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Coexistence of Ampicillin Resistance Gene and Class I Integron in Virulent *Aeromonas* Spp. Isolated from Temsah Lake, Egypt

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Abstract: In a view of increasing evidence supporting the role of *Aeromonas* in human disease, studies on the presence of virulence, antibiotic resistance determinants among *Aeromonas* spp. isolates from fresh water palatable Nile tilapia intended for human consumption in Temsah Lake (Egypt) were done, involved their association with plasmids which can be attributed to horizontal gene transfer of mobile genetic elements. *Aeromonas hydrophila* and *Aeromonas sobria* were isolated from different organs from Nile tilapia *(Oreochromis niloticus)* from Temsah Lake (94.7%), (1.3%). *Lip*ase gene was common in *A. hydrophila* isolated from tilapia (50%), all *A. sobria* isolates in the study were non virulent. The isolates showed phenotypic antibiotic resistance to the frequently used antibiotics for human infections in testing the antibiotic sensitivity using disc diffusion method, gentamycin; ciprfloxacin were successfully combat the bacterial growth in vitro (100% sensitive), while ampicillin failed to combat *Aeromonas* spp. One to four resistant plasmids (29/32) were isolated from multi-drug resistance; (16/29) virulent, (13/29) non virulent, while (3/32) virulent *Aeromonas* spp. were not plasmid encoded with a molecular weight ranging from (3.3 kbp to 20 kbp), $bla_{TEM\text{-}1b}$ resistance gene was detected in 15/19 (78.9%) of the *Aeromonas* plasmid encoded strains from tilapia, all non virulent strains were $bla_{TEM\text{-}1b}$ positive also variable Bands of class 1 integronplasmid born were detected in 16/19 (84.2%) isolates that confer multiple antibiotic resistance; variable sizes between (0.3 - 1.6kbp) were found. Co-existence of both determinants in most of the plasmid bearing isolates was observed. This study can be considered as a reference for further studies about these recently introduced antimicrobial resistance genes.

Key words: *Aeromonas* spp. · Virulence · Antibiotic Resistance · Plasmid · $Bla_{tem\text{-}1b}$ · Class I Integron

INTRODUCTION

Motile Aeromonas Septicemia (MAS) is a more drastic one and distributed world-wide, affecting various species of fish and shellfish as well as farmed fish in both freshwater and marine water, Azad *et al.* [1]. The causative agent of MAS, *Aeromonas hydrophila*, is also an opportunistic pathogen of humans causing illness that ranged from mild to dysentery-like diarrhea to meningitis and septicemia particularly when fish is eaten raw or improperly cooked [2]. *Aeromonas* species are ubiquitous Gram negative bacilli, classified within the new Aeromonadaceae family [3].

A wide range of putative virulence factors have been detected and studied in several *Aeromonas* spp. [4]; they play a pivotal role in the establishment of infection. Several studies have reported the detection and characterization of virulence factors in *Aeromonas* spp.

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isolated from freshwater fish, Tilapia (*Oreochromis niloticus*), humans [5]. The genes for lipases were present in 97% of the strains of presumptive *Aeromonas* spp. were isolated from frozen (Tilapia, *Oreochromis niloticus*) purchased in local markets in Mexico City [6], also lipase virulence gene in *A. hydrophila* isolates obtained from fish of the São Francisco River Valley [7]. Motile Aeromonads due to their ubiquitous distribution are considered as bacterial indicators of freshwater environment, especially for harbouring resistance genes [8]. Wastewater has been considered an important environmental reservoir of antibiotic-tolerant bacteria [9]. The acquisition of new genetic material by susceptible bacteria from resistant strains often facilitates the incorporation of the multiple resistance genes into the host's genome or plasmids [10]. Plasmids containing multiple antimicrobial resistance determinants could potentially be transferred in natural microenvironments between bacterial pathogens of fish, humans [11] which proved by a number of plasmids have been found in association with both clinical and environmental *Aeromonas* isolates [8, 12]. The presence of bacteria with genes that code for resistance determinants has potential implications for human health when fish are consumed and farm run-offs get into the environment [13]. β-lactam antibiotics are commonly used in the treatment of bacterial infections but they are hydrolyzed by β*eta*-lactamases [14] which are enzymes produced by resistant bacteria that inactivate β*eta*-lactam drugs by hydrolyzing the β*eta*-lactam ring of the β*eta*-lactam molecules. Most βeta-lactamases inactivate either penicillins or cephalosporins, but some can inactivate both classes of drugs [15]**.** The TEM β-lactamase, conferring resistance to penicillin family antibiotics such as ampicillin, is encoded by the *bla- Tem* gene, which is found in a group of closely related transposons that represent three of the earliest bacterial resistance transposons to be identified Tn1**.**TEM enzymes are important determinants of resistance in gram-negative bacteria and more than 180 variants derived from the TEM-1 or TEM-2 β-lactamase have been recorded by Bush and Jacoby [16].

