2.1. Introduction

The genus *Aeromonas* belongs to the family *Aeromonadaceae* with in the *Gammaproteobacteria* and comprises Gram-negative, non spore-forming, motile bacilli or coccobacilli with rounded ends which measure 1-3.5 µm in diameter. They are facultative anaerobic, oxidase and catalase positive, able to reduce nitrate to nitrite, glucose-fermenting and are generally resistant to the vibriostatic agent O/129. They grow optimally within a temperature range between 22 and 35°C, but growth occurs in a temperature range from 0 to 45°C for some species (Igbinosa *et al.*, 2012). They tolerate a pH range from 4.5 to 9.0, but the optimum pH range is from 5.5 to 9.0 (Isonhood and Drake, 2002).
In the past, aeromonads could be broken down roughly into two major groupings, based upon growth characteristics and other biochemical features. The mesophilic group, typified by *Aeromonas hydrophila*, consists of motile isolates that grew well at 35 to 37°C. The second group, referred to as psychrophilic strains, were nonmotile and had optimal growth temperature of 22 to 25°C. This group consists of isolates that currently reside within the species *Aeromonas salmonicida*. Mesophilic aeromonads have been also described as motile aeromonads; these two characteristics of *Aeromonas* are usually interconnected and have been commonly used for separation of the group from the psychrophilic and nonmotile *A. salmonicida* (Janda and Abbot, 2010).

The taxonomy of the genus is complex and has undergone numerous changes with the description of many new species. The genus includes 14 well-recognized species (Joseph and Carnahan, 2000) to which eleven new species have been added since 2002, which includes *A. piscicola, A. fluvialis, A. taiwanensis, A. sanarellii, A. rivuli, A. tecta, A. aquariorum, A. bivalvium, A. sharmana, A. molluscorum, A. simiae* and *A. culicicola* (Janda and Abbott, 2010; Martinez-Murcia et al., 2011).

*A. hydrophila, A. salmonicida, A. veronii* bt. *sobria, A. caviae, A. jandaei* and *A. schuberti* have been reported as pathogens of various fish species (Nielsen et al., 2001; Rahman et al., 2002; Kozinska, 2007; Chen et al., 2012) and most of them are recognized as human and animal pathogens (Janda and Abbott, 2010). However, there are pathogenic as well as non-pathogenic strains belonging to these groups.
Several extracellular enzymes and toxins including haemolysins, proteases, lipases, DNases and cytotoxins have been reported as virulence factors of motile Aeromonas (Erdem et al., 2010; Oliveira et al., 2012; Cai et al., 2012) but the role of each single factor in relation to pathogenesis varies.

The proteolytic activity of Aeromonas has been correlated with its ability to induce pathology in fish (Castro-Escarpulli et al., 2003). Proteases contribute to host tissue invasion by digesting or destroying cell membranes and by degrading host surface molecules. There is also some evidence that hydrolytic enzymes are able to attack cells and molecules of the host immune system to avoid or resist antimicrobial activity. Aeromonas spp. produce a lipase, glycerophospholipid cholesterol acyltransferase (GCAT), that results in production of cholesteryl esters and phospholipase activity that digests plasma membrane of host cells with extensive host cell destruction (Buckley, 1983). Lipases may provide bacterial nutrients and constitute virulence factors by interacting with human leucocytes or by affecting several immune system functions through free fatty acids generated by lipolytic activity. Nucleases have not yet been confirmed in terms of its association with pathogenicity, but reports have indicated that it participates in the development of host infection. They are shown to be important virulence factors in other genera such as genus Streptococcus (Gavin et al., 2003; Kirov et al., 2004). They may aid in the release of bacteria from disintegrating host cells in inflammatory lesions by digesting host DNA and reducing viscosity. The action of DNase may also make nucleotides available for bacterial utilization.
2.2. Review of Literature
2.2.1. Media for the culturing and isolation of *Aeromonas*

In view of the potential losses to the commercial and sport fishing industries and the serious implications for human health, definitive isolation and identification of *Aeromonas* species is necessary to develop therapeutic strategies. Aeromonads grow readily on ordinary media, but separation from other organisms in mixed population and eventual identification is more complex and difficult.

A huge number of selective and differential isolation media have been developed for the recovery of *Aeromonas* species from the environment, food, and clinical specimens (Villari *et al.*, 1999). Studies suggest that no single medium results in optimum recovery of aeromonads and combinations of different isolation media and methods are frequently employed by direct plating, membrane filtration or multiple tube tests for determining most probable numbers (Igbinosa *et al.*, 2012). Several culture media have been evaluated for the recovery of aeromonads (McMahon and Wilson, 2001). Starch ampicillin agar (SAA), bile salts inositol brilliant green agar (BIBG) and *Aeromonas* Medium (Ryan’s Medium) are recommended (Igbinosa *et al.*, 2012). Starch glutamate ampicillin penicillin (SGAP-10) medium was used in the isolation of aeromonads from sewage sludge (APHA, 1998). This medium is highly selective, and it has been used to detect aeromonads from food and other samples. *Aeromonas* species grow readily in blood culture media and on 5% sheep blood agar used in clinical laboratories, but if screening is based on haemolysis, approximately 10% of *Aeromonas* isolates will be missed because they are nonhaemolytic (Janda and Abbott, 2010). Isolation of aeromonads from contaminated samples such as faeces require the use of selective and
differential media such as MacConkey agar, cefsulodin irgasan novobiocin (CIN) agar, or blood ampicillin agar (10 mg/L ampicillin) (APHA, 1998; USEPA, 2001). Several culture enrichment broth such as alkaline peptone water (APW) or tryptose broth containing ampicillin (TSB-30, ampicillin 30mg/L) have been recommended for the recovery of *Aeromonas* from contaminated sources, particularly when aeromonads are present in small numbers compared to other bacteria present (McMahon and Wilson, 2001).

2.2.2. Identification of *Aeromonas*

Diagnosing disease and identifying the infectious agents included are important for managing any disease. In the past, *Aeromonas* species were placed alongside *Vibrio* species and *Plesiomonas shigelloides* in the family *Vibrionaceae*. Resistance to vibriostatic compound O/129 (150 µg) and variable presence of ornithine decarboxylase activities differentiates *Aeromonas* from *Vibrio* and *Plesiomonas* (Joseph and Carnahan, 2000). The aeromonads share many biochemical characteristics with members of the *Enterobacteriaceae*, from which they are primarily differentiated by being oxidase positive (Saavedra *et al*., 2006). Other important distinguishing qualities include their inability to grow in the presence of 6.5% sodium chloride; ability to liquefy gelatin; inability to ferment inositol; negative string test. Some phenotypic characteristics include inability to grow on thiosulfate citrate bile salts sucrose agar and ability of most but not all *Aeromonas* species to ferment D-mannitol and sucrose (USEPA, 2006). Carnahan *et al.* (1991) have examined a large number of *Aeromonas* isolates from diverse clinical and geographic sources and used the frequency matrix of test results from a numerical taxonomy analysis to develop a highly discriminative subset of
tests. These tests were then used to construct a flexible dichotomous identification key (Aerokey), that allowed identifying *Aeromonas* isolates to the species level. Awan *et al.* (2009) have made use of this key for the identification of *Aeromonas* from food samples and Abulhamd (2010), for motile aeromonads from aquatic environment.

