1.1. Ornamental fishes and its trade

Enchanting beauty of nature is brought into our living room or office space in the form of aquaria. The gentle sound of flowing water and the graceful movement of fishes in aquaria brings peaceful ambience to our home. It relaxes our mind and pleases our eyes. They are an utterly stylish complement to our drawing room. Aquarium keeping or keeping colourful and fancy fishes known as ornamental fishes is one of the oldest and most popular hobbies in the world.
In India, the practice of ornamental fish keeping started in 1951 with the opening of the Taraporevale Aquarium at Mumbai and the establishment of several aquarium societies in the city. Since then the practice has become widespread in India, with more than hundred varieties of indigenous species and even more of exotic ones (Natarajan et al., 2009).

Ornamental fishes or “live jewels” are among the most popular and fastest growing categories of pets. They form an important commercial component of aquaculture providing for aesthetic requirements and upkeep of the environment. Their beauty, vibrant colours, entertaining behaviour and their unique charms make them the most in demand. The growing interest in aquarium fishes has resulted in a steady increase in aquarium fish trade globally. The global scope of the ornamental fish trade and growing popularity of pet fishes are strong indicators of the myriad economic and social benefits the pet industry provides.

Over one billion ornamental fishes 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).

Aquaculture is an emerging industrial sector which requires continued research with scientific and technical developments, and innovation. 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. The low production cost and higher returns within a very short time, growing demand for fishes both from domestic and international market are the major attractions of this sector when compared to any other sector. Unlike seasonal work in agriculture, earning a regular income from this provides further motivation (Shaleesha and Stanley, 2000). Our country has a rich and unique biodiversity with a variety of indigenous ornamental fishes. The Western Ghats of India is a gold mine of tropical ornamental fishes and it is one of the 25 "hotspot" areas of the world. It exhibits exceptional mega biodiversity and high degree of endemism with respect to fresh water fishes. Altogether 210 primary fishes (excluding the marine migrants) are found in the inland waters, of which 53 species are endemic. Majority of these fish species have ornamental value also.

Establishment of an ornamental fish culture industry would enable Indian producers to win market share, both locally and internationally. Indian aquaculture has demonstrated a six and half fold growth over the last two decades, with freshwater aquaculture contributing over 95 percent of the total aquaculture production. Aquaculture in India, in general, is practised with the utilisation of low to moderate levels of inputs, especially organic-based fertilizers and feed. India utilises only about 40 percent of the available 2.36 million hectares of ponds and tanks for freshwater aquaculture and hence there is room for both horizontal and vertical expansion of this sector (http://www.fao.org.).

Kerala occupies one of the foremost positions in the aquatic biodiversity. The long coastline and the extensive inland waters of the State have brought people belonging to different cultural groups in contact with fishing. The State
is endowed with total area of about 2,26,274 hectares of fresh water resources consisting of rivers, fresh water lakes, reservoirs, minor irrigation tanks, ponds etc. Of these, about 1,30,000 hectares area is ideally suited for fresh water fish culture (Harikumar and Rajendran, 2007). In Kerala, 21 out of 44 rivers surveyed in 2005, 142 species were reported, out of which 51% are considered as possible ornamentals (http://www.fisheries.kerala.gov.in). Considering the substantial contribution aquaculture makes towards socio-economic development in terms of income and employment through the use of unutilised and underutilised resources, eco-friendly aquaculture has been accepted as a vehicle for rural development.

Ornamental fish trade is growing at rapid pace in Kerala, with many small scale investors involved in breeding, rearing and marketing of ornamental fishes. The State has immense potential for developing the ornamental fish industry as it is bestowed with several small and large natural fresh water bodies. Utilization of a small share of these water bodies for the development of the ornamental fish industry would bring in economic growth to the State.

In order to make ornamental fisheries an export oriented industry, Matsyafed, and Marine Products Export Development Authority (MPEDA) are providing assistance for ornamental fish breeding and export. The ornamental fish trade is promoted by the State Government by organizing International aqua shows and seminars on biannual basis ensuring participation of scientists, administrators, breeders, traders and entrepreneurs even from foreign countries. A major derivative of this initiative was formation of aqua technology park-KAVIL (Kerala Aqua Ventures International Ltd) by the Government of Kerala with public private
participation which provides a unique platform for the investment and trade opportunities (http://www.fisheries.kerala.gov.in).

