

Research Report

Open Access

## Distribution, Extracellular Virulence Factors and Drug Resistance of Motile Aeromonads in Fresh Water Ornamental Fishes and Associated Carriage Water

Nifty John<sup>✉</sup>, A. A. M. Hatha<sup>✉</sup>

Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Lakeside Campus, Cochin University of Science and Technology, Cochin-682 016, Kerala, India

✉ Corresponding author email: mohamedhatha@gmail.com; ✉ Authors

International Journal of Aquaculture, 2013, Vol.3, No.17 doi: 10.5376/ija.2013.03.0017

Received: 30 May, 2013

Accepted: 3 Jun., 2013

Published: 20 Jun, 2013

**Copyright** © 2013 John. This is an open access article published under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

### Preferred citation for this article:

John, 2013, Distribution, Extracellular Virulence Factors and Drug Resistance of Motile Aeromonads in Fresh Water Ornamental Fishes and Associated Carriage Water, International Journal of Aquaculture, Vol.3, No.17 92-100 (doi: 10.5376/ija.2013.03.0017)

**Abstract** During last decades there has been a continuous growth of aquaculture industries all over the world and taking into consideration the spurt in freshwater ornamental fish aquaculture and trade in Kerala, the present study was aimed to assess the prevalence of various motile *Aeromonas* spp. in fresh water ornamental fishes and associated carriage water. The extracellular virulence factors and the antibiogram of the isolates were also elucidated. Various species of motile aeromonads such as *Aeromonas caviae*, *A. hydrophila*, *A. jandaei*, *A. schubertii*, *A. sobria*, *A. trota* and *A. veronii* were detected. *Aeromonas sobria* predominated both fish and water samples. Extracellular enzymes and toxins produced by motile aeromonads are important elements of bacterial virulence. The production of extracellular virulence factors - proteases, lipase, DNase and haemolysin by the isolates were studied. All the isolates from both fish and water samples produced gelatinase and nuclease but the ability to produce lipase, caseinase and haemolysins was found to vary among isolates from different sources. Among the 15 antibiotics to which the isolates were tested, all the isolates were found to be sensitive to chloramphenicol, ciprofloxacin and gentamicin and resistant to amoxicillin. Local aquarists maintain the fish in crowded stressful conditions, which could trigger infections by the obligate/ opportunistic pathogenic members among motile aeromonads.

**Keywords** Ornamental fish; Motile aeromonads; Antibiotic resistance; Diseases

### Introduction

Keeping colourful and fancy fishes known as ornamental fishes, aquarium fishes, or live jewels is one of the oldest and most popular hobbies in the world. The growing interest in aquarium fishes has resulted in a steady increase in aquarium fish trade globally. The global scope and scale of the ornamental fish trade and growing popularity of pet fish are strong indicators of the myriad economic and social benefits the pet industry provides. Culture of ornamental fish in the backyards of households requires very little space, skill and time, and has the potential to improve the economic condition of the household. 'Earning a regular income, unlike seasonal work in agriculture, provides further motivation (Shaleesha and Stanley, 2000). Aquaculture is an emerging industrial sector which requires continued research with scientific and technical developments, and innovation. Over one billion ornamental fish comprising more than 4000 freshwater and 1400 marine species are traded internationally each year, making it one of the most

important components of the global fish trade. Freshwater species make up 90% of this trade as they are the most popular and widely kept aquarium pets worldwide (Krishnakumar et al., 2009). The trade in ornamental (pet) fish is greater than 1 billion animals per year globally. More than 45 million fish per year are imported into the United Kingdom (UK) alone from a wide range of countries, in particular those in South East Asia (Wittington and Chong, 2007).

Fish diseases are a scourge of ornamental fish industry bringing huge economic loss. Bacterial organisms may be the primary cause of disease, or they may be secondary invaders. The majority of bacterial fish pathogens are natural inhabitants of the aquatic environment. Infections caused by motile members of the genus *Aeromonas*, are amongst the most common and troublesome diseases diagnosed in cultured warm water fishes and have been referred to by various names, including motile aeromonad septicemia (MAS), motile aeromonad infection (MAI), hemorrhagic

septicemia, red pest, and red sore. *Aeromonas* bacteria causing these infections are called aeromonads (Camus et al., 1998). *Aeromonas* infections are a serious threat to fresh water fish production, bringing enormous economic loss to ornamental fish industry.

The detection of virulence factors in *Aeromonas* is a key component in the determination of potential pathogenicity, because more than two virulence factors act multifunctionally and multifactorially it seems necessary to continue surveying the distribution of known virulence determinants in currently circulating *Aeromonas* strains.

The disease problems are treated with antibiotics, the indiscriminate use of which can result in the rapid spread of multi-drug resistant pathogens across the system. It is also important for the ornamental fish industry to recognize the extent to which the bacteria associated with ornamental fish have developed antimicrobial resistance. This fact along with the financial crisis caused by the mortality of ornamental fishes makes the study of different geographical isolates of aeromonads important.

Aquaculture is in a phase of rapid growth and development. Fish diseases are among the most important problems and challenges confronting fish culturing. Among the etiological agents of bacterial fish disease, the motile *Aeromonas* group is considered important. *Aeromonas* spp. is ubiquitous in natural waters and comprises mesophilic motile and psychrophilic non motile gram-negative bacteria. Worldwide studies have demonstrated that *Aeromonas* spp. are universally distributed and widely isolated from clinical, environmental and animal sources, food samples and aquatic environment (Janda and Abbott, 2010). In aquatic environments, they are found in ground water, surface water, estuarine environments, sewage effluents, lakes and rivers (Galindo and Chopra, 2007) and in public drinking water and tap water (Pablos et al., 2009; Kivanc et al., 2011).