The most commonly utilized molecular technique in the clinical laboratory for genus and species identification of bacteria is 16S rRNA gene sequencing (Janda and Abbott, 2007). Polymerase chain reaction (PCR) methods have been developed to detect the presence of *Aeromonas* species in a wide range of samples (Igbinosa *et al*., 2012). A number of species-specific probes have been developed for some genomic groups including *A. hydrophila*, *A. trota*, *A. schubertii* and *A. jandaei* (Janda, 2001). Two probes, one designed to detect glycerophospholipid-cholesterol acyltransferase and the other directed at an outer membrane protein, do detect all the members of the genus (Chacon *et al*., 2002; Khushiramani *et al*., 2009). The digoxigenin-labeled genus specific DNA probe reported by Chacon *et al.* (2002) appears to pick up > 98% of aeromonads and is nonreactive in colony hybridization assays against phenotypically similar bacteria, such as *Vibrio* species and *P. shigelloides*. A digoxigenin-labeled DNA probe directed against an OmpA homologue produced a positive reaction in colony hybridization assays against all *Aeromonas* isolates, while the probe remained unreactive against several other Gram-negative pathogens, including *Vibrio* species (Khushiramani *et al*., 2009).

### 2.2.3. Distribution of motile aeromonads

Widespread in various habitats, *Aeromonas* are mainly present in aquatic environments (Di Bari *et al*., 2007; Figueira *et al*., 2011) but also in soil, food
and animals (Janda and Abbott, 2010). They are ubiquitous inhabitants of both freshwater and estuarine aquatic environment. They are isolated from ground water, surface water, waste water, chlorinated and non chlorinated drinking water (Soler et al., 2002) and in some countries, in bottled mineral water (Massa et al., 2001; Pandove et al., 2013). They are known to be fish pathogens since 1894 (Emmerich and Weibel, 1894; Kirkan et al., 2003; Patil et al., 2011).

Isolation of Aeromonas from tropical seafood (squid, prawn and mussel), sediment and water samples from aquafarms and associated mangroves in Kerala has been reported (Joseph et al., 2013). The isolates belonged to A. hydrophila, A. enteropelogenes, A. caviae, A. punctata and A. aquarorium. Kumar and Ramulu (2013) isolated A. hydrophila, A. sobria and A. caviae from different organs of Pangasius hypophthalmus in culture ponds of India, and found A. hydrophila to be the dominant species in skin, liver and kidney samples of these fishes.

Shayo et al. (2012) isolated A. hydrophila, A. veronii and A. caviae from four anatomical sites namely kidney, liver, gills and skin from normal and diseased Tilapia with cutaneous ulcerative signs, in a study conducted in Tanzania. In a study conducted in Nigeria, Ashiru et al. (2011) reported A. caviae to be the predominant species on the body surface and intestinal tract of Tilapia fish while A. hydrophila and A. sobria were predominant in Catfish. Suhet et al. (2011) evaluated Aeromonas in samples of Nile Tilapia reared in net-cage. A. hydrophila was the most occurring species in fish body surface, followed by A. caviae and A. sobria. While in the fish kidneys, the most occurring species were A. hydrophila and A. veronii. Eissa et al. (2008) have
shown that the prevalence of motile aeromonad septicaemia in cultured and wild Nile Tilapia (*Oreochromis niloticus*) was 10.0% and 2.5% respectively; it was 18.75% and 6.25% in cultured and wild Karmout Catfish, respectively. Hatha *et al.* (2005) isolated motile aeromonads from the intestines of farm-raised freshwater fishes such as *Catla catla*, *Labeo rohita* and *Ctenopharyngodon idella* and characterized them to species level, which revealed 61% *A. hydrophila*, 30% *A. caviae* and 7% *A. sobria*.

*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). In a study conducted in China, Li and Cai (2011) identified *A. sobria* as the pathogenic agent of tail-rot disease in juvenile tilapia. In a study conducted by Nam and Joh (2007), *A. sobria* was detected in all the intestinal samples from diseased Trout. In addition, they were detected in lesions on the body (89%). *A. sobria* has been identified as a causative agent of disease in farmed Perch *Perca fluviatilis* L. in Switzerland (*Wahli et al.*, 2005).

Cai *et al.* (2012) reported *A. veronii* bv. *veronii* as the pathogenic agent of ulcerative syndrome in Chinese longsnout catfish. *A. veronii* has been isolated from the ascitic fluid of Oscar *Astronotus ocellatus* showing signs of infectious dropsy in India (*Sreedharan et al.*, 2011). *A. schubertii* were isolated from diseased Snakeheads, suffering high mortality in a farm in Southern China by Chen *et al.* (2012).

*A. hydrophila* was isolated from haemorrhagic diseased freshwater fishes in China (*Ye et al.*, 2013). Citarasu *et al.* (2011) have isolated *A. hydrophila* from Goldfish (*Carrassius auratus*) and Koi (*Cyprinus carpio koi*)
in South India during massive fish disease outbreaks in various infected ornamental fish hatcheries. Occurrence of *A. hydrophila* in marine fish species was reported by Al-Maleky and Haneff (2013).

The frequencies of the identified *Aeromonas* species from water and Rainbow trout samples from Turkish coastal regions were 38.33% for *A. sobria*, 23.33% for *A. hydrophila* and 10% for *A. veronii* (Onuk et al., 2013). In a study conducted by Hu et al. (2012), *Aeromonas* isolates from diseased fish, healthy fish and water environment in China, were identified to species levels. *A. veronii* and *A. hydrophila* were the species most commonly isolated from diseased fish, while *A. veronii* was the most common species in healthy fish and water samples. Dias et al. (2012) isolated *Aeromonas* spp. from ornamental fishes and also from water samples. A total of 288 strains grouped in seven different species-*A. veronii, A. media, A. jandaei, A. hydrophila, A. caviae, A. culicicola* and *A. aquariorum* were isolated. In a study conducted by Sreedharan et al. (2012), fish samples and water samples from two ornamental fish culture systems were analyzed for the presence of *Aeromonas*. The isolates were clustered and 3 clusters were identified: *A. caviae, A. jandaei* and *A. veronii* biovar *sobria*. Sugita et al. (1995) reported distribution of *A. veronii* (22%), *A. caviae* (18%), *A. hydrophila* (13%), *A. sobria* (8%), *A. jandaei* (7%) and other *Aeromonas* spp. (33%) in the intestinal tracts of diseased fish and in water from a fish farm.

*A. hydrophila* has frequently been found in fish and shellfish. In a retail survey of seafoods, motile *Aeromonas* were found in 66% of shellfish and 34% of finfish (Aberoum and Jooyandeh, 2010). Seafoods probably become contaminated by *Aeromonas* spp. through the growing water and the animals
themselves, with many fish species containing *Aeromonas* spp. in their gut. Thayumanavan *et al.* (2007) examined samples of finfish and prawns for the presence of *Aeromonas* sp. Aeromonads were detected in 37.3% of finfish and 35.6% of prawn samples. Vivekanandhan *et al.* (2005) examined samples of fish and prawn from the major fish markets of Coimbatore, South India, over a period of two years for the presence of aeromonads (reported as *A. hydrophila*). *Aeromonas* sp. was detected in 33.6% and in 17.6% of fish and prawn samples respectively. During the period of the study, seasonal variation was observed in the prevalence levels of *Aeromonas* sp. in fish and prawns with a higher prevalence during monsoon seasons. In a study conducted by Yucel *et al.* (2005), a total of 132 market fish (64 fresh water and 68 seawater) samples were collected from Turkey and investigated for the presence of *Aeromonas* spp. and they observed a variation in the distribution of *Aeromonas* spp. depending on the samples (gill, intestine, liver, kidney) examined. In freshwater samples, the predominant species was *A. caviae*, followed by *A. hydrophila* and *A. veroni bv. sobria*. In seawater samples, the predominant species was *A. veroni bv. sobria*, followed by *A. hydrophila* and *A. caviae*. Neyts *et al.* (2000) reported the presence of *Aeromonas* species in 72% of fish and shrimp samples.

