Distribution, extracellular virulence factors and antibiogram of motile aeromonads in fresh water ornamental fishes and immune response of *Cyprinus carpio* against *Aeromonas hydrophila* infection

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By

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Certificate

This is to certify that the thesis entitled **"Distribution, extracellular virulence factors and antibiogram of motile aeromonads in fresh water ornamental fishes and immune response of *Cyprinus carpio* against *Aeromonas hydrophila* infection"** is an authentic record of research work carried out by Mrs. Nifty John under my supervision and guidance in the Department of the Marine Biology, Microbiology and Biochemistry, School of Marine sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements of the degree of Doctor of Philosophy in Microbiology of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any University or Institution.

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Declaration

I hereby declare that the thesis entitled **"Distribution, extracellular virulence factors and antibiogram of motile aeromonads in fresh water ornamental fishes and immune response of *Cyprinus carpio* against *Aeromonas hydrophila* infection"** is a genuine record of research work done by me under the supervision and guidance of Dr. A. A Mohamed Hatha, Associate Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology and no part thereof has been presented for the award of any other degree, diploma or associateship in any University or Institution earlier.

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This is to certify that all relevant corrections and modifications suggested by the audience during the presynopsis seminar and recommended by the doctoral committee of Mrs. Nifty John has been incorporated in the thesis entitled "Distribution, extracellular virulence factors and antibiogram of motile aeromonads in fresh water ornamental fishes and immune response of *Cyprinus carpio* against *Aeromonas hydrophila* infection"

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Abbreviations

%	Percentage
°C	Degree Celsius
A_{260}	Absorbance at 260 nm
ANOVA	Analysis of variance
APHA	American Public Health Association
APW	Alkaline peptone water
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Cfu/ml	Colony forming units per millilitre
DC	Differential count
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPX	Distyrene plasticizer and xylene
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agriculture Organization
g	gram
h	hour
H &E	Haematoxylin and Eosin
IP	intra peritoneal
L	Litre
LC_{50}	Lethal concentration causing 50% mortality
LD_{50}	Lethal dose causing 50% mortality
M	molar
MAR	Multiple antibiotic resistance
mg	Milligram
mg/L	Milligram per litre
min	minutes
ml	Millilitre
mM	Milli Molar

MAS	Motile aeromonad septicaemia
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
OD	Optical density
OECD	Organization for economic co-operation and development
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pm	picomoles
ppm	Parts per million
PRIMER v 6	Plymouth Routines in Multivariate Ecological Research, version 6
RBC	Red blood corpuscles
rpm	Revolutions per minute
RPS	Relative percentage survival
rRNA	Ribosomal ribonucleic acid
sec	Seconds
SAA	Starch ampicillin agar
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SPSS	Statistical Programme for Social Sciences version 13.0
TLC	Total leucocyte count
UIA-N	Un ionized ammonia-Nitrogen
w/v	Weight by volume
WBC	White blood corpuscles
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micro Molar

Chapter 1

General Introduction

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 (Arulvasu *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 (*Natemigonus 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 *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 *Aeromonas* at the species level (Figueras *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 *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 *gyrB* (encoding the B-subunit of DNA gyrase, a type II DNA topoisomerase) and *rpoD* (encoding σ^{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 *et al*., 2004).

The sequence analysis of the polymerase chain reaction amplicons of the *gyrB* gene was viewed as a better phylogenetic chronometer than the 16S ribosomal gene. Yanez *et al.* (2003) have documented that the *gyrB* gene agree with the 16S ribosomal data which led to placement of the genus *Aeromonas* in the family *Aeromonadaceae*, and *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 *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), immunoevasion (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 is said to be pathogenic because it possess all the requirements of pathogenic bacteria. It attaches and enters into 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 contributes 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 secretions systems has been demonstrated, including the presence of several virulence factors such as enolase. Several extracellular products are elaborated, including cytotoxic and cytotonic 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); cytotonic 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 sub-therapeutic 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.

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.

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 NH_4^+ (ionized) and NH_3 (non-ionized/ un-ionized), with the latter form being more toxic since it can more easily diffuse across lipid bilayers. The NH_4^+ to NH_3 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 NO_2^- has an affinity for the branchial Cl^- uptake mechanism, presumably

the Cl^-/HCO_3^- exchanger; thus, whenever nitrite is present in the ambient water, a part of the Cl^- uptake will be shifted to NO_2^- 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, Photorhodobacterium, 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*.,

2010; Nayak, 2010; El-Ezabi *et al*. 2011; Lin *et al*., 2012; Bloch *et al*., 2013). 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*., 2006 a; 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), lactococossis (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.

Chapter 2

Prevalence, distribution and extracellular virulence factors of motile aeromonads in fresh water ornamental fishes and associated carriage water, in a small-scale farm in Kerala

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. schubertii* 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 aeromomads (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. Occurance 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.

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

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.

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 acetylmethyl 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

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.

$$H_2NCO\ N\ H_2 \rightarrow 2NH_3 + CO_2$$

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

Esculin 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 glyceryl 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^{o}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^{o}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^{o}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).

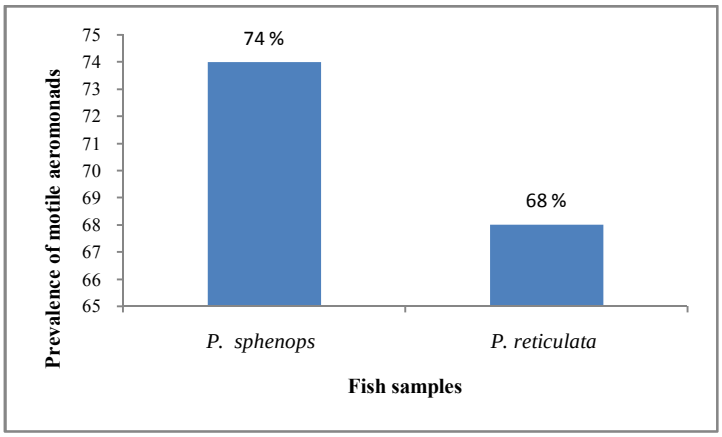


Figure 2.1. Prevalence of motile aeromonads in *P. sphenops* and *P. reticulata*

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 2.1. Prevalence of motile aeromonads in different body parts of *P. sphenops* and *P. reticulata*

Body parts	*P. sphenops* (%)	*P. reticulata* (%)
Body surface	45.31	33.33
Gill	34.37	30.86
Intestine	20.31	35.80

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.

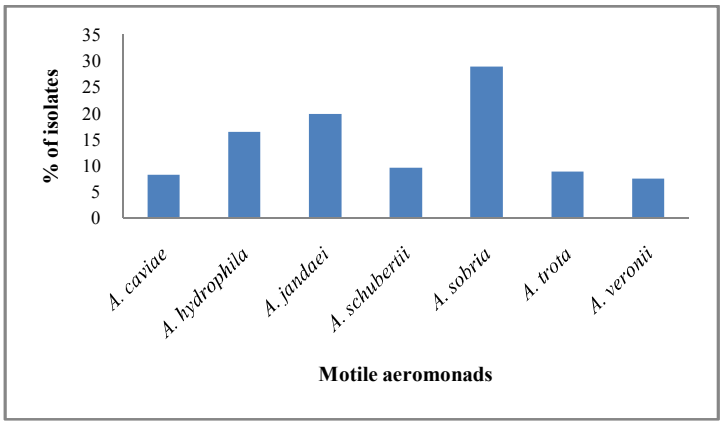


Figure 2.2. Overall distribution of different species of motile aeromonads in fish samples

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.

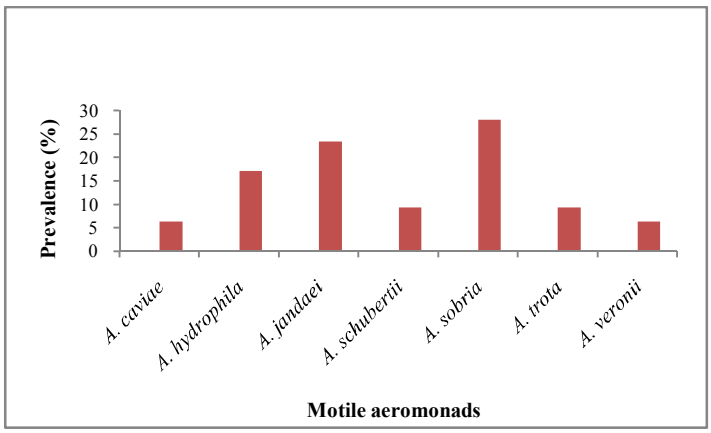


Figure 2.3. Distribution of different species of motile aeromonads in *Poecilia sphenops*

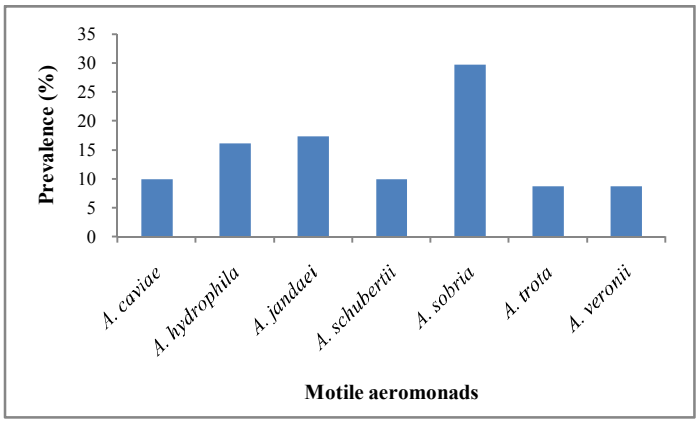


Figure 2.4. Distribution of different species of motile aeromonads in *Poecilia reticulata*

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.*

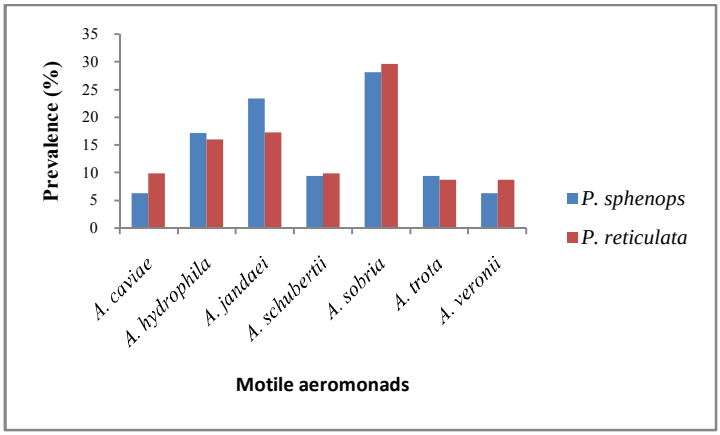


Figure 2.5. Relative prevalence of different species of motile aeromonads in *P. sphenops* and *P. reticulata*

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.

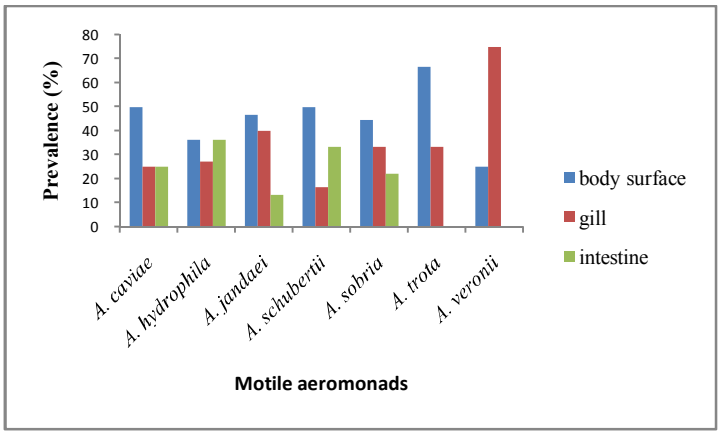


Figure 2.6. Distribution of different species of motile aeromonads in various body parts of *Poecilia sphenops*

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.

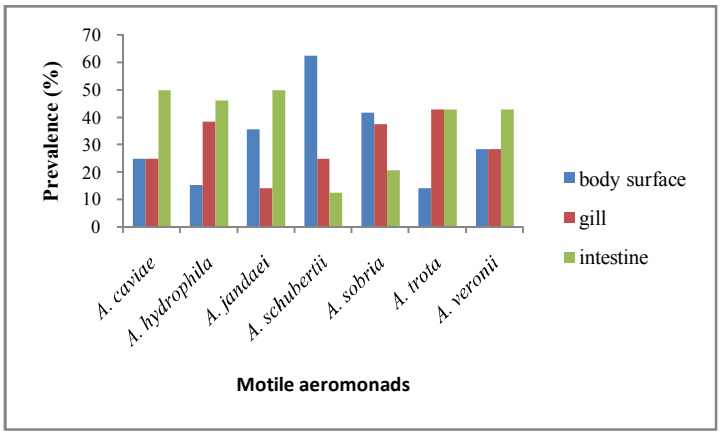


Figure 2.7. Distribution of different species of motile aeromonads in various body parts of *P. reticulata*

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

Diversity indices	*P. sphenops*			*P. reticulata*		
	Body surface	Gill	Intestine	Body surface	Gill	Intestine
Richness (d)	2.29	2.43	1.94	2.34	2.36	2.28
Evenness (J')	0.97	0.97	0.98	0.96	0.97	0.98
Diversity (H')	1.89	1.89	1.57	1.87	1.9	1.91
Dominance (D)	0.91	0.91	0.9	0.9	0.91	0.91

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.

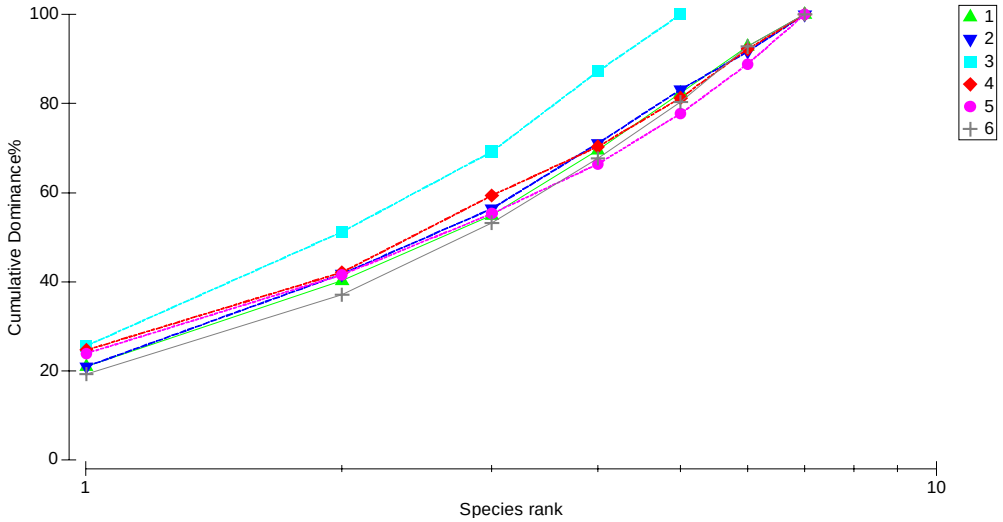


Figure 2.8. k dominance curve of motile aeromonads in various body parts of *P. sphenops* and *P. reticulata*
1-body surface, 2-gill, 3-intestine of *P. sphenops* and
4-body surface, 5-gill, 6-intestine of *P. reticulata*

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

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

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%).

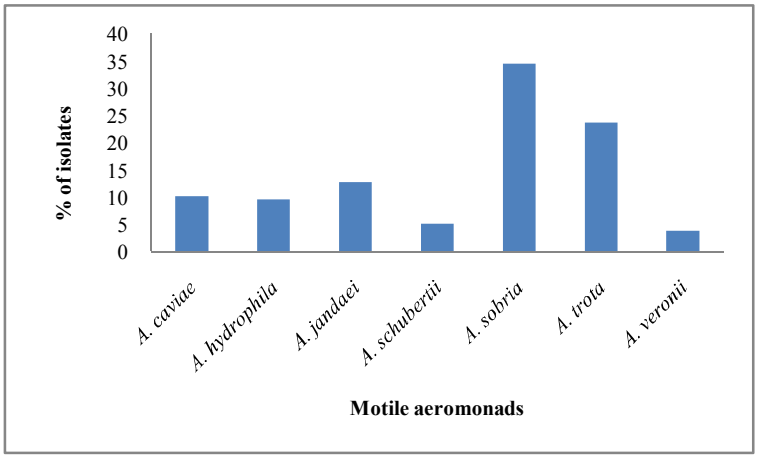


Figure 2.9. Distribution of different species of motile aeromonads in water samples

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.

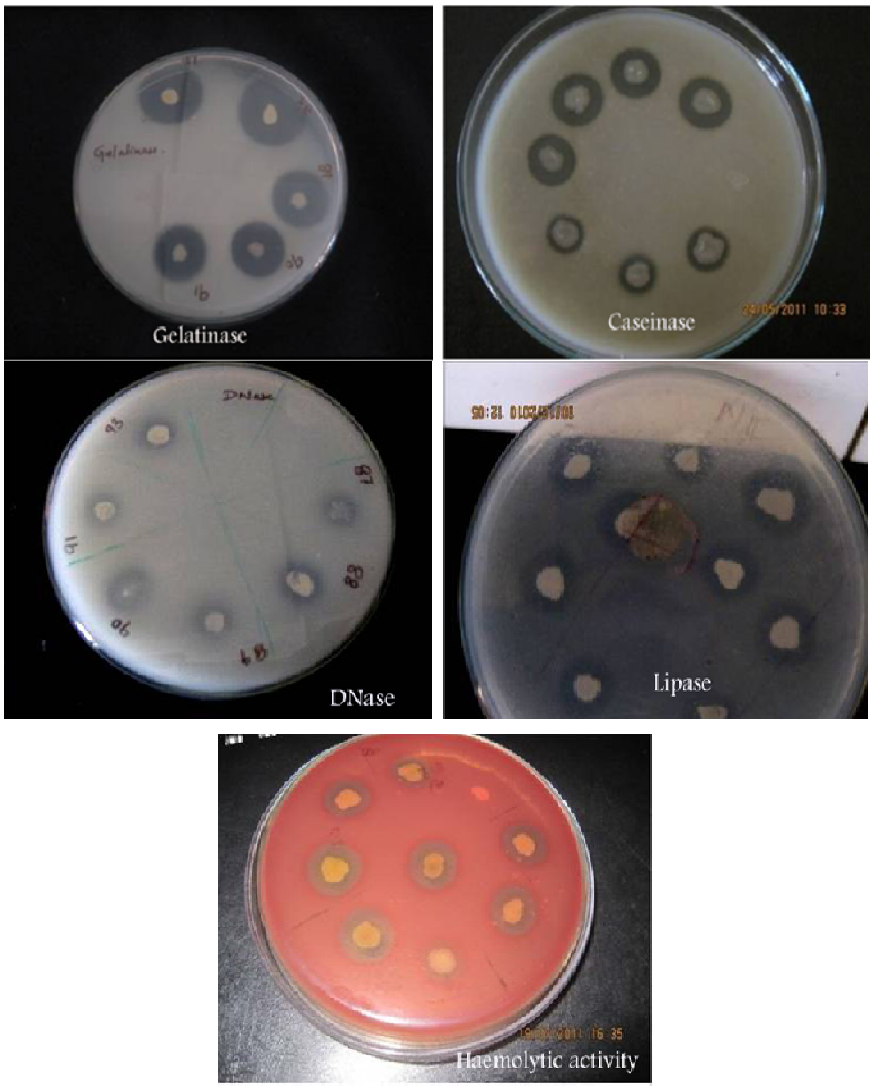


Plate 2.2. Extracellular virulence factors produced by *Aeromonas*

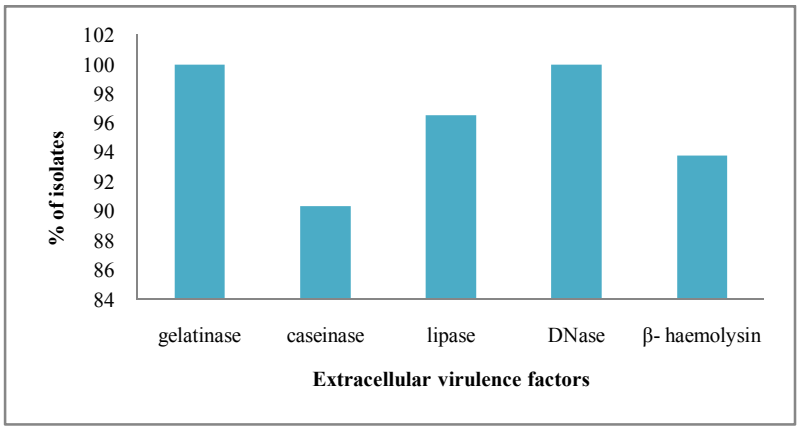


Figure 2.10. Distribution of extracellular virulence factors in *Aeromonas* isolates from fish samples

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).

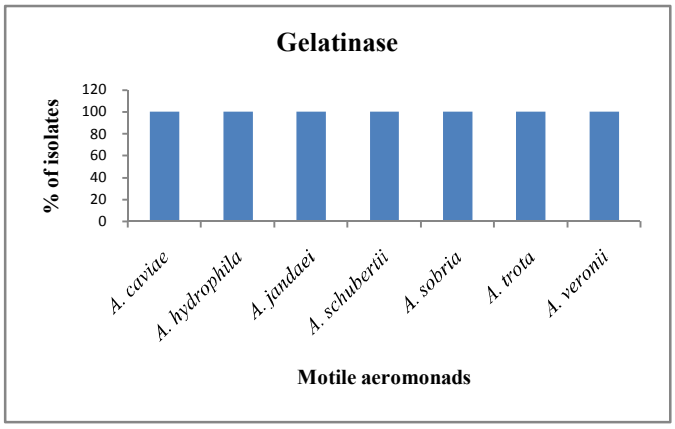


Figure 2.11. Production of gelatinase by different *Aeromonas* spp.

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.

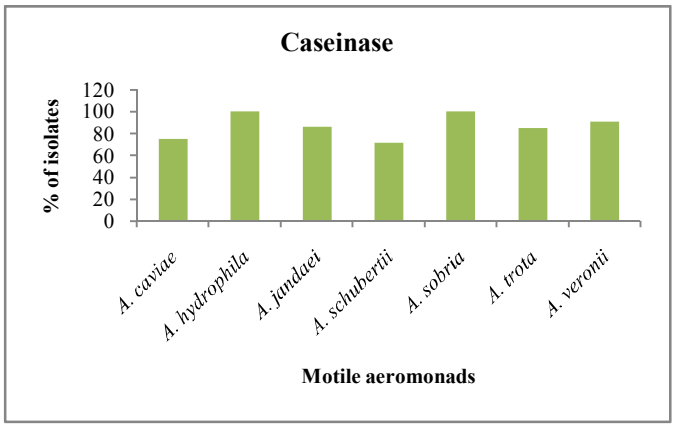


Figure 2.12. Production of caseinase by different *Aeromonas* spp.

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.

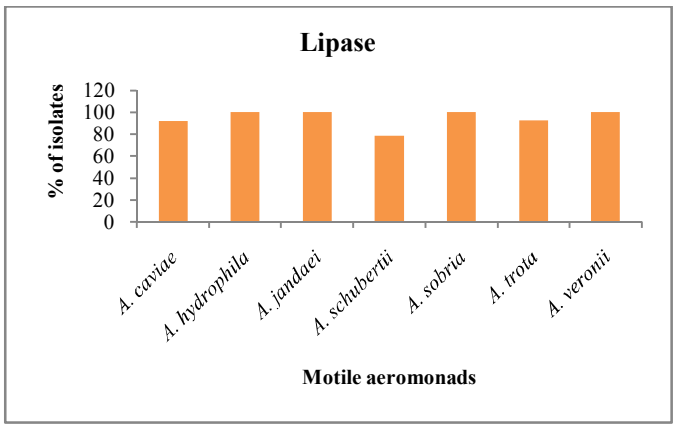


Figure 2.13. Production of lipase by different *Aeromonas* spp.

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.

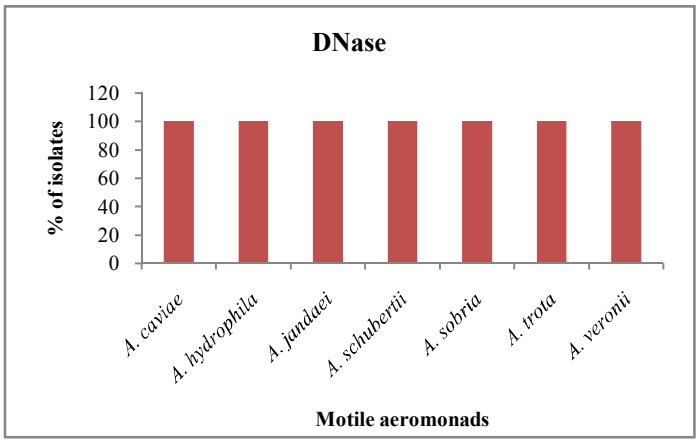


Figure 2.14. Production of DNase by different *Aeromonas* spp.

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.

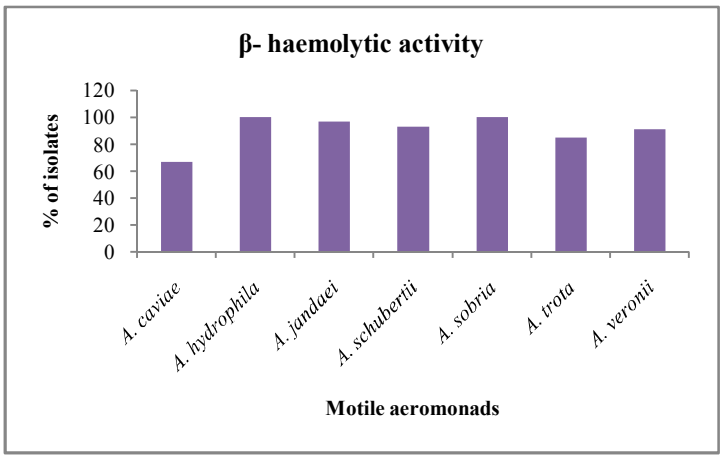


Figure 2.15. Production of haemolysin by different *Aeromonas* spp.

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.

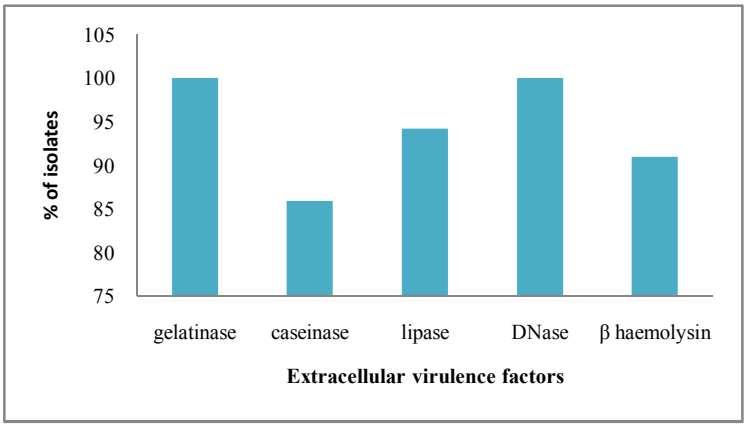


Figure 2.16. Distribution of extracellular virulence factors in *Aeromonas* isolates

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

***Aeromonas* spp.**	**Percentage of motile aeromonads producing extracellular virulence factors**				
	gelatinase	**caseinase**	**lipase**	**DNase**	**β-haemolysin**
A. caviae	100	62.5	87.5	100	68.75
A. hydrophila	100	93.33	100	100	100
A. jandaei	100	85	95	100	90
A. schubertii	100	62.5	75	100	87.5
A. sobria	100	96.29	98.14	100	100
A. trota	100	83.78	91.89	100	86.48
A. veronii	100	83.33	100	100	83.33

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).

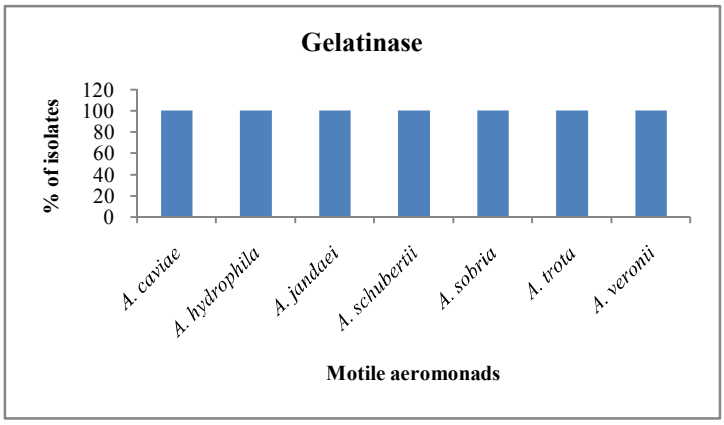


Figure 2.17. Production of gelatinase by different *Aeromonas* spp. from water samples

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.

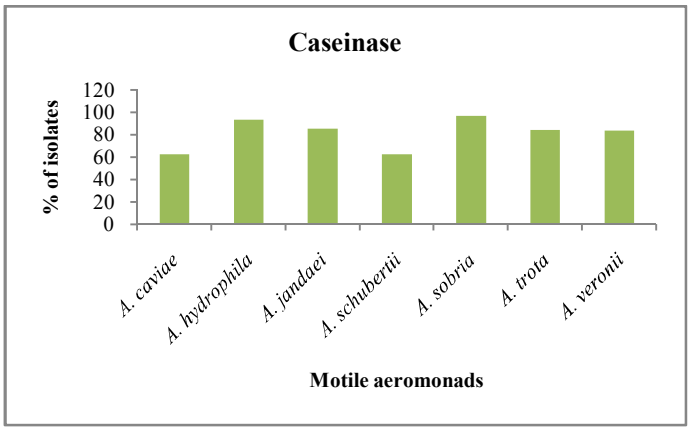


Figure 2.18. Production of caseinase by different *Aeromonas* spp**.** from water samples

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.

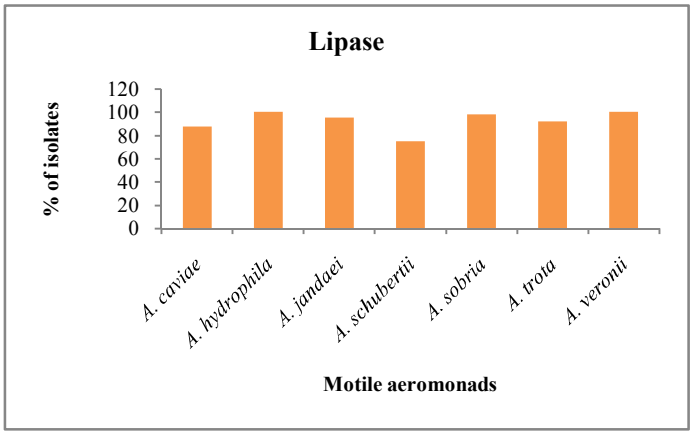


Figure 2.19. Production of lipase by different *Aeromonas* spp. from water samples

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).

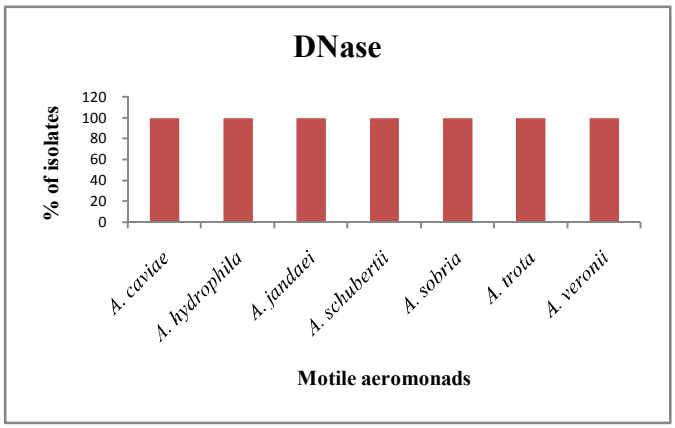


Figure 2.20. Production of DNase by different *Aeromonas* spp. from water samples

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.

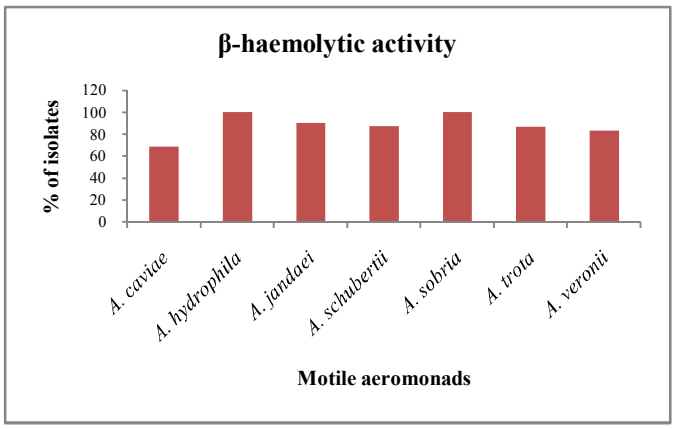


Figure 2.21. Production of Haemolysin by different *Aeromonas* spp. from water samples

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, Phillipines, 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 pathogenecity 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 extracellualr 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 syndome (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

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

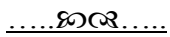
All the isolates encountered in the present study from the water samples were producers of gelatinase and nuclease. Production of caseinase and lipase has also been identified in strains isolated from water. The results suggest potentially virulent nature of the motile aeromonads from the farm water 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

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.



Chapter 3

Prevalence, distribution and extracellular virulence factors of motile aeromonads in fresh water ornamental fishes and associated carriage water in commercial retail aquaria

3.1. Introduction

Aquarium keeping is a popular hobby around the world, with about 60 million enthusiasts worldwide (Natarajan *et al*., 2009). More than 90% of live non domesticated wildlife imported to the United States was freshwater and marine ornamental fishes originating largely from Southeast Asia. On an average, 187 million live aquarium fishes were imported annually, 99% of which were intended for commercial sale in the pet industry (Smith *et al*., 2012).

Carriage and aquarium tank water associated with ornamental fishes provide primary conditions for bacterial growth and most fishes in trade require the same warm, nutrient-rich and aerated environments that favour bacterial growth. Aquarium fishes which are usually maintained at a constant

water temperature can develop *Aeromonas* infection at any time (Jayavignesh *et al*., 2011). *Aeromonas* infections are probably the most common bacterial disease diagnosed in cultured warm water fishes with disease outbreaks being increasingly recognized as a significant constraint on aquaculture production, export and trade, consequently affecting the economic development of the sector in many countries (Joseph *et al*., 2013).

Aeromonas species secretes many extracellular proteins, including amylase, chitinase, elastase, aerolysin, nuclease, gelatinase, lecithinase, lipase and protease. These proteins are known as virulence factors that cause disease in fish and humans (Nam and Joh, 2007). These virulence factors are useful in distinguishing between potentially pathogenic and non-pathogenic strains (Pandove *et al*., 2013).

3.2. Review of literature

3.2.1. Distribution of motile aeromonads

The presence of *Aeromonas* in aquaria is reported in early literature. Sanyal *et al*. (1987) have sampled a total of 100 tropical aquaria and isolated *Aeromonas* species from 98 tanks. Rose *et al*. (2013) has reported the occurrence of *Aeromonas* in ornamental fishes imported to North America from Colombia, Singapore and Florida.

Hossain (2008) isolated *A. veronii* and *A. hydrophila* from skin ulcerative symptomous Gourami (*Colisa lalia*) from an ornamental pet shop, imported to Korea from South and South-east Asian countries, especially Bangladesh, India, Thailand, Singapore and Hong Kong. Musa *et al*. (2008) reported that bacteria isolated from sick freshwater ornamental fish from aquarium shops in Malaysia were mostly *A. hydrophila* (60%). *A. hydrophila* was the dominant

species (89.66%) found as the cause of bacterial disease in ornamental fish investigated in a study by Jongjareanjai *et al*. (2009). *A. hydrophila* has been reported as the most common bacteria associated with aquatic animal disease (Barker, 2001).

Dias *et al*. (2012) isolated *Aeromonas* spp. from ornamental fishes and 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 India, Patil *et al*. (2011) isolated *Aeromonas* from the diseased blue Gourami and from the aquarium water that housed the fish. Rathore *et al.* (2005) isolated motile aeromonads from fish as well as water samples and identified different species such as *A. hydrophila*, *A. sobria, A. veronii, A. schubertii* and *A. caviae*. 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. In Kerala, Sreedharan *et al*. (2012) isolated *A. caviae* (33.3%), *A. jandaei* (38.3%) and *A. veronii* biovar *sobria* (28.3%) from ornamental fish and associated carriage water samples.

3.2.2. Extracellular virulence factors in motile aeromonads

A number of putative virulence factors (aerolysin/haemolysin, proteases, lipases, DNases) have been described in several species of the genus *Aeromonas* (Soler *et al*., 2002; Castro-Escarpulli *et al*., 2003). These virulence factors are used as survival means, self defense mechanism and establishment of pathogenicity (Odeyemi *et al*., 2012). They may play an important role in the development of disease, either in humans or in fish.

Proteases contribute to host tissue invasion by digesting or destroying cell membranes and by degrading host surface molecules. The nuclease gene is known to be associated with the development of pathogenicity (Nam and Joh, 2007). Lipases have been considered to be important for bacterial nutrition and also play role as virulence factors by interacting with human leukocytes or by affecting several immune system functions through free fatty acids generated by lipolytic activity. Extracellular lipases secreted by *Aeromonas* spp. actively participate in the alteration of the host plasma membrane and thus increase the severity of infection (Onuk *et al*., 2013).

Among the *Aeromonas* isolates obtained from fresh water fish samples, protease activity was found in all the isolates of *A. hydrophila* and *A. veronii* bv. *sobria*, but in 81% of *A. caviae* isolates (Erdem *et al*., 2010). Illanchezian *et al*. (2010) observed that 98% of *A. hydrophila* isolated from seafood samples in India, exhibited protease activity. Among the *Aeromonas* isolates obtained from fish samples, Castro-Escarpulli *et al*. (2003) observed that all the isolates exhibited lipase and DNase activity; 96% exhibited gelatinase activity and 61% caseinase activity. All the *A. hydrophila* isolates from acute abdominal dropsy and ulcerative disease in Indian major carps exhibited gelatinase activity, where as only 66.65% exhibited caseinase activity (Shome *et al*., 1999). Vadivelu *et al*. (1995) stated that protease from bacteria was required to cause disease in host cell as 94 % of tested *Aeromonas* were positive for protease production *in vitro*.

The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads. Beta haemolysin has been reported as a virulence factor in motile aeromonads (Erdem *et al*., 2010).

Haemolytic activity of *Aeromonas* spp. from various origins was studied by Orozova *et al*. (2010). The strains have been isolated from drinking water and fish samples in Bulgaria, as well as from intensively bred Trout fish in France, Sweden, Denmark, Spain, Scotland and England. They have observed that 60% of isolates from water and 90% of isolates from fish samples possessed haemolytic activity. Among the *Aeromonas* isolates obtained from fresh water fish samples, strains identified as *A. hydrophila* and *A. veronii* bv. *sobria* were the stronger producers of haemolysin, whereas *A. caviae* strains were non haemolytic (Erdem *et al*., 2010). Illanchezian *et al*. (2010) observed that 86% of *A. hydrophila* isolates from seafood samples in India was haemolytic. Notermans *et al*. (1986) have determined that 93% of *A. hydrophila* and 63% *A. sobria* isolates from drinking water possessed haemolytic activity.

3.3. Objectives of the study

Evidence from the literature suggest ornamental fishes and aquarium tank water harbouring them, is an understudied source for microbial communities and pathogens that pose potential risks to the pet industry, fishes in trade, humans and other species (Smith *et al*., 2012). Aquarium water has been suggested as a source of aeromonads resulting in gastrointestinal infection and many opportunistic infections of wounds (Huddleston *et al*., 2006). The wide distribution of *Aeromonas* in water environments and its pathogenesis led several species of *Aeromonas* already identified to be considered as a public health risk (Tokajian and Hashwa, 2004). Hence, it is important to study the prevalence of *Aeromonas* and the distribution of different species. Also the study of potential virulence factors associated with them is important.

Very often, ornamental fishes are kept in crowded conditions at retail vendor level, being kept in large numbers in small tanks with sometimes several species in the same tank. This is highly stressful to the fishes, suppressing their immunity and making them more prone to diseases by opportunistic and obligate pathogens. *Aeromonas* are a group of bacteria capable of taking advantage of a breach in fish's immune system and causing infection in them, bringing heavy economic loss to the sector. Hence the study has been taken up with the following specific objectives:

- To study the prevalence of motile aeromonads among the ornamental fishes maintained by retail aquarium vendors.
- To study the prevalence of motile aeromonads in the associated carriage water.
- 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.

3.4. Material and Methods

3.4.1. Sample collection

3.4.1.1. Ornamental fish samples

Live, healthy ornamental fish samples were collected from three different aquarium vendors, Cochin. 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.

3.4.1.2. 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 10ºC until analysis and were analyzed within 4 hours of collection. A total of one hundred samples were analyzed.

3.4.1.2.1. Physico-chemical analysis of water samples

Physico-chemical analysis of water samples was carried out as described in section 2.4.1.2.1**.**

3.4.2. Bacteriological Analysis

Bacteriological analysis of the fish and water samples were carried out as described in section 2.4.2.

3.4.3. 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. These isolates (175 isolates from the fish and 182 isolates from the water samples) were than subjected to an array of biochemical tests as described in section 2.4.3.

3.4.4. Characterization of the isolates to species level

Identification of the isolates to species level was done according to Aerokey II (Section 2.4.4).

3.4.5. Study of Extracellular virulence factors

Extracellular virulence factors were analyzed as per the methods described in section 2.4.5.

3.4.6. Statistical Analysis

Statistical Analysis was carried out as described in section 2.4.6.

3.5. Results

3.5.1. Prevalence of motile aeromonads in different fish samples

Motile aeromonads were isolated from 84% of *Poecilia sphenops* and 80% of *P. reticulata* samples collected.

3.5.2. Prevalence of motile aeromonads in different body parts of fish samples

Table 3.1 shows the prevalence of motile aeromonads in different body parts of *P. sphenops* and *P. reticulata*. Gill samples showed a higher prevalence of motile aeromonads in both *P. sphenops* and *P. reticulata*.

Table 3.1. Prevalence of motile aeromonads in different body parts of *P. sphenops* and *P. reticulata*

Name of fish	% occurrence of motile aeromonads in		
	Body surface	Gill	Intestine
P. sphenops	35.49	40.86	23.65
P. reticulata	29.68	36.58	34.14

3.5.3. 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 3.1. One hundred and seventy five isolates from the samples were characterized to species level. *Aeromonas sobria* was the predominant species (40.57%) followed by *A. caviae* (31.43%). Prevalence of other species of motile aeromonads was less than 10%.

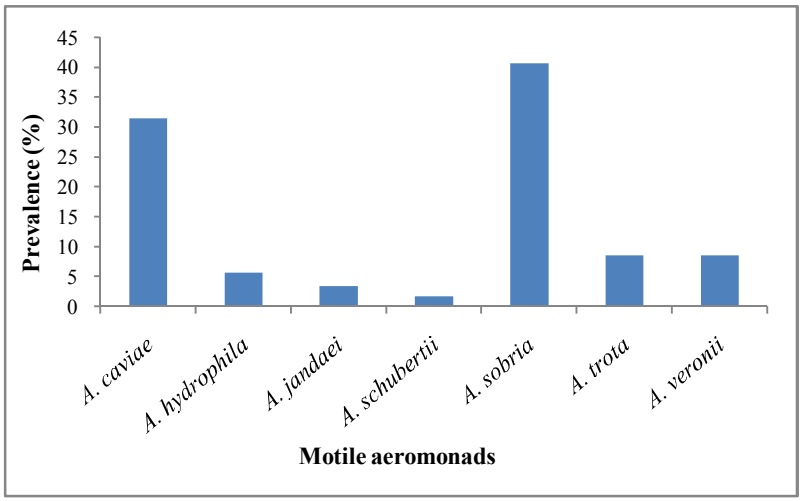


Figure 3.1. Overall distribution of motile aeromonads in fish samples

3.5.3.1. 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 3.2 and 3.3 respectively. *A. sobria* was the predominant motile aeromonad in both *P. sphenops* and *P. reticulata* followed by *A. caviae*. Prevalence of other species of motile aeromonads was less than 10%.

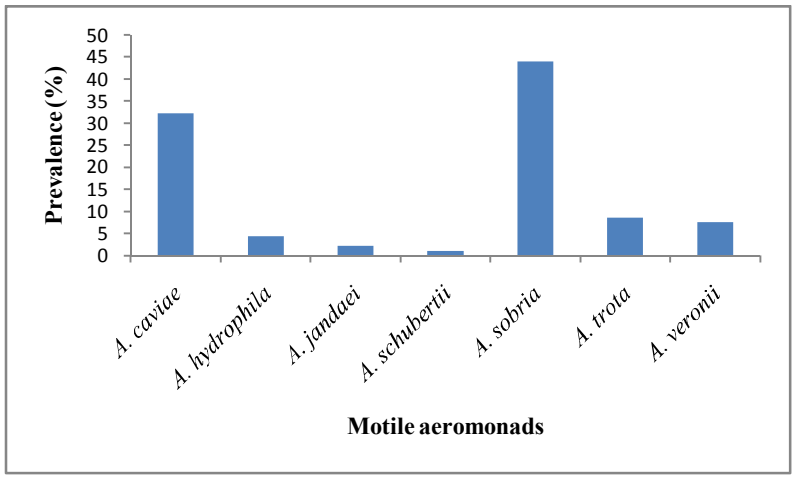


Figure 3.2. Prevalence of different species of motile aeromonads in *P. sphenops*

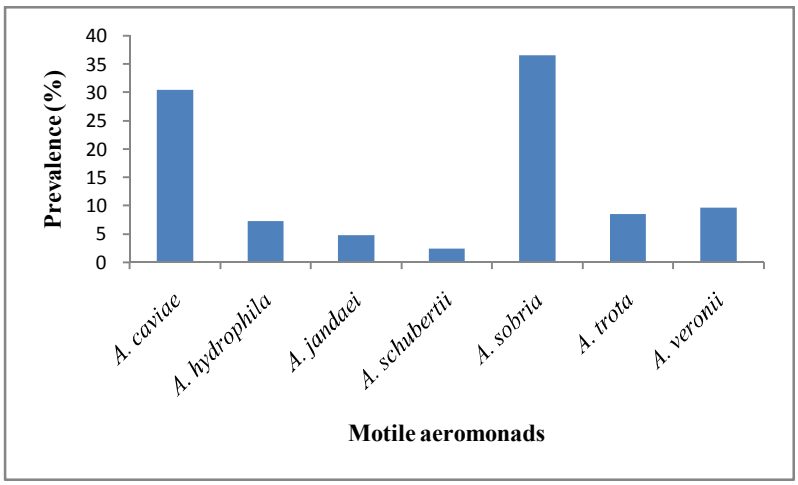


Figure 3.3. Prevalence of different species of motile aeromonads in *P. reticulata*

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

Figure 3.4 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 *Aeromonas* in *P. sphenops* and *P. reticulata*. Prevalence of *A. sobria* was relatively higher in *P. sphenops*, while the prevalence of *A. jandaei* and *A. hydrophila* was relatively higher in *P. reticulata*.

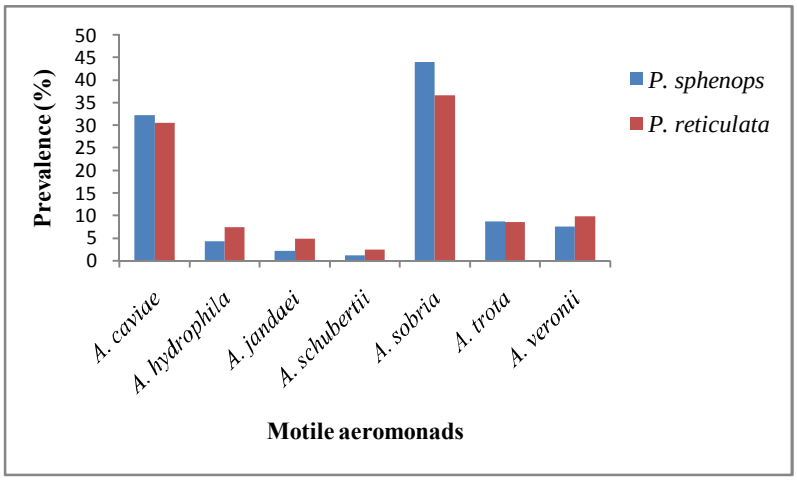


Figure 3.4. Relative prevalence of different species of motile aeromonads in *P. sphenops* and *P. reticulata*

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

Figure 3.5 gives a graphical representation of the distribution of different species of motile aeromonads in various body parts of *Poecilia sphenops*. Prevalence of different motile aeromonads was found to be high on the body surface and gills, compared to intestine. *A. schubertii* was found to occur only on the body surface.

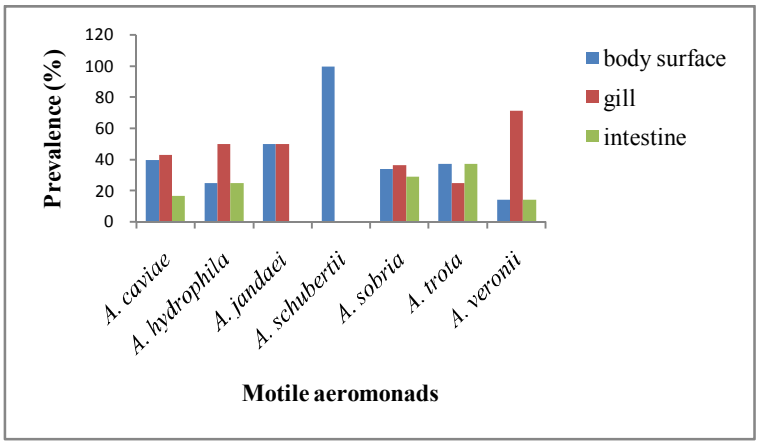


Figure 3.5. Distribution of different species of motile aeromonads in various body parts of *P. sphenops*

Figure 3.6 gives a graphical representation of the distribution of different species of motile aeromonads in the body parts of *P. reticulata.* Occurrence of motile aeromonads was found to be relatively high in the gill and intestine. Prevalence of *A. hydrophila, A. jandaei* and *A. trota* was found to be high in the gills.

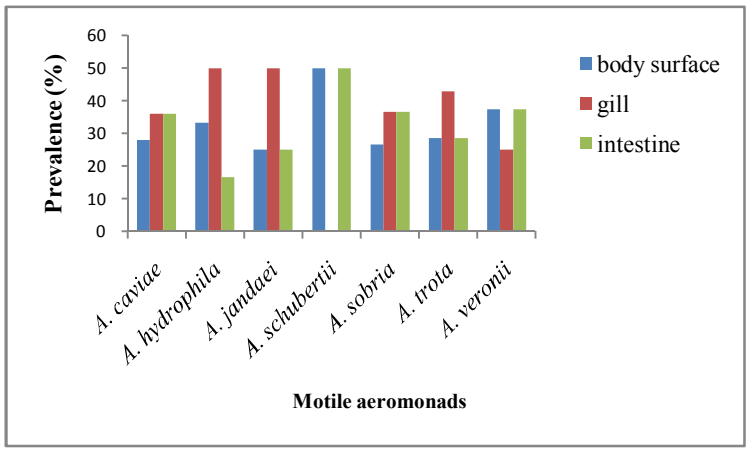


Figure 3.6. Distribution of different species of motile aeromonads in various body parts of *P. reticulata*

3.5.5. Diversity indices

Table 3.2 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 *Poecilia sphenops* and *P. reticulata*. Here the Margalef 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 3.2. Diversity indices of various body parts of the two different fishes

Diversity indices	*P. sphenops*			*P. reticulata*		
	Body surface	**Gill**	**Intestine**	**Body surface**	**Gill**	**Intestine**
Richness (d)	1.72	1.37	1.29	1.78	1.47	1.8
Evenness (J')	0.71	0.78	0.76	0.8	0.87	0.78
Diversity (H')	1.37	1.41	1.22	1.56	1.55	1.52
Dominance (D)	0.7	0.72	0.66	0.75	0.77	0.75

3.5.6. k-dominance plot

The k-dominance visually represented the species abundance, richness and species evenness (Figure 3.7). 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.