1.2. Fish diseases-a scourge on ornamental fish industry

Fish diseases are among the most important problems and challenges confronting commercial aquaculture. It is a major risk factor in ornamental fish industry, with millions of dollars lost annually (Citarasu et al., 2011; Al-Maleky and Haneff, 2013). A variety of microbial agents (viruses, bacteria, fungi, parasites etc.) can cause diseases in aquaculture system. Bacteria constitute the most economically significant group of pathogenic agents. Bacterial diseases are responsible for heavy mortality in both cultured and wild fishes throughout the world. The majority of bacterial infections are caused by Gram-negative organisms belonging to the genera: *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium* (*Flexibacter*), *Mycobacterium*, *Pseudomonas* and *Vibrio*. Among the Gram-positive bacteria, *Streptococcus* has been shown to cause disease in ornamental fishes. In Asian countries fish culture continues to be ravaged by bacterial diseases such as Motile Aeromonad Septicaemia (MAS), Furunculosis and Edwardsiellosis. Bacterial organisms may be the primary cause of disease, or they may be secondary invaders, taking advantage of a breach in the fish's integument or compromise of its immune system. The majority of bacterial fish pathogens are natural inhabitants of the aquatic environment, whether it is freshwater or marine.

1.3. Motile aeromonads as fish pathogens

Infections caused by members of the bacterial genus *Aeromonas*, with a relatively high antibiotic resistance, are among the most common and
troublesome diseases of fish (Kalyankar et al., 2013; Okolie and Chenia, 2013; Saad et al., 2014). *Aeromonas* cause an assortment of diseases in fish, including MAS, haemorrhagic septicaemia, fin rot, soft tissue rot and red sore resulting in major die-offs and fish kills around the globe (Joseph et al., 2013; Yadav et al., 2014). It is one of the most common bacterial diseases diagnosed in marine and cultured freshwater fish (Pandey et al., 2010; Kozińska and Pękala, 2012). Other pathologic conditions attributed to members of the motile aeromonad complex may include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, haemorrhagic septicaemia, red sore disease, red rot disease and scale protrusion disease. In the acute form of disease, a fatal septicaemia may occur so rapidly that fish die before they have time to develop anything but a few gross signs of disease. When clinical signs of infection are present, affected fish may show exophthalmia, reddening of the skin and an accumulation of fluid in the scale pockets. The abdomen may become distended as a result of an oedema and the scales may bristle out from the skin to give a “washboard” appearance. *Aeromonas* bacteria causing these infections are called aeromonads.

Many species of *Aeromonas* have been implicated in fish disease, including *A. hydrophila*, *A. veronii*, *A. sobria*, *A. schubertii*, *A. allosaccharophila* and *A. salmonicida* which cause haemorrhagic septicaemia, red sore disease and ulcerative infections in fishes (Turska-Szewczuk et al., 2013).

### 1.4. Motile aeromonads—a public health risk

*Aeromonas*, once considered mainly an opportunistic pathogen in immunocompromised humans, is now implicated as the etiologic agent involving immunocompetent individuals of all age groups. They are
recognized as etiological agents of a wide spectrum of diseases in man and animals (Kumar et al., 2012; Ghenghesh et al., 2013). The literature has indicated that some motile Aeromonas spp. are emerging food and water-borne pathogens of increasing importance (Al-Maleky and Haneff, 2013). These organisms have been associated with several food-borne outbreaks. Exposure to Aeromonas spp. through ingestion of food and water is constant, and case reports suggest that susceptible individuals may acquire gastrointestinal illness from chronic exposure to high numbers of aeromonads.

The ability of these microorganisms to grow well at refrigeration temperatures (Janda and Abbott, 2010) could be important in their role as food-poisoning agents. They are increasingly being isolated from patients with traveler's diarrhoea (Sarkar et al., 2013). Associations of aeromonads with human disease were reported very early by Von Graevenitz and Mensch (1968) providing evidence for their recognition as human pathogens and suggesting that some aeromonads may be associated with gastrointestinal disease. Aeromonas spp. is isolated most frequently from fecal specimens from children under five years of age, while isolation of aeromonads from other body sites typically occurred in adult populations. They have been recognized as agents of both intestinal (gastroenteritis, child diarrhea, traveler’s diarrhea, dysentery) and life-threatening extraintestinal infections (septicaemia, wound infections, urinary tract infections and occasionally, meningitidis and peritonitis). In many non-intestinal infections, the organism gains entrance from contaminated water through wounds (Afizi et al., 2013). A. hydrophila, A. veronii, A. sobria, A. jandaei, A. schubertii and A. caviae are most commonly implicated in human infections (Turska-Szewczuk et al., 2013). The presence of enteropathogenic Aeromonas spp. in potable and
domestic water supplies and their ability to withstand killing by chlorination in biofilms and to many antibiotics could cause serious clinical threats (Sen and Rogers, 2004).

1.5. *Aeromonas hydrophila*-most notorious *Aeromonas* sp.

