aeromonas species* were isolated, *A. hydrophila* was isolated with an incidence of 99.3 %. *Vibrio parahemolyticus* showed high resistance for amoxicillin and colistin followed by cefotaxime and streptomycin. Meanwhile, *A. hydrophila* were highly resistant to amoxicillin and tetracycline followed by streptomycin, cefotaxime, and colistin. Five isolates of *V. Parahemolyticus* and *A. hydrophila* were screened using PCR for detection of 4 antibiotic resistance genes β-lactamase resistance gene (*bla*TEM); aminoglycosides (*aad*A1); tetracycline-resistant A *tet*A (A) and polymyxin resistance (*mcr*1) which were distinguished in all five *V. parahaemolyticus* and *A. hydrophila* isolates. The high detection of *V. parahaemolyticus* and *A. hydrophila* antibiotic resistance genes in our study could pose a potential economic problem as it may overlap the control of fish diseases and hence the economy.

1. INTRODUCTION

The speedy expansions of fish culturing and escalating fish requirement results in the extension of aquaculture, increasing stressors on fish, and thus intensify the hazard of diseases (Reverter *et al*., 2014). Aquaculture is regarded as the major food source that provides a protein of animal source proper for the consumption of the populace in the developing countries (Abbas *et al*., 2017). Infectious diseases are the chief problem in fish farms, causing huge economic costs due to the serious practices of fish farming (Bulfon *et al*., 2015).

Several *Vibrio* species are well recognized for their severity to cause fish disease, besides, causing mortality in reared fish is very common during early larval stages and can occur suddenly, leading sometimes to the death of the population (Thompson *et al*., 2004). *Aeromonas hydrophila* is considered a major pathogen producing outbreaks in fish aquaculture with extreme mortality rates; causing severe economic losses to the aquaculture all over the world (Fang *et al*., 2004).

Antibiotics has habitually been collaborated as immersion baths or feed additives to stimulate the fast growth of fish, treat bacterial infections, and also prevent the water plants' growth (Abu Bakar *et al*., 2010). Multidrug resistance (MDR) developed from the uncontrolled massive antibiotics usage in fish culture to control the bacterial infection and prevent the rapid spread of disease. Besides, the misuse of antibiotics not only improves the antibiotic-resistant bacteria and the spreading of the antibiotic-resistant genes but also results in the existence of antibiotic remains in aquatic animals such as fish (Miranda *et al*., 2018). Direct transmission of resistant bacteria through food to humans and the transfer of resistance genes to other bacteria happen, thus causing a possible hazard to human wellbeing (Kim *et al*., 2013). Therefore, this study aimed to study the prevalence of *V. parahemolyticus* and *A. hydrophila* in some fish farms and to assess the putative risk of possible antibiotic resistance could be transmitted to human through farm fish.

2. MATERIAL AND METHODS

2.1. Collection of samples:

A total of 100 clinically diseased fish samples,50 Nile tilapia (*Oreochromis niloticus*) and 50 mullet fish (*Mugil cephalus*) were gathered from various fish farms at Kafr el-sheik Governorate at the period from January to October (2019). The fish farms were complaining of high mortality rate and fish showed signs of septicemia including unilateral and bilateral exophthalmia, skin ulcers, and

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hemorrhages. The examined diseased fish samples were taken in a sterile strong plastic bag with half of its volume pumped water with pressured oxygen and transferred alive with a minimum delay to the bacteriology unit of Animal Health Research Institute, Tanta branch, Egypt for clinical and bacteriological examination. Three hundred and five lesion samples were amassed from 100 diseased fishes; 157 samples from 50 Nile tilapia (*O. niloticus*) and 148 samples from 50 mullet fish (*Mugil cephalus*), where the samples were taken from apparently path-gnomic lesions in liver, kidneys, spleen, heart, anterior intestine, and gills.

2.2. Isolation of Vibrio and Aeromonas species using the conventional cultural method:

The samples were taken by a sterilized loopful from the lesions and inoculated in peptone broth 1% (Oxoid) for *Aeromonas* isolation and 1% peptone broth + 3% NaCl for *Vibrio* isolation and incubated aerobically at 37°C for 18-24 hours. An inoculum from the cultured broth was streaked onto selective diagnostic agar media: *Aeromonas* selective agar (BSIBG agar, HIMEDIA, M1890-55G) for *Aeromonas* spp. and Cholera medium TCBS (Oxoid, UK) for *Vibrio* spp. and incubated for 24 hours at 37ºC. One separated typical colony from each selected agar medium was picked up and purified onto the same agar medium. After that one separated typical colony from agar medium was picked up and transferred into the nutrient broth (Oxoid, UK) with 15% glycerol was aerobically incubated at 37 ºC for 18-24hrs, then preserved in the refrigerator at -85 ºC (Quinn *et al*., 2002 and Markey *et al*., 2013).