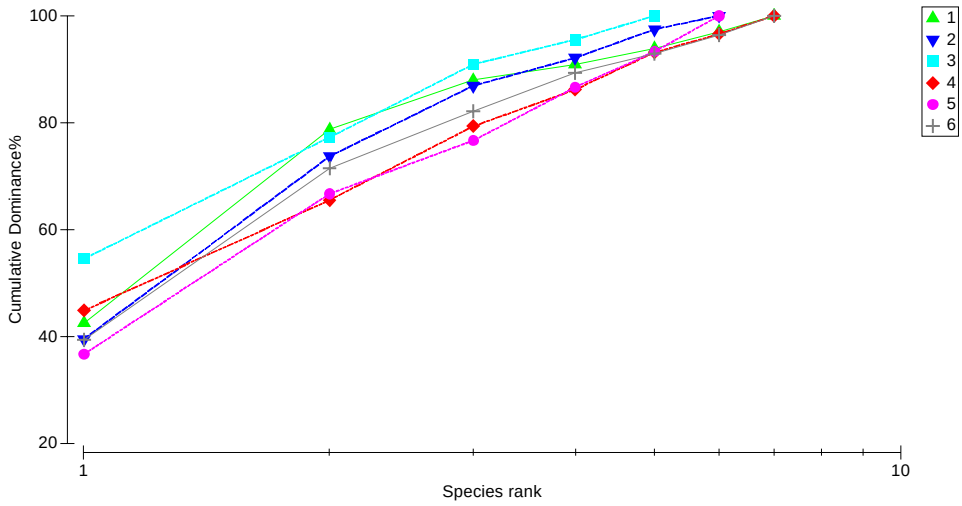


Figure 3.7. k dominance curve of motile aeromonads in various body parts of *P. sphenops* and *P. reticulata*
1-body surface, 2-gill, 3-intestine of *P. sphenops* and
4-body surface, 5-gill, 6-intestine of *P. reticulata*

3.5.7. Physico-chemical characteristics of water samples

Physico-chemical characteristics of water samples analyzed are given in Table 3.3. Ammonia-N, nitrite and nitrate were found to be higher than the permissible range for ornamental fish culture.

Table 3.3. Physico-chemical characteristics of water samples

Parameters	Average	Range
Temperature (°C)	27	26-28
pH	7.15	7-7.8
Dissolved oxygen (mg/L)	6.5	3.8-9.78
Total ammonia nitrogen (mg/L)	3.012	0.057-6.5
Nitrite (mg/L)	1.2	0.532-2.08
Nitrate (mg/L)	25.07	0.098-50.55

3.5.8. Prevalence and distribution of motile aeromonads in water samples

Motile aeromonads were isolated from 84% of the water samples collected from retail aquarists. One hundred and eighty two isolates from the water samples were characterized to species level. The distribution of motile aeromonads in water samples is given in Figure 3.8. *A. sobria* was the predominant species in water samples also (34.80%) followed by *A. caviae* but it's occurrence was much less than that encountered in fish samples. Distribution of *A. hydrophila* and *A. veronii* were almost equal in water samples.

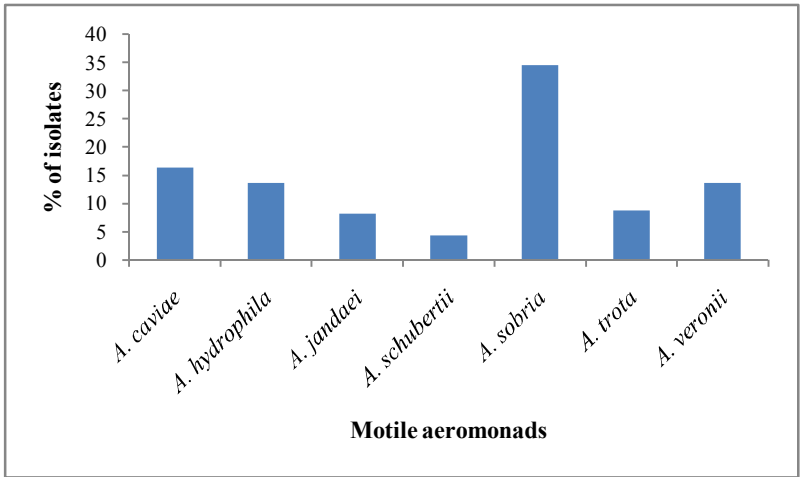


Figure 3.8. Distribution of different species of motile aeromonads in water samples

3.5.9. Production of extracellular virulence factors in motile aeromonads from fish samples

Production of extracellular virulence factors of motile *Aeromonas* isolates in fish samples from retail aquarists is given in Figure 3.9. All the isolates were capable of producing gelatinase and DNase. Lipase was produced by 94.85% of

the isolates. Haemolytic activity was detected in 89.71% of the isolates while caseinase was detected in 80.57% of the isolates.

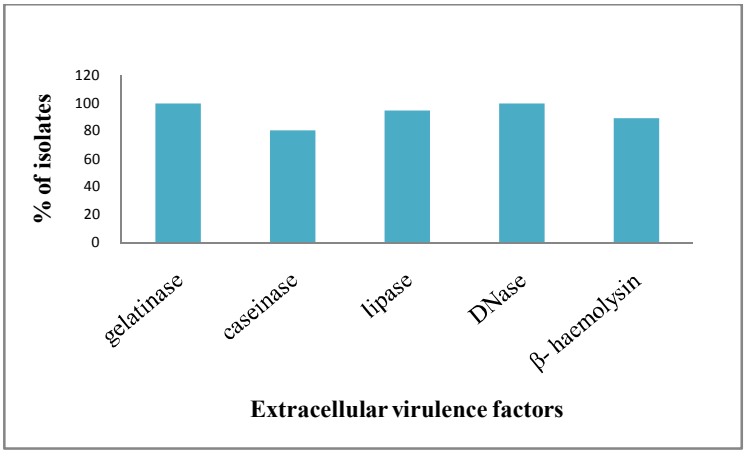


Figure 3.9. Production of extracellular virulence factors in motile aeromonads

3.5.10. Production of extracellular virulence factors in different species of *Aeromonas* from fish samples

The production of extracellular virulence factors by different species of *Aeromonas* from aquarium fishes is shown in Table 3.4. Gelatinase and DNase were produced by members of all the species identified. All the isolates of *Aeromonas sobria* produced caseinase, while only 90% of *A. hydrophila* were capable of producing caseinase. Lipase production was more frequent in the isolates tested. All the isolates of *A. jandaei, A. hydrophila* and *A. veronii* produced lipase and all the isolates of *A. sobria, A. hydrophila, A. jandaei* and *A. schubertii* were β-haemolytic.

Table 3.4. 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	52.73	89.09	100	72.72
A. hydrophila	100	90	100	100	100
A. jandaei	100	83.33	100	100	100
A. schubertii	100	66.67	66.6	100	100
A. sobria	100	100	98.59	100	100
A. trota	100	80	93.33	100	86.66
A. veronii	100	86.66	100	100	93.33

3.5.10.1. Production of proteases by motile aeromonads from aquarium fish samples

Gelatinase and casienase were the two enzymes screened for the detection of protease activity. All the isolates of *Aeromonas* spp. obtained from fish samples exhibited gelatinase activity and is given in Figure 3.10.

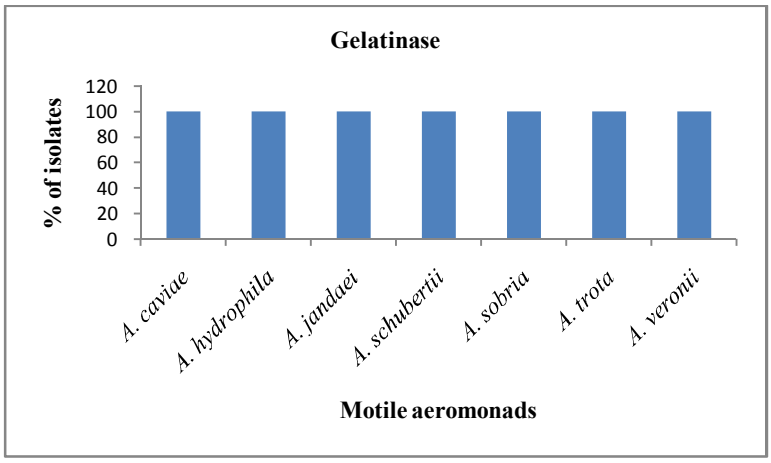


Figure 3.10. Production of gelatinase by different species of *Aeromonas*

All the isolates of *Aeromonas sobria* exhibited caseinase production and 90% of *A. hydrophila* isolates were caseinase producers (Figure 3.11).

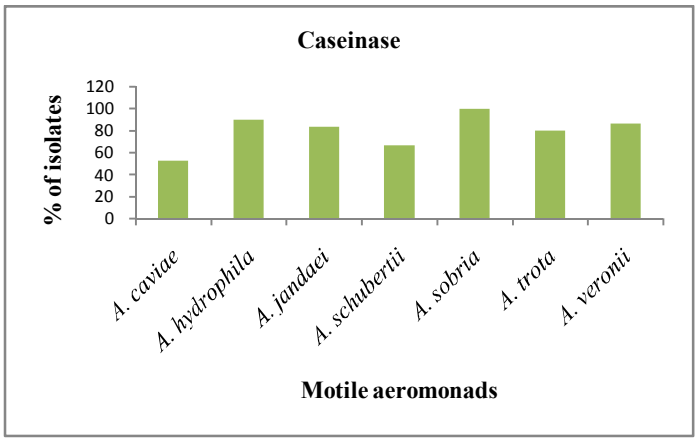


Figure 3.11. Production of caseinase by different *Aeromonas* spp.

3.5.10.2. Production of Lipase in motile aeromonads from fish samples

Lipase was produced by all the isolates of *A. hydrophila*, *A. jandaei* and *A. veronii* while 98.59% of *A. sobria* were capable of producing lipase. Production of lipase by different species of *Aeromonas* is given in Figure 3.12.

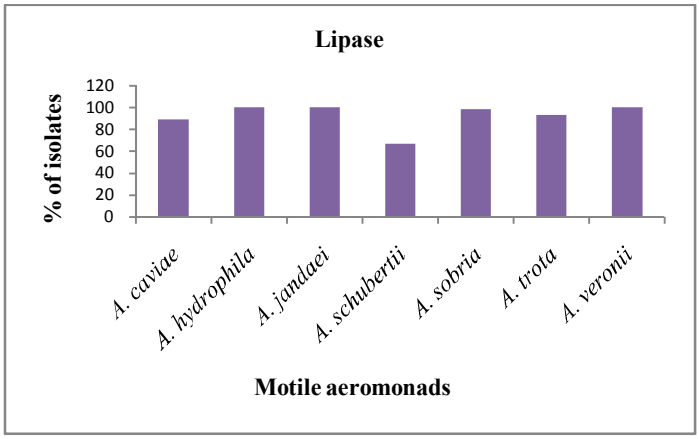


Figure 3.12. Production of lipase by different *Aeromonas* spp.

3.5.10.3. Production of DNase in motile aeromonads from fish samples

All the isolates of *Aeromonas* spp. obtained from fish samples exhibited DNase activity (Figure 3.13).

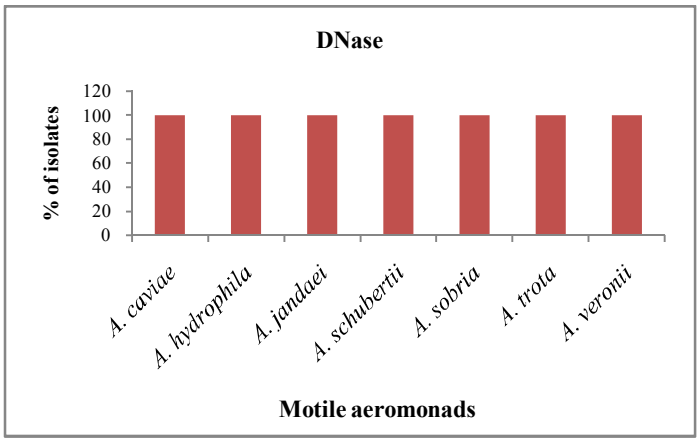


Figure 3.13. Production of DNase by different *Aeromonas* spp.

3.5.10.4. Production of Haemolysin in motile aeromonads from fish samples

All the isolates of *Aeromonas hydrophila, A. jandaei, A. sobria* and *A. schubertii* exhibited β-haemolysin production. β-haemolysin production was infrequent in *A. caviae* (72.72%). Production of haemolysin by different species of *Aeromonas* is given in Figure 3.14.

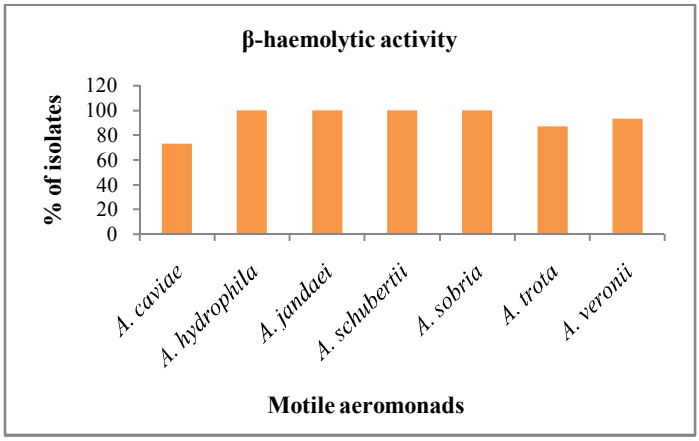


Figure 3.14. Production of haemolysin by different *Aeromonas* spp**.**

3.5.11. Extracellular virulence factors of motile aeromonads in water samples

Production of extracellular virulence factors of *Aeromonas* isolates in water samples is given in Figure 3.15. Gelatinase and DNase production was detected in all the isolates. Lipase was produced by 96.7% of the isolates and haemolytic activity was detected in 91.75% of the isolates.

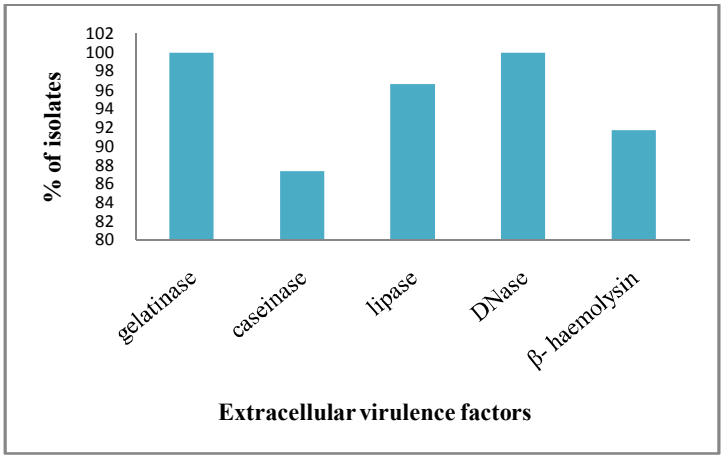


Figure 3.15. Production of extracellular virulence factors in *Aeromonas* isolates

3.5.12. Production of extracellular virulence factors in different species of *Aeromonas* from water samples

The production of extracellular virulence factors by different species of *Aeromonas* is shown in Table 3.5. Gelatinase and DNase were produced by all the isolates of different species of *Aeromonas.* All the isolates of *Aeromonas jandaei* and *A. sobria* exhibited caseinase production. All the isolates of *A. hydrophila, A. jandaei* and *A. veronii* and 98.4% of *A. sobria* produced lipase. β-haemolysin was produced by all the isolates of *A. hydrophila, A. jandaei, A. sobria* and 92% of *A. veronii*.

Table 3.5. Production of extracellular virulence factors in different species of *Aeromonas* from water samples

***Aeromonas* spp.**	**Percentage of motile aeromonads producing extracellular virulence factors**				
	gelatinase	**caseinase**	**lipase**	**DNase**	**β-haemolysin**
A. caviae	100	60	90	100	70
A. hydrophila	100	88	100	100	100
A. jandaei	100	100	100	100	100
A. schubertii	100	62.5	87.5	100	75
A. sobria	100	100	98.4	100	100
A. trota	100	87.5	93.75	100	87.5
A. veronii	100	88	100	100	92

3.5.12.1. Production of proteases by motile aeromonads from aquarium 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 3.16).

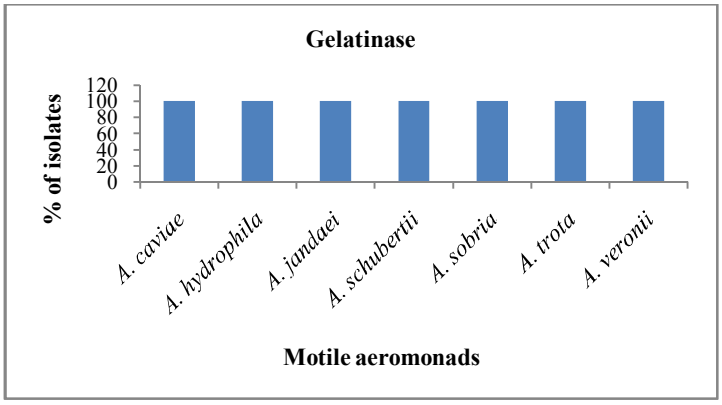


Figure 3.16. Production of gelatinase by different *Aeromonas* spp.

Production of caseinase by different species of *Aeromonas* is given in Figure 3.17. All the isolates of *Aeromonas jandaei* and *A. sobria* exhibited caseinase production while 88% of *A. hydrophila* and *A. veronii* isolates were caseinase producers. Caseinase production was relatively low in *A. caviae* and *A. schubertii* isolates.

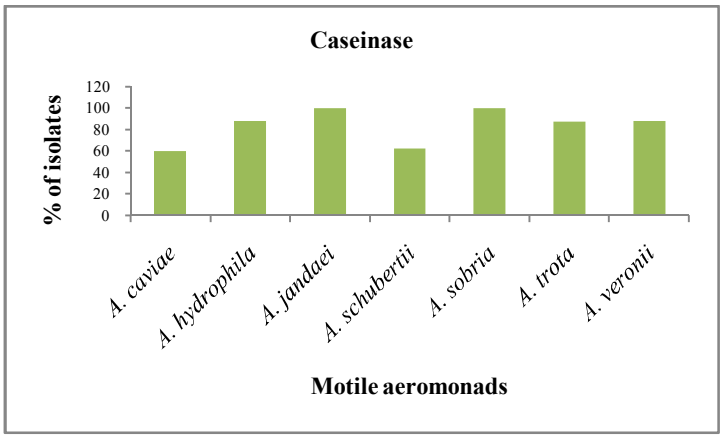


Figure 3.17. Production of caseinase by different *Aeromonas* spp.

3.5.12.2. Production of Lipase in motile aeromonads from water samples

Lipase was produced by all the isolates of *A. hydrophila, A. jandaei* and *A. veronii*, but their production among the isolates of other spp. varied. Production of lipase by different species of *Aeromonas* is given in Figure 3.18.

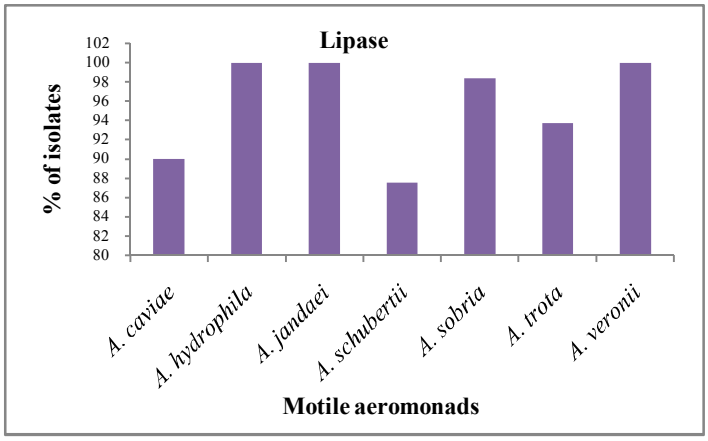


Figure 3.18. Production of lipase by different *Aeromonas* spp.

3.5.12.3. Production of DNase in motile aeromonads from water samples

All the isolates of *Aeromonas* spp. obtained from water samples exhibited DNase activity (Figure 3.19).

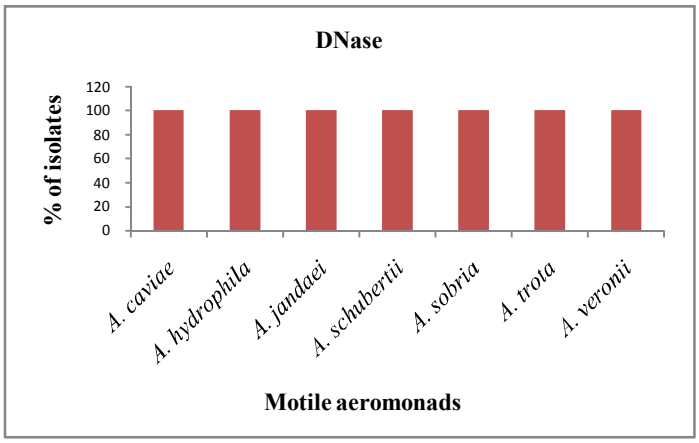


Figure 3.19. Production of DNase by different *Aeromonas* spp.

3.5.12.4. Production of Haemolysin in motile aeromonads from water samples

All the isolates of *Aeromonas hydrophila*, *A. jandaei* and *A. sobria* exhibited β-haemolysin production. Though most of motile aeromonads were capable of elaborating β-haemolysin, the ability was found to vary among different species. Production of haemolysin by different species of *Aeromonas* is given in Figure 3.20.

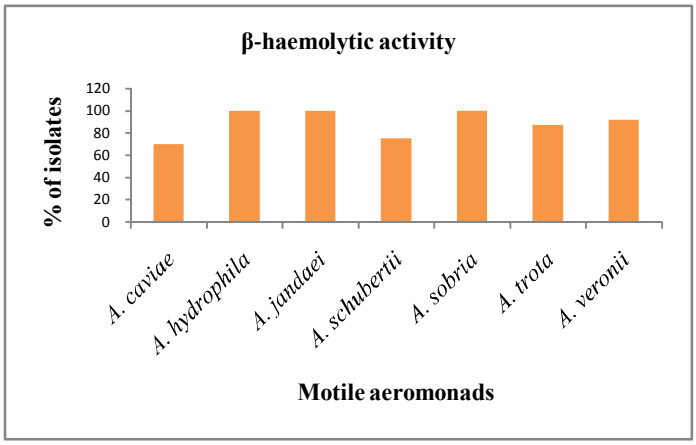


Figure 3.20. Production of β-haemolysin by different *Aeromonas* spp.

3.5.13. Relative prevalence of motile aeromonads in fish samples from farm and retail aquaria

Figure 3.21 gives relative prevalence of motile aeromonads in the two different fish samples from farm and retail aquaria. Prevalence of motile aeromonads was high in *P. sphenops* and *P. reticulata* from retail aquaria, compared to those from the farm.

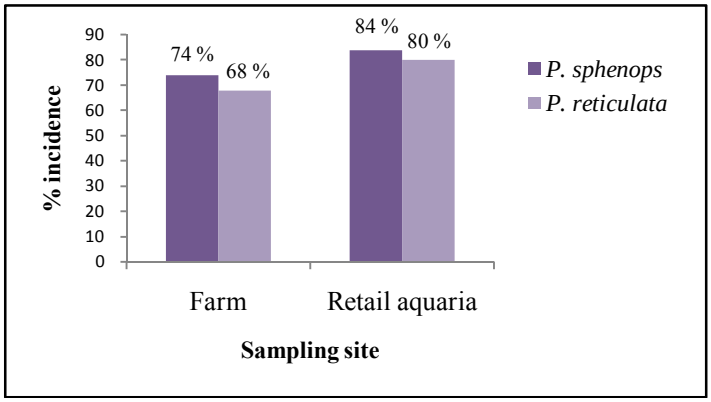


Figure 3.21. Relative prevalence of motile aeromonads in fish samples from farm and retail aquaria

3.5.14. Relative prevalence of motile aeromonads in different body parts of fish samples from farm and retail aquaria

Relative prevalence of motile aeromonads in different body parts of *P. sphenops* and *P. reticulata* from farm and retail aquaria is given in Table 3.6. Prevalence of motile aeromonads was higher in the gill samples of fish from retail aquaria.

Table 3.6. Relative prevalence of motile aeromonads in different body parts of fish samples

Body parts	Farm		Retail aquaria	
	P. sphenops **(%)**	***P. reticulata*** **(%)**	***P. sphenops*** **(%)**	***P. reticulata*** **(%)**
Body surface	39.06	33.33	35.49	29.68
Gill	37.5	30.86	40.86	36.58
Intestine	23.44	35.80	23.65	34.14

3.5.15. Relative prevalence of different species of motile aeromonads in fish samples from farm and retail aquaria

Relative prevalence of motile *Aeromonas* isolates in fish samples from farm and retail aquarium vendors is given in Figure 3.22. In fish samples from both farm and aquarium vendor's *A. sobria* was the predominant species followed by *A. jandaei* and *A. hydrophila.* While isolation of *A. caviae* was relatively frequent in samples from aquarium vendors it was found to be very low in samples from farm. *A. trota* and *A. veronii* were present in comparable levels in samples from both farm and retail aquarium vendors.

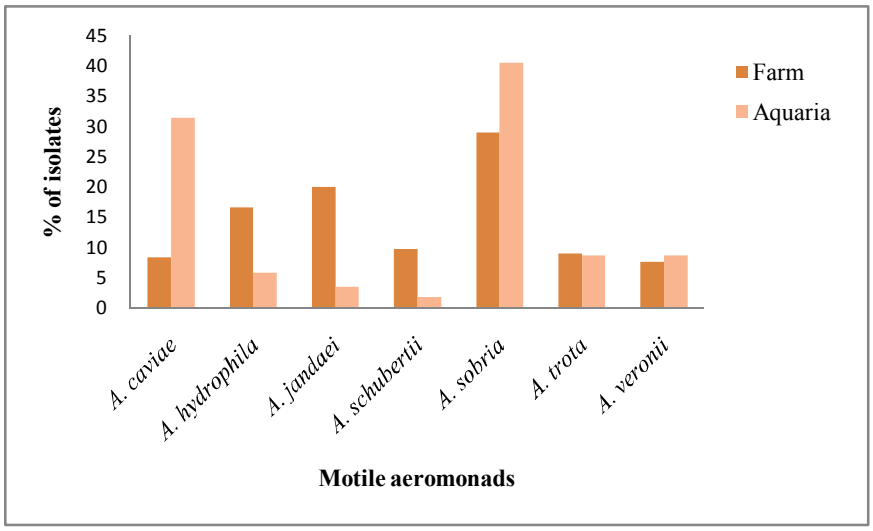


Figure 3.22. Relative prevalence of different species of motile *Aeromonas* in fish samples

3.5.16. k-dominance plot

The k-dominance curve of fish samples from farm and retail aquaria is given in Figure 3.23. Ornamental fish samples from farm exhibited more diversity and less dominance, whereas samples from retail aquaria exhibited high dominance contributed mainly by *A. sobria* and *A. caviae*.

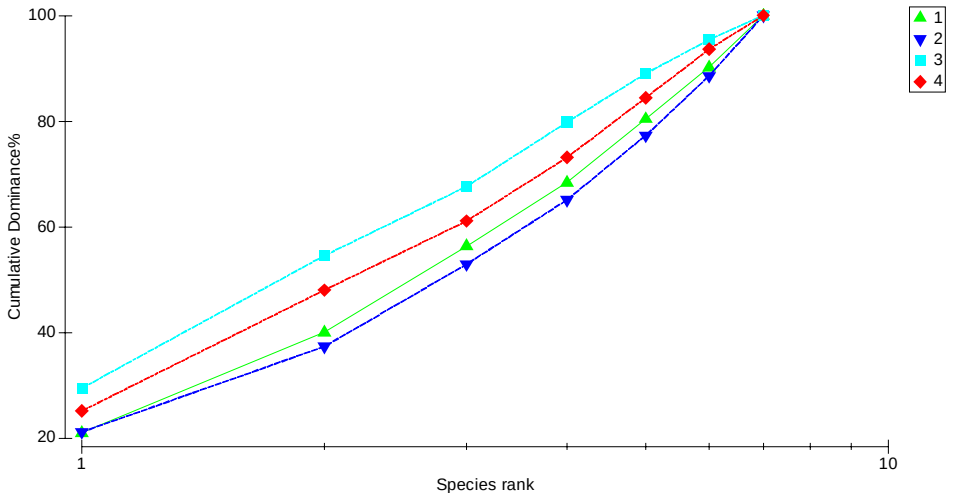


Figure 3.23. k dominance curve of motile aeromonads in *P. sphenops* and *P. reticulata* from farm and retail aquaria
1- *P. sphenops* and 2- *P. reticulata* from farm
3- *P. sphenops* and 4- *P. reticulata* from retail aquaria

3.5.17. Relative prevalence of motile aeromonads in water samples from farm and retail aquaria

Motile aeromonads were isolated from 68 and 84% of the water samples from farm and retail aquaria respectively.

3.5.18. Relative prevalence of different species of motile aeromonads in water samples from farm and retail aquaria

Relative prevalence of different species of motile *Aeromonas* in water samples from farm and retail aquarium vendors is given in Figure 3.24. In water samples from both farm and aquarium vendor's *A. sobria* was the predominant species. *A. trota* was the second dominant species in farm but its presence in samples from aquarium vendor was less than 10%. *A. caviae* was the second dominant species in water samples from aquarium vendors, while its prevalence was less in samples from farm.

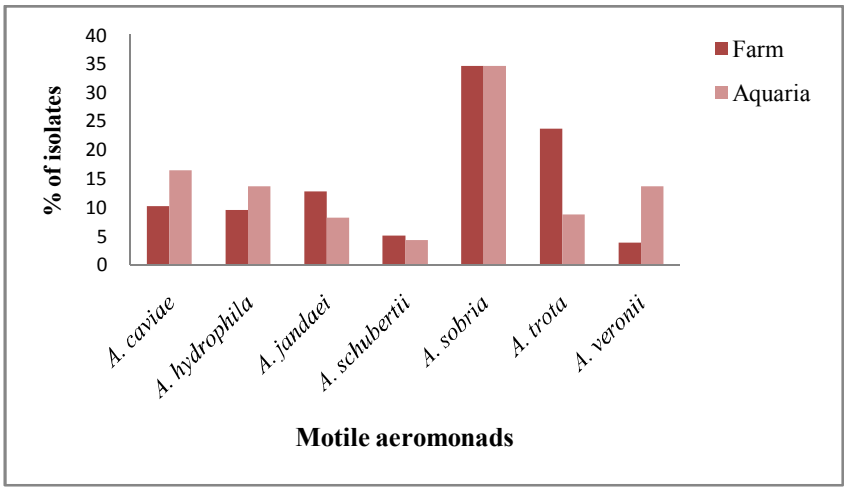


Figure 3.24. Relative prevalence of different species of motile *Aeromonas* in water samples

3.5.19. k-dominance plot

The k-dominance curve of water samples from farm and retail aquaria is given in Figure 3.25. Water samples from farm exhibited less diversity and high dominance, contributed mainly by *A. sobria* and *A. trota*.

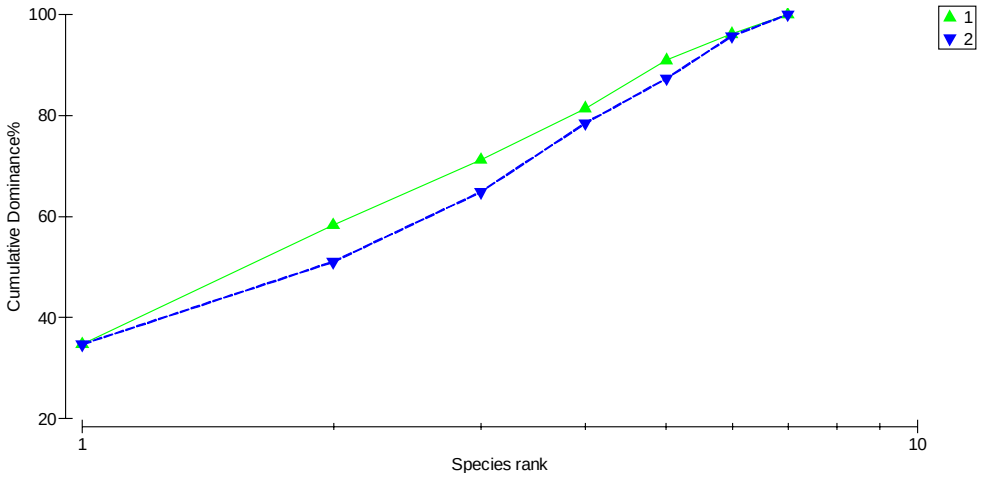


Figure 3.7. k dominance curve of motile aeromonads in water samples from farm and retail aquaria

1-water samples from farm; 2- water samples from retail aquaria

3.5.20. Relative prevalence of extracellular virulence factors in motile aeromonads in fish samples from farm and retail aquarium vendors

Figure 3.26 represents a comparison of the production of extracellular virulence factors in motile aeromonads from fish samples. Gelatinase and DNase were produced by all the isolates from farm and retail aquaria. No significant variation (p>0.05) was observed in the production of extracellular virulence factors in motile aeromonads from farm and retail aquarium vendors.

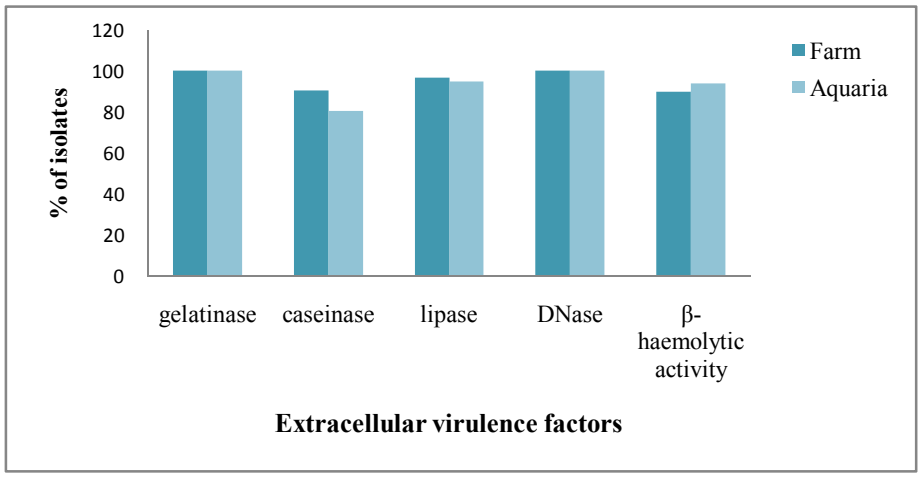
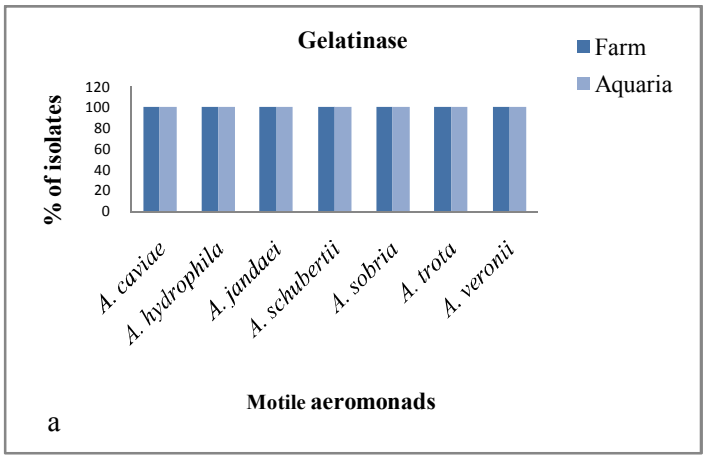
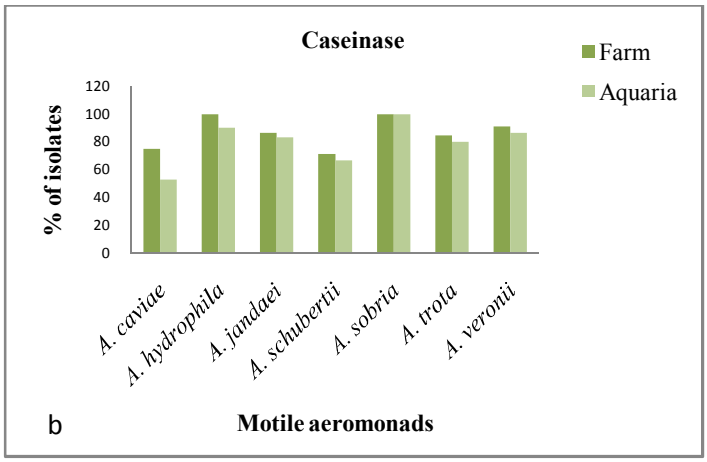


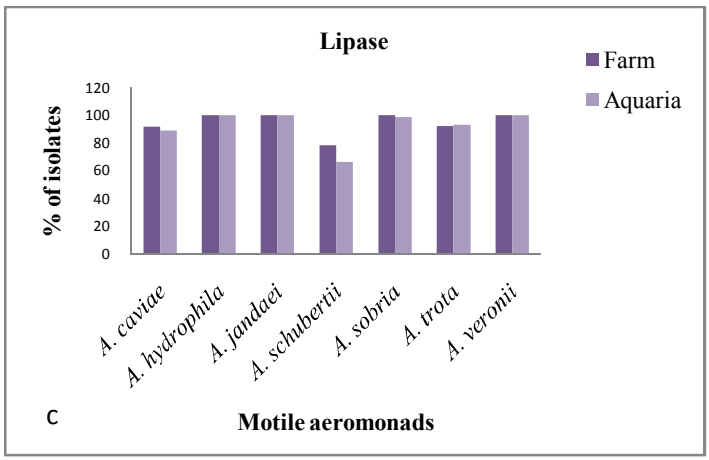
Figure 3.26. Relative prevalence of extracellular virulence factors in motile aeromonads from fish samples

3.5.21. Relative prevalence of extracellular virulence factors in different species of motile aeromonads in fish samples from farm and retail aquarium vendors

Relative prevalence of various extracellular virulence factors produced by different species of *Aeromonas* in fish samples from farm and retail aquarium vendors is represented in Figure 3.27 (a-e).


Gelatinase
Farm
Aquaria
% of isolates
120
100
80
60
40
20
0
A. caviae
A. hydrophila
A. jandaei
A. schubertii
A. sobria
A. trota
A. veronii
Motile aeromonads
a


Caseinase
Farm
Aquaria
% of isolates
120
100
80
60
40
20
0
A. caviae
A. hydrophila
A. jandaei
A. schubertii
A. sobria
A. trota
A. veronii
b
Motile aeromonads


Lipase
Farm
Aquaria
% of isolates
120
100
80
60
40
20
0
A. caviae
A. hydrophila
A. jandaei
A. schubertii
A. sobria
A. trota
A. veronii
c
Motile aeromonads

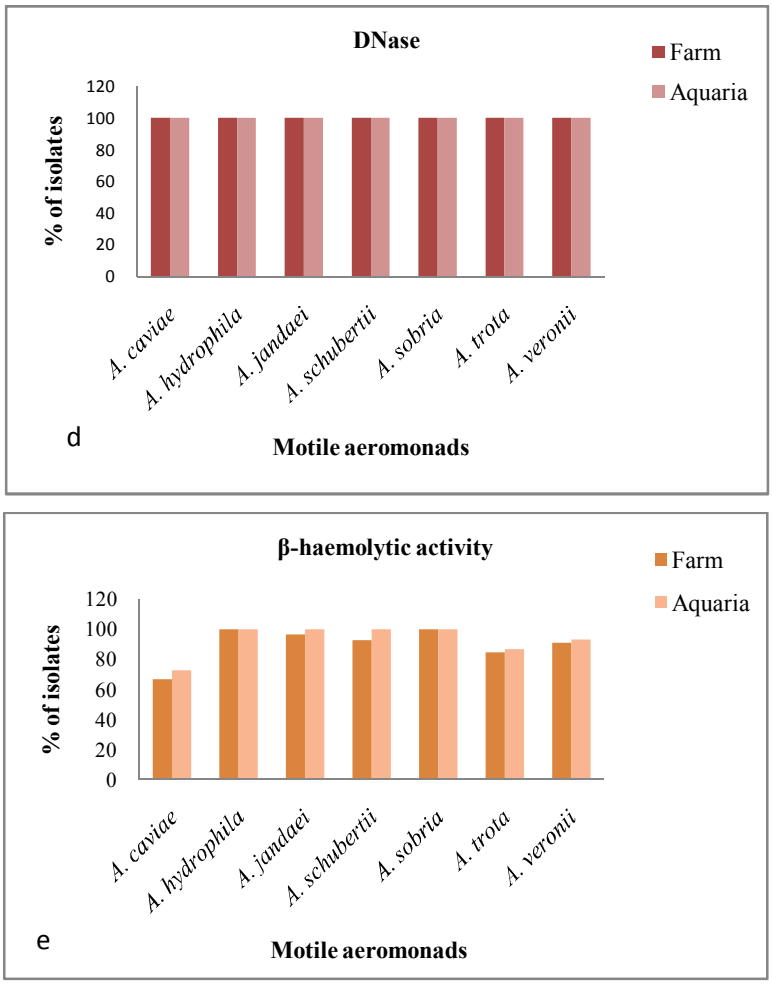


Figure 3.27 (a-e). Relative prevalence of various extracellular virulence factors produced by different species of motile aeromonads in fish samples from farm and retail aquarium vendors

Gelatinase was produced by all the isolates from both farm and aquarium vendors. Caseinase was produced by all the isolates of *A. sobria* from fish samples of both farm and aquarium vendors, where as all the isolates of *A. hydrophila* from farm and 90% of *A. hydrophila* from aquarium vendors produced the enzyme.

Lipase production was frequent in motile *Aeromonas* isolates from both farm and aquarium vendors except in isolates of *A. schubertii* in which case less than 80% of the isolates were producers of lipase. This enzyme was produced by all the isolates of *A. hydrophila, A. jandaei* and *A. veronii* from both farm and aquarium vendors. DNase was produced by all the motile aeromonads from both farm and aquarium vendors.

β-haemolytic activity was found to be more frequent in motile aeromonads from aquarium vendors. All the isolates of *A. hydrophila* and *A. sobria* from farm were β-haemolytic, while all the isolates of *A. hydrophila, A. jandaei, A. schubertii* and *A. sobria* from aquarium vendors were β-haemolytic. Production of β- haemolysin was found to be less common in isolates of *A. caviae.*

3.5.22. Relative prevalence of extracellular virulence factors in motile aeromonads in water samples from farm and retail aquarium vendors

Figure 3.28 represents a comparison of the production of extracellular virulence factors in motile aeromonads from water samples. The production of extracellular virulence factors by motile aeromonads in water samples from farm and aquarium vendors did not differ significantly (p>0.05). Gelatinase and DNase were produced by all the isolates tested and 91% of isolates from both farm and aquarium vendors were β-haemolytic. More than 85% of the isolates from both farm and aquarium vendors produced all the virulence factors.

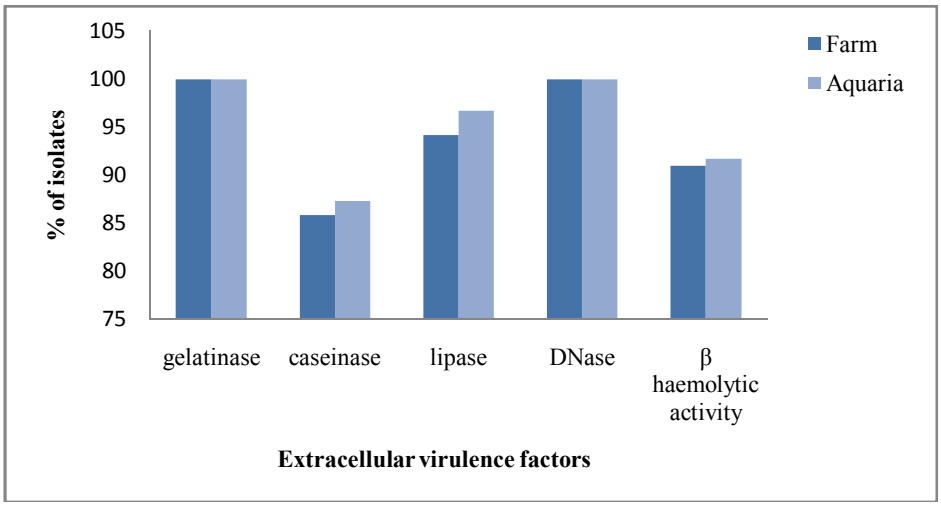


Figure 3.28. Relative prevalence of extracellular virulence factors in motile aeromonads from water samples

3.5.23. Relative prevalence of extracellular virulence factors in different species of motile aeromonads in water samples from farm and retail aquarium vendors

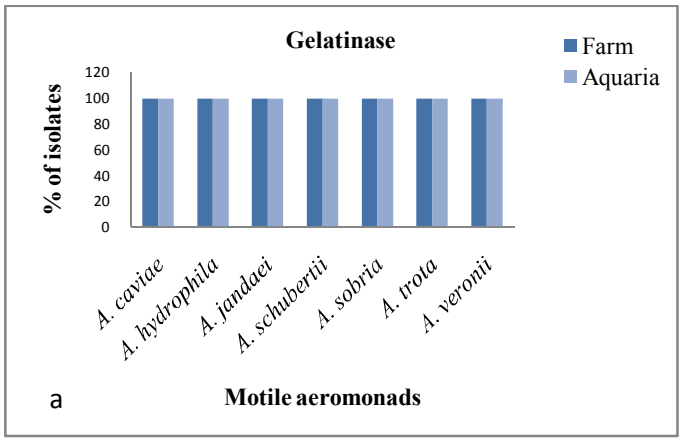
Relative prevalence of various extracellular virulence factors produced by different species of *Aeromonas* in water samples from farm and retail aquarium vendors is represented in Figure 3.29 (a-e). Gelatinase was produced by all the isolates from both farm and aquarium vendors.

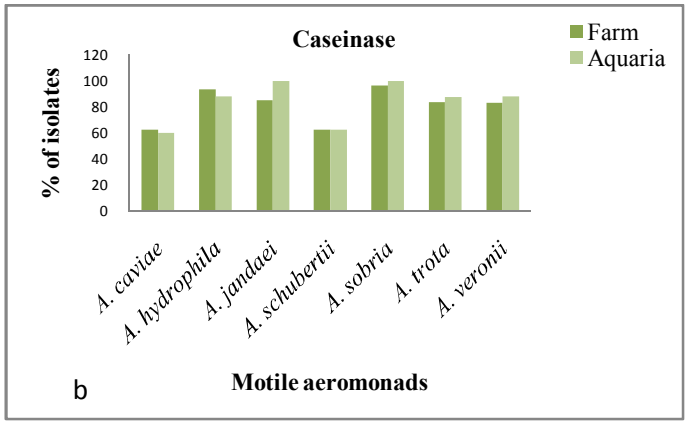
All the isolates of *A. jandaei* and *A. sobria* from aquarium vendors exhibited caseinase production. Production of this enzyme was found to be less in *A. caviae* and *A. schubertii.*

Lipase was produced by all the isolates of *A. hydrophila* and *A. veronii* in water samples from farm while all the isolates of of *A. hydrophila A. jandaei* and *A. veronii* from aquarium vendors were

lipolytic. DNase was produced by all the isolates from both farm and aquarium vendors.

All the isolates of *A. hydrophila* and *A. sobria* from farm were β-haemolytic and all the isolates of *A. hydrophila, A. jandaei* and *A. sobria* from aquarium vendors were β-haemolytic.





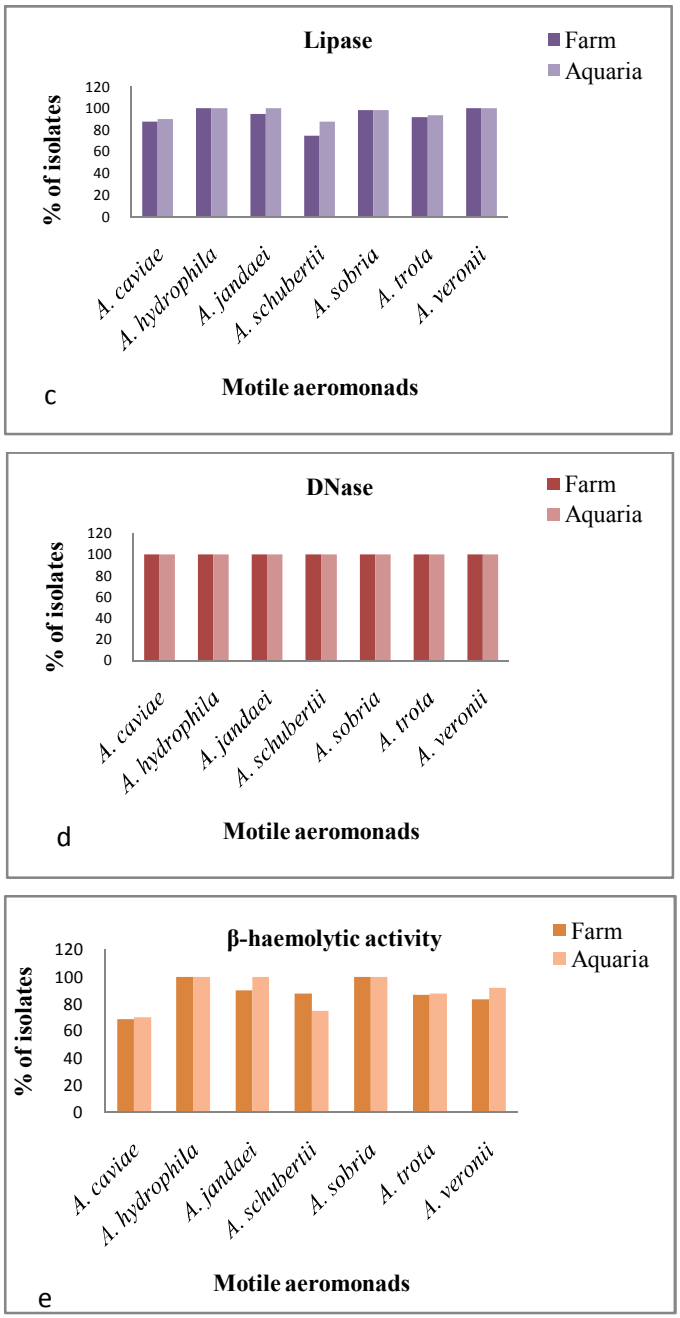


Figure 3.29 (a-e). Relative prevalence of various extracellular virulence factors produced by different species of motile aeromonads in water samples from farm and retail aquarium vendors

3.6. Discussion

3.6.1. Distribution and characteristics of motile aeromonads from aquarium vendors

Aeromonas was present in 84% of *Poecilia sphenops* and 80% of *P. reticulata* samples tested in the present study and such a high prevalence of *Aeromonas* in aquarium samples is evident from early literature (Sanyal *et al*., 1987). They have sampled a total of 100 tropical aquaria and isolated *Aeromonas* species from 98 tanks. However, the prevalence level was higher than those reported by Erdem *et al*. (2010) who reported 65% of fresh water fish samples from Turkey to be positive for *Aeromonas* spp.

Aeromonas sobria was the predominant species isolated from both *P. sphenops* and *P. reticulata* followed by *A. caviae*. Nearly 10% of the isolates were found to be *A. trota* and *A. veronii*. Other motile aeromonads encountered were *A. hydrophila, A. jandaei* and *A. schubertii*. Our results reveal a lower prevalence of *A. hydrophila* when compared to that of Rathore *et al*. (2005) who have reported *A. hydrophila* to be the predominant sp. in fish samples collected from aquaria in India. They have included both diseased as well healthy fishes in their study. However, they also reported species such as *A. sobria* (13%), *A. veronii* (13%), *A. caviae* (9%) and *A. schubertii* (9%), similar to the observations in the present study though the relative prevalence of these species varied.

Of the *Aeromonas* isolates obtained from the aquaria by Sanyal *et al*. (1987), 82 % belonged to *A. hydrophila* and *A. sobria* species. *A. hydrophila* and *A. sobria* have frequently been isolated from EUS lesions in fish (Rathore *et al*., 2005; Hossian *et al*., 2011; Kumar and Ramulu, 2013). Jongjareanjai

et al. (2009) reported *A. hydrophila* as the predominant agent of bacterial disease in ornamental fish. Musa *et al*. (2008) also reported that bacteria isolated from sick freshwater ornamental fish from aquarium shops in Malaysia were mostly *A. hydrophila* (60%). *A. veronii* biotype *sobria* was found to be more pathogenic followed by *A. hydrophila* and *A. jandaei* (Iqbal *et al*., 1999). *A. veronii* has been identified from diseased Gourami collected from a pet shop and may constitute an important causative agent of epizootic ulcerative disease in Korea (Hossain, 2008). In our study, we have analyzed live and apparently healthy fishes for the prevalence of motile aeromonads, assuming that poor water quality put them under stress leading to infection by motile aeromonads.