In a study conducted in Brazil, by Evangelista-Barreto *et al.* (2010) *Aeromonas* were identified in 63% of water samples analyzed. Suhet *et al.* (2011) evaluated *Aeromonas* in samples of lake water and found *A. hydrophila* as the most occurring species, followed by *A. caviae* and *A. sobria*. Bagyalakshmi *et al.* (2009) studied the distribution of *Aeromonas* spp. isolated from Bhavani river, South India. The predominant strain was identified as *A. hydrophila*, while the other strains belonged to the species *A. sobria* and
A. caviae. In Turkey, Koksal et al. (2007) reported the prevalence of Aeromonas such as A. hydrophila (46%), A. sobria (34%), A. caviae (8%), A. veronii (3%) and A. jandaei (3%), in water samples. Aeromonas density can vary depending on pollution, changes in temperature and nutritional status (Koksal et al., 2007).

2.2.4. Extracellular virulence factors of motile aeromonads

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, including aerolysin/haemolysin, 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; Serrano et al., 2002). The high rate of haemolytic 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).

Aeromonas caviae, A. jandaei and A. veronii biovar sobria isolates obtained by Sreedharan et al. (2012) from ornamental fish culture systems in Kerala, produced highly active hydrolytic enzymes, haemolytic activity and slime formation in varying proportions. In fish samples collected from Turkey by Yucel et al. (2005), all the Aeromonas isolated were examined for haemolytic, lipolytic and proteolytic activity. More than 80% (A. veroni bv. sobria, A. hydrophila) were positive for haemolytic activity. Lipolytic and proteolytic activity was found to be low.
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Extracellular products of *A. veronii* from Chinese longsnout catfish contained gelatinase, lecithinase, elastase, lipase and lipopolysaccharide (Cai *et al.*, 2012). Chen *et al.* (2012) reported that some of the *A. schubertii* isolated from diseased Snakeheads, were positive for haemolysin, elastase, lipase and lecithinase. Singh *et al.* (2010) screened isolates of *A. hydrophila* from fish muscle and water samples for the presence of virulence factors such as aerolysin, haemolysin and lipase. All the isolates produced lipase, whereas only 60% of isolates produced haemolysis with RBCs.

All the *Aeromonas* isolates obtained from tropical seafood (squid, prawn and mussel), sediment and water samples from aquafarms and associated mangroves in Kerala by Joseph *et al.* (2013) were haemolytic on blood agar. Thayumanavan *et al.* (2007) studied the incidence of *A. hydrophila* in retail sea food outlets in Coimbatore. Of the isolates they have obtained, 84.9% of the strains were haemolysin producers. Hatha *et al.* (2005) isolated *A. hydrophila*, *A. caviae* and *A. sobria* from the intestine of farm-raised freshwater fishes. Haemolytic activity was detected mostly in *A. hydrophila*, while only half of the *A. sobria* and *A. caviae* showed this activity.

Suhet *et al.* (2011) isolated *Aeromonas* from samples of Nile Tilapia reared in net-cage and from the lake water, of which 57% exhibited haemolytic activity. Monfort and Baleux (1990) reported that all the isolates of *A. hydrophila* and *A. sobria* were haemolysin producers; whereas among the *A. caviae* isolates, 96% were non haemolytic.

Motile *Aeromonas* strains were isolated from samples of water and sediment collected at different sites along a river by Paniagua *et al.* (1990). Caseinases, haemolysins and vero cytotoxins were produced by 100, 91, and
94.59%, respectively of *A. hydrophila* strains. *A. sobria* isolates showed relatively lower caseinolytic activity. The researchers however reported that there was no correlation between these activities and the degree of virulence of the strains for fish. In a report by Bagyalakshmi *et al.* (2009), occurrence of haemolysin, lipase, protease, gelatinase and caseinase was established as virulence factors in *Aeromonas* spp. isolated from Bhavani River, India. Review of literature reveals dearth of information about the prevalence of motile aeromonads and their virulence potential from ornamental fish farm from Cochin area.

### 2.3. Objectives of the study

Considering the market potential of ornamental fishes, many small scale entrepreneurs have set up small scale fish farms in their courtyards/ponds/water bodies available nearby. *Aeromonas* being a normal flora of natural waters is likely to be associated with these fishes. They are also a normal inhabitant of the gastrointestinal tract of fresh water fishes, but opportunistic in nature, capable of causing infections in fishes under stress or with compromised immune system. The prevalence, distribution of different species and potential virulence factors produced by *Aeromonas* are relatively underexplored/unexplored in the study area. The information is important on the health management of these fishes, a fall in which would result in disease outbreak from such opportunistic pathogens causing heavy economic loss to the farmers involved. Hence the study has been taken up with the following specific objectives:

- To study the prevalence of motile aeromonads among the ornamental fishes maintained in an ornamental fish farm.
To study the prevalence of motile aeromonads in the associated carriage water from the farm.

To characterize the isolated motile aeromonads to species level and study the distribution of different species in ornamental fish and carriage water samples.

To study the extracellular virulence factors of different species of motile aeromonads associated with ornamental fishes and associated carriage water from the farm.

2.4. Material and Methods

2.4.1. Description of farm

The farm is located at Edavanakkad, Cochin, with around 20 concrete tanks, 16 mud ponds and 80 glass tanks of different sizes for breeding, rearing and stocking fresh water ornamental fishes. There is individual water inlet and outlet for each tank and is equipped with aeration. The farm is blessed with natural water supply throughout the year with no water treatment required.

![Plate 2.1. Ornamental fish farm at Edavanakkad](image)

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Sample collection

2.4.1.1. Ornamental fish samples

Live, healthy ornamental fish samples were collected from the fish farm. The fishes collected include *Poecilia sphenops* (Black molly) and *Poecilia reticulata* (Guppy). Fifty samples of each fish were collected over a period of two years (2008-2010). They were transported to the laboratory in sterile polythene bags and analyzed within 4 hours of collection.

2.4.1.2. Water samples

Water samples which housed the ornamental fishes were collected from the same farm, at 20 cm from the surface, using sterile bottles that were labelled. The bottles were placed in an ice box to keep the temperature below 10°C until analysis and were analyzed within 4 hours of collection. A total of one hundred samples were analyzed.

2.4.1.2.1. Physico-chemical analysis of water samples

Temperature and pH of water samples were recorded *in situ*, at the time of sampling using a mercury bulb thermometer and a hand held pH probe respectively. Dissolved oxygen, total ammonia nitrogen (phenate method), nitrite and nitrate were estimated by APHA (1998) method.

2.4.2. Bacteriological Analysis

2.4.2.1. Fish samples

Fishes were anesthetized using MS 222 (Sigma, US) and bacteria were isolated from different parts of the body (body surface, gill and intestine) of fish samples. 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 (composition of alkaline peptone water per litre: peptone, 10 g; NaCl, 10 g, pH 8.4) which was used as an enrichment medium. After incubation at 37°C for 18 h, a loopful of the alkaline peptone water (APW) culture was streaked on Starch Ampicillin Agar plates (composition of Starch Ampicillin Agar per litre: peptone, 10 g; NaCl, 5 g; soluble starch, 1 g; phenol red, 0.018 g; agar 15 g, pH 7.4±0.2; ampicillin, 0.01 g), used as the selective isolation medium (Palumbo et al., 1985 a). 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 coloured colonies were isolated. The isolated cultures were then purified by repeated streaking on nutrient agar plates and subjected to further phenotypic/biochemical characterization.

2.4.2.2. Water samples

The water samples collected were serially diluted using sterile distilled water. Using a sterile pipette, 0.1 ml aliquot of the sample was placed on Starch Ampicillin Agar (SAA) plates and plating was done by the spread plate method. The plates were incubated at 37°C for 18-24 h and isolation of pure culture was carried out as described in 2.4.2.1 and subjected to further phenotypic/biochemical characterization.

