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VIRULENCE POTENTIAL AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF MOTILE AEROMONADS ASSOCIATED WITH FRESHWATER ORNAMENTAL FISH CULTURE SYSTEMS: A POSSIBLE THREAT TO PUBLIC HEALTH

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ABSTRACT

Aeromonas spp. are ubiquitous aquatic organisms, associated with multitude of diseases in several species of animals, including fishes and humans. In the present study, water samples from two ornamental fish culture systems were analyzed for the presence of *Aeromonas*. Nutrient agar was used for *Aeromonas* isolation, and colonies (60 No) were identified through biochemical characterization. Seven clusters could be generated based on phenotypic characters, analyzed by the programme NTSYSpc, Version 2.02i, and identified as: *Aeromonas caviae* (33.3%), *A. jandaei* (38.3%) and *A. veronii* biovar *sobria* (28.3%). The strains isolated produced highly active hydrolytic enzymes, haemolytic activity and slime formation in varying proportions. The isolates were also tested for the enterotoxin genes (*act*, *alt* and *ast*), haemolytic toxins (*hlyA* and *aerA*), involved in type 3 secretion system (TTSS: *ascV*, *aexT*, *aopP*, *aopO*, *ascF–ascG*, and *aopH*), and *glycerophospholipid-cholesterol acyltransferase* (*gcat*). All isolates were found to be associated with at least one virulent gene. Moreover, they were resistant to frequently used antibiotics for human infections. The study demonstrates the pathogenic potential of *Aeromonas*, associated with ornamental fish culture systems suggesting the emerging threat to public health.

Key words: *Aeromonas*, antibiotic susceptibility, ornamental fish culture systems, virulence

INTRODUCTION

Species of *Aeromonas* are autochthonous microflora of aquatic environments and have been considered important pathogens for cold or warm blooded animals (52). They are regarded as important pathogens of aquatic animals, causing significant economic losses in the aquaculture industry worldwide (45). Recent works have emphasized their emergence as primary human pathogens as well, since they have been related to a variety of local and systemic infections, even in immunologically competent hosts (31). It has been suggested that the high prevalence of *Aeromonas* sp. in the

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environment be considered a threat to public health, as infections caused by these pathogens are generally the result of ingestion of contaminated water or food (3, 24).

The virulence of *Aeromonas* is complex and involves multiple virulence factors such as various hydrolytic enzymes, cytotoxic and cytotonic enterotoxins, haemolytic toxins and TTSS (31). These virulence factors enable the bacteria to colonize, gain entry, establish, replicate, and cause damage in host tissues and to evade the host defense system and spread, eventually killing the host (73). Another important factor is the increasing incidence of multidrug resistance amongst *Aeromonas* spp. worldwide (30, 41, 64). Antibiotic-resistant bacteria present in an aquaculture setting may be transferred to humans through wound infections, following the exposure to contaminated water or fish (56).

Because *Aeromonas* spp. are pathogenic to fishes and humans, their presence in culture environment is of concern (10). Aquarium water has been suggested as the source of aeromonads resulting in gastrointestinal infection (58). In the realm of aquaculture, aquarium fish industry constitutes a large segment of the pet animal industry (71) having global marketing network. Alike in any aquaculture practice, the intensification of the ornamental fish culture has led to the emergence of diseases and mortality with varied manifestations. In our study undertaken in this background, we could find that *Aeromonas* spp. were the associated bacterial flora of majority of disease outbreaks (64). *Aeromonas* sp. has been identified as the aetiology of diseases in freshwater ornamental fishes with a variety of clinical signs such as fin rot/tail rot, ulceration, exophthalmia, dropsy etc. (17, 46, 64). Moreover, there have been several reports on zoonoses acquired following injuries from handling fish, working in aquaculture systems, or keeping fish as pets (22, 42). Even though, several studies on the distribution pattern of aeromonads in different aquaculture systems have been reported (2, 55), those from ornamental fish culture systems are scanty. In view of the limited reports, the present study was undertaken to investigate the prevalence of *Aeromonas* spp. in freshwater ornamental fish culture environments, their antimicrobial susceptibility pattern, and the presence of virulent factors. This information turns out to be the reflection of the normal flora of *Aeromonas* in ornamental fish culture systems.

MATERIALS AND METHODS

Collection of water samples

Water samples (100 mL) were collected from two ornamental fish culture systems located at Thrissur District, Kerala, India, in which gold fishes were mass reared. The samples were collected during the month of November 2007. The water samples were collected in sterile bottles according to the Standard Methods for Examination of Water and Wastewater (7), transported in ice box and analyzed within 24 hr.

Isolation of *Aeromonas*

The water samples were subjected to 10-fold serial dilution in 0.5% saline, and aliquots of 200 μL samples from each dilution were spread plated onto nutrient agar (gL^{-1} peptone - 5.0; beef extract - 5.0; NaCl -5.0; agar-20.0; pH 7.5 ±0.3) plates. The plates were incubated for 48 hr at 28ºC. Colonies were randomly picked from the plates, sub-cultured in nutrient agar slants, and subjected for further characterization.

Phenotypic characterization

The isolates were examined for Kovac's cytochrome oxidase, O/129 sensitivity (Oxoid), catalase, production of hydrogen sulphide in TSI, arginine dihydrolase, lysine and ornithine decarboxylase, indole production, methyl red test, Voges-Proskauer reaction (acetoin production), citrate utilization, urease production, phenylalanine deaminase, gluconate oxidation, nitrate reduction, ONPG (β-galactosidase) production, and acid production from sugars as described by

Collee *et al*. (18). The isolates were also tested for hydrolysis of esculin (70), production of alkylsulfatase (32), pyrazinamidase (13), and utilization of DL-lactate (32), malonate and acetate (21).

The identification was accomplished following Aerokey II devised by Carnahan *et al*. (14).

Clustering of the isolates were achieved by the programme NTedit, Version 1.1b (Applied Biostatics Inc), and analyzed by the programme NTSYSpc, Version 2.02i (Applied Biostatics Inc). Similarities were calculated by sequential agglomerative hierarchical nested cluster method (SAHN), and cluster analysis was performed by mean of the unweighted paired group method using arithmetic average (UPGMA).