The widespread distribution of these bacteria in the aquatic environment and the stress induced by intensive culture practices predisposes fish to infections. A number of putative virulence factors that may play an important role in the development of disease, have been described in several species of the genus *Aeromonas*, which includes haemolysins,

cytotoxins, enterotoxins, proteases, lipases, DNases and adhesins (Sen and Rodgers, 2004). The pathogenesis of *Aeromonas* infections is multifactorial, and no single virulence factor can be unequivocally pinpointed as responsible for particular symptoms or disease stages. Pathological conditions attributed to members of the motile aeromonad complex may include dermal ulceration, tail or fin rot, ocular ulceration, hemorrhagic septicemia and scale protrusion disease. Outbreaks of motile aeromonad septicaemia can reach epidemic proportions in farmed aquatic animals, with high rates of mortality (Liles et al., 2011). The disease problems are treated with antibiotics, but the emergence of antibiotic-resistant bacteria imposes a substantial burden on aquaculture. Antibiotic resistance is particularly relevant in pathogenic *Aeromonas* species in which, besides the classical resistance to  $\beta$ -lactam antibiotics, multiple-resistance has been frequently identified (Goñi-Urriza et al., 2000; Vila et al., 2002; 2003). These bacteria can receive and transfer antibiotic resistance genes to other Gram negative bacteria (Marchandin et al., 2003).

Diseases in intensive freshwater aquaculture have brought great economic loss to India in recent years. Infections due to *Aeromonas* are common and pose a threat to the development of the aquaculture enterprise. Therefore the present study was carried out to assess the prevalence of various motile *Aeromonas* spp. in fresh water ornamental fishes and associated carriage water. The extracellular virulence factors and the antibiogram of the isolates were also studied.

## 1 Results

### 1.1 Distribution of *Aeromonas* Species

Motile *Aeromonas* spp. was isolated from ornamental fish samples and associated carriage water samples. One hundred and seventy five isolates from the fish samples and one hundred and eighty two isolates from the water samples were characterized to species level, *Aeromonas sobria* predominated in both cases - 40.57% in fish samples and 34.80% in water samples. *A. caviae* was the second dominant spp. in both samples but its percentage of occurrence was much higher in fish samples, (31.43%), when compared to water samples (16.57%). *A. hydrophila*, *A. jandaei*, *A. schubertii* and *A. veronii* predominated in water samples when compared to fish samples as shown in Figure 1.

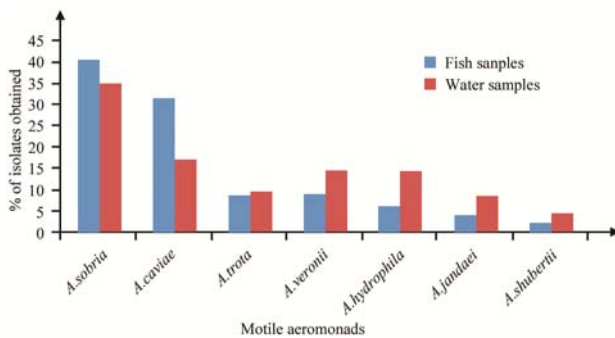


Figure 1 Distribution of *Aeromonas* spp. in fish and water samples

Prevalence of motile aeromonads at various parts of the body of fresh water ornamental fishes is given in Figure 2. *A. hydrophila*, *A. veronii* and *A. jandaei* were frequently encountered on gill surface when compared to body surface and intestine.

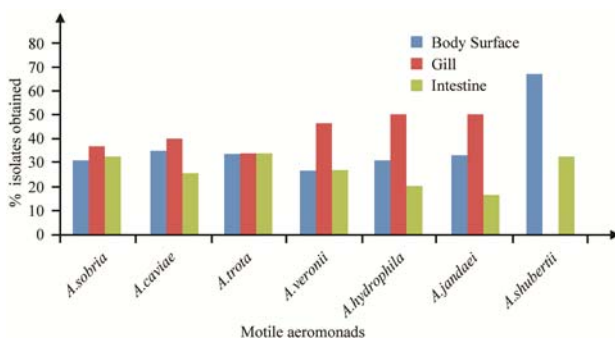


Figure 2 Distribution of *Aeromonas* spp. in the body surface, gill and intestine of fishes

## 1.2 Extracellular Virulence Factors of *Aeromonas* Species

All the isolates of *Aeromonas* spp. obtained exhibited gelatinase and DNase activity. All the isolates of *Aeromonas sobria* exhibited caseinase and  $\beta$  haemolysin production. Similarly all the isolates of *A. hydrophila* and *A. jandaei* were capable of producing  $\beta$  haemolysin.  $\beta$  haemolysin production was infrequent in *A. caviae*. Lipase was produced by all the isolates of *A. hydrophila*, *A. jandaei* and *A. veronii* but their production among the isolates of other spp. varied. The production of extracellular virulence factors by the *Aeromonas* isolates obtained from fish and water samples are given Table 1 and Table 2 respectively.