2.4.3. Phenotypic/biochemical characterization

All the isolates obtained were initially screened by using the following tests: Gram staining, oxidase test, catalase test, motility test and glucose fermentation. Only those strains that were Gram negative rods, oxidase and catalase positive, motile and glucose fermenting were considered as presumptive aeromonads.
2.4.3.1. Kovac’s Oxidase test (Cytochrome oxidase activity)

This test is used to determine whether an organism is capable of producing the enzyme cytochrome oxidase. The detection of cytochrome oxidase activity is used as a differentiating test mainly for the aerobic and facultatively anaerobic groups of Gram-negative bacteria. The reagent (impregnated into strips of filter paper) contains tetramethyl-p-phenylene diamine dihydrochloride (TPDD) which serves as an alternate substrate for the cytochrome oxidase reaction. In the reduced state the reagent is colourless, but when oxidised it becomes purple.

The organism was freshly grown on nutrient agar. Using a platinum loop/wooden applicator, a colony was picked and a compact smear was made on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride. A positive reaction is indicated by the development of an intense deep violet/purple colour within ten seconds. Negative reaction is indicated by the absence of the characteristic colour within ten seconds.

2.4.3.2. Catalase test

This test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The bubbles resulting from the production of oxygen gas indicated a positive catalase result.

The test organisms were grown on a slope of nutrient agar. A thick smear of the organism was made from a 24 h culture on a clean slide and a drop of hydrogen peroxide was placed on it. Immediate development of effervescence was considered as a positive result.
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2.4.3.3. Motility assay

a) **Soft agar method**

Motility test was performed in soft agar medium (composition of soft agar medium per litre: peptone, 5 g; NaCl, 5 g; beef extract, 3 g; agar, 3 g, pH 7.4±0.2). The pure cultures were stab inoculated into the medium and incubated at 28±0.5°C for 24 to 48 hours. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility. A thick growth along the line of inoculation was considered negative.

b) **Hanging drop method**

Petroleum jelly was placed near the four corners of the cover slip using a toothpick. A loopful of 16 to 18 hour-old bacterial culture was placed in the centre of the cover slip. The cavity slide was placed with the concave surface facing down over the cover slip so that the depression covers the drop of culture. Slide was gently pressed to form a seal between the slide and the cover slip. Preparation was inverted quickly so that the drop of culture was seen hanging from the cover slip. The slide was placed under high power objective (40 X) and the edge of the hanging drop was focused and observed for actual movement of the cells that could very well be differentiated from Brownian movement.

2.4.3.4. Oxidation Fermentation reaction

The purpose of this test is to determine whether an organism attack sugars (in this case glucose) by fermentation or oxidation. O/F medium (Hi Media Laboratories, Mumbai) was employed for this test. This reaction was determined by inoculating the organisms into agar media deeps prepared by supplementing the basal media with 1% glucose. The organism was inoculated by stabbing the butt and streaking the slant. The tubes were incubated at 28±0.4°C.
The results were recorded as follows:

- **O** - Oxidation (acid production indicated by yellow coloration in the slant)
- **F** - Fermentation (yellow coloration in both butt and slant)
- **FG** - Fermentation with gas production
- **Alk / N** - alkaline reaction (blue coloration in the slant and no reaction in the butt).

The presumptive aeromonads (145 isolates from the fish and 156 isolates from the water samples) were then subjected to an array of biochemical tests, listed in the following section.

### 2.4.3.5. Production of Indole

Indole formation depends exclusively on the development of bacteria producing enzymes called tryptophanases, that oxidize the L-tryptophan producing indole, skatole (methyl indole) and indoleacetate.

Test organism was grown in tryptone water (composition of tryptone water per litre: tryptone, 20 g; NaCl, 5 g, pH 7.5±0.2) for 24 h at 28°C. Indole production was determined by adding a few drops of Kovac’s reagent (composition of Kovac’s reagent: ρ-dimethyl amino benzaldehyde, 5 g; amyl alcohol, 75 ml; Con.HCl, 25 ml) to 5 ml of culture medium. A positive test was indicated by the development of a red colour in the reagent layer.

### 2.4.3.6. Methyl Red and Voges-Proskauer tests

MR-VP broth (Hi Media Laboratories, Mumbai) was used for the test. The cultures were incubated at 28±0.5°C for 48 h and the respective reagents were added.
Methyl Red test

This test is used to check the ability of an organism to produce and maintain stable acid end products from glucose fermentation. The stable production of enough acid to overcome the phosphate buffer results in a pH less than 4.4. On addition of the pH indicator-methyl red, if the culture broth has a pH below 4.4, a red colour appears. If the MR turns yellow, the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized. The indicator was prepared by dissolving 0.1g methyl red in 300 ml 95% ethyl alcohol, which was then diluted to 500 ml with distilled water.

Voges-Proskauer test (acetoin production)

Some organisms, after producing acids from glucose, are capable of converting acids to acetyl methyl carbinol or 2, 3-butanediol, which are neutral substances. Aeration in the presence of alkali then converts the products to diacetyl, which in turn reacts with the peptone constituents producing a pink colouration.

To a 48 hour old culture broth, 0.6 ml of 5% solution of alpha naphthol in absolute ethanol was added followed by 0.2 ml of 40% KOH, and then mixed well. A positive reaction was indicated by the development of a pink colour in 2-5 min, becoming crimson in 30 min. The tubes were shaken at intervals to ensure maximum aeration.

2.4.3.7. Production of Urease

Christensen’s Urea Agar Base (Hi Media Laboratories, Mumbai) supplemented with 2% urea was used for the test. The test is used to detect the ability of an organism to produce the enzyme urease. Urease is an enzyme that
Prevalence, distribution and extracellular virulence factors of motile aeromonads in fresh water….

breaks the carbon-nitrogen bond of amides to form carbon dioxide and ammonia. The production of ammonia raises the pH of the media above 8.4 and the pH indicator, phenol red, turns from yellow to pink.

\[ \text{H}_2\text{NCO} \text{ N H}_2 \rightarrow 2\text{NH}_3 + \text{CO}_2 \]

The test culture was inoculated onto Christensen’s Urea Agar slants and incubated for 24 h at 28±0.5°C. Urease activity was indicated by the change in colour of the medium from yellow to pink (pinkish red).

2.4.3.8. Nitrate reduction test

Nitrate Broth is used for this test. The test is used to determine if an organism is capable of reducing nitrate (NO\(^3\)) to nitrite (NO\(^2\)) or other nitrogenous compounds via the action of the enzyme nitrate reductase.

Sterile nitrate broth (composition of nitrate broth per litre: peptone, 5 g; beef extract, 5 g; yeast extract, 1 g; KNO\(_3\), 1 g; NaCl, 30 g, pH 7±0.2) in 5ml quantity was inoculated with the test culture and incubated at 28±0.4°C for 48 h. Nitrate reduction tests were conducted adding Griess reagent to the surface of the medium (Griess reagent consists of 2 solutions- solution A: sulphanilic acid, 8 g; 5N glacial acetic acid, 1 litre and solution B: Dimethyl α-naphthylamine, 5 g; 5N glacial acetic acid, 1 litre).

The presence of nitrite could be determined by adding 0.5 ml of Griess reagent A, followed by 0.5 ml of Griess reagent B to 5 ml of the culture. If nitrite is present in the media, then it will react with the reagents to form a red compound. This is considered a positive result.
2.4.3.9. Hydrolysis of Esculin

Escurin is a glycoside. Hydrolysis of esculin yields esculetin and dextrose. The ability of the microorganisms to hydrolyze this glycoside can be investigated by incorporating 0.1% esculin into nutrient agar. Ferric citrate is added to the medium at a concentration of 0.05%. In the presence of an iron salt, esculetin forms a brown-black complex that diffuses into the surrounding medium. A positive reaction is shown by the development of a brownish black colour.