Phenotypic expression of virulence – *In vitro* assays

All isolates were tested for the production of DNase (26), caseinase, chitinase, phospholipase (lecithinase), gelatinase (protease), and degradation of tributyrin (for lipase) (18). Elastase activity on solid medium was detected by spot inoculating the organisms on Luria Bertani (LB) medium supplemented with 0.2% elastin-congo red (Sigma-Aldrich Co.) with clear zone around the growth and diffusion of Congo red into the clear zone, and haemolytic activity on LB agar containing 5% (vol/vol) human blood (65). Brain-heart infusion agar plates were supplemented with 0.8 gL^{-1} Congo red (Sigma- Aldrich). Following incubation at 30ºC for 24 hr, slime production was indicated by the development of black colonies, whereas the absence of slime led to non-pigmented colonies (23).

Extraction of total DNA

Cell suspension (1 mL) grown in LB medium was centrifuged at 10000g for 10 min at 4ºC, pellet resuspended in 500 µL TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and centrifuged at 10000g for 10 min at 4ºC. The pellet was resuspended in 500 µL lysis buffer (Tris-HCl 0.05 mM, pH 8.0, EDTA 0.05 mM, NaCl, 0.1 mM, SDS 2%, PVP 0.2% and mercaptoethanol 0.1%) (40) and 10 µL Proteinase K was added and incubated initially for 1 hr at 37ºC and then for 2 hr at 55ºC. Further extraction was carried out by phenol-chloroform extraction method as described by Sambrook & Russell (57).

PCR detection of virulent genes

The representative cultures, were subjected for PCR to detect virulent genes such as enterotoxins (*act, alt* and *ast*), haemolytic toxins (*hlyA* and *aerA*), genes involved in type 3 secretion system (TTSS: *ascV*, *aexT*, *aopP*, *aopO*, *ascF–ascG*, and *aopH*), and *glycerophospholipid-cholesterol acyltransferase* (*gcat*).

PCR was performed in a DNA thermal cycler (Eppendorf AG, Hamburg, Germany) having the reaction mixture (final volume 25 µL) containing 2.5 µL 10X buffer, 1.5 µL 25 mM $MgCl_2$, 1.0 µL of 10 pmol of each oligonucleotide primer, 1.0 µL of DNA template, 2 µL of 2.5 mM each deoxynucleoside triphosphate and 1 µL of Taq DNA polymerase.

The previously described primers and PCR conditions were used for the specific amplification of virulent genes. Characteristics of primers used for the PCR amplification of virulent genes are summarized in Table 1. The PCR products were analyzed by electrophoresis on 1% agarose gel prepared in 1X TAE buffer. The gels were stained with ethidium bromide (0.5 μgmL^{-1}), visualized on a UV light transilluminator, and documented.

Antibiotic susceptibility test

Susceptibility to selected antibiotics was tested on nutrient agar plates by the disc diffusion method of Baur *et al*. (9). Briefly, the nutrient agar plates were swabbed with overnight grown cultures of the isolates. Readymade antibiotic discs from HiMedia Laboratories, India, were aseptically placed on the swabbed plates. The plates were incubated at 28 ± 1^0C for 18 hr and the clearing zone formed around the discs recorded using Hi Antibiotic Zone Scale (Himedia). The multiple antibiotic resistance (MAR) index (number of antibiotics to which the isolate was resistant/total number of antibiotics tested) was determined for each isolate (37).

Table 1. Primers used for the amplification of virulent genes

Virulent genes	Primers	DNA sequences (5'-3')	Product size (bp)	Reference
act	F	AGAAGGTGACCACCAAGAACA	232	36
	R	AACTGACATCGGCCTTGAACTC		
ast	F	TCTCCATGCTTCCCTTCCACT	331	36
	R	GTGTAGGGATTGAAGAAGCCG		
alt	F	TGACCCAGTCCTGGCACGGC	442	36
	R	GGTGATCGATCACCACCAGC		
aerA	F	CCCGCCGATCTGCAACCGGG	489	50
	R	CTGGTCTGGATAGACGGGCTCTGCC		
hlyA	F	GGCCGGTGGCCCGAAGATACGGG	597	28
	R	GGCGGCGCCGGACGAGACGGG		
aexT	F	GGCGCTTGGGCTCTACAC	535	12
	R	GAGCCCGCGCATCTTCAG		
ascV	F	GCCCGTTTTGCCTATCAA	807	12
	R	GCGCCGATATCGGTACCC		
*aop*P	F	GAGAGTTGGCTAGCGGTGAG	490	12
	R	TCCTCATGGAGCGCATCCAG		
*aop*O	F	CGAGACAGACAAGTTTGC	401	12
	R	TGTCGTTGTGGACTATCC		
*aop*H	F	TCAATCAGGACGATGTCG	518	12
	R	GTTGGCATTGAGATCTGC		
*asc*F – *asc*G	F	ATGAGGTCATCTGCTCGCGC	789	72
	R	GGAGCACAACCATGGCTGAT		
gcat	F	CTCCTGGAATCCCAAGTATCAG	237	49
	R	GGCAGGTTGAACAGCAGTATCT		

RESULTS AND DISCUSSION

Isolates of *Aeromonas* from two freshwater ornamental culture systems were characterized phenotypically and evaluated for the presence of virulence markers. Sixty colonies were randomly picked (30 from each source), and subjected for Gram staining, Kovac's oxidase activity, glucose fermentation using marine oxidation fermentation medium (MOF), motility using semi-solid agar, and the test of resistance to O/129. Those isolates, which were Gram-negative, rods, motile, oxidase-positive, glucose fermenting, resistant to O/129 were designated to aeromonads. Table 2 indicates the phenotypic characteristics of the isolates.

Table 2. Phenotypic characterization of *Aeromonas* spp. recovered from freshwater ornamental fish culture systems

Phenotypic Characters[a]	Cluster 1 (n=8)	Cluster 2 (n=9)	Cluster 3 (n=6)	Cluster 4 (n=7)	Cluster 5 (n=12)	Cluster 6 (n=10)	Cluster 7 (n=8)
MOF	F	F	F	F/G	F	F	F
Voges-Proskaur reaction	-	+	+	+	+	+	+
Methyl red test	+	-	-	-	-	-	-
Utilization of:							
Citrate	-	+	+	+	+	+	+
Acetate	+	+	-	-	+	+	+
Gluconate oxidation	-	+	+	+	+	+	+
Production of Alkyl sulfatase	-	+	-	-	-	-	-
Hydrolysis of esculin	+	-	-	-	+	-	-
Lysine decarboxylase	-	+	+	+	-	+	+
Acid production from:							
1. Sucrose	+	+	-	-	+	-	+
2. D-mannose	-	+	+	+	+	+	+
3. Glycerol	+	+	+	+	-	+	+
4. Salicin	+	-	-	-	+	-	-
5. D-cellobiose	+	-	-	-	+	-	-
6. L-arabinose	+	-	-	-	-	-	-
Identity	*A. caviae*	*A. veronii* biovar *sobria*	A. *jandaei*	A. *jandaei*	*A. caviae*	A. *jandaei*	*A. veronii* biovar *sobria*

MOF-Marine Oxidation Fermentation; F-Fermentative; F/G-Fermentative with gas production

[a] Following results were uniform to all;

Positive Results: kovac's oxidase, catalase, indole production, nitrate reduction, oxidation of ONPG (ortho-nitrophenyl-β-D-galactopyranoside), production of pyrazinamidase and arginine dihydrolase, acid production from fructose, D-maltose, trehalose, dextrin, starch, D-galactose and D-ribose, tolerance to 0 and 3% NaCl.