*Aeromonas hydrophila* is the most widespread pathogen and it can be easily spread through accidental abrasions. MAS caused by *A. hydrophila*, is one of the most common and challenging diseases in freshwater fishes causing heavy mortality (Das *et al.*, 2011; Ye *et al.*, 2013; Hoque, 2014). It has been recognized to be the aetiological agent of several distinct pathological conditions including tail/fin rot, MAS or haemorrhagic septicaemia, as a primary pathogen or in association with other pathogens, like *Aphanomyces* spp. causing epizootic ulcerative syndrome (EUS) (Kumar and Ramulu, 2013). EUS is a globally distributed disease and has become an epidemic affecting a wide variety of wild and cultured fish species, especially in Southeast Asia including Pakistan and India. Many strains of *A. hydrophila* exacerbate diseases such as spring viraemia of Carp, *Saprolegnia declina* infections, Myxobacterial and other protozoan infections (Harikrishnan and Balasundaram, 2005).

Fish infected with *A. hydrophila* may have many different symptoms. These range from sudden death in otherwise healthy fish to lack of appetite, swimming abnormalities, pale gills, bloated appearance and skin ulcerations. This bacterium causes haemorrhagic septicaemia in fishes, characterized by presence of small superficial lesion, focal haemorrhages, particularly in the gills and opercula, ulcers, abscesses, exophthalmia and abdominal distension (Aruvassu *et al.*, 2013).
Most cultured and feral fishes such as Brown trout (*Salmo trutta*), Rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*), Ayu (*Plecoglossus altivelis*), Carp (*Cyprinus carpio*), Japanese eel (*Anguilla japonica*), American eel (*Anguilla rostrata*), Gizzard shad (*Dorosoma cepedianum*), Goldfish (*Carassius auratus*), Golden shiner (*Notemigonus crysoleucas*), Snakehead fish (*Ophiocephalus striatus*) and Tilapia (*Tilapia nilotica*) are susceptible to *A. hydrophila* infection. In Southeast Asia, fish kills due to *A. hydrophila* contribute a substantial economic loss to the fish farming industry (Harikrishnan and Balasundaram, 2005; Citarasu *et al*., 2011). The bacterial haemorrhagic septicaemia disease outbreak due to *Aeromonas hydrophila* in a fresh water Carp pond in south India was reported by Ahamad *et al.* (2013).

*Aeromonas hydrophila* has increasingly been implicated as a virulent and antibiotic resistant etiologic agent in various human diseases (Al-Fatlawy and Al-Ammar, 2013; Grim *et al*., 2013). It is commonly involved in causing human infections such as septicaemia, bacterial endocarditis, gastroenteritis, cellulitis, wound sepsis with necrosis, gangrene, localized infections of eyes and throat as well as pneumonia and traveler’s diarrhea. Diseases can be caused by ingestion of contaminated fishes, fish foods and drinking water, or direct contact with recreational waters.

Isolation of *A. hydrophila* from water and food sources, and the increasing resistance of this organism to antibiotics and chlorination in water, presents a significant threat to public health (Venkataiah *et al*., 2013). *A. hydrophila* has been placed on the US Environmental Protection Agency’s Contaminant Candidate List of emerging pathogens in drinking water (Pandove *et al*., 2013).
1.6. Distribution and characteristics of *Aeromonas*

Worldwide studies have demonstrated that *Aeromonas* spp. are universally distributed and widely isolated from clinical, environmental and animal sources, food samples including fresh grocery produce, seafood, raw meats, packaged ready-to-eat meats, cheese, milk and from aquatic environment. In aquatic environment, they are found in ground water, surface water, estuarine environment, sewage effluents, lakes and rivers (Galindo and Chopra, 2007), well water (Mansour *et al.*, 2014) and tap water (Pablos *et al.*, 2009; Kivanc *et al.*, 2011). The prevalence of *Aeromonas* species in the aquatic environment has been recognized as a potential health risk and some countries have adopted aeromonad counts as an additional indicator of water quality (Pandove *et al.*, 2013). They can also be found in soil. The wide diversity of aeromonads’ habitat can clearly be seen by the recently isolated strain of *A. caviae* from an explored sulfur spring in Orissa, India (Patra *et al.*, 2007). In developing countries, *Aeromonas* spp. appears to be common in milk and milk products including pasteurized milk (Ghenghesh *et al.*, 2008). While *Aeromonas* spp. is not considered faecal bacteria, they are present in the faeces of healthy animals and humans. Motile aeromonads were isolated from the intestine of poultry (Yehia, 2013), and canines (Prashant *et al.*, 2013). *Aeromonas* spp. were detected even in cockroaches and houseflies. Since cockroaches and houseflies are clearly very mobile, it seems quite likely that they can carry *Aeromonas* sp. from hospitals into neighboring communities, and *vice versa*. In addition, isolation of *A. culicicola* from the midgut of mosquitos is also reported (Pidiyar *et al.*, 2004).

Members of the genus *Aeromonas* are Gram-negative non spore-forming straight rods, which occur singly, in pairs or in short chains. They are oxidase
and catalase positive, glucose-fermenting, facultative anaerobic and resistant to vibriostatic agent O/129. Most of the mesophilic species within this genus are motile and have a single polar flagellum. Swarming motility with the production of lateral flagella has also been described (Kirov et al., 2002; Andrade et al., 2006). Although strains of *A. salmonicida* are capable of producing lateral flagella, they are non-motile. This is thought to be a result of inactivation of the laf A (flagellin gene) by transposase 8 (IS3 family) (Al-Maleky and Haneff, 2013).