## 1.3 Antimicrobial Resistance

All the aeromonad isolates tested were resistant to amoxicillin and sensitive to ciprofloxacin, chloramphenicol and gentamicin regardless of their source. While all the *Aeromonas* isolates from water were sensitive to nitrofurantoin, 1.14% of isolates from fish samples was resistant to nitrofurantoin. Resistance to ceftazidime was found in 13.33% of *A. veronii* and 5.63% of *A. sobria* isolates from fish samples and 8% of *A. veronii* isolates from water samples. All the other isolates were sensitive to this antibiotic. All the isolates except 3.63% of *A. caviae* from fish samples and 20% of *A. hydrophila*, 12.69%

Table 1 Prevalence of extracellular virulence factors among motile aeromonads from ornamental fish samples

<i>Aeromonas</i> spp.	Percentage of motile aeromonads producing virulence factors				
	Gelatinase	Caseinase	Lipase	DNase	Haemolytic activity
<i>A. sobria</i>	100	100.00	98.59	100	100.00
<i>A. caviae</i>	100	52.73	89.09	100	72.72
<i>A. jandaei</i>	100	83.33	100.00	100	100.00
<i>A. trota</i>	100	80.00	93.33	100	86.66
<i>A. veronii</i>	100	86.66	100.00	100	93.33
<i>A. hydrophila</i>	100	90.00	100.00	100	100.00
<i>A. shubertii</i>	100	66.67	66.60	100	100.00

Table 2 Prevalence of extracellular virulence factors among motile aeromonads from carriage water samples

<i>Aeromonas</i> spp.	Percentage of motile aeromonads producing virulence factors				
	Gelatinase	Caseinase	Lipase	DNase	Haemolytic activity
<i>A. sobria</i>	100	100.0	98.40	100	100.0
<i>A. caviae</i>	100	60.0	90.00	100	70.0
<i>A. jandaei</i>	100	100.0	100.00	100	100.0
<i>A. trota</i>	100	87.5	93.75	100	87.5
<i>A. veronii</i>	100	88.0	100.00	100	92.0
<i>A. hydrophila</i>	100	88.0	100.00	100	100.0
<i>A. shubertii</i>	100	62.5	87.50	100	75.0

of *A. sobria* and 8% of *A. veronii* from water samples were sensitive to sulfafurazole. Resistance exhibited

to other antibiotics by the isolates from fish and water samples are given in Table 3 and Table 4 respectively.

Table 3 Antibiotic resistance of motile aeromonads from fish samples

Name of antibiotics	Percentage of strains exhibiting resistance						
	<i>A. sobria</i>	<i>A. caviae</i>	<i>A. veronii</i>	<i>A. hydrophila</i>	<i>A. trota</i>	<i>A. jandaei</i>	<i>A. shubertii</i>
Amoxicillin (30)	100.00	100.00	100.00	100	100.00	100.00	100
Carbenicillin (100)	76.05	60.00	80.00	70	66.66	100.00	100
Cefpodoxime (10)	18.30	43.63	13.33	80	6.66	0	0
Ceftazidime (30)	5.63	0	13.33	0	0	0	0
Cephalothin (30)	18.30	56.36	0	100	26.66	33.33	0
Chloramphenicol (30)	0	0	0	0	0	0	0
Ciprofloxacin (5)	0	0	0	0	0	0	0
Gentamicin (10)	0	0	0	0	0	0	0
Nalidixic acid (30)	35.21	63.63	73.33	40	53.33	66.66	0
Nitrofurantoin (100)	0	3.63	0	0	0	0	0
Streptomycin (10)	28.16	18.18	33.33	30	20.00	0	0
Sulphafurazole (300)	0	3.63	0	0	0	0	0
Tetracycline (30)	29.57	38.18	13.33	40	20.00	50.00	0
Trimethoprim (5)	0	9.09	0	10	6.66	0	0

Table 4 Antibiotic resistance of motile aeromonads isolates from water samples

Name of antibiotics	Percentage of strains exhibiting resistance						
	<i>A. sobria</i>	<i>A. caviae</i>	<i>A. veronii</i>	<i>A. hydrophila</i>	<i>A. trota</i>	<i>A. jandaei</i>	<i>A. shubertii</i>
Amoxicillin (30)	100.00	100.00	100	100	100.00	100.00	100.0
Carbenicillin (100)	71.42	60.00	88	80	87.50	100.00	62.5
Cefpodoxime (10)	42.85	36.66	36	88	31.25	26.66	0
Ceftazidime (30)	0	0	8	0	0	0	0
Cephalothin (30)	0	40.00	0	100	18.75	53.33	0
Chloramphenicol(30)	0	0	0	0	0	0	0
Ciprofloxacin (5)	0	0	0	0	0	0	0
Gentamicin (10)	0	0	0	0	0	0	0
Nalidixic acid (30)	28.57	33.33	56	92	62.50	0	37.5
Nitrofurantoin (100)	0	0	0	0	0	0	0
Streptomycin (10)	26.98	0	0	0	18.75	0	12.5
Sulphafurazole (300)	12.69	0	8	20	0	0	0
Tetracycline (30)	25.39	16.66	0	36	12.50	33.33	25.0
Trimethoprim (5)	14.28	20.00	8	28	0	0	0

## 2 Discussion

The prevalence and distribution of bacteria belonging to the genus *Aeromonas* in aquatic environments is of great public health concern since *Aeromonas* spp. can cause infections and epizootics in a variety of animals. Motile aeromonads have been recognized as occasional pathogens of cultured fishes and the most common bacteria in freshwater habitats throughout the world. It is the etiological agent for motile aeromonad septicaemia (MAS) in fish. *Aeromonas* has also been

frequently isolated from the lesions of epizootic ulcerative syndrome (EUS) fishes (Torres et al., 1990; Subasinghe et al., 1990; Roberts et al., 1990). This disease is a serious threat to the freshwater fish production of Southeast Asian countries. It causes mass mortalities in both cultured and wild fish species every year. The prevalence of different species of *Aeromonas* is likely to vary with geographical locations. In the present study *Aeromonas sobria* was the predominant species isolated from fish samples

(40.57%) followed by *A. caviae*. Nearly 10% of the isolates were found to be *A. trola* and *A. veronii*. Other motile aeromonads included *A. hydrophila*, *A. jandaei* and *A. schubertii*. *A. sobria* associated with epizootic ulcerative syndrome (EUS) has resulted in great damage to fish farms in parts of Southeast Asia such as Bangladesh and India (Rahman et al., 2002). *A. sobria*, has been identified as a causative agent of disease in farmed perch *Perca fluviatilis* L. in Switzerland (Wahli et al., 2005). *Aeromonas veronii* has been isolated from the ascitic fluid of Oscar *Astronotus ocellatus* showing signs of infectious dropsy in India (Sreedharan et al., 2011). Hatha et al., (2005) reported *A. hydrophila* to be the predominant species in the intestine of farm- raised fresh water fish followed by *A. caviae* and *A. sobria*. In the present study *A. sobria* was frequently encountered in the intestinal samples of the ornamental fishes.