In the present study, when the prevalence of *Aeromonas* in different body parts were considered, it was found that the prevalence was high in gills in both *Poecilia sphenops* and *P. reticulata* but, *A. schubertii* was isolated only from body surface in *P. sphenops*. k-dominance curve showed that intestine of *P. sphenops* represented a less diverse distribution of species.

Results of the present study revealed that, *Aeromonas* was present in 84% of the water samples tested. In terms of prevalence and abundance in water samples, the most predominant species was found to be *Aeromonas sobria* (34.80%) followed by *A. caviae* (16.57%). Distribution of *A. hydrophila* and *A. veronii* was found to be equal (13.81%), while prevalence of *A. trota, A. jandaei* and *A. schubertii* were less than 10%. *A. caviae* was found to be second most predominant species; *A. schubertii* was the least predominant species. Rathore *et al*. (2005) reported *A. hydrophila* to be the predominant sp. in water samples collected from aquaria in India. However, they also reported

species such as *A. sobria*, *A. veronii, A. caviae* and *A. schubertii.* Smith *et al*. (2012) have identified *A. schubertii, A. veronii* and *A. hydrophila* in aquarium tank water harbouring ornamental fishes. According to them, very few studies have characterized the overall microbial communities or potential pathogens associated with ornamental fishes or their water.

Physico-chemical characteristics of water samples revealed that ammonia, nitrite and nitrate are higher than the permissible range for ornamental fish culture, and is prone to induce stress in fishes. This is very important as stress induced debilitation and subsequent immune system functioning is a major trigger causing infections by opportunistic pathogens. Our results revealed nearly 80% prevalence of motile aeromonads in the ornamental fishes analyzed during the study. Motile aeromonads are known for their role as opportunistic/obligate pathogens (Turska-Szewczuk *et al*., 2013).

3.6.2. Distribution of extracellular virulence factors in motile *Aeromonas* in samples from aquarium vendors

Gelatinase and DNase production was exhibited by all the isolates from fish samples in the present study. All *A. sobria* and nearly 90% of *A. hydrophila* isolates were capable of elaborating caseinase. Proteases act as virulence factors by facilitating host tissue invasion by destroying cell membranes and degrading host surface molecules. DNases may aid in the release of bacteria from disintegrating host cells in inflammatory lesions by digesting host DNA. Lipase production was also wide spread among the isolates. Wide spread production of such virulence factors in *Aeromonas* is reported earlier (Castro-Escarpulli *et al*., 2003; Kozinska, 2007; Erdem *et al*., 2010). Lipases act as

virulence factors by affecting several immune system functions through free fatty acids generated by lipolytic activity.

The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads. All the isolates of *A. hydrophila*, *A. jandaei, A. sobria* and *A. schubertii* from fish samples encountered in the present study were haemolysin producers. Nearly 90% of *A. trota* and *A. veronii* isolates were also haemolytic. The results are in agreement with the observations of Singh and Sanyal (1997), who reported that haemolysin production can also be extended to *A. jandaei* and *A. trota* as reported earlier for *A. hydrophila* and *A. sobria.* Production of haemolysins in most of the isolates of *Aeromonas* was reported earlier in fishes (Esther and Gideon, 2006) and in seafoods (Ullmann *et al*., 2005). In a study conducted in South India, 89% of *A. hydrophila* isolates from retail seafood outlets were found to be haemolysin producers (Thayumanavan *et al*., 2007). However, only 70% of *A. caviae* in the present study had the capability of haemolysin production. Haemolysins act as virulence factors by exerting lytic activities on red blood cells.

Results from studies on experimental infection in Blue Gourami using *Aeromonas* isolated from the diseased fishes by Patil *et al*. (2011) indicated only few cases of mortality, suggesting that these strains were opportunistic in nature, and that they can survive on fish or water surface and may cause disease under unfavourable conditions such as overcrowding or poor water quality. In the present investigation, we have analyzed only the live ones. However, we have seen mortality in aquarium at the retail vendor level. In most cases this was observed during the morning hours when they reopen the

shops and change the water. We tried to isolate motile aeromonads from these fishes, but recovery was modest as most of them were in a decomposed state and had been dominated by the spoilage flora.

All the motile aeromonads from water samples encountered in the present study exhibited gelatinase and DNase production. Caseinase and lipase production was also broadly seen in the isolates. Similar to our observation, Bagyalakshmi *et al*. (2009) reported all the *Aeromonas* isolates to be DNase producing. According to Paniagua *et al.* (1990), caseinase was produced by all the isolates of *A. hydrophila*, while little or no caseinase activity was displayed by *A. sobria* isolates in water samples from a river. In contrast to their observations, in South India Bagyalakshmi *et al*. (2009) reported 100% of *A. caviae* and *A. sobria* and 50% of *A. hydrophila* isolates to be caseinolytic.

In the present study, all the isolates of *A. hydrophila, A. jandaei* and *A. sobria* from water samples were haemolysin producers. Nearly 90% of *A. trota* and *A. veronii* isolates were also haemolytic. In a study conducted in South India, 89% of *Aeromonas* isolates from domestic water samples (Alavandi and Ananthan, 2003) were found to be haemolysin producers.

Though extracellular virulence factors cannot be considered as definite marker for pathogenicity of the isolates, the poor water quality conditions of the aquaria maintained at retail aquarists in this region might trigger disease outbreak by opportunistic pathogens.

3.6.3. Distribution of motile aeromonads-Farm v/s Retail aquaria

Worldwide studies have demonstrated that *Aeromonas* spp. is universally distributed. They are widely isolated from clinical, environmental and animal

sources, food samples and aquatic environment (Aberoum and Jooyandeh, 2010; Janda and Abbott, 2010). In developing countries, potentially pathogenic *Aeromonas* spp. is very common in different types of foods, drinking water and nosocomial outbreaks (Ghenghesh *et al*., 2008).

Present study revealed the prevalence of motile aeromonads in ornamental fish and carriage water samples from an ornamental fish farm and retail aquarium vendors. Prevalence was high in samples from aquarium vendors, compared to the samples from farm. In samples from farm, 71% of the fish samples and 68% of the water samples were positive for motile aeromonads whereas, in samples from retail aquarium vendors, 82% of the fish samples and 84% of the water samples were positive for motile aeromonads.

A. sobria was the most frequently isolated species in fish and water samples collected from both farm and aquarium vendors. Similar to the observation in the present study, Onuk *et al*. (2013) reported the predominance of *A. sobria* from water and Rainbow trout samples from Turkish coastal regions. Nam and Joh (2007) reported *A. sobria* to be the dominant species in fish and water samples collected from Trout farms in all seasons, in the Republic of Korea. Contrary to these observations, Figueras (2005) and Ottaviani *et al*. (2011) reported *A. hydrophila* and *A. veronii* as the most prevalent species in fish samples as well as in water samples.

Present study revealed *A. jandaei* and *A. trota* as other important motile *Aeromonas* species that are frequently encountered next to *A. sobria* in samples from farm, but *A. caviae* was the second dominant species in samples from aquarium vendors. The occurrence of *A. caviae* was found to be less than 10% in samples from farm. *A. hydrophila* was less frequently isolated (<10%) in samples

from aquarium vendors and it's percentage of occurrence was very low when compared to *A. sobria* and *A. caviae*. Similar to the observation in the present study, a higher prevalence of *A. jandaei* was reported in samples from ornamental fish farm (Suhet *et al.*, 2011; Sreedharan *et al*., 2012). Diversity indices and k dominace curve of distribution of *Aeromonas* species in fish samples demonstrated that a diverse distribution of *Aeromonas* was seen in samples from farm, whereas, more dominance was seen in samples from retail aquaria contributed mainly by *A. sobria* and *A. caviae*. This is again a reflection of change in the water quality leading to selection of some of the species. The relatively good water quality at farm level resulted in a more or less diverse distribution of motile aeromonads in water and fish samples from farm.

As the distribution of motile *Aeromonas* species in aquatic environment is significantly related to levels of pollution in waters, the high occurrence of *A. caviae* in samples from retail aquarium vendors, when compared to samples from farm indicates that water samples in farm is not polluted. There are reports that *A. caviae* predominated in waters with a high degree of faecal pollution while *A. sobria* predominated in waters with low or no faecal pollution (Imziln, 2001; Ashiru *et al*., 2011). Dumontet (2000) reported *A. caviae* to be predominant in the coastal waters of Southern Italy which was submitted to high faecal pollution. Large numbers of aeromonads, especially *A. caviae* is considered to be indicative of nutrient-rich conditions of water (Abulhamd, 2009). The small difference in the percentages and isolated species strengthens the hypothesis that the micro biota reflects the microbiological quality of the environment from where the fish come from (Suhet *et al*., 2011).

Physico-chemical analysis of water samples revealed that the concentrations of ammonia, nitrite and nitrate were within the permissible range for ornamental fish culture in samples from farm where as it was higher than the permissible range and at a level to induce stress in fish, in samples from retail aquaria.

3.6.4. Prevalence of extracellular virulence factors among motile aeromonads-Farm v/s Retail aquaria

Present study revealed frequent production of extracellular virulence factors in motile aeromonads from both farm and aquarium vendors. Gelatinase and DNase were produced by all the isolates from both farm and retail aquarists. Caseinase was produced by all the isolates of *A. sobria* from fish samples of both farm and aquarium vendors, where as all the isolates of *A. hydrophila* from farm and 90% of *A. hydrophila* isolates in fish samples from aquarium vendors, produced the enzyme. In water samples, all the isolates of *A. jandaei* and *A. sobria* from aquarium vendors exhibited caseinase production.

Lipase production was also widespread in motile *Aeromonas* isolates. All the isolates of *A. hydrophila, A. jandaei* and *A. veronii* from both farm and aquarium vendors and all the isolates of *A. sobria,* from farm and 98.5% from aquarium vendors produced lipase. In water samples, lipase was produced by all the isolates of *A. hydrophila* and *A. veronii* from farm, and all the isolates of *A. hydrophila, A. jandaei* and *A. veronii* from aquarium vendors. *Aeromonas* isolates exhibiting high levels of protease, lipase and nuclease production is reported by several authors (Shome *et al*., 1999; Sechi *et al*., 2003; Kozin´ska, 2007; Bagyalakshmi *et al*., 2009; Erdem *et al*., 2010). Proteases and phospholipases cause damage to host cells leading to cell death.

Proteases exert their virulence action by causing tissue damage and enhanced invasiveness. The lipases are considered important for bacterial nutrition and constitute as virulence factors by interacting with leucocytes or by affecting several immune system functions through free fatty acids generated by lipolytic activity. DNase may aid in the release of bacteria from disintegrating host cells in inflammatory lesions by digesting host DNA.

The results indicate that the poor water quality of the aquatic environment do not necessarily affect the virulence potential *per se*, however, it affects the distribution of different species of motile aeromonads.

β-haemolytic activity in fish samples was found to be more frequent in isolates from aquarium vendors. All the isolates of *A. hydrophila* and *A. sobria* isolates from farm were β-haemolytic, where as in samples from aquarium vendors all the isolates of *A. hydrophila*, *A. jandaei*, *A. schubertii* and *A. sobria* were β-haemolytic. In water samples all the isolates of *A. hydrophila* and *A. sobria* from farm and all the isolates of *A. hydrophila, A. jandaei* and *A. sobria* from aquarium vendors were β-haemolytic. β-haemolysins act as virulence factors by exerting lytic activities on red blood cells and inducing anaemia.

Similar to the observations in the present study high haemolytic activity for *A. hydrophila, A. jandaei* and *A. veronii* (Suhet *et al*., 2011); for *A. hydrophila* and *A. sobria* (Monfort and Baleux, 1990; Yucel and Citak, 2003; Erdem *et al*., 2010) is already reported.

There are reports of most of the *Aeromonas* isolates exhibiting these virulence factors in fish samples (Kozinska, 2007) and in water samples

(Bagyalakshmi *et al*., 2009). Yadav *et al*. (2014) also reported incidence of motile *Aeromonas* spp. with virulence potential, from water and fish samples.

In general, the prevalence of various virulence factors such as gelatinase, caseinase, DNase, lipase and β-haemolysin production was high among the isolates tested indicating their capability to act as opportunistic pathogens in the environment. This will be all the more important in retail aquaria as the water quality was found to be poor.

Chapter 4

Prevalence of antibiotic resistance and resistance profiles of motile aeromonads in ornamental fish and carriage water samples in a small scale farm and commercial aquaria

4.1. Introduction

Many ornamental fishes are very valuable as individual fish, so bacterial infections, if untreated bring heavy economic loss and financial crisis to the farmers and retail vendors involved in the sector. *Aeromonas, Citrobacter, Edwardsiella, Flavobacterium, Mycobacterium, Pseudomonas, Vibrio, Yersinia* and *Streptococcus* are the bacterial pathogens commonly involved in infections in ornamental fishes (Musa *et al*., 2008).

4.1.1. Use of antibiotics in aquaculture

Antibiotics are routinely used to treat infections caused by pathogens. Use of antibiotics can be categorized as therapeutic, prophylactic or metaphylactic.

Therapeutic use corresponds to the treatment of established infections. Metaphylaxis is a term used for group-medication procedures that aim to treat sick animals while also medicating others in the group to prevent disease. Prophylaxis means the preventative use of antimicrobials in either individuals or groups to prevent the development of infections. The concern on the overuse of antibiotics in the ornamental fish industry and its possible effect on the increasing drug resistance in both commensal and pathogenic organisms in these fish are greater than ever (Rose *et al*., 2013). Though officially there are no Food and Drug Administration (FDA)-approved antibiotics for treating ornamental fishes (Yanong, 2006), they are often used illegally. Some of the antibiotics used in the ornamental fish trade are:

4.1.1.1. β-Lactam antibiotics

The β-Lactam antibiotics include the penicillins, cephalosporins, carbapenems and monocyclic β-Lactam (monobactams). All of the members in this family possess a highly reactive β-Lactam ring. Different groups within the family are distinguished by the structure of the ring and the side chain attached to the β-lactam ring. Penicillins differ from cephalosporins in that penicillins contain a 5-membered thiazolidine ring complex and cephalosporins contain a 6-membered dihydrothiazine β-Lactam ring complex.

The penicillins, including penicillin, amoxicillin and ampicillin are most effective against Gram-positive bacteria; *Aeromonas* have an intrinsic resistance towards this antibiotic. The cephalosporins are broad-spectrum antibiotics. An added advantage of the cephalosporins is that they are relatively stable in the presence of β-lactamases. An example of a potent, naturally occurring cephalosporin is cefoxitin.

4.1.1.2. Erythromycin

Erythromycin is most effective against Gram-positive bacteria, such as *Streptococcus* species. The vast majority of bacteria that cause disease in fish are Gram-negative, so erythromycin should only be used after culture and sensitivity test results confirm it as effective.

4.1.1.3. Tetracyclines

Oxytetracycline and related antibiotics are considered broad-spectrum antibiotics (effective against a wide variety of bacteria), and they work well when mixed with food. Terramycin ® is a brand of oxytetracycline that is FDA approved for use in the production of salmonids, channel catfish and lobsters. There is evidence suggesting that oxytetracycline can suppress immune functions in Carp, Rainbow trout, Turbot and Atlantic cod (Romero *et al*., 2012).

4.1.1.4. Aminoglycosides

The aminoglycosides including gentamicin, neomycin, kanamycin and amikacin are very effective against Gram-negative bacterial infections when administered by injection. Unfortunately, this group has also been shown to cause kidney damage in fish when administered by this technique. As a group, these antibiotics are not considered effective when used in oral or bath treatments. A couple of exceptions may be kanamycin and neomycin, both of which may be effective against external infections if used in bath treatments.

4.1.1.5. Quinolones

The quinolones, including nalidixic acid and oxolinic acid are considered broad-spectrum antibiotics, like the tetracyclines, and they work against a wide variety of bacteria. Quinolones are excreted unaltered and are very persistent in

the environment. They have been shown to damage the nervous system of other animals. Fluoroquinolones are categorized as of high regulatory concern by the FDA.

4.1.1.6. Nitrofurans

The nitrofurans, including nitrofurantoin, nitrofurazone, furanace and furazolidone are commonly used in the ornamental fish trade, but the FDA strictly forbids their use by producers of food fish.

4.1.1.7. Sulfa drugs

The sulfa drugs, including Romet®, are also considered to be broad-spectrum antibiotics. The more common sulfa drugs are not as effective as they once were, due to their misuse or overuse resulting in the creation of many bacteria that are now resistant to them. Romet® works well when mixed with feed, but it does not work well as a bath treatment.

4.1.2. Major Routes of Administration of antibiotics

4.1.2.1. Injection

Injection is very labor intensive and impractical for commercial aquaculture, though it is the most direct and effective method for getting antibiotics into the blood stream. However, for small numbers of fish, or for important or expensive fish, injection may be the best method.

4.1.2.2. Oral administration

The most cost effective and commonly used method of administering antibiotics in aquaculture is by mixing them with feed. The proper dose of antibiotic is mixed with the feed during preparation, or it is added after preparation, using a binding agent. The mixture is then fed to the fish for the

prescribed number of days. Oral administration of antibiotics requires that fishes must be able to take feed, so only fishes that are able to consume feed will be treated.

4.1.2.3. Bath treatments

Although bath treatments are a popular method of administering antibiotics, much more drug is required to achieve the desired result as compared to oral treatments or injections. In many cases, even a large amount of antibiotic in the water does not guarantee that enough of it will get into the fish for an effective treatment. At the same time, excessive amounts of antibiotic in the water can increase the likelihood of water-borne bacteria developing resistance to the drug. Bath treatments should be considered only when majority of the fish are unable to consume food, and should be switched to oral medications as soon as the fishes resume eating.

4.1.3. Mechanism of action

β-lactam antibiotics (penicillins, cephalosporins) act by inhibiting bacterial cell wall synthesis. They block the synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer.

Aminoglycosides, tetracyclines and chloramphenicol work by inhibiting protein synthesis. These antibacterial drugs take advantage of the structural differences between bacterial and eukaryotic ribosomes to selectively inhibit bacterial growth. Aminoglycosides and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit.

Quinolones exert their antibacterial effects by disrupting DNA synthesis and causing lethal double-strand DNA breaks during DNA replication. For

example, the bactericidal action of the fluoroquinolone, ciprofloxacin results from the inhibition of topoisomerase II (DNA gyrase) and topoisomerase IV (both Type II topoisomerases), which are required for bacterial DNA replication, transcription, repair and recombination.

Nitrofurantoin acts by inactivating or altering bacterial ribosomal proteins and other macromolecules. As a result of such inactivation, the vital biochemical processes of protein synthesis, aerobic energy metabolism, DNA synthesis, RNA synthesis and cell wall synthesis are inhibited.

Sulfonamides and trimethoprim (TMP) block the pathway for folic acid synthesis, which ultimately inhibits DNA synthesis. Sulfonamides block the conversion of para-aminobenzoic acid to dihydrofolic acid by inhibiting the enzyme dihydrofolate synthetase. Trimethoprim blocks the conversion of dihydrofolic acid to tetrahydrofolic acid by inhibiting dihydrofolate reductase.

4.1.4. Development and mechanism of resistance

Several mechanisms have been developed by bacteria to deal with antibiotics but all require either the modification of existing genetic material or the acquisition of new genetic material. Originally it was believed that all resistance was acquired through spontaneous mutation. The widespread development of multi-drug resistance in many species of bacteria led to the notion that another mechanism beyond spontaneous mutation was responsible for the acquisition of antibiotic resistance. Lateral or horizontal gene transfer (HGT) is a process whereby genetic material contained in small packets of DNA can be transferred between individual bacteria. There are three possible mechanisms of HGT. These are transduction, transformation or conjugation.

Different mechanisms of resistance in a bacterial cell may cooperate and determine the final level of resistance. These mechanisms are: efflux pumps, reduced permeability of cell membranes, inactivation of the antibiotic by enzymes, alteration of the target of the antibiotic reducing its affinity for it and duplication of the drug target with a resistant form (Acar and Moulin, 2012).

4.2. Review of literature

4.2.1. Use/misuse of antibiotics in aquaculture: a public health concern

Intensive aquaculture has led to rising problems with bacterial diseases, the treatment of which now requires the routine use of antimicrobial agents (Rawn *et al*., 2009). The major concerns with treating fish diseases with antibiotics are the potential impact of antimicrobials on the aquatic environment, both marine and fresh water, and the risks associated with the development of antimicrobial resistance by fish pathogens as well as commensals. Treatments with antimicrobial agents create an inevitable selection on resistant bacteria. When resistant, bacteria survive and multiply, to create even more resistant bacteria. In addition, sub-therapeutic doses of antibiotics used for growth promotion could promote resistance (Marshall and Levy, 2011). It is well documented that fish pathogens and other aquatic bacteria can develop resistance as a result of antimicrobial exposure. Examples include *Aeromonas salmonicida, A. hydrophila, Edwardsiella tarda, Yersinia ruckeri, Photobacterium damselae* and *Vibrio anguillarum* (Romero *et al*., 2012).

Genetic determinants of antibiotic resistance that have been described in aquaculture environment are regularly located on mobile genetic elements. Indeed, resistance genes have been found located on transferable plasmids and integrons in pathogenic bacteria such as *Aeromonas* spp., *Edwardsiella* spp.

and *Vibrio* spp. (Defoirdt *et al*., 2011). Resistance genes efficiently circulate among bacterial genus. Due to the exchange of resistance genes, non-pathogenic bacteria can become host of potential dangerous resistance genes. When non-pathogenic bacteria exchange these genes to pathogenic bacteria, (multi) resistant bacteria can develop (Bogaard and Stobberingh, 2000).

A number of investigations have indicated that the wide and irrelevant antibiotics usage leads to the selection of genes encoding the resistance. A danger of resistance exists among 5-10% of infections treated, which leads to a lack of success or a prolonged treatment (Orozova *et al*., 2010). According to a number of authors, the infections caused by resistant bacteria increase the risk of a higher degree of death and a wider spreading as a result of their adaptation towards different aquatic environment (Kollef *et al*., 1999; Helms *et al*., 2002; Cosgrove *et al*., 2003; Orozova *et al*., 2010). The continuous use of antimicrobial agents in aquaculture has resulted in more resistant bacterial strains in the aquatic environment (Madhuri *et al*., 2012).

Bacterial pathogens that are capable of infecting both humans and animals are considered as the major offenders for the origin and spread of antibiotic resistance in the environment (Prashant *et al*., 2013). Studies have established that plasmids, integrons and associated antimicrobial resistance (AMR) genes of bacteria recovered from the aquatic environment can share very high nucleotide sequence homology to clinically important strains (Schmidt *et al*., 2001; Sørum *et al*., 2003; McIntosh *et al*., 2008; Verner-Jeffreys *et al*., 2009). Therefore, the increasing rates of antimicrobial resistance observed among all types of bacterial communities are perceived as a potential danger for human health. Global problem with antibiotic resistance,

raises concern that treatments for fish diseases may not work when needed, creating yet another mechanism for exposing humans to antibiotic-resistant bacteria (Rose *et al*., 2013). It is of great fear that we will go back to the period where there was no antibiotics available, if the number of resistant bacteria continues to increase (Figure 4a).

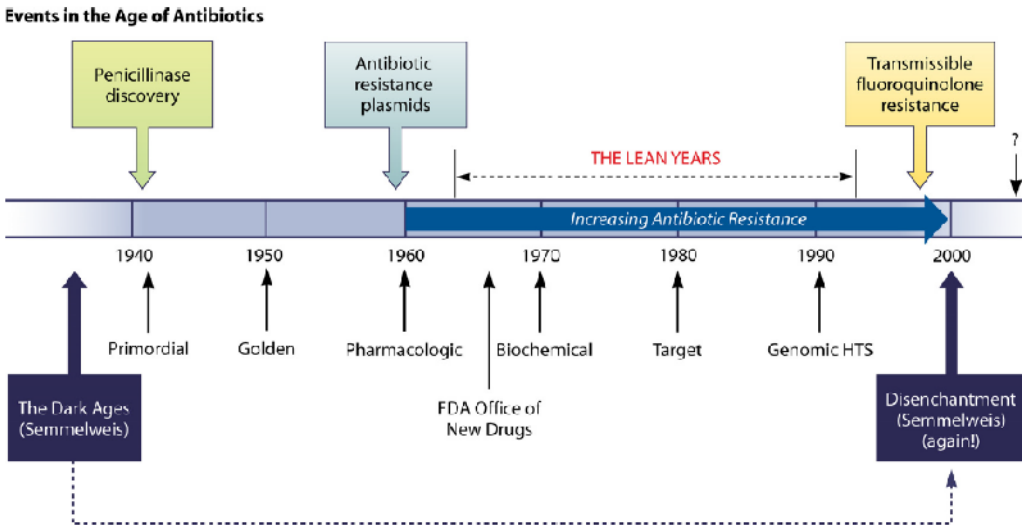


Figure 4a. The course of an era before antibiotics, through a period of little antibiotic resistance towards a period where a fear is developing, that no antibiotics are being effective against bacteria, resulting in the same effects as in the era of no antibiotics (Davies and Davies, 2010).

Currently, antibiotics are only partially effective due to the emergence of resistant bacteria and therapeutic treatments may have limited success at controlling infectious bacterial diseases (Romero *et al*., 2012).

Antibiotics enter the environment as a result of leaching from faeces and uneaten antibiotic feed and it has been estimated that a minimum of 75% of most of the antibiotics in feed used in aquaculture systems are exported to the surrounding environment and accumulate in the sediment (Romero *et al*.,

2012; Olatoye and Basiru, 2013). Several investigations report an increase in the prevalence of resistant bacteria isolated from water, sediments or wild animals near fish farms (Schmidt, *et al*., 2001; Giraud, 2004). Antimicrobials in waste water are increasingly found and potentially have an important role in the rise and selection of antimicrobial resistance in the environment (Kummerer and Henninger, 2003). Dissemination of antibiotics and antibiotic resistance in the environment is depicted in Figure 4b.

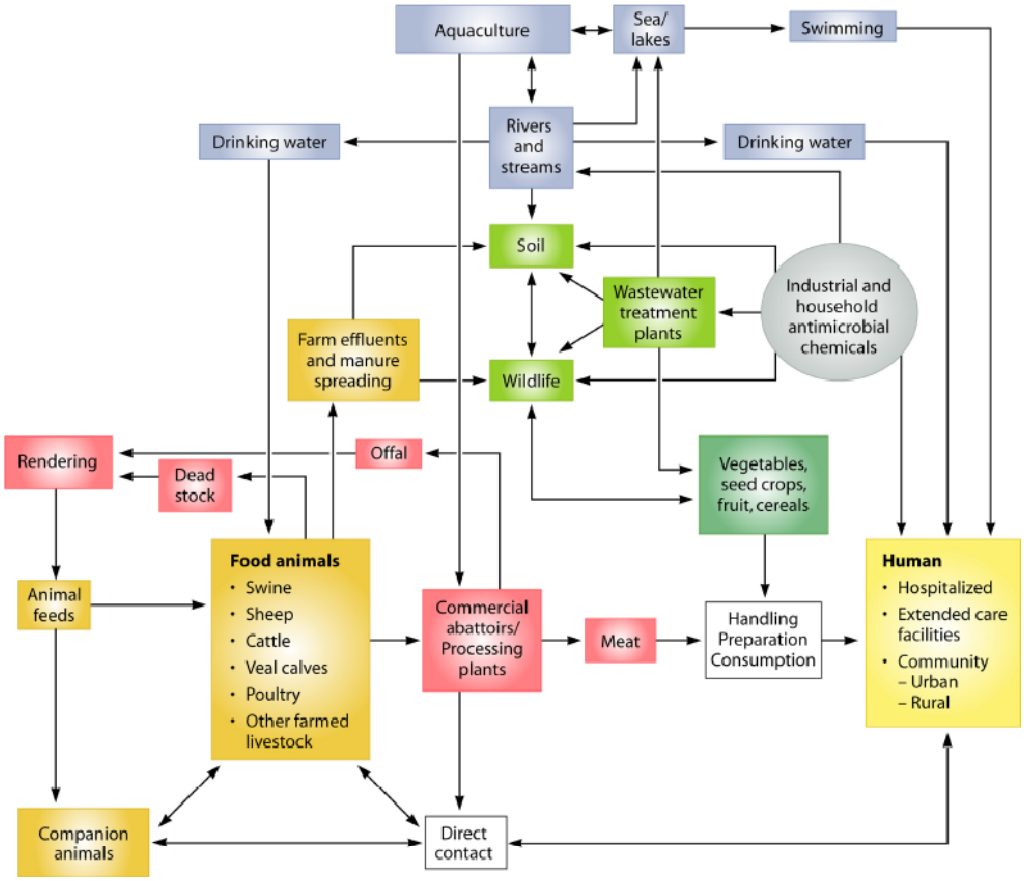


Figure 4b. Dissemination of antibiotics and antibiotic resistance within agriculture, community, hospital, wastewater treatment and associated environment (Davies and Davies, 2010).

Alderman and Hastings (1998) noted that controls on antibiotic use in aquaculture vary widely from country to country. In developed countries such as members of the European Union, the United States, Canada and Norway, regulatory control is strong and use of antibiotics is declining because of improvements in management and development of effective vaccines. However, 90% of aquaculture production occurs in developing countries, where regulatory controls are weak and use of antibiotics appears to be widespread (Akinbowale *et al*., 2006).

There are no precise reports to indicate the accurate use of antimicrobials in the ornamental fish industry, and it is suggested that the use of antimicrobials is widespread and not through the consult of a veterinarian (Van Waart, 2010). Increased use of antimicrobial agents has resulted in a rise in the occurrence of antimicrobial resistance in several bacterial populations (Rose *et al*., 2013), especially in developing countries where antimicrobials are readily available without prescription (Olatoye and Basiru, 2013), and the situation is more panicking in India, due to adverse effect of antibiotic resistant organism on human health resulting from non-human usage of antimicrobials (Prashant *et al*., 2013).

4.2.2. Antibiotic profile of motile aeromonads-world scenario

High level of antibiotic resistance against cephalexin, chloramphenicol, erythromycin, nitrofurantoin, sulfamethoxazole/trimethoprim in *Aeromonas hydrophila, A. sobria* and *A. caviae* is reported by Afizi *et al*. (2013); the isolates were sensitive to gentamicin and ciprofloxacin. Antibiotic resistant *Aeromonas* isolates in ornamental fishes imported to North America from Colombia, Singapore and Florida is also reported by Rose *et al*. (2013).

Antibiotic resistance profile of different *Aeromonas* species such as *A. veronii, A. media, A. jandaei, A. hydrophila, A. caviae, A. culicicola* and *A. aquariorum* isolated from the skin of ornamental fishes imported to Portugal was studied by Dias *et al*. (2012). Results revealed resistance levels of more than 80% to the antibiotics tetracycline, ticarcillin, carbenicillin, ampicillin and erythromycin. Ciprofloxacin and norfloxacin resistance was more prevalent among *A. hydrophila* isolates (43% and 34% respectively) than the other species. All the strains presented multi-resistance to the tested antibiotics. Antibiotic susceptibility tests of *A. schubertii* isolated from diseased Snakeheads in China by Chen *et al*. (2012) showed that it was susceptible to cefoxitin, cefoperazone and chloramphenicol, however, resistant to policyclin, cefotamine, penicillin G, clindamycin and oxacillin.

In a study conducted in Nigeria, by Ashiru *et al*. (2011), *Aeromonas caviae*, *A. sobria* and *A. hydrophila* from fish samples were reported to be highly susceptible to pefloxacin, ofloxacin and ciprofloxacin, while they were randomly susceptible to ceftriazone, gentamicin, cotrimoxazole and amoxicillin but resistant to tetracycline, nitrofurantoin and augmentin. Čížek *et al*. (2010) investigated the antibiotic susceptibility of *Aeromonas* isolates from ornamental carps and found that 50% of the isolates were resistant to oxytetracycline, 25% to ciprofloxacin, 15% to trimethoprim and 7% to chloramphenicol and florfenicol.

Antimicrobial sensitivity patterns of the *Aeromonas* isolates studied by Abulhamd (2010) revealed that 100% were sensitive to gentamicin, 80% to sulfamethoxazole–trimethoprim, 70% to chloramphenicol, 50% to ciprofloxacin, 40% to neomycin, 30% to tetracycline, 20% to streptomycin and

10% to erythromycin. All the isolates were resistant to novobiocin and bacitracin. In another study in Malaysia, undertaken by Lee *et al*. (2009) all the *Aeromonas* isolates from Asian seabass were found to be sensitive to nitrofuratoin, furazolidone, tetracycline, doxycycline, flumequine, oxolinic acid and florfenicol.

Aeromonas isolates from food samples such as fish, water, fruits and vegetables in Abu Dhabi were investigated for antibiotic susceptibility profile by Awan *et al*. (2009). Most strains were resistant to penicillins (ticarcillin, mezlocillin, oxacillin, piperacillin), sulfamethoxazole, trimethoprim and macrolides (erythromycin, vancomycin, clindamycin) but sensitive to tetracycline, chloramphenicol, nitrofurantoin, aminoglycosides (amikacin, gentamicin, tobramycin), cephalosporins (cefuroxime, ceftrioxone, cefazolin, cephalexin, cephalothin, cefoxitin, cefotaxime), quinolone (ciprofloxacin), colistin sulfate and trimethoprim-sulfamethoxazole. The degree of antibiotic resistance in Catfish and Eel farms in the southern part of Netherlands was examined by Penders and Stobberingh (2008). The prevalence of resistance was as follows: ampicillin and oxytetracycline 100%; sulfamethoxazole 24%; trimethoprim 3%; and ciprofloxacin and chloramphenicol 0%. The majority of samples showed a high degree of oxytetracycline resistance.

Akinbowale *et al.* (2006) reported that resistance to ampicillin, amoxycillin, cephalexin and erythromycin was widespread among *Aeromonas* isolates in aquaculture systems in Australia. Resistance to oxytetracycline, tetracycline, nalidixic acid and sulfonamides was common but resistance to chloramphenicol, florfenicol, ceftiofur, cephalothin, cefoperazone, oxolinic acid, gentamicin, kanamycin and trimethoprim was less common. All strains

were susceptible to ciprofloxacin. Multiple antibiotic resistance was also observed by them. All the aeromonads (*A. hydrophila, A. veroni bv. sobria, A. caviae*) isolated from market fish samples collected from Turkey by Yucel *et al.* (2005) were susceptible to ciprofloxacin and ceftriaxone, but were resistant to ampicillin, cephalothin and trimethoprim.

Kudinha *et al*. (2004) tested *Aeromonas* isolates from various origins for susceptibility to antimicrobial drugs. All the *Aeromonas* isolates were sensitive to neomycin and gentamicin and resistant to ampicillin. Overall resistance to antibiotics was significantly higher in *A. hydrophila* and *A. sobria* isolates than *A. caviae.* Multiple drug resistance occurred more in *A. hydrophila* isolates than *A. sobria* and *A. caviae.* The most common multi-drug resistance in all three *Aeromonas* species was the combination of tetracycline, streptomycin and furazolidone, accounting for about 20% of all isolates. Findings of this study suggest that *A. hydrophila* isolates are more resistant to antibiotics than the other two.

Oxytetracycline resistance frequencies are reported to be high among environmental *Aeromonas* isolates (Schmidt *et al*., 2000). Huys *et al*. (2000) found oxytetracycline resistant strains in water from fish farms. Goni-Urriza *et al*. (2000) found as many as 49% tetracycline-resistant *Aeromonas* species in their study. There are significantly more resistant *Aeromonas* detected in samples of outlet water, compared to inlet water of some fresh water fish farms (Schmidt *et al*., 2000) indicating some sort of selection on the way. Ko *et al*. (1996) reported high resistance to tetracyclines, trimethoprim-sulfamethoxazole, some extended-spectrum cephalosporins (ceftriaxone, cefotaxime, cefixime), and tobramycin. Development of antibiotic resistance as a

major problem in treating *Aeromonas* infections is reported in early literature (Aoki and Egusa, 1971; Mitchell and Plumb, 1980).

4.2.3. Antibiotic profile of motile aeromonads–Indian scenario

Aeromonas isolates from tropical seafood (squid, prawn and mussel), sediment and water samples from aquafarms and associated mangroves in Kerala were screened for antibiotic sensitivity by Joseph *et al*. (2013). The isolates were susceptible to gentamicin, streptomycin, trimethoprim, cefixime and chloramphenicol and exhibited varying degrees of resistance to ampicillin, nalidixic acid, tetracycline and co-trimaxozole. MAR index value greater than 0.2, indicative of the high risk environment was shown by 20% of *Aeromonas* spp. *Aeromonas* isolates obtained from ornamental fish samples in Kerala, exhibiting resistance to different antibiotics is reported by Sreedharan *et al*. (2012). Patil *et al*. (2011) isolated *Aeromonas* from diseased Blue gourami and from the aquarium water that housed the fish. Antimicrobial susceptibility assay indicated that the strains were sensitive to nitrofurantoin, enrofloxacin and furazolidone, and significantly resistant to erythromycin, oxytetracycline and tetracycline.

In a study conducted by Kaskhedikar and Chhabra (2010), fourteen antibacterial agents belonging to nine different groups' viz. aminoglycosides, cephalosporins, nitrofurantoin, fluroquinolones, chloramphenicol, sulphonamides, tetracyclines, penicillin and polymixin were used for *in vitro* sensitivity testing of *Aeromonas hydrophila* isolated from samples of fish, collected from retail shops in India. They observed complete sensitivity to ciprofloxacin, cefuroxime, ceftriaxone, cephotaxime, chloramphenicol, gentamicin, kanamycin, nitrofurantoin, nalidixic acid and ofloxacin. All the isolates were resistant to ampicillin and colistin

antibiotics. Multiple drug resistance was also observed in all *A. hydrophila* isolates.

Thayumanavan *et al*. (2007) reported that all the isolates of *Aeromonas hydrophila* obtained from retail sea food outlets in Coimbatore were sensitive to chloramphenicol and ciprofloxacin and resistant to bacitracin. Different species of motile aeromonads such as *A. hydrophila, A. veronii, A. sobria, A. caviae* and *A. schubertii* obtained from fish and water samples by Rathore *et al*. (2005) were sensitive to gentamicin and ciprofloxacin and resistant to erythromycin, furazolidone and penicillin. Oxytetracycline was found to be effective against only 60% of the isolates.

Aeromonas spp. of fresh water fish farm were reported to have relatively lower level of resistance to chloramphenicol, gentamicin and nalidixic acid (Hatha *et al*., 2005). However, a higher level of resistance to ampicillin, novobiocin, amoxicillin and oxytetracycline was exhibited by the isolates. *Aeromonas* spp. associated with ornamental fishes was sensitive to chloramphenicol, ciprofloxacin and gentamicin, but exhibited resistance towards co-trimoxazole, nitrofurantoin and oxytetracycline (Sasmal *et al*., 2004).

Antimicrobial resistance of *Aeromonas* isolates from water samples from a river in India was done by Bagyalakshmi *et al.* (2009). All the isolates were resistant to ampicillin, carbenicillin and cephalothin. The highest resistances encountered were 91.9% to streptomycin, 90.8% to polymyxin-B and 85% to rifampicin. In contrast, all the strains were sensitive to cefotaxime.

Ye *et al.* (2013) studied the antibiotic susceptibility of *Aeromonas hydrophila* from hemorrhagic diseased freshwater fishes in China and found that all the

isolates were sensitive to chloramphenicol, streptomycin, and norfloxacin and resistant to penicillin. In an investigation by Odeyemi *et al*. (2012), all the *A. hydrophila* isolates were resistant to 6-10 antibiotics. The antibiotics tested were vancomycin, polymycin, ciprofloxacin, tetracycline, neomycin, novobiocin, ampicillin, kanamycin, oxytetracycline and bacitracin.

Antibiotic sensitivity test for *Aeromonas hydrophila* isolated from diseased Catfish was studied by Jayavignesh *et al*. (2011). The isolate screened was found to be sensitive to chloramphenicol, sulfamethazole, ciprofloxacin, rifampicin, neomycin and pefloxacin and resistant to ampicillin, amoxicillin, erythromycin, clindamycin, cloxacillin, oxytetracycline and streptomycin.

The *in vitro* antibiotic susceptibility of *A. hydrophila* isolated from various naturally infected fishes collected from both fresh and brackish water to different antibiotics was evaluated by El-Barbary (2010). Oxytetracycline only was an effective antibiotic for all tested isolates. All the isolates were resistant to amoxicillin, ampicillin and penicillin.

In a study by Jongjareanjai *et al*. (2009), chloramphenicol showed the highest efficacy against the *A. hydrophila* isolates tested. Other effective antibiotics included sulfamethoxazole-trimetroprim and amikacin. All the isolates showed resistance to metronidazole and 92.31 % were resistant to penicillin and amoxicillin.

A. hydrophila isolates from wild *Piaractus mesopotamicus* and *Oreochromis niloticus* in Brazil were found highly resistant to amoxicillin, ampicillin, lincomycin, novobiocin, oxacillin, penicillin and trimetoprim-sulfametoxazole (Belem-Costa and Cyrino, 2006). Multiple antibiotic resistance has been

registered for *A. hydrophila* isolated from freshwater fish farms in association with a variety of drugs, commonly used as feed additives (Son *et al*., 1997; Vivekanandhan *et al*., 2002).

4.3. Objectives of the study

Emerging antibiotic resistance to diverse groups of antibiotics seems to be common in *Aeromonas* (Carvalho *et al*., 2012). The potential of aeromonads to develop and disseminate antibiotic resistance either in the aquatic environment (Huddleston *et al*., 2006; Blasco *et al*., 2008; Cattoir *et al*., 2008; Gordon *et al*., 2008; Lamy *et al*., 2009; Abulhamd, 2010; Arias *et al*., 2010; Figueira *et al*., 2011; Joseph *et al*., 2013) or in ornamental fish samples (Sasmal *et al*., 2004; Cizek *et al*., 2010; Dias *et al*., 2012; Sreedharan *et al*., 2012; Rose *et al*., 2013) is well documented. *Aeromonas* spp. is environmental reservoirs of resistance genes to different classes of antibiotics. Most *Aeromonas* isolates have intrinsic or chromosomally mediated resistance against ampicillin (Ghenghesh *et al*., 2013). This can lead to transfer of resistance genes to other commensal or pathogenic bacteria.

In ornamental fish culture systems antimicrobials are used by owners and retailers to directly control bacterial infections. They are also added to the water these fishes are transported to suppress the growth of potential pathogens during transport. As a consequence, antibiotic tolerant bacteria have likely been selected for and proliferate in the trade (Verner-Jeffreys *et al*., 2009). Complicating matters is the fact that many antibiotics used to fight fish bacteria are the same ones widely used on humans. The antibiotic classes for the sensitivity testing are selected on the basis of their importance to human health and misusage in aquaculture systems. Use of antibiotics for treatment or

better feed conversion is bound to vary according to local practises. So it is important to recognize the extent to which the bacteria associated with ornamental fishes have developed antimicrobial resistance in this locality.

The objectives of the present study are to investigate the prevalence of antibiotic resistance among motile aeromonads from two systems of production such as a small scale organic farm where antibiotics are not used and the other from commercial ornamental fish trade where the fishes are from large production farms elsewhere outside Kerala state. The specific objectives are as follows:

1) To find out the prevalence of antibiotic resistance in motile aeromonads isolated from ornamental fish and water samples from the farm.
2) To determine the variation in prevalence of antibiotic resistance among different species of motile aeromonads from ornamental fish and water samples from the farm.
3) To perform MAR indexing and determine the antibiotic resistance pattern of motile aeromonads from the farm.
4) To find out the prevalence of antibiotic resistance in motile aeromonads isolated from ornamental fish and water samples from aquaria.
5) To determine the variation in prevalence of antibiotic resistance among different species of motile aeromonads from ornamental fish and water samples from aquaria.
6) To perform MAR indexing and determine the antibiotic resistance pattern of motile aeromonads from aquaria.

4.4. Material and Methods

4.4.1. Bacterial isolates

Aeromonas isolates obtained from ornamental fish and water samples collected from an ornamental fish farm and retail aquarium vendors were used for the antimicrobial susceptibility test. From the farm, 145 isolates of motile aeromonads from fish samples and 156 isolates from water samples were used for the test. From the aquaria, 175 isolates from fish samples and 182 isolates from water samples were used. The number of isolates belonging to different species is given in Table 4.1.

Table 4.1. Number of motile aeromonads belonging to different species used in the antimicrobial susceptibility test

***Aeromonas* spp.**	**Farm samples**		**Aquarium samples**	
	Fish	**Water**	**Fish**	**Water**
A. caviae	12	16	55	30
A. hydrophila	24	15	10	25
A. jandaei	29	20	6	15
A. schubertii	14	8	3	8
A. sobria	42	54	71	63
A. trota	13	37	15	16
A. veronii	11	6	15	25

4.4.2. Antimicrobial susceptibility test

Susceptibility to antimicrobial agents was performed for the identified *Aeromonas* isolates by the disc diffusion method (Bauer *et al*., 1966). Pure cultures of *Aeromonas* were inoculated into nutrient broth and incubated at 37^0C for 6-8 h. The cultures were then seeded onto sterile Mueller Hinton agar plates using a sterile cotton swab. After the inoculums has dried, antibiotic discs were

placed on the surface of the agar with sterile forceps and pressed down gently to ensure even contact. Diameter in mm of clear zones surrounding the antibiotic discs indicating bacterial growth inhibition was measured after 16-18 h incubation at 37^0C and susceptibility/resistance interpretation was performed according to the manufacturer's interpretative table supplied by the Hi-media laboratories, Bombay as a matching criteria.

A panel of 14 antibiotics which belonged to 9 different classes was included in this study (Table 4.2).

Table 4.2. Antibiotics used and their concentration

Antibiotics	Code	Concentration (mcg)
Penicillins		
Amoxicillin	Amx	30
Carbenicillin	Cb	100
Cephalosporins		
Cephalothin	Cep	30
Cefpodoxime	Cpd	10
Ceftazidime	Caz	30
Aminoglycosides		
Gentamicin	Gen	10
Streptomycin	S	10
Tetracyclines		
Tetracycline	Te	30
Chloramphenicol		
Chloramphenicol	C	30
Quinolones		
Nalidixic acid	Na	30
Fluroquinolones		
Ciprofloxacin	Cip	5
Nitrofurantoin		
Nitrofurantoin	Nit	100
Sulfonamides		
Sulfafurazole	Sf	300
Trimethoprim	Tr	5

4.4.3. Multiple Antibiotic Resistance (MAR) indexing

The MAR index was calculated for each isolate separately and was determined as a/b, where 'a' is the number of antibiotics towards which the isolate is resistant, and 'b' is the number of antibiotics, towards which the isolate was subjected to susceptibility testing. The MAR index, which is higher than 0.2 identifies bacteria isolated from environment with higher risk of contamination, where antibiotics has been often used. The MAR index less than 0.2 identify strains from the environment, where antibiotics are rarely used or are not used at all (Krumperman, 1983).

4.4.4. Statistical analysis

Statistical analysis was performed by Chi-Square test. Difference was considered significant when $p<0.05$.

4.5. Results

4.5.1. Antibiotic resistance of motile aeromonads in samples from an ornamental fish farm

4.5.1.1. Prevalence of antibiotic resistance in motile aeromonads from ornamental fish samples

Figure 4.1 represents the prevalence of antibiotic resistance in motile aeromonads from ornamental fish samples. All the *Aeromonas* isolates tested exhibited resistance to amoxicillin, while, all the isolates were sensitive to ceftazidime, ciprofloxacin, chloramphenicol, gentamicin, sulfafurazole and trimethoprim.

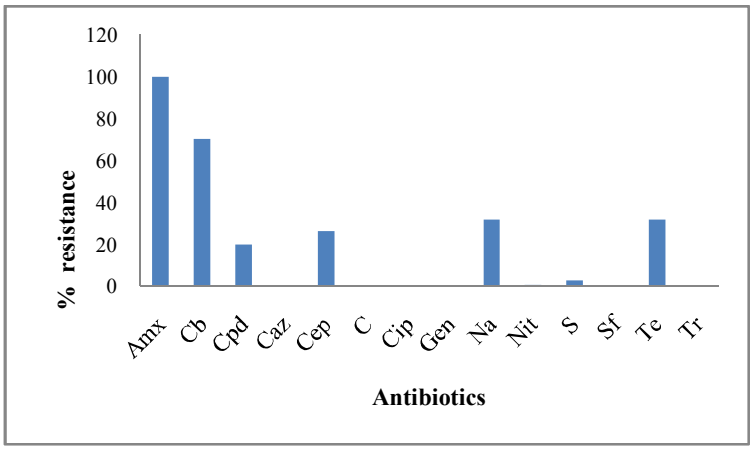


Figure 4.1. Prevalence of antibiotic resistance in motile aeromonads from ornamental fish samples

4.5.1.2. Prevalence of antibiotic resistance in motile aeromonads from farm water samples

Figure 4.2 represents the prevalence of antibiotic resistance in motile aeromonads from water samples. Resistance to amoxicillin was exhibited by all the *Aeromonas* isolates tested, whereas all of them were susceptible to ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin and streptomycin.

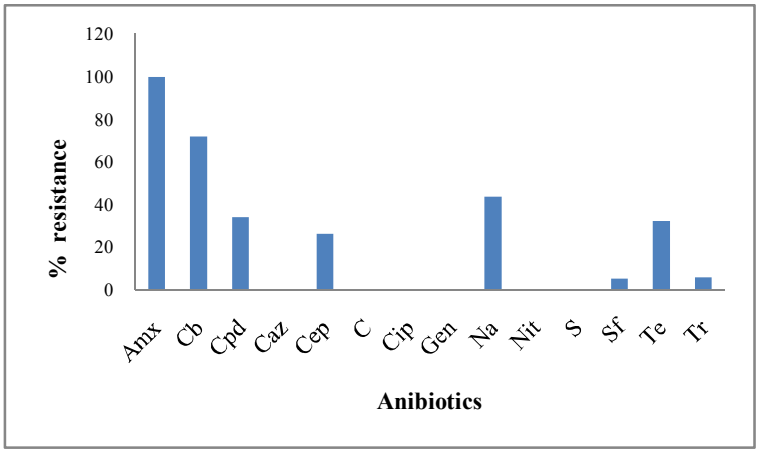


Figure 4.2. Prevalence of antibiotic resistance in motile aeromonads from water samples

4.5.1.3. Variation in prevalence of antibiotic resistance among various species of motile aeromonads in fish samples from farm

Table 4.3 represents the resistance of different species of *Aeromonas* to the antibiotics tested. All the *Aeromonas* isolates tested exhibited resistance to amoxicillin. A high degree of resistance to cefpodoxime was exhibited by *Aeromonas caviae* and *A. hydrophila* and a lower level of resistance to this drug by *A. jandaei* (17.24%) and *A. veronii* (9.09%); all the other species were susceptible to this antibiotic. Among the different species tested only *Aeromonas caviae* (8.3%) and *A. sobria* (9.5%) was resistant to nitrofurantoin and streptomycin respectively. All the isolates tested were susceptible to ceftazidime, ciprofloxacin, chloramphenicol, gentamicin, sulfafurazole and trimethoprim.

Table 4.3. Variation in prevalence of antibiotic resistance among different species of motile aeromonads in ornamental fish samples from farm

Antibiotic code	% of resistance among various species of motile aeromonads						
	A. caviae	***A. hydrophila***	***A. jandaei***	***A. schubertii***	***A. sobria***	***A. trota***	***A. veronii***
Amx	100	100	100	100	100	100	100
Cb	58.33	70.83	82.75	64.28	71.42	53.84	72.72
Cpd	58.33	66.66	17.24	0	0	0	9.09
Caz	0	0	0	0	0	0	0
Cep	41.66	100	31.03	0	0	0	0
C	0	0	0	0	0	0	0
Cip	0	0	0	0	0	0	0
Gen	0	0	0	0	0	0	0
Na	41.66	29.16	17.24	28.57	35.71	23.07	63.63
Nit	8.33	0	0	0	0	0	0
S	0	0	0	0	9.52	0	0
Sf	0	0	0	0	0	0	0
Te	16.66	37.5	17.24	14.28	54.76	15.38	27.27
Tr	0	0	0	0	0	0	0

4.5.1.4. Variation in prevalence of resistance among various *Aeromonas* spp. in water samples from farm

Table 4.4 represents the resistance of different species of *Aeromonas* to the antibiotics tested. Resistance to amoxicillin was exhibited by all the *Aeromonas* isolates tested. Except *Aeromonas schubertii,* all the other species exhibited resistance to cefpodoxime; a high degree of resistance to this drug was exhibited by *A. hydrophila* (66.66%) and *A.caviae* (50%) and a lower level of resistance by other species (≤ 35%). All the isolates of *A. hydrophila* were resistant to cephalothin; and around 50% of the isolates of *A. caviae* and *A. jandaei* and 24.3% of the isolates of *A. trota* were also resistant to this drug. All the species of *Aeromonas* except *A. jandaei* and *A. veronii* exhibited resistance to tetracycline. Among the different species tested, only *A. sobria* (around 15%) was resistant to sulfafurazole and trimethoprim. All the isolates tested were susceptible to ceftazidime, ciprofloxacin, chloramphenicol, gentamicin, nitrofurantoin and streptomycin.