Negative Results: O/129 sensitivity, utilization of malonate and DL-lactate, production of urease and ornithine decarboxylase, acid production from D-sorbitol, L-rhamnose, D-melibiose, m-inositol, raffinose, D-lactose, adonitol and inulin, tolerance to 6, 8 and 10% NaCl.

From the Table 2, it is apparent that the isolates formed a heterogeneous population, as they differed in the characters such as: gas production from glucose, methyl red test, Voges-Proskaur reaction, citrate and acetate utilization, gluconate oxidation, production of alkyl sulfatase and lysine decarboxylase, esculin hydrolysis, acid production from sucrose, D-mannose, glycerol, salicin, D-cellobiose and L-arabinose. Clustering of the isolates was achieved at 80% similarity based on the phenotypic characters examined, and seven clusters could be generated having a common origin (Figure 1). Cluster 1 and 5 were identified as *Aeromonas caviae* (33.3%), cluster 3, 4 and 6 as *A. jandaei* (38.3%), and cluster 2 and 7 as *A. veronii* biovar *sobria* (28.3%). It has been reported that *A. hydrophila*, *A. veronii* biovar *sobria*, *A. caviae* and *A. jandei* are the species most commonly implicated in human intestinal infections (33), accounting for >85% of the clinical isolates of this genus (62.).

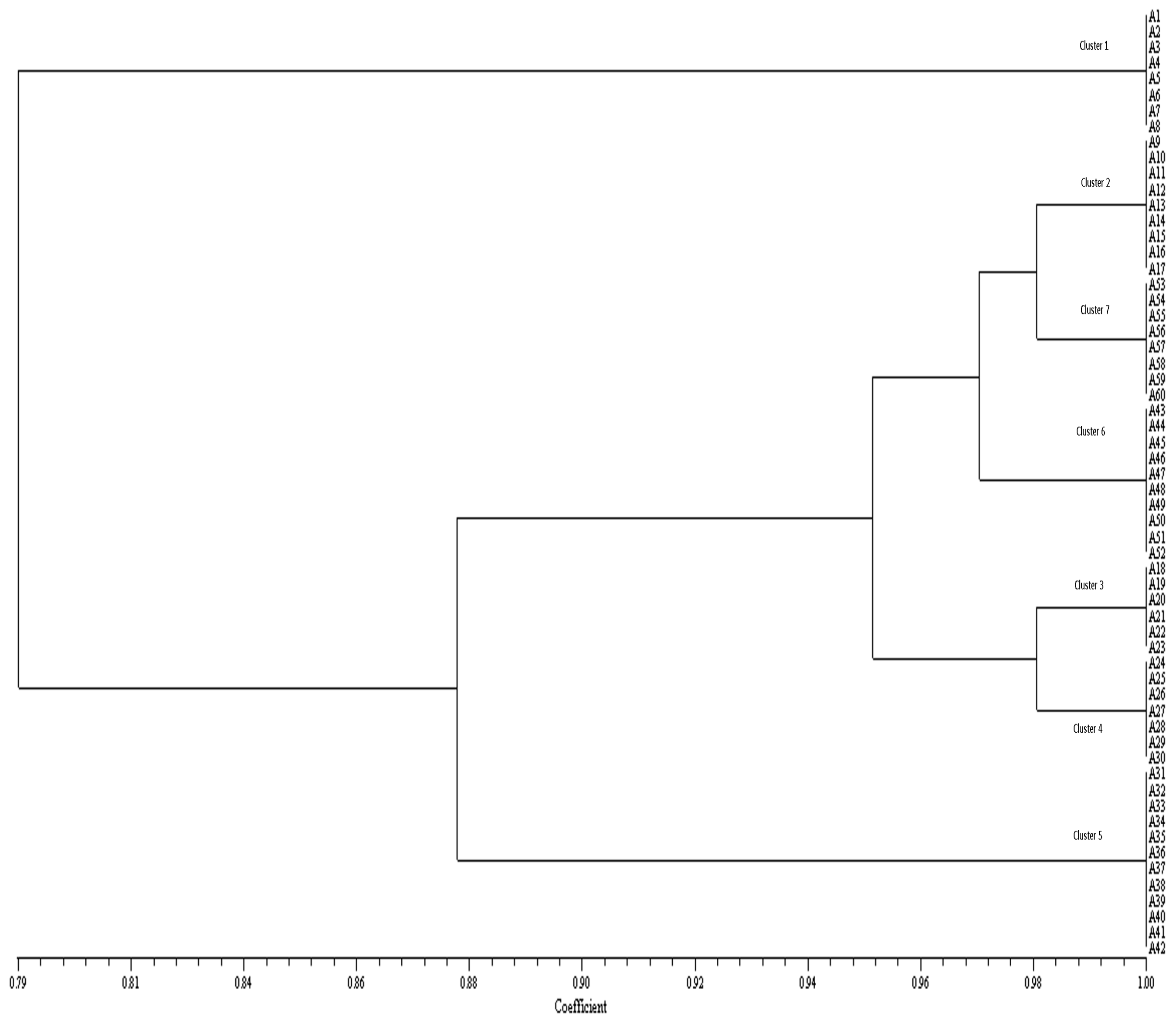

Figura 1. Dendrogram of *Aeromonas* isolates from freshwater ornamental fish culture systems based on phenotypic characters

All isolates were subjected to a few phenotypic expression assays, which indirectly correlated with the virulence. Among the hydrolytic enzymes tested, all isolates produced amylase, lipase, caseinase, chitinase and gelatinase, but not elastase. However, only 48.3% of the isolates displayed lecithinase, DNase and slime formation. Haemolytic activity was observed in 47 isolates (78.3%) (Table 3). All these activities were reported as virulence-associated factors, and have been suggested that virulence level is correlated to the amount of enzymes and toxins produced. It was reported that extra cellular proteases aid the organism in overcoming the initial host defense mechanism such as resistance to serum killing (43), and are needed for the maturation of exotoxins such as aerolysin (29). Lipases play an important role in invasiveness and establishment of infections (67), while secreted phospholipases act as both haemolysins and glycerophospholipid-cholesterol acyl-transferases (61). The association of nucleases with pathogenicity has not yet been confirmed, but reports have indicated that it participates in the development of host infection (47). Slime production reflects the microorganism's capacity to adhere to specific host tissues and thereby to produce invasive micro colonies (44,) and diverse illness (66). The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads (59).