Motile *Aeromonas* species are ubiquitous bacteria in aquatic environments. These bacteria can be found in both polluted and unpolluted fresh water, in sewage, in drinking water, private wells, in unchlorinated as well as chlorinated water. In recent years, the presence of *Aeromonas* spp. in municipal drinking water supplies has become an emerging public health problem (Haunainen, 1994). In the present study, in terms of prevalence and abundance in water samples, the most predominant species was found to be *Aeromonas sobria* (34.80%) followed by *A. caviae* (16.57%). Distribution of *A. hydrophila* and *A. veronii* was found to be equal (13.81%), while prevalence of *A. trola*, *A. jandaei* and *A. schubertii* were less than 10%. *A. schubertii* was the least predominant sp. in both water and fish samples in our study, *A. caviae* was found to be second most predominant motile aeromonad. High prevalence of *A. caviae* in water is reported previously by Evangelista-Barreto et al. (2010). In a study conducted in Turkey, Koksal et al., (2007) reports the prevalence of *Aeromonas* such as *A. hydrophila* (46%), *A. sobria* (34%), *A. caviae* (8%), *A. veronii* (3%) and *A. jandaei* (3%). Rathore et al. (2005) reports *A. hydrophila* to be the predominant sp. in water and fish samples collected from aquaria in India, which is contrary to our observations. However, they also reported similar species such as *A. hydrophila*, *A. sobria*, *A. veronii*, *A. caviae* and *A. schubertii*, though the relative prevalence of these species was found to vary.

The pathogenesis of *Aeromonas* infections is multifactorial, as aeromonads produce a wide variety of virulence factors. Several virulence factors have been studied in *Aeromonas* including aerolysin/hemolysin, enterotoxins, proteases, lipases and deoxyribonucleases (Chopra et al., 2000; Janda, 2001; Chacón et al., 2003). Nevertheless, it is apparent that some exo-enzymes are important pathogenicity factors. The haemolytic and proteolytic activities of motile and mesophilic aeromonads were reported in most studies as virulence-associated factors (Esteve et al., 1995; Gonzalez-Serrano et al., 2002; Rahman et al., 2002). The high rate of hemolytic activity detected in *Aeromonas* spp. is remarkable. The haemolytic activity is strongly associated with enterotoxin production in members of the genus *Aeromonas* (Burke et al., 1983) *A. hydrophila* strains producing cytotoxins, proteases and aerolysin were commonly isolated from both healthy and moribund fish (Cahill, 1990). Potentially pathogenic *Aeromonas* species are present in diseased as well as healthy fish.

Widespread haemolytic, caseinolytic and gelatinolytic activity was encountered among the motile aeromonads isolated in the present study. These factors were considered as pathogenicity markers by (Koziońska, 2007). The isolates obtained from healthy fishes in this study are also potentially pathogenic as revealed by the production of extracellular virulence factors by the isolates. Though extracellular virulence factors cannot be considered as definite marker for pathogenicity of the isolates, the poor water quality conditions of the aquaria maintained by local farmers in this region might trigger disease outbreak by opportunists. Shome et al. (1999) report that the production of enzymes or toxins is not reflective of biological virulence and they do not satisfy a strain to be virulent or avirulent even though these appear to enhance the disease process *in-vivo*. The whole process of pathogenesis is a complex interaction among the host, agent and environmental determinants. Yucel et al., (2003) reports *A. hydrophila* and *A. sobria* to be stronger producers of hemolysin, and *A. caviae* strains to be non haemolytic. In the present study also, *A. sobria*, *A. hydrophila*, *A. schubertii* and *A. jandaei* isolates from fish samples are 100% haemolysin producers. Hatha et al. (2005) reports 100% of *A. hydrophila* 77.8% of *A. caviae* and 50% of *A. sobria* isolated from fresh water fishes in South

India to be  $\beta$  haemolytic.

Equal distribution of  $\alpha$  and  $\beta$  haemolytic activity among the *A. hydrophila* isolates from fish samples in India, was reported earlier (Illanchezian et al., 2010). While around 90% of *A. veronii* and *A. trota* isolated in the present study were able to produce haemolysin, only 72% of *A. caviae* had this capability. All the isolates in the present study produced gelatinase and nuclease. Similarly all *A. sobria* isolates were capable of elaborating caseinase. In general, caseinase production potential was widespread among most of the motile aeromonads encountered in this study. Castro-Escarpulli et al. (2003) observed comparable levels of extracellular virulence factors among the motile aeromonads from frozen fish samples in Mexico. In contrast to their finding, in the present study 100 % of *A. hydrophila*, *A. veronii* and *A. jandaei* isolates are lipase producers. Possibility of caseinolytic (Mateos et al., 1993) and gelatinolytic activity (Shome et al., 1999) with virulence is substantiated by the above research groups who observed that all the *A. hydrophila* isolates from diseased fishes with dropsy and EUS had caseinolytic and gelatinolytic activity.