Table 4.4. Variation in prevalence of antibiotic resistance among different species of motile aeromonads in water samples from farm

Antibiotic code	% of resistance among various species of motile aeromonads						
	A. caviae	***A. hydrophila***	***A. jandaei***	***A. schubertii***	***A. sobria***	***A. trota***	***A. veronii***
Amx	100	100	100	100	100	100	100
Cb	62.5	73.33	80	75	70.37	70.27	83.33
Cpd	50	66.66	25	0	35.18	27.02	16.66
Caz	0	0	0	0	0	0	0
Cep	43.75	100	50	0	0	24.32	0
C	0	0	0	0	0	0	0
Cip	0	0	0	0	0	0	0
Gen	0	0	0	0	0	0	0
Na	31.25	33.33	25	37.5	40.74	67.56	50
Nit	0	0	0	0	0	0	0
S	0	0	0	0	0	0	0
Sf	0	0	0	0	14.81	0	0
Te	12.5	40	0	12.5	55.55	29.72	0
Tr	0	0	0	0	16.66	0	0

4.5.1.5. MAR index of motile aeromonads in fish samples from ornamental fish farm

Figure 4.3 shows the MAR index of motile aeromonads isolated from fish samples of farm. MAR index value greater than 0.2 was exhibited by 59.31% of the isolates. The value ranged from 0.21 to 0.43.

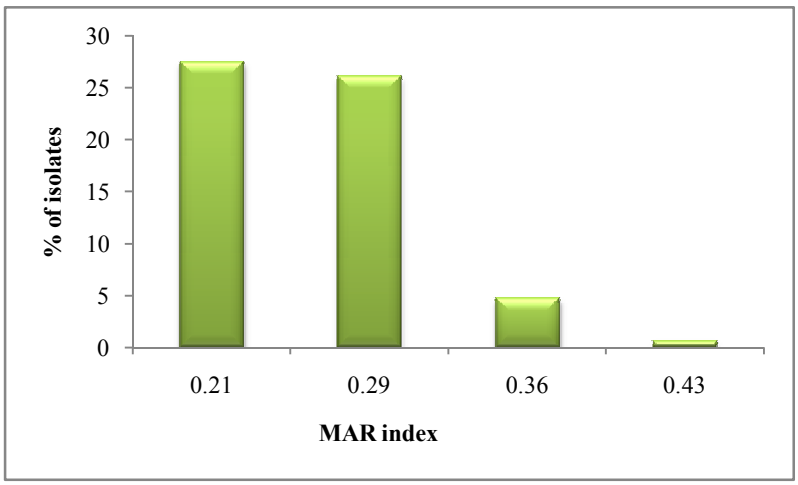


Figure 4.3. MAR index of motile aeromonads in ornamental fish samples from farm

4.5.1.6. MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads from ornamental fish samples

Table 4.5 shows the MAR index, resistance pattern and prevalence of specific resistance pattern among different species of motile aeromonads (MAR index ≥ 0.21) from ornamental fish samples of farm. Among the different species *A. hydrophila* was found to exhibit resistance towards more number of antibiotics (6) with a MAR index value of 0.43.

Table 4.5. MAR index, resistance pattern and prevalence of specific resistance pattern among different species of motile aeromonads in ornamental fish samples from farm

***Aeromonas* spp.**	**MAR index**	**Resistance pattern**	**Prevalence of specific pattern (%)**
A. caviae			
	0.21	AmxCpdTe	16.7
	0.21	AmxCbCpd	8.33
	0.21	AmxCbNa	8.33
	0.29	AmxCpdCepNa	25
	0.29	AmxCbCpdCep	8.33
	0.36	AmxCbCepNaNit	8.33
A. hydrophila			
	0.21	AmxCpdCep	29.2
	0.29	AmxCbCepTe	16.7
	0.29	AmxCbCpdCep	25
	0.36	AmxCbCepNaTe	16.7
	0.36	AmxCbCpdCepNa	8.33
	0.43	AmxCbCpdCepNaTe	4.17
A. jandaei			
	0.21	AmxCbTe	13.8
	0.21	AmxCbCep	17.2
	0.29	AmxCbCpdCep	13.8
	0.29	AmxCbCpdTe	3.45
A. schubertii			
	0.21	AmxCbNa	14.3
	0.29	AmxCbNaTe	14.3
A. sobria			
	0.21	AmxCbTe	23.8
	0.21	AmxCbS	4.76
	0.29	AmxCbNa Te	31
A. trota			
	0.21	AmxCbTe	7.69
	0.21	AmxCbNa	15.4
	0.29	AmxCbNaTe	7.69
A. veronii			
	0.21	AmxCbTe	9.09
	0.21	AmxCbNa	18.2
	0.29	AmxCbNaTe	18.2
	0.29	AmxCbCpdNa	9.09

4.5.1.7. MAR index of motile aeromonads from water samples of farm

Figure 4.4 shows the MAR index of motile aeromonads isolated from water samples of farm. Of the total isolates 65.38% exhibited MAR index value greater than 0.2. The value of MAR index ranged from 0.21 to 0.36.

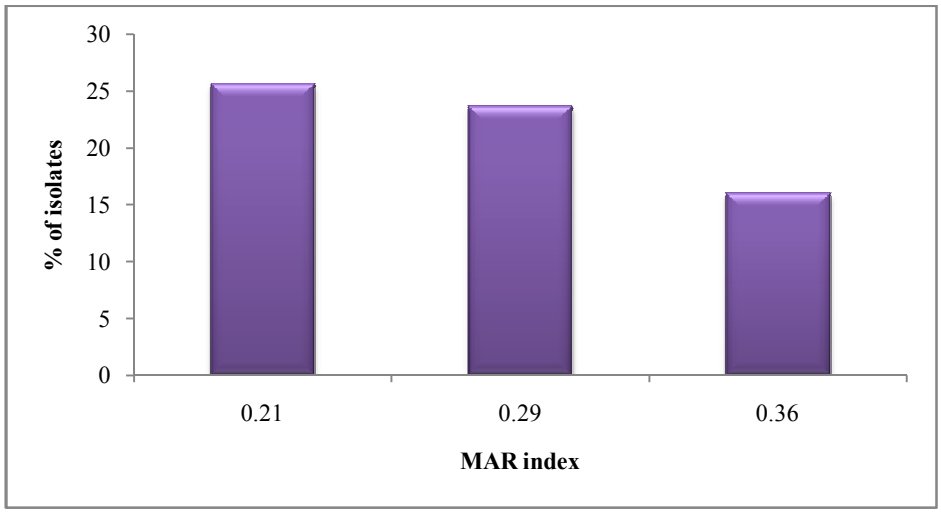


Figure 4.4. MAR index of motile aeromonads in water samples from farm

4.5.1.8. MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads from water samples

Table 4.6 shows the MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads (MAR index ≥ 0.21) in water samples from farm.

Table 4.6. MAR index, resistance pattern and prevalence of specific resistance pattern among different species of motile aeromonads in water samples from farm

Aeromonas spp.	MAR index	Resistance pattern	Prevalence of specific pattern (%)
A. caviae			
	0.21	AmxCbNa	6.25
	0.21	AmxCpdTe	12.5
	0.21	AmxCbCpd	6.25
	0.29	AmxCpdCepNa	18.75
	0.29	AmxCbCpdCep	6.25
	0.29	AmxCbCepNa	6.25
A. hydrophila			
	0.21	AmxCpdCep	26.66
	0.29	AmxCbCepTe	13.33
	0.29	AmxCbCpdCep	20
	0.36	AmxCbCpdCepNa	13.33
	0.36	AmxCbCepNaTe	20
	0.36	AmxCbCpdCepTe	6.66
A. jandaei			
	0.21	AmxCbCep	5
	0.21	AmxCbCpd	5
	0.29	AmxCbCpdCep	20
	0.29	AmxCbCepNa	15
A. schubertii			
	0.21	AmxCbNa	25
	0.29	AmxCbNaTe	12.5
A. sobria			
	0.21	AmxCbNa	3.70
	0.21	AmxCbTe	11.1
	0.21	AmxCpdNa	1.85
	0.29	AmxCbNaTe	25.9
	0.29	AmxCbCpdTr	1.85
	0.36	AmxCbCpdSfTr	3.70
	0.36	AmxCbCpdNaTe	7.40
	0.36	AmxCpdSfTeTr	11.11
A. trota			
	0.21	AmxCbNa	18.91
	0.21	AmxCbCep	13.51
	0.21	AmxCpdNa	8.10
	0.21	AmxCbTe	5.40
	0.29	AmxCbNaTe	5.40
	0.29	AmxCbCepNa	2.70
	0.36	AmxCbCpdNaTe	18.9
A. veronii			
	0.21	AmxCbNa	33.33
	0.29	AmxCbCpdNa	16.66

4.5.2. Antibiotic resistance of motile aeromonads in samples from retail aquarium vendors

4.5.2.1. Prevalence of antibiotic resistance in motile aeromonads from ornamental fish samples

Figure 4.5 represents the prevalence of antibiotic resistance in motile aeromonads in ornamental fish samples from aquarium vendors. All the *Aeromonas* isolates tested exhibited resistance to amoxicillin, while all the isolates were sensitive to chloramphenicol, ciprofloxacin and gentamicin.

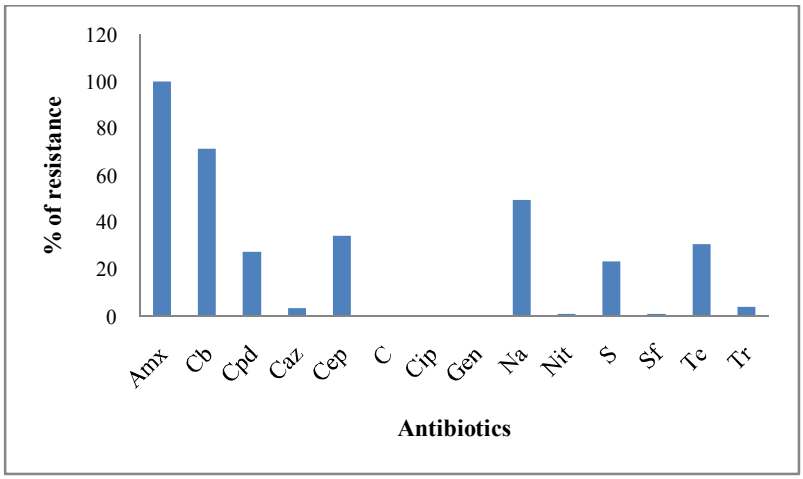


Figure 4.5. Prevalence of antibiotic resistance in motile aeromonads from ornamental fish samples

4.5.2.2. Prevalence of antibiotic resistance in motile aeromonads from water samples

Figure 4.6 represents the prevalence of antibiotic resistance in motile aeromonads in water samples from aquarium vendors. Resistance to amoxicillin was exhibited by all the *Aeromonas* isolates tested whereas all of them were susceptible to chloramphenicol, ciprofloxacin, gentamicin and nitrofurantoin.

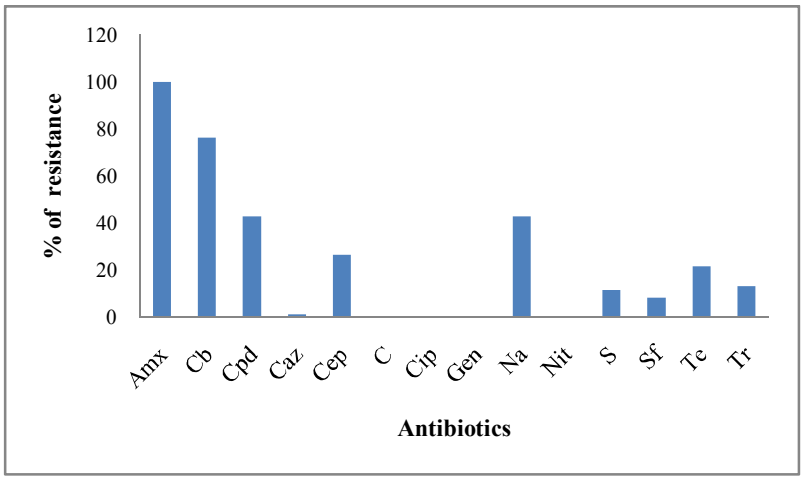


Figure 4.6. Prevalence of antibiotic resistance in motile aeromonads from water samples

4.5.2.3. Variation in prevalence of antibiotic resistance among different species of motile aeromonads in fish samples from retail aquarium vendors

Table 4.7 represents the resistance of different species of *Aeromonas* to the antibiotics tested. All the isolates tested were resistant to amoxicillin. Except *Aeromonas schubertii*, all the other species exhibited resistance to nalidixic acid and tetracycline. A high degree of resistance to cefpodoxime was exhibited by *A. hydrophila* (80%) and *A. caviae* (43.63%) and a lower level of resistance by other species (< 20%). All the isolates of *A. jandaei* and *A. schubertii* were sensitive to this drug. Resistance to ceftazidime was exhibited only by *A. veronii* (13.33%) and *A. sobria* (5.63%); all the other species were susceptible to this antibiotic. Among the different species tested, resistance to nitrofurantoin and sulfafurazole was exhibited only by *A. caviae*. Except *A. jandaei* and *schubertii*, all the other species exhibited resistance to streptomycin. Less than 10% of the isolates of *A. hydrophila*, *A. caviae* and

A. trota exhibited resistance to trimethoprim. All the isolates tested were susceptible to ciprofloxacin, chloramphenicol and gentamicin.

Table 4.7. Variation in prevalence of antibiotic resistance among different species of motile aeromonads in ornamental fish samples from retail aquarium vendors

Antibiotic code	% of resistance among different species of motile aeromonads exhibiting antibiotic resistance						
	A. caviae	***A. hydrophila***	***A. jandaei***	***A. schubertii***	***A. sobria***	***A. trota***	***A. veronii***
Amx	100	100	100	100	100	100	100
Cb	60	70	100	100	76.05	66.66	80
Cpd	43.63	80	0	0	18.30	6.66	13.33
Caz	0	0	0	0	5.63	0	13.33
Cep	56.36	100	33.33	0	18.30	26.66	0
C	0	0	0	0	0	0	0
Cip	0	0	0	0	0	0	0
Gen	0	0	0	0	0	0	0
Na	63.63	40	66.66	0	35.21	53.33	73.33
Nit	3.63	0	0	0	0	0	0
S	18.18	30	0	0	28.16	20	33.33
Sf	3.63	0	0	0	0	0	0
Te	38.18	40	50	0	29.57	20	13.33
Tr	9.09	10	0	0	0	6.66	0

4.5.2.4. Variation in prevalence of antibiotic resistance among various *Aeromonas* spp. in water samples from retail aquarium vendors

Table 4.8 represents the resistance of different species of *Aeromonas* to the tested antibiotics. Resistance to amoxicillin was exhibited by all the *Aeromonas* isolates tested, whereas all of them were susceptible to chloramphenicol, ciprofloxacin, gentamicin and nitrofurantoin.

Table 4.8. Variation in prevalence of antibiotic resistance among different species of motile aeromonads in water samples from retail aquarium vendors

Antibiotic code	% of resistance among different species of motile aeromonads exhibiting antibiotic resistance						
	A. caviae	***A. hydrophila***	***A. jandaei***	***A. schubertii***	***A. sobria***	***A. trota***	***A. veronii***
Amx	100	100	100	100	100	100	100
Cb	60	80	100	62.5	71.42	87.5	88
Cpd	36.66	88	26.66	0	42.85	31.25	36
Caz	0	0	0	0	0	0	8
Cep	40	100	53.33	0	0	18.75	0
C	0	0	0	0	0	0	0
Cip	0	0	0	0	0	0	0
Gen	0	0	0	0	0	0	0
Na	33.33	92	0	37.5	28.57	62.5	56
Nit	0	0	0	0	0	0	0
S	0	0	0	12.5	26.98	18.75	0
Sf	0	20	0	0	12.69	0	8
Te	16.66	36	33.33	25	25.39	12.5	0
Tr	20	28	0	0	14.28	0	8

4.5.2.5. MAR index of motile aeromonads in ornamental fish samples from retail aquarium vendors

Figure 4.7 shows the MAR index of motile aeromonads isolated from ornamental fish samples. MAR index value greater than 0.2 was exhibited by 62.85% of the isolates. The value ranged from 0.21 to 0.57.

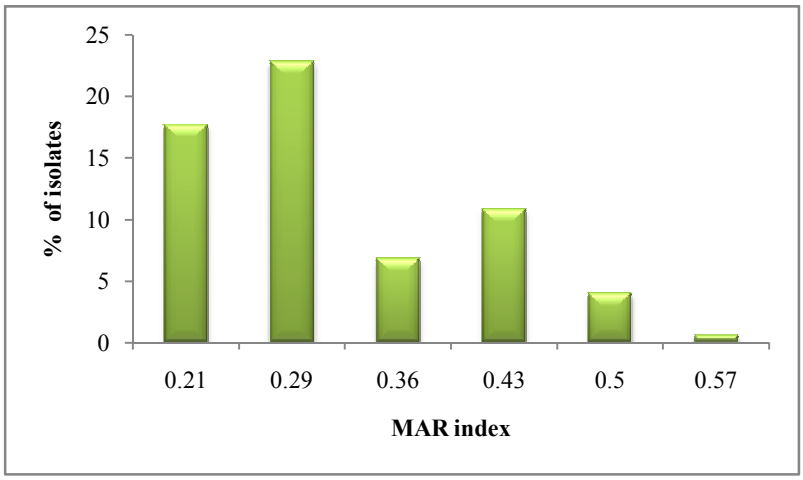


Figure 4.7. MAR index of motile aeromonads in ornamental fish samples from retail aquarium vendors

4.5.2.6. MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads from ornamental fish samples

Table 4.9 shows the MAR index, resistance pattern and prevalence of specific resistance pattern among different species of motile aeromonads (MAR index ≥ 0.21) from ornamental fish samples collected from retail aquarium vendors. Among the different species, 20% of *A. hydrophila* and 1.82% of *A. caviae* exhibited resistance towards 7 and 8 antibiotics respectively.

Table 4. 9. MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads in ornamental fish samples from retail aquarium vendors

Aeromonas spp.	MAR index	Resistance pattern	Prevalence of specific pattern (%)
A. caviae			
	0.21	AmxCbNa	3.64
	0.21	AmxCpdCep	3.64
	0.21	AmxNaS	1.82
	0.21	AmxSTe	1.82
	0.21	AmxCepNa	1.82
	0.29	AmxCbCpdNa	5.45
	0.29	AmxCepNaS	3.64
	0.29	AmxCepSTe	3.64
	0.29	AmxCpdCepNa	1.82
	0.29	AmxCbCepTe	1.82
	0.36	AmxCbCpdNaTe	1.82
	0.36	AmxCbCpdCepTe	5.45
	0.36	AmxCbCpdCepNa	3.64
	0.43	AmxCbCpdCepNaTe	14.5
	0.43	AmxCbCepNaSTe	1.82
	0.5	AmxCbCpdCepNaTeTr	7.27
	0.5	AmxCbCpdCepNaNitSf	1.82
	0.57	AmxCbCpdCepNaNitSfTr	1.82
A. hydrophila			
	0.21	AmxCpdCep	10
	0.29	AmxCbCpdCep	20
	0.43	AmxCbCpdCepTeTr	10
	0.43	AmxCbCpdCepNaTe	10
	0.43	AmxCbCpdCepNaS	10
	0.5	AmxCbCpdCepNaSTe	20
A. jandaei			
	0.21	AmxCbCep	16.7
	0.29	AmxCbNaTe	50
	0.29	AmxCbCepNa	16.7

A. sobria			
	0.21	AmxCbTe	7.04
	0.21	AmxCbNa	2.82
	0.21	AmxNaS	4.23
	0.21	AmxCepS	2.82
	0.21	AmxCepTe	1.41
	0.29	AmxCbNaTe	5.63
	0.29	AmxCbCepTe	4.23
	0.29	AmxCbNaS	12.7
	0.29	AmxCbCpdCaz	2.82
	0.29	AmxCbCpdCep	1.41
	0.36	AmxCbCpdCazNa	2.82
	0.36	AmxCbCpdSTe	1.41
	0.36	AmxCbCpdCepTe	2.82
	0.36	AmxCpdCepSTe	1.41
	0.43	AmxCbCpdNaSTe	5.63
A. trota			
	0.21	AmxCbNa	13.3
	0.21	AmxCbS	6.67
	0.21	AmxSTr	6.67
	0.21	AmxCbTe	6.67
	0.29	AmxCbNaTe	6.67
	0.29	AmxCbCepNa	13.3
	0.43	AmxCbCpdCepNaTe	6.67
A. veronii			
	0.21	AmxCbNa	20
	0.21	AmxNaS	6.67
	0.29	AmxCbNaS	20
	0.43	AmxCbCpdCazNaTe	13.3

4.5.2.7. MAR index of motile aeromonads from water samples of retail aquarium vendors

Figure 4.8 shows the MAR index of motile aeromonads isolated from water samples. MAR index value greater than 0.2 was exhibited by 62.63% of the isolates. The value ranged from 0.21 to 0.57.

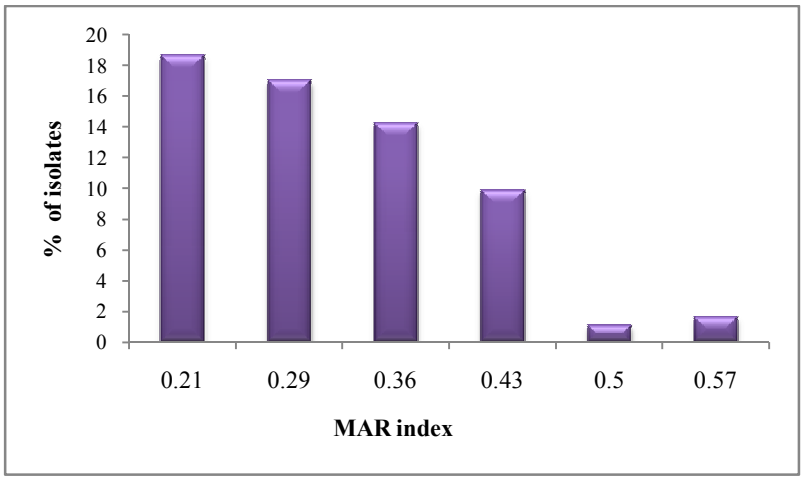


Figure 4.8. MAR index of motile aeromonads in water samples from retail aquarium vendors

4.5.2.8. MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads from aquarium water samples

Table 4.10 shows the MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads (MAR index ≥ 0.21) from water samples collected from retail aquarium vendors. Among the different species, 12% of *A. hydrophila* exhibited resistance towards 8 antibiotics.

Table 4.10. MAR index, resistance pattern and prevalence of specific pattern among different species of motile aeromonads in water samples from retail aquarium vendors

Aeromonas spp.	MAR index	Resistance Pattern	Prevalence of specific pattern (%)
A. caviae			
	0.21	AmxCbCep	16.7
	0.21	AmxCpdTe	3.33
	0.29	AmxCpdNaTe	13.3
	0.36	AmxCpdCepNaTr	20
A. hydrophila			
	0.29	AmxCbCpdCep	8
	0.29	AmxCepNaTe	12
	0.29	AmxCpdCepNa	4
	0.36	AmxCbCpdCepNa	32
	0.43	AmxCbCpdCepNaTe	8
	0.43	AmxCbCpdCepNaTr	16
	0.43	AmxCbCpdCepNaSf	4
	0.43	AmxCpdCepNaSfTe	4
	0.57	AmxCbCpdCepNaSfTeTr	12
A. jandaei			
	0.21	AmxCbCep	53.3
	0.21	AmxCbTe	6.67
	0.29	AmxCbCpdTe	26.7
A. schubertii			
	0.21	AmxCbNa	12.5
	0.29	AmxCbNaTe	12.5
	0.36	AmxCbNaSTe	12.5
A. sobria			
	0.21	AmxCpdTr	1.59
	0.21	AmxCbNa	6.35
	0.29	AmxCbNaS	4.76
	0.29	AmxCbCpdS	6.35
	0.29	AmxCpdNaS	1.59
	0.36	AmxCpdNaSTe	4.76
	0.36	AmxCbCpdSfTr	1.59
	0.36	AmxCbCpdNaS	1.59
	0.36	AmxCpdSfTeTr	4.76
	0.36	AmxCb CpdNaTe	1.59
	0.43	AmxCbCpdNaSTe	7.94
	0.43	AmxCbCpdSfTeTr	6.35

A. trota			
	0.21	AmxCbNa	25
	0.21	AmxCbS	12.5
	0.29	AmxCbCpdNa	18.8
	0.29	AmxCbNaTe	6.25
	0.36	AmxCbCpdCepNa	6.25
	0.43	AmxCbCpdCepNaTe	6.25
A. veronii			
	0.21	AmxCbNa	20
	0.21	AmxCbCpd	8
	0.29	AmxCbCpdNa	16
	0.36	AmxCbCpdCazNa	4
	0.5	AmxCbCpdCazNaSfTr	8

4.5.3. Relative prevalence of antibiotic resistance among motile aeromonads in ornamental fish samples from farm and retail aquarium vendors

Resistance to amoxicillin was exhibited by all the isolates from both farm and retail aquarium vendors and resistance to carbenicillin by around 70% of the isolates from both sampling stations. Resistance to ceftazidime, sulfafurazole and trimethoprim was exhibited only by the isolates from retail aquarium vendors and resistance to trimethoprim was found to be significantly different ($p<0.05$). A large number of isolates from retail aquarium vendors were resistant to streptomycin and nalidixic acid when compared to the isolates from farm and the difference was significant ($p<0.05$). Around 30% of the isolates from both farm and retail aquarium vendors were resistant to tetracycline. Relative prevalence of antibiotic resistance in isolates from both sampling sites is given in Figure 4.9.

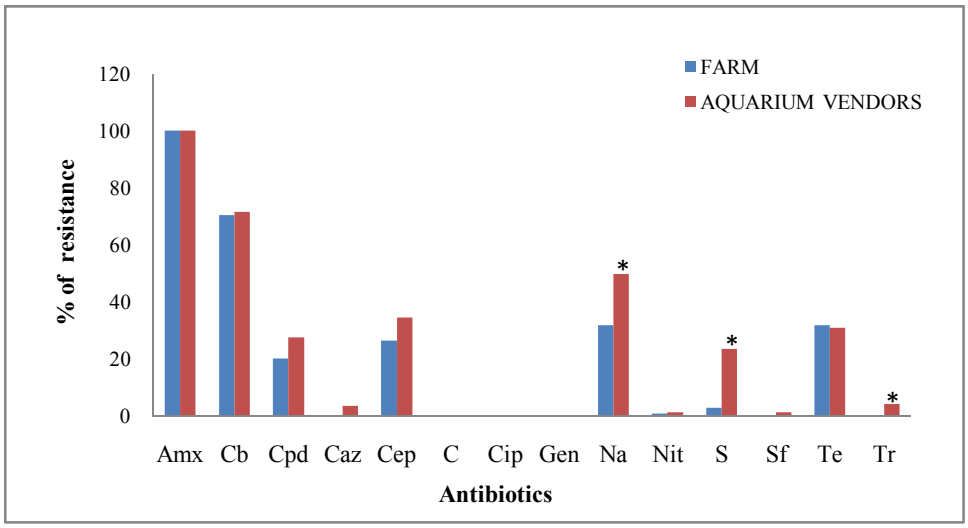


Figure 4.9. Relative prevalence of antibiotic resistance in motile aeromonads in fish samples from farm and retail aquarium vendors

4.5.4. Relative prevalence of antibiotic resistance among motile aeromonads in water samples from farm and retail aquarium vendors

All the isolates from both farm and retail aquarium vendors were resistant to amoxicillin and around 70% of the isolates from both sampling sites, resistant to carbenicillin. Resistance to ceftazidime and streptomycin was exhibited only by the isolates from retail aquarium vendors and resistance to streptomycin was found to be significantly different ($p<0.05$). Resistance to sulfafurazole and trimethoprim was exhibited by isolates from both farm and retail aquarium vendors. More number of isolates from farm (32.05%) was resistant to tetracycline when compared to the isolates from aquarium vendors (21.42%). A comparison of antibiotic resistance in isolates from both sampling sites is given in Figure 4.10.

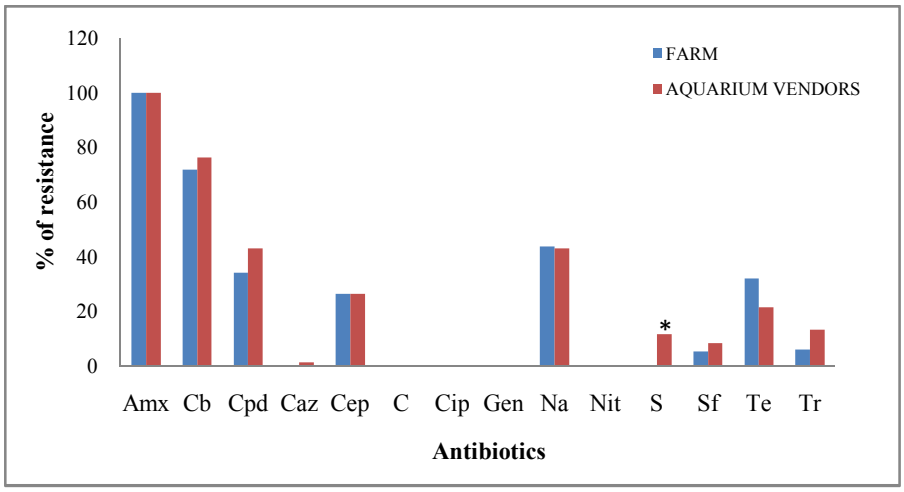


Figure 4.10. Relative prevalence of antibiotic resistance in motile aeromonads in water samples from farm and retail aquarium vendors

4.5.5. Relative MAR index of motile aeromonads in ornamental fish samples from farm and retail aquarium vendors

Among the motile aeromonads isolated from fish samples, nearly 60% of the isolates from both farm and aquarium samples were resistant to 3 or more antibiotics.

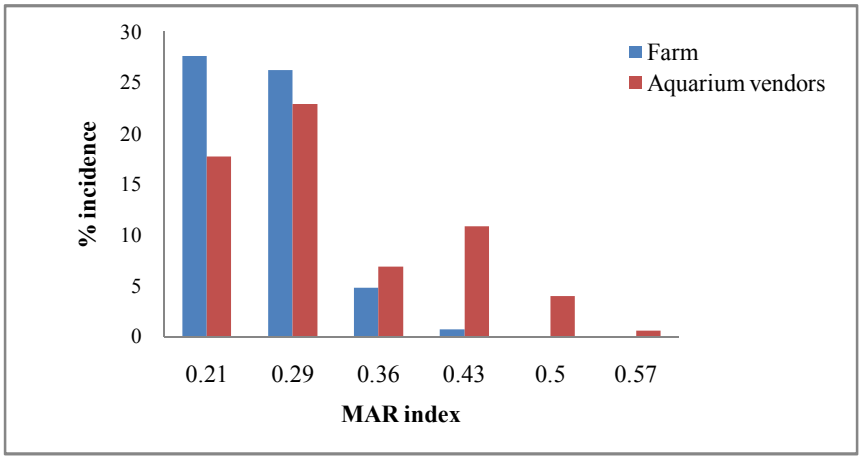


Figure 4.11. Relative MAR index of motile aeromonads in ornamental fish samples from farm and retail aquarium vendors

The MAR index value ranged from 0.21 to 0.43 in isolates from farm and 0.21 to 0.57 in isolates from aquarium vendors. A comparison of MAR index value is given in Figure 4.11. A MAR index value of 0.5 and 0.57 is seen only in isolates from aquaria.

4.5.6. Relative MAR index of motile aeromonads in water samples from farm and retail aquarium vendors

Among the motile aeromonads isolated from water samples, greater than 60% of the isolates from both farm and aquarium samples were resistant to 3 or more antibiotics. The MAR index value ranged from 0.21 to 0.36 in isolates from farm and 0.21 to 0.57 in isolates from aquarium vendors. A comparison of MAR index value is given in Figure 4.12. A MAR index value of 0.43, 0.5 and 0.57 was seen only in isolates from aquaria.

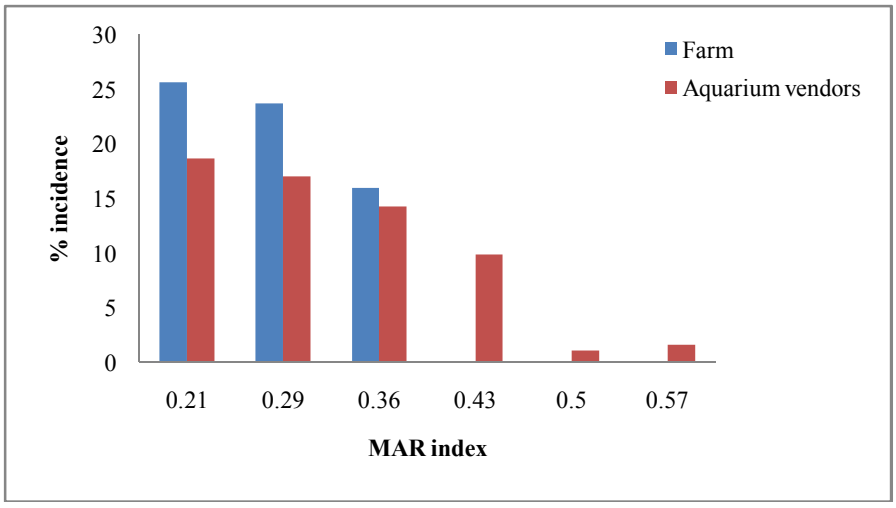


Figure 4.12. Relative MAR index of motile aeromonads in water samples from farm and retail aquarium vendors

4.6. Discussion

As early as antibiotics have been in use, microbial antibiotic resistance was developed (Igbinosa and Okoh, 2012). Resistance of *Aeromonas* spp. to commonly used antibiotics is an emerging problem in the ornamental fish industry (Dias *et al*., 2012). The spread of antibiotic resistance among this genus is of prime concern, because of the emergence of these organisms as a primary human pathogen (Janda and Abbott, 2010).

4.6.1. Antibiotic resistance of motile aeromonads from fish samples

Resistance to penicillins

The resistance of all *Aeromonas* spp. to amoxicillin observed in this study are in agreement with the reports of Sreedharan *et al*. (2012), who reported complete resistance to amoxicillin by motile aeromonads from ornamental fish culture system in Kerala. Suhet *et al*. (2011) also reported 100% resistance to amoxicillin by different species of *Aeromonas* such as *Aeromonas caviae*, *A. hydrophila*, *A. jandaei*, *A. veronii* and *A. sobria* isolated from fishes; same is the report of Verner-Jeffreys *et al*. (2009), among *Aeromonas* spp. isolated from ornamental fishes. On the contrary, Hatha *et al*. (2005) encountered a comparatively lower level of amoxicillin resistance (<50%) among the motile aeromonads isolated from farm raised fresh water fishes in India.

More than 70% of the isolates in the present study, from both farm and commercial aquarium samples exhibited resistance to carbenicillin. More than 90% of the *Aeromonas* spp. isolated from the skin of ornamental fishes, exhibiting resistance against carbenicillin is reported by Dias *et al*. (2012). All

the *Aeromonas* isolates investigated by Castro-Escarpully *et al*. (2003), showed resistance to carbenicillin. As it is clear that penicillins are not effective in treating *Aeromonas* infections, the use of this class in aquaculture must be prudent to avoid negative effects on human treatments due to the possible transfer of commensal bacteria and resistant pathogens from aquatic environment (Heuer *et al*., 2009).

Resistance to cephalosporins

Varying levels of resistance towards cephalosporins was shown by the motile aeromonads in the present study. Ceftazidime was the most effective among the cephalosporins tested as none of the isolates obtained from the fish samples from farm was resistant to ceftazidime. Resistance to first and second-generation cephalosporins (cephalothin and cefoxitin respectively) has been reported in motile aeromonads (Dias *et al*., 2012). Similar to their observation, around 30% of the isolates from aquarium samples in the present study was found to be resistant to cephalothin and cefpodoxime. However, resistance to these antibiotics among motile aeromonads from farm was found to be slightly lesser (26% and 20% respectively). In samples from aquarium vendors, 5% of *A. sobria* and nearly 10% of *A. veronii* were resistant to this antibiotic. Orozova *et al*. (2010) who studied antibiotic resistance of *Aeromonas* spp. isolated from fish samples in Bulgaria, as well as from intensively bred Trout fish in different countries reported that *Aeromonas* spp. has typically been susceptible to third generation of cephalosporins. Complete resistance to cephalothin and sensitivity to ceftriaxone (Yucel *et al*., 2005), cefotaxime and cefuroxime (Castro-Escarpully *et al*., 2003) are also reported.

Resistance to aminoglycosides

Different species of motile aeromonads obtained in this study was completely sensitive to gentamicin, regardless of the source. Except 9.5% of *A. sobria* in fish samples from farm, all the isolates from farm was sensitive to streptomycin, but varying levels of resistance to streptomycin was exhibited by different species of *Aeromonas* from commercial aquarium vendors. The number of isolates exhibiting resistance to streptomycin was found to be significantly higher ($p<0.05$) in samples from retail aquarium vendors. Afizi *et al*. (2013) reported complete sensitivity of *Aeromonas* to gentamicin. In India, various species of *Aeromonas* exhibiting complete sensitivity to the aminoglycosides, gentamicin and streptomycin is reported by Sreedharan *et al*. (2012). All the isolates of motile aeromonads obtained from fish samples by Rathore *et al*. (2005) were also sensitive to gentamicin. Hatha *et al*. (2005) observed complete sensitivity of motile aeromonads to streptomycin, but a small percentage (<10%) of the isolates were resistant to gentamicin. On the contrary, higher levels of resistance from 13.3% (Matyar *et al*., 2010) to even 31% (Verner-Jeffreys *et al*., 2009) were also observed. Variation in resistance to antibiotics among different species of motile aeromonads was also observed among the isolates of the present study. Variation in resistance to antibiotics is possible as it is a function of selection pressure in the respective environment. Hence it is important to monitor specifically each of the environment of concern to determine the exact level of prevalence of antibiotic resistance.

All the *A. hydrophila* isolates from hemorrhagic diseased freshwater fishes were sensitive to streptomycin (Ye *et al*., 2013); more than 90% of *A. hydrophila* isolates from retail seafood outlets was sensitive to gentamicin

and streptomycin (Thayumanavan *et al*., 2007). Castro-Escarpulli *et al*. (2003) reported all the isolates of *A. hydrophila* and *A. veronii* bv *sobria* to be resistant to streptomycin and 75% of *A. veronii* bv *sobria* isolates to be resistant to gentamicin, while all the isolates of *A. hydrophila* were sensitive to this antibiotic.

Resistance to tetracyclines

Nearly 30% of motile aeromonads from both farm and commercial aquarium vendors encountered in the present study were resistant to tetracycline. Similar to our findings, antimicrobial sensitivity patterns of the *Aeromonas* isolates studied by Abulhamd (2010) revealed 30% of isolates to be resistant to tetracycline. Among *Aeromonas* isolates from ornamental Carps, 50% were reported to be resistant to oxytetracycline (Čížek *et al*., 2010). Significant resistance to tetracycline is also reported by Patil *et al*. (2011).

Kaskhedikar and Chhabra (2010) reported 50% of *A. hydrophila* isolates from samples of fish, collected from retail shops in India to be resistant to this antibiotic. A total of 41% of *A. hydrophila* isolated from fish samples in India were resistant to tetracycline (Vivekanandhan *et al*., 2002). There are reports of 100% tetracycline resistance by *A. caviae, A. jandaei, A. veronii* bv *sobria* and *A. hydrophila* (Ashiru *et al*., 2011; Sreedharan *et al*., 2012). On the contrary, Suhet *et al*. (2011) reported all the members of *A. caviae*, *A. hydrophila*, *A. jandaei* and *A. sobria* and 50% of *A. veronii* to be sensitive to this antibiotic. El-Barbary (2010) reported oxytetracycline as an effective antibiotic for all tested isolates of *A. hydrophila* obtained from various naturally infected fishes.

Resistance to chloramphenicol

All the isolates of motile aeromonads from both farm and retail aquaria in the present study were sensitive to chloramphenicol. Complete sensitivity to chloramphenicol by motile aeromonads is reported in India by several authors (Sasmal *et al*., 2004; Kaskhedikar and Chhabra, 2010; Sreedharan *et al*., 2012). All the *A. hydrophila* isolates from hemorrhagic diseased freshwater fishes (Ye *et al*., 2013) and retail seafood outlets (Thayumanavan *et al*., 2007) were sensitive to chloramphenicol. Though chloramphenicol is found to be very effective against *Aeromonas,* it is banned for use in aquaculture and veterinary medicine worldwide (FAO, 2005). It is only used at therapeutic doses for the treatment of serious human infectious diseases due to a number of adverse effects (Schwarz *et al*., 2004).

Resistance to quinolones

Nearly 30% of isolates from fish samples from farm and 50% from commercial aquarium vendors in the present study showed resistance to this antibiotic and the difference was found to be significant ($p<0.05$). Similar to the present observation, a high level of resistance to nalidixic acid by *Aeromonas* isolates from ornamental fish farm is reported by Sreedharan *et al*. (2012). On the contrary, complete susceptibility of *Aeromonas* spp. to nalidixic acid has also been reported (Castro-Escarpulli *et al*., 2003; Suhet *et al*., 2011).

Among the different species of motile aeromonads, high level of resistance to nalidixic acid was observed for *A. veronii* (63%). Nearly 40% of *A. caviae* and 30% of *A. hydrophila, A. schubertii* and *A. sobria* were resistant to this antibiotic. Kaskhedikar and Chhabra (2010) observed complete susceptibility of

A. hydrophila isolates to this antibiotic. In another study, among the motile aeromonads isolated from fish samples in India, varying levels of resistance to nalidixic acid by different species is reported by Hatha *et al*. (2005).

All the isolates of motile aeromonads obtained in the present study were sensitive to ciprofloxacin. Such a high level of sensitivity to ciprofloxacin by various species of motile aeromonads from fish samples is reported by several workers (Sasmal *et al*., 2004; Rathore *et al*., 2005; Yucel *et al*., 2005; Suhet *et al*., 2011; Sreedharan *et al*., 2012). On the contrary, resistance to ciprofloxacin is also reported (Abulhamd, 2010; Čížek *et al*., 2010). Dias *et al*. (2012) reported that ciprofloxacin resistance was more prevalent among *A. hydrophila* isolates than the other species. Castro-Escarpulli *et al*. (2003) reported 50% of *A. veronii* bv *sobria* isolates to be resistant to ciprofloxacin. In another study in India, Thayumanavan *et al*. (2007) reported that all the isolates of *A. hydrophila* obtained from retail seafood outlets in Coimbatore were sensitive to ciprofloxacin.

Though ciprofloxacin is found to be very effective against *Aeromonas*, it's use is not recommended in aquaculture as it is widely used in human medicine. Quinolones are excreted unaltered and are very persistent in the environment. They have been shown to damage the nervous system of other animals.

Resistance to nitrofurantoin

High level of susceptibility to nitrofurantoin was exhibited by the motile aeromonads in the present study. Among the different species of motile aeromonads, *A. caviae* was the only species found to exhibit resistance to this

antibiotic. In the fish samples, 8.33% of *A. caviae* from the farm and 3.63% from aquarium vendors were resistant to this antibiotic. Members of all the other spp. were completely sensitive. High level of susceptibility to nitrofurantoin by the isolates from fish samples is reported by several workers (Castro-Escarpulli *et al*., 2003; Lee *et al*., 2009; Kaskhedikar and Chhabra, 2010; Sreedharan *et al*., 2012). On the contrary, study conducted by Ashiru *et al*. (2011) reported all the isolates of *A. caviae*, *A. hydrophila* and *A. sobria* to be resistant to nitrofurantoin.

Resistance to trimethroprim and sulfamethoxazole

Among the different *Aeromonas* species encountered in fish samples from farm in the present study, none of the isolates were resistant to sulfafurazole and trimethoprim. In samples from commercial aquarium vendors, nearly 1 and 4% of the total isolates were resistant to sulfafurazole and trimethoprim respectively. Difference in resistance towards trimethoprim among motile aeromonads in samples from retail aquarists was found to be significantly high ($p<0.05$) when compared to the isolates from farm. Among the different species, only *A. caviae* (3.6%) was resistant to sulfafurazole and nearly 10% of *A. caviae, A. hydrophila* and *A. trota* showed resistance to trimethoprim. Complete susceptibility of *Aeromonas* spp. to trimethroprim and sulfamethoxazole is reported by several authors (Awan *et al*., 2009; Orozova *et al*., 2010; Sreedharan *et al*., 2012). On the contrary, Yucel *et al.* (2005) reported all aeromonads (*A. hydrophila, A. veronii bv. sobria* and *A. caviae*) from retail fishes to be resistant to trimethoprim. Antimicrobial sensitivity patterns of the *Aeromonas* isolates studied by Abulhamd (2010) revealed only 80% sensitivity to sulfamethoxazole–trimethoprim. Čížek *et al*. (2010)

investigated the antibiotic susceptibility of *Aeromonas* isolates from ornamental Carps and found that, only 15% were resistant to trimethoprim. Complete sensitivity of *A. hydrophila* isolates from diseased Catfishes to sulfamethoxazole–trimethoprim was also reported (Jayavignesh *et al.*, 2011).

4.6.2. Antibiotic resistance of motile aeromonads from water samples

Resistance to penicillins

All the isolates of motile aeromonads in water samples from both farm and retail aquaria in the present study were resistant to amoxicillin. Total resistance to amoxicillin by motile aeromonads from water samples is documented (Verner-Jeffreys *et al*. 2009; Suhet *et al*., 2011). Akinbowale *et al.* (2006) also reported widespread resistance to amoxicillin in *Aeromonas* species.

More than 70% of the isolates in the present study, from both farm and commercial aquarium water samples exhibited resistance to carbenicillin. Very high resistance to carbenicillin among *Aeromonas* species in water samples housing ornamental fishes is reported by Dias *et al*. (2012). Complete resistance to carbenicillin among *Aeromonas* isolates exhibiting from river water samples in India is reported by Bagyalakshmi *et al*. (2009).

Resistance to cephalosporins

Ceftazidime was the most effective among the cephalosporins tested as none of the isolates in water samples from the farm was resistant to ceftazidime. In samples from commercial aquarium vendors, nearly 10% of *A. veronii* in water samples were resistant to this antibiotic. Koksal *et al*. (2007) reported 32% of *A. hydrophila* and *A. sobria* and 18% of *A. caviae* to be resistant to ceftazidime whereas all the isolates of *A. jandaei* and *A. veronii* were sensitive. Higher level

of resistance to cephalothin and cefpodoxime was detected among the isolates in the present study. High resistance to cephalothin has been detected in motile aeromonads (Dias *et al*., 2012). All the isolates of *A. hydrophila, A. sobria* and *A. caviae* from river water samples in India, exhibiting resistance to cephalothin is reported by Bagyalakshmi *et al*. (2009).

Resistance to aminoglycosides

Different species of motile aeromonads obtained in this study was completely sensitive to gentamicin, regardless of the source. All the isolates of motile aeromonads in water samples from farm were sensitive to streptomycin, but varying levels of resistance to this antibiotic was exhibited by the isolates from commercial aquarium vendors and the difference was found to be significant. Various species of *Aeromonas* from water samples exhibiting complete sensitivity to the aminoglycosides, gentamicin and streptomycin is reported in India (Joseph *et al*., 2013). Similar results of sensitivity to gentamicin for motile aeromonads from water samples in India is reported earlier (Rathore *et al*., 2005). Koksal *et al*. (2007) reported all the isolates of *A. caviae, A. jandaei, A. sobria* and *A. veronii* from water samples to be sensitive to gentamicin, and 1% of *A. hydrophila* to be resistant. Antimicrobial sensitivity patterns of the *Aeromonas* isolates studied by Abulhamd (2010) revealed that 100% was sensitive to gentamicin, but only 20% was sensitive to streptomycin.

Resistance to tetracyclines

In the present study, nearly 30% of isolates in water samples from farm and 20% isolates from commercial aquarium vendors were found to be resistant to tetracycline. Significant resistance to tetracycline in *Aeromonas* from aquaculture systems or aquarium water is reported (Jacobs and Chenia,

2007; Verner-Jeffreys *et al*., 2009; Patil *et al*., 2011). Resistance to tetracycline and oxytetracycline is common in *Aeromonas* species (Schmidt *et al*., 2000; Akinbowale *et al.,* 2006). Research from the Southern part of the Netherlands also reported an oxytetracycline resistance level of 65% of motile *Aeromonas* in Catfish and Eel farms (Penders and Stobberingh, 2008). Huys *et al*. (2000) found oxytetracycline resistant strains in water from fish farms. Goni-Urriza *et al*. (2000) found as many as 49% tetracycline-resistant *Aeromonas* species in their study.

Among the different species of motile aeromonads encountered in the present study, tetracycline resistance was found to be high in *A. hydrophila* and *A. sobria* isolates in water samples from farm. In samples from commercial aquarium vendors, tetracycline resistance was found to be high in *A. hydrophila* and *A. jandaei* isolates. McPhearson *et al*. (1991) reported 43% and 62% of *A. hydrophila* isolated from Catfish ponds to be resistant to tetracycline and oxytetracycline respectively. On the contrary, Sreedharan *et al*. (2012) reported all the members of *A. caviae*, *A. jandaei* and *A. veronii* bv *sobria* to be resistant to this antibiotic.

Resistance to chloramphenicol

All the isolates of motile aeromonads obtained from water samples in the present study were sensitive to chloramphenicol. Complete sensitivity to chloramphenicol in *Aeromonas* isolates from water samples in aquafarms is reported in India (Joseph *et al*., 2013). Different species of *Aeromonas* such as *A. caviae, A. hydrophila, A. trota, A. sobria* and *A. veronii* isolated from water samples was found to be completely sensitive to chloramphenicol by Evangelista-Barreto *et al*. (2010). Very high susceptibility of *Aeromonas* to

chloramphenicol has been reported by various researchers (Goni-Urriza *et al.,* 2000; Akinbowale *et al*., 2006; Penders and Stobbering, 2008). On the contrary, a higher level of chloramphenicol resistance at 56% among *Aeromonas* species in the water housing ornamental fishes was also observed (Verner-Jeffreys *et al*., 2009).

Resistance to quinolones

Nearly 40% of isolates from water samples from both farm and aquarium vendors in the present study exhibited resistance to nalidixic acid. In India, Joseph *et al*. (2013) reported 20% of *Aeromonas* isolates to be resistant to nalidixic acid. In water samples, Evangelista-Barreto *et al*. (2010) reported 13% of the strains to be resistant to nalidixic acid; and 2% by Koksal *et al*. (2007). Suhet *et al*. (2011) reported complete susceptibility of *Aeromonas* spp. to nalidixic acid. Resistance level encountered in the present study was relatively higher than most of the previous reports.

Complete sensitivity to ciprofloxacin by various species of motile aeromonads from water samples is reported by several workers (Rathore *et al*., 2005; Akinbowale *et al.,* 2006; Penders and Stobbering, 2008; Suhet *et al*., 2011). Different species of *Aeromonas* such as *A. caviae, A. hydrophila, A. trota, A. sobria* and *A. veronii* isolated from water samples was found to be completely sensitive to ciprofloxacin (Evangelista-Barreto *et al*., 2010). Koksal *et al*. (2007) reported 1% of *A. hydrophila* to be resistant and all the isolates of *A. caviae, A. jandaei, A. sobria* and *A. veronii* from water samples to be sensitive to ciprofloxacin. These results are in accordance with our findings for all the isolates in water samples, from both farm and aquarium vendors, which were found to be sensitive to ciprofloxacin. On the contrary,

antimicrobial sensitivity patterns of the *Aeromonas* isolates studied by Abulhamd (2010) revealed that only 50% was sensitive to ciprofloxacin.