Several authors have suggested that the presence of aeromonads capable of producing virulence factors in water is a threat to public health (11, 38). Involvement of virulent genes on the pathogenesis of *Aeromonas* sp. has been demonstrated (34), which encode for secreted enzymes and toxins that contribute to the pathogenicity of the organism (4). In the present study, one representative strain from each cluster was chosen for screening virulent genes, and designated them as *Aeromonas* MCCB 143, 144, 145, 146, 147, 148 and 149. Table 3 indicates the distribution of virulent genes in *Aeromonas* isolates recovered from ornamental fish culture systems. The significant observation was that, in all isolates, at least one virulent gene could be amplified. Cytotoxic enterotoxin (Act) was produced by 58.3% of the *Aeromonas* strains isolated, while 28.3% were able to produce heat-labile enterotoxin (Alt). Altogether, 28.3% produced both Act and Alt. However, none of the isolates possessed *ast* gene and haemolytic toxin genes. Only 20.0% of the isolates possessed *ascF-ascG* gene. Surprisingly, *gcat* gene could be amplified in all isolates.

Table 3. Incidence of virulent markers in *Aeromonas* isolated from freshwater ornamental fish culture systems

Virulent factor	MCCB 143 *A. caviae*	MCCB 144 *A. veronii* biovar *sobria*	MCCB 145 A. *jandaei*	MCCB 146 A. *jandaei*	MCCB 147 *A. caviae*	MCCB 148 A. *jandaei*	MCCB 149 *A. veronii* biovar *sobria*
DNase	-	+	-	-	+	-	+
Lecithinase	-	+	-	-	+	-	+
Slime formation	-	+	-	-	+	-	+
β-haemolysis	+	+	-	-	+	+	+
alt	-	+	-	-	-	-	+
ast	-	-	-	-	-	-	-
act	+	+	-	-	-	+	+
aer A	-	-	-	-	-	-	-
hly A	-	-	-	-	-	-	-
aex T	-	-	-	-	-	-	-
*asc*F – *asc* G	-	-	-	-	+	-	-
aop P	-	-	-	-	-	-	-
aop O	-	-	-	-	-	-	-
aop H	-	-	-	-	-	-	-
gcat	+	+	+	+	+	+	+

In the present study, the production of a wide array of virulence factors by *Aeromonas* species is indicative of their potential to cause diseases in fishes and humans. Among the enterotoxins, *Act* is one of the most significant virulence factors, which have hemolytic, cytotoxic, and enterotoxic activities, and *Alt* is associated with diarrhea that induced fluid secretion in the ligated small intestinal loops of animals (16). TTSS, which delivers toxins directly to the cytosol of eukaryotic host, is a virulent trait that correlates with bacterial pathogenicity, and their presence can be used as a general indicator of virulence (68). The gene *ascF-ascG* encodes the needle complex and a chaperone, respectively (25). It is recognized that *gcat* has lipase or phospholipase activity, which mediate erythrocyte lysis by digesting their plasma membrane (54).

The isolates were individually tested against 70 antibiotics. The results were obtained by measuring the inhibition zones after 24 hours. The percentage of antimicrobial resistance of isolates of *Aeromonas* to the antibiotics is shown in Table 4.

Table 4. Antibiotic susceptibility pattern of *Aeromonas* spp. from freshwater ornamental fish culture systems

Class	Antibiotic (Disc content)	Resistant (%)		
β-lactam antibiotics		*A.caviae* (n=20)	*A.veronii* biovar *sobria* (n=17)	*A.jandaei* (n=23)
1.Penicillins	Amoxicillin (10 µg), Ampicillin (10 µg), Methicillin (5 µg), Penicillin G (10 U), Cloxacillin (1 µg), Ticarcillin (75 µg)	100	100	100
	Oxacillin (1 µg)	100	100	26.1
	Piperacillin (100 µg), Carbenicillin (100 µg)	0	0	0
2.Cephalosporins				
1st generation	Cephalexin (30 µg)	0	52.9	30.4
	Cefazolin (30 µg)	0	53.0	30.4
	Cephradine (25µg)	0	53.0	30.4
	Cephaloridine (10 µg)	100	100	100
	Cephalothin (30 µg)	40.0	100	30.4
	Cephadroxil (30 µg)	0	53.0	0
2nd generation	Cefaclor (30 µg)	0	53.0	0
	Cephoxitin (30 µg)	0	53.0	30.4
	Cefamandole (30 µg)	0	0	0
3rd generation	Cefriaxone (10 µg), Ceftazidime (30 µg), Cefoperazone (75 µg), Ceftizoxime (30 µg)	0	0	0
3.Carbapenem	Imipenam (10 µg)	0	0	0
Aminoglycosides	Amikacin (10 µg), Gentamycin (10 µg), Kanamycin (30 µg), Neomycin (30 µg), Netillin (10 µg), Streptomycin (10 µg), Tobramycin (10 µg)	0	0	0
Macrolides	Azithromycin (15 µg), Tylosine (15 µg)	0	0	0
	Clarithromycin (15 µg)	0	53.0	0
	Erythromycin (10 µg)	100	100	100
	Oleandomycin (15 µg)	40.0	53.0	56.5
	Spiramycin (30 µg)	40.0	0	0
Tetracyclines	Doxycycline HCl (10 µg), Oxytetracycline (30 µg), Tetracycline (10 µg)	100	100	100
	Chlortetracycline (30 µg), Minocycline (30 µg)	0	0	0
Chloramphenicol	Chloramphenicol (10 µg)	0	0	0
Rifamycins	Rifampicin (2 µg)	0	53.0	0
Lincosamides	Clindamycin (2 µg), Lincomycin (2 µg)	100	100	100
Steroids	Fusidic acid (10 µg)	100	100	100
Nitrofurans	Nitrofurazone (100 µg)	0	0	0
	Furazolidone (50 µg), Furaxone (100 µg)	0	53.0	0
Sulfonamides	Trimethoprim (5 µg), Sulfadiazine (100 µg), Sulfafurazole (300 µg), Sulfaphenazole (200 µg)	0	0	0
Quinolones/ Fluoroquinolones	Ciprofloxacin (5 µg), Enrofloxacin (5 µg), Floxidine (20 µg), Nitroxoline (30 µg), Norfloxacin (10 µg), Ofloxacin (2 µg), Pefloxacin (5 µg), Sparfloxacin (5 µg)	0	0	0
	Pipemidic acid (20 µg), Nalidixic acid (30 µg)	100	53.0	26.0
Aminocoumarins	Novobiocin (30 µg)	60.0	0	0
Nitrofurantoin	Nitrofurantoin (100 µg)	0	0	0
Polypeptides	Polymixin B (50 U)	100	100	100
	Colistin (10 µg)	100	47.0	74.0
	Bacitracin (10 U)	100	100	100
Fosfomycin	Fosfomycin (50 µg)	0	0	0
Glycopeptides	Vancomycin (5 µg)	100	100	26.0

In the present study, all the isolates showed varying degree of resistance to the β- lactam antibiotics. Except for piperacillin and carbenicillin, all isolates displayed some degree of resistance to penicillins tested. *A. veronii* biovar *sobria* was the only species to exhibit any significant cephalosporin resistance. Among the cephalosporins tested, 100% resistance was only noticed against cephaloridine, although partial resistance against cephalexin, cephalothin, cefazolin, cephradine, cephadroxil, cefaclor and cephoxitin was observed. Conversely, all isolates were susceptible to the third generation cephalosporins and imipenam. One characteristic feature of *A. veronii* biovar *sobria* was the resistance to cephalothin. In fact, susceptibility to cephalothin is one of the specific characteristics of *A. veronii* biovar *sobria* (1); therefore, the variability observed compromises the classical use of this phenotypic character for species delineation (1).

It is known that *Aeromonas* spp. are among the few microorganisms harboring different chromosomal β-lactamase genes, including *cphA*, *cepH* and *ampH*, encoding class B, C and D β-lactamases, respectively (8). Among the clinical populations of Gram-negative microorganisms, *bla*TEM-1 is the most frequently detected antimicrobial resistance gene. Although its expression results in penicillin resistance, diverse point mutations in the *bla*TEM-1 gene have contributed to the emergence of TEM-type extended-spectrum β-lactamases, resulting in simultaneous resistance to penicillins and broad-spectrum cephalosporins (69).

While the isolates subjected for study here displayed decreased susceptibility to the 1st generation quinolones such as nalidixic acid and pipemidic acid, they were highly susceptible to the newer generation fluoroquinolones such as ciprofloxacin, enrofloxacin, sparfloxacin, norfloxacin, pefloxacin, ofloxacin, floxidine and nitroxoline. In the present study, 100% of *A.caviae*, 53% of *A.veronii* biovar *sobria* and 26% of *A.jandaei* were found to be resistant to nalidixic acid. However, Guz and Kozinska (27) reported susceptibility to nalidixic acid by *Aeromonas* isolates from carp suffering from motile aeromonad septicemia.

Although fluoroquinolones have been reported as the treatment of choice for *Aeromonas* infections (63), it is well established that nalidixic acid resistance predicts the development of fluoroquinolone resistance during therapy, as well as therapeutic failure (53). Quinolone resistance in Gram-negative bacteria is primarily attributable to mutations in the quinolone resistance determining regions (QRDRs) consisting of the *gyr*A and *par*C genes, which are the subunits of the target enzymes of quinolones, DNA gyrase subunit A and topoisomerase IV, respectively (5).

Except azithromycin and tylosine, all isolates showed varying degrees of resistance to macrolides. Jacobs and Chenia (30) reported that 10.8% of the *Aeromonas* strains isolated from South African aquaculture systems were resistant to azithromycin. In the present study, all isolates were resistant to erythromycin. However, Orozova *et al.* (51) reported sensitivity of *Aeromonas* strains to this antibiotic. Surprisingly, all isolates were resistant to lincosamides tested. However, Guz and Kozinska (27) reported susceptibility to licomycin by *Aeromonas* isolates from carp suffering from motile aeromonad septicemia. There are a number of inactivating enzymes that act on the macrolides and lincosamides. Esterases act on erythromycin and the nucleotidyltransferases confer resistance to the lincosamides (6).

All isolates displayed 100% susceptibility to trimethoprim and chloramphenicol and 100% resistance to fusidic acid, while 60% of *A.caviae* isolates displayed novobiocin resistance. This result differed from the study of Chang *et al.* (15) who reported trimethoprim resistance in *Aeromonas* strains from food borne outbreak and environmental sources in Taiwan. All isolates were sensitive to aminoglycosides. However, Guz and Kozinska (27) reported resistance of *Aeromonas* isolates from carp suffering from motile aeromonad septicemia against kanamycin, neomycin and streptomycin.

In the present study, all isolates exhibited uniform

resistance to polymixin B and bacitracin, and varying degree of resistance to colistin. Fifty three per cent of *A.veronii* biovar *sobria* isolates displayed resistance to rifampicin, furazolidone and furaxone. However, other isolates showed susceptibility to these antibiotics. All isolates displayed 100% susceptibility to nitrofurantoin, nitrofurazone and fosfomycin, while 100% resistance to vancomycin was shown by *A. veronii* biovar *caviae* and *A.caviae* isolates. Vancomycin resistance is attributable to Van A, B, C, D, E, and G phenotypes. The resistance phenotype is accomplished using multiple proteins specified in gene clusters and each result in the production of a modified peptidoglycan (19).

A high degree of resistance towards tetracyclines has been displayed by the isolates. They showed 100% resistance to oxytetracycline, tetracycline and doxycycline. However, the percentage of tetracycline-resistant *Aeromonas* spp. strains in our study was more when compared with the results of other studies on antibiotic resistance in aquaculture farms (2, 60). Indeed, for several decades, tetracycline has been widely used in clinical medicine, veterinary and agriculture (26), contributing to higher levels of microbial resistance, especially among the genus *Aeromonas* (20, 30, 48). The resistance to tetracyclines occurs through the presence of *tet* genes in the bacterial DNA.

The multiple antibiotic resistance (MAR) pattern of *Aeromonas* spp. was calculated and the. MAR index presented in Table 5. It was observed that all the isolates showed MAR index of more than 0.2 (ranged from 0.243 to 0.457), indicating indiscriminate use of antibiotics. A MAR index of 0.2 or more is said to have originated from high risk sources of contamination (37) where antibiotics are often used.

The rapid emergence of antibiotic resistance among bacteria is, to a great extent, due to the dissemination of antibiotic resistance genes by horizontal transfer mediated by plasmids, transposons and integrons (39). The isolation of multiresistant aquatic *Aeromonas* species from freshwater in other parts of the world along with our own findings warrant the need to take proper measures to prevent the introduction of resistant *Aeromonas* into water sources used by humans, as the contact with contaminated water and fish may result in resistance gene transfer from fish to the human intestinal microbiota. Likewise, the increase in antimicrobial resistance poses a growing challenge in the treatment of *Aeromonas* infections in fish as well as in humans. If such antibiotic resistant aeromonads, which are true human and aquatic pathogens, happen to multiply within fresh water ornamental fish culture systems, they obviously may turn out to be a threat to public health.

Table 5. MAR index of *Aeromonas* spp. isolated from freshwater ornamental fish culture systems

MAR index	% occurrence		Total % (n=60)
	Source 1*(n=30)	Source 2* (n=30)	
0.243	0	43.3	21.7
0.286	0	33.3	16.7
0.300	23.3	0	11.7
0.314	0	23.3	11.7
0.328	16.7	0	8.3
0.343	26.6	0	13.3
0.457	33.3	0	16.7

*Source 1 includes isolates belonging to cluster 1, 2, 3 and 4 and Source 2 includes isolates belonging to cluster 5, 6 and 7.

CONCLUSION

The data generated suggest that the ornamental fresh water fishes are always under the threat of an infection caused by *Aeromonas* because, they live in an environment with a normal flora of *Aeromonas* equipped with at least a couple of virulent

genes having the capability of their expression during moments of stress. Besides, *Aeromonas* spp. might also pose a threat to public health especially to those who come into contact with such diseased fishes or ornamental fish culture systems, as their virulence factors including antibiotic resistance genes, could be transmitted to humans, leading to diverse local and systemic infections.

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REFERENCES

1. Abbott, S.L.; Cheung, W.K.W.; Janda, J.M. (2003). The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. *J.Clin. Microbiol*., 41, 2348–2357.
2. Akinbowale, O.L.; Peng, H.; Grant, P.; Barton, M.D. (2007). Antibiotic and heavy metal resistance in motile aeromonads and pseudomonas from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *Int. J. Antimicrob. Agents*, 30, 177–182.
3. Alavandi, S.V.; Ananthan, S. (2003). Biochemical characteristics, serogroups and virulence factors of *Aeromonas* species isolated from cases of diarrhoea and domestic water samples in Chennai. *Indian. J. Med. Microbiol.,* 21, 233-238.
4. Albert, M.J.; Ansaruzzaman, M.; Talukder, K.A.; Chopra, A.K.; Rahman, K.M.; Faruque, A.S.; Islam, M.S.; Sack, R.B.; Mollby, R. (2000). Prevalence of enterotoxin genes in *Aeromonas* spp. isolated from children with diarrhea, healthy controls, and the environment. *J.Clin.Microbiol.,* 38(10), 3785-3790.
5. Alcaide, E.; Blasco, M.D.; Esteve, C. (2010). Mechanisms of quinolone resistance in *Aeromonas* species isolated from humans, water and eels. *Res. Microbiol.,* 161, 40-45.
6. Alekshun, M.N.; Stuart, B.L. (2007). Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell,* 128, 1037-1050.
7. APHA. (1995). Standard Methods for the Examination of Water and Wastewater, 20th edition. American Public Health Association/American Water Works Association/ Water Environment Federation, Washington, DC, USA.
8. Balsalobre, L.C.; Dropa, M.; de Oliveira, D.E.; Lincopan, N.; Mamizuka, E.M.; Matté, G.R.; Matté, M.H. (2010). Presence of BLA_{TEM}-116 gene in environmental isolates of *Aeromonas hydrophila* and *A. jandaei* from Brazil. *Braz. J. Microbiol*., 41, 718-719.
9. Bauer, A.W.; Kirby, W.M.M.; Sherris, J.C.; Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. *Am .J .Clin. Pathol.,* 45, 493-496.
10. Bomo, A.M.; Husby, A.; Stevik, T.K.; Hanssen, J.F. (2003). Removal of fish pathogenic bacteria in biological sand filters. *Water Res.,* 37(11), 2618-2626.
11. Bondi, M.; Messi, P.; Guerrieri, E.; Bitonte, F. (2000). Virulence profiles and other biological characters in water isolated *Aeromonas hydrophila*. *New Microbiologica.,* 23(4), 347-356.
12. Burr, S.E.; Frey, J. (2007). Analysis of type III effector genes in typical and atypical *Aeromonas salmonicida*. *J. Fish. Dis.,* 30, 711-714.
13. Carnahan, A.M.; Hammontree, L.; Bourgeois, L.; Joseph, S.W. (1990). Pyrazinamidase activity as a phenotypic marker for several *Aeromonas* spp. isolated from clinical specimens. *J. Clin. Microbiol.,* 28, 391-392.
14. Carnahan, A.M.; Behram, S.; Joseph, S.W. (1991). Aerokey II: a flexible key for identifying clinical *Aeromonas* species. *J .Clin. Microbiol.,* 29(12), 2843-2849.
15. Chang, Y.C.; Daniel, Y.C.S.; Jan, Y.W.; Shang, S.Y. (2007). Molecular characterization of class 1 integrons and antimicrobial resistance in *Aeromonas* strains from food borne outbreak-suspect samples and environmental sources in Taiwan. *Diagn. Microbiol. Infect. Dis*., 59, 191–197.
16. Chopra, A.K.; Houston, C.W. (1999). Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microbes Infect.,* 1, 1129-1137.
17. Cizek, A.; Dolejska, M.; Sochorova, R.; Strachotova, K.; Piackova, V.; Vesely, T. (2010). Antimicrobial resistance and its genetic determinants in aeromonads isolated in ornamental (koi) carp (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio*). *Vet. Microbiol.,* 142 (3-4), 435-439.
18. Collee, J.G.; Fraser, A.G.; Marmion, B.P.; Simmons, A. (1996). Practical medical microbiology. Churchil Livingstone.
19. Courvalin, P. (1994). Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. *Antimicrob. Agents Chemother*., 38, 1447–1451.
20. Evangelista-Barreto, N.S.; de Carvalho, F.C.T.; Vieira, R.H.S.D.F.; Reis, C.M.F.D.; Macrae, A.; Rodrigues, D.D.P. (2010). Characterization of *Aeromonas* species isolated from an estuarine environment. *Braz. J. Microbiol*., 41, 452-460
21. Ewing, W.H. (1986). Edwards and Ewing's identification of

Enterobacteriaceae, 4th ed. Elsevier Science Publishing Co., Inc., New York, N.Y.

22. Filler, G.; Ehrich, J.H.; Strauch, E.; Beutin, L. (2000). Acute renal failure in an infant associated with cytotoxic *Aeromonas sobria* isolated from patient's stool and from aquarium water as suspected source of infection. *J. Clin .Microbiol.,* 38(1), 469-470.
23. Freeman, D.J.; Elakliner, F.R.; Keane, C.T. (1989). New method for detecting slime producing by coagulase negative *Staphylococci. J.Clin.Pathol.,* 42, 872-874.
24. Garibay, R.I.A.; Aguilera-Arreola, G.; Ocana, A.N.; Cerezo, S.G.; Mendoza, M.S.; Lopez, J.M.; Campos, C.E.; Cravioto, A.; Castro-Escarpulli, G. (2006). Serogroups, K1 antigen, and antimicrobial resistance patterns of *Aeromonas* spp. strains isolated from different sources in Mexico. *Mem. Inst. Oswaldo. Cruz*., 101, 157-161.
25. Ghosh, P. (2004). Process of protein transport by the type III secretion system. *Microbiol. Mol. Biol.Rev.,* 68, 771–795
26. Gilchrist, M.J.; Greko, C.; Wallinga, D.B.; Beran, G.W.; Riley, D.G.; Thorne, P.S. (2007). The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environ. Health Perspect.,* 115, 313-316.
27. Guz, L.; Kozinska, A. (2004). Antibiotic susceptibility of *Aeromonas hydrophila* and *A. sobria* isolated from farmed carp (*Cyprinus carpio* L.) *Bull .Vet .Inst .Pulawy.,* 48, 391-395.
28. Heuzenroeder, M.W.; Wong, C.Y.F.; Flower, R.L.P. (1999). Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: Correlation with virulence in a suckling mouse model. *FEMS. Microbiol.Lett.,* 174(1), 131-136.
29. Howard, S.P.; Buckley, J.T. (1985). Protein export by a gram-negative bacterium, production of aerolysin by *Aeromonas hydrophila*. *J. Bacteriol.,* 161(3), 1118-1124.
30. Jacobs, L.; Chenia, H.Y. (2007). Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *Int. J. Food Microbiol.,* 114, 295–306.
31. Janda, M.; Abbott, S.L. (2010). The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection. *Clin. Microbiol. Rev.,* 23 (1), 35-73.
32. Janda, J.M.; Abbott, S.L.; Khashe, S.; Kellogg, G.H.; Shimada, T. (1996). Further studies on biochemical characteristics and serologic properties of the genus *Aeromonas*. *J .Clin. Microbiol.,* 34, 1930–1933.
33. Janda, M.; Abbott, S.L. (1998). Evolving concepts regarding the genus *Aeromonas*: An expanding panorama of species, disease presentations, and unanswered questions. *Clin. Infect. Dis.,* 27, 332-344.
34. Janda, J.M. (2002). *Aeromonas* and *Plesiomonas*. In: Sussman, M. (ed.). *Molecular Medical Microbiology*. Academic Press, San Diego. 1237-1270.
35. Jefferies, C.D.; Holtman, D.E.; Guse, D.G. (1957). Rapid method for the determining the activity of microorganisms on nucleic acids. *J. Bacteriol.,* 73, 590.
36. Kingombe, C.I.B.; Huys, G.; Tonolla, M.; Albert, M.J.; Swings, J.; Peduzzi, R. (1999). PCR detection, characterization and distribution of virulence genes in *Aeromonas* spp. *Appl. Environ. Microbiol.,* 65, 5293–5302.
37. Krumperman, P.H. (1985). Multiple antibiotic indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol*., 46, 165– 170.
38. Kuhn, I.; Huys, G.; Coopman, R.; Kersters, K.; Janssen, P. (1997). A 4-year study of the diversity and persistence of coliforms and *Aeromonas* in the water of a Swedish drinking water well. *Can. J. Microbiol.,* 43 (1), 9-16.
39. Lachmayr, K.L.; Kerkhof, L.J.; Dirienzo, A.G.; Cavanaugh, C.M.; Ford, T.E. (2009). Quantifying nonspecific TEM beta-lactamase (bla_{TEM}) genes in a wastewater stream. *Appl. Environ. Microbiol.,* 75(1), 203-11.
40. Lee, Y.K.; Kim, H.W.; Liu, C.L.; Lee, H.K. (2003). A simple method for DNA extraction from marine bacteria that produce extracellular materials. *J. Microbiol. Methods.,* 52, 245-250.
41. Lee M.F.; Peng C.F.; Lin Y.H.; Lin S.R.; Chen Y.H. (2008). Molecular diversity of class 1 integrons in human isolates of *Aeromonas* spp. isolates from Southern Taiwan. *Jpn. J. Infect. Dis*., 61, 343-349.
42. Lehane, L.; Rawlin, G.T. (2000). Topically acquired bacterial zoonoses from fish: a review. *Med.J.Aust.,* 173(5), 256-259.
43. Leung, K.Y.; Stevenson, R.M.W. (1988). Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. *J. Gen .Microbiol.,* 134, 151-160.
44. Lilenbaum, W.; Nunes, E.L.C.; Azeredo, M.A.I. (1998). Prevalence and antimicrobial susceptibility of *Staphylococci* isolated from the skin surface of clinically normal cats. *Lett. Appl. Microbiol.,* 27, 224-228.
45. Maniati, M.; Petinaki, E.; Maniatis, K.A.N. (2005). Antimicrobial susceptibility of *Aeromonas* sp., *Vibrio* sp. and *Plesiomonas shigelloides* isolated in the Philipines and Thailand. *Int. J. Antimicrob. Agents.,* 25, 345-353.
46. Martınez-Murcia, A.J.; Saavedra, M.J.; Mota, V.R.; Maier, T.; Stackebrandt, E.; Cousin, S. (2008). *Aeromonas aquariorum sp. nov*., isolated from the aquaria of ornamental fish. *Int.J.Syst Evol. Microbiol*., 58, 1169-1175.
47. Nam, I.Y.; Myung, H.; Joh, K. (2004). Molecular cloning, purification, and characterization of an extracellular nuclease from *Aeromonas hydrophila* ATCC 14715. *J. Microbiol. Biotechnol., 14*, 178-181.
48. Nawaz, M.; Sung, K.; Khan, S.A.; Khan, A.A.; Steele, R. (2006). Biochemical and molecular characterization of tetracycline-resistant *Aeromonas veronii* isolates from catfish. *Appl. Environ. Microbiol.,* 72, 6461-6466.
49. Nerland, A.H. (1996). The nucleotide sequence of the gene encoding GCAT from *Aeromonas salmonicida* ssp. *salmonicida*. *J .Fish. Dis.,* 19, 145–150.

50. Ormen, O.; Ostensvik, O. (2001). The occurrence of aerolysin-positive *Aeromonas* spp. and their cytotoxicity in Norwegian water sources. *J. Appl. Microbiol.,* 90, 797-802.
51. Orozova, P.; Chikova, V.; Kolarova, V.; Nenova, R.; Konovska, M.; Najdenski, H. (2008). Antibiotic resistance of potentially pathogenic *Aeromonas* strains. *Trakia Journal of Sciences.*, 6 (1), 71-77.
52. Palu, A.P.; Gomes, L.M.; Miguela, M.A.L.; Balassiano, I.T.; Queiroz, M.L.P.; Almeida, A.C.F.; de Oliveira, S.S. (2006). Antimicrobial resistance in food and clinical *Aeromonas* isolates. *Food Microbiol*., 23, 504–509.
53. Park, C.H.; Robicsek, A.; Jacoby, G.A.; Sahm, D.; Hooper, D.C. (2006). Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin modifying enzyme. *Antimicrob. Agents Chemother.*, 50, 3953-3955.
54. Pemberton, J.M.; Kidd, S.P.; Schmidt, R. (1997). Secreted enzymes of *Aeromonas*. *FEMS Microbiol. Lett.,* 152, 1-10.
55. Penders, J.; Stobberingh, E.E. (2008). Antibiotic resistance of motile aeromonads in indoor catfish and eel farms in the southern part of The Netherlands. *Int. J. Antimicrob. Agents.,* 31, 261–265.
56. Petersen, A.; Dalsgaard, A. (2003). Antimicrobial resistance of intestinal *Aeromonas* spp. and *Enterococcus* spp. in fish cultured in integrated broiler-fish farms in Thailand. *Aquaculture.,* 219, 71–82.
57. Sambrook, J.; Russell, D.W. (2001). Molecular cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
58. San Joaquin, V.H.; Pickett, D.A.; Welch, D.F.; Finkhouse, B.D. (1989). *Aeromonas* species in aquaria: a reservoir of gastrointestinal infections? *J.Hosp.Infect.,* 13(2), 173-177.
59. Santos, J.A.; Gonzalez, C.J.; Otero, A.; Garcia-Lopez, M.L. (1999). Hemolytic and siderophore production in different *Aeromonas* species isolated from fish. *Appl. Environ. Microbiol.,* 65, 5612-5614.
60. Schmidt, A.S.; Bruun, M.S.; Dalsgaard, I.; Larsen, J.L. (2001). Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile *Aeromonas* from a fish farming environment. *Appl. Environ. Microbiol.,* 67, 5675-5682.
61. Scoaris, D.D.O.; Colacite, J.; Nakamura, C.V.; Nakamura, T.U.; Filho, B.A.A.; Filho, B.P.D. (2008). Virulence and antibiotic susceptibility of *Aeromonas* spp. isolated from drinking water. *Antonie van Leeuwenhoek,* 93, 111-122.
62. Sen, K.; Rodgers, M. (2004). Distribution of six virulence factors in *Aeromonas* species isolated from U.S. drinking water utilities, a PCR identification. *J. Appl. Microbiol.,* 97, 1077-1086.
63. Sinha, S.; Shimada, T.; Ramamurthy, T.; Bhattacharya, S.K.; Yamasaki, S.; Takeda, Y.; Nair, G.B. (2004). Prevalence, serotype distribution, antibiotic susceptibility and genetic profiles of mesophilic *Aeromonas* species isolated from hospitalized diarrhoeal cases in Kolkata, India. *J .Med.Microbiol.,* 53, 527–534.
64. Sreedharan, K. (2008). *Aeromonas* associated with Freshwater Ornamental fish Culture Systems: Characterization, Pathogenicity and Management. Doctoral Thesis, Cochin University of Science and Technology, India.
65. Swift, S.; Lynch, J.M.; Fish, L.; Kirke, D.F.; Tomas, J.M.; Stewart, G.S.A.B.; Williams, P. (1999). Quarum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. *Infect. Immun.,* 67(10), 5192-5199.
66. Tenover, F.C.; Lancaster, M.V.; Hill, B.C. (1988). Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *J .Clin. Microbiol.,* 36, 1020-1027.
67. Timpe, J.M.; Holm, M.M.; Vanlenberg, S.L.; Basrur, V.; Lafontaine, E.R. (2003). Identification of a *Moraxella catarrhalis* outer membrane protein exhibiting both adhesion and lipolytic activities. *Infect. Immun.,* 71, 4341-4350.
68. Vilches, S.; Urgell, C.; Merino, S.; Chacon, M.R.; Soler, L.; Castro-Escarpulli, G.; Figueras, M.J.; Tomas, J.M. (2004). Complete type III secretion system of a mesophilic *Aeromonas hydrophila* strain. *Appl. Environ. Microbiol*., 70(11), 6914-6919.
69. Weldhagen, G.F.; Poirel, L.; Nordmann, P. (2003). Ambler class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. *Antimicrob. Agents Chemother.*, 47(8), 2385-92.
70. Wilcox, M.H.; Cook, A.M.; Eley, A.; Spencer, R.C. (1992). *Aeromonas* spp.as a potential cause of diarrhoea in children. *J .Clin. Pathol.,* 45, 959–963.
71. Winfree R.A. (1989). Tropical fish, their production and marketing in the United States. *World Aquacult*., 20, 24-30.
72. Wu, C.J.; Wu, J.J.; Yan, J.J.; Lee, H.C.; Lee, N.Y.; Chang, C.M.; Shih, H.I.; Wu, H.M.; Wang, L.R.; Ko, W.C. (2007). Clinical significance and distribution of putative virulence markers of 116 consecutive clinical *Aeromonas* isolates in southern Taiwan. *J. Infect.,* 54, 151-158.
73. Yu, H.B.; Zhang, Y.L.; Lau, Y.L.; Yao, F.; Vilches, S.; Merino, S.; Tomas, J.M.; Howard, S.P.; Leung, K.Y. (2005). Identification and characterization of putative virulence genes and gene clusters in *Aeromonas hydrophila* PPD/134/91. *Appl. Environ. Microbiol*., 71(8), 4469-4477.

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