2.4.3.10. Sodium chloride tolerance test

Growth at 0% and 6.5% (w/v) NaCl was tested by inoculating the culture in 1% sterile tryptone broth at pH 7.3±0.3 containing the desired concentration of analytical grade NaCl and incubating at 28±0.4°C for 18-24 hours. Growth was detected visually by observing turbidity.

2.4.4. Characterization of the isolates to species level

Identification of the isolates to species level was done according to Aerokey II (Carnahan et al., 1991; Appendix 1).

2.4.5. Study of Extracellular virulence factors
2.4.5.1. Detection of protease activity

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 h. Zone of clearance around the colonies after the plates were flooded with saturated solution of ammonium sulphate indicated that gelatin has been hydrolyzed.
**Production of Caseinase:** The test organisms were spot inoculated on skim milk agar plates and the plates incubated at 28°C for 24-48 h. Caseinase production was detected by the presence of clear zones around the test colonies.

**2.4.5.2. Production of Lipase**

Tributyrin or glycercyl tributyrate is commonly used for studying lipolytic activities. The test organisms were spot inoculated on tributyrin agar plates (nutrient agar supplemented with 1% tributyrin) and the plates were incubated at 28°C for 24-48 h. A positive result was indicated by a zone of clearance around the colonies of lipolytic organisms, where the tributyrin has been hydrolyzed (Rhodes, 1959).

**2.4.5.3. Production of DNase**

A plate test for the demonstration of bacterial decomposition of nucleic acid was performed. The test organisms were spot inoculated on DNA agar plates (0.2% DNA) and the plates were incubated at 28°C for 24-48 h. 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.

**2.4.5.4. β– 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 β-haemolytic activity was recorded as clear zone around the colonies after incubation at 37°C for 24 h.
2.4.6. Statistical Analysis

Statistical analysis of data was performed by Chi-Square test. Difference was considered significant when \( p < 0.05 \). The software programme PRIMER v 6 (Plymouth Routines in Multivariate Ecological Research, version 6.1.9) was also used for analysis of data.

2.4.7. Diversity indices

The diversity indices like Shannon-Wiener index (\( H' \)), Pielou’s evenness index (\( J \)), Simpson’s index (\( D \)) and Margalef species richness index (\( d \)) were calculated using the PRIMER 6 Statistical software.

2.4.8. k-dominance curve

Diversity profiles are also presented using k-dominance curves (Lambshead et al., 1983). The purpose of this distributional representation is to extract information on patterns of relative species abundance and dominance. The curves presented are cumulative ranked abundance plotted against species rank (logged axis). Here, the percentage dominance of the organisms was plotted against their rank individually and cumulatively. In the present study, k-dominance plots were constructed using statistical software PRIMER 6.

2.5. Results

2.5.1. Prevalence of motile aeromonads in different fish samples

Motile aeromonads were isolated from 74% of Poecilia sphenops and 68% of P. reticulata samples collected (Figure 2.1).
2.5.2. Prevalence of motile aeromonads in different body parts of fish samples

Table 2.1 shows the prevalence of motile aeromonads in different body parts such as body surface, gill and intestine of *P. sphenops* and *P. reticulata*. Prevalence of motile aeromonads in the body surface of *P. sphenops* was high compared to gill and intestine, while the prevalence was more or less equal on various body parts of *P. reticulata*.

<table>
<thead>
<tr>
<th>Body parts</th>
<th><em>P. sphenops</em> (%)</th>
<th><em>P. reticulata</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body surface</td>
<td>45.31</td>
<td>33.33</td>
</tr>
<tr>
<td>Gill</td>
<td>34.37</td>
<td>30.86</td>
</tr>
<tr>
<td>Intestine</td>
<td>20.31</td>
<td>35.80</td>
</tr>
</tbody>
</table>

2.5.3. Distribution of different species of motile aeromonads in fish samples

Overall distribution of different species of motile *Aeromonas* in ornamental fish samples is given in Figure 2.2.
Figure 2.2. Overall distribution of different species of motile aeromonads in fish samples

One hundred and forty five isolates were characterized to species level. *Aeromonas sobria* was the predominant species (29%) followed by *A. jandaei* (20%) and *A. hydrophila* (16%). Distribution of other species was less than 10%.

2.5.3.1. Distribution of different species of motile aeromonads in *Poecilia sphenops* and *P. reticulata*

Distribution of different species of motile aeromonads in *Poecilia sphenops* and *P. reticulata* is given in Figure 2.3 and 2.4 respectively.

Figure 2.3. Distribution of different species of motile aeromonads in *Poecilia sphenops*
Distribution pattern of various species of motile aeromonads were similar in both the fishes. *A. sobria* was the predominant motile aeromonad in both *P. sphenops* and *P. reticulata* followed by *A. jandaei* and *A. hydrophila*.

2.5.3.2. Relative prevalence of different species of motile aeromonads in *Poecilia sphenops* and *P. reticulata*

Figure 2.5 represents relative prevalence of different species of motile aeromonads in *Poecilia sphenops* and *P. reticulata*.
No significant difference ($p>0.05$) was observed in the prevalence of different species of motile *Aeromonas* in the fishes. Prevalence of *A. jandaei* and *A. hydrophila* was relatively higher in *P. sphenops*.

2.5.4. Distribution of various species of motile aeromonads in different parts of the body of fresh water ornamental fishes

Figure 2.6 gives a graphical representation of the distribution of different species of motile aeromonads in the body parts of *Poecilia sphenops*. Distribution of different species was found to be high in the body surface except in the case of *A. veronii*, where it was found to be high in the gill. *A. hydrophila* was equally distributed in the body surface and intestine.

![Figure 2.6. Distribution of different species of motile aeromonads in various body parts of Poecilia sphenops](image)

Figure 2.7 gives a graphical representation of the distribution of different species of motile aeromonads in the body parts of *P. reticulata*. Occurrence of *A. schubertii* was found to be high in the body surface where as the occurrence of other motile aeromonads was found to be relatively high in the intestine.
Prevalence, distribution and extracellular virulence factors of motile aeromonads in fresh water ….

**Figure 2.7.** Distribution of different species of motile aeromonads in various body parts of *P. reticulata*

### 2.5.5. Diversity indices

Table 2.2 shows the diversity indices namely the Shannon-Wiener Diversity index (H'), Margalef richness index (d), Pielou’s evenness index (J') and Simpson dominance index (D) of various body parts of *P. sphenops* and *P. reticulata*. Species diversity is the relative abundance of different species at each site of time reduced to a single index. Margalef richness index is the indicator of species richness in a specified location or time. Here the species richness index was lower in the intestine of *P. sphenops*, where Shannon diversity index was also lower, which means that as the species richness is lower, diversity is also lower.

**Table 2.2.** Diversity indices of various body parts of the two different fishes

<table>
<thead>
<tr>
<th>Diversity indices</th>
<th><em>P. sphenops</em></th>
<th><em>P. reticulata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body surface</td>
<td>Gill</td>
</tr>
<tr>
<td>Richness (d)</td>
<td>2.29</td>
<td>2.43</td>
</tr>
<tr>
<td>Evenness (J')</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>Diversity (H')</td>
<td>1.89</td>
<td>1.89</td>
</tr>
<tr>
<td>Dominance (D)</td>
<td>0.91</td>
<td>0.91</td>
</tr>
</tbody>
</table>
2.5.6. k- dominance plot

The k-dominance visually represented the species abundance, richness and species evenness (Figure 2.8). Species evenness is derived from the slope of the line that fits the graph. A steep gradient indicates low evenness. A shallow gradient indicates high evenness as the abundances of different species are similar. The intestine of *P. sphenops* represented a less diverse and balanced distribution of species.