Resistance to nitrofurantoin

None of the isolates from water samples from both farm and retail aquaria in the present study exhibited resistance to nitrofurantoin. High level of susceptibility to nitrofurantoin by the isolates from water samples is reported by several workers (Koksal *et al*., 2007; Evangelista-Barreto *et al*., 2010; Patil *et al*., 2011). On the contrary, Matyar *et al*. (2010) found 23.3% of *Aeromonas* isolates from water samples to be resistant to nitrofurantoin.

Resistance to trimethroprim and sulfamethoxazole

Among the different *Aeromonas* species isolated from farm in the present study, nearly 15% of *A. sobria* in water samples exhibited resistance to sulphfurazole and trimethoprim; all the other species were sensitive. Among isolates from commercial aquarium vendors, nearly 15% of *A. sobria,* 8% of *A. veronii* and 20% of *A. hydrophila* exhibited resistance to sulfafurazole and trimethoprim. Koksal *et al*. (2007) reported 18% of *A. hydrophila,* 14% of *A. sobria* and 1% of *A. caviae* to be resistant to sulfamethoxazole–trimethoprim where as all the isolates of *A. jandaei* and *A. veronii* were sensitive. Imziln (2001) reported 38% of *A. hydrophila,* 23% of *A. sobria* and 46% of *A. caviae* from water samples to be resistant to sulfamethoxazole and less than 10% of of *A. hydrophila* and *A. sobria* and 27% of *A. caviae* to be resistant to trimethoprim. Only less than 5% of all the species were resistant to a combination of sulfamethoxazole–trimethoprim. Among *Aeromonas* isolates from environmental sources belonging to different species, including *A. caviae, A. hydrophila, A. jandaei, A. sobria* and *A. veronii,* Huddleston *et*

al. (2006) reported 26% of total isolates to be resistant to trimethoprim and none of the isolates to be resistant to sulfamethoxazole. Complete susceptibility of *Aeromonas* spp. to trimethroprim and sulfamethoxazole from aquatic environment is reported by Jongjareanjai *et al.* (2009). Antimicrobial sensitivity patterns of the *Aeromonas* isolates studied by Abulhamd (2010) revealed 80% sensitivity to sulfamethoxazole–trimethoprim.

4.6.3. Multiple antibiotic resistance in motile aeromonads

Multiple antibiotic resistance is a common occurrence in the genus *Aeromonas.* Approximately 60% of the isolates in the present study from both farm and commercial aquarium vendors exhibited multiple antibiotic resistance, with a MAR index value greater than 0.2. Dias *et al*. (2012) observed that all the *Aeromonas* spp. isolated from the skin of ornamental fishes presented multiresistance to the tested antibiotics; same was the case with isolates from water samples. Orozova *et al*. (2010) reported all strains of *Aeromonas* spp. investigated by them to be multi-resistant. Multiple antibiotic resistance in *Aeromonas* is also reported by Radu *et al*. (2003) and Taylor (2003). Joseph *et al*. (2013) reported 20% of *Aeromonas* sp. from tropical seafood and water samples from aquafarms in India to be multiple drug resistant.

On comparing the MAR index values of isolates in fish samples from farm and aquarium vendors, it was observed that isolates from aquarium vendors were resistant to more number of antibiotics than isolates from farm. That is, number of isolates having MAR index value greater than 0.29 was more predominant in samples from commercial aquarium vendors. Isolates exhibiting resistance to 7 and 8 antibiotics (MAR index values of 0.5 and 0.57) was seen

only in isolates from commercial aquarium vendors. In water samples, resistance towards 6 antibiotics (MAR index value greater than 0.36) was seen only in isolates from commercial aquarium vendors.

The diversity of antibiotic resistance pattern exhibited by the motile aeromonads encountered in the present study reflects the diversity among the isolates. The antibiotic resistance pattern of different species of *Aeromonas* showed that *A. caviae* and *A. hydrophila* are the two species that are resistant to higher number of antibiotics when compared to other spp. Among the *A. caviae* isolates encountered from fish samples from retail aquaria, a resistance pattern of AmxCbCpdCepNaNitSfTr (resistance to 8 antibiotics) was exhibited by 1.82% of the isolates. Resistance pattern of AmxCbCpdCepNaTe (resistance to 6 antibiotics) was exhibited by 14.5% of the isolates and 7.27% exhibited a resistance pattern of AmxCbCpdCepNaTeTr (resistance to 7 antibiotics). Among the *A. hydrophila* isolates, 20% exhibited a resistance pattern of AmxCbCpdCepNaSTe (resistance to 7 antibiotics). In water samples, 20% of *A. caviae* exhibited a resistance pattern of AmxCpdCepNaTr. Among *A. hydrophila* isolates from water samples, 12% exhibited a resistance pattern of AmxCbCpdCepNaSfTeTr (resistance to 8 antibiotics). A resistance pattern of AmxCbCpdCepNa (resistance to 5 antibiotics) was exhibited by 32% of the *A. hydrophila* isolates. Odeyemi *et al*. (2012) investigated antibiotics resistance profile of *A. hydrophila* and results revealed that all the isolates were resistant to between 6 and 10 antibiotics. Eight of the isolates showed resistance to 8 antibiotics. Kudinha *et al*. (2004) also suggested *A. hydrophila* isolates to be more resistant to antibiotics than *A. caviae* and *A. sóbria*. Multiple resistance to antibiotics was observed in *A. caviae*, *A. media*, *A. sóbria* and *A. veronii* bv. so*bria* by Evangelista-Barreto *et al*.

(2010) and reported that *A. caviae* showed the highest multiple resistance. Numerous findings of fish pathogens resistant to one or several antibiotic agents have been reported, sometimes associated with serious economic losses, when widespread antimicrobial resistance impairs an effective medical treatment of clinical outbreaks (Schmidt *et al*., 2001).

Indian ornamental fish industry is emerging into a major export oriented one, as the biodiversity of the country and considerable demand from outside offer lots of scope. Green certification of the ornamental fish production centres is also becoming mandatory in order to ensure minimum/no use of antibiotics during production, maintenance and marketing. Results of the present study reveal considerable antibiotic resistance among the motile aeromonads from ornamental fish production facility such as small scale/commercial farms. This needs to be taken care of and steps should be initiated to reduce the antibiotic resistance level in such production facility.

Success of aquaculture should rely on maintaining fishes in good health, capable of competing infectious agents by keeping good water quality and reducing stress on fishes rather than the misuse of antibiotics and spreading of antibiotic resistance. Therefore, before antibiotics are even considered, sources of stress such as poor water quality, nutrition, genetics, crowding and handling or transport must be removed or reduced.

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Chapter 5

Molecular characterization, growth characteristics and virulence potential of a representative strain of *Aeromonas hydrophila*

5.1. Introduction

Aeromonas hydrophila is a ubiquitous Gram-negative rod-shaped bacterium which is motile by a single polar flagellum. It is catalase-positive, oxidase-positive and fermentative in nature (Pandove *et al*., 2013). It is commonly isolated from fresh water fishes and ponds and is a normal inhabitant of the gastro intestinal tract of fishes. It is a widely distributed opportunistic pathogen throughout the world.

A. hydrophila infection in fishes has been reported to occur from time to time in Asian countries including China, Phillipines, Thailand and India (Ebanks *et al*., 2004), and it leads to a decrease in production and economic losses (Hoque, 2014). The disease caused by this bacterium primarily affects

freshwater fish such as Cat fish, several species of Bass, and many species of tropical or ornamental fish (Kumar and Ramulu, 2013). In intensive fish culture systems fishes are more prone to infection by these bacteria (Sarkar and Rashid, 2012). It is a common aquatic bacterium that has increasingly been implicated in serious human infections also (Grim *et al*., 2013).

5.2. Review of literature

5.2.1. Taxonomy and classification

Although *Aeromonas* was initially positioned in the family *Vibrionaceae*, successive phylogenetic analysis 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* (Igbinosa *et al*., 2012). The genera of the family *Aeromonadaceae* now include *Aeromonas*, *Oceanimonas*, *Oceanisphaera*, and *Tolumonas*. Until the late 1970s, aeromonads were divided into two major groups based on physiological properties and host range. The two groups were *A. hydrophila* and *A. salmonicida.* Thereafter, the genus *Aeromonas* has advanced with the addition of new species and the reclassification of preexisting taxa. The genus now includes several species: *A. hydrophila*, *A. bestiarum, A. salmonicida*, *A. caviae*, *A. media, A. eucrenophila, A. sobria, A. veronii*, *A. jandaei, A. trota, A. schubertii, A. encheleia, A. allosaccharophila, A. popoffi, A. culicicola*, *A. simiae, A. molluscorum*, *A. sharmana*, *A. bivalvium*, *A. aquariorum*, *A. tecta, A. piscicola, A. fluvialis, A. taiwanensis, A. sanarellii* and *A. rivuli* (Kozinska, 2007; Janda and Abbot, 2010; Martinez-Murcia *et al*., 2011).

Identification and characterization of the *Aeromonas* species, has long been controversial due to their phenotypic and genomic heterogeneities. Since

biochemical properties do not accurately reflect the genomic complexity of a given species, molecular methods are used for the identification of *Aeromonas* isolates (Abulhamd, 2009). Genetic classification of aeromonads has been accomplished by mol% G+C composition, DNA–DNA relatedness studies, 16S rDNA sequence analysis and multilocus sequence typing analysis. Varieties of other molecular methods has been employed for the taxonomic purposes of *Aeromonas*, and include plasmid analysis, restriction enzyme analysis, ribotyping, restriction fragment length polymorphism and amplified fragment length polymorphism. In addition, the use of pulsefield–gel electrophoresis, species specific probes, and RLFP-PCR of 16S rDNA has been proposed (Carnahan and Joseph, 2005). But, 16S rRNA gene sequencing is the most commonly utilized molecular technique for genus and species identification of bacteria (Janda and Abbot, 2010).

5.2.2. Growth characteristics

Motile aeromonads are adapted to environments that have a wide range of conductivity, turbidity, pH, salinity and temperature. Temperature is considered as the major controlling factor in the distribution of *Aeromonas* spp. in natural environment. In fresh water or seawater within temperate latitudes, aeromonads were found in high numbers in late summer/early autumn when the temperature was around 20-25°C and were rarely detected during cold seasons (Kersters *et al*., 1995; Gavriel *et al*., 1998). The growth temperature range for aeromonads is from 4-44°C, but individual strains typically have a restricted growth range according to their ecological niche and growth of strains at both extremes of the range are rare (Guz and Sopińska, 2008). Shayo *et al*. (2012) reported the highest prevalence of *Aeromonas* during the dry season when temperatures were higher (27.2°C).

According to Pandove *et al*. (2013), *A. hydrophila* grow over a wide temperature range, 0 to 45°C, with a temperature optimum of 22 to 32°C. Guz and Sopińska (2008) reported that *A. hydrophila* isolated from motile aeromonad septicaemia diseased carp, grew better at 28°C than at 18 and 38°C. The maximum growth temperature for most strains of *A. hydrophila* appears to be at least 42°C with most enterotoxigenic strains capable of growth at 43°C. Jayavignesh *et al*. (2011) reported that *A. hydrophila* isolated from diseased Catfish was able to grow well at the optimum temperature of 37°C, but it also had the ability to grow at 4°C. According to Holt *et al*. (1994), the optimal temperature range for multiplication of *A. hydrophila* lies between 22 and 28ºC. The growth of *A. hydrophila* at different temperatures ranging from 4 to 42°C and 5 to 35°C have been reported by Palumbo *et al*. (1985 b) and Callister and Agger (1987) respectively. The effect of temperature on the growth kinetics of strains of *A. hydrophila* was evaluated by several workers (Knochel, 1990; Stecchini *et al*., 1993; Santos *et al*., 1996; Sautour *et al*., 2003; Wang and Gu, 2005). Temperature is responsible for the increases in the number of *A. hydrophila* in natural aquatic habitats (Mateos *et al*., 1993).

Aeromonas spp. is fairly sensitive to low pH and are able to tolerate pH up to 9.8. They prefer alkaline pH (Wang and Gu, 2005). Jayavignesh *et al*. (2011) reported that *A. hydrophila* was able to grow well at a pH range of 5 to 9, but minimum growth was found at pH 5 when compared to other ranges (5-9). According to Sautour *et al.* (2003), *A. hydrophila* can grow at pH 5.8 or higher, and may survive at pH 4.6, but tolerate high pH well. All *Aeromonas* resist pH ranges from 4.5 to 9 but the optimum pH range is 5.5 to 9 (Isonhood and Drake, 2002). Incubation of *A. hydrophila* at different pH values, *i.e*. 6.0,

6.5, 7.0 and 7.5 did not significantly affect the growth rates (Buncic and Avery, 1995).

Tolerance to moderate concentrations of sodium chloride by *Aeromonas* species is reported by several authors (Palumbo, 1988; Knochel 1990; Beuchat, 1991; Delamare *et al*., 2000). Palumbo *et al*. (1991) determined that *A. hydrophila* was able to grow in the presence of 0·6 M NaCl. Saline habitats had a much higher density of *A. hydrophila* than did freshwater habitats (Hazen *et al*., 1978). Vivekanandhan *et al*. (2003) reported that NaCl concentration of 0.5, 1.0 and 2% favoured the growth of *A. hydrophila.* According to Isonhood and Drake (2002), the optimum sodium chloride concentration range for the growth of *Aeromonas* is 0 to 4%. The growth rate of *A. hydrophila* is optimal at 30°C at pH 7 and when water activity is 0.99 (Sautour *et al.,* 2003).

5.2.3. Virulence genes

Evaluation of *Aeromonas* virulence requires the assessment of virulence phenotypes and complete virulence genes set. Different combinations of virulence determinants normally exist in *Aeromonas* species. Toxins with haemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas* spp.; and while a number of toxins are produced by different species, single isolates often carry the genes encoding multiple toxins. Aerolysin (Aer), cytotonic enterotoxin (Alt), cytotoxic enterotoxin (Act), temperature-sensitive protease (EprCAI) and serine protease (Ahp) are indicated in the pathogenesis of *Aeromonas* (Xu *et al*., 1998; Heuzenroeder *et al*., 1999; Albert *et al*., 2000; Li and Cai, 2011) but, haemolytic toxins (Joseph and Carnahan, 2000; Abrami *et al*., 2003) play an important role in

their virulence. Two haemolytic toxins have been described in *A. hydrophila*: the AHH1 haemolysin and aerolysin. Most *Aeromonas* haemolysins described are related to one of these two toxins (Heuzenroeder *et al.*, 1999).

Aerolysin has been studied for at least 3 decades and thus is well characterized. The 54-kDa pore-forming toxin (PFT) is secreted as pro-aerolysin that binds with high affinity to glycosylphosphatidylinositol (GPI)-anchored proteins on target cells to integrate into the plasma membrane. The proteins build stable heptameric aerolysin complexes that form β-barrel pores (Bucker *et al.*, 2011). Analysis of the nucleotide sequence showed that the haemolysin is homologous with aerolysin (*A. hydrophila* haemolysin). The overall homology in amino acid sequence between the haemolysin and aerolysin was 68.5% identity. The two toxins have similar modes of action (Fujii *et al.*, 2008). In addition, a gene encoding the cytolytic enterotoxin (Act) from *A. hydrophila* has been sequenced and shown to possess haemolytic, cytolytic and enterotoxic activities. Sequence analysis revealed that the *act* gene shared 89 and 93% DNA and amino acid homologies respectively, with the *A. hydrophila* aerolysin gene. Mutagenesis studies indicated that aerolysin mutant strains were less virulent in assays of toxicity *in vivo* and *in vitro* (Abrami *et al.*, 2003; Fadl *et al.*, 2007) and the haemolytic activity of *A. hydrophila* is related to both the haemolysin and the aerolysin genes (Wang *et al.*, 2003).

Screening for specific cytotoxin and haemolysin genes appears to be the most effective way of detecting and characterizing *Aeromonas* virulence factors (Wang *et al.*, 2003). Direct detection of the haemolytic genes *aer A* and *hly A* has been suggested as a reliable approach for identifying potentially pathogenic *Aeromonas* strains (Heuzenroeder *et al.*, 1999).

5.2.4. Pathogenicity

Pathogenicity of *A. hydrophila* in fishes was studied by several authors (El-Barbary, 2010; Citarasu *et al*., 2011; Hu *et al*., 2012; Sarkar and Rashid, 2012; Saad *et al*., 2014). Santos *et al*. (1991) have determined the LD_{50} of *A. hydrophila* to several fish species: *Salmo trutta (*$2x10^5$ cells/ml), *Anguilla japonica* (>10^8 cells/ml), *Plecoglossus altivelis* ($8,6x10^4$ cells/ml), *Lepomis macrochirus* (>10^8 cells/ml). Oliveira *et al.* (2011) have found that the 96-h LD_{50} value of *A. hydrophila* to matrinxã is 6.66 x 10^{11} cells/ml. *A. hydrophila* injected at 10^7 cfu/ml caused nearly cent percent mortality in *Clarias batrachus* (Thune *et al*., 1982) and *Carassius auratus* fingerlings (Citarasu *et al*., 2011). Lethal doses of *A. hydrophila* to *Channa punctatus* was found to be 10^9 cfu/ml (Yesmin *et al*., 2004) and to *R. quelen* was $1.3x10^9$ and $3.5x10^8$ cfu/ml (Oliveira *et al.,* 2011). Pathogenicity of *A. hydrophila* recovered from naturally diseased Shing fish was investigated against Catfishes (*Heteropneustes fossilis* and *Clarias batrachus*), Carps (*Labeo rohita, Catla catla* and *Cirrhinus cirrhosus*) and Perch (*Anabas testudineus*) by Sarkar and Rashid (2012) and pathogenicity was confirmed by mortality of 60 to 100% of all the tested fishes within 2-11 days.

A. hydrophila was isolated from haemorrhagic diseased freshwater fishes in China (Ye *et al*., 2013). In the Indian major carp, *Catla catla*, a highly virulent strain of *A. hydrophila* has been isolated from Andaman during 1996–1998 (Shome and Shome, 1999). *A. hydrophila* has been known as the most possible cause of the disease of common Carp (Sumawidjaja *et al*., 1981). Epizootic ulcerative syndrome, involving both cultured and wild fishes in Burma, Indonesia, Malaysia, Singapore and

Thailand was found to be associated primarily with *A. hydrophila* (Harikrishnan and Balasundaram, 2005). *A. hydrophila* is considered to be the principal cause of bacterial haemorrhagic septicaemia in fresh water fish and has been reported in association with various ulcerative syndrome and red spot disease (Kumar and Ramulu, 2013; Hoque *et al*., 2014). These infections can cause high mortalities in fish hatcheries and in natural fresh water fish population.

5.3. Objectives of the study

In India, *A. hydrophila* has been isolated and characterized from aquatic environment and various fishes (Illanchezian *et al*., 2010; Kaskhedikar and Chhabra, 2010; Sarkar *et al*., 2013). Citarasu *et al*. (2011) have isolated *A. hydrophila* from Goldfish (*Carrassius auratus*) and Koi (*Cyprinus carpio koi*), during massive fish disease outbreaks from various ornamental fish hatcheries in South India. The onset of infection depends on the virulence of the pathogen, the possible route of entry of the bacteria (Roberts, 1993), their distribution within the fish (Rey *et al*., 2009), the fish species and resistance, environmental conditions and the season.

Wide spectrum of plasticity to environmental conditions, worldwide distribution, opportunistic nature and high virulence potential increases the risk of *A. hydrophila* as a menace to aquatic animal health and bring heavy economic loss to the aquaculture industry. Since we have encountered considerable share of *A. hydrophila* among the fresh water ornamental fishes from farm and retail aquariums, we decided to characterize in detail a representative strain of *A. hydrophila*. The specific objectives were as follows:

1) Molecular identification of a representative strain of *Aeromonas hydrophila.*

2) Study of the effect of environmental parameters on the growth of selected *A. hydrophila* strain.

3) Molecular detection of virulence genes in the selected *A. hydrophila* strain.

4) Determination of its virulence potential *in vivo.*

5.4. Material and Methods

5.4.1. Source of *Aeromonas hydrophila*

A representative strain of *Aeromonas hydrophila* NJ 87 isolated from the body surface of *Poecilia sphenops* (John and Hatha, 2013) and identified by phenotypic and biochemical methods (section 2.4.3.) was selected for further molecular characterization.

5.4.2. Molecular characterization

Molecular characterization of the isolate was done by 16S rRNA gene sequencing, which involves extraction of genomic DNA, PCR amplification of the DNA and sequencing of the PCR product.

5.4.2.1. Extraction of genomic DNA

Pure culture of *Aeromonas hydrophila* NJ 87 was inoculated in Luria Bertanii broth and incubated at 28°C, 120 rpm for 12 hours. 1 ml aliquot of the culture was centrifuged at 8000 rpm for 10 minutes at 4°C and the resultant pellet was resuspended in 500 µl TEN buffer (Tris-HCl 10 mM, pH 8.0, EDTA 1 mM, NaCl 0.15 mM) and centrifuged again at 8000 rpm for

10 minutes at 4°C. Subsequently, the pellet was resuspended in 500 µl Lysis buffer (Tris-HCl 0.05 mM, pH 8.0, EDTA 0.05 mM, NaCl 0.1 mM, SDS 2%, PVP 0.2% and 0.1% mercaptoethanol) (Lee *et al.,* 2003). Proteinase K (20 µg/ml) was added and incubated first at 37°C for 1 h and then at 55°C for 2 h. After this step, the DNA was further extracted by standard phenol-chloroform method of Sambrook and Russell (2001). The sample was deproteinated by adding an equal volume of phenol (tris-equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and aqueous layers were separated by centrifugation at 15000 rpm for 15 minutes at 4°C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once again. After this, an equal volume of chloroform:isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15000 rpm for 15 minutes at 4°C to separate the aqueous phase which was transferred to a fresh tube. Then the DNA was precipitated by incubation at –20°C overnight after adding equal volume of ice-cold absolute alcohol and 0.1% (v/v) 3M sodium acetate. The precipitated DNA was collected by centrifugation at 15000 rpm for 15 minutes at 4°C and the pellet was washed in ice-cold 70% ethanol. Centrifugation was repeated once again and the supernatant decanted and the tubes were left open until the pellet dried. DNA pellet obtained was resuspended in Tris- EDTA (TE) buffer (Tris-HCl 0.5 mM, EDTA 0.5 mM, pH 8.0).

The isolated DNA was quantified spectrophotometrically (A_{260}) and the purity of DNA was assessed by calculating the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}), the value of which determined the amount of protein impurities in the sample. Electrophoresis was done using 0.8 % agarose gel. Concentration of DNA was calculated from the following formula:

Conc. of DNA (µg/ml) = OD at 260 nm x 50 x dilution factor.

5.4.2.2. 16S rRNA gene amplification

The universal bacterial primers, F: 5′-GAGTTTGATCCTGGCTCA -3′and R: 5′ -ACGGCTACCTTGTTACGACTT -3′, were used to amplify the 16S rRNA genes of the isolate (Reddy *et al.,* 2000). The amplification reaction was performed by using a DNA thermal cycler (Bio-Rad laboratories, USA).

Bacterial DNA (50 ng) was amplified by polymerase chain reaction (PCR) in a total volume of 25 µl containing 2.5 µl of 10X PCR buffer, 0.5 U Taq DNA Polymerase (New England Biolabs), 10 pmol each of the two primers, and 200 µM each of dATP, dCTP, dGTP and dTTP. PCR program comprised an initial denaturation step of 95°C for 5 minutes, 35 cycles of 94°C for 20 sec, 58°C for 30 sec and 68°C for 2 min, followed by a final extension of 10 min at 68°C. The amplification was carried out in a thermocycler (Bio-Rad laboratories). PCR products were analyzed by electrophoresis on 1% agarose gel prepared in 1X TAE buffer and stained with ethidium bromide. PCR product was purified using Gen Elute PCR clean up kit (Sigma).

5.4.2.3. Nucleotide sequencing

Nucleotide sequencing was performed with AB1 PRISM 3700 Big Dye Sequencer at Sci Genom Labs, Kakkanad, Cochin.

5.4.2.4. 16S rRNA gene sequence similarity and Phylogenetic analysis

DNA sequence data were compiled and analyzed. Sequence analysis was completed by using BLAST network services, Clustal W and MEGA 5. The Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.,*

1990) was used to search the GenBank database for homologous sequences (http:// www.ncbi.nlm.nih.gov). The sequences were multiple aligned using the programme Clustal W (Thompson *et al.,* 1994). Then the aligned 16S-rDNA gene sequences were used to construct a phylogenetic tree using the neighbour-joining (NJ) method (Saitou and Nei, 1987) using the MEGA 5 package (Tamura *et al.,* 2011). Bootstrap analysis was based on 1000 replicates.

5.4.3. Study of the effect of environmental parameters on the growth of *Aeromonas hydrophila* NJ 87

The growth of the isolate under different environmental conditions was studied. The different environmental parameters tested were temperature, pH and salinity.

5.4.3.1. Effect of temperature on the growth

Nutrient broth, in 5 ml aliquots was prepared and sterilized. The nutrient broth was inoculated with 25 µl of a 24 h old of *A. hydrophila* NJ 87 culture, incubated at different temperatures for 18 h and OD_{610} values were determined. Different temperature selected for the study was 5, 10, 15, 25, 30, 35, 40 and 45°C. The experiment was conducted in triplicate.

5.4.3.2. Effect of pH on the growth

Nutrient broth with different pH values, in 5 ml aliquots was prepared and sterilized. The different pH range selected for the study was 3, 4, 5, 6, 7, 8, 9, 10 and 11. The nutrient broth was inoculated with 25 µl of a 24 h old bacterial culture, incubated at 28°C for 18 h and OD_{610} values were determined. The experiment was conducted in triplicate.

5.4.3.3. Effect of salinity on the growth

Nutrient broth with different salinity, in 5 ml aliquots was prepared and sterilized. The salinity range selected for the study were 0%, 0.5%, 1%,1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5% and 6% NaCl w/v. The nutrient broth was inoculated with 25 µl of a 24 h old bacterial culture, incubated at 28°C for 18 h and OD_{610} values were determined. The experiment was conducted in triplicate.

5.4.4. PCR Amplification of virulence genes

Genomic DNA extraction was carried out as described in the previous section 5.4.2.1.Virulence genes selected for the study were aerolysin (*Aer A*), haemolysin (*hly A*) and cytotoxin (*Act*). Details of primers used for the detection of these virulence genes are given in Table 5.1.

5.4.4.1. Detection of aerolysin gene

The PCR reaction mixture consisted of a final volume of 25 µl and contained 200 µM of each deoxynucleotide dATP, dCTP, dGTP and dTTP, 15 pmol of primers Aer A1 and Aer A2 (sequences for each primer is given in Table 5.1), 5 µl of 10X PCR buffer, 1.5 µl of template DNA, 1.5mM of $MgCl_2$ and 1 unit of Taq DNA Polymerase (New England Biolabs) and Milli Q water (to a final volume of 25 µl). PCR was performed in 0.2ml PCR tubes.

The PCR program comprised an initial denaturation step of 94°C for 5 min followed by 35 cycles of 0.5 min at 94°C, annealing for 0.5 min at 52°C and 2 min extension at 72°C with a 10 min final extension at 72°C in a thermocycler (Bio-Rad laboratories, USA).

5.4.4.2. Detection of haemolysin gene

The PCR reaction mixture consisted of a final volume of 25 µl and contained 200 µM of each deoxynucleotide, dATP, dCTP, dGTP and dTTP, 15 pmol of primers Hly H1 and Hly H2 (sequences for each primer is given in Table 5.1), 5 µl of 10X PCR buffer, 1.5 µl of template DNA, 1.5mM of $MgCl_2$ and 1 unit of Taq DNA Polymerase (New England Biolabs) and Milli Q water (to a final volume of 25 µl). PCR was performed in 0.2ml PCR tubes.

The amplification protocol consisted of an initial denaturation step of 94°C for 5 min followed by 35 cycles of 0.5 min at 94°C, annealing for 0.5 min at 62°C and 2 min extension at 72°C with a 10 min final extension at 72°C in a thermocycler (Bio-Rad laboratories, USA).

5.4.4.3. Detection of cytotoxin gene

PCR was performed by using 25 µl of a PCR mixture containing 1.5 µl of template DNA, 200 µM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 2.5 µl of 10 X PCR buffer, 2.5 µl of a 25 mM $MgCl_2$ solution, 0.25 µl of a 200 mM solution of primers Act F and Act R (sequences for each primer is given in Table 5.1), and 1 unit of Taq DNA Polymerase (New England Biolabs) and Milli Q water (to a final volume of 25 µl). PCR was performed in 0.2ml PCR tubes.

PCR amplification was performed by using the following temperature program: 1 cycle of denaturation for 5 min at 95°C; 25 cycles of melting at 95°C for 15 sec, annealing at 66°C for 30 sec, and elongation at 72°C for 30 sec; and a final extension round at 72°C for 10 min in a thermocycler (Bio-Rad laboratories, USA).

Table 5.1 Primers used for PCR detection of virulence genes

Primers	Virulence genes	DNA sequences (5´ - 3´)	PCR product (bp)	References
Aer A1	*A. hydrophila* aerolysin gene	gcctgagcgagaaggt	**416**	Heuzenroeder *et al*. (1999)
Aer A2		cagtcccacccacttc		
HlyA H1	*A. hydrophila* haemolysin gene	ggccggtggcccgaagatacggg	**597**	Heuzenroeder *et al*. (1999)
HlyA H2		ggcggcgccggacgagacggg		
Act F	*A. hydrophila* cytotoxin gene	gagaaggtgaccaccaagaaca	**232**	Kingombe *et al*. (1999)
Act R		aactgacatcggccttgaactc		

5.4.5. Gel Electrophoresis

The PCR amplicons were electrophoresed on 1.5% agarose gel prepared in 1X TBE buffer and stained with ethidium bromide. The gel image was visualized through a Gel Doc system (Bio-Rad Gel Doc^{TM} Imager, USA).

5.4.6. Determination of virulence potential *in vivo*

5.4.6.1. Experimental Fish and its maintenance

Fresh water ornamental fish, Koi carp (*Cyprinus carpio*), collected from an aquarium shop in Cochin, Kerala, India were used for the LD_{50} determination. Fishes weighing ~1.5g ± 0.2g were brought to the laboratory, acclimatized in tanks containing dechlorinated water over a period of three weeks. The number of fishes stocked in each tank was according to Organization for Economic Co-operation and Development (OECD) guide lines. Faeces and uneaten feed residues were siphoned out of the tank together with about one third of the water volume of the aquarium each day and replaced with fresh dechlorinated tap water before the morning feed. The water temperature ranged from 25±2°C, dissolved oxygen concentration from 6.8-7.4 ppm, pH 7-7.5 and salinity 0 ppt. Fishes were fed on pelleted commercial feed *ad libitum.* After the period of acclimatization, the fishes were transferred to the respective experimental tanks.

5.4.6.2. Bacterial strain

Pure culture of *Aeromonas hydrophila* NJ 87 was grown in nutrient broth for 24 h at 28°C. The broth cultures were harvested by centrifugation at 5000 × g for 15 min at 4°C. The bacterial pellet was washed by resuspension in sterile phosphate buffered saline (PBS-pH 7.4) and centrifugation as above and the final pellet was resuspended in PBS to get a cell density of 1 x 10^8 cells/ml and serially diluted to get 10^7 to 10^4 cells/ml. The viable counts of the suspension were confirmed by spread plate technique.

5.4.6.3. Determination of 50% lethal dose (LD_{50}) value

LD_{50} of the bacterium was determined by intra-peritoneal (IP) injection of experimental fishes in each group with one of the different dilutions (ranging from 10^4 to 10^8 cfu/ml) of *Aeromonas hydrophila* NJ 87. For the inoculation, fishes were previously anesthetized with clove oil (80ppm). Eight fishes from each group were injected with 0.1 ml of one of the saline suspension of 10^4, 10^5, 10^6, 10^7 and 10^8 cfu/ml of the specific isolate. The experiment was conducted in triplicate. Control group was injected with 0.1ml saline. Fishes were recovered from anesthetized condition, distributed into respective tanks. Mortalities were recorded daily for 7 days. The pathogen was reisolated from dead fish samples to confirm the cause of mortality. The LD_{50} was calculated following Reed and Muench (1938).

5.4.7. Statistical analysis

Statistical analysis of data was performed using one way Analysis of Variance (ANOVA) with post- hoc multiple comparison analysis using Tukey's HSD. Mean of the results was compared using SPSS 13.0 package

for windows at a significance level of $p<0.05$. Data are presented as mean ± standard deviation (SD).

5.5. Results

5.5.1. Molecular identification

16S rRNA gene amplification of the representative strain using universal primers resulted in an amplicon with a product size of 1500bp (Figure 5.1). This product was partially sequenced and the 16S rRNA gene sequences of the isolate has been submitted to the GenBank data base and compared using the BLAST algorithm. The sequences showed a high similarity (99% identity, 100% query coverage) to that of *A. hydrophila* type strain - LMG 19562T. The sequences were deposited in GenBank, and were allotted with an Accession No. JX987236 (Appendix 2).

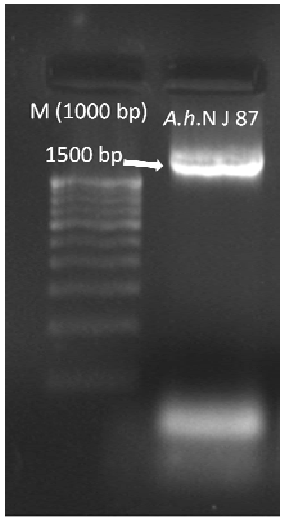

Figure 5.1. PCR product of 16S rRNA gene of the representative strain
M - DNA ladder, *A.h.* NJ 87–1500 bp PCR product of 16S rRNA gene of the isolate

5.5.2. Phylogenetic tree

The phylogenetic tree (Figure 5.2) constructed from 16S rRNA sequences of the isolates and 20 homologous sequences, using the neighbour-joining method, clustered the isolate with *A. hydrophila* type strain-LMG 19562T (Accession No. NR 042155).

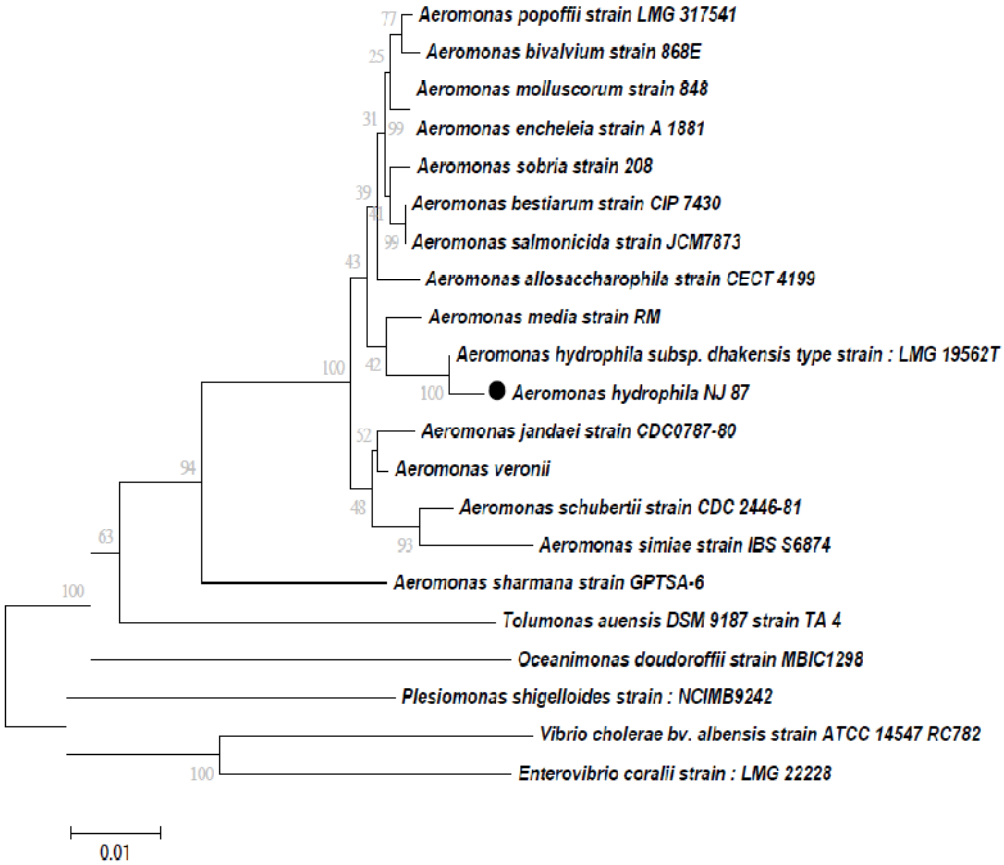

Figure.5.2. The phylogenetic tree, based on 16S rRNA sequences, generated using the neighbour-joining method; 1000 bootstrap replicates. The bootstrap values (%) are shown besides the clades and scale bars represent distance values

5.5.3. Effect of environmental parameters on the growth of *Aeromonas hydrophila* NJ 87

5.5.3.1. Effect of temperature

The isolate was found to grow over a wide temperature, ranging from 10 to 45°C (Figure 5.3). Maximum growth occurred at 30°C, while there was no significant difference (p<0.05) in growth at 25 and 30°C (Appendix 3.1).

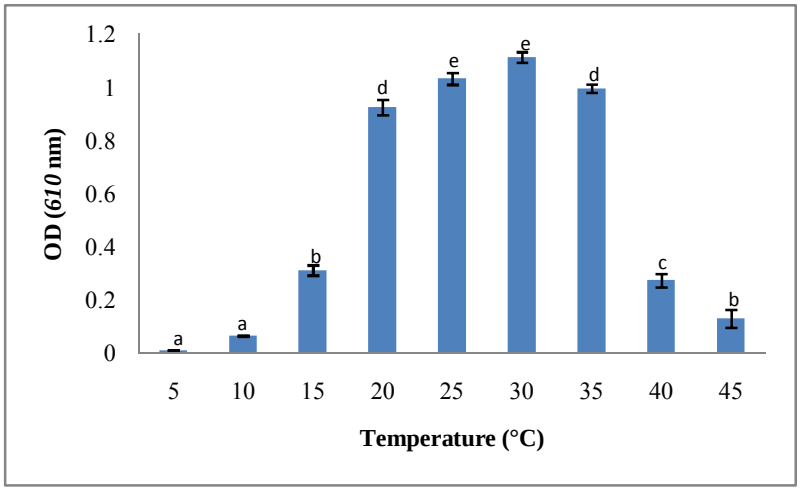

Figure 5.3. Effect of temperature on the growth of *A. hydrophila* NJ 87

* Values with different superscripts denote significant differences. Values with same superscripts denote no significant differences. Error bars represent standard error of the mean.

5.5.3.2. Effect of pH

The isolate was found to grow over a wide range of pH, ranging from 5 to 10. Maximum growth occurred at pH 7, though there was no significant difference (p<0.05) in growth at pH 6 and 7 (Appendix 3.2). Effect of pH on the growth of the isolate is given in Figure 5.4.

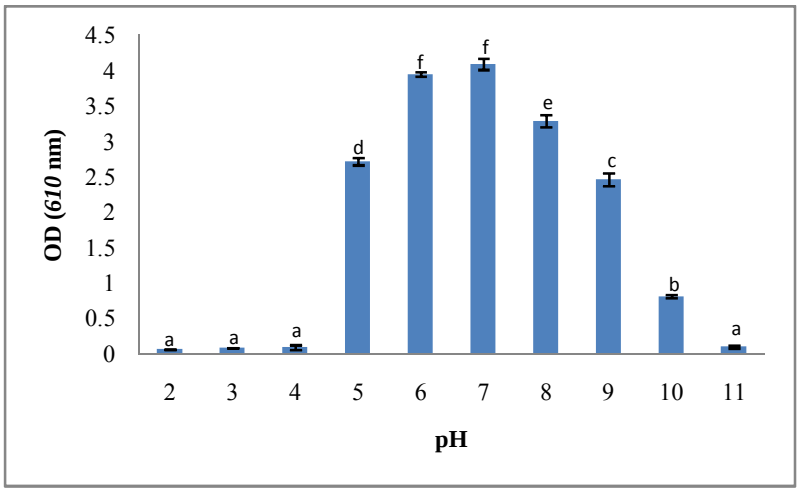

Figure 5.4. Effect of pH on the growth of *A. hydrophila* NJ 87

***** Values with different superscripts denote significant differences. Values with same superscripts denote no significant differences. Error bars represent standard error of the mean.

5.5.3.3. Effect of salinity

The isolate was found to grow over a wide range of salinity, with NaCl content in the medium ranging from 0 to 4.5% (w/v). The optimum NaCl content for growth was found to be 0.5 and 1%, though no significant difference ($p<0.05$) in growth was observed in salinity ranges from 0.5 to 2% (Appendix 3.3). NaCl content higher than 4.5% was found to inhibit the growth of *A. hydrophila* NJ 87 (Figure 5.5).

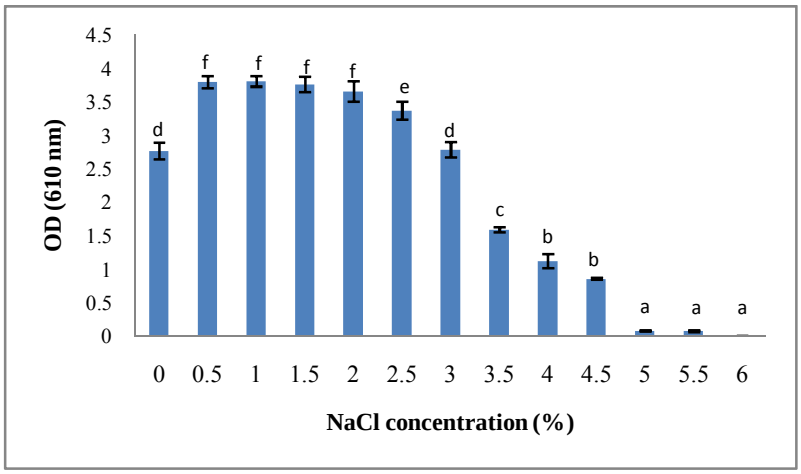

Figure 5.5. Effect of salinity on the growth of *A. hydrophila* NJ 87

***** Values with different superscripts denote significant differences. Values with same superscripts denote no significant differences. Error bars represent standard error of the mean.

5.5.4. Virulence genes in *A. hydrophila* NJ 87

5.5.4.1. Aerolysin gene

PCR amplification performed using primers Aer A1 and Aer A2 resulted in an amplicon with a product size of 416 bp (Figure 5.6) reflecting the presence of aerolysin genes in *A. hydrophila* NJ 87.

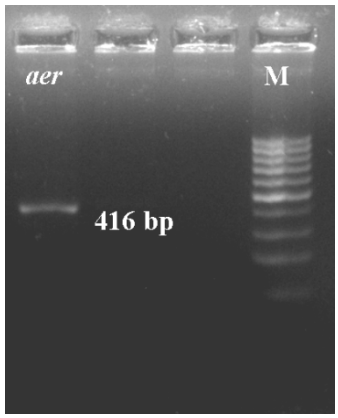

Figure 5.6. Gel image showing aerolysin gene

*M- DNA ladder, *aer*- 416 bp PCR product of aerolysin gene of the isolate

5.5.4.2. Haemolysin gene

PCR using primers Hly H1 and Hly H2 resulted in an amplification of a 597 bp product (Figure 5.7) reflecting the presence of haemolysin genes in *A. hydrophila* NJ 87.

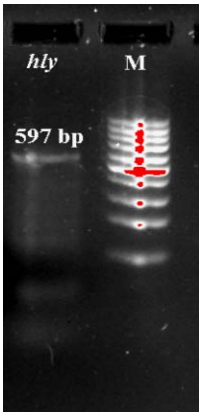

Figure 5.7. Gel image showing haemolysin gene
*M - DNA ladder, *hly* -597 bp PCR product of haemolysin gene of the isolate

5.5.4.3. Cytotoxin gene

PCR amplification performed using primers Act F and Act R resulted in an amplification of a 232 bp product (Figure 5.8) reflecting the presence of cytotoxin genes in *A. hydrophila* NJ 87.

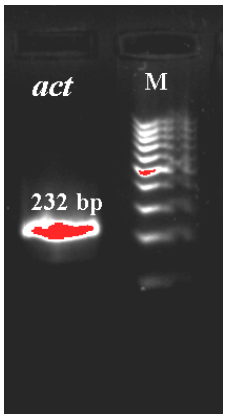

Figure 5.8. Gel image showing cytotoxin gene
*M - DNA ladder, *act*- 232 bp PCR product of cytotoxin gene of the isolate

5.5.5. Virulence potential *in vivo*

The virulence of *Aeromonas hydrophila* NJ 87 was assessed *in vivo* from the LD_{50} values. When the fishes were injected with 10^8 and 10^7 cfu/ml of the isolate, there was 100% mortality, while no mortality was observed on injection with10^4 cfu/ml. LD_{50} of the isolate was found to be $10^{6.1}$cfu/ml (Appendix 4) and it displayed the virulent nature of the isolate.

Skin ulcerations, tail and fin rot, haemorrhagia, protruded abdomen, bristling out of scales from skin and exophthalmia were observed in moribund fishes and *A. hydrophila* was reisolated from all the dead fish samples. The pathogen was not present in samples from survivors, and mortalities did not occur in the control groups injected with sterile saline.

5.6. Discussion

Mesophilic *Aeromonas* species, most notably *Aeromonas hydrophila*, have been linked to major fish disease outbreak around the world over the past decade, resulting in enormous economic losses (Janda and Abbott 2010). *A. hydrophila* is the causative agent of motile aeromonad septicaemia (Afizi *et al*., 2013), and in Asian countries fish culture continues to be ravaged by this disease. Therefore a representative strain of *A. hydrophila* isolated from the body surface of the fish *Poecilia sphenops* and identified by phenotypic and biochemical methods was selected for virulence studies. Growth characteristics of the isolate were also studied. It was first confirmed as *A. hydrophila* by 16S rRNA gene sequencing. The strain showed 99% similarity to the gene sequences of a type strain of *A. hydrophila*-LMG 19562T. 16S rRNA gene sequencing is considered as a reliable tool for

identification of bacteria. The result of 16S rRNA gene sequencing confirmed the result of phenotypic and biochemical studies.

5.6.1. Growth characteristics

Temperature dependent seasonal variations have been observed for *Aeromonas* spp. with the highest population in summer and the lowest in winter (Guz and Sopińska, 2008). Shayo *et al*. (2012) reported the highest prevalence of *Aeromonas* during the dry season when temperatures were higher (27.2°C). They grow optimally at temperature ranges between 22°C and 35°C, but growth can also occur at 0–45°C in a few species (Ghenghesh *et al*., 2008). Rouf and Rigney (1971) noticed that various strains of *Aeromonas* have a wide range of growth temperature.

The *A. hydrophila* isolate in the present study exhibited growth in a wide range of temperature. The optimum temperature requirement of the isolate was 25-30°C. Pandove *et al*. (2013), reported that *A. hydrophila* grow over a wide temperature range, 0-45°C, with a temperature optimum of 22 to 32°C. Our results are also in agreement with the findings of Khalil and Mansour (1997), who found that the optimum temperature for *A. hydrophila* growth was 30°C. Guz and Sopińska (2008) reported that *A. hydrophila* isolated from motile aeromonad septicaemia diseased Carp, grew better at 28°C than at 18 and 38°C. The maximum growth temperature for most strains of *A. hydrophila* appears to be 42°C. Jayavignesh *et al*. (2011) reported that *A. hydrophila* isolated from diseased Catfish was able to grow well at the optimum temperature of 37°C, while growth at temperature range of 41°C was found to be minimum when compared to other ranges. The isolate was not able to grow at a temperature of 50°C. Hazen *et al*. (1978) reported that the thermal

optimum for most strains of *A. hydrophila* is 35°C, and the thermal maximum is very close to 45°C which is very similar to our findings.

The isolate *A. hydrophila* NJ 87 in the present study was found to grow over a wide range of pH from 5 to 10. However, maximum growth occurred at pH 7 though there was no significant difference in growth between pH 6 and 7. This was similar to the observation of Buncic and Avery (1995), who reported that incubation of *A. hydrophila* at different pH values from 6-7.5 did not significantly affect the growth rates. Sautour *et al.* (2003) also reported that pH 7 is optimum for the growth of *A. hydrophila.* It can grow at pH 5.8 or higher, and may survive at pH 4.6, but tolerate high pH well. It is reported that *A. hydrophila* was able to grow well at a pH range of 5 to 9, but the growth at pH range of 5 was found to be minimum (Jayavignesh *et al*., 2011). We have also observed very minimal growth at pH values less than 5 and greater than 9. It is reported that *A. hydrophila* show more or less similar growth at pH 7.0, 8.0 and 9.0 at 30°C (Vivekanandhan *et al*., 2003). All *Aeromonas* resist pH ranges from 4.5 to 9 but the optimum pH range is 5.5 to 9 (Isonhood and Drake, 2002). Wang and Gu (2005) suggested a strong suppressing effect of acidity on *Aeromonas* growth. Hazen *et al*. (1978) observed that *A. hydrophila* growth is unaffected by pH's from 5 to 9 and that it is incapable of growth at a pH lower than 4 or higher than 10.

Our results on the effect of NaCl on the growth of *A. hydrophila* NJ 87 revealed its ability to grow over a NaCl content in the medium ranging from 0 to 4.5% (w/v). The influence of salt concentration on the growth of *A. hydrophila* was studied by Vivekanandhan *et al*. (2003), and the results revealed that NaCl concentration of 0.5, 1.0 and 2.0% favoured the growth of

this organism at 30°C. Similar to their observation, the optimum NaCl content for growth of the isolate in present study ranged from 0.5 to 2%. Increase in the salt concentration resulted in decrease in the growth of this organism. While, 3.5-4.5% salt concentration supported moderate growth of the organisms in the medium, at 5.0% NaCl concentration there was no growth. Isonhood and Drake (2002) reported that the optimum sodium chloride concentration range for the growth of *Aeromonas* is 0 to 4%.

Sodium chloride (NaCl) or salt is commonly used in aquaculture for the control of microbial infections. In addition, salt is applied to improve fish survival during transportation (Velasco-Santamaria and Cruz-Casallas, 2008). Harpaz *et al*. (2005) stated that addition of 4% salt to the fish diet lead to better feed utilization. Surplus 0.1% salt to water is recommended for fresh-water fish as stress reducing from low temperature (Koeypudsa and Jongjareanjai, 2010).

5.6.2. Virulence and pathogenicity of *A. hydrophila* NJ 87

The pathogenic and virulence characteristics of *A. hydrophila* are associated with a range of different exotoxins (haemolysin, enterotoxins and cytotoxins) and exoenzymes (eg., proteases and lipases) (Yogananth *et al*., 2009). The cytotonic enterotoxins Ast and Alt (Chopra *et al*., 1996; Albert *et al*., 2000; Sha *et al*., 2002), and the cytotoxin encoded by the *act* gene (Xu *et al*., 1998), play an important role in the pathogenesis of *Aeromonas*. However, among the several *Aeromonas* toxins, the aerolysin/haemolysin group (which include the Act toxin) is the most important for pathogenesis (Carnahan and Joseph, 2005). The isolate in the present study possessed both haemolysin and aerolysin gene, signifying its role as a pathogen.

Since previous studies (Wong *et al*., 1998) have suggested that the combined effect of aerolysin (AerA) and a *Vibrio cholerae*-HlyA-like haemolysin (HlyA) contributes to virulence in *A. hydrophila*, a different approach for the identification of potentially pathogenic *Aeromonas* isolates is the PCR detection of the genes for the haemolysins *Aer* A and *Hly* A (Serrano *et al*., 2002).

The cytotoxic enterotoxin encoded by the *act* gene of *A. hydrophila* has multifunctional activities: it has cytotoxic and haemolytic activities, in addition to having enterotoxic activity (Xu *et al*., 1998; Chopra and Houston, 1999). The β-haemolysin-related aerolysin and the cytotoxic enterotoxin (Act) are pore-forming toxins able to alter cell permeability.