Aeromonas species have been shown to possess integrons, which are capable of antibiotic resistance gene acquisition**.** A strong association between integron presence and phenotypic multiple antibiotic resistance has been observed [17, 18]. Integrons are not mobile but tend to be associated with conjugative plasmids which can serve as vehicles for their transmission [19]. The increasing incidence of integrons and other resistance determinants among veterinary microorganisms makes alarm on the therapeutic options for both human and animal diseases due to an increased prevalence of resistant zoonotic pathogens, which could subsequently cause human infections during processing and preparation procedures [20].

MATERIALS AND METHODS

Isolation and Identification of Bacterial Isolates: Presumptive *Aeromonas* spp. cultures were isolated from kidney, liver, muscle gathered from 2oo apparently diseased alive (Nile tilapia, *Oreochromis niloticus*) in Temsah Lake, Egypt. Bacterial colonies were grown on Trypticase Soya Broth (TSB) and kept at 37°C/18-24hrs for bacterial refreshment then cultured on Bile Irgasan Aeromonas agar37°C/18-24hrs, presumptively identified *Aeromonas* spp. isolates were maintained on BHI agar for further purification and other biochemical tests. All isolates subjected to the gram stain, oxidase, catalase, vogues proskauer, bile esculine hydrolysis, indole, oxidation- fermentation,citrate utilization and sugar fermentation [21].

Antibiotic Susceptibility Testing: Antibiotic susceptibility to 12 antimicrobial agents was determined using Binoanalyse and Oxoid antibiotic disks, on Muller-Hinton (Oxoid) agar plates following CLSI, 2011, the tested organisms were flooded on surfaces of the Muller Hinton agar by a sterile cotton swap by immersion the bacterial suspension adjusted to an optical density of 0.5 McFarland standard units then the discs gently pressed using sterile forceps, then the plates were incubated and the zone diameter and interpretation of the results were recorded [22].

Molecular Characterization of Lipase Virulent Gene in Aeromonas Spp. Isolates, Polymerase Chain Reaction Analysis: Genomic DNA was extracted according to a Bacterial DNA Extraction Kit (Spin-column), BioTeke Corporation, China from 38 isolates from an overnight TSB broth/ 37 °C then the isolates run by PCR through a thermocycler machine to detect the positive isolates of lipase virulent genes. A 760 bp*lip* fragment was amplified by PCR using the *lip*ase gene primer set [23] (Table 1). PCR amplifications were carried out in a DNA thermal cycler (Eppendorf). PCR cycling parameters consisted of 40 cylces of 94°C for1min; 62°C for 1min; 72°C for 1.5min. An initial denaturation step of 95°C for 5min and a final elongation step of 72°C for 5min were included in the reaction.6 µl of PCR product with 2-4 µl of gel loading dye and loaded the 1% agarose gel stained with ethidium

Table 1: Primer pairs and amplicon sizes of virulent and resistance genes

Gene	Primer name	Primer Sequence (5'-3')	Product length (bp)	Reference
Lip	lip-F lip-R	AACCTGGTTCCGCTCAAGCCGTTGTTGCTCGCCTCGGCCCAGCAGT	760	[23]
bla*TEM-1b* gene	(b*la*TEM)-F (*bla*TEM)- R	5'-ATGAGTATTCAACATTTCCG-3' 5'-ACCAATGCTTAATCAGTGAG-3'	859	[24]
ClassI integron	Class 1 intg F Class 1 intgR	GGC ATC CAA GCA GCA AG GGC ATC CAA GCA GCA AG	Variable	[42]

promide with 6 µl of molecular weight marker in a separate lane in each gel running at 100 volts for 30 min till the blue front (tracking dye) nears about 2 cm from the edge of the gel (3/4 of the gel) and viewed by UV trans-illumination.