2.3. Biochemical identification of Vibrio and Aeromonas isolates

The biochemical identification for the recovered isolated from the examined fish samples were performed according to (Quinn *et al*., 2002; Nicky, 2004 and Markey *et al*., 2013) by application of oxidase, catalase, indole production, citrate utilization, urease test, triple sugar iron, and methyl red tests.

2.3. Antibiotic susceptibility testing:

An in-Vitro sensitivity test was done on the isolated *V. parahemolyticus* and *A. hydrophila* strains to study their sensitivity for different antibiotics using the disc diffusion method of Koneman *et al*. (1997) using different antimicrobial agents (Oxoid, UK): amoxicillin (AML/10), cefotaxime (CTX/30), ciprofloxacin (CIP/5), colistin sulfate (CT/10), gentamicin (GEN/10), streptomycin (S/10) and tetracycline (TE/30). Mueller Hinton broth tubes were inoculated with at least 4-5 colonies of each isolated *V. parahemolyticus* and *A. hydrophila* strains and incubated at 37°C for 24 hrs. Then the plates of Mueller Hinton agar were covered by one ml of the inoculated broth then incubated at 37°C for 24 hrs. The interpretation of results was carried out according to CLSI, (2016).

2.4. Molecular detection of antibiotic resistance genes by the polymerase chain reaction

PCR was used for the detection of antibiotic resistance-associated genes by primers targeting different resistant genes to β-lactams (*bla*TEM), tetracycline (*tet*A (A)), aminoglycosides (*aad*a1), and polymyxin resistant (*mcr*1) (Metabion, Germany) (Table 1).

The extraction of DNA was performed by QIAamp® DNA Mini Kit (Catalogue no. 51304) according to the manufacturer's instructions.

The cycling condition for each gene was performed according to the references and Emerald Amp GT PCR Master Mix (Takara, Cat PR310A). The primary denaturation was done at 94˚C/ 5 min and the secondary denaturation was occurred at 94˚C/ 30 sec for all genes. The annealing process was done at 54˚C/40 sec (*bla*TEM and *aad*A1), at 50˚C/40 sec (*tet*A (A)) and 60˚C/ 30 sec (*mcr*1). The extension process was done at 72˚C/ 45 sec for all genes except the *mcr*1 gene at 72˚C/ 30 sec. the final extension was occurred at 72˚C/ 10 min except the *mcr*1 gene at 72˚C/ 7 min.

The amplification was performed on Eppendorf Master Cycler® (Eppendorf AG, Hamburg, Germany) in a total reaction volume of 25 µl containing 12.5 µl Emerald Amp GT PCR Master Mix, 1 µl of each forward and reverse primers, 4.5 µl molecular biology grade water, and 6 µl test DNA.

The PCR amplicons were analyzed by electrophoresis using a 1.5 % agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH = 8.3). A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

Table 1 Oligo-nucleotide primers and cycling conditions of the primers during conventional PCR

Target genes	Oligonucleotide sequence (5′ → 3′)		Product size (base pairs)	References
*bla*TEM	F	ATCAGCAATAAACCAGC	516	Colom et al., 2003
	R	CCCCGAAGAACGTTTTC		
*aad*A1	F	TATCAGAGGTAGTTGGCGTCAT	484	Randall et al. 2004
	R	GTTCCATAGCGTTAAGGTTTCATT		
*tet*A(A)	F	GGTTCACTCGAACGACGTCA	576	
	R	CTGTCCGACAAGTTGCATGA		
*mcr*1	F	CGGTCAGTCCGTTTGTTC	308	Newton-Foot et al., 2017
	R	CTTGGTCGGTCTGTAGGG		

3. RESULTS

3.1 The prevalence of Vibrio parahemolyticus in different tissue samples in diseased fishes: Out of 100 diseased fish samples (50 from O. niloticus and 50 from Mugil cephalus), 65 isolates of Vibrio species were isolated and identified, V. parahemolyticus was isolated with a prevalence of 55.4% (36/65) and isolated from liver, kidneys, spleen, heart, intestine, and gills with a prevalence of 30.5(11/36), 16.7(6/36), 11.1(4/36), (3/36) 8.3, 2.8(1/36) and 30.5% (11/36), respectively (Table 2).