![K dominance curve](image)

**Figure 2.8.** k dominance curve of motile aeromonads in various body parts of *P. sphenops* and *P. reticulata*

1-body surface, 2-gill, 3-intestine of *P. sphenops* and 4-body surface, 5-gill, 6-intestine of *P. reticulata*

2.5.7. Physico-chemical characteristics of water samples

Physico-chemical characteristics of water samples are given in Table 2.3 and were found to be within the permissible range for ornamental fish culture.
Table 2.3. Physico-chemical characteristics of water samples

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>28.5</td>
<td>28-29</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>6.6-8</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/L)</td>
<td>7.17</td>
<td>3.4-9.36</td>
</tr>
<tr>
<td>Total ammonia nitrogen (mg/L)</td>
<td>0.19</td>
<td>0.022-0.418</td>
</tr>
<tr>
<td>Nitrite (mg/L)</td>
<td>0.0158</td>
<td>0.0013-0.05</td>
</tr>
<tr>
<td>Nitrate (mg/L)</td>
<td>0.1926</td>
<td>0.02-0.462</td>
</tr>
</tbody>
</table>

2.5.8. Prevalence and distribution of different species of motile aeromonads in water samples

Motile aeromonads were isolated from 68% of the water samples collected. One hundred and fifty six isolates from the samples were characterized to species level. The distribution of *Aeromonas* spp. in water samples is given in Figure 2.9. As observed in the case of fish samples, *A. sobria* was the predominant species in water samples also (34.61%) followed by *A. trota* (23.71%).

![Distribution of different species of motile aeromonads in water samples](image)

2.5.9. Extracellular virulence factors of motile aeromonads in fish samples

Many of the motile aeromonads were capable of producing various extracellular virulence factors (Plate 2.2). Gelatinase and DNase production was
detected in all the isolates. Lipase was produced by 96.55% of the isolates. β-haemolytic activity was detected in 92.79% of the isolates. Caseinase was detected in 90.34% of the isolates. Prevalence of the production of extracellular virulence factors by motile aeromonads from fish samples is given in Figure 2.10.

Plate 2.2. Extracellular virulence factors produced by *Aeromonas*
Figure 2.10. Distribution of extracellular virulence factors in *Aeromonas* isolates from fish samples

2.5.10. Production of extracellular virulence factors in different species of motile aeromonads from fish samples

The production of extracellular virulence factors by different species of *Aeromonas* is shown in Table 2.4. Gelatinase and DNase were produced by members of all the species identified. All the isolates of *Aeromonas hydrophila* and *A. sobria* exhibited caseinase production, while 90% of *A. veronii* produced caseinase. Lipase production was more frequent in the isolates tested. All the isolates of *A. hydrophila*, *A. jandaei*, *A. sobria* and *A. veronii* produced lipase and all the isolates of *A. hydrophila* and *A. sobria* were β-haemolytic. More than 90% of *A. jandaei*, *A. schubertii* and *A. veronii* exhibited β-haemolytic activity.
Table 2.4. Production of extracellular virulence factors in different species of *Aeromonas* from fish samples

| *Aeromonas* spp. | Percentage of motile aeromonads producing extracellular virulence factors |  |  |  |  |
|------------------+-------------------------------------------------+-----------------+-----------------+-----------------+-----------------|
|                  | gelatinase | caseinase | lipase | DNase | β-haemolysin |
| *A. caviae*      | 100        | 75        | 91.66  | 100   | 66.66         |
| *A. hydrophila*  | 100        | 100       | 100    | 100   | 100           |
| *A. jandaei*     | 100        | 86.20     | 100    | 100   | 96.55         |
| *A. schubertii*  | 100        | 71.42     | 78.57  | 100   | 92.85         |
| *A. sobria*      | 100        | 100       | 100    | 100   | 100           |
| *A. trota*       | 100        | 84.61     | 92.30  | 100   | 84.61         |
| *A. veronii*     | 100        | 90.90     | 100    | 100   | 90.90         |

2.5.10.1. Production of proteases by motile aeromonads from fish samples

Gelatinase and casienase are the two enzymes screened for the protease activity. All the isolates of *Aeromonas* spp. obtained from fish samples exhibited gelatinase activity (Figure 2.11).

![Figure 2.11. Production of gelatinase by different *Aeromonas* spp.](image)

Production of caseinase by different species of *Aeromonas* is given in Figure 2.12. All the isolates of *A. sobria* and *A. hydrophila* and 90% of *A. veronii* exhibited caseinase production.
2.5.10.2. Production of Lipase in isolates from fish samples

Lipase was produced by all the isolates of *A. hydrophila*, *A. jandaei*, *A. sobria* and *A. veronii*, while 90% of *A. caviae* and *A. trota* produced the enzyme. Production of lipase by different species of *Aeromonas* is given in Figure 2.13.

![Figure 2.13. Production of lipase by different Aeromonas spp.](image)

2.5.10.3. Production of DNase in isolates from fish samples

All the isolates of *Aeromonas* spp. obtained from fish samples exhibited DNase activity and the results are given in Figure 2.14.

![Figure 2.14. Production of DNase by different Aeromonas spp.](image)
2.5.10.4. Production of Haemolysin in isolates from fish samples

All the isolates of *A. hydrophila* and *A. sobria* and about 90% of *A. jandaei*, *A. schubertii* and *A. veronii* exhibited β-haemolysin production. β-haemolysin production was infrequent in *A. caviae* (66%). Production of haemolysin by different species of *Aeromonas* is given in Figure 2.15.

![Figure 2.14. Production of DNase by different Aeromonas spp.](image)

**Figure 2.14.** Production of DNase by different *Aeromonas* spp.

![Figure 2.15. Production of haemolysin by different Aeromonas spp.](image)

**Figure 2.15.** Production of haemolysin by different *Aeromonas* spp.
2.5.11. Extracellular virulence factors of motile aeromonads in water samples

Production of extracellular virulence factors of *Aeromonas* isolates in water samples is given in Figure 2.16. Gelatinase and DNase production was detected in all the isolates. Lipase was produced by 94.23% of the isolates. Haemolytic activity was detected in 91.02% of the isolates. Caseinase was elaborated by 85.89% of the isolates.

![Figure 2.16. Distribution of extracellular virulence factors in *Aeromonas* isolates](image)

2.5.12. Production of extracellular virulence factors in different species of motile aeromonads from water samples

The production of extracellular virulence factors by different species of *Aeromonas* is shown in Table 2.5. Gelatinase and DNase were produced by all the isolates of different species of *Aeromonas*. Ability to produce caseinase was relatively lower. All the isolates of *A. hydrophila* and *A. veronii* and 98% of *A. sobria* produced lipase. β-haemolysin was produced by all the isolates of *A. hydrophila* and *A. sobria*.
Table 2.5. Production of extracellular virulence factors in different species of *Aeromonas* from water samples

<table>
<thead>
<tr>
<th><em>Aeromonas</em> spp.</th>
<th>Percentage of motile aeromonads producing extracellular virulence factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gelatinase</td>
</tr>
<tr>
<td><em>A. caviae</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. hydrophila</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. jandaei</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. schubertii</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. sobria</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. trota</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. veronii</em></td>
<td>100</td>
</tr>
</tbody>
</table>

2.5.12.1. Production of proteases by motile aeromonads from water samples

Gelatinase and casienase were the two enzymes screened for the detection of protease activity. All the isolates of *Aeromonas* spp. obtained from water samples exhibited gelatinase activity (Figure 2.17).