Plasmid DNA Analysis: Plasmid DNA was isolated from 32 Bacterial isolates which were a mixture of virulent and non virulent isolates showed antibiotic resistance to one or more antibiotics using a high purity plasmid DNA mini-preparation kit (Spin-Column technique) following an overnight growth of isolates in TrypticaseSoy Broth. Plasmid DNA was visualized following electrophoresis in 0.8% agarose gels, ethidium bromide staining then the gel run at 80 volts for 3o min and UV illumination.

Characterization of Ampicillin Resistance Gene in *Aeromonas* Spp. Plasmid DNA: A 859 bpbla*TEM-1b* fragment was amplified with each of two degenerate primer sets [24], Table (1). PCR cycling parameters consisted of 30 cycles of 95°C for 30 s, 59°C for 30 s., 72°C for 1min in a PCR thermal cycler (Eppendorf). An initial denaturation step 95°C for 5 min and a final elongation step 72°C for 5 min were included in the reaction. Fifteen microliters were mixed with 3 µl loading dye and 6 µl molecular weight markers in a separate lane in each agarose gel 0.8%, ethidium bromide staining then the gel run at 80 volts for 3o min and viewed by UV trans-illumination.

Identification of Integrons in *Aeromonas* Spp. Plasmid DNA: The presence of integrons was determined by PCR analysis using primer sets described in Table (1). Nineteen isolates were tested for Class I integrons by amplifying the 5' CS conserved region (Table 1), PCR cycling parameters consisted of 30 cycles of 95°Cfor 45 s.;56°C for 60 s. and 72°C for 90 S. in a PCR thermal cycler (Eppendorf). An initial denaturation step of 94°C for 5 min and a final elongation step of 72°C for 10 min were included in the reaction. Fifteen micro-liter of PCR reaction mixture was subjected to electrophoresis in 0.8% agarose gels, stained in Ethidium Bromide and viewed by UV trans-illumination.

RESULTS AND DISCUSSION

The identification process for *Aeromonas hydrophila* was carried out firstly through biochemical tests it showed, gram-negative, positive Oxidase and Catalase, gave black color on bile esculine hydrolysis, form an acid using glucose with a pink to dark red color in vogues proskauer, methyl red tests. Similarly it was found most of the isolates related to *Aeromonas spp.* gave acidic reaction at the bottoms with a gas, H_2S production, positive citrate utilization and indole fermentation [25].

Rathore *et al*. [26] recorded higher prevalence of aeromonads in water and fish with *A. hydrophila* as a predominant species (43%) followed by *A. sobria* and *A. veronii* (13% each), this result is in tune with the present study on Nile tilapia where *A. hydrophila* isolates were about 216/228 identified isolates (94.7%) from tilapia in Temsah Lake in Egypt with a higher rate than *A. sobria*3/228 (1.3%). The observed clinical signs in the examined fish suffering from Motile Aeromonas Septicemia (MAS) were previously reported by Okpkowassili and Okpkowassili [27] who reported that septicemia, ascitis, erosion, ulceration, detachment of scales and muscular necrosis are the most predominant clinical signs of MAS in Nile tilapia, these findings were in tune with the present study with additional signs of pin point hemorrhages at the caudal peduncle. The postmortem findings are supported by those of Eissa *et al*.[28] who found that, the parenchymatus organs suffered from congestion with focal lesions in the liver, spleen and kidney as well as, serosanguinous fluids filling the abdominal cavity which is in correlation with post mortem finding with another signs of corrosive and hemorrhagic liver.