3.2. The prevalence of Aeromonas hydrophila in different tissue samples in diseased fishes: Out of 100 diseased fish samples, 72 Aeromonas species isolates from positive samples were identified with a prevalence of 90.3(65/72) and was detected in the liver, kidney, spleen, heart, intestine, and gills with a prevalence of 32.3(21/65),

27.7(18/65), 6.2(4/65), 17.0(11/65), 6.2(4/65) and 10.8% (7/65), respectively (Table 2).

3.3. The antibiotic susceptibility tests for the isolated bacteria:

The in-vitro sensitivity tests for the isolated V. parahemolyticus strains (n=36) showed high resistance for amoxicillin 91.7% (33/36) and colistin 63.9% (23/36) followed by cefotaxime 58.3% (21/36) and streptomycin 52.7% (19/36) (Table 3).

The sensitivity tests for the isolated A. hydrophila revealed that the isolated A. hydrophila (n= 65) were highly resistant for amoxicillin 100.0% (65/65) and tetracycline 87.7% (57/65) followed by streptomycin 63.1% (41/65), cefotaxime 57.0% (37/65) and colistin sulfate 54.0% (35/65) (Table 4).

Table 2 Distribution of *Vibrio parahemolyticus* and *Aeromonas hydrophila* species isolated from the examined organs.

Bacterial species	***V. parahemolyticus***				***A. hydrophila***			
Fish type	*O. niloticus*	*Mugil cepahlus*	Total		*O. niloticus*	*Mugil cephalus*	Total	
lesion samples	No.	No.	No.	%	No.	No.	No.	%
Liver	4	7	11	30.5	8	13	21	32.3
Kidney	5	1	6	16.7	5	13	18	27.7
Spleen	3	1	4	11.1	0	4	4	6.2
Heart	1	2	3	8.3	3	8	11	17
Intestine	1	0	1	2.8	4	0	4	6.2
Gills	6	5	11	30.5	6	1	7	10.8
Total	20	16	36	55.4	26	39	65	90.3

Percentage in relation to the total number of *Vibrio* (65) and *Aeromonas* species isolated (72).

Table 3 In-Vitro antimicrobial sensitivity test for isolated *V. parahemolyticus* strains

Antimicrobial agents	**Disk Concentrations**	**Sensitive**		**Intermediate**		**Resistant**	
		No.	%	No.	%	No.	%
Amoxicillin (AML)	10 µg	3	8.3	0	0.0	33	91.7
Cefotaxime (CTX)	30 µg	5	14.0	10	27.7	21	58.3
Ciprofloxacin (CIP)	5 µg	11	30.6	19	52.8	6	16.6
Colistin (CT)	10 µg	13	36.1	-	-	23	63.9
Gentamicin (GEN)	10 µg	27	75.0	9	25.0	0	0.0
Streptomycin (S)	10 µg	5	14.0	12	33.3	19	52.7
Tetracycline (TE)	30 µg	31	86.1	2	5.6	3	8.3

No.: Number of isolates. %: Percentage in relation to the total number of *V. parahemolyticus* isolates (36).

Table 4 In-Vitro anti-microbial sensitivity test for isolated *A. hydrophila* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Amoxicillin (AML)	10 µg	0	0.0	0	0.0	65	100.0
Cefotaxime (CTX)	30 µg	13	20.0	15	23.0	37	57.0
Ciprofloxacin (CIP)	5 µg	54	83.1	9	13.8	2	3.1
Colistin sulfate (CT)	10 µg	30	46.0	-	-	35	54.0
Gentamicin (GEN)	10 µg	49	75.4	11	17.0	5	7.6
Streptomycin (S)	µg	10	15.4	14	21.5	41	63.1
Tetracycline (TE)	µg	1	1.5	7	45.5	57	87.7

No.: Number of isolates. %: Percentage in relation to the total number of *A. hydrophila* isolates (65)

3.4. Molecular investigation of antibiotic resistance genes in Vibrio parahemolyticus and Aeromonas hydrophila species:

Five random isolates from each Vibrio parahemolyticus and Aeromonas hydrophila were subjected to PCR amplification targeting the antimicrobial resistance determinants β-lactamase (blaTEM), tetracycline resistance (tetA (A)), and aminoglycosides (aada1) and polymyxin resistant (mcr1) genes which were amplified in all five tested A. hydrophila and all five V. parahemolyticus studied strains giving a product of 516, 576, 484 and 308 bp., respectively. (Figures 1 to 4).