Ye *et al*. (2013) reported that, among the *A. hydrophila* isolates tested by them, aerolysin and cytotoxin were present in 85% and 35% of the strains respectively. Hu *et al*. (2012) evaluated the frequency of the aerolysin (*aer*) and cytotoxic enterotoxin (*act*), in *Aeromonas* and observed that *act* genes were present in most strains (87%), while the *aer* gene was present in only 47%. *Act* gene was present in 58.3% of the *Aeromonas* strains isolated by Sreedharan *et al*. (2012). Homogeneous distribution of aerolysin and cytotonic enterotoxin genes in *A. hydrophila* complex strains was also observed by Castilho *et al*. (2009). They observed that the most common genotype found in *A. hydrophila* strains was hly^+ (85%) and $aer\ A^+$ (78.7%). Serrano *et al.* (2002) tested eleven strains of *A. hydrophila* from freshwater fish and one strain of *A. hydrophila* from human diarrhoea, for the presence of the haemolytic genes *aer A* and *hly A*, and found that ten *A. hydrophila* isolates were $aer\ A^+\ hly\ A^+$ while two were $aer\ A^+ hly\ A^-$. Heuzenroeder *et al*. (1999) also noted that $aer\ A^+$ $hly\ A^+$ genotype was the most common in *A. hydrophila*. They suggested that

haemolytic and cytotoxic activities of *A. hydrophila* isolate in their study were conferred by two haemolytic toxins, HlyA and AerA. It was proposed that most virulent aeromonads may have at least two haemolytic toxins. When the genotypes of known virulent strains were compared, it was apparent that all *A. hydrophila* isolates with the *hly* A^+ *aer* A^+ genotype were virulent.

A. hydrophila isolated from the fish *Poecilia sphenops* in the present study was both *hly* A^+ and *aer* A^+. A 232 bp fragment of *act* gene was also amplified in the isolate. The presence of all the three genes in the isolate indicates the highly virulent nature of the strain. This assumes significance as most of the aquarium vendors do not provide ideal environmental conditions for the ornamental fishes kept for selling and the resultant stress could make them prone to infections by virulent strains of *A. hydrophila* easily.

The isolate was further evaluated for its virulence potential *in vivo*. The infectivity study resulted in fish mortality depending on the load of pathogen injected. At 10^8 and 10^7 cfu/ml of the isolate, there was 100% mortality, while at 10^4 cfu/ml there was no mortality. Pathogenicity of the isolate was assessed by estimating the LD_{50} value. LD_{50} value of the isolate was found to be $10^{6.1}$cfu/ml. The LD_{50} value is used to indicate the degree of virulence of a strain (Angka *et al*., 1995). Strains that exhibited $LD_{50} \geq 10^8$ cfu/fish were considered avirulent (Santos *et al*., 1988; Esteve *et al*., 1993). Hence the isolate in the present study was found to be virulent in nature.

Sarkar and Rashid (2012) reported that *A. hydrophila* recovered from naturally diseased Shing fish was pathogenic against Catfishes (*Heteropneustes fossilis* and *Clarias batrachus*), Carps (*Labeo rohita, Catla catla* and *Cirrhinus cirrhosus*) and Perch (*Anabas testudineus*) when injected experimentally. A

mortality of 60 to 100% of all the tested fishes was observed within 2-11 days. *A. hydrophila* injected at 10^7 cfu/ml, caused nearly cent percent mortality in *Clarias batrachus* (Thune *et al*., 1982) and *Carassius auratus* fingerlings (Citarasu *et al*., 2011). *A. hydrophila* isolates exhibiting variable levels of pathogenicity to *O. niloticus* is reported by El-Barbary (2010). In a challenge study conducted by Paniagua *et al*. (1990), 72% of *A. hydrophila* isolates were virulent for fish. Great variations in the virulence test conditions (water temperature, fish size and injection route) used by different authors make it difficult to compare quantitative virulence results.

Infected fishes showed clinical signs such as skin ulcerations, tail and fin rot, haemorrhagia, protruded abdomen, bristling out of scales from skin and exophthalmia. Similar symptoms are reported in Carps with *A. hydrophila* infection (Faktorovich, 1969; Cipriano, 2001; Roberts, 2001). *A. hydrophila* was reisolated from all the dead fish samples. The pathogen was not present in samples from survivors, and mortalities did not occur in the control groups injected with sterile saline.

The distribution of *A. hydrophila* in many aquatic systems globally indicates the successful adaptation of the bacteria to such environment. Adaptations to a wide spectrum of environmental parameters by *A. hydrophila* NJ 87 isolated in the present study imply their possible long term survival in water. The virulent nature of the isolate adds to its potential threat of causing diseases in fishes especially under stress conditions, which are very often encountered at retail outlets of aquarium fishes.

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Chapter 6

Effect of stress factors on the survival and immune response of *Cyprinus carpio* against *A. hydrophila* infection

6.1. Introduction

Diseases occur in natural populations of fishes and the presence of pathogens can occur even in the absence of disease. Since removal of the pathogen is an unrealistic option, it is important to know how to minimize the risk of infectious diseases. The aetiology of disease involves an often complex interaction between three main factors: the status of the host organism, the environment and pathogens. When one or more of these factors is unfavourable the host must adapt its physiology and/or behaviour to compensate. These adaptive responses, stress, impair normal physiological functioning and reduce the host's chance of survival. Essentially anything, whether it is external or internal that disturbs the "normal" physiological balance can be considered as stress.

Stress is a normal and natural phenomenon and it is impossible for life to exist without it. In its benevolent form it strengthens an animal's ability to survive. In its malevolent form it weakens the host to the point where their normal physiological processes no longer can protect the host against the onslaught of pathogenic organisms. The term stressor (stress factor) means the stimulus that inflicts stress on fish. Stressors cause a series of morphological, biochemical and physiological changes to occur in fish. Four distinct stages of stress are identifiable: 1) alarm reaction where the fish tries to escape the stressor; 2) resistance to the stressor through physiological adaptation; 3) fatigue where the fish is noticeably weak but responsive to stimuli, and 4) exhaustion where the fish's physiology is unable to sufficiently adapt to a persisting stress condition, and it can no longer respond to stimuli.

The impact of stress on fish depends on the duration and magnitude of the stress condition. Death is the ultimate result, but sub-lethal stress can cause reduced growth, low yield, poor feed conversion, poor health and diseases. The stress can be classified into acute and chronic type. The acute type are typically physical disturbances such as those caused by handling, transport *etc*. Chronic stress is due to poor water quality, overcrowding, malnutrition *etc*. and in particular, it lowers the resistance of fishes to infectious agents.

6.1.1. Influence of stress factors on disease resistance and immunity

Environmental circumstances (poor water quality, changes in temperature, poor nutrition, crowding, transporting *etc.*) usually produced in intensive fish farming systems can induce stress, making fish more susceptible to a wide variety of pathogens (Reno, 1998). Infectious diseases are caused by pathogenic organisms (parasites, bacteria, viruses and fungi) present in the

environment or carried by other fish. In fact, fish are usually exposed to pathogens or potential pathogens, and infectious disease results from a series of complex interacting variables of the pathogen, host and environment. Many characteristics of pathogens are directly relevant in the disease development (Hedrick, 1998). The occurrence of the disease upon interaction of the pathogen with the fish depends on several host factors such as age, size, developmental stage, nutritional and reproductive status and immunological defences of the host. Moreover, there are many other predisposing factors which contribute to the development of the infectious disease.

In various animals, the immune system appears to be particularly sensitive to the harmful effects of chemicals of environmental concern (Skouras *et al*., 2003). Following factors influence the direction (enhancing versus suppressive) of the effects of stress on immune function:

1) Duration: acute or short-term stress experienced at the time of activation of an immune response enhances innate and adaptive immune responses. Chronic or long-term stress can suppress immune response.

2) Leukocyte distribution: compartments (*e.g*., skin), that are enriched with immune cells show immuno-enhancement, while those that are depleted of leukocytes (*e.g*., blood) show immuno-suppression, during acute stress.

3) The differential effects of physiologic versus pharmacologic stress hormones: endogenous hormones in physiological concentrations

can have stimulating effect on immune function. Endogenous hormones at pharmacologic concentrations are immuno-suppressive.

4) Timing: immuno-enhancement is observed when acute stress is experienced during the early stages of an immune response while immuno-suppression may be observed at late stages. The type of immune response determines whether the effects of stress are ultimately beneficial or harmful to the organism (Dhabhar, 2009). Acute stress-induced immune response may serve to increase immuno-protection during exposure to infectious agents. In contrast to acute stress, chronic stress has been shown to suppress the immune system by altering the cytokine balance (Glaser *et al*., 2001; Glaser and Kiecolt-Glaser, 2005) and accelerating immunosenescence (Epel *et al*., 2004) and to suppress immunity by decreasing numbers, trafficking (Dhabhar and McEwen, 1997) and functioning of protective immune cells while increasing suppressor T cells (Saul *et al*., 2005). Psychological and physiological resilience mechanisms (Figure 6a) are crucial for determining how quickly an organism's physiological response returns to resting conditions following the activation of a stress response (Dhabhar, 2009).

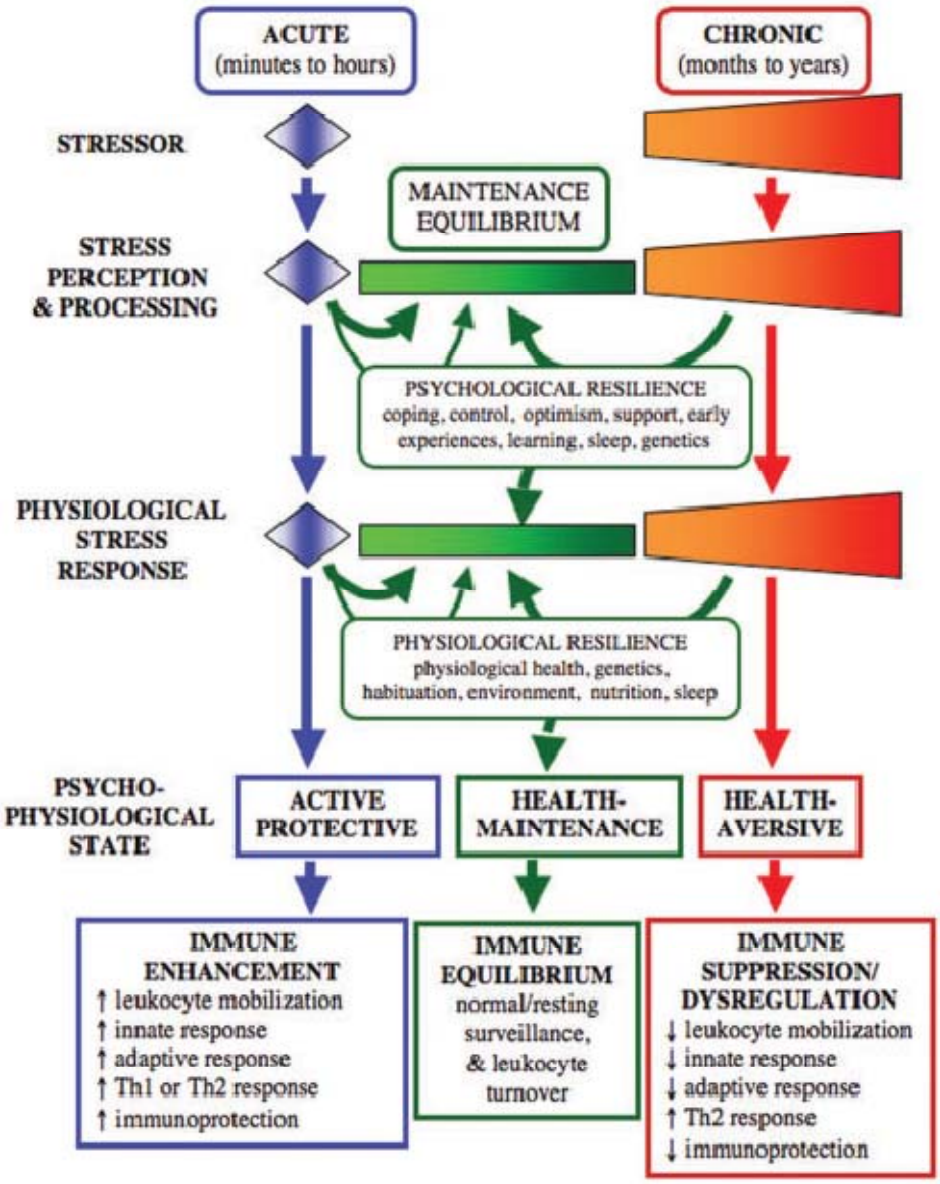

Figure 6a. The Stress spectrum model (Dhabhar, 2009).

Haematological and biochemical parameters are used as health indicators to detect the structural and functional status of fish under stress condition (Thrall, 2004; Pimpao *et al*., 2007; Suvetha *et al*., 2010). Fish blood

is widely used in toxicological research and environmental monitoring as a promising indicator of physiological and pathological changes (Mulcahy, 1975). Haematological parameters such as haemoglobin, haematocrit, red blood cell count, white blood cell count, mean corpuscular haemoglobin concentration and biochemical parameters like plasma glucose, protein and glycogen (muscle and liver) are widely used to evaluate the effect of environmental contaminants on host (El-Sayed *et al*., 2007; Kavitha *et al*., 2010). Leukocyte profiles are altered by stress and can be directly related to stress hormone levels and therefore, are particularly useful in the field of conservation physiology. Leukocytes make up the primary line of defence in the innate immune system of vertebrates, and stress causes alterations in their numbers. It is well-established that neutrophils, being phagocytic, proliferate in circulation to combat infections and the increase in this cell type alone can cause increases in Neutrophil:Lymphocyte (N:L) ratios during infections. Indeed, the two factors are closely related: stress leads to disease susceptibility, and infections or diseases can cause increase in stress (Davis *et al*., 2008).

Any change in water quality is rapidly reflected in fish gill structure and function, since gills are continuously exposed to ambient water. Gills are the primary sites of gas exchange, acid-base regulation and ion transfer (Randall, 1990). The gill epithelium consists mainly of three types of cells such as pavement or respiratory cells, mucus cells and chloride cells (Laurent and Perry, 1995). Gills are the most delicate structures of the fish body and their vulnerability has thus considerable importance because of their external location and necessarily intimate contact with water, which means that they are liable to damage by any irritant material in the water whether dissolved or

suspended. Gills, therefore, are potentially useful to monitor the health of fish (Pawert *et al*., 1998). The gills in fish can be a valuable model for assessing the effects of toxicants on cells and tissues (Mallatt *et al*., 1995). Gills can be exposed directly to known concentrations of toxicant and effects can be related precisely to toxicant concentration.

6.2. Review of literature

The concept of fish health management whereby the host, pathogen and environment are proactively managed to better suit the optimal conditions for sustained growth and health of the fish, has to be developed to facilitate the growth of the aquaculture industry. Fish production can be increased by identifying environmental stress factors and providing an appropriate environment for the fish to grow (Imanpoor *et al.*, 2011; Farhangi and Rostami-Charati, 2012). The most important factor involved in fish health management is water quality. Poor water quality has been pointed out as one of the major causes for the high mortality of ornamental fishes (Oliveira *et al*., 2008). Because fish are totally dependent upon water to breathe, feed and grow, excrete wastes, maintain a salt balance and reproduce, maintaining good water quality is critical to successful aquaculture. Intensive culture practices and conversion of wetlands into aquaculture ponds has resulted in increase in nutrients and organic wastes, leading to general deterioration of water quality.

Nitrogenous wastes including ammonia-N, nitrite-N and nitrate-N are increasingly becoming a global issue in aquatic ecosystems. These pollutants are interrelated via the nitrification cycle, with the direct metabolic product ammonia-N generally being the most toxic with high species specificity. Generally, ammonia-N, nitrite-N and nitrate-N remain very low in most aquatic

ecosystems (<2 mg/L total nitrogen) since there are natural processes that dilute these nutrients as well as being continuously removed by various aquatic plants and microbes. However, due to increasing human activities associated with population growth and industrialization, pollution levels are escalating within fresh water systems globally (Dolomatov *et al*., 2013; Romano and Zeng, 2013; Sachar and Raina, 2014). The increase in concentrations of nitrogenous wastes are a ubiquitous concern in aquaculture particularly since the trend in aquaculture is a move toward more intensive culture systems with a reliance on higher feed inputs (Bouwman *et al*., 2011; FAO, 2011).

6.2.1. Ammonia as a stress factor

Ammonia is a metabolic by-product of fish. Most nitrogen in fish feed that is not converted to fish flesh enters the water as ammonia, either by direct excretion from fish or by bacterial action on wastes (Abbas, 2006). High levels of ammonia can act as a cause of the death in fishes (Ogbonna and Chinomso, 2010; El-Shebly and Gad, 2011; Farhangi and Rostami-Charati, 2012). It's mechanisms of action include causing water and mineral imbalances, decreasing blood pH, altering cardiac function and affecting ATP levels (Evans *et al*., 2006). Clinical signs of ammonia toxicity include mortalities, neurologic and behavioural abnormalities, lethargy, anorexia, poor growth and secondary infections (Wildgoose, 2001).

In China, 100 tonnes of fishes were poisoned in Fuhe river in central Hubei province, due to discharge of ammonia into the river by a chemical factory (www.cnn.com, 2013). In a study conducted to ascertain the cause of the death of fishes from a pond located in Nigeria, Ogbonna and Chinomso (2010) indicated that among the parameters determined, the concentration of

ammonia exceeded the maximum limit of 0.2 mg/L for aquatic life. The ammonia concentrations from test results ranged from 0.25 mg/L to 0.5 mg/L. Higher values of pH, electrical conductivity, chloride, nitrate and calcium were seen in the sample with the highest level of ammonia (0.5 mg/L). The death of the fishes was more evident in samples having high concentration of ammonia. So it was confirmed that ammonia concentration above 0.20 mg/L in fish ponds has a tendency to harm the fishes. Chronic exposure to sub-lethal ammonia significantly reduced growth in *Carassius auratus* (Yang *et al*., 2010).

El-Sherif and El-Feky (2008) studied the effect of ammonia toxicity in Tilapia and found that the growth performance was significantly decreased with increasing concentration of un ionized ammonia (UIA)-Nitrogen. El-Shafai *et al*. (2004) showed that the lowest-observable effect concentration of ammonia on the growth performance in Tilapia is 0.144 mg/L UIA-N. Sublethal effects of ammonia on Rainbow trout (*Oncorhynchus mykiss*) were investigated by Vosylienë and Kazlauskienë (2004) at all stages of its development (embryos, larvae and adult fish). Data demonstrated adverse effects of ammonia in sublethal concentrations on fish at different stages of their development. The total body mass of Rainbow trout larvae significantly decreased on exposure to ammonia. Ninety six hour LC_{50} of UIA-N to Shortnose sturgeon fingerlings was 0.58 ± 0.213 mg/L (Isely and Tomasso, 1998) and 28-day LC_{50} for Turbot (*Scophthalmus maximus*) was found to be 0.95 mg/L. Daud *et al*. (1988) reported 6.6 mg/L NH_3-N as 48-h LC_{50} in hybrid Tilapia species. On the other hand, Redner and Stickney (1979) found that the 48-h LC_{50} for Tilapia was 2.4 mg/L NH_3-N.

The relative percent survival (RPS) of Rainbow trout (*Oncorhynchus mykiss* Walbaum), exposed for one month to low, medium and high concentrations of ammonia and then challenged with a virulent *Streptococcus iniae* strain was examined by Hurvitz *et al*. (1997). The RPS of fish exposed to the high ammonia level was significantly lower, but there was no difference in the RPS of Trout maintained at low and medium ammonia levels. Prolonged exposure of trout to medium ammonia concentrations reduced the RPS as compared with control fish.

Channel catfish, *Ictalurus punctatus*, injected with *Aeromonas hydrophila* and kept under stressed conditions had significantly higher total bacterial counts than non-stressed controls. *A. hydrophila* was isolated from 67% of the stressed fish and 9% of the control fish. *Edwardsiella tarda*, apparently endemic in the population, was isolated from 43% of the stressed fish and 7% of the control fish (Walters and Plumb, 1980). Exposure to sublethal UIA-N concentrations can also increase susceptibility to fungal and parasitic diseases such as columnaris (Amin *et al*., 1988), saprolegniosis (Carballo *et al*., 1995), and trichodiniasis (Hassan, 1999).

In a study conducted by Verghese *et al*. (2007) using Indian Spiny Lobster *Panulirus homarus*, significant reduction in total haemocyte count and phenol oxidase activity were observed at higher ammonia-N concentrations than their respective optimal conditions. Thus, suggesting that extreme environmental parameters can induce modifications in the immune system of the Spiny Lobster which may enhance their susceptibility to opportunistic pathogens. Liu and Chen (2004), challenged white shrimp *Litopenaeus vannamei* with *V. alginolyticus*, and then placed in water containing different

concentrations of ammonia. Mortality of shrimp in water with ammonia was significantly higher than those in the control solution. No significant difference in total haemocyte count, hyaline cells and granular cells were observed among shrimps at different ammonia-N concentrations, but phagocytic activity and clearance efficiency to *V. alginolyticus* significantly decreased among shrimps exposed to higher ammonia concentrations. In another experiment conducted by Cheng *et al.* (2004), mortality of Taiwan Abalone *Haliotis diversicolor supertexta* injected with *Vibrio parahaemolyticus* and placed in water containing different concentrations of ammonia-N, increased directly with ambient ammonia-N concentration. The Abalone when exposed to 3.16 mg/L ammonia-N had decreased phenoloxidase activity, phagocytic activity and blood clearance efficiency after 24 h. The susceptibility of freshwater prawn *M. rosenbergii*, which had been challenged with *Enterococcus* increased significantly, when the prawns were exposed to increasing ammonia concentration (Cheng and Chen, 2002).

Haematological responses of fishes on exposure to ammonia were studied by Tilak *et al.* (2007) and Yang *et al.* (2010). Both these research groups reported a continuous decrease in red blood cell and total haemoglobin. Ajani (2008) assessed the haematological responses of *Clarias gariepinus* to sub-lethal ammonia toxicity at different exposure hours. Packed cell volume and red blood cell counts were decreased after 48 h. A significant reduction in white blood cell count and lymphocytes, and a slight increase in monocyte numbers were observed. Vosylienë and Kazlauskienë (2004) observed that long-term exposure to sublethal concentrations of ammonia induced a significant decrease in parameters of red (erythrocyte count, haemoglobin

concentration and haematocrit level) and white (leukocyte count and lymphocyte percentage) blood cells in Rainbow trout (*Oncorhynchus mykiss*).

Ramesh and Saravanan (2008) stated that on exposure to stress, changes in the leucocyte system in *Cyprinus carpio*, manifest in the form of leucocytosis with heterophilia and lymphopenia which are characteristic leucocytic response in animals exhibiting stress. Observations by McLeay and Gordon (1977), Tomasso *et al*. (1983), Wedemeyer *et al*. (1983) and Peters *et al*. (1988) also reported an increase in the leucocrit of stressed fish. The greater volume of the leucocytes resulted from an increase in number and a hypertrophy of granulocytes. In fact, the effect of disease on leukocyte profiles is similar to that of stress in that neutrophilia and lymphopenia are commonly observed. In addition to causing relative neutrophilia and lymphopenia, infections commonly cause general increase in monocytes, which also phagocytize foreign particles and infectious agents, and general increase in total WBC count (Davis *et al*., 2008).

Histopathological changes of the effect of ammonia on the gills of fresh water fish *Cyprinus carpio* was studied by Chezhian *et al*. (2012) and they noticed several changes in the fish gills like lamellar fusion, hyperplasia, chloride cell proliferation and fusion in secondary lamella as compared with control. In a study conducted by El- Shebly and Gad (2011) on Nile tilapia (*Oreochromis niloticus*), exposure to different concentrations of ammonia revealed various degrees of pathological lesions on gills which became severe with increasing concentrations of ammonia. Exposure to 0.2 mg/L UIA-N concentration revealed epithelial hyperplasia, congestion of central vein and secondary lamellae showed telangiectasis with vacuolation of gill lamellae.

Exposure to 0.4 mg/L UIA-N concentration, showed hyperplastic interlamellar occlusion represented by clear fusion of the adjacent secondary lamellae, clear erosion of secondary lamellae, telangiectasia of gill lamellae and gill hyperplasia. The fish exposed to the highest UIA-N concentration (0.6 mg/L) exhibited gill hyperplasia, degeneration of epithelium lining the secondary lamellae, sloughing of the lamellar epithelium and telangiectasia of gill lamellae. Kirk and Lewis (1993) reported that the gills of Rainbow trout exposed to 0.1 mg/L ammonia for 2 h exhibited deformation of the gill lamellae. Smart (1976) and Smith and Piper (1975) found that the most characteristic feature for chronic exposure of Rainbow trout to ammonia was the appearance of swollen, rounded secondary gill lamellae or telangiectatic capillaries in the secondary lamellae.

Histopathological observations on *Cyprinus carpio* exposed to different ammonia concentrations were reported by Peyghan and Takamy (2002). They observed that the most important lesions that encountered in the gill were hyperemia, oedema and aneurysm. In the kidney, degenerative changes of tubules and glomeruli, expansion of Bowman's capsules, hyperemia, congestion and haemorrhage were the most prominent lesions. There were hyperaemia, degeneration and the presence of some necrotic area in the liver.

Sink (2010) studied UIA-N toxicity to Golden shiners in response to ammonia source, pH, calcium concentration, and salinity. No difference in toxicity of ammonium chloride salt or aqueous ammonia hydroxide to Golden shiners was found. UIA toxicity increased as pH increased. An increase in environmental calcium decreased the toxicity of UIA at pH 8, whereas salinity had no effect on UIA toxicity.

6.2.2. Nitrite as a stress factor

When feed is digested by fish, the excess nitrogen is converted into ammonia, which is then excreted as waste into the water. Total ammonia nitrogen is then converted to nitrite (NO_2), which under normal conditions, is quickly converted to non-toxic nitrate (NO_3) by naturally occurring bacteria, for example, *Nitrosomonas* in the first step and *Nitrobacter* in the second step. Blood appears to be the primary target of nitrite action, within the red blood cells it oxidises haemoglobin to methaemoglobin, resulting in methaemoglobinemia and hypoxia. Apart from methaemoglobinemia, nitrite has multiple physiological effects disrupting ion regulatory, respiratory, endocrine and excretory processes (Jensen, 2003; Kroupova *et al.*, 2005). Nitrite is actively taken up across the gills and accumulates in plasma, gills, liver, brain and muscle (Gisbert *et al.*, 2004). Gills may appear pale or tan in color. In severe cases, gills and blood may show brown discoloration due to the methaemoglobin (Wildgoose, 2001).

Studies in a number of fishes have established that a higher nitrite level in water is one of the important factors causing considerable stress in fish. Its uptake, toxicity mechanism and physiological effects have been extensively studied in many fishes (Stormer *et al.*, 1996; Grossel and Jensen, 1999; Huang and Chen, 2002; Jensen, 2003; Ajani *et al.*, 2007; Dolezalova *et al.*, 2011; Dolomatov *et al.*, 2013; Zuskova *et al.*, 2013).

Kroupova *et al.* (2010) studied the effect of chronic exposure of nitrite on common carp larvae and embryos. LC_{50} (29 days) was found to be 88 mg/L NO_2, lowest-observed-effect concentration was 28 mg/L NO_2 and no-observed-effect concentration was 7 mg/L NO_2. Fish from all the concentrations showed a

dose-related delay in development compared with the controls. Lordosis, scoliosis and body shortening were observed at all concentrations. The incidence of these malformations was positively correlated with nitrite concentration. Lethal effects of nitrogenous compounds on *Cyprinus carpio* were studied by Tilak *et al.* (2007) and he investigated the 24-h LC_{50} of nitrite as 171.36 (ppm). The 96-h LC_{50} nitrite-N for different fish species was studied by several authors. It was found to be 10.4 mg/L for fingerlings of *Cirrhinus mrigala* (Ham.) (Das *et al*., 2004 a); 11.3 ± 8.17 mg/L for Shortnose sturgeon fingerlings (Isely and Tomasso, 1998); 7 mg/L for Channel catfish (*Ictalurus punctatus*) (Palachek and Tomasso, 1984) and 22 mg/L for Rainbow trout (*Oncorhynchus mykiss*) (Russo and Thurston, 1977). Effects of chronic nitrite exposure on growth in juvenile Atlantic Cod, *Gadus morhua* was studied by Siikavuopio and Saether (2006) and they observed clear negative effects of moderate concentrations of nitrite on growth in young Cod.

Nitrite can decrease the resistance to bacteria and parasites and even cause high mortality in aquatic animals (Jiang *et al*., 2013). Bunch and Bejerano (1997) examined the effect of oxygen and nitrite concentration on the infection of Tilapia with *Streptococcus* sp. and found that stress associated with these factors resulted in significant increases in mortality. Rainbow trout exposed for 24 hours to 0.24 mg/L NO_2^- were challenged after nitrite exposure with *Saprolegnia parasitica* causing mycotic dermal infection. The acute stress response provoked by nitrite exposure accounts for the main contribution to the increase in saprolegniosis susceptibility, representing approximately a 100% increase in the percentage of infected fish when compared with the control group (Carballo *et al*., 1995).

The effect of elevated nitrite concentration on the haematological profile of Rainbow trout was studied by Zuskova *et al*. (2013). Nitrite exposure was associated with a significant increase in methaemoglobin, mean corpuscular haemoglobin and plasma nitrite concentration. Kroupova *et al*. (2008) studied the effect of increasing concentration of nitrite in Rainbow trout and observed significantly lower haemoglobin levels in fish exposed to 0.1, 0.6, and 1.0 mg/L NO_2^- compared to the control. Lower haematocrit values were observed in 0.6 and 1.0 mg/L NO_2^- treated groups and higher leucocyte counts were found in fish exposed to 0.1 and 0.6 mg/L NO_2^-. Other indices such as red blood cell counts, mean corpuscular volume and mean haemoglobin concentration did not differ from the control. Ajani *et al*. (2007) assessed the haematological responses of *Clarias gariepinus* to sublethal nitrite toxicity at different exposure hours (0, 6, 24, 48, 72, 96 h). Packed cell volume and haemoglobin were elevated after 6 h of exposure and decreased after 48 h. Red blood cell count decreased significantly at 6 h followed by a significant increase at 24 h. A reduction in white blood cell count, lymphocytes and monocytes were also observed. The erythrocytes of nitrite treated carp showed a significantly higher number of elongated erythrocytes with the nucleus located in one cell pole, and all erythrocytes had remarkably pale cytoplasm compared to the control group (Svobodova *et al*., 2005). An increase in red blood cell counts and haemoglobin concentration in *Brycon cephalus* on exposure to high nitrite is reported by Avilez *et al*. (2004). A sublethal nitrite toxicity study was conducted for a period of 96 h with fingerlings of *Catla catla* (Das *et al*., 2004 b) and *Labeo rohita* (Das *et al*., 2004 c). The study revealed that exposure to nitrite caused changes in almost all the haematological parameters in the fingerlings depending on the concentration

as well as exposure period. Total leukocyte count increased significantly after 12 h, signifying development of a protective response of the body to nitrite stress.

Considerable importance is given to studies of tissue-specific toxicity of nitrites, the results of which indicate a high sensitivity to nitrites by epithelium of the gill apparatus, as well as tissues of liver, brain and kidney of fish (Dolomatov *et al*., 2013). Kroupova *et al*. (2008) revealed focal hyperplasia and oedema of the respiratory epithelium of secondary lamellae in the gill of Rainbow trout at a concentration of 0.01 mg/L NO_2^-, the lowest level of exposure. At higher concentrations, oedema and hyperplasia of the respiratory epithelium of secondary lamellae was observed. Increasing concentrations of nitrite also resulted in an increased number of chloride cells. Hyperplasia, vacuolisation and elevated numbers of chloride cells were the main histological lesions that occurred in the gills of nitrite treated carp (*Cyprinus carpio*) (Svobodova *et al*., 2005). Michael *et al*. (1987) observed hyperplasia and hypertrophy in the gills of *Clarias lazera* chronically exposed to nitrite. Nitrite accumulation causing tissue damage has been reported in fish (Das *et al*., 2004 d).

6.2.3. Crowding as a stress factor

High stocking density as a stress factor affecting growth and survival of fishes is reported by several authors (Can, 2013; Kumar *et al*., 2013; Offem and Ikpi, 2013). Effect of stocking density on the growth and survival rate of Goldfish (*Carassius auratus*) was studied by Hassan *et al*. (2013) and the results indicated that stocking density significantly affected the growth, but had no significant effect on survival. Similar results were obtained by Mensah

et al. (2013) with *Oreochromis niloticus.* Jha and Barat (2005) studied the effect of different stocking densities on the growth and survival rate of Koi carp, *Cyprinus carpio* vr. *Koi.* Weight gain for Koi carps stocked at low stocking density was significantly higher than that of fish in the other treatments. There was a significant difference in survival rates of Koi carps among the treatments ranging from 62.43 to 93.26%. Increased/decreased water temperatures have been reported to cause increased pathogenicity of the bacterium in cultured fish (Yambot, 1998). These authors found that changes in water temperature, along with the increased stocking density, act as major factors in the occurrence of disease outbreaks by *A. hydrophila* in fish.

Crowding stress causing immune suppression and higher susceptibility to pathogens is reported by several authors (Gornati *et al*., 2004; Costas *et al*., 2008; Di Marco *et al*., 2008). The effect of various environmental stressors on the disease resistance of Catfish, when artificially challenged with *Aeromonas hydrophila* was studied by Crumlish *et al*. (2003). Fishes exposed to high stocking density stress, exhibited highest percentage mortalities when challenged with *A. hydrophila.* Mortalities in fish groups receiving both stress and bacterial challenge was higher, when compared with fishes receiving stress only or bacterial challenge only. Overcrowding resulting in outbreaks of infectious diseases is reported by Angka *et al*. (1995). The effect of crowding condition on the non-specific immune response and disease resistance of fancy Carp (*Cyprinus carpio* L.) was studied by Yin *et al*. (1995). The fish were stressed for 1, 7, 14 and 30 days. Their susceptibility to infectious disease was tested by challenging with the bacterium *A. hydrophila*. Disease resistance of the stressed fish was significantly reduced on day 7. However, no great difference in resistance against *A. hydrophila* was found between day 7 and

day 30. No mortality was encountered in either stressed or unstressed fish, when not challenged with the pathogen. This suggests that, chronically stressed fish might have an adaptation capability that allowed them to survive under a stable crowding condition with a lower level of non-specific immune parameters. Nevertheless, these chronically stressed fish may still be more vulnerable to infectious diseases if the intensity of stress is suddenly increased.

Peters *et al*. (1988) subjected Rainbow trout (*Oncorhynchus mykiss*) to social stresses of cohabitation with Cohorts and then exposed these fish to infection by *A. hydrophila*. When compared, Rainbow trout showed physical evidence of stress based on elevated plasma glucose concentrations and increased leukocyte volumes. Following exposure to the pathogen, the bacterium was recovered from more organs and with greater prevalence among the stressed Trouts than from the Cohorts. Rainbow trout inoculated with *Aeromonas salmonicida* and subjected to handling and anoxic stress, showed a higher mortality rate when compared with the unstressed fish (Angelidis *et al*., 1987). An increase in the proliferation of the bacterial pathogens, *Vibrio anguillarum* and *A. salmonicida* was seen from 24 h in the plasma of Atlantic cod (*Gadus morhua*) stressed by short term exposure to overcrowding (Caipang *et al*., 2009), indicating a decrease in the antibacterial activity of serum in stressed fishes.

Effect of crowding and transportation stress on the haematology of African catfish (*Clarias gariepinus*) was studied by Adeyemo *et al.* (2009). No significant differences was observed in the haematocrit, white blood cell, eosinophil and haemoglobin of the stressed fish relative to the baseline values, but, significant differences were observed in the values of the

neutrophil and lymphocyte counts of the stressed fish. The effect of transport stress on the biochemical and haematological profiles of common carp was investigated by Dobšíková *et al*. (2009). Significant increases in haematocrit and metamyelocyte count were found. Mean corpuscular volume and counts of monocytes, band neutrophils and segmented neutrophils were significantly changed independently of the transport duration. The movement of immune cells during stressful periods is influenced by stress hormones; therefore the mobilization of neutrophils and macrophages that form the first line of defence may be important for survival (Ruane *et al.*, 2002). Ortuno *et al.* (2001) demonstrated that intense acute crowding led to leukocyte migration into the blood from the head kidney. Wendelaar (1997) also reported that stress caused a rapid increase of neutrophils and a reduction of lymphocytes in peripheral blood. Immunosuppression, as shown by decreasing circulating lymphocytes in Sea bream as an effect of crowding stress is reported by Tort *et al.* (1996).

6.3. Objectives of the study

Motile aeromonad septicaemia is a major devastating disease in cultured fish and caused mainly by *Aeromonas hydrophila* (Afizi *et al*., 2013). The environmental stresses are an important factor contributing to outbreaks of disease due to *A. hydrophila* in ornamental and other cultured fresh water fishes. This is a common inhabitant of healthy fish and the aquatic system; it is also an established opportunistic pathogen infecting fish under physiological or environmental stress (Citarasu *et al*., 2011). Under conditions of stress, it is even likely that some strains of motile aeromonads that are ordinarily part of the normal gut flora become pathogenic. Unfavourable environmental conditions or poor management practices in aquaculture farms or tanks can induce stress

in fish and trigger motile aeromonad infections. This can result in a significant constraint on fish production and economics. Hence the study has been taken up with the following specific objectives:

1) To determine the 96h Median lethal concentration (LC_{50}) of un-ionized ammonia nitrogen (UIA-N) for *Cyprinus carpio.*

2) To study the effect of UIA-N on the survival and immune response of *C. carpio*, post challenge with *A. hydrophila.*

3) To determine the 96h Median lethal concentration (LC_{50}) of nitrite–N for *C. carpio.*

4) To study the effect of nitrite-N on the survival and immune response of *C. carpio*, post challenge with *A. hydrophila.*

5) To study the effect of stocking density on the survival and immune response of *C. carpio*, post challenge with *A. hydrophila*

6.4. Material and Methods

6.4.1. Experimental Fish and its maintenance

Fresh water ornamental fish, Koi carp (*Cyprinus carpio*), collected from an aquarium shop in Cochin, Kerala, India was used for the study. Fishes weighing ~1.5g ± 0.2g were brought to the laboratory, acclimatized in tanks containing dechlorinated water over a period of three weeks. The number of fishes stocked in each tank was according to OECD guide lines. Faeces and uneaten feed residues were siphoned out of the tank together with about one third of the water volume of the aquarium each day and replaced with fresh dechlorinated tap water before the morning feed. The physico chemical parameters of water were estimated according to the procedure of APHA

(1998) and maintained as follows: Water temperature 25- 27°C, dissolved oxygen concentration 6.8-7.4 ppm, pH 7-7.5, un-ionized ammonia 0.1-0.14 mg/L and salinity 0 ppt. Fishes were fed on commercial diet *ad libitum.* Feeding was stopped 24 hour before the commencement of the bioassay (Ajani, 2008). After the period of acclimatization, the fishes were transferred to the respective experimental tanks.

6.4.2. Experimental design for challenge with *Aeromonas hydrophila*

6.4.2.1. Bacterial culture

Pure culture of *Aeromonas hydrophila* (Genbank accession number: JX987236) originally isolated from aquarium fish samples (John and Hatha, 2013) was used for challenge experiment. The isolate was grown in nutrient broth for 24 h at 28°C. The broth cultures were harvested by centrifugation at 5000 × g for 15 min at 4°C. The bacterial pellet was washed by resuspension in sterile phosphate buffered saline (PBS-pH 7.4) and centrifugation as above and the final pellet was resuspended in PBS to get a cell density of 1×10^6 cells/ ml. The viable count of the suspension was confirmed by spread plate technique.

6.4.3. Stress factor: Ammonia

6.4.3.1. Bioassay

Semi-static bioassay method was chosen for the study and each test solution was renewed daily.

6.4.3.2. Preparation of test solution

The stock solution of ammonia was prepared by dissolving 38.2 g of NH_4Cl (Merck reagent grade) in one litre distilled water to achieve a

concentration of 10,000 mg/L ammonia-N and further diluted to desired concentrations with the tap water.

6.4.3.3. Determination of 96h Median lethal concentration (LC_{50}) of un-ionized ammonia-nitrogen (UIA-N)

Experimental fishes were exposed to six different ammonia concentrations in water and a control with ammonia-free water for 96 hours. During the experiment, water temperature was maintained at 25-27°C, dissolved oxygen concentration at 6.8-7.4 ppm and pH at 8. Triplicate tanks of eight fishes each were used for each of the six different un-ionized ammonia (UIA) concentrations (1.44, 1.72, 2.01, 2.3, 2.58 and 2.87 mg/L). The amount of UIA was calculated by multiplying the total ammonia nitrogen (TAN) by the appropriate conversion factor according to the measured water temperature and pH (Emerson *et al*., 1975). Fish mortality occurring at each ammonia concentration was noted for 96 hours. The LC_{50} values were calculated using PROBIT analysis.

6.4.3.4. Challenge experiment

Fishes were exposed to control, 1/5 and 1/10 concentration of 96- h LC_{50} (*i.e*., 0, 0.21 and 0.41 mg/L) un-ionized ammonia-N for 120 h that was renewed daily. The experiment was carried out in triplicate for each concentration of ammonia-N. Following 120 h of ammonia exposure fishes were injected intra-peritoneally (IP) with 0.1 ml dose (1×10^6 cfu/ml) of the bacterial pathogen. All the groups were kept under observation for 7 days to record mortality. The cause of death was confirmed by reisolating the organism from body parts of dead fishes (10% of dead fishes were used for reisolation) using starch ampicillin agar.

6.4.4. Stress factor: nitrite

Experimental Fishes, maintenance and acclimatization and bioassay method were the same as described in 6.4.1.

6.4.4.1. Preparation of test solution

A test solution of nitrite-N was prepared by dissolving 4.93 g of sodium nitrite (Merck) in one litre of distilled water to make 1000 mg/L, and further diluted to desired concentrations with the tap water.

6.4.4.2. Determination of 96h Median lethal concentration (LC_{50}) of nitrite-N

Experimental fishes were exposed to five different nitrite concentrations in water and a control with nitrite free water for 96 hours. Three replicate tanks of eight fishes each were used for each of the five different nitrite concentrations (20, 40, 60, 80, 100 mg/L). Fish mortality occurring at each nitrite concentration was noted for 96 hours. The LC_{50} values were calculated using PROBIT analysis.

6.4.4.3. Challenge experiment

Fishes were exposed to control, 1/5 and 1/10 concentration of 96- h LC_{50} (*i.e.*, 0, 4.68 and 9.37 mg/L nitrite-N) for 120 h that was renewed daily. The experiment was carried out in triplicate for each concentration of nitrite-N. Following 120 h of nitrite exposure fishes were injected intra-peritoneally (IP) with 0.1 ml dose (1×10^6 cfu/ml) of the bacterial pathogen (6.4.2.1). All the groups were kept under observation for 7 days to record mortality. The cause of death was confirmed by reisolating the organism from body parts of dead fishes (10% of dead fishes were used for reisolation) using starch ampicillin agar.

6.4.5. Stress factor: stocking density

Apparently healthy *Cyprinus carpio* were stocked at four different stocking densities with 12, 18, 24 and 30 fishes in 10 L water per each aquarium, with 2 aquaria per stocking density. Fish were fed on commercial diet *ad libitum.* Faeces and uneaten feed residues were siphoned out of the tank together with about one third of the water volume of the aquarium each day and replaced with fresh dechlorinated tap water before the morning feed. Fish mortality occurring at each stocking density was noted for 21 days.

6.4.5.1. Challenge experiment

In a separate experiment, fishes were stocked at four different stocking densities with 12, 18, 24 and 30 fishes in 10 L water per each aquarium, for 7 days, with 3 aquaria per stocking density. Fishes were injected intra-peritoneally (IP) with 0.1 ml dose (1 x 10^6 cfu/ml) of the bacterial pathogen (6.4.2.1). All the groups were kept under observation for 7 days to record mortality. The cause of death was confirmed by re-isolating the organism from body parts of dead fishes (10% of dead fishes were used for reisolation) using starch ampicillin agar.

6.4.6. Percentage Survival

Fish mortality was recorded for 7 days following bacterial challenge and percentage survival was calculated employing the following formula:

$$\% \text{ survival} = \frac{\text{No. of surviving fish after challenge}}{\text{No. of fish injected with the pathogen}} \times 100$$

6.4.7. Blood and serum collection

Fishes were anaesthetized with clove oil in ethanol at ratio of 1:10 (v/v) and added to water to get a final strength of 80 ppm.The point at which the fish lost sensitivity to touch was used for blood collection. Blood was collected by tail ablation. Using a haematocrit tube, blood was taken from caudal vein. The extracted blood was divided into two sets of Eppendorf tubes. One set contained a pinch of ethylene diamine tetra acetic acid (EDTA) used as an anticoagulant for haematological analysis (blood clearance efficiency, total leucocyte count and differential cell count). The second set was left to clot at room temperature for one hour, then kept at 4^{o}C for three hours and centrifuged at 3,000 × g for 15 min at 4^{o}C. The collected serum was used for determining serum bactericidal efficiency. Blood and serum samples pooled from a random sample of fish in each experimental tank were used.

6.4.7.1. Serum bactericidal efficiency

Aeromonas hydrophila was grown in nutrient broth for 24 h at 28°C. The broth cultures were harvested by centrifugation at 5000 × g for 15 min at 4°C. The pellet was washed and suspended in PBS to make a concentration of 1×10^{5} cfu/ml. The sera and bacteria were mixed at 1:1 dilution, incubated in a micro vial for 1 hour at 37^{o}C. In the control group, PBS solution was used instead of serum. After incubation, the number of viable bacteria was determined by counting the colonies grown on starch ampicillin agar plates for 24 hours at 28^{o} C. The bactericidal activity of test serum was expressed as percentage of colony forming units in test group to that in the control group (Aly *et al*., 2008; Maqsood *et al*., 2010).

6.4.7.2. Pathogen clearance efficiency of blood

Bacterial load in blood samples of infected fishes were determined 24 hours post challenge. Appropriately diluted blood samples (100µl) from each group were spread plated on starch ampicillin agar plates and incubated at 28^{o}C and the colonies were counted after 24 hours. Clearance efficiency, defined as percentage inhibition (PI) of *A. hydrophila* was calculated as:

PI =100-{(cfu in test group)/(cfu in control group)}x100

6.4.7.3. Total leucocyte count (TLC) and differential cell count (DC)

Total leucocyte was counted under a light microscope using a Neubauer haemocytometer following the method described by Praful and Darshan (2003).

Methanol fixed blood smears were stained with Giemsa stain for differential leukocyte count according to Feldman *et al*. (2000).

6.4.8. Statistical analysis

Statistical analysis was performed using one-way Analysis of Variance (ANOVA). Means were compared using Duncan's multiple range tests. Difference was considered significant when $p<0.05$. Standard deviations of the treatment means were also estimated. All statistics were carried out using SPSS 13.

6.4.9. Histopathological analysis

Gill tissues from the body of infected fishes were removed and fixed in 10% buffered formalin for 24 hours. Tissues were then washed in running tap water overnight. Tissues were dehydrated in ascending grade of alcohol; for

30 minutes in 50% alcohol and for 45 minutes each in 80 and 90% alcohol and then transferred to absolute alcohol (two changes) for one hour each.

Tissues were cleared in xylene until they became translucent. They were then transferred to a mixture of xylene and paraffin wax and left overnight. The tissues were infiltrated in 2-3 changes of molten paraffin wax of melting point 60-62°C for one hour each. They were then embedded in paraffin wax of melting point 60-62°C. Paraffin blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns.

6.4.9.1. Staining technique using Haematoxylin-Eosin stain

The slides containing the section were processed serially as follows:

a) The slides were rinsed twice in xylene: 5min each

b) The slides were transferred to xylene: absolute alcohol mixture (1:1): 1min

c) Slides were passed sequentially through absolute alcohol, 80%-alcohol and 50% alcohol: 1 min each

d) Washed in running tap water: 5 min

e) The slides were stained using haematoxylin: 2 min

f) Washed in running tap water: 5 min

g) Dipped once in acid: absolute alcohol mixture (0.5:100)

h) Washed in running tap water: 5 min

i) The slides were stained using eosin: 1 min.

j) Slides were dehydrated sequentially through 50%, 80% and absolute alcohol: 1 min each

k) Dipped once in running tap water

l) Blotted slides on filter paper

m) Rinsed in xylene: 5 min

n) The slides were cleared in xylene (2 changes) and mounted in D.P.X and observed under the microscope (Leica DM/LS type) with camera attachment and photographed.

6.5. Results

6.5.1. Effect of ammonia (UIA-N) on the survival and immune response of *Cyprinus carpio*

6.5.1.1. 96-h Median lethal concentration (LC_{50}) of un-ionized ammonia (UIA)-N for *Cyprinus carpio*

LC_{50} (96-h) of un-ionized ammonia (UIA)-N for *Cyprinus carpio* was assessed by the semi-static bioassay method, and it was found to be 2.05 mg/L by PROBIT analysis. For the challenge experiments fishes were exposed to1/10 and 1/5 of the 96-h LC_{50} values.

6.5.1.2. Effect of ammonia (UIA-N) on the percentage survival of *Cyprinus carpio*

Figure 6.1 shows the effect of ammonia on the percentage survival of *Cyprinus carpio,* when challenged with *A. hydrophila.* A significant reduction ($p<0.05$) in the percentage survival of *Cyprinus carpio* is observed with increasing concentration of ammonia, compared to the control (Appendix 5.1). Survival rate reduced from 75% in the control to 45.83% in the highest ammonia concentration.

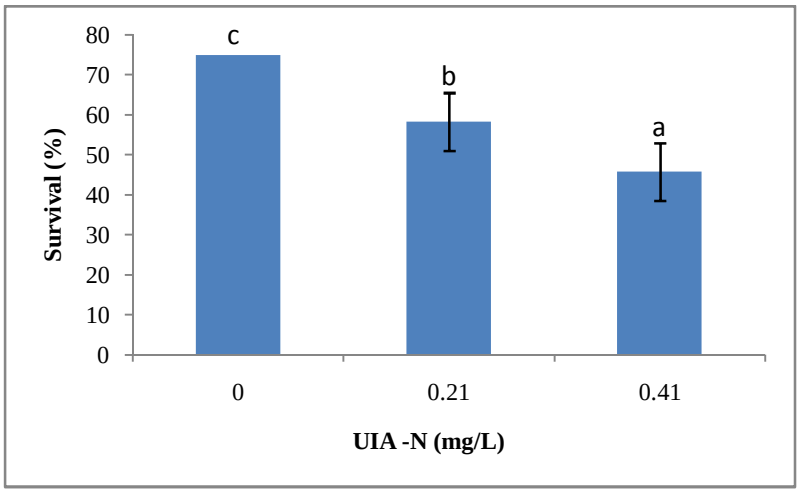

Figure 6.1 Effect of ammonia on the percentage survival of *C. carpio*

* a,b,c denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean.

6.5.1.3. Effect of ammonia (UIA-N) on the immune parameters of *Cyprinus carpio*

6.5.1.3. a. Serum bactericidal efficiency

Bactericidal efficiency of serum of *Cyprinus carpio* exposed to different ammonia concentrations and control (without ammonia), when challenged with *A. hydrophila* is given in Figure 6.2. Bactericidal efficiency of serum is found to decrease significantly ($p<0.05$) with increasing concentration of ammonia as evident from the survival rate of bacteria (Appendix 5.2). Survival rate of bacteria on incubation with serum increased to 67.9% in fishes treated with 0.41mg/L UIA-N compared to 47.3% in the control group.

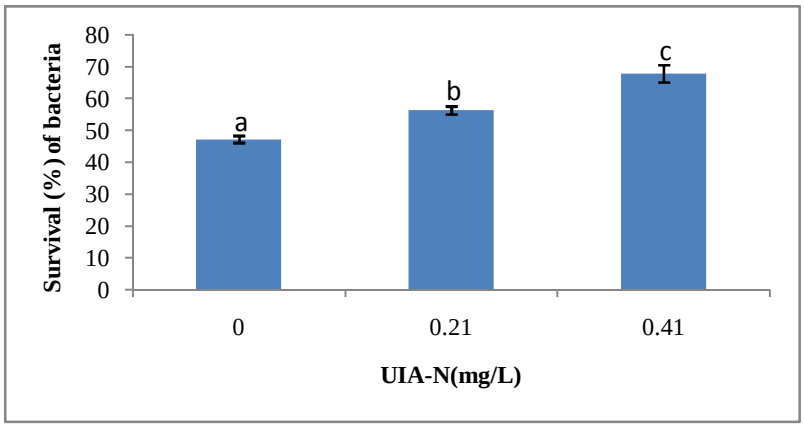

Figure 6.2. Effect of ammonia on the serum bactericidal efficiency of *C. carpio*

* a,b,c denote significant difference (p<0.05) between different treatments. Error bars represent standard deviation of the mean.