In this study A high prevalence of lipase gene in genomic DNA of *Aeromonas* isolates was detected 19/38 (50%). Similarly, aeromonads produce a wide variety of virulence factors like lipases that act as disease cause in the host [29]. It has been predicted that the occurrence of human diseases is related to the production of a variety of extracellular toxins from *Aeromonas* such as proteases, lipases, elastase, lecithinase, chitinases and hemolysins

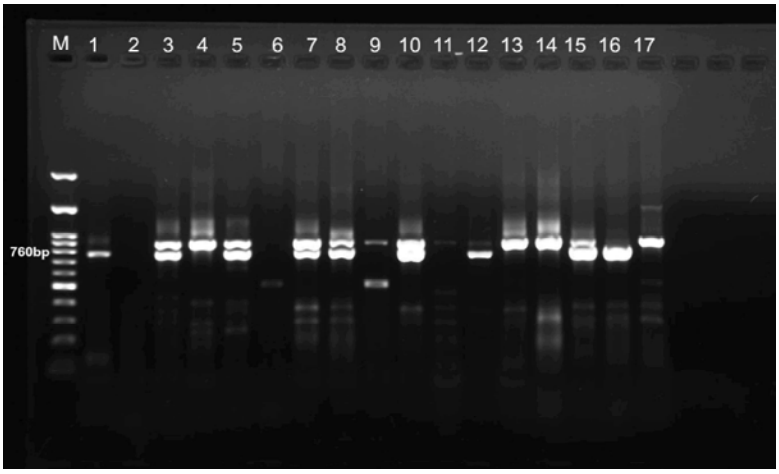

Fig. 1: *Agarose gel electrophoresis of PCR products of encoded Lipase gene from Aeromonas strains from Nile tilapia, Temsah Lake.(M 100 bp DNA Ladder H3 RTU, Gene Direx*(100. 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 3000 bp); *lane* 1, positive control *Aeromonas* isolate from muscle; *lane* 2, negative control isolate. *lane* 3, 5, 7, 8, 10, 12, 15, 16 are positive lipase virulent *Aeromonas hydrophila* isolates. *lane* 4, 6, 9, 11, 13, 14, 17 were non virulent lipase isolates; *lane* 9,11 were non virulent *Aeromonas sobria*.).

Table 2: Interpretation of antibiotic sensitivity test for 21*Aeromonas* isolates isolated from Nile tilapia organs and their percentages

	Resistant		Intermediate		Sensitive	
Antimicrobial agent	No.	%	No.	%	No.	%
Ampicillin	21	100	-	-	-	-
Lincomycin	21	100	-	-	-	-
Methicillin	21	100	-	-	-	-
Ceftazidime/Clavulanic acid	21	100	-	-	-	-
Streptomycin	-	-	6	28.6	15	71.4
Imipenem	-	-	13	61.9	8	38.1
Kanamycin	-	-	5	23.8	16	76.2
Ciprofloxacin	-	-	-	-	21	100
Gentamycin	-	-	-	-	21	100
Nalidixic acid	5	23.8	14	66.7	2	9.52
Tetracycline	1	4.8	9	42.8	11	52.4
Chloramphenicol	-	-	8	38.1	13	61.9

[23]. So, detection of virulence genes of *A. hydrophila* by PCR amplification of DNA has a great potential for rapid identification of this bacterium because it has proved to be highly specific. However, all *A. sobria* isolates in this study appeared to be nonvirulent (Figure 1).