The aeromonads can grow at a temperature ranging from 5 to 44°C. The optimum temperature for growth is 22-28°C. Most isolates of clinical significance will grow readily at 37°C. The pH range for growth is 5.5-9.0. Growth is inhibited in 6.5% salt broth.

1.7. Taxonomy and molecular identification of *Aeromonas*

Over the years from 1890’s to present, significant changes in the nomenclature of genus *Aeromonas* have taken place. Although *Aeromonas* was initially positioned in the family *Vibrionaceae*, successive phylogenetic analyses point out that the genus *Aeromonas* is not closely related to vibrios resulting in the relocation of *Aeromonas* from the family *Vibrionaceae* to a new family, the *Aeromonadaceae*. The genera of the family *Aeromonadaceae* now include *Oceanimonas*, *Aeromonas*, *Tolumonas* (incertaesedis) and *Oceanisphaera*.

Taxonomy of the genus *Aeromonas* is heavily debated, as species distinction is often difficult to achieve (Martino *et al.*, 2013). The number of proposed *Aeromonas* species has shown a logarithmic increase over the past
two decades. The genus comprises the following species: *Aeromonas hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (biovars *sobria* and *veronii*), *A. jandaei*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. popoffii* and the two DNA homology groups (HG), *Aeromonas* sp. HG11 (now included in *A. encheleia*) and *Aeromonas* sp. HG13 (formerly Enteric Group 501) that has been named as *A. diversa*. The species *A. enteropelogenes* and *A. ichthiosmia* are now considered to be synonyms of *A. trota* and *A. veronii*, respectively. Eleven new species, *A. culicicola*, *A. simiae*, *A. molluscorum*, *A. bivalvium*, *A. aquariorum*, *A. tecta*, *A. piscicola*, *A. fluvialis*, *A. taiwanensis*, *A. sanarellii*, and *A. rivuli* have been described. Two additional potential new species, “*A. cavernicola*” and “*A. lusitana*”, have also been recognized (Martinez-Murcia *et al.*, 2011).

Conventional identification scheme based on biochemical characteristics may often lead to misidentification due to lack of uniformity of some biochemical characteristics and atypical reactions. Although other conventional identification techniques such as serotyping, phage typing and whole-cell protein electrophoresis have been used, a lack of sensitivity to identify species exactly was also been reported (Onuk *et al.*, 2013). For the identification of bacteria, the speed and precision of molecular approaches are attractive to many investigators, and such approaches can be used as either complements or alternatives to biochemical identification.

The first attempts to identify aeromonads to genotype relied upon differences in 16S ribosomal DNA sequences. Phylogenetic analyses based on 16S rRNA genes indicated that aeromonads are a very complex group of
species (Martinez-Murcia \textit{et al.}, 1992). In almost all species of the genus, rDNA-derived relationships correlated well with DNA–DNA hybridization. DNA probes and RFLP profiles designed from 16S rDNA diagnostic regions have served to identify \textit{Aeromonas} at the species level (Figueras \textit{et al.}, 2000). However, there are reported discrepancies between different sets of DNA–DNA hybridization data and the fact that 16S rRNA is highly conserved brings the latest descriptions of some species into question (Yanez \textit{et al.}, 2003). The study of two or more housekeeping genes could be useful to improve the reliability of the phylogenies. It has been reported that \textit{gyrB} (encoding the B-subunit of DNA gyrase, a type II DNA topoisomerase) and \textit{rpoD} (encoding $\sigma^{70}$ factor which is one of the sigma factors that confer promoter-specific transcription initiation on RNA polymerase) could be suitable phylogenetic markers for bacterial systematics. They seem to be good index genes for determining the course of genome evolution because they are essential single-copy genes on which horizontal gene transfer seldom occurs (Soler \textit{et al.}, 2004).

The sequence analysis of the polymerase chain reaction amplicons of the \textit{gyrB} gene was viewed as a better phylogenetic chronometer than the 16S ribosomal gene. Yanez \textit{et al.} (2003) have documented that the \textit{gyrB} gene agree with the 16S ribosomal data which led to placement of the genus \textit{Aeromonas} in the family \textit{Aeromonadaceae}, and \textit{gyrB} gene sequences were useful in resolving discrepancies between 16S ribosomal gene sequences and DNA-DNA hybridization results.