![Figure 2.17. Production of gelatinase by different *Aeromonas* spp. from water samples](image)

Production of caseinase by different species of *Aeromonas* is given in Figure 2.18. More than 90% of *A. hydrophila* and *A. sobria* exhibited caseinase production.
2.5.12.2. Production of Lipase in isolates from water samples

Lipase was produced by all the isolates of *A. hydrophila* and *A. veronii* and 98.14% of *A. sobria*. More than 90% of *A. jandaei* and *A. trota* also produced this enzyme. Production of lipase by different species of *Aeromonas* is given in Figure 2.19.
2.5.12.3. Production of DNase in isolates from water samples

All the isolates of *Aeromonas* spp. obtained from water samples exhibited DNase activity (Figure 2.20).

![Figure 2.20. Production of DNase by different *Aeromonas* spp. from water samples](image)

2.5.12.4. Production of Haemolysin in isolates from water samples

All the isolates of *Aeromonas hydrophila* and *A. sobria* exhibited β-haemolysin production. Production of haemolysin by different species of *Aeromonas* is given in Figure 2.21.

![Figure 2.21. Production of Haemolysin by different *Aeromonas* spp. from water samples](image)
2.6. Discussion
2.6.1. Prevalence and distribution of motile Aeromonas in fish samples

Aeromonas are the most common bacteria in the aquatic environment and have been recognized as opportunistic pathogens of cultured and wild fishes throughout the world (Abulhamd, 2010). The fish may succumb to these bacteria when they are exposed to stressful conditions prevailing in intensive culture systems of Southeast Asian countries. It is the etiological agent for motile aeromonad septicaemia (MAS) in fish (Turska-Szewczuk et al., 2013; Yadav et al., 2014). Aeromonas has also been frequently isolated from the lesions of epizootic ulcerative syndrome (EUS) fishes (Torres et al., 1990; Roberts et al., 1990; Rahman et al., 2002; Nam and Joh, 2007; Hossian et al., 2011; Kumar and Ramulu, 2013). This disease is a serious threat to the freshwater fish production of Southeast Asian countries. A. hydrophila infection in fishes has been reported to occur from time to time in Asian countries including China, Philippines, Thailand and India (Ebanks et al., 2004). Occurrence of potential pathogenic Aeromonas species in tropical seafood and aquafarms off Cochin coast in South India is also reported (Joseph et al., 2013).

In the present study, motile Aeromonas were isolated from 74% of Poecilia sphenops and 68% of P. reticulata samples tested. Similar to the present observation, Erdem et al. (2010) reported 65% of fresh water fish samples from Turkey to be positive for Aeromonas spp. Kumar and Ramulu (2013) reported around 55% of Pangasius hypophthalmus in culture ponds of Kaikalur and Mudinepalli mandals of Andhra Pradesh in India to be positive for Aeromonas.
Aeromonas sobria was the predominant species isolated from *P. sphenops* and *P. reticulata* in the present study followed by *A. jandaei*. Nearly 16% of the isolates were *A. hydrophila*. Other motile aeromonads encountered were *A. caviae, A. trota, A. veronii* and *A. schubertii* each of which contributed less than 10%. In a study conducted by Suhet *et al.* (2011) on fish and water samples, eight different species of *Aeromonas* were isolated which included *A. hydrophila, A. caviae, A. sobria, A. veronii, A. jandaei, A. salmonicida, A. eucrenophila* and *A. schubertii*. Similar to the observations in the present study, Onuk *et al.* (2013) reported predominance of *A. sobria* in trout samples from Turkey. The frequencies of the identified *Aeromonas* species from water and rainbow trout samples from Turkish coastal regions reported by them were 38.33% for *A. sobria*, 23.33% for *A. hydrophila*, and 10% for *A. veronii*. The dominant strain isolated from diseased fish samples in a trout farm in the Republic of Korea was also *A. sobria* (Nam and Joh, 2007). *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). It also has resulted in mass mortality in fishes in China (Li and Cai, 2011) and has been identified as a causative agent of disease in farmed perch *Perca fluviatilis* L. in Switzerland (Wahli *et al.*, 2005). Similar to the present observation, a high prevalence of *A. jandaei* in the fresh water fish samples tested is reported Suhet *et al.* (2011). Sreedharan *et al.* (2012) reported a predominance of *A. jandaei* (38.3%) in fish samples from ornamental fish culture systems in Kerala.

Hossian *et al.* (2011) have isolated *A. hydrophila, A. sobria* and *A. schubertii* from different fish samples such as Silver carp, Glass barb, Rohu, Mrigal *etc.* in Bangladesh. Ye *et al.* (2013) isolated *A. hydrophila* from
haemorrhagic diseased freshwater fishes in China. A haemorrhagic disease due to *A. hydrophila* infections in aquaculture causing huge economic losses was already reported in China (Maiti *et al*., 2009; Beaz-Hidalgo *et al*., 2010). Shayo *et al*. (2012) reported that *A. hydrophila* and other motile aeromonads constitute an important causative agent of bacterial ulcerative diseases in Tanzania. Erdem *et al*. (2010) reported *A. hydrophila* to be the dominant species in edible Carp followed by *A. caviae* and *A. veronii* bv. *sobria*.

The prevalence of *Aeromonas veronii* encountered in both *Poecilia sphenops* and *P. reticulata* was found to be <10%. *A. veronii* has been isolated from Oscar *Astronotus ocellatus* showing signs of infectious dropsy in India (Sreedharan *et al*., 2011) and from Catfishes in China (Cai *et al*., 2012). The prevalence of *A. caviae* was also found to be <10%. Ashiru *et al*. (2011) reported *A. caviae* to be the predominant species in Tilapia fish while *A. hydrophila* and *A. sobria* were predominant in Catfish and there was complete absence of *A. hydrophila* in Tilapia fish.

In the present study, high incidence of *Aeromonas* was observed on the body surface of *Poecilia sphenops* which was similar to the findings of Erdem *et al*. (2010) who also observed highest *Aeromonas* incidence on the skin of fish samples tested. High incidence of *A. jandaei* and *A. schubertii* on the body surface of fish samples is in agreement with the observation of Suhet *et al*. (2011). *A. jandaei* was also isolated by Hirsch *et al*. (2006), on the body surface of Nile tilapia in Alto Rio Grande, Minas Gerais.

On the contrary, in *Poecilia reticulata*, there was an almost equal distribution of motile aeromonads in body surface, gill and intestine. Kumar and Ramulu (2013) also isolated *Aeromonas* from different organs such as
skin, liver and kidney of *Pangasius hypophthalmus* in culture ponds of India. They found *A. hydrophila* to be the dominant species in fish samples. In a previous study Hatha *et al.* (2005) also reported *A. hydrophila* to be the predominant species in the intestine of farm-raised fresh water fish followed by *A. caviae* and *A. sobria*. K dominance curve showed that the intestine of *P. sphenops* represented a less diverse and balanced distribution of species. Except for the report of Sreedharan *et al.* (2011 and 2012), most other reports are about isolation of motile aeromonads from farm-raised edible fish and the mortality caused by them. Our results highlight considerable prevalence of motile aeromonads in ornamental fishes, which might pose threat to emerging ornamental fish industry in the study area.

### 2.6.2. Prevalence and distribution of motile *Aeromonas* in water samples

Motile *Aeromonas* species are ubiquitous bacteria in aquatic environment. 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 since *Aeromonas* spp. can cause infections and epizootics in several species of animals (Pandove *et al.*, 2013).

The prevalence of different species of *Aeromonas* is likely to vary with geographical locations (Sinha *et al.*, 2004) and with pollution in the aquatic environment (Imziln, 2001). While *A. sobria* can be found in unpolluted waters, *A. caviae* predominates in waters with a high degree of faecal pollution. In less polluted waters, *A. caviae* and *A. hydrophila* were almost equally distributed. Large numbers of aeromonads, especially *A. caviae*, could
therefore be considered to be indicative of nutrient-rich conditions of water (Abulhamd, 2009).