6.5.1.3. b. Pathogen clearance efficiency of blood

Pathogen clearance efficiency of blood of *Cyprinus carpio* exposed to different ammonia concentrations and control (without ammonia), when challenged with *A. hydrophila* is given in Figure 6.3.

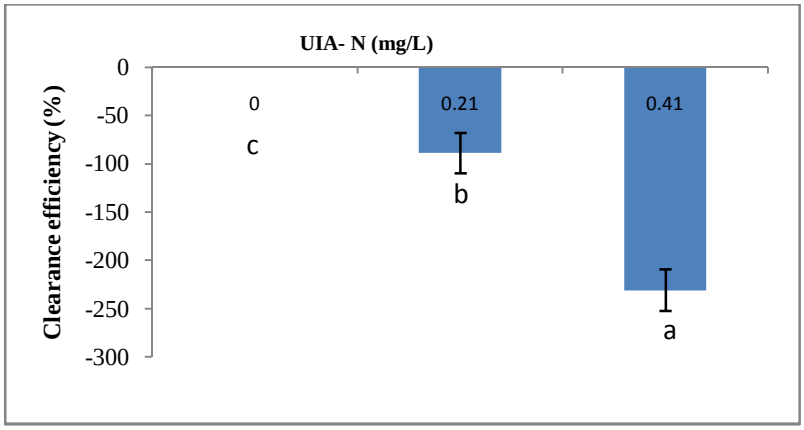

Figure 6.3. Effect of ammonia on the pathogen clearance efficiency of blood of *C. carpio*

* a,b,c denote significant difference (p<0.05) between different treatments. Error bars represent standard deviation of the mean.

Bacterial load in the blood of fishes in different groups showed that pathogen clearance efficiency of blood is found to decrease significantly ($p<0.05$) with increasing concentration of ammonia (Appendix 5.3). It was decreased to 230% in fishes exposed to 0.41 mg/L UIA-N, compared to the control.

6.5.1.3. c. Total leucocyte count and differential cell count

Total leucocyte count was found to increase significantly ($p<0.05$) with increasing concentration of ammonia and severity of infection. A significant reduction ($p<0.05$) in the lymphocyte count (%) and increase in the neutrophil count (%) was also observed (Table 6.1) (Appendix 5.4).

Table 6.1. Total leucocyte count and differential count (%) of control and experimental fishes

UIA-N (mg/L)	Total leucocyte count (x 10^3/mm^3)	Lymphocyte (%)	Neutrophil (%)
0	31.83 ± 0.57^a	89.66 ± 0.57^c	10.33 ± 0.57^a
0.21	43.33 ± 1.52^b	85.33 ± 1.15^b	14.66 ± 1.15^b
0.41	47.66 ± 0.76^c	79.66 ± 1.15^a	20.33 ± 1.15^c

* a,b,c denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean

6.5.1.4. Effect of ammonia on the pathology of gill tissues

Increasing concentration of ammonia depressed the immune system of *Cyprinus carpio* and increased the severity of infection with *A. hydrophila*. This is evident with increasing severity of pathological lesions in the gill tissues (Plate 6.1).

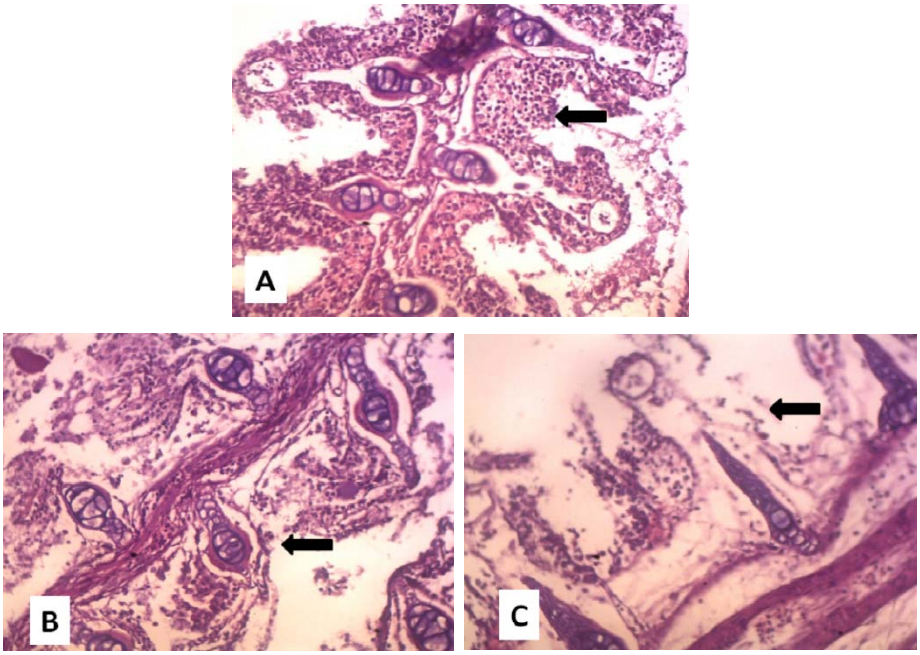

Plate 6.1. Effect of increasing concentration of ammonia on the pathology of gill tissues of *Cyprinus carpio*

A. Photomicrograph of gill of *C. carpio*, injected with *A. hydrophila* (control). Arrow indicates damaged gill filaments (H&E X 400)

B. Photomicrograph of gill of *C. carpio* exposed to ammonia (0.21 mg/L) and injected with *A. hydrophila*. Arrow showing necrosis of gill filaments (H&E X 400)

C. Photomicrograph of gill of *C. carpio* exposed to ammonia (0.41mg/L) and injected with *A. hydrophila* showing complete necrosis of gill filaments (H&E X 400)

6.5.2. Effect of nitrite-N on the survival and immune response of *Cyprinus carpio*

6.5.2.1. 96-h Median lethal concentration (LC_{50}) of nitrite-N for *Cyprinus carpio*

LC_{50} (96-h) of nitrite-N for *Cyprinus carpio* was assessed by the semi-static bioassay method, and it was found to be 46.85 mg/L by PROBIT analysis. For the challenge experiments fishes were exposed to 1/10 and 1/5 of the 96-h LC_{50} values.

6.5.2.2. Effect of nitrite-N on the percentage survival of *Cyprinus carpio*

Figure 6.4 shows the effect of nitrite-N on the percentage survival of *Cyprinus carpio,* when challenged with *A. hydrophila.* A significant reduction ($p<0.05$) in the percentage survival of *Cyprinus carpio* is observed with increasing concentration of nitrite, compared to the control (Appendix 6.1). Rate of survival decreased from 79% in the untreated control to 66.66% in fishes treated with 4.68 mg/L nitrite-N and further to 50% in fishes treated with 9.37 mg/L nitrite-N.

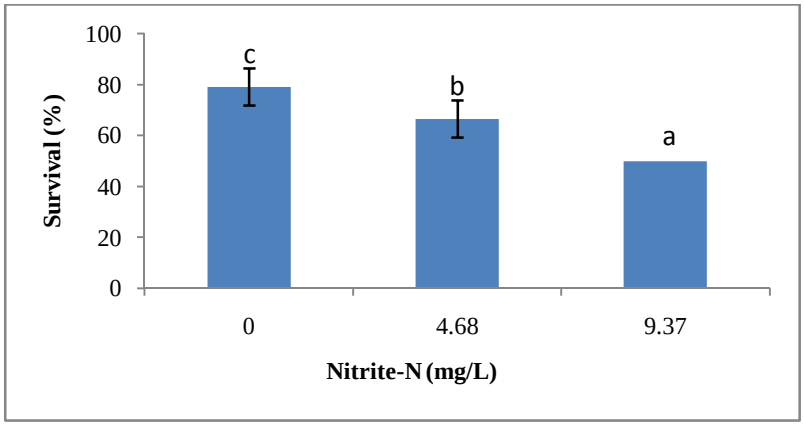

Figure 6.4. Effect of nitrite on the percentage survival of *C. carpio*

* a,b,c denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean.

6.5.2.3. Effect of nitrite-N on the immune parameters of *Cyprinus carpio*

6.5.2.3. a. Serum bactericidal efficiency

Bactericidal efficiency of serum of *Cyprinus carpio* exposed to different nitrite concentrations and control (without nitrite), when challenged with *A. hydrophila* is given in Figure 6.5. Bactericidal efficiency of serum is found to

decrease significantly ($p<0.05$) with increasing concentration of nitrite as evident from the survival rate of bacteria, on incubation with serum (Appendix 6.2).

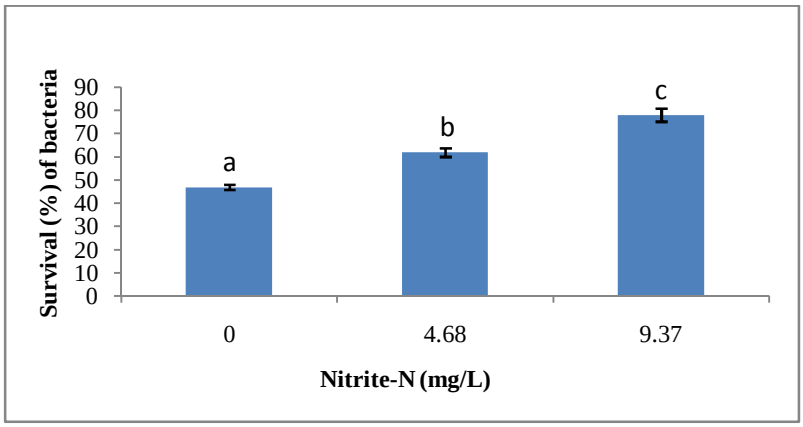

Figure 6.5. Effect of nitrite on the serum bactericidal efficiency of *C. carpio*

* a,b,c denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean.

6.5.2.3. b. Pathogen clearance efficiency of blood

Pathogen clearance efficiency of blood of *Cyprinus carpio* exposed to different nitrite concentrations and control (without nitrite), when challenged with *A. hydrophila* is given in Figure 6.6. Pathogen clearance efficiency is found to decrease significantly ($p<0.05$) with increasing concentration of nitrite (Appendix 6.3). It was decreased to 354.1% in fishes exposed to 9.37 mg/L nitrite-N, compared to the control.

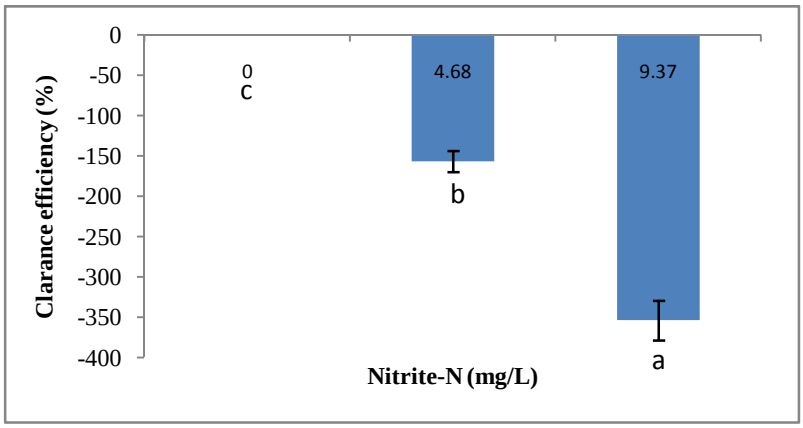

Figure 6.6. Effect of nitrite on the pathogen clearance efficiency of blood of control and experimental fishes

* a,b,c denote significant difference (p<0.05) between different treatments. Error bars represent standard deviation of the mean.

6.5.2.3. c. Total leucocyte count and differential cell count

Total leucocyte count was found to increase significantly (p<0.05) with increasing concentration of nitrite and severity of infection. A significant reduction (p<0.05) in the lymphocyte count (%) and increase in the neutrophil count (%) was also observed (Table 6.2) (Appendix 6.4).

Table 6.2. Total leucocyte count and differential count (%) of control and experimental fishes

Nitrite-N (mg/L)	Total leucocyte count (x 10^3/mm^3)	Lymphocyte (%)	Neutrophil (%)
0	31.16 ± 1.04^a	90.33 ± 0.57^c	9.66 ± 0.57^a
4.68	41.33 ± 1.25^b	84.66 ± 1.52^b	15.33 ± 1.52^b
9.37	45.83 ± 0.76^c	81.33 ± 0.57^a	18.66 ± 0.57^c

* a,b,c denote significant difference (p<0.05) between different treatments. Error bars represent standard deviation of the mean

6.5.2.4. Effect of nitrite on the pathology of gill tissues

Histopathological analysis of gill tissues of *Cyprinus carpio* revealed degeneration of epithelium lining the primary lamellae and necrosis of secondary gill lamellae. The tissue damages were found to become severe as the concentrations of nitrite increased (Plate 6.2).

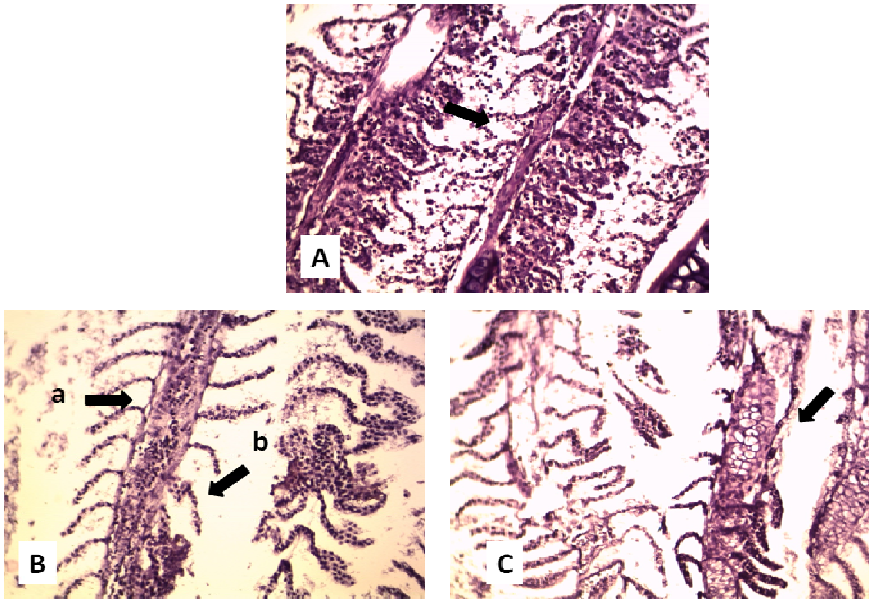

Plate 6.2. Effect of increasing concentration of nitrite on the pathology of gill tissues of *Cyprinus carpio*

A. Photomicrograph of gill of *C. carpio*, injected with *A. hydrophila* (control). Arrow indicates loss of normal gill architecture (H&E X 400)

B. Photomicrograph of gill of *C. carpio*, exposed to nitrite (4.68 mg/L) and injected with *A. hydrophila* showing epithelial desquamation (a) and damaged secondary lamella (b) (H&E X 400)

C. Photomicrograph of gill of *C. carpio*, exposed to nitrie (9.37 mg/L) and injected with *A. hydrophila* showing complete necrosis of secondary lamella (H&E X 400)

6.5.3. Effect of stocking density on the survival and immune response of *Cyprinus carpio*

Stocking density up to 30 fishes/10L water was not found to adversely affect the survival of *Cyprinus carpio* as no mortality was observed for 21 days in any of the experimental tanks of 12, 18, 24 and 30 fishes in 10 L water.

6.5.3.1. Effect of stocking density on the percentage survival of *Cyprinus carpio* post challenge with *A. hydrophila*

Effect of stocking density on the percentage survival of *Cyprinus carpio,* when challenged with *A. hydrophila* is given in Figure 6.7. A significant reduction ($p<0.05$) in the percentage survival of *C. carpio* is observed with increasing stocking densities, compared to the optimum (Appendix 7.1).

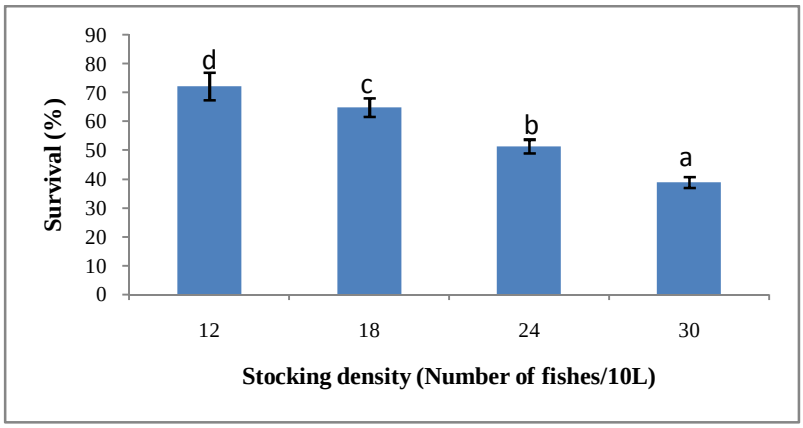

Figure 6.7. Effect of stocking density on the percentage survival of *C. carpio* exposed to different stocking densities

* a,b,c denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean.

6.5.3.2. Effect of stocking density on the immune parameters of *Cyprinus carpio*

6.5.3.2. a. Serum bactericidal efficiency

Bactericidal efficiency of serum of *Cyprinus carpio* exposed to different stocking densities, when challenged with *A. hydrophila* is given in Figure 6.8. Bactericidal efficiency of serum is found to decrease significantly ($p<0.05$) with higher stocking densities as evident from the survival rate of bacteria, on incubation with serum (Appendix 7.2).

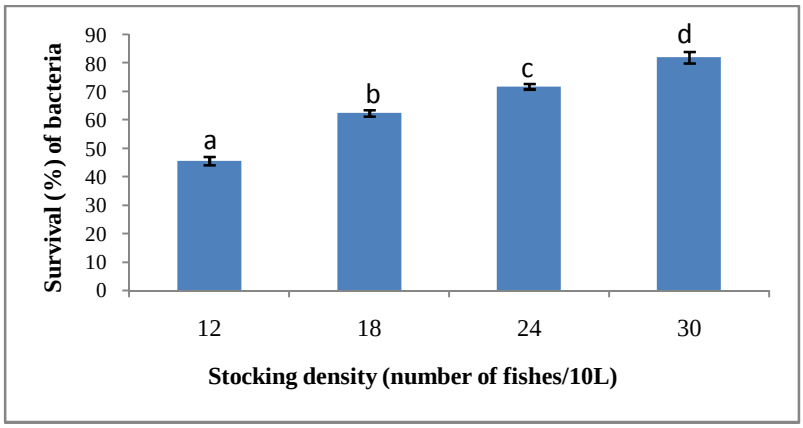

Figure 6.8. Effect of stocking density on the serum bactericidal efficiency of *C. carpio*

* a,b,c denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean.

6.5.3.2. b. Pathogen clearance efficiency of blood

Pathogen clearance efficiency of blood of *Cyprinus carpio* exposed to different stocking densities, when challenged with *A. hydrophila* is given in Figure 6.9. Pathogen clearance efficiency is found to decrease significantly ($p<0.05$) with higher stocking densities (Appendix 7.3).

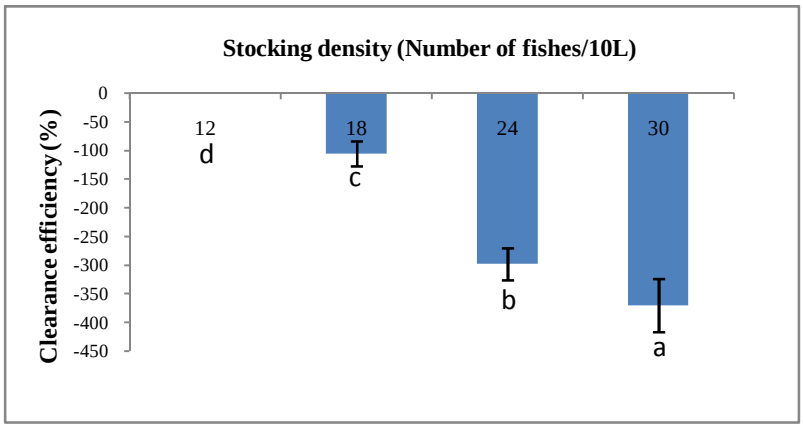

Figure 6.9. Effect of stocking density on the pathogen clearance efficiency of blood of *C. carpio*

* a,b,c denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean.

6.5.3.2. c. Total leucocyte count and differential cell count

Total leucocyte count was found to increase significantly ($p<0.05$) with higher stocking densities. A significant reduction ($p<0.05$) in the lymphocyte count (%) and increase in the neutrophil count (%) was also observed (Table 6.3) (Appendix 7.4)

Table 6.3. Total leucocyte count and differential count (%) of control and experimental fishes

Stocking density (number of fishes/10L)	Total leucocyte count (x 10^3/mm^3)	Lymphocyte (%)	Neutrophil (%)
12	28.66 ± 1.04^{a}	89.33 ± 0.57^{d}	10.66 ± 0.57^{a}
18	41.66 ± 0.76^{b}	85.66 ± 1.52^{c}	14.33 ± 1.52^{b}
24	51.5 ± 1.32^{c}	82.66 ± 1.15^{b}	17.33 ± 1.15^{c}
30	60.83 ± 0.76^{d}	77.66 ± 0.57^{a}	22.33 ± 0.57^{d}

* a,b,c, denote significant difference ($p<0.05$) between different treatments. Error bars represent standard deviation of the mean.

6.5.3.3. Effect of stocking density on the pathology of gill tissues

Histopathological analysis of gill tissues of *Cyprinus carpio* revealed lamellar fusion and necrosis of primary and secondary gill lamellae which became severe with higher stocking densities (Plate 6.3).

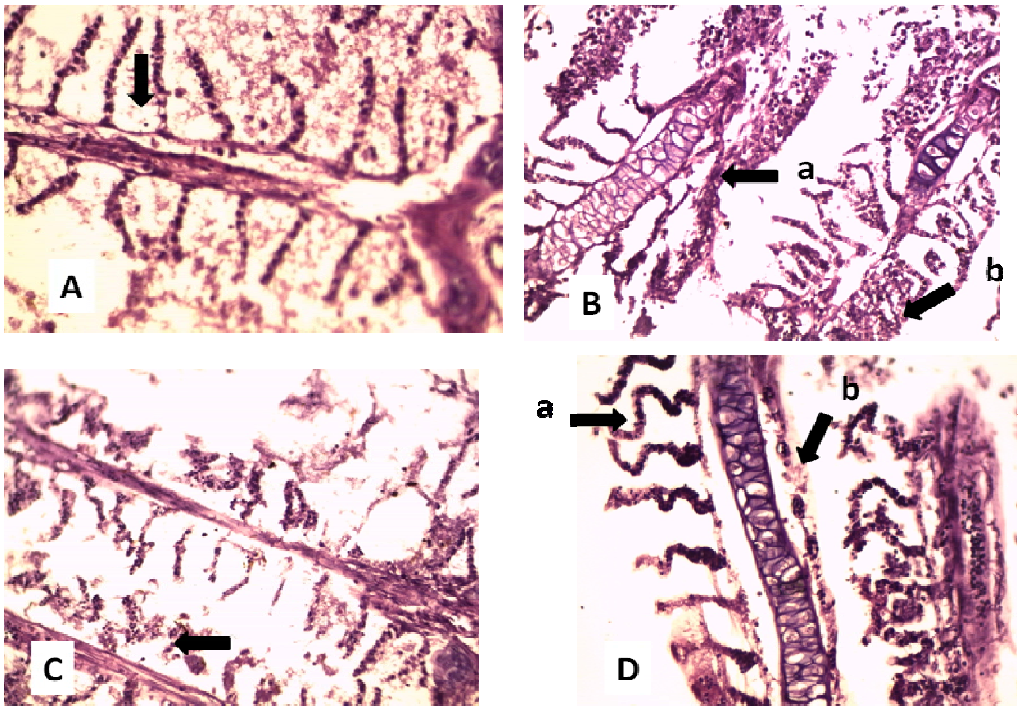

Plate 6.3. Effect of stocking density on the pathology of gill tissues of *Cyprinus carpio*

A. Photomicrograph of gill of *C. carpio*, under optimal stocking density (control) injected with *A. hydrophila.* Arrow indicates epithelial desquamation (H&E X 400); (B-D) Photomicrograph of gill of *C. carpio*, under increasing stocking density and injected with *A. hydrophila* (H&E X 400).

B. showing damaged secondary lamella (a) and lamellar fusion (b).

C. showing damaged secondary lamella.

D. showing lamellar curling (a) and complete necrosis of secondary lamella (b)

6.6. Discussion

Aeromonas bacteria that are members of the gut micro flora in fish and other aquatic animals may cause various diseases under environmental stress conditions (Turska-Szewczuk *et al*., 2013). Outbreaks of motile aeromonad septicaemia due to the potential opportunistic pathogen *Aeromonas hydrophila* are experienced, particularly when fish are exposed to both physical and environmental stress factors (Crumlish *et al*., 2003; Abulhamd, 2010). Immunomodulation is often associated with a stress response in exposed fish (Tort *et al*., 1996), leaving them immunocompromised and susceptible to disease (Fries, 1986).

6.6.1. Ammonia as a stress factor influencing the survival and immune response

High levels of ammonia is reported as a cause of death in fishes by several authors (Ogbonna and Chinomso, 2010; El-Shebly and Gad, 2011; Farhangi and Rostami-Charati, 2012). The toxicity of ammonia in different fish species has been extensively studied (El-Shafai *et al*., 2004; Lamarie *et al*., 2004; Aysel and Koksal 2005; Ajani, 2008). We have evaluated the ammonia level in the water at the aquarium vendor and found that the concentration was at a high level and hence chosen this as a stress factor.

In the present study, 96-h LC_{50} of un-ionized ammonia (UIA)-N for Koi carp (*Cyprinus carpio*) was found to be 2.1mg/L. Similar to our observation, Guan *et al*. (2010) reported the 96-h toxicity of un-ionized ammonia for Common carp (*Cyprinus carpio* L.) to be 2.33 mg N/L. On the contrary, Abbas (2006) reported it to be 0.93 mg N/L. Peyghan and Takamy (2002) estimated the 24-h LC_{50} values of common carp (*Cyprinus carpio*), as 123 mg/L total

ammonia-N. LC_{50} (96-h) of un-ionized ammonia ranges from 0.32 mg N/L in Rainbow trout *(Oncorhynchus mykiss)* to 3.1 mg N/L in Channel catfish *(Ictalarus punctatus)*. It is 1.01 mg N/L for striped bass (*Morane saxatilis*) and 0.64 mg N/L for another freshwater dicentrarchid, the hybrid striped bass (*M. saxatilis* x *M. chrysops)* (Coeurdacier and Dutto, 1999). The toxicity of ammonia depends on the experimental conditions and the type and size of fishes.

A link between the environmental stressor UIA and mortalities among wild populations of fish is suggested by several authors (Eldar *et al*., 1995; Evans *et al*., 2006; El-Shebly and Gad, 2011). Chronic or sublethal exposure to UIA can cause increased disease susceptibility as a result of post exposure stress and immune impairment (Evans *et al*., 2006). A significant reduction in survival of *Cyprinus carpio* with increasing concentration of ammonia–N was observed in the present study. Rate of survival decreased from 75% in the untreated control to 58.3% in fishes treated with 0.21 mg/L UIA-N. It was further decreased to 45.83% in fishes treated with 0.41 mg/L UIA-N.

Similar to the observation in the present study, mortality of Taiwan abalone *Haliotis diversicolor supertexta* injected with *Vibrio parahaemolyticus* increasing directly with ambient ammonia-N concentration was observed by Cheng *et al.* (2004). Similar observations were also made by Hurvitz *et al*. (1997). The effect of sublethal concentrations of ammonia on the survival of Rainbow trout (*Oncorhynchus mykiss* Walbaum), challenged with *Streptococcus iniae*, was examined by them and found that the survival of fish exposed to the high ammonia level was significantly lower. Long term exposure of Trout to medium ammonia concentrations reduced the survival as compared with control.

Ammonia caused a depression in immune parameters such as serum bactericidal efficiency and blood bacterial clearance efficiency. The survival rate of bacteria on incubation with serum was found to increase significantly in serum collected from fishes exposed to higher concentration of UIA-N compared with control, indicating a reduction in bactericidal efficiency of serum. Similarly, the pathogen clearance efficiency of blood collected from fishes exposed to increasing concentration of UIA-N was found to decrease significantly compared with control fishes. It was decreased to 230% in fishes exposed to 0.41 mg/L UIA-N, compared to the control. Similar to the observation in the present study, Walters and Plumb (1980) observed that in Channel catfish (*Ictalurus punctatus*) injected with a sublethal dose of *A. hydrophila* and then exposed to stress conditions, the trunk kidneys of surviving stressed fish had significantly higher total bacterial counts than non-stressed controls. *A. hydrophila* was isolated from more number of the stressed fish than of the control fish. *Edwardsiella tarda*, apparently endemic in the population, was also isolated from more number of stressed fish than from the control fish. Bacterial clearance efficiency of blood of Taiwan abalone *Haliotis diversicolor supertexta* injected with *Vibrio parahaemolyticus* was reported to decrease directly with increasing ammonia-N concentration (Cheng *et al.*, 2004). They noticed that increasing ammonia concentration caused a depression in immune parameters.

Haematological profiles have often been used as stress indicators and the fastest way to detect the manifestation of stress is the evaluation of blood parameters. Leucocytes are involved in the regulation of immunological function and they make up the primary line of defence in the innate immune system of vertebrates. Stress causes alterations in their numbers and their

response varies with the type and severity of the stress and duration of exposure. An increase in total leucocyte count and neutrophil count and a reduction in lymphocyte count with increasing concentration of ammonia were observed in the present study. Total leucocyte count increased from $31.83\pm0.57x10^3/mm^3$ in the untreated control fishes, injected with *A. hydrophila* to, $43.33\pm1.52x10^3/mm^3$ (0.21 mg/L) and $47.66\pm0.76x10^3/mm^3$ (0.41 mg/L) in the ammonia exposed fishes. Results of the present study are in accordance with the observations of Ramesh and Saravanan (2008) who stated that on exposure to stress, changes in the leucocyte system in *Cyprinus carpio*, manifest in the form of leucocytosis with heterophilia and lymphopenia which are characteristic leucocytic response in animals exhibiting stress. Observations by McLeay and Gordon (1977), Tomasso *et al*. (1983), Wedemeyer *et al*. (1983) and Peters *et al*. (1988) also reported an increase in the leucocrit of stressed fish. The increased volume of the leucocytes results from an increase in number of granulocytes. The increase of leucocyte population could also be related to the tissue damage such as necrosis in fish (Oliveira *et al*., 2002) and it can also be due to a depletion of circulating differentiated cells (Fink and Saliban, 2005).

Lymphocytopenia and neutrophilia are reported secondary effects of stress in fish, as a consequence of stress-related release of catecholamines. The stress-induced elevation of plasma cortisol has a direct cytolytic effect on lymphocytes (Dobšíková *et al*., 2009). Lymphocytopenia is observed in the present study with increasing concentrations of UIA-N. Lymphocyte count decreased from 89.66±0.57% in the untreated control fishes to 85.33±1.15 (0.21 mg/L) and 79.66±1.15% (0.41 mg/L) in the ammonia treated fishes. Fathima (2011) stated that cortisol secreted during stress reaction in fish

shortens the lifespan of lymphocytes and promotes their apoptosis and reduces their proliferation, so a decrease in lymphocyte count as well as their activity is often observed as effects of stress, irrespective of the stressing agent. Lymphocytopenia in stressed fishes is observed by several workers (Pickford *et al*., 1971; Esch and Hazen, 1980; Peters *et al*., 1980; Pickering *et al*., 1982). Furthermore, the remaining lymphocytes seem handicapped in their function (Peters *et al*., 1988). Lymphocytopenia in stressed fish may also be due to the extravasations of the cells and their penetration into the epithelium of gills, skin or intestine. The movement of immune cells during stressful periods is influenced by stress hormones; therefore the mobilization of neutrophils and macrophages that form the first line of defence may be important for survival (Ruane *et al.*, 2002; Dobšikova *et al.*, 2009).

In fact, the effect of disease on leukocyte profiles is similar to that of stress in that neutrophilia and lymphopenia are commonly observed. It is well-established that neutrophils, being phagocytic, proliferate in circulation to combat infections. Same was the observations in the present study, there was marked lymphocytopenia and neutrophilia. Neutrophil count increased from 10.33±0.57% in the untreated control fishes to 14.66±1.15 (0.21 mg/L) and 20.33±1.15% (0.41 mg/L) in the ammonia treated fishes. In addition to causing relative neutrophilia and lymphopenia, infections commonly cause general increase in monocytes, which also phagocytize foreign particles and infectious agents, and general increase in total WBC counts (Davis *et al*., 2008).

On exposure to sub-lethal level of ammonia for 96 hours, Ajani (2008) observed a significant reduction in white blood cell count and lymphocytes,

but there was a slight increase in monocyte numbers. Vosylienë and Kazlauskienë (2004) also observed that long-term exposure to sublethal concentrations of ammonia induced a significant decrease in parameters of white (leukocyte count and lymphocyte percentage) blood cells in rainbow trout (*Oncorhynchus mykiss*). Abbas (2006) stated that studies of blood parameters in fishes subjected to conditions of elevated environmental ammonia have resulted in conflicting results.

Histopathological studies of fish exposed to pollutants revealed that fish organs were efficient indicators of water quality (Cengiz *et al*., 2001). The gills are important organs in fish for respiration, osmotic regulation, acid base balance and nitrogenous waste excretion. Any change in water quality is rapidly reflected in fish gill structure, since gills are continuously exposed to water. Gills are the most delicate structures of the fish body and because of their external location and necessarily intimate contact with water, they are liable to damage by any stressing agent in the water whether dissolved or suspended. Gills, therefore, are potentially useful to monitor the health of fish (Pawert *et al*., 1998).

Histopathological analysis of gill tissues of *Cyprinus carpio* in the present study revealed damage and necrosis of primary and secondary gill lamellae which became severe with increasing concentrations of ammonia. Major changes observed were erosion of secondary lamellae, degeneration of epithelium lining the primary lamellae and sloughing of the lamellar epithelium. Chezhian *et al*. (2012) studied the effect of ammonia on the gills of fresh water fish *Cyprinus carpio*. They noticed several changes in the fish gills like lamellar fusion, hyperplasia, chloride cell proliferation and fusion in

secondary lamella as compared with control. In the histopathologic study by Peyghan and Takamy (2002), the most important lesions encountered in the gill were hyperemia, oedema and aneurysm. In a study conducted by El-Shebly and Gad (2011), Nile tilapia (*Oreochromis niloticus*) was exposed to different concentrations of ammonia. Histopathological investigations of gills by them also revealed various degrees of pathological lesions which became severe with increasing concentrations of ammonia. Major changes noticed by them were also similar to the observations in the present study such as fusion of the adjacent secondary lamellae, erosion of secondary lamellae, gill hyperplasia, degeneration of epithelium lining the secondary lamellae and sloughing of the lamellar epithelium.

6.6.2. Nitrite as a stress factor influencing the survival and immune response

Nitrite levels in water at the aquarium vendors were evaluated in the present study and found the concentrations to be at a higher level and hence nitrite as a stress factor was chosen. Accumulation of nitrite in aquaculture environment can lead to stress condition in aquatic animals, increasing the mortality and eventually causing serious production loss in aquaculture (Jiang *et al*., 2013). Studies in a number of fish have established that a higher nitrite level in water is one of the important factors causing considerable stress in fish (Stormer *et al*., 1996; Grossel and Jensen, 1999; Huang and Chen, 2002; Jensen, 2003; Das *et al*., 2004 a, b, c; Acharya *et al*., 2005; Ajani *et al*., 2007; Dolezalova *et al*., 2011; Dolomatov *et al*., 2013; Zuskova *et al*., 2013).

In the present study, 96-h LC_{50} of nitrite-N for *Cyprinus carpio* was found to be 46.85 mg/L. Toxicity of nitrite in fish varies widely with the fish species. It also depends on the size and age of fish. Very small fish seems less

sensitive to nitrite than fish of intermediate or large size (Kroupova *et al.*, 2005). The 96-h LC_{50} for smaller fish (average weight 4.4 ± 1.50 g) was 81 mg/L N-NO_2^- and larger fish (90.7 ± 16.43 g) demonstrated a 96-h LC_{50} of 8 mg/L N-NO_2^- (Atwood *et al.*, 2001). 24-h LC_{50} of nitrite for *Cyprinus carpio* was found to be171.36 (ppm) by Tilak *et al.* (2007). The 96-h LC_{50} nitrite for fingerlings of *Cirrhinus mrigala* (Ham.), was found to be 10.4 mg/L by Das *et al*. (2004 a). The 96-h LC_{50} of nitrite-N to Shortnose sturgeon fingerlings was 11.3 ± 8.17 mg/L (Isely and Tomasso, 1998). Palachek and Tomasso (1984) reported the 96-h LC_{50} of nitrite-N for Channel catfish *Ictalurus punctatus* to be 70 mg/L, and Russo and Thurston (1977) reported the 96-h LC_{50} of nitrite-N for Rainbow trout *Oncorhynchus mykiss* to be 22 mg/L.

A significant reduction in survival of *Cyprinus carpio* with increasing concentration of nitrite-N was observed in the present study. Rate of survival of fishes injected with *A. hydrophila*, decreased from 79% in the untreated control to 66.66% in fishes treated with 4.68 mg/L nitrite-N and further to 50% in fishes treated with 9.37 mg/L nitrite-N. Similar to the present observation, Bunch and Bejerano (1997) noticed that stress associated with dissolved oxygen and nitrite resulted in significant increases in mortality of Tilapia on infection with *Streptococcus* sp. Nitrite stress is reported to favour infection of aquarium fishes by *Saprolegnia*, which is a major disease of ornamental fishes (Carballo *et al*., 1995).

Nitrite caused a depression in immune parameters such as serum bactericidal efficiency and blood bacterial clearance efficiency. The survival rate of bacteria on incubation with serum increased from 46.85% in the control

group to 61.89 (4.68 mg/L) and 77.97% (9.37 mg/L nitrite-N) in the nitrite treated groups, indicating a reduction in bactericidal efficiency of serum. The pathogen clearance efficiency of blood decreased to 156.7 (4.68 mg/L) and 354.1% (9.37 mg/L nitrite-N) in the nitrite treated groups, compared to the control.

The results revealed an increase in total leucocyte count and neutrophil count and a reduction in lymphocyte count with increasing concentration of nitrite-N. Total leucocyte count increased from $31.16\pm1.04x10^3/mm^3$ in the untreated control fishes, injected with *A. hydrophila* to, $41.33\pm1.25\ x10^3/mm^3$ (4.68 mg/L) and $45.83\pm0.76x10^3/mm^3$ (9.37 mg/L nitrite-N) in fishes exposed to nitrite. Observations of the present study are in accordance with the results of Kroupova *et al*. (2008) who studied the effect of increasing concentration of nitrite in Rainbow trout and observed higher leucocyte counts in fish exposed to high concentration of nitrite. A sublethal nitrite toxicity trial was conducted for a period of 96 h with fingerlings of *Catla catla* (Das *et al*., 2004 b) and *Labeo rohita* (Das *et al*., 2004 c). The study revealed that exposure to nitrite caused changes in almost all the haematological parameters in the fingerlings depending on the concentration as well as exposure period. Total leukocyte count was found to increase significantly after 12 h. The total leucocyte counts increases due to the stimulated lymphoperisis and/or enhanced release of lymphocytes from lymphoid tissue or may be associated with the nitrite induced tissue damage (Das *et al*., 2004 c). Contrary to the above observations, Ajani *et al*. (2007) reported a decrease in the value of white blood cells, in *Clarias gariepinus* on exposure to sublethal concentration of nitrite, but similar to the observation in the present study, she reported a decrease in the value of lymphocytes. Literature on the effect of nitrite on the

immune parameters is scarce. Most of the literature dealing with the nitrite toxicity mainly focuses on the methaemoglobin formation and a very few deals with the effect on other haematological parameters (Das *et al*., 2004 c).

Nitrite accumulation causing tissue damage has been reported in fish (Dolomatov *et al*., 2013). In the histopathological analysis of gill tissues of *Cyprinus carpio* in the present study, degeneration of epithelium lining the primary lamellae and necrosis of secondary gill lamellae were observed and the tissue damages were found to become severe as the concentrations of nitrite increased. Hyperplasia, vacuolisation and elevated numbers of chloride cells were the main histological lesions that occurred in the gills of nitrite treated Carp (*Cyprinus carpio*) (Svobodova *et al*., 2005). Kroupova *et al*. (2008) observed hyperplasia and oedema of the respiratory epithelium of secondary lamellae in the gills of nitrite treated Rainbow trout. Increasing concentrations of nitrite also resulted in an increased number of chloride cells. Michael *et al*. (1987) observed hyperplasia and hypertrophy in the gills of *Clarias lazera* chronically exposed to nitrite.

6.6.3. Crowding as a stress factor influencing the survival and immune response

Ornamental fishes at the retail aquarium vendor level are usually kept at very high stocking density leading to considerable stress and hence stocking density was considered as a stress factor in the present study. Fishes were stocked at different stocking densities for seven days and then challenged with *A. hydrophila.* The effect of crowding condition on the non-specific immune response and disease resistance of fancy Carp (*Cyprinus carpio* L.) was studied by Yin *et al*. (1995). The fish were stressed for different days (1, 7, 14

and 30 days) and then challenged with *A. hydrophila*. Disease resistance of the stressed fish was significantly reduced on day 7. However, no great difference in resistance against *A. hydrophila* was found between day 7 and day 30. Based on this report fishes were stressed for 7 days in the present study and a significant reduction in the survival of *Cyprinus carpio* with increasing stocking density was observed.

Survival rate of *Cyprinus carpio* challenged with *A. hydrophila* decreased from 72.2% in groups with a stocking density of 12 fishes/10L to 38.8% in groups with a stocking density of 30 fishes/10L. These results are also in agreement with the observations of Crumlish *et al*. (2003), who reported that fishes exposed to higher stocking densities, exhibited higher percentage mortalities when challenged with *A. hydrophila.* Mortalities in fish groups receiving both stress and bacterial challenge was higher, when compared with fishes receiving stress only or bacterial challenge only. Crowding stress causing increased mortality in hybrid striped bass in culture facilities is also reported by Stoffregen *et al*. (1996). Rainbow trout inoculated with *A. salmonicida* and subjected to handling and anoxic stress, showing a higher mortality rate when compared with the unstressed fish is also reported (Angelidis *et al*., 1987).

Higher socking densities causing significant reduction in immune parameters such as serum bactericidal efficiency and bacterial clearance efficiency of blood was evident in the present study. Pathogen clearance efficiency of blood decreased to 106%, 297.9% and 370.2% when the stocking density increased to 18, 24 and 30 fishes respectively in 10L water compared to the control. Similar to this observation, subjecting Rainbow trout

(*Oncorhynchus mykiss*) to social stresses of cohabitation with Cohorts and then exposing these fish to infection by *A. hydrophila*, Peters *et al*. (1988) observed that, the bacterium was recovered from more organs and with greater prevalence from the subordinate Rainbow trouts which were under stress than from the non stressed, dominant Cohorts. The number of bacteria in the blood in the stressed fishes was also higher, when compared to non stressed fishes.

The survival rate of bacteria on incubation with serum increased from 45.61% in the control group to 62.42 (18 fishes/10L), 71.80 (24 fishes/10L) and 82.01% (30 fishes/10L) with increasing stocking densities, indicating a reduction in bactericidal efficiency of serum. Similar to the results in the present study, an increase in the proliferation of the bacterial pathogens, *Vibrio anguillarum* and *Aeromonas salmonicida* was seen from 24 h in the plasma of Atlantic Cod (*Gadus morhua*) stressed by short term exposure to overcrowding (Caipang *et al*., 2009), indicating a decrease in the antibacterial activity of serum in stressed fishes. Crowding stress causing immune suppression and higher susceptibility to pathogens is reported by several authors (Gornati *et al*., 2004; Costas *et al*., 2008; Di Marco *et al*., 2008) and this might explain why severe outbreaks of infectious diseases often occur in intensive fish culture when there is a drastic change in the temperature or water quality (Angka *et al*., 1995).

An increase in total leucocyte count and neutrophil count and a reduction in lymphocyte count with increasing stocking density were observed in the present study. Total leucocyte count increased from 28.66±1.04 $x10^3/mm^3$ in the control fishes injected with *A. hydrophila* to 60.83±0.76 $x10^3/mm^3$ in groups stocked at 30 fishes in 10 L water. Similar observations were made by Peters *et al*.

(1988) who subjected Rainbow trout (*Oncorhynchus mykiss*) to social stresses of cohabitation with Cohorts and then exposed these fish to infection by *A. hydrophila*. When compared, Rainbow trout which showed physical evidence of stress also exhibited increased leukocyte volumes. In another investigation by Peters and Schwarzer (1985), it was found that neutrophilic granulocytes and their precursor cells developed into large macrophage like cells in Rainbow trout exposed to social stress. Their morphological appearance suggested that after a transient stage of stimulation these cells progressively degenerate. Thus, a reduction in their functional capacity was expected, affecting not only the phagocytic destruction of pathogens, but also the presentation of antigen to the antibody-producing lymphocytes (Peters *et al.*, 1988). In view of the observed structural alteration of phagocytes expressed in the increased leucocrit in stressed fish, they consider the functional disturbances of these cells to be of major significance within the context of the host-pathogen interaction.

Neutrophilia and lymphopenia was observed with increasing stocking densities and neutrophil count increased from 10.66±0.57% in the control fishes, injected with *A. hydrophila* to 22.33±0.57% in groups stocked at 30 fishes in 10 L water. Lymphocyte count decreased from 89.33±0.57 (control) to 77.66±0.57% (30 fishes/10 L water). These results are in accordance with the observations of Ellsaesser and Clem (1986) who noticed that in Channel catfish eighteen hours after the induction of stress, there was neutrophilia with increases of up to 30% of the circulating leucocytes. There was also a marked lymphopenia which appeared to be the result of a reduction in the number of both T and B lymphocytes in the circulation. Wendelaar (1997) reported that stress caused a rapid increase of neutrophils and a reduction of lymphocytes in

peripheral blood. Ortuno *et al*. (2001) demonstrated that intense acute crowding led to leukocyte migration into the blood from the head kidney. Significant differences in the values of the neutrophil and lymphocyte counts of African catfish (*Clarias gariepinus*) (Adeyemo *et al.*, 2009) and in the neutrophil counts of common carp (Dobšíková *et al*., 2009) exposed to crowding stress is reported.

Histopathological analysis of gill tissues of *Cyprinus carpio* in the present study revealed lamellar fusion and necrosis of primary and secondary gill lamellae which became severe with higher stocking densities. Degeneration of epithelium lining the primary lamellae was also observed.

These findings suggest that short-term crowding stress modulates the immune responses in fishes and therefore husbandry procedures should consider these effects while addressing better management strategies. Considering the mortality and reduction in immune parameters in *Cyprinus carpio* caused by the three stress factors studied (UIA-N, nitrite-N and stocking density) it was found that ammonia (UIA-N) is the most important one causing high mortality. Histopathological analysis of gill tissues also revealed the same results.

6.7. Recommendations/suggestions to overcome stress related issues

Ornamental fishes have market value only as live, healthy ones; therefore it is necessary to maintain them in a healthy, stress free environment. The present study highlights the adverse impact of environmental stressors on fish immunity and points the need for the management of these factors in fish culture ponds. Aquaculture has the advantage that it is possible to control many of the potential hazards at the production level by using good fish farm management practices.

Aquaria should not be overcrowded and the stocking density should be maintained at optimum. Physico-chemical characteristics of the water have to be routinely checked and maintained within the recommended range for each fish species. Frequent water changes (30–50%) are the mainstay of treatment of ammonia and nitrite toxicity. Feeding should be reduced or temporarily stopped. Oxygenation should also be improved. An increase towards the alkaline range of normal pH increase ammonia toxicity, hence the pH should be regularly evaluated and maintained.

Chapter 7

Effect of probiotic on survival and immune response of *Cyprinus carpio* against *A. hydrophila* infection

7.1. Introduction

Prevention of diseases is a primary goal in aquaculture. The application of hygienic and preventive measures of the environment, such as fish health management, sanitation and disease control procedures are critical factors to prevent fish disease. When faced with disease problems, the common response has been to turn to antimicrobial drugs. Use of antibiotics in aquaculture may lead to the development of drug resistant bacteria and transfer of resistant genes between bacteria (Witte, 2000; Schwarz *et al*., 2001), the accumulation of residual antibiotics in aquaculture products (Cabello 2006; Hoque, 2014), environmental pollution (Kumari *et al*., 2007) and detrimental effect on the microbial biodiversity (Zhou *et al*., 2010). It also has resulted in trade restrictions in export markets. The drawbacks of using antibiotics in

aquaculture evoke a keen interest in probiotics (Wang *et al*., 2008; Watson *et al*., 2008; Bloch *et al*., 2013). They are also an alternative to the use of vaccines to protect the fish from infectious diseases, as vaccines cannot be relied as a universal disease control measure in aquaculture due to their limited availability in few countries and their pathogen specific action of protection against certain bacterial and viral diseases (El-Ezabi *et al*., 2011).

The term "probiotics" was originally used by Lilley and Stillwell (1965) to describe a substance(s) that stimulate the growth of other microorganisms. The term has now been redefined. The definition of probiotic differs greatly depending on the source, but the most widely quoted definition for probiotics was made by Fuller (1989). He defined probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". It is a live microbial dietary additive which confers health advantages and had its long history of use in humans and animals (Ige, 2013). It can also be defined as "a viable microorganism which when ingested through the oral cavity in a sufficient quantity confer on the host a beneficial effect due to an improvement in the intestinal microbial balance" (Giorgio *et al*., 2010). Merrifield *et al*. (2010) defined probiotic for aquaculture as "a live, dead or component of a microbial cell that when administered via the feed or to the rearing water benefits the host by improving either disease resistance, health status, growth performance, feed utilization, stress response or general vigour, which is achieved at least in part via improving the hosts microbial balance or the microbial balance of the ambient environment". Another proposed definition of probiotics used in aquaculture is "live microbial cultures added to feed or environment (water) to increase viability (survival) of the host" (Ringo *et al*., 2010). Probiotics are

biopreparations containing living microbial cells that optimize the colonization and composition of the growth and gut microflora in animals and stimulate digestive processes and immunity (Dhanaraj *et al*., 2010). Another definition considered appropriate for aquaculture by Ige (2013) is "any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish". Probiotics may reduce the incidence of disease or lessen the severity of outbreaks in aquaculture.

In aquaculture, the range of probiotics evaluated for use is considerably wider than in terrestrial agriculture. Several probiotics either as monospecies or multispecies supplements are commercially available for aquaculture practices (Decamp and Moriarty 2006; Ghosh *et al*., 2007). Probiotics protect fish by various mechanisms such as blocking adhesion sites for pathogens, production of organic acids (formic acid, acetic acid, lactic acid) to lower pH and alter protein structure, production of hydrogen peroxide and reactive oxygen species to damage enzyme systems in pathogens and by the activation of innate and adaptive immune responses to amplify killing of pathogenic agents (Bloch *et al*., 2013). Probiotic microorganisms will of course, have to be non-pathogenic and non-toxic in order to avoid undesirable side-effects when administered to fish. Studies have shown that probiotics are important for weight gain in fish (Aly *et al*., 2008; Al-Dohail *et al*., 2009; Sharma *et al*., 2013).

Enhancement of the immune system seems to be the most promising method for preventing fish diseases. The immune systems of fish have two integral components: the innate, natural or nonspecific defence system and the

adaptive, acquired or specific immune system. The normal micro biota in the gastrointestinal system influences the innate immune system, which is of vital importance for the disease resistance of fish. It is reported that the non-specific immune system can be stimulated by probiotics (Lara-flores, 2011).

The success of probiotics, has laid the foundation for other concepts like "prebiotics" which are the non-digestible food ingredients that selectively stimulate the growth and/or activity of one or limited microbes and "synbiotics", the nutritional supplements combining probiotics and prebiotics. The obvious potential advantages of such approaches are that they promote specific microbe(s) in the intestine for restoring the intestinal microbial balance and exerting numerous beneficial effects in host (Nayak, 2010).

7.2. Review of literature

7.2.1. Bacteria used as probiotics in aquaculture

In aquaculture practices, probiotics are used for a quite long time but in last few years probiotics became an integral part of the culture practices for improving growth and disease resistance. This strategy offers innumerable advantages to overcome the limitations and side effects of antibiotics and other drugs and also leads to high production through enhanced growth and disease prevention (Mishra *et al*., 2001; Das *et al*., 2008; Sahu *et al*., 2008; Nayak, 2010).