Molecular identification, albeit currently in vogue as a means of bacterial identification, has limited applications in the microbiology laboratory with regards to \textit{Aeromonas}. This is principally due to the low frequencies of
human *Aeromonas* infections reported in the United States and other industrialized nations, limited data suggesting a need for definitive identification past the complex level, and no significant correlation between species and concentration in the gastrointestinal tract and the disease state. Molecular identifications, however, are still useful under certain circumstances. These circumstances include definitive identification of isolates with aberrant biochemical properties, for cases of recurrent disease (e.g., biliary), in the description of new disease settings or resistance patterns associated with aeromonads, for public health surveillance activities, and for publication purposes (Janda and Abbott, 2010).

### 1.8. Virulence and pathogenicity

Virulence provides a quantitative measure of the pathogenicity of an organism or the likelihood of causing disease. Virulence factors refer to the factors (i.e., gene products) that enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease. These factors enable a pathogen to achieve the following: colonisation of a niche in the host (this includes adhesion to cells), immuno evasion (evasion of the host's immune response), immunosuppression (inhibition of the host's immune response) and obtain nutrition from the host. Factors contributing to virulence include toxins, proteases, haemolysins, lipases, adhesins, agglutinins, and various hydrolytic enzymes. Virulence factors are present in two forms, cell-associated structures, and extracellular products. Among the cell-associated structures are pili, flagella, outer membrane proteins, lipopolysaccharide and capsules. The major extracellular products include cytotoxic, cytolytic, haemolytic and enterotoxic proteins.
Aeromonas species is said to be pathogenic because it possesses all the requirements of pathogenic bacteria. It attaches and enters host cells through the production of flagella, pili and adhesions. Multiplication in host tissue is assisted by the production of siderophores and outer membrane proteins, while production of capsule, S-layer, lipopolysaccharide and porins contribute to their resistance to host defense mechanisms. Enterotoxins, proteases, phospholipases and haemolysins cause damage to host cells leading to cell death. Haemolysins are exotoxins and the lytic activities on red blood cells are reported to be important for nutrient acquisition or for causing anaemia. The occurrence of both Type II and Type III secretion systems has been demonstrated, including the presence of several virulence factors such as enolase. Several extracellular products are elaborated, including cytotoxic and cytotoxic enterotoxins, haemolysins and various hydrolytic enzymes. Aeromonas spp. produces a wide variety of extracellular hydrolytic enzymes such as arylamidases, amylase, deoxyribonuclease, esterases, peptidases, elastase, chitinase and lipase, some of which are thought to contribute to pathogenesis.

Proteases cause tissue damage, enhanced invasiveness and provision of nutrients. The lipases are considered important for bacterial nutrition and have been found to damage the host plasma membrane. DNase 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.

The pathogenic potential of A. hydrophila has been related to several virulence factors including the cytotoxic enterotoxin Act (Chopra et al., 1999),
which has haemolytic, cytotoxic and enterotoxic activities; a variety of proteases (Khajanchi et al., 2010; Hu et al., 2012); cytotoxic enterotoxins Ast and Alt (Sha et al., 2002); type 3 secretion systems (T3SSs) (Yu et al., 2004); and motility factors such as lateral and polar flagella (Sen and Lye, 2007). The important incidence of motile Aeromonas spp. with virulence potential, from water and fish samples is reported by Yadav et al. (2014).

Identification of virulence factors by molecular methods such as PCR is an approach for determination of potentially pathogenic Aeromonas spp. isolates. Although numerous virulence factors contribute to pathogenesis of fish and human diseases caused by Aeromonas, none of them alone can be responsible for all symptoms of disease stages. Because of the complexity in pathogenesis of Aeromonas spp., actually due to its multifactorial nature, identification of multiple virulence factors of this genus become important.

1.9. Use of antibiotics in aquaculture and emergence of antibiotic resistance

Heavy economic loss brought by bacterial infections is a major problem faced by the ornamental fish industry. Infections are treated with antibiotics, but there has been growing concern about the overuse of antibiotics in the ornamental fish industry and its possible effect on the increasing drug resistance in bacteria associated with these fishes (Rose et al., 2013). The success of antibiotic treatment depends on a number of factors: sensitivity of the bacteria involved to the antibiotic chosen, proper dosage and treatment intervals being used and other contributing stress factors involved in infection. Officially there are no Food and Drug Administration (FDA) approved
antibiotics for treating ornamental fish (Yanong, 2006), but they are often used off-label (Akinbowale et al., 2006).

Wide spread use of antibiotics for treating bacterial diseases and subtherapeutic use for growth promotion are held responsible for the emergence of antibiotic resistance. Farmers involved in uncontrolled administration of antibiotics for aquaculture production is reported by Olatoye and Basiru (2013). Aquaculture has been implicated as potential environment to the development and selection of resistant bacteria and a source of these pathogens to other animals and humans (Madhuri et al., 2012). It can also act as a reservoir of antimicrobial resistance genes that may eventually be transferred to clinically relevant bacteria (Miranda et al., 2013). In recent years development of drug resistant or multidrug resistant pathogens has become a key problem in India and many other countries. In India, the situation is more upsetting due to less stringent regulatory control of antibiotics with extensive use of antibiotics in animal husbandry and aquaculture (Prashant et al., 2013). In addition to the development and spread of drug resistance, the use of antibiotics in aquaculture can lead to the accumulation of residual antibiotics in aquatic environment (Cabello, 2006; Kumari et al., 2007; Hoque, 2014), accumulation in the food chain (Chen et al., 2010) and detrimental effect on the microbial biodiversity (Zhou et al., 2010).