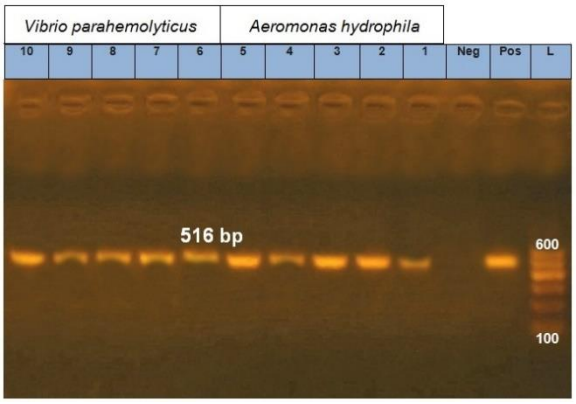

Figure 1 PCR amplification of *bla*TEM resistance gene of *A. Hydrophila* and *V. parahemolyticus* on agarose gel 1.5%. Lane L: 100-600bp. DNA Ladder. Neg.: Negative control (*Enterobacteriaceae*), Pos.: Positive control (local strain obtained from Central lab for quality control of poultry production, El-Giza, Egypt, CLQP) (at 516 bp for *bla*TEM gene). Lane 1, 2, 3, 4 and 5: *A. hydrophila* (*bla*TEM gene) positive. Lane 6, 7, 8, 9 and 10: *V. parahemolyticus* (*bla*TEMgene) positive.

4. DISCUSSION

Intensive aquaculture production leads to the development of infectious disease outbreaks. Bacterial diseases are the most common diseases in intensive fish raring facilities (Kusuda and Salati, 1999).

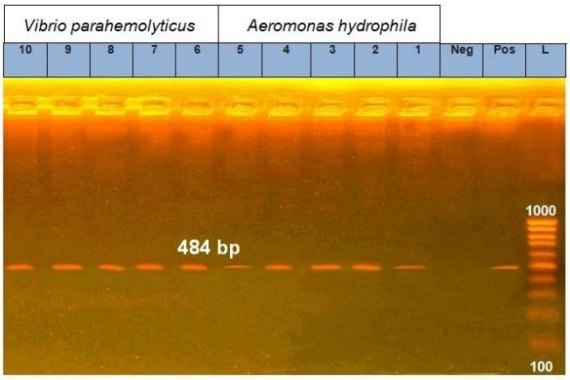

Figure 2 PCR amplification of *aad*A1 resistance gene of *A. Hydrophila* and *V. parahemolyticus* on agarose gel 1.5%. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (Enterobacteriaceae). Pos: Positive control (local strain obtained from CLQP) (at 484 bp for *aad*A1 gene). Lane 1, 2, 3, 4 and 5: *A. hydrophila* (*aad*A1 gene) positive. Lane 6, 7, 8, 9 and 10: *V. parahemolyticus* (*aad*A1 gene) positive

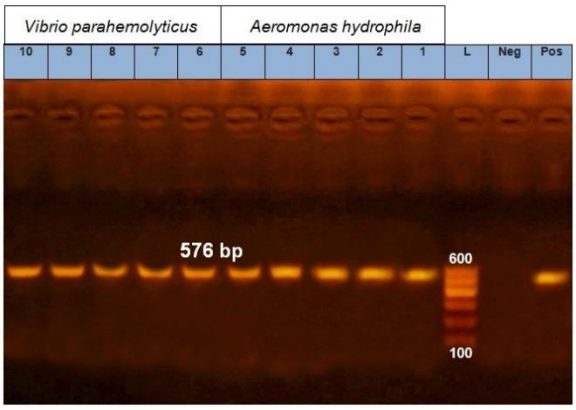

Figure 3 PCR amplification of *tet*A (A) resistance gene of *A. Hydrophila* and *V. parahemolyticus* on agarose gel 1.5%. Lane L: 100-600 bp. DNA Ladder. Neg.: Negative control (*Enterobacteriaceae*). Pos.: Positive control (local strain obtained from CLQP) (at 576 bp for tetA(A) gene). Lane 1, 2, 3, 4 and 5: *A. hydrophila* (*tet*A(A) gene) positive. Lane 6, 7, 8, 9 and 10: *V. parahemolyticus* (*tetA*(A) gene) positive