All the isolates from the water samples in the present study are producers of gelatinase and nuclease.  $\beta$  haemolysin was produced by 100% of *A. hydrophila* and *A. sobria* (Tables 1 and 2).  $\beta$  haemolytic activity among all the isolates of *A. hydrophila* and *A. sobria* and  $\alpha$  haemolytic activity among all the isolates of *A. caviae* from water sample are reported earlier (Gibotti et al., 2000). The extracellular virulence factors among *Aeromonas* spp. isolated from Bhavani river, South India was studied by Bagyalakshmi et al. (2009). Several of these virulence factors have been identified in strains isolated from water (Sechi et al., 2003). In the present study also, all these virulence factors have been identified in strains isolated from water.

There have been conflicting reports on the susceptibility of motile *Aeromonas* to commonly used antibiotic agents. High level of resistance against amoxicillin, and carbenicillin and 100% sensitivity to chloramphenicol, ciprofloxacin and gentamicin was noticed in the isolates from both fish and water samples. Motile aeromonads isolated from fish and water samples, in India exhibiting 100% sensitivity to,

ciprofloxacin and gentamicin and high level of susceptibility to chloramphenicol is reported by Rathore et al. (2005). Complete sensitivity to chloramphenicol, ciprofloxacin and gentamicin by the isolates is reported by Penders and Stobberingh (2008). All the isolates from water samples in the present study were susceptible to nitrofurantoin and only a 3.63% of *A. caviae* isolates from fish samples exhibited resistance to the antibiotic. High level of susceptibility to nitrofurantoin by the isolates is also reported by Castro-Escarpulli et al. (2003) but in contrast to our finding he has reported 100% sensitivity to nalidixic acid and more than 50% resistance to gentamicin and varying degrees of resistance to chloramphenicol and ciprofloxacin.

Among the motile aeromonads isolated from fish samples in India, relatively low level (<10%) of resistance to nalidixic acid, gentamicin and ciprofloxacin is reported by Hatha et al., (2005). However, resistance to nalidixic acid was considerably high among the motile aeromonads encountered in the present study (49.71%), while all the isolates were sensitive to gentamicin and ciprofloxacin. Varying levels of resistance to nalidixic acid, gentamicin and ciprofloxacin has been reported among motile aeromonads from water samples (Koksai et al., 2007; Evangelista-Barreto et al., 2010). Similar to the findings of these researchers, we also observed complete sensitivity of our isolates to nitrofurantoin, chloramphenicol and ciprofloxacin. The development of antibiotic resistant strains is a great extent attributed to the misuse of antibiotics in culture systems for treatment and for better feed conversion, these levels are bound to vary according to local practises.

Resistance to ceftazidime among the isolates from water and fish are found to vary from 8 to 15% among *A. veronii* and *A. sobria*. Except 3.63% of *A. caviae* isolates, all the other aeromonad isolates from fish samples were susceptible to sulfafurazole. While all *A. caviae* isolates from water were susceptible to sulfafurazole, 12.69% of *A. sobria*, 8% of *A. veronii* and 20% of *A. hydrophila* isolates were resistant to this drug. Resistance to trimethoprim was found to vary among the different species of motile aeromonads such as *A. caviae*, *A. hydrophila*, *A. trota* from fish samples, however all the isolates of *A. trota*, *A. jandaei* and *A. schubertii* from water was sensitive to this drug.

The present study reveals the prevalence of motile aeromonads in ornamental fishes and associated carriage water. Most of the motile aeromonads were capable of producing extracellular virulence factors and had acquired multidrug resistance. Poor water management practices could trigger infections caused by these potential pathogens/opportunists which might spell doom for this emerging industry in Kerala. Prudent use of antibiotics and proper water management practices may be promoted for sustainable development of ornamental fish industry, which can sustain the livelihoods of large number of rural people, who are currently engaged in this industry.

### 3 Materials and Methods

#### 3.1 Collection of Samples

Live, healthy ornamental fish samples (*Poecilia sphenops* and *Poecilia reticulata*) and associated carriage water samples were collected from three different aquarium vendors in and around Cochin. The fish samples were transported to the laboratory in sterile polythene bags and water samples in sterile bottles. The samples were then analyzed for aeromonads within 4 hours of collection.

##### 3.1.1 Isolation and identification of *Aeromonas*

Different parts of the body (body surface, gill and intestine) of fish samples were analyzed for motile aeromonads. The body surface and the gill of the fishes were repeatedly swabbed using sterile cotton swabs. Using a pair of scissors, an incision was made near the vent of the fish facilitating the swabbing of intestine. The swabs were then transferred to alkaline peptone water (APW pH 8.4) which was used as the enrichment medium. After incubation at 37°C for 18 h, a loopful of the APW culture was streaked on Starch Ampicillin Agar (Ampicillin 10 mg/L) which was used as the selective isolation medium and incubated at 37°C for 18-24 h (Palumbo et al., 1985).

The water samples were serially diluted and bacterial isolation was done by spread plate method using Starch Ampicillin Agar plates. The plates were incubated at 37 °C for 18-24 h and then flooded with approximately 5 ml of Lugol's iodine solution and amylase positive yellow to honey colored colonies were isolated. The isolated cultures were then purified by repeated streaking on nutrient agar plates. Those

strains that were gram negative bacilli, motile, oxidase and catalase positive, glucose fermenting, nitrate reducing, urease negative and which do not grow in 6% NaCl were further tested for arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase activity. Additional tests include acid production from mannitol, sucrose, arabinose, Voges-Proskauer reaction, hydrolysis of esculin and indole production. The isolates were identified to the species level according to Aerokey II (Carnahan et al., 1991).