Since Aeromonas spp. has been implicated in human and animal diseases and many members of the organisms are inherently resistant to penicillins (penicillin, ampicillin, carbenecillin and ticarcillin), they may play important role in emergence of antimicrobial resistance (Igbinosa et al., 2012).
Aeromonas isolates exhibiting resistance to different antibiotics is reported in ornamental fishes (Čížek et al., 2010; Dias et al., 2012; Rose et al., 2013) and aquaculture systems (Ashiru et al., 2011; Cai et al., 2012; Chen et al., 2012; Dias et al., 2012; Ye et al., 2013). In India, Aeromonas isolates exhibiting resistance to various antibiotics in fish samples and aquatic environment, is reported by several authors (Hatha et al., 2005; Thayumanavan et al., 2007; Kaskhedikar and Chhabra, 2010; Sreedharan et al., 2012; Joseph et al., 2013). Aeromonas spp. is reported as environmental reservoir of resistance genes to different classes of antibiotics (Igbinosa et al., 2012). These resistance genes can be transferred to other bacteria through horizontal gene transfer.

Heavy use of antibiotics in aquaculture needs to be reduced and replaced with alternative method for treating fish diseases to avoid the emergence of antibiotic resistance in pathogenic and environmental bacteria. Fish health management is a better alternative for reducing infections. It includes improved husbandry, nutrition, water quality, optimal stocking density and use of vaccines, immunostimulants and probiotics (Reneshwary et al., 2011).

**1.10. Factors causing disease outbreaks**

Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose part of the normal intestinal microflora of healthy fish. Therefore, the presence of these bacteria, by itself, is not indicative of disease and consequently, stress is often considered to be a contributing factor in outbreaks of disease caused by these bacteria. Such stressors are most commonly associated with environmental and physiological parameters that adversely affect fish under intensive culture.
General Introduction

Environmental stress factors, particularly those associated with poor water quality conditions, enhance the development of disease. These factors include high water temperatures, high ammonia and nitrite levels, pH disturbances and low dissolved oxygen levels. Heavy parasite infestation, overcrowding, high organic loads in the water, rough handling and transport also may lead to outbreaks of disease (Hoque, 2014; Sachar and Raina, 2014).

1.10.1. Common water quality related environmental stress factors

Fishes have a very intimate relationship with their surrounding aquatic environment, performing all their bodily functions in water and therefore poor water quality kills more number of fishes than does infectious agents. Adverse environmental conditions may acutely or chronically stress the fish, hampering their metabolic machinery and affecting the physiological status thus suppressing their innate and adaptive immune responses (Sachar and Raina, 2014). All fish species have optimal environmental requirements and when they are cultured under these conditions maximum growth rates are achieved with reduced disease susceptibility.

1.10.1.1. Temperature

Aquatic animals take on the temperature of their environment and are intolerant of rapid temperature fluctuations. Temperature tolerances of fish are broadly categorized into cold water, cool water, warm water and tropical water. For each species, there is a minimum and maximum tolerance limit, as well as an optimal temperature range for growth. This optimal temperature range may vary with each species, and with each development stage of the fish. Fish generally experience stress and disease breakout when temperature is chronically near their maximum tolerance or fluctuates suddenly.
Chapter 1

1.10.1.2. pH

Water pH plays a crucial role in metabolism, maintenance of homeostasis and physiological well being of aquatic animals (Wood et al., 1989; Parra and Baldisserotho 2007). Extreme increase and decrease in pH value in an aquatic medium are reported to cause disturbance in acid–base and iron regulation (Evans et al., 2005), impair fish growth and reproduction and in some cases leads to mortality (Zaniboni-Filho et al., 2009). Optimal water pH varies with species. Most aquarium fish live in water with a pH ranging from 5.5 to 8.5. Fish in freshwater aquaria generally do best with a neutral pH. Human activities such as indiscriminate dumping of domestic wastes and persistent discharge of industrial and municipal effluents into the water body have been reported to increase its acidity level (Chindah et al., 2000) while some studies observed nutrient enrichment as the reason to the reduced pH in the aquatic medium (Alwan et al., 2009). The pH can increase during algal blooms and in heavily planted ponds/aquaria due to carbon dioxide usage (Roberts and Palmeiro, 2008).