It is extremely dangerous as a number of microorganisms in aquatic environments carry a diversity of antimicrobial resistance genes. From antimicrobial susceptibility data obtained in the present study, the indiscriminate use of ampicillin would result in treatment failure, given the high percentage of resistant strains; the β-lactam resistance phenotype (penicillins and many of the early cephalosporins) may be explained in part by *Aeromonas* spp naturally occurring β-lactamase production [30] and inducible β-lactamase activity [31]. Although the expected high levels of resistance to β-lactam agents were observed, isolates in Jacobs and Chenia [32] study appeared to be susceptible for most part to aminoglycosides, fluoroquinolones, 2nd and 3rd generation cephalosporins and carbapenems moreover aeromonads in Hatha *et al.* [33] study appeared to have susceptibility profiles which displayed susceptibility to amikacin, gentamicin, chloramphenicol, 2ndand 3rd generation cephalosporins, carbapenems, these findings were partially accepted to the present study findings where *Aeromonas* isolates were susceptible toquinolones (Ciprofloxacin), aminoglucosides (Gentamycin, Streptomycin, kanamycin), chloramphenicol, tetracycline and lower sensitivity to first type quinolones (nalidixic acid) and carpamenems (Imipenem) while complete resistance to β-lactam antibiotics (ampicillin, lincomycin, methicillin), 2nd generation cephalosporins mixed with clavulinic acid (ceftazidime/clavulanic) acid Table (2).

Fig. 2: *Agarose gel electrophoresis of plasmid DNA in Aeromonas* spp. *isolates (lane a*: 1000 bp DNA Ladder (Bio Teke corporation), *lane b*: 23 kbpëDNA /Hind Marker (Bio Teke corporation). *lanes k,M,L, kidney, muscle, liver; lanes k3, L4were non virulent Aeromonas sobria; Lane k5: no plasmid, remaining lanes positive plasmid from A. hydrophila)*.

Plasmids were extracted and used as template for amplification of integrons and ampicillin resistance gene. The isolated plasmids in this study were ranged in size from 3.3 to 20 kb, they were isolated from tilapia skinlesions also it was found there is no significance difference between *Aeromonas* strains plasmid DNA size and number obtained from different organs of Nile tilapia plasmid associated ampicillin and tetracycline resistance was associated with a plasmid in a single strain only this was not correlated with our finding that plasmid associated resistance to one or more antibiotic resistance [34]. Plasmids were also detected in *A. hydrophila* and *A. veroniibiovarsobria* freshwater fish isolates, respectively, ranging in size from 2.3 to15.7 kb, with 56.6% overall prevalence of plasmids [35]. In contrast the findings of this study showed higher plasmid rate 29/32 **(**90.6%) in *Aeromonas* spp. isolates. Similarly, the plasmids detected in *Aeromonas* spp. isolates showed differences in size and intensity, which may be related to their copy number in the host cell. Some of the isolates also appeared to carry multiple plasmids [32], these results were nearer to the present finding in presence of multiple plasmid reaching from one to four plasmid at the same isolate, Figure 2.

The presence of β-lactamase gene in *Aeromonas* has been reported in several studies also in gram-negative bacteria which primarily mediated by β-lactamases leading to hydrolyzing the β-lactam ring and inactivate the antibiotic. Many different β-lactamases have been described; however, TEM-, SHV- OXA-, CMY- and CTX M- β-lactamases are the most dominant in gram-negative bacteria [36], *Aeromonas hydrophilia* isolated in Limpopo Province of South Africa was found to be positive for *blaTEM* gene [37]. The study of Igbinosa and Okosh [38] was the first report on the presence of *blaTEM* gene in *Aeromonas* isolates from waste water treatment plant in Eastern Cape Province of South Africa with a lower detection rate of 8.3% for *blaTEM* gene. However, the identified $bla_{TEM\text{-}1b}$was in a higher rate 15/19 (78.9%) of the *Aeromonas* plasmid encoded isolates from tilapia, Figure (3). These results were agree with that of Ash *et al.* [39] who identified ampicillin resistance genes in 70% of the plasmids however they were not incoordinance with Ndi and Barton [13] who mentioned in their studies bla_{TEM} was not detected in any of *Aeromonas* isolates from Australia origin although there was a phenotype β- lactam resistance. Moreover in another study three *Aeromonas hydrophila* strains isolated from patients blood in Taiwan were found to harbor *blaTEM* gene, another report of *blaTEM* gene positive *Aeromonas hydrophilia* from an aged patient with necrotizing fasciitis [40]. The presence of β-lactamase gene in both clinical and environmental isolates of *Aeromonas* species is complicated as it tends to limit treatment options in *Aeromonas* infections.