#### 3.2 Study of Extracellular Virulence Factors

##### 3.2.1 Production of gelatinase

Pure cultures of the isolates were spot inoculated on gelatin agar plates (2%w/v gelatin), and the plates were incubated at 28 °C for 24-48 hrs. Zone of clearance around the colonies, after the plates were flooded with saturated solution of ammonium sulphate indicated that gelatin has been hydrolyzed.

##### 3.2.2 Production of caseinase

The test organisms were heavily spot inoculated on skim milk agar plates and the plates incubated at 28°C for 24 hrs -48 hrs. Caseinase production was detected by the presence of clear zones around the test colonies.

##### 3.2.3 Production of lipase

Tributyryl or glyceryl tributyrates is commonly used for studying lipolytic activities. The test organisms were heavily spot inoculated on tributyrin agar plates and the plates incubated at 28°C for 24 hrs -48 hrs. A positive result was indicated by a zone of clearance around the colonies of lipolytic organisms, where the tributyrin has been hydrolyzed (Rhodes, 1959).

##### 3.2.4 Production of DNase

A plate test for the demonstration of bacterial decomposition of nucleic acid was performed. The test organisms were heavily spot inoculated on DNA agar plates and the plates incubated at 28°C for 24 hrs -48 hrs. After incubation the plates were flooded with 1M HCl. The appearance of clear zone around the colonies indicated that the bacteria has elaborated DNase and hydrolyzed the DNA. The rest of the plate with the intact DNA turned opaque white, on addition of 1M HCl.

##### 3.2.5 $\beta$ - Haemolytic assay

Haemolytic activity was determined using blood agar

medium containing 5% human blood. Pure cultures of bacterial isolates were spot inoculated onto blood agar plates and  $\beta$  haemolytic activity was recorded as clear zone around the colonies after incubation at 37°C for 24 h.

### 3.3 Antimicrobial Susceptibility Test

Susceptibility to antimicrobial agents was performed for the identified *Aeromonas* spp. by the disc diffusion method (Bauer et al., 1966). The antibiotics used and the concentrations tested include amoxicillin (30 mcg), carbenicillin (100 mcg), cefpodoxime (10mcg), ceftazidime (30 mcg), cephalothin (30 mcg), chloramphenicol (10 mcg), ciprofloxacin (5 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), nitrofurantoin (100 mcg), streptomycin (10 mcg), sulphafurazole (300 mcg), tetracycline (30 mcg) and trimethoprim (5 mcg).

Pure cultures of *Aeromonas* were inoculated into nutrient broth and incubated at 37°C for 6 h-8 h. The cultures were then seeded onto Mueller Hinton agar plates. Antibiotic discs were placed on the surface of the agar with sterile forceps and pressed down gently to ensure even contact. Zone of inhibition around the antibiotic discs was measured after 16 h-18 h incubation at 37 °C and susceptibility/resistance interpretation was performed according to the manufacturer's interpretative table supplied by the Hi-media laboratories, Bombay as a matching criteria.

#### Authors' contributions

N.J. contributed considerably during collection, analysis and interpretation of data, analysis of the results and write-up of the manuscript. A.A.M.H. have made substantial contributions to the design and acquisition of data, analysis and interpretation of results and have been involved in revising the manuscript critically for important intellectual content; and have given final approval of the version to be published. Both the authors read and approved the final manuscript.

#### Acknowledgements

First author is thankful to Cochin University of Science and Technology for awarding research fellowship. Authors are also thankful to the Department of Marine Biology, Microbiology and Biochemistry for providing the facilities to carry out the research.

#### References

Bagyalakshmi B., Sridhar D., Ponnurugan P., Smitha A. J., Arti K., Vidyashri T., Vinothini P., Sowmya R., 2009, Characterization, hemolysis and multidrug resistance among *Aeromonas* spp. isolated from bhavani river, erode, south India, Recent Research in Science and Technology, 1(1): 14-19

Bauer A. W., Kirby W. M., Sherris J. C., and Turck M., 1966, Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol., 45(4):493-496, PMID:5325707

Burke V., Gracey M., Robinson J., Peck D., Beaman J., and Bundell C., 1983, The microbiology of childhood gastroenteritis: *Aeromonas* species and other infective agents, J. Infect. Dis., 148(1): 68-74, <http://dx.doi.org/10.1093/infdis/148.1.68>

Cahill M. M., 1990, A review, virulence factors in motile *Aeromonas* species, J. Appl. Bacteriol., 69(1): 1-16, <http://dx.doi.org/10.1111/j.1365-2672.1990.tb02905.x>

Camus A. C., Durborow R. M., Hemstreet W. G., Thune R. L., and Hawke J. P., 1998, *Aeromonas* Bacterial Infections - Motile Aeromonad Septicemia, SRAC Publication No. 478

Carnahan A. M., Behram S., and Joseph S.W., 1991, Aerokey II: a flexible key for identifying clinical *Aeromonas* species, J. Clin. Microbiol., 29(12): 2843-2849, PMID:1757558

Castro-Escarpulli G., Figueras M. J., Aguilera-Arreola G., Soler L., Fernández-Rendón E., Aparicio G.O., Guarro J., and Chacon M.R., 2003, Characterisation of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico, Int. J. Food Microbiol., 84(1): 41-49, [http://dx.doi.org/10.1016/S0168-1605\(02\)00393-8](http://dx.doi.org/10.1016/S0168-1605(02)00393-8)

Chacón M. R., Figueras M. J., Castro-Escarpulli G., Soler L., and Guarro J., 2003, Distribution of virulence genes in clinical and environmental isolates of *Aeromonas* spp., Antonie Van Leeuwenhoek, 84(4): 269-278, <http://dx.doi.org/10.1023/A:1026042125243>