1.10.1.3. Dissolved oxygen

Oxygen is one environmental parameter that exerts a tremendous effect on growth and production through its direct effect on feed consumption and metabolism and its indirect effect on environmental conditions. Oxygen affects the solubility and availability of many nutrients. Dissolved oxygen, the volume of oxygen contained in water, is often the critical parameter in the health and well-being of livestock. Lack of dissolved oxygen can be directly harmful to culture organisms or cause a substantial increase in the level of toxic metabolites. It is therefore important to continuously maintain dissolved oxygen at optimum levels which is usually above 5 mg/L.
1.10.1.4. Ammonia

Ammonia is the primary nitrogenous waste product of fish and also originates from the decay of complex nitrogenous/protein compounds (Romano and Zeng, 2013). In aquatic environment, ammonia-N exists in two forms as NH4+ (ionized) and NH3 (non-ionized/ un-ionized), with the latter form being more toxic since it can more easily diffuse across lipid bilayers. The NH4+ to NH3 ratios are dependent on salinity, temperature and to a greater extent, pH. Ammonia is more toxic in warmer water, at higher pH and at lower salinity.

Ammonia toxicity can result from overcrowding, overfeeding, build-up of organic debris, infrequent water changes and immature/inadequate biological filtration. Ammonia toxicity is one of the most common water quality problems affecting aquarium fish and can cause acute mortality or chronic sublethal stress.

1.10.1.5. Nitrite

Nitrite is an intermediate product in the biological oxidation of ammonia to nitrate. Nitrite toxicity frequently occurs in intensive fish culture pond. Nitrite oxidises the haemoglobin in blood to methaemoglobin leading to methaemoglobinemia, which severely affects fish health. Elevated nitrite concentrations cause great problems in intensive culture of commercial fish species and ornamental fish (Kroupova et al., 2005). Factors that affect the nitrification process include pH, temperature, concentration of dissolved oxygen, number of nitrifying bacteria and the presence of inhibiting compounds. Problems with nitrite in freshwater animals stem from the fact that NO2− has an affinity for the branchial Cl− uptake mechanism, presumably...
the Cl⁻/HCO₃⁻ exchanger; thus, whenever nitrite is present in the ambient water, a part of the Cl⁻ uptake will be shifted to NO₂⁻ uptake (Jensen, 2003).

1.10.1.6. Crowding

With the rapid development in aquaculture industry, intensive fish culture practices have become more common. Fishes are often been exposed to crowding stress, being kept in large numbers in small tanks with sometimes several species in the same tank. Crowding is a common husbandry practice in aquaculture, as it is reducing the water level needed or increasing the stocking density. Stocking density is one of the factors that have a strong impact on the final results of rearing fish. Crowding, which includes several stressors has been shown to decrease growth in various fish species (Szczepkowski et al., 2011). Infections can flare up rather easily in such a crowded environment.

1.11. Use of probiotics in aquaculture

Outbreaks of diseases are being increasingly recognized as a significant constraint in aquaculture production, especially for the farming of ornamental fish. Traditional disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance and due to concerns over environmental impacts (Lin et al., 2012). Probiotics have been characterized as ecofriendly alternative measures of disease control in aquaculture (Ige 2013; Kamgar et al., 2013).

The first generally accepted definition for probiotic was proposed by Fuller (1989). He defined a probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”.

Based on the intricate relationship an aquatic organism has with the external environment when compared with that of terrestrial animals, the definition of a probiotic for aquatic environment was modified. Gatesoupe (1999) redefined probiotics for aquaculture as “Microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health”. The definition of Gatesoupe focuses on the oral delivery of the probiotics and its ability to improve the health of the host as a result of its presence in the digestive tract. Verschuere et al. (2000) suggested the definition for probiotic as a “a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment”.

The varieties of probiotic microorganisms available for use in aquaculture include both Gram-positive (Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Micrococcus, Carnobacterium, Shewanella, Bacillus) and Gram-negative bacteria (Aeromonas, Vibrio, Alteromonas, Photobacterium, Pseudomonas, Clostridium), yeasts (Debaryomyces, Saccharomyces) and unicellular algae (Tetraselmis) (Mozhdeh et al., 2010; Ige 2013; Oke et al., 2013).

The use of probiotics as dietary live microbial supplements in commercial fish culture improves the growth (Gatesoupe, 1999; Irianto and Austin, 2002; Watson et al., 2008; Al-Dohail et al., 2009; El- Nobi et al. 2009; Zhou et al. 2010; Sharma et al., 2013) and enhance the disease resistance of fishes by suppressing the pathogens or enhancing immunity (Merrifield et al.,
In the last decade there has been increasing interest in the modulation of the non-specific immune system of fish as both a treatment and prophylactic measure against disease resistance (Misra et al., 2006a; Marzouk et al., 2008) and probiotics can stimulate the non-specific immune system of fish.

