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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Submitted: December 11, 2010; Approved: January 16, 2012.

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 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 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 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 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 g L⁻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.

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 µg mL⁻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 |
|---------------|---------|----------------------|------------------|-----------|
| act           | F       | AGAAGGTGACCCACAAAGAAC | 232              | 36        |
|               | R       | AACTGACATCGCCCTTGTCAC |                  |           |
| ast           | F       | TCTCCATGCTTCCCTTCCCTC | 331              | 36        |
|               | R       | GTTGAGGTAGTGAAGAAGCGG |                  |           |
| alt           | F       | TGACCCAGTCTGGCAAGCCGC | 442              | 36        |
|               | R       | GTGATCAATCACAACCACGC |                  |           |
| aerA          | F       | CCCGCAGTCTGGCAACCAGG | 489              | 50        |
|               | R       | CTGGTCGATAGACCCGGCTG |                  |           |
| hlyA          | F       | GCCGGTGCCGAAGATACCGG  | 597              | 28        |
|               | R       | GGCAGGCGAGCAAGACGCCG  |                  |           |
| aexT          | F       | GGCCTTGAGGCTTCAAC     | 535              | 12        |
|               | R       | GAGCCCGAGCTTTTGCA     |                  |           |
| ascV          | F       | GCCCGTTTGGCTTCATCAA   | 807              | 12        |
|               | R       | GCGCGATATCGGTATCC     |                  |           |
| aopP          | F       | GAGAGTGCTAGCGGTAGG    | 490              | 12        |
|               | R       | TCCCTCAGGGAGCCATCCAG  |                  |           |
| aopO          | F       | CGAGAGTAGAGACTTCGC    | 401              | 12        |
|               | R       | TGGGCTATGAGTCGTG      |                  |           |
| aopH          | F       | TCAATCGAGACGTGCTG     | 518              | 12        |
|               | R       | GTTGGCCTTAGAGATCTGC   |                  |           |
| ascF – ascG   | F       | ATGAGGGTACTGTCGGCGC   | 789              | 72        |
|               | R       | GGAGCACAACGACTGCTGAT  |                  |           |
| gcat          | F       | CTCTGAGAAATCACAATCGA  | 237              | 49        |
|               | R       | GGCAGGGTGAACAGAGATCT  |                  |           |

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 | 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/G             | F               | F                | F                | F               |
| Voges-Proskauer reaction | -               | +               | +               | +               | +                | +                | +               |
| Methyl red test       | +               | -               | -               | -               | -                | -                | -               |
| Utilization of:       |                 |                 |                 |                 |                  |                  |                  |
| Citrate               | -               | +               | +               | +               | +                | +                | +               |
| Acetate               | +               | +               | -               | +               | +                | +                | +               |
| Gluconeate 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      | A. jandaei      | A. caviae       | A. jandaei      | A. caviae        | A. jandaei       | A. veronii      |
| biivar sobria         |                 | biivar sobria   |                 | biivar sobria   | biivar sobria    | biivar sobria    | biivar 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, dextin, 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.).

**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 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             | +                  | +                                 | -                  | -                  | +                  | +                  | +                                 |
| aex T           | -                  | +                                 | -                  | -                  | -                  | -                  | -                                 |
| ascF – asc G    | -                  | -                                 | -                  | +                  | -                  | -                  | -                                 |
| aop P           | -                  | -                                 | -                  | -                  | -                  | -                  | -                                 |
| aop O           | -                  | -                                 | -                  | -                  | -                  | -                  | -                                 |
| aop H           | -                  | -                                 | -                  | -                  | -                  | -                  | -                                 |
| gcat            | +                  | +                                 | +                  | +                  | +                  | +                  | +                                 |