More than 100 different antibiotic resistance gene cassettes have been found within integrons and a majority encodes antibiotic resistance phenotypes [32]. It was observed their site-specific recombination system enables them to collect multiple antimicrobial resistance

Fig. 3: Agarose gel electrophoresis of PCR products of plasmid encodedbla $_{TEM\text{-}1b}$ gene from *Aeromonas strains. (*Lane M: 100 bp DNA Ladder H3 RTU, Gene Direx; positive 859 bp bands of $bla_{TEM\text{-}1b}$gene in *Aeromonas* spp. at all plasmid DNA isolated from kidney, liver, muscle except three isolates have no $bla_{TEM\text{-}1b}$ resistance gene *lane 2, 3, 5)*.

Fig. 4: PCR amplification of Class I integron 5' CS mediated plasmid isolated from *Aeromonas* spp. *Lane M: 100 bp DNA Ladder H3 RTU, Gene Direx; lane 1-19*: positive Class 1 integron isolated from different *Aeromonas s*pp. from kidney, muscle and liver of Nile tilapia from Temsah Lake varied in sizes from 0.3-1.6 kbp.; *lane 5, 12, 18*: negative, *A. hydrophila*; *lane 7,13,15,16*: positive Class 1 integron in *Aeromonas hydrophila*; *lane*13 was *Aeromonas sobria*.

gene cassettes. Although not mobile themselves, they are often associated with conjugative plasmids or transposons. Integrons form an important source for the spread of antibiotic resistance [41]. Although not mobile themselves, they are often associated with conjugative plasmids or transposons. It's observed a higher detection rate of Class I integron in 65% of antibiotic resistant *A. salmonicida* from North Europe and North America [12] also in Nawaz *et al.* [42] studies 48.0% that confer resistance to multiple antibiotics, (43.5%) of *Aeromonas* spp. isolates carry Class I integrons also 5 integrons were plasmid encoding in Sarria-Guzma'n *et al.* [43] and in Peña [44] 42% *Aeromonas* isolates which is nearly agreed with our higher integrons detection rate from 16/19 (84.2%) plasmid *Aeromonas* strains from Nile tilapia fish (Figure 4). The finding of this study detected varied sizes of multiple Class I integrons amplicons plasmid mediated ones were (0.3 - 1.6 kbp), The primer was able to amplify the integrons from 19/32 (59.4%) plasmid, (virulent and non virulent isolates). The same plasmid DNA bearing $bla_{TEM\text{-}1b}$gene and Class I integron were in the same isolates except in 2/19 (10.5%) were (virulent *A. hydrophila*), not

contained bla_{TEM-1b} genebut harboured integrons and were plasmid DNA encoded, all non virulent *Aeromonas hydrophila* contained both bla_{TEM-1b} gene and Class I integron in their plasmid.

The sequencing results showed 100% similarity of plasmid encoded bla_{TEM-1b} resistant gene in two strains were isolated from liver and muscle of *Oreochromis niloticus* collected from Lake Temsah in Ismailia governorate, Egypt with *Klebsiella pneumoniae* plasmid SSLT5 extended spectrum βeta-lactamase gene isolated from hospitalized neonates in Basra city, Iraq, extended spectrum *βeta*-lactamase gene, partial cds in the Genebank under accession number GenBank: KP240605.1 (http://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_746822998).This similarity indicates that plasmids may be co-transferred between this. All the isolates in the present study had been obtained from a tilapia (*Oreochromis niloticus*) Temsah Lake, not previously been exposed to antibiotics for therapeutic purposes nor in feed.

CONCLUSION

The present study showed a high frequency of virulence and multiple drug resistance among *Aeromonas* spp. isolated from *O. niloticus* in Temsah Lake and suggesting domestic wastewater as a reservoir of antibiotic resistance determinants in the study community. Virulence and resistance were encoded by genes previously widespread in other Aeromonadacae. Ciprofloxacin, Gentamycin were drugs of choice to *Aeromonas* spp. while Ampicillin resistance was the prevalent between all isolates which then investigated by molecular studies.

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