Protection against many diseases including Edwardsiellosis (Chang and Liu, 2002; Taoka et al., 2006), enteric red mouth disease (Raida et al., 2003), epizootic ulcerative syndrome (Sharma et al., 2013), furunculosis (Balcazar et al., 2007), motile aeromonad septicaemia (Lin et al., 2011), lactococcosis (Kim et al., 2012) and streptococcosis (Kamgar et al., 2013) are successfully accomplished through feeding probiotics. Furthermore, treatment with probiotics leads to better protection of fish from multiple diseases. Apart from protection against bacterial pathogens, probiotics can protect against viral and protozoan infections as well (Nayak, 2010).

When looking at probiotics intended for an aquatic usage it is important to consider certain influencing factors that are fundamentally different from terrestrial based probiotics. Aquatic animals have a much closer relationship with their external environment. Potential pathogens are able to maintain themselves in the external environment (water) and proliferate independently of the host animal (Verschuere et al., 2000). These potential pathogens are taken up constantly by the animal through the processes of osmoregulation and feeding (Watson et al., 2008). Therefore, in aquaculture, the use of probiotics to improve the quality of water in which fish are cultured (usually named bioremediation or biocontrol when they act only in water) has considerable importance (Wang et al., 2008; Al-Dohail et al., 2009).
1.12. Significance of the study

Ornamental fish culture is poised for good development in the state of Kerala as it has got excellent potential to use back yard ponds and other water bodies for culture of ornamental fish. Yet, the demand of ornamental fishes in Kerala is mostly met by supplies from other states. State Govt. and MPEDA is taking proactive steps to increase the production of ornamental fishes.

Diseases and mortality occur at the vendor/farm level due to stressful maintenance conditions which induce infections due to obligate/opportunistic pathogens in the environment. *Aeromonas* infections are a serious threat to fresh water fish production, bringing enormous economic loss to ornamental fish industry (Saad *et al.*, 2014). 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 (Yadav *et al.*, 2014). Hence it is necessary to continue surveying the distribution of known virulence determinants in currently circulating *Aeromonas* strains.

There has been growing concern about the overuse of antibiotics in the ornamental fish industry and its possible effect on the increasing drug resistance in bacteria associated with these fishes (Rose *et al.*, 2013). Emergence of antibiotic resistant pathogens is a major hindrance to treat the infections in aquaculture. Commercial production systems in other states depend heavily on antibiotics to treat diseases while the small scale organic farmers are likely to depend more on maintenance of water quality to control diseases. Present study compares the extent of antibiotic resistance among motile aeromonads from the two systems of production such as commercial
and small scale farm (in Kerala), thus it indirectly identify the extent / type of antibiotics used in ornamental fish production. Monitoring antibiotic resistance among the bacteria offers a tool to indirectly look at the selection pressure.

Unfavourable environmental conditions or poor management practices in aquaculture farms or tanks can induce stress in fish. Stressed fish population becomes vulnerable to potential pathogens either from the environment or a carrier fish and ultimately succumbs to the infection. Therefore, only studies involving the characteristics of potential pathogenic microorganisms, and a better understanding of the environmental factors affecting the health and immunity of fishes, will allow the application of adequate measures to prevent and control the major diseases limiting the production of ornamental fishes. Prevention of diseases can be achieved in part by the use of probiotics which are an eco-friendly tool for boosting the immunity of host (Ige, 2013).

1.13. Objectives of the study

The presence of motile aeromonads in ornamental fishes and associated carriage water is well documented. Though aeromonads are a part of autochthonous flora of natural waters, disease outbreak occurs as a result of environmental stress on the cultured species and virulence of the pathogens. While ornamental aquaculture in many parts of the world is highly organized and practiced scientifically, it is highly unorganized in India. The culture ponds/tanks are often maintained in very poor manner and the fishes are subjected to high degree of stress during transportation from the production facility to retail vendors. The situation is no better at retail outlets, where fishes are maintained in crowded condition without proper aeration or food. All these could result in high prevalence of diseases caused by motile aeromonads.
No systematic study has been carried out to understand the prevalence of motile aeromonads in ornamental fishes and carriage water in the study area which includes an organic fish farm, located at Edavanakkad, Cochin and different retail aquarium shops in and around Cochin. Hence the present study has been taken up with the following broad objectives:

- To study the prevalence, distribution and extracellular virulence factors of motile aeromonads among the ornamental fishes and associated carriage water from an ornamental fish farm.
- To study the prevalence, distribution and extracellular virulence factors of motile aeromonads in the ornamental fishes and carriage water maintained by retail aquarists.
- To study the prevalence of antibiotic resistance among motile aeromonads from ornamental fishes and carriage water collected from farm and retail aquarium vendors.
- To confirm the identity of a representative strain of *Aeromonas hydrophila* by molecular methods and study its growth characteristics and virulence potential.
- To study the survival and immune response of *Cyprinus carpio* to challenge infection with *A. hydrophila* following exposure to different environmental stress factors.
- To study the survival and immune response of *C. carpio* to challenge infection with *A. hydrophila* following probiotic treatment.