Most probiotics proposed as biological control agents in aquaculture belong to the Lactic acid bacteria (*Lactobacillus, Lactococcus* and *Carnobacterium*), such as *Lactococcus lactis, Lactobacillus plantarum*, *L. rhamnosus, L. sakei, L. delbrueckii, Leuconostoc mesenteroides, Carnobacterium divergens* and

C. inhubens (Balcázar *et al*., 2007; Balcazar *et al*., 2008; Hagi and Hoshino, 2009; Merrifield *et al*., 2010; Pérez-Sánchez *et al*., 2011; Ige 2013).

Another important group involves *Bacillus,* such as *Bacillus subtilis*, *B. clausii*, *B. cereus*, *B. coagulans, B. circulans* and *B. licheniformis* (Salinas *et al*., 2005; Ochoa-Solano and Olmos-Soto, 2006; Balcázar and Rojas-Luna, 2007; Newaj-Fyzul *et al*., 2007; Bandyopadhyay and Das Mohapatra, 2009; Nakayama *et al*., 2009; Sun *et al*., 2010; Ai *et al*., 2011; Olmos *et al*., 2011; Kamgar *et al*., 2013).

Vibrio such as *Vibrio fluvialis, V. alginolyticus* (Fjellheim *et al*., 2007; Thompson *et al*., 2010), *Pseudomonas* (Chythanya, 2002; Das *et al*., 2006; Preetha *et al*., 2007; Abd El-Rhman *et al*., 2009; Ström-Bestor and Wiklund, 2011), and *Aeromonas* such as *Aeromonas hydrophila, A. media* (Irianto *et al*., 2003; Lategan *et al*., 2004; Lategan *et al*., 2006) are also used as probiotics.

These probiotics have been used in different aquatic organisms and have been shown to be successful, not only for their ability to prevent disease, but also for improving digestion and growth. Many of these applications have been targeted at the early stages of development of the aquatic organisms, such as the larval stages, because these stages are more susceptible to infections (Bricknell and Dalmo, 2005; Vine *et al*., 2006; Fjellheim *et al*., 2007; Dierckens *et al*., 2009; Fjellheim *et al*., 2010; Avella *et al*., 2011).

7.2.2. Probiotic treatments and enhancement of disease resistance and immune response

Bacillus subtilis used as an agent for the control of streptococcosis in Rainbow trout hatchery is reported by Kamgar *et al*. (2013). Mrigal fed with

probiotics showed protection from epizootic ulcerative syndrome (Sharma *et al.,* 2013). Chabrillón *et al*. (2006) observed that, the mortality of Gilthead seabream (*Sparus aurata*) receiving a diet supplemented with potential probiotic was significantly lower, when compared with fishes receiving non-supplemented commercial diet, on challenge with *Listonella anguillarum.* Lategan *et al*. (2004) studied the control of saprolegniosis in fish by probiotics. Morbidity due to saprolegniosis was low in probiotics treated tanks, in comparison to the non-treated control tanks.

Sharifuzzaman and Austin (2009) observed a significant reduction in mortality in Rainbow trout fed with *Kocuria* supplemented diet when challenged intraperitoneally with *Vibrio anguillarum*. Marzouk *et al*. (2008) observed a high resistance to challenge with *Pseudomonas fluorescens* in *Oreochromis niloticus* fed with *Bacillus subtilis* and *Saccharomyces cerevisiae.* Effects of probiotics on survival in Japanese flounder (*Paralichthys olivaceus*) were evaluated by Taoka *et al*. (2006). Pathogen challenge tests with *V. anguillarum* resulted in significantly higher survival in the probiotics treated groups than the control group. Gram *et al*. (1999) studied the effect of probiotic against infection by *V. anguillarum,* in Rainbow trout (*Oncorynchus mykiss* Walbaum), and found a significant reduction in mortality in the probiotic fed fishes compared to the control. Gildberg *et al*. (1997) fed Atlantic cod fry with feed containing *Carnobacterium divergens* and then challenged with a virulent strain of *V. anguillarum*. An improved disease resistance was observed in the probiotic fed fishes.

Balcazar *et al*. (2007) analysed the effect of probiotic strains on the immune responses of Rainbow trout (*Oncorhynchus mykiss*). Rainbow trout

fed with probiotic supplemented diet exhibited survival rates ranging from 97.8 to 100%, whereas survival was 65.6% in fish not treated with the probiotics, when challenged with *A. salmonicida.* Newaj-Fyzul *et al*. (2007) observed that in Rainbow trout, the survival rate of fish challenged with *Aeromonas* spp. ranged from 65 to 100% for the probiotic fed as compared to 5 to 15% in the control fish. Austin *et al*. (1995) evaluated the effect of administering *Vibrio alginolyticus* strain in a bath treatment to Atlantic salmon and the results revealed that application of *Vibrio* led to a reduction in mortality after exposure to *A. salmonicida*.

Kamgar *et al*. (2013) observed significant difference in the serum total protein, serum albumin, IgM and lysozyme of probiotic fed Rainbow trout, compared to control. Kim *et al*. (2012) investigated the effect of a probiotic, *Enterococcus faecium*, on the immune responses against infection with *Lactococcus garvieae* in Olive flounder (*Paralichthys olivaceus*). The lysozyme activity, complement activity and antiprotease activity was found elevated on probiotic treatment. El-Ezabi *et al.* (2011) investigated the effect of *Bacillus subtilis* and *Lactobacillus plantarum,* a mixture of both bacterial isolates and the yeast, *Sacchromyces cerevisiae* on the immune response of the Nile tilapia (*Oreochromis niloticus*). The results showed significantly higher phagocytic activity, acid phosphatase activity, lysozyme activity and total immunoglobulin activity in probiotic fed fish as compared with the control. Al-Dohail *et al*. (2011) observed the protective effect of probiotics in African catfish against infection by *Staphylococcus xylosus, Aeromonas hydrophila* and *Streptococcus agalactiae*. They found that serum total immunoglobulins concentration was significantly higher in fishes fed with probiotic supplemented diet, compared to the control.

Marzouk *et al*. (2008) observed that *Bacillus subtilis* and *Saccharomyces cerevisiae* improved the non specific immune response of *O. niloticus,* through the stimulation of macrophage cells and increased phagocytic activity. Probiotic fed fishes exhibited an increase in the number of lymphocytes, monocytes and total white blood cell count and also a high resistance to the challenge with *Pseudomonas fluorescens.*

Balcazar *et al*. (2007) analysed the effect of probiotic strains on the cellular and humoral immune responses of Rainbow trout (*Oncorhynchus mykiss*). The fish supplemented with probiotic diets were more resistant to challenge with *A. salmonicida.* In comparison to untreated control fish, the phagocytic activity of leukocytes and the alternative complement activity in serum were significantly greater in the probiotic fed group. Panigrahi *et al*. (2007) studied the immune modulation including the expression of cytokine genes following dietary administration of probiotic bacteria, *Lactobacillus rhamnosus, Enterococcus faecium* and *Bacillus subtilis* to Rainbow trout (*Oncorhynchus mykiss*). Production of superoxide anions and leukocytes were found to be enhanced after feeding with probiotics. There was also an improvement in the alternate complement activity of serum, in the probiotic fed fishes. Besides this, there was an up regulation of cytokine genes, in this group. Newaj-Fyzul *et al*. (2007) observed that in Rainbow trout, probiotic feeding provided protection against *Aeromonas* challenge and it also stimulated the immune parameters like serum and gut lysozyme activity, peroxidase activity and phagocytic activity besides resulting in an increase in lymphocyte populations.

Effects of probiotics on growth, stress tolerance and non-specific immune response in Japanese flounder (*Paralichthys olivaceus*) were evaluated by Taoka

et al. (2006). Growth and survival against *Vibrio anguillarum* challenge, of Flounder treated by supplying commercial probiotics either in the diet, or into the rearing water, were higher compared to the control group. They have also observed an increase in plasma lysozyme activity in the probiotic treated group. Sakata (2006) investigated the effect probiotics on the non-specific immune system of Tilapia (*Oreochromis niloticus*). Probiotics were introduced by feeding either in the form of live or dead cells, or supplying live cells to the rearing water in a closed recirculating system. The probiotics treatment enhanced non-specific immune parameters such as lysozyme activity, migration of neutrophils and plasma bactericidal activity, resulting in improvement of resistance to *Edwardsiella tarda* infection. Sakai *et al*. (1995) observed that, oral administration of *Clostridium butyricum* bacteria to Rainbow trout enhanced the resistance of fish to vibriosis by increasing the phagocytic activity of leucocytes.

Administration of probiotics can also cause alteration in the number of immunohaematological parameters in fishes. Selvaraj *et al*. (2005) studied the effects of yeast glucan administration on immune modulations in *Cyprinus carpio* against the bacterial pathogen *A. hydrophila,* and they observed significant increase in total blood leucocyte counts and an increase in the proportion of neutrophils and monocytes.

Al- Dohail *et al*. (2009) noticed an elevation in red blood cell count, white blood cell count and packed cell volume in African cat fish fingerling fed on a diet containing *Lactobacillus acidophilus* compared with the normal control. Mehrim (2009) recorded a similar observation in Nile tilapia fed on probiotics compared with the normal control. Tantawy *et al*. (2009) found that

application of *Lactobacillus acidophilus* in common carp feed showed a significant increase in total and differential leucocytic count.

Infections cause haematological changes in fishes. Study by Haniffa and Mydeen, (2011) evaluated the haematological changes in *Channa striatus* intramuscularly administered with *A. hydrophila*. Fish injected with *A. hydrophila* showed a higher mean corpuscular volume and packed cell volume than control fish. White blood cells and lymphocytes numbers increased significantly in fish injected with *A. hydrophila* when compared to non-injected control. Zorriehzahra *et al*. (2010) observed an increase in total leucocyte count and a decrease in lymphocytes and neutrophils in diseased Rainbow trout *(Oncorhynchus mykiss*) compared with control. Thoria (2010) noticed a significant decrease in red blood cell count, haemoglobin concentration and packed cell volume with leukocytosis in Nile Cat fish experimentally infected with *A. hydrophila* compared with normal non infected group. The study of Harikrishnan *et al*. (2003) in Carp infected by *A. hydrophila* showed an increase in the leukocyte number. But the opposite was found in Pacu (*Piaractus mesopotamicus*) experimentally infected with *A. hydrophila* (Garcia *et al.,* 2007). Rafiq *et al*. (2001) did not observe any alteration in the differential counts of white blood cells in Tilapia challenged with *A. hydrophila*. Stoskopf (1993) found leukocytosis, neutrophilia, monocytosis and lymphopenia in naturally infected common carp with *A. hydrophila* compared with the normal non infected group.

Martins *et al.* (2008; 2009) have reported that Tilapia injected with *Enterococcus* showed an increase in white blood cells, lymphocytes and thrombocytes when compared to non-injected fishes. In *Oreochromis aureus*

infected with *Corynebacterium* sp., Silveira-Coffigny *et al*. (2004) observed an increased number of lymphocytes. Pathiratne and Rajapakshe (1998) observed high number of neutrophils in Chichlids with epizootic ulcerative syndrome. Lamas *et al.* (1994) and Balfry *et al.* (1997) found a reduced number of lymphocytes in the blood of Rainbow trout and Tilapia, infected with *V. anguillarum* and *V. parahaemolyticus,* respectively.

7.2.3. Control of *Aeromonas* infections in fishes through probiotics

The protective effects of oral administration of *Bacillus coagulans* and chitosan oligosaccharides, single or combined, on the resistance of *Cyprinus carpio* koi against *A. veronii* were observed by Lin *et al*. (2012). The control of *A. hydrophila* infection in *Cyprinus carpio* by bacteria *Enterococcus faecium* isolated from fish *Mugil cephalus* intestine was observed by Gopalakannan and Arul (2011). Feeding Nile Tilapia with the probiotic *Micrococcus luteus* was found to exert an inhibitory effect against *A. hydrophila* (Abd El-rhman *et al*., 2009). Zhou *et al*. (2010) observed inhibitory ability of probiotic, *Lactococcus lactis*, against *A. hydrophila in vitro.* Kumar *et al.* (2008) assessed the use of probiotic *Bacillus subtilis* in *Labeo rohita* against *A. hydrophila* infection and found it to be effective in controlling infection. The *in vitro* antimicrobial assay by Aly *et al*. (2008) showed that *Bacillus subtilis* and *Lactobacillus acidophilus* inhibited the growth of *A. hydrophila*. They have observed that *Tilapia nilotica* (*Oreochromis niloticus*) fed with this bacterial mixture exhibited better protection against *A. hydrophila* challenge infection. Abdel-Tawwab *et al*. (2008) showed that the addition of probiotic *Saccharomyces cerevisiae* in diet decreased the mortality of Nile Tilapia challenged by *A. hydrophila*. Newaj-Fyzul *et al*.

(2007) observed that *Bacillus subtilis* controls *Aeromonas* infection in Rainbow trout.

In another study, Vendrell *et al*. (2008) showed that feeding Rainbow trout with *Lactobacillus rhamnosus* supplemented feed reduced fish mortality caused by *A. salmonicida.* The beneficial effect of probiotic strains, *Lactococcus lactis, Leuconostoc mesenteroides* and *Lactobacillus sakei* in Rainbow trout against *A. salmonicida* infection was noticed by Balcazar *et al*. (2007). Protective effect of probiotics against *A. salmonicida* infection in Rainbow trout has been reported (Irianto and Austin, 2002; Brunt *et al*., 2007). It has been found that the addition of a probiotic bacteria *L. rhamnosus* to Rainbow trout diet could reduce mortality of fish challenged with a virulent strain of *A. salmonicida* (Nikoskelainen *et al*., 2001). A strain of *Carnobacterium* sp. isolated from the intestine of Atlantic salmon was effective in controlling infections caused by *A. salmonicida,* in fry and fingerlings of Salmonids (Robertson *et al*., 2000). Application of the probiotic *V. alginolyticus* led to a reduction in mortality after exposures to *A. salmonicida* in Atlantic salmon (Austin *et al*., 1995). The use of combination of putative strains of *A. sobria* and *Brochothrix thermosphacta* was able to prevent fin rot caused by *A. bestiarum* in Rainbow trout (Pieters *et al*., 2008).

7.3. Objectives of the study

Review of literature amply reflects the role of *A. hydrophila* and *A. sobria* as fish pathogens and the role of probiotics in combating/controlling the disease caused by them. Unlike in developed countries regulatory control over the use/misuse of antibiotics in aquaculture system is weak in India. The same is reflected in our studies on prevalence of antibiotic resistance among

motile aeromonads from ornamental fishes in the study area. Hence the study has been taken up with the following objectives:

1) To study the effect of probiotic treatment (*Bacillus* NL 110) on the survival of *Cyprinus carpio*, post challenge with *A. hydrophila.*

2) To study the effect of probiotic treatment (*Bacillus* NL 110) on the immune parameters of *C. carpio*, post challenge with *A. hydrophila.*

3) To study the effect of probiotic treatment (*Bacillus* NL 110) on the histopathology of *C. carpio*, post challenge with *A. hydrophila.*

7.4. Material and Methods

7.4.1. Experimental fishes

Koi carp (*Cyprinus carpio*), the fresh water ornamental fish, obtained from an aquarium shop in Kerala, India was used for the study. Fishes weighing ~1.5g ± 0.2g were brought to the laboratory, acclimatized in tanks containing dechlorinated water over a period of two weeks until feed consumption and general behaviour became normal. The tanks in which the fishes were maintained had water temperature ranging from 27 to 29^{o}C, dissolved oxygen concentrations from 6.8 to 7.8 mg/L, pH from 7.0 to 7.5, and unionized ammonia concentration from 0.04 to 0.14mg/L. After the period of acclimatization, the fishes were transferred to the experimental tanks and were allowed to acclimatize for another week.

7.4.2. Experimental diet

Pure culture of *Bacillus* NL110 (probiotic strain, lab stock, Rahiman *et al*., 2010) was used for experimental diet preparation. *Bacillus* NL110 was inoculated into sterile nutrient broth and incubated on a shaking incubator for

24 hours at 37°C. The cells were then harvested by centrifugation at 3,000 rpm for 15 minutes, washed thrice and resuspended in physiological saline. The cells were thoroughly mixed with commercial fish feed to obtain 10^9 cells/g feed. The feed was aseptically spread out and dried overnight at 37°C. Feed thus prepared was stored at 4°C and fed to the fishes maintained in respective experimental tanks (Shariffuzaman and Austin 2009; Rahiman *et al*., 2010).

7.4.3. Addition of probiotic through water

Pure culture of *Bacillus* NL110 was inoculated into sterile nutrient broth and incubated on a shaking incubator for 24 hours at 37°C. The cells were then harvested by centrifugation at 3,000 rpm for 15 minutes, washed thrice, resuspended in distilled water, added to the experimental tank to obtain a final concentration of ~10^6 cells/ ml.

7.4.4. Experimental design

After acclimatization, fishes were randomly divided into two groups. One group was kept as control and was fed with commercial feed. The other group was fed with experimental feed (probiotic supplemented diet). Each group consisted of 12 animals in each tank. Experiment was conducted in triplicate. Fishes in each group were fed at 2% of the body weight per day for a period of 14 days (Shariffuzaman and Austin 2009).

Each aquarium was supplied with compressed air using aquarium air pumps. Fish wastes settled at the bottom of the tanks were siphoned out daily along with three quarters of the aquarium water, which was replaced by aerated water from the storage tank. In the experimental tank, probiotic was incorporated in water to get a final concentration of ~10^6 cells/ml. The basic physico-

chemical parameters of water viz. temperature, dissolved oxygen, NH_3-N, NO_2-N and NO_3-N were maintained at optimal levels.

7.4.5. Challenging with *Aeromonas hydrophila*

7.4.5.1. Bacterial culture

Pure culture of *Aeromonas hydrophila* (Genbank accession number: JX987236) originally isolated from aquarium fish samples (John and Hatha, 2013) was used for challenge experiment. The isolate was grown in nutrient broth for 24 h at 28°C. The broth cultures were harvested by centrifugation at 5000 × g for 15 min at 4°C. The bacterial pellet was washed by resuspension in sterile phosphate buffered saline (PBS-pH 7.4) and centrifugation as above and the final pellet was resuspended in PBS to get a cell density of 7.5 x 10^6 cells/ ml. The viable count of the suspension was confirmed by spread plate technique.

7.4.5.2. Challenge experiment

At the end of 14 days feeding, fishes from each experimental group were injected intra-peritoneally (IP) with 0.1 ml dose (7.5 x 10^6 cfu/ml) of *Aeromonas hydrophila.* All groups were kept under observation for 7 days to record clinical signs of the disease and mortality. The cause of death was confirmed by reisolating the organism from body parts of dead fishes (10% of dead fishes were used for reisolation) using starch ampicillin agar.

7.4.6. Relative Percentage Survival

Fish mortality was recorded for 7 days following bacterial challenge and percentage survival was calculated employing the following formula:

$$\% \text{ survival} = \frac{\text{No. of surviving fish after challenge}}{\text{No. of fish injected with the pathogen}} \times 100$$

Relative Percentage Survival (RPS) (Amend, 1981) was determined using the following equation

RPS = 1- (% mortality in treated group / % mortality in control group) x 100

7.4.7. Serum collection

The procedure for serum collection was same as described in section 6.4.7. Serum bactericidal efficiency was determined as described in section 6.4.7.1.

7.4.8. Statistical analysis

Statistical analysis was performed using student's *t* test to determine differences between treatments, at significance level of 0.05. Standard errors of treatment means were also estimated. All statistics were carried out using SPSS 13.

7.4.9. Histopathological analysis

Gill, liver and intestine from the body of infected fishes were removed and fixed in 10% buffered formalin for 24 hours. Tissues were then washed in running tap water overnight. Tissues were dehydrated in ascending grade of alcohol; for 30 minutes in 50% alcohol and for 45 minutes each in 80 and 90% alcohol and then transferred to absolute alcohol (two changes) for one hour each.

Tissues were cleared in xylene until they became translucent. They were then transferred to a mixture of xylene and paraffin wax and left overnight. The tissues were infiltrated in 2-3 changes of molten paraffin wax of melting

point 60-62°C for one hour each. They were then embedded in paraffin wax of melting point 60-62°C. Paraffin blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns.

Staining technique using Haematoxylin-Eosin stain was carried out as described in 6.3.9.1.

7.5. Results

7.5.1. Survival of *Cyprinus carpio* treated with probiotic NL110

After the challenge with *A. hydrophila* significant increase in survival ($p<0.05$) was observed in *Cyprinus carpio* fed with the probiotic diet compared to the control (Figure 7.1) (Appendix 8.1).

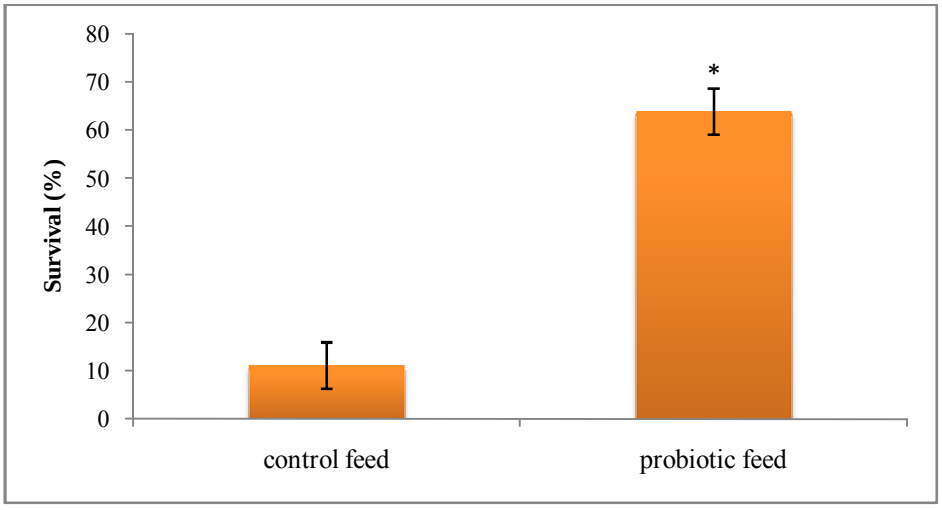

Figure 7.1. Survival (%) of *Cyprinus carpio* in control and experimental group after challenge with *A. hydrophila*

* Represents significant difference ($p<0.05$) between control and experimental group

Relative percentage survival observed was 59%. RPS values over 50 indicate positive effect of the probiotics (Amend, 1981).

7.5.2. Serum bactericidal efficiency

Bactericidal efficiency of serum was significantly (p<0.05) higher in the probiotic treated group compared to the control as is evident from the survival rate of bacteria after incubation with serum (Figure 7.2) (Appendix 8.2).

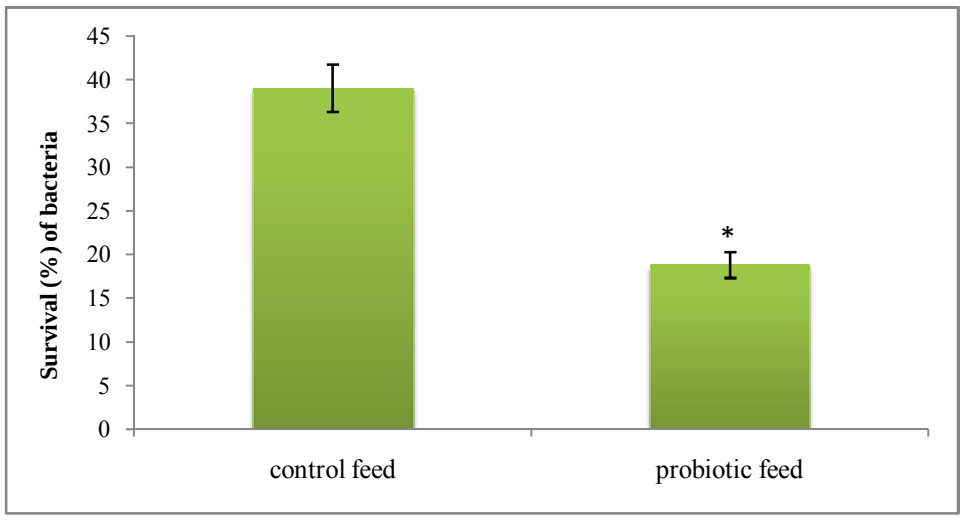

Figure 7.2. Serum bactericidal efficiency in control and experimental groups after challenge with *A. hydrophila*

* Represents significant difference (p<0.05) between control and experimental groups

7.5.3. Histopathological analysis

Histopathological analysis of gill tissues of fishes injected with *A. hydrophila* exhibited lamellar hyperplasia, clubbing and epithelial desquamation in the probiotic treated group. However, the pathological changes were more severe in fishes fed with control diet, these fishes exhibited severe damage of secondary lamella. Fishes injected with saline showed normal gill architecture (Plate 7.1 A-C).

Histopathological analysis of liver tissues is given in Plate 7.2 A-C. Fishes injected with saline showed normal architecture of liver. Fishes injected with

A. hydrophila exhibited areas of necrosis in the probiotic treated group, vacuolization and pyknosis of nuclei was more prominent in fishes fed with control diet.

Histopathological analysis of intestinal tissues is given in Plate 7.3 A-C. Fishes injected with saline showed normal architecture of intestine. Fishes injected with *A. hydrophila* exhibited shrinkage of enterocytes in the probiotic treated groups; severe necrosis of enterocytes was seen in fishes fed with control diet.

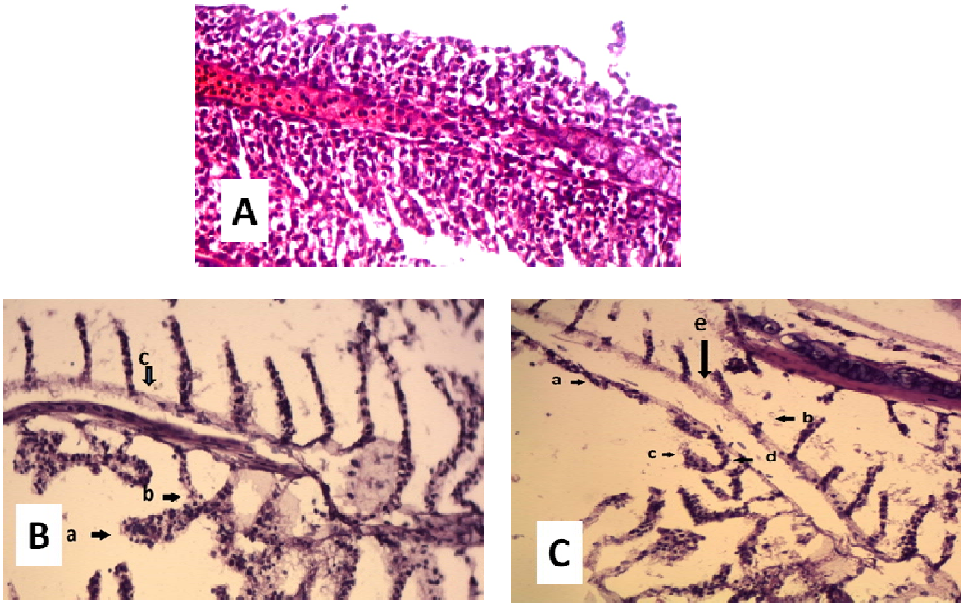

Plate 7.1.

A. Photomicrograph of gill of *C. carpio* injected with saline (control) showing normal gill architecture (H&E X 400)
B. Photomicrograph of gill of *C. carpio* fed on probiotic diet and injected with *A. hydrophila* showing hyperplasia (a), lamellar clubbing (b) and epithelial desquamation (c) (H&E X 400)
C. Photomicrograph of gill of *C. carpio* fed on normal diet and injected with *A. hydrophila* showing damaged secondary lamella (a), lamellar shortening (b), hyperplasia (c), lamellar curling (d) and epithelial desquamation (e) (H&E X 400)

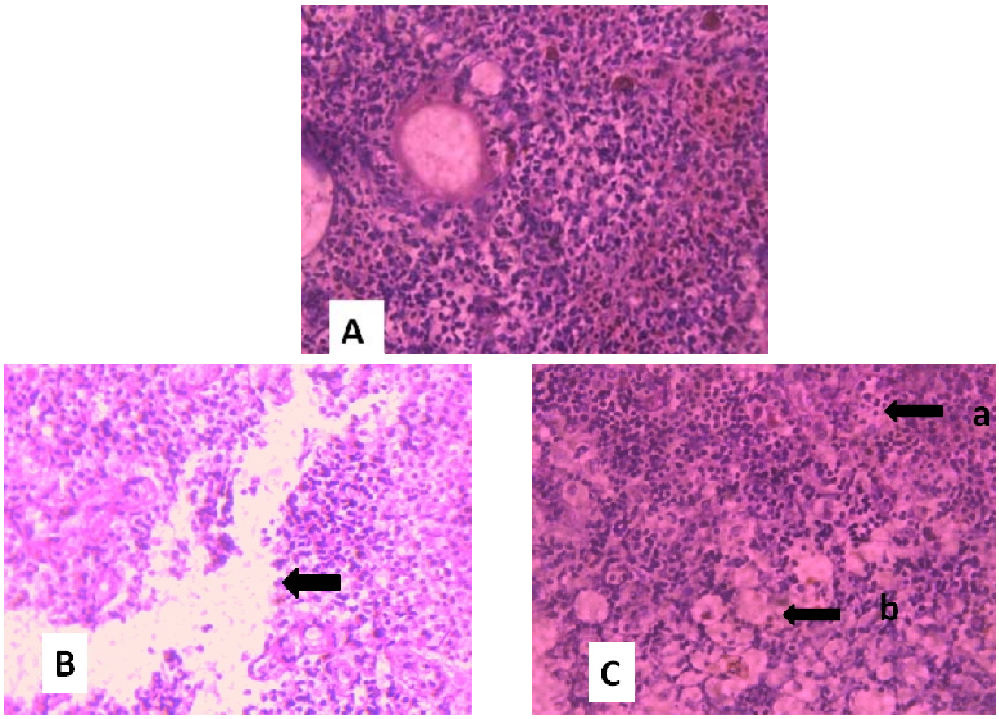

Plate 7.2.

A. Photomicrograph of liver of *C. carpio* injected with saline (control) showing normal architecture (H&E X 400)

B. Photomicrograph of liver of *C. carpio* fed on probiotic diet and injected with *A. hydrophila* showing focal areas of necrosis between the hepatocytes (H&E X 400)

C. Photomicrograph of liver of *C. carpio* fed on normal diet and injected with *A. hydrophila* showing pyknotic nuclei (a) and prominent vacuolization in hepatocytes (b) (H&E X 400)

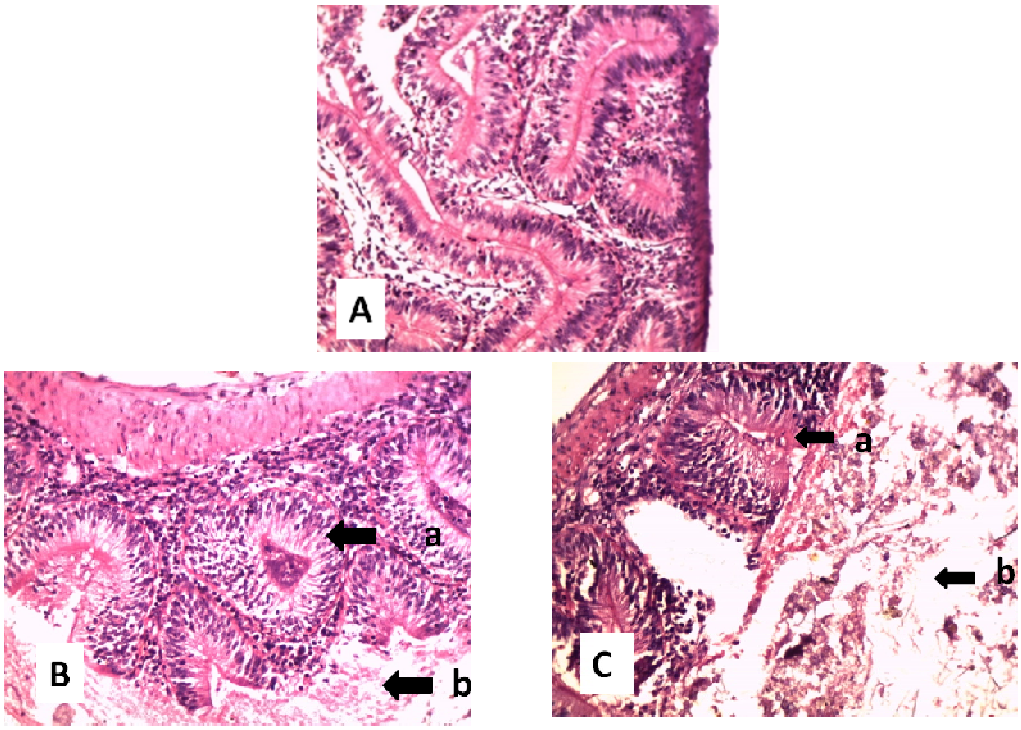

Plate 7.3

A. Photomicrograph of intestine of *C. carpio* injected with saline (control) showing normal architecture (H&E X 400)

B. Photomicrograph of intestine of *C. carpio* fed on probiotic diet and injected with *A. hydrophila* showing shrinkage (a) and necrosis (b) of enterocytes (H&E X 400)

C. Photomicrograph of intestine of *C. carpio* fed on normal diet and injected with *A. hydrophila* showing architectural loss (a) and complete necrosis (b) of enterocytes (H&E X 400)

7.6. Discussion

With the increasing intensification and commercialization of aquaculture production, disease is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries (Oke *et al*., 2013). Prevention or control of diseases is essential to the success of the large-scale, intensive production of fish in culture. The enhancement of the immune system of fish is considered to be the most promising method of preventing fish diseases in aquaculture. Modulation of host immune system is one of the most commonly purported benefits of the probiotics. Probiotics either individually or in combination can enhance both systemic as well as local immunity in fish (Nayak, 2010).

Influence of probiotic feeding duration on disease resistance and immune parameters in Rainbow trout was evaluated by Sharifuzzaman and Austin (2009). The results revealed that a two-week feeding regime led to the maximum reduction in mortalities and higher disease protection, with protection linked to stimulation of immune parameters compared to one, three and four week feeding regimes. Based on this literature and several other reports (Balcazar *et al*., 2007; Newaj-Fyzul *et al*., 2007; Kumar *et al.,* 2008; Kim *et al*., 2012) a feeding regime of 14 days was selected in this study and the results showed that feeding *Cyprinus carpio* with *Bacillus* NL110 had significantly improved the survival rate of fishes against *Aeromonas hydrophila* infection.

In the present study, *Cyprinus carpio* fed on feed supplemented with *Bacillus* NL110 for two weeks exhibited survival rate of 63%, whereas survival was only 11% in fish not treated with the probiotics, when challenged with *A. hydrophila.* Similar to the present study, Kumar *et al.* (2008)

administered *Bacillus subtilis* to *Labeo rohita* for two weeks and challenged intraperitoneally with *A. hydrophila*. The results suggested that *B. subtilis* can enhance immune responses in the fishes and improve the survival rate. Improvements in immunity and disease resistance in *Cyprinus carpio* koi against *A. veronii* on oral administration of *Bacillus coagulans* and chitosan oligosaccharides, single or combined was reported by Lin *et al*. (2012). In another study, Vendrell *et al*. (2008) showed that feeding rainbow trout with *Lactobacillus rhamnosus* supplemented feed reduced fish mortality caused by *A. salmonicida.* Balcazar *et al*. (2007) and Newaj-Fyzul *et al*. (2007) observed high survival rates in Rainbow trouts (*Oncorhynchus mykiss*) administered with probiotics compared to control fishes when challenged with *Aeromonas.* The control of *A. hydrophila* infection in fishes on feeding with probiotics and immunostimulants is reported by several authors (Selvaraj *et al*., 2005; Abdel-Tawwab *et al*., 2008; Aly *et al*., 2008; Abd El-rhman *et al*., 2009; Maqsood *et al*., 2010; Gopalakannan and Arul 2011). Protection against *A. salmonicida* infection is also reported (Austin *et al*., 1995; Robertson *et al*., 2000; Nikoskelainen *et al*., 2001; Irianto and Austin, 2002; Brunt *et al*., 2007).

Significant increase in the serum bactericidal activity in the probiotic treated group in comparison to the control is observed in the present study. The survival rate of bacteria after incubation with serum was found to be 39% in the case of control fish, whereas it was 18.8% in the case of probiotic fed fishes. This is similar to the observation of Maqsood *et al*. (2010), who reported increase in the serum bactericidal activity in common carp challenged with *A. hydrophila* on feeding with the immunostimulant chitosan. Reneshwary *et al*. (2011) reported that the increase in resistance against *A. hydrophila* in fish fed with *Bacillus thuringiensis* can be explained on the basis

of increased bactericidal activity of serum. Kumar *et al*. (2008) indicated significant increase in the serum bactericidal activity of *Labeo rohita* fed with *Bacillus subtilis*. Similar observations are also made by Nayak *et al*. (2007) and Aly *et al*. (2008). Taoka *et al*. (2006) reported that the probiotic treatment enhanced the non-specific immune parameters such as plasma bactericidal activity, resulting in the improvement of fish resistance against *Edwardsiella tarda* infection. The increased serum bactericidal activity in *Achyranthes* treated *Labeo rohita* infected with *A. hydrophila* indicated that various humoral factors involved in innate and/or adaptive immunities are elevated in the serum to protect the host effectively from infection (Rao *et al.,* 2006). Misra *et al.* (2006 b) mentioned that, serum bactericidal activity in the fish injected with different dosages of β-glucan was significantly higher than in controls.

Probiotics also modulate various immunohaematological parameters in fishes; interact with the immune cells such as mononuclear phagocytic cells (monocytes, macrophages) and polymorphonuclear leucocytes (neutrophils) and natural killer cells to enhance innate immune responses. Probiotics, in both *in vitro* and *in vivo* conditions, actively stimulate the proliferation of B lymphocytes in fish (Nayak, 2010).

Histopathological analysis of gill tissues of *Cyprinus carpio* in the present study revealed severe architectural loss of gill filaments in the control fishes compared to the probiotic treated fishes. In the liver, vacuolization and pyknosis of nuclei was more prominent in the fishes fed with control diet. In the intestine, necrosis of enterocytes was more prominent in the fishes fed with control diet. The histopathological studies by Nouh *et al*. (2009) revealed no remarkable pathological alterations in the gill arch and lamellae of Nile tilapia

infected with *A. hydrophila*. The liver revealed congestion and vacuolation of some hepatic cells with nuclear pyknosis. In the intestine mucinous degeneration in the epithelial lining was observed. Focal epithelial desquamation was also seen in the intestine.

When looking at probiotics for an aquatic usage, it is important to consider certain influencing factors that are fundamentally different from terrestrial based probiotics. Aquatic animals have much closer relationship with their external environment. Therefore there are many differences between terrestrial and aquatic animals in the level of interaction between the intestinal microbiota and the surrounding environment. The larval forms of most fish and shellfish are released in the external environment at an early ontogenetic stage. These larvae are highly exposed to gastrointestinal microbiota-associated disorders, because they start feeding even when the digestive tract is not yet fully developed, and the immune system is still incomplete (Lara-flores, 2011). Thus, probiotic treatments are particularly desirable during the larval stages (Gatesoupe, 1999).

Improved water quality has especially been associated with probiotics. Research also shows that the use of commercial probiotics in aquaculture ponds can reduce concentrations of nitrogen and phosphorus and increase the production yield (Wang *et al*., 2008). The multipronged attack by probiotics is more efficient than just relying on antibiotics to disrupt cell wall structures and/or poison metabolic pathways in pathogenic agents.

Chapter 8

Summary

The thesis deals with the prevalence and distribution of motile aeromonads in selected ornamental fishes. It also gives an account of the production of extracellular virulence factors and the antibiogram of the different species of motile aeromonads isolated. The growth characteristics and virulence potential of a representative strain of *Aeromonas hydrophila* is also studied. The nucleotide sequencing of the strain was carried out and sequences deposited in Genbank. Survival and immune response of *Cyprinus carpio* under different stress conditions and on probiotic treatment with *Bacillus* NL110, when challenged with *A. hydrophila* is also dealt within this thesis.

Salient findings of the study are summarized as follows

- Motile aeromonads were isolated from 74% of *Poecilia sphenops* and 68% of *Poecilia reticulata* samples collected from ornamental fish farm. They were isolated from different body parts of fish samples such as body surface, gill and intestine. In *P. sphenops,* body surface showed greater prevalence (39.06%), while in *P. reticulata,* intestine showed greater prevalence (35.8%). One

hundred and forty five isolates were characterized to species level. *Aeromonas sobria* was the predominant species (29%) and *A. jandaei*, the second dominant sp. (20%) in both fish 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. *A. sobria* was the predominant species in water samples also (34.61%) and *A. trota* was the second dominant species (23.71%).
- In samples from retail aquaria, motile aeromonads were isolated from 84% of *Poecilia sphenops* and 80% of *Poecilia reticulata* samples collected. In both *P. sphenops* and *P. reticulata* samples, gills showed greater prevalence; 40.86 and 36.58% respectively. One hundred and seventy five isolates from the samples were characterized to species level. *Aeromonas sobria* was the predominant species (40.57%) and *A. caviae,* the second dominant sp. (31.43%).
- Motile aeromonads were isolated from 84% of the water samples collected. One hundred and eighty two isolates from the samples were characterized to species level. *A. sobria* was the predominant species (34.80%) and *A. caviae,* the second dominant species in water samples also.
- The k-dominance curve of ornamental fish samples from farm and retail aquaria showed that samples from retail aquaria exhibited less diversity and high dominance contributed mainly by *A. sobria* and *A. caviae*.

- The k-dominance curve of water samples showed that samples from farm exhibited high dominance, contributed mainly by *A. sobria* and *A. trota.*
- There was an extensive production of extracellular virulence factors such as gelatinase (100%), DNase (100%), caseinase (>80%) and lipase (>90%) among the motile aeromonads.
- β-haemolytic activity was also wide spread among the isolates, with all the isolates of *A. hydrophila* and *A. sobria* haemolytic. *A. caviae* was found to be relatively less haemolytic.
- No significant variation ($p>0.05$) was observed in the production of extracellular virulence factors in motile aeromonads from farm and retail aquarium vendors.
- All the isolates from fish and water samples (both farm and retail aquaria) exhibited resistance to amoxicillin. All the isolates (both fish and water samples) from fish farm were sensitive to ceftazidime, chloramphenicol, ciprofloxacin and gentamicin.
- All the isolates (both fish and water samples) from retail aquaria were sensitive to chloramphenicol, ciprofloxacin and gentamicin. All the isolates in water samples were also sensitive to nitrofurantoin.
- Number of motile aeromonads exhibiting resistance to nalidixic acid, streptomycin and trimethoprim was found to be significantly higher ($p<0.05$) in fish samples from retail aquaria, compared to that of farm.

- In water samples, significantly higher (p<0.05) resistance towards streptomycin was exhibited by motile aeromonads in samples from retail aquaria.
- Isolates exhibiting resistance to 7 or more antibiotics (MAR index values greater than 0.43) was seen only in fish samples from commercial aquarium vendors. The MAR index ranged from 0.21 to 0.43 in isolates from farm and 0.21 to 0.57 in isolates from aquarium vendors.
- In water samples, resistance to 6 or more antibiotics (MAR index value greater than 0.36) was seen only in isolates from commercial aquarium vendors. The MAR index value ranged from 0.21 to 0.36 in samples from farm and 0.21 to 0.57 in isolates from aquarium vendors.
- The species *A. hydrophila* exhibited a wide production of virulence factors and MAR indexing revealed that this species is resistant to higher number of antibiotics; therefore a representative strain of *A. hydrophila* identified by biochemical methods was used for further studies and has been confirmed to be the same by 16S rRNA gene sequencing (GenBank Accession No. JX987236).
- The strain was found to grow over a wide range of temperature (10-45°C), pH (5-10) and salinity (0-4.5%).
- Virulence genes-*aerolysin* (416bp), *haemolysin* (597 bp) and *cytotoxin* (232 bp) were detected in the isolate and LD_{50} of the isolate was found to be $10^{6.1}$cfu/ml.

- Physico-chemical analysis of water samples from farm and retail aquarium vendors revealed higher concentrations of ammonia and nitrite in samples from commercial aquarium vendors compared to the farm. The concentration of these factors in retail aquaria were also found to be above the optimal range for ornamental fish culture. In retail aquaria fishes are often stocked at higher densities sometimes with several species in the same tank. Therefore these factors were considered as stress factors in further studies.

- LC_{50} (96-h) of un-ionized ammonia (UIA)-N for *Cyprinus carpio* was found to be 2.05 mg/L. Survival (%) of *C. carpio* reduced from 75% (control) to 58.33 and 45.83 in the 1/10 and 1/5 of the LC_{50} values respectively of UIA-N when challenged with *A. hydrophila*.

- LC_{50} (96-h) of nitrite-N for *C. carpio* was found to be 46.85 mg/L. Survival (%) of *C. carpio* reduced from 79% (control) to 66.66 and 50 in the 1/10 and 1/5 of the LC_{50} values respectively of nitrite-N, when challenged with *A. hydrophila*.

- With increasing stocking densities of *C. carpio,* 12, 18, 24 and 30 fishes in 10 litre water, survival (%) reduced from 72.2 to 64.81, 51.38 and 38.88% respectively when challenged with *A. hydrophila*.

- Significant reduction ($p<0.05$) in immune response of *C. carpio* was also observed in comparison to the control and with increasing concentration of UIA-N, nitrite-N and stocking densities when challenged with *A. hydrophila,* as evidenced from pathogen clearance efficiency of blood and serum bactericidal efficiency.

- Total leucocyte count was found to increase significantly with increasing concentration of stress factors and severity of infection. A significant reduction in the lymphocyte count (%) and increase in the neutrophil count (%) was also observed.
- Histopathological analysis revealed increasing severity of pathological changes in the gill tissues with increasing dose of stress factors.
- Of the three stress factors studied, un-ionized ammonia (UIA)-N was found to be more harmful to *C. carpio* in terms of survival, immune response and histopathological changes.
- On probiotic treatment with *Bacillus* NL110, significant increase in survival ($p<0.05$) was observed in *C. carpio* challenged with *A. hydrophila* compared to the control. Relative percentage survival was found to be 59%.
- Bactericidal efficiency of serum was also significantly ($p<0.05$) higher in the probiotic treated group compared to the control.
- Histopathological analysis of gill, liver and intestinal tissues of *C. carpio* revealed protective effect of probiotic *Bacillus* NL110 on challenge with *A. hydrophila.*

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www.cnn.com

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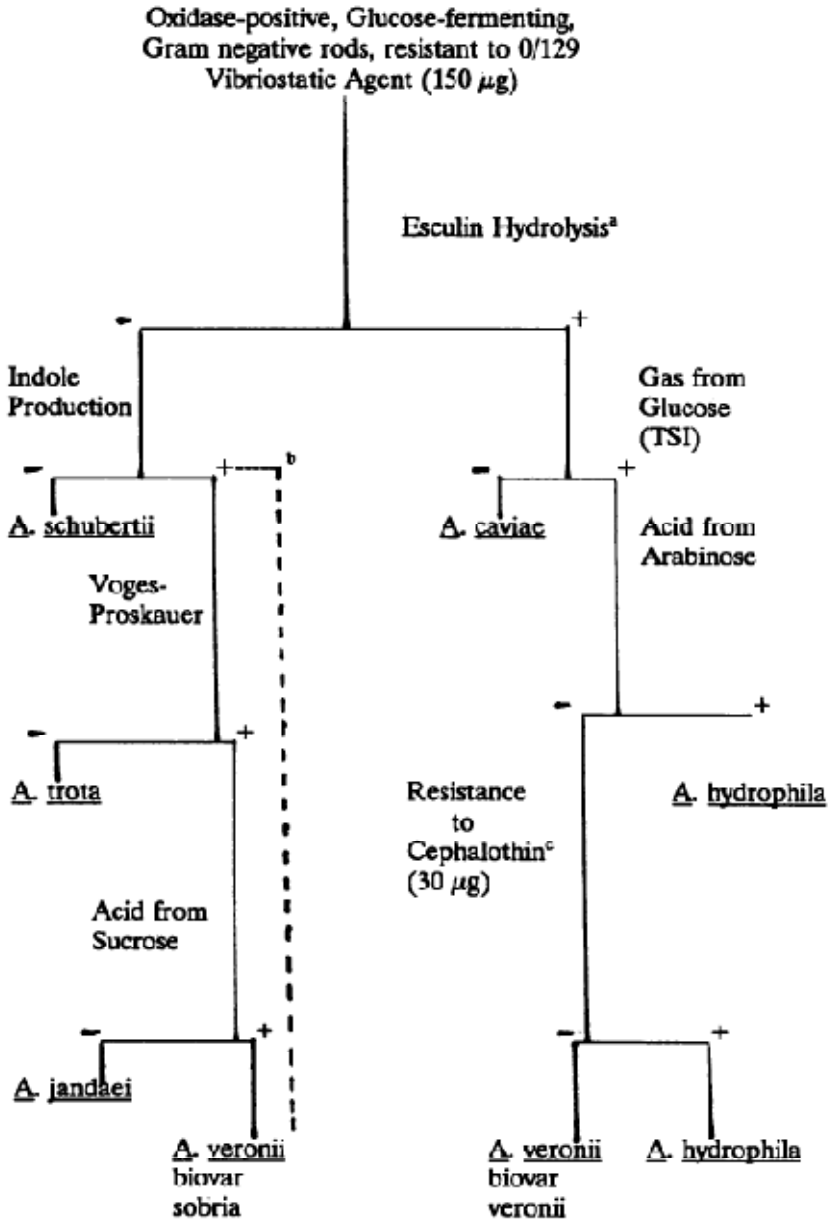
Appendix 1

AERO KEY II



Appendix 2

GENBANK SUBMISSION

Aeromonas hydrophila strain NJ87 16S ribosomal RNA gene, partial sequence

GenBank: JX987236.1

FASTA Graphics

```
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VERSION     JX987236.1  GI:429327095
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  ORGANISM  Aeromonas hydrophila
            Bacteria; Proteobacteria; Gammaproteobacteria; Aeromonadales;
            Aeromonadaceae; Aeromonas.
REFERENCE   1  (bases 1 to 1342)
  AUTHORS   John,N. and Mohamed Hatha,A.
  TITLE     Molecular characterization of a hydrophila isolated from fish
            samples
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1342)
  AUTHORS   John,N. and Mohamed Hatha,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-OCT-2012) Dept. of Marine Biology, Microbiology &
            Biochemistry, Cochin University of Science and Technology, Fine
            Arts Avenue, Cochin, Kerala 682016, India
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                     /strain="NJ87"
                     /host="ornamental fish"
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      121 ggggaccttc gggccttgcg cgattggata tgcccaggtg ggattagcta gttggtgagg
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```

Appendix 3.1

Table 3.1(a). Anova for the effect of temperature on the growth of *Aeromonas hydrophila* NJ 87

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.221	8	.653	1420.725	.000
Within Groups	.008	18	.000		
Total	5.229	26			

Table 3.1(b) Homogeneous Subsets for the effect of temperature on the growth of *Aeromonas hydrophila* NJ 87

Tukey HSD

temperature	N	Subset for alpha = .05				
		1	2	3	4	5
5.00	3	.0107				
10.00	3	.0660				
45.00	3		.1307			
40.00	3			.2743		
15.00	3			.3120		
20.00	3				.9250	
35.00	3				.9663	
25.00	3					1.0790
30.00	3					1.1100
Sig.		.096	1.000	.472	.359	.699

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 3.2

Table 3.2(a) Anova for the effect of pH on the growth of *Aeromonas hydrophila* NJ 87

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	78.471	9	8.719	3199.744	.000
Within Groups	.054	20	.003		
Total	78.525	29			

Table 3.2(b) Homogeneous Subsets for the effect of pH on the growth of *Aeromonas hydrophila* NJ 87

Tukey HSD

pH	N	Subset for alpha = .05					
		1	2	3	4	5	6
2.00	3	.0693					
3.00	3	.0870					
4.00	3	.0970					
11.00	3	.1047					
10.00	3		.8147				
9.00	3			2.4633			
5.00	3				2.7853		
8.00	3					3.2913	
6.00	3						3.9480
7.00	3						4.0890
Sig.		.997	1.000	1.000	1.000	1.000	.079

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 3.3

Table 3.3(a) Anova for the effect of Salