

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

Krishnan Sreedharan¹; Rosamma Philip²; Isaac Sarojani Bright Singh^{1*}

¹National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin-682 016, Kerala, India; ²Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Fine Arts Avenue, Cochin-682 016, Kerala, India.

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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

*Corresponding Author. Mailing address: National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin-682 016, Kerala, India.; Tel/Fax.: +91-484-2381120.; E-mail: isbsingh@gmail.com

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 cytotoxic 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 μ L 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 \pm 0.3) plates. The plates were incubated for 48 hr at 28°C. 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 Biostatistics Inc), and analyzed by the programme NTSYSpc, Version 2.02i (Applied Biostatistics 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 30°C 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 4°C, 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 4°C. 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 37°C and then for 2 hr at 55°C. 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 MgCl₂, 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°C 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
<i>act</i>	F	AGAAGGTGACCACCAAGAACA	232	36
	R	AACTGACATCGGCCTTGAACCT		
<i>ast</i>	F	TCTCCATGCTTCCCTTCCACT	331	36
	R	GTGTAGGGATTGAAGAAGCCG		
<i>alt</i>	F	TGACCCAGTCCTGGCACGGC	442	36
	R	GGTGATCGATCACCACCAGC		
<i>aerA</i>	F	CCCGCCGATCTGCAACCGGG	489	50
	R	CTGGTCTGGATAGACGGGCTCTGCC		
<i>hlyA</i>	F	GGCCGGTGGCCCGAAGATACGGG	597	28
	R	GGCGGCGCCGGACGAGACGGG		
<i>aexT</i>	F	GGCGCTTGGGCTCTACAC	535	12
	R	GAGCCCGCGCATCTTCAG		
<i>ascV</i>	F	GCCCGTTTTGCCTATCAA	807	12
	R	GCGCCGATATCGGTACCC		
<i>aopP</i>	F	GAGAGTTGGCTAGCGGTGAG	490	12
	R	TCCTCATGGAGCGCATCCAG		
<i>aopO</i>	F	CGAGACAGACAAGTTTGC	401	12
	R	TGTCGTTGTGGACTATCC		
<i>aopH</i>	F	TCAATCAGGACGATGTCC	518	12
	R	GTTGGCATTGAGATCTGC		
<i>ascF – ascG</i>	F	ATGAGGTCATCTGCTCGCGC	789	72
	R	GGAGACAACCATGGCTGAT		
<i>gcat</i>	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	<i>A. caviae</i>	<i>A. veronii</i> biovar <i>sobria</i>	<i>A. jandaei</i>	<i>A. jandaei</i>	<i>A. caviae</i>	<i>A. jandaei</i>	<i>A. veronii</i> biovar <i>sobria</i>

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. jandaei* are the species most commonly implicated in human intestinal infections (33), accounting for >85% of the clinical isolates of this genus (62.).

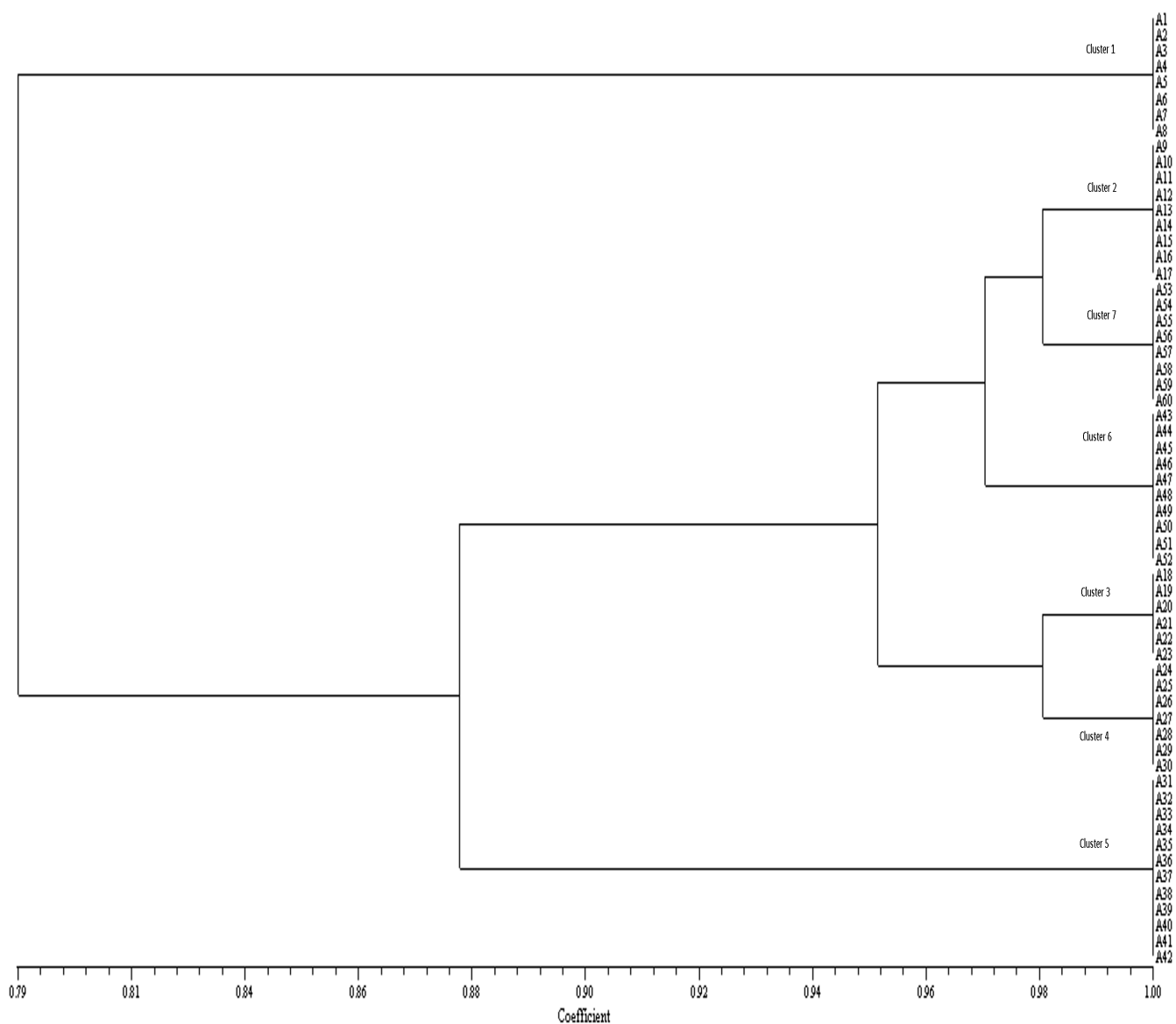


Figure 1. Dendrogram of *Aeromonas* isolates from freshwater ornamental fish culture systems based on phenotypic characters

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 <i>A. caviae</i>	MCCB 144 <i>A. veronii</i> biovar <i>sobria</i>	MCCB 145 <i>A. jandaei</i>	MCCB 146 <i>A. jandaei</i>	MCCB 147 <i>A. caviae</i>	MCCB 148 <i>A. jandaei</i>	MCCB 149 <i>A. veronii</i> biovar <i>sobria</i>
DNase	-	+	-	-	+	-	+
Lecithinase	-	+	-	-	+	-	+
Slime formation	-	+	-	-	+	-	+
β-haemolysis	+	+	-	-	+	+	+
<i>alt</i>	-	+	-	-	-	-	+
<i>ast</i>	-	-	-	-	-	-	-
<i>act</i>	+	+	-	-	-	+	+
<i>aer</i> A	-	-	-	-	-	-	-
<i>hly</i> A	-	-	-	-	-	-	-
<i>aex</i> T	-	-	-	-	-	-	-
<i>ascF – asc</i> G	-	-	-	-	+	-	-
<i>aop</i> P	-	-	-	-	-	-	-
<i>aop</i> O	-	-	-	-	-	-	-
<i>aop</i> H	-	-	-	-	-	-	-
<i>gcat</i>	+	+	+	+	+	+	+

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 β-lactam antibiotics	Antibiotic (Disc content)	Resistant (%)			
		<i>A.caviae</i> (n=20)	<i>A.veronii</i> biovar <i>sobria</i> (n=17)	<i>A.jandaei</i> (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 1 st generation	Cephalexin (30 µg)	0	52.9	30.4	
	Cefazolin (30 µg)	0	53.0	30.4	
	Cephadrine (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	
	2 nd generation	Cefaclor (30 µg)	0	53.0	0
		Cephoxitin (30 µg)	0	53.0	30.4
		Cefamandole (30 µg)	0	0	0
	3 rd generation	Ceftriaxone (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 cephalixin, 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 piperimidic acid, they were highly susceptible to the newer generation fluoroquinolones such as ciprofloxacin, enrofloxacin, sparfloxacin, norfloxacin, pefloxacin, ofloxacin, flolidine 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 *gyrA* and *parC* 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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