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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)                      | *A. caviae* (n=20) | *A. veronii biovar sobria* (n=17) | *A. jandaei* (n=23) |
|------------------------|------------------------------------------------|--------------------|-----------------------------------|---------------------|
| **β-lactam antibiotics** |                                                |                    |                                   |                     |
| 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                |                                   |                     |
|                        | **Piperacillin (100 µg)**, Carbenicillin (100 µg) | 0                  | 0                                 | 0                   |
| 2. Cephalosporins      | **Cephalaxin (30 µg)**                           | 0                  | 52.9                              | 30.4                |
| 1st generation         | **Cefazolin (30 µg)**                            | 0                  | 53.0                              | 30.4                |
|                        | **Cephradine (25 µg)**                           | 0                  | 53.0                              | 30.4                |
|                        | **Cephaloridine (10 µg)**                        | 100                | 100                               | 100                 |
|                        | **Cephalothin (30 µg)**                          | 40.0               | 100                               | 30.4                |
|                        | **Cephalosporin (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         | **Ceftriaxone (10 µg)**                          | 0                  | 0                                 | 0                   |
|                        | **Cefazidime (30 µg)**                           | 0                  | 0                                 | 0                   |
|                        | **Cefoperazone (75 µg)**                         | 0                  | 0                                 | 0                   |
|                        | **Ceftizoxime (30 µg)**                          | 0                  | 0                                 | 0                   |
| 3. Carbapenem          | **Imipenam (10 µg)**                             | 0                  | 0                                 | 0                   |
| Aminoglycosides        | **Amikacin (10 µg)**                             | 0                  | 0                                 | 0                   |
|                        | **Gentamycin (10 µg)**                           | 0                  | 0                                 | 0                   |
|                        | **Kanamycin (30 µg)**                            | 0                  | 0                                 | 0                   |
| Macrolides             | **Neomycin (30 µg)**                             | 0                  | 53.0                              | 0                   |
|                        | **Netilin (10 µg)**                              | 0                  | 53.0                              | 0                   |
|                        | **Tobramycin (10 µg)**                           | 0                  | 0                                 | 0                   |
| 1st generation         | **Azithromycin (15 µg)**                         | 0                  | 0                                 | 0                   |
|                        | **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                   |
| 3rd generation         | **Tetracycline HCl (10 µg)**                     | 100                | 100                               | 100                 |
|                        | **Oxytetracycline (30 µg)**                      | 0                  | 0                                 | 0                   |
|                        | **Tetracycline (10 µg)**                         | 0                  | 0                                 | 0                   |
| Chloramphenicol        | **Chloramphenicol (10 µg)**                      | 0                  | 0                                 | 0                   |
| Rifamycins             | **Rifampicin (2 µg)**                            | 0                  | 53.0                              | 0                   |
| Lincosamides           | **Clindamycin (2 µg)**                           | 100                | 100                               | 100                 |
| Steroids               | **Fusidic acid (10 µg)**                         | 100                | 100                               | 100                 |
| Nitrofurans            | **Nitrofurazone (100 µg)**                       | 0                  | 0                                 | 0                   |
|                        | **Furazolidone (50 µg)**                         | 0                  | 53.0                              | 0                   |
| Sulfonamides           | **SulfaDiazine (100 µg)**                        | 0                  | 0                                 | 0                   |
|                        | **SulfaFurazole (300 µg)**                       | 0                  | 0                                 | 0                   |
|                        | **SulfaPhenazole (200 µg)**                      | 0                  | 0                                 | 0                   |
| Quinolones/            | **Ciprofloxacin (5 µg)**                         | 0                  | 0                                 | 0                   |
| Fluoroquinolones       | **Enrofloxacin (5 µg)**                          | 0                  | 0                                 | 0                   |
|                        | **Floxidine (20 µg)**                            | 0                  | 0                                 | 0                   |
|                        | **Nitroxoline (30 µg)**                          | 0                  | 0                                 | 0                   |
|                        | **Norfloxacin (10 µg)**                          | 100                | 53.0                              | 26.0                |
|                        | **Oxofloxin (2 µg)**                             | 60.0               | 0                                 | 0                   |
|                        | **Pefloxacin (5 µg)**                            | 0                  | 0                                 | 0                   |
|                        | **Sparfloxacin (5 µg)**                          | 0                  | 0                                 | 0                   |
| Aminocoumarins         | **Novobiocin (30 µg)**                           | 100                | 100                               | 100                 |
| 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 cephalexin, cephalothin, cefazolin, cephadrine, cephadroxil, cefaclor and cephotxin 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, floridine 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 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 macrodilides and lincosamides. Esterases act on erythromycin and the nucleotidylytransferases 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 | % occurrence | Total % (n=60) |
| 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.

ACKNOWLEDGEMENTS

The authors thank the Marine Products Export Development Authority (MPEDA), Ministry of Commerce & Industry, Govt. of India (Project code: 3/3/OFD/HO/2003 dated 25-02-2004), and Department of Biotechnology (DBT), Govt. of India (Project Code: BT/PR4012/AAQ/03/204/2003), for financial support. The first author thanks MPEDA for fellowship.

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