In the present study, in terms of prevalence and abundance in water samples, the most predominant species was found to be *Aeromonas sobria* (34.61%) followed by *A. trota* (23.71%) which is indicative of relatively good water quality in the study area. Similar to the observation in the present study, Nam and Joh (2007) reported *A. sobria* to be the dominant spp. in water samples collected from trout farms in all seasons, in the Republic of Korea. On the contrary, a high prevalence of *A. caviae* in water has also been reported (Evangelista-Barreto et al., 2010). Dumontet (2000) reported *A. caviae* to be the predominant sp. compared to *A. sobria* in the coastal waters of Southern Italy which was submitted to high fecal pollution. In a study conducted in Turkey, Koksal et al. (2007) reported the prevalence of *Aeromonas* as *A. hydrophila* (46%), *A. sobria* (34%), *A. caviae* (8%), *A. veronii* (3%) and *A. jandaei* (3%).

It is difficult to compare the level of *Aeromonas* incidence published by different authors because of the obvious differences in methods used in sampling period, geographical location, the origin of the samples and methodology for analysis (Aberoum and Jooyandeh, 2010). However, the present data clearly confirm the widespread distribution of motile *Aeromonas* in the aquatic environment. Less number of *A. caviae* in the water samples tested reflects the unpolluted water used in the farm. Physico-chemical characteristics of water samples analysed showed that all the parameters tested were within the permissible range for ornamental fish culture and not causing any stress to the fishes being stocked.
2.6.3. Distribution of extracellular virulence factors in motile Aeromonas from fish samples

Several authors have reported the isolation of different species of Aeromonas potentially pathogenic to tropical fishes such as Aeromonas hydrophila (Kozinska, 2007), A. sobria (Rahman et al., 2002), A. veronii (Kozinska, 2007; Orozova et al., 2009) and A. jandaei (Santos et al., 1999) from apparently healthy samples. Potentially pathogenic Aeromonas species are present in diseased as well as healthy fish. The pathogenesis of Aeromonas infections is multifactorial, as aeromonads produce a wide variety of virulence factors. The expression of two or more virulence-associated factors can be considered as pathogenicity indicators (Serrano et al., 2002; Kozinska, 2007). Several virulence factors have been studied in Aeromonas including aerolysin/haemolysin, 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; Serrano et al., 2002; Rahman, 2002; Kozinska, 2007).

Widespread proteolytic and nuclease activity was encountered among the motile aeromonads isolated in the present study. Castro-Escarpulli et al. (2003) observed comparable levels of extracellular virulence factors among the motile aeromonads from frozen fish samples in Mexico. 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 epizootic ulcerative syndrome (EUS) had caseinolytic and gelatinolytic
activity. Erdem et al. (2010) suggested that proteases, more than haemolysin, may be important virulence factors in Aeromonas infections, because majority of the isolates obtained in their study produced protease; all the isolates of A. hydrophila and A. veronii bv. sobria and 81.8% of A. caviae were producers of protease. These factors were considered as pathogenicity markers by Kozinska (2007). There are reports that strains of the high virulent group A. hydrophila were also powerful producers of the enzyme protease and that their culture filtrate caused haemorrhages and mortalities in Carp and partially purified enzyme revealed lethal characteristics (Shome et al., 2005; Jayavignesh et al., 2011). 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.

In the present study, all the isolates of A. hydrophila and A. sobria from fish samples were haemolysin producers and only 66% of A. caviae were haemolytic. Nearly 95% of A. jandaei and 90% of A. veronii were haemolysin producers. These results are substantiated by the findings of Farag (2006) who showed a significant difference between the haemolysin production of A. hydrophila and A. veronii bv. sobria as compared with that of A. caviae. Yucel and Citak (2003) reported A. hydrophila and A. sobria to be stronger producers of haemolysin, and A. caviae strains to be non haemolytic. Erdem et al. (2010) reported that nearly 90% of A. veronii bv. sobria and A. hydrophila from edible fish samples were beta-haemolytic on sheep blood agar plates, while none of the A. caviae strains were haemolytic. These findings are also in agreement with the results recorded by Janda et al. (1984), who showed similar levels of beta-haemolytic among Aeromonas strains such as A. veronii bv. sobria and A. hydrophila. Orozova et al. (2010) reported 90% of
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*Aeromonas* isolates from fish samples to be haemolytic. Our findings revealed considerably higher levels of beta-haemolytic activity among *A. caviae* from ornamental fishes such as *P. sphenops* and *P. reticulata*.

Hatha *et al.* (2005) reported all the isolates of *A. hydrophila*, 77.8% of *A. caviae* and 50% of *A. sobria* isolated from farm-raised edible fresh water fishes in South India to be β haemolytic. Equal distribution of α and β-haemolytic activity among the *A. hydrophila* isolates from fish samples in India, was reported earlier (Illanchezian *et al.*, 2010). The haemolytic activity is also reported to be strongly associated with enterotoxin production in members of the genus *Aeromonas* (Burke *et al.*, 1983). The high rate of haemolytic activity detected in *Aeromonas* spp. is remarkable. The whole process of pathogenesis is a complex interaction among the host, pathogens and environmental determinants.

2.6.4. Distribution of extracellular virulence factors in motile *Aeromonas* from water samples

All the isolates encountered in the present study from the water samples were producers of gelatinase and nuclease. Production of caseinase and lipase has also been identified in strains isolated from water. The results suggest potentially virulent nature of the motile aeromonads from the farm water samples. The extracellular virulence factors among *Aeromonas* spp. isolated from Bhavani river South India was studied by Bagyalakshmi *et al.* (2009). Similar to the observation in the present study, they also have observed nuclease production by all the isolates and gelatinase production by 94% of the isolates. Caseinase and lipase have also been produced by many of the isolates. Several of these virulence factors have also been identified in strains
Prevalence, distribution and extracellular virulence factors of motile aeromonads in fresh water….isolated from water by Sechi et al. (2003). Considerable differences between the number, types and quantities of proteases produced by aeromonads have been reported and attributed to own strain variation, origin, incubation temperature or culture media (Cahill, 1990; Mateos et al., 1993; Santos et al., 1996).

β-haemolysin was produced by all the isolates of *A. hydrophila* and *A. sobria* and 90% of the isolates of *A. jandaei*. Nearly 85% of *A. schubertii*, *A. trota* and *A. veronii* and 68% of *A. caviae* were haemolysin producers. β-haemolytic activity among all the isolates of *A. hydrophila* and *A. sobria* and α haemolytic activity among all the isolates of *A. caviae* from water sample are reported earlier (Gibotti et al., 2000). Similar to the observation in the present study, high haemolytic activity for *A. jandaei* is already reported by Suhet et al. (2011), who observed *A. jandaei* as the species with the highest haemolytic activity (100%), followed by *A. veronii* (90%), *A. hydrophila* (74%) and *A. sobria* (50%) in water samples. Our results are also in agreement with the observations of Monfort and Baleux (1990) who have reported haemolysin production in 100% of the strains of *A. hydrophila* and *A. sobria* isolated from brackish water samples of a sewage treatment lake in the South of France.

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, and therefore it seems necessary to continue surveying the distribution of known virulence determinants in currently circulating *Aeromonas* strains.
Our results revealed relatively good water quality in the fresh water ponds of the farm which is reflected in the distribution pattern of motile aeromonads in the water. However, the prevalence of various virulence factors was considerably high, which offer them an advantage to turn as pathogens once there is deterioration of water quality and results in stress on the ornamental fishes maintained in these farms. The results also highlight the possibility of developing small scale ornamental fish farm which could be maintained without considerable mortality and crop loss.