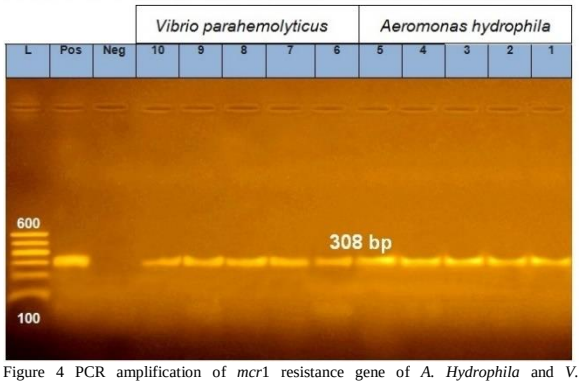

Figure 4 PCR amplification of *mcr*1 resistance gene of *A. Hydrophila* and *V. parahemolyticus* on agarose gel 1.5%. Lane L: 100-600 bp. DNA Ladder. Neg.: Negative control (*Enterobacteriaceae*). Pos.: Positive control (local strain obtained from CLQP) (at 308 bp for *mcr*1 gene). Lane 1, 2, 3, 4 and 5: *A. hydrophila* (*mcr*1gene) positive. Lane 6, 7, 8, 9 and 10: *V. parahemolyticus* (*mcr*1) positive

The present bacteriological examination revealed that *V. parahemolyticus* were isolated mainly from the liver and gills. Reveres results recorded by Aly *et al*., (2020) where the highest intensities of *V. parahemolyticus* which isolated from Gilthead Seabream were mainly from kidneys followed by spleen and liver, this may be due to difference in fish species or season. *Aeromonas hydrophila* was isolated mainly from the liver and kidneys. Nearly similar results were recorded by Enany *et al*., (2019) and Algammal *et al*., (2020). The in-vitro sensitivity tests for the isolated *V. parahemolyticus* showed high resistance for amoxicillin and colistin followed by cefotaxime and streptomycin. These results agreed with those reported by Lee *et al*., (2018) and Lopatek *et al*., (2018). In contrast, Xu *et al*., (2016) reported that most *V. parahemolyticus* isolates were resistant to streptomycin and Ashrafudoulla *et al*., (2019) reported that the isolates were highly resistant to tetracycline. These changes may be due to differences in geographical distribution or treatment regimes. The in-vitro susceptibility tests for the isolated *A. hydrophila* showed that the tested *A. hydrophila* strains were highly resistant to amoxicillin may suggest the production of beta-lactamase, which is constant with the findings of Daood, (2012) and Revina *et al*., (2017). The results of PCR for amplification of *bla*TEM gene in *V. parahaemolyticus* strains showed that the *bla*TEM gene was amplified in all 5 *V. parahaemolyticus* studied strains similar to the results reported by Cardoso *et al*., (2018) and Faja *et al*., (2019). However, the results were not in agreement with Hu *et al*., (2020) and Jeamsripong *et al*., (2020) who failed to detect *bla*TEM virulent genes in these strains. Meanwhile, the *aad*A1 and the *tet*A (A) gene were amplified in all 5 *V. parahaemolyticus* studied strains. These results were agreed with those of Faja *et al*., (2019). Otherwise, Jiang *et al*., (2014) cannot detect the *tet*A gene in any of the isolates. The *mcr*1 gene was amplified in all 5 *V. parahaemolyticus* studied strains. Lei *et al*., (2019) firstly reported the occurrence of plasmid-encoded *mcr*-1 in virulent *V. parahaemolyticus* strain where the *mcr*-1 gene was detected in one colistin-resistant *V. parahaemolyticus* isolate. The *bla*TEM gene was amplified in all 5 studied *A .hydrophila* strains, similar results were obtained by Ibrahim (2015) and Okolie (2015). However, the results were not in agreement with (Ndi and Barton, 2011) who failed to detect *the bla*TEM virulent gene in these strains. The *aad*A1 gene also was amplified in all 5 *A. hydrophila* strains which agreed with those of Ndi and Barton (2011) and Okolie (2015). The *tet*A(A) gene was amplified in all 5 *A. hydrophila* studied strains. These results were agreed with those of Ndi and Barton (2011) and Ibrahim (2015). The results were not in agreement with Igbinosa and Okosh, (2012) who failed to detect *tet* virulent genes in these strains.

5. CONCLUSIONS

Our study exposed an elevated prevalence of *Vibrio* and *Aeromonas* species in aquaculture in the examined farms. The isolated strains displayed a prominent multiple antibiotic resistance associated with high antibiotic resistance genes make aquaculture a reservoir for antibiotic-resistant bacteria which is considered a putative risk on public health and more preventive measures for water pollutant factors must be taken.

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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