Chopra A. K., Xu X. J., Ribardo D., Gonzalez M., Kuhl K., Peterson J. W., and Houston C. W., 2000, The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages, Infect. Immun., 68(5): 2808-2818, <http://dx.doi.org/10.1128/IAI.68.5.2808-2818.2000>

Esteve C., Amaro C., Garay E., Santos Y., Toranzo A. E., 1995, Pathogenicity of live bacteria and extracellular products of motile *Aeromonas* isolated from eels, J. Appl. Bacteriol., 78(5): 555-562, <http://dx.doi.org/10.1111/j.1365-2672.1995.tb03099.x>

Evangelista-Barreto N. S., Carvalho F. C. T. de, Vieira R. H. S. dos F., Reis M. F. dos, Macrae A., and Rodrigues D. dos P., 2010, Characterization of *Aeromonas* species isolated from an estuarine environment, Braz. J. Microbiol., 41(2): 452-460, <http://dx.doi.org/10.1590/S1517-83822010000200027>

Galindo C. L., Chopra A. K., 2007, *Aeromonas* and *Plesiomonas* species, Food Microbiology: Fundamentals and Frontiers, 3rd ed. ASM Press, Washington, D.C., pp.381-400

Gibotti A., Saridakis H. O., Pelayo J. S., Tagliari K. C., and Falcao D. P., 2000, Prevalence and virulence properties of *Vibrio cholerae* non-O1, *Aeromonas* spp. and *Plesiomonas shigelloides* isolated from CambeÂ Stream (State of ParanaÂ , Brazil), J Appl. Microbiol., 89: 70-75, <http://dx.doi.org/10.1046/j.1365-2672.2000.01077.x>

Goñi-Urriza M., Pineau L., Capdepuy M., Roques C., Caumette P., and Quentin C., 2000, Antimicrobial resistance of mesophilic *Aeromonas* spp. isolated from two European rivers, J. Antimicrob. Chemother., 46(2): 297-301, <http://dx.doi.org/10.1093/jac/46.2.297>

Hatha M., Vivekanandhan A. A., Joice G. J., Christol, 2005, Antibiotic resistance pattern of motile aeromonads from farm raised fresh water fish, Int. J. Food. Microbiol., 98(2): 131-134, <http://dx.doi.org/10.1016/j.jifoodmicro.2004.05.017>

Haunnen M. L., 1994, Phenotypic characteristics of the three hybridization groups of *Aeromonas hydrophila* complex isolated from different sources, J. Appl. Bact., 76(5): 455-462, <http://dx.doi.org/10.1111/j.1365-2672.1994.tb01102.x>

Illanchezian S., Jayaraman S., Manoharan M. S., and Valsalam S., 2010, Virulence and cytotoxicity of seafood borne *Aeromonas hydrophila*, Braz. J. Microbiol., 41(4): 978-983, <http://dx.doi.org/10.1590/S1517-83822010000400016>

Janda J. M., 2001, *Aeromonas* and *Plesiomonas*, In M. Sussman (ed.), Molecular Medical Microbiology, Academic Press, San Diego, USA, pp.1237-1270

Janda J. M., and Abbott S. L., 2010, The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection, Clinical microbiology reviews, 23(1): 35-73, <http://dx.doi.org/10.1128/CMR.00039-09>



- Kivanc M., Yilmaz M., and Demir F., 2011, The occurrence of *Aeromonas* in drinking water, tap water and the porsuk river, Braz. J. Microbiol., 42(1): 126-131, <http://dx.doi.org/10.1590/S1517-83822011000100016>
- Koksall F., Oguzkurt N., Samast M., and Altas K., 2007, Prevalence and antimicrobial resistance patterns of *Aeromonas* strains isolated from drinking water samples in Istanbul, Turkey, Chemotherapy, 53(1): 30-35, <http://dx.doi.org/10.1159/000098248>
- Kozińska A., 2007, Dominant pathogenic species of mesophilic aeromonads isolated from diseased and healthy fish cultured in Poland, J. Fish. Dis., 30(5): 293-301, <http://dx.doi.org/10.1111/j.1365-2761.2007.00813.x>
- Krishnakumar K., Raghavan R., Prasad G., Bijukumar A., Sekharan M., Pereira B., and Ali A., 2009, When pets become pests – exotic aquarium fishes and biological invasions in Kerala, India, Current science, 97(4): 474-476
- Liles M. R., Hemstreet W., Waldbieser G. C., Griffin M. J., Hossain M. J., Williams M. A., Hayden K., and Terhune J. S., 2011, Comparative genomics of *Aeromonas hydrophila* isolates from an epidemic in channel catfish, American Society for Microbiology Meeting No.1489
- Marchandin H., Godreuil S., Darbas H., Jean-Pierre H., Jumas-Bilak E., Chanal C., and Bonnet R., 2003, Extended-spectrum  $\beta$ -lactamase TEM-24 in an *Aeromonas* clinical strain: acquisition from the prevalent *Enterobacter aerogenes* clone in France, Antimicrob. Agents. Chemother., 47(12): 3994-3995, <http://dx.doi.org/10.1128/AAC.47.12.3994-3995.2003>
- Mateos D., Anguita J., Naharro G., and Paniagua C., 1993., Influence of growth temperature on the production of extracellular virulence factors and pathogenicity of environmental and human strains of *Aeromonas hydrophila*, J. Appl. Bacteriol., 74(2): 111-118, <http://dx.doi.org/10.1111/j.1365-2672.1993.tb03003.x>
- Pablos M., Rodríguez-Calleja J. M., Santos J. A., Otero A., and García-López M. L., 2009, Occurrence of motile *Aeromonas* in municipal drinking water and distribution of genes encoding virulence factors, Int. J. Food Microbiol., 135(2): 158-164, <http://dx.doi.org/10.1016/j.ijfoodmicro.2009.08.020>
- Palumbo S. A., Maxino F., Williams A. C., Buchanan R. L., and Thayer D. W., 1985, Starch-Ampicillin Agar for the Quantitative Detection of *Aeromonas hydrophila*, Appl. Environ Microbiol., 50(4): 1027-1030, PMID:16346899
- Penders J., and 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(3): 261-265, <http://dx.doi.org/10.1016/j.ijantimicag.2007.10.002>
- Rahman M., Colque-Navarro P., Kühn I., Huys G., Swings J., and Mollby R., 2002, Identification and characterization of pathogenic *Aeromonas veronii* biovar *sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh, Appl. Environ. Microbiol., 68(2): 650-655, <http://dx.doi.org/10.1128/AEM.68.2.650-655.2002>
- Rathore G., swaminathan T. R., Abidi R., Mahanta P. C., and Kapoor D., 2005, Isolation and characterization of motile aeromonads from aquatic environment, Indian J. Fish., 52(2): 241-248
- Rhodes M. E., 1959, The characterisation of *Pseudomonas fluorescens*, J. Gen. Microbiol., 21(1): 221-263, <http://dx.doi.org/10.1099/00221287-21-1-221>
- Roberts R. J., Frerichs G. N., and Millar S. D., 1990, Epizootic ulcerative syndrome the current position, In "Diseases in Asian aquaculture I" (ed. by Shariff M., Subasinghe R.P and Arthur J.R.), Fish Health Section, Asian Fisheries Society, Manila, pp.431-436
- Sechi L. A., Deriu A., Falchi M. P., Fadda G., and Zanetti S., 2003, Distribution of virulence genes in *Aeromonas* spp. isolated from Sardinian waters and from patients with diarrhea, J. Appl. Microbiol., 92(2): 221-227, <http://dx.doi.org/10.1046/j.1365-2672.2002.01522.x>
- Sen K., and Rodgers M., 2004, Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification, J. Appl. Microbiol., 97(5): 1077-1086, <http://dx.doi.org/10.1111/j.1365-2672.2004.02398.x>
- Gonzalez-Serrano C. J., Santos J. A., Garcia-Lo'pez M. L., and Otero A., 2002, Virulence markers in *Aeromonas hydrophila* and *Aeromonas veronii* biovar *sobria* isolates from freshwater fish and from diarrhoea case, J. Appl. Microbiol., 93(3): 414-419, <http://dx.doi.org/10.1046/j.1365-2672.2002.01705.x>
- Shaleesha A., and Stanley V. A., 2000, Involvement of Rural Women in Aquaculture: An Innovative Approach, The ICLARM Quarterly, 23(3):13-17
- Shome R., Shome B. R., and Ram N., 1999, Study of virulence factors of *Aeromonas hydrophila* isolates causing acute abdominal dropsy and ulcerative diseases in Indian major carps, Indian J. Fish., 46(2): 133-140
- Sreedharan K., Philip R., and Singh S. B., 2011, Isolation and characterization of virulent *Aeromonas veronii* from ascitic fluid of oscar *Astronotus ocellatus* showing signs of infectious dropsy, Dis. Aquat. Org., 94(1): 29-39, <http://dx.doi.org/10.3354/dao02304>
- Subasinghe R. P., Jayasinghe L. P., Balasuriya K. S. W., and Kulathilake M., 1990, Preliminary investigations into the bacterial and fungal pathogens associated with the ulcerative fish disease syndrome in Sri Lanka, In "The second Asian fisheries forum" (ed. by R. Hirano and Hanyu), The Asian Fisheries Society, Manila, pp.655-657
- Torres J. L., Shariff M., and Law A. T., 1990, Identification and virulence screening of *Aeromonas* spp. isolated from healthy and epizootic ulcerative syndrome (EUS)-infected fish, In "The second Asian fisheries forum" (ed. by Hirano R. and Hanyu I.), The Asian Fisheries Society, Manila, pp.663-666
- Vila J., Marco F., Soler M., Chacón M., and Figueras M. J., 2002, In vitro antimicrobial susceptibility of clinical isolates of *Aeromonas caviae*, *Aeromonas hydrophila*, and *Aeromonas veronii* biotype *sobria*, J. Antimicrob. Chemoth., 49(4): 701-702, <http://dx.doi.org/10.1093/jac/49.4.701>
- Vila J., Ruiz J., Gallardo J., Vargas M., Soler L., Figueras M. J., Gascón J., 2003, *Aeromonas* spp. and traveler's diarrhea: clinical features and antimicrobial resistance, Emerg. Infect. Dis., 9(5): 552-555, <http://dx.doi.org/10.3201/eid0905.020451>
- Wahli T., Burr S. E., Pugovkin D., Mueller O., and Frey J., 2005, *Aeromonas sobria*, a causative agent of disease in farmed perch, *Perca fluviatilis* L. J. Fish. Dis., 28(3): 141-150, <http://dx.doi.org/10.1111/j.1365-2761.2005.00608.x>
- Wittington R. J., and Chong R., 2007, Global trade in ornamental fish from an Australian perspective: The case for revised import risk analysis and management strategies, Preventive Veterinary Medicine, 81(1-3): 92-116, <http://dx.doi.org/10.1016/j.prevetmed.2007.04.007>
- Yucel N., and Citak S., 2003, The occurrence, hemolytic activity and antibiotic susceptibility of motile *Aeromonas* spp. isolated from meat and milk samples in Turkey, J. Food Safety., 23: 189-200, <http://dx.doi.org/10.1111/j.1745-4565.2003.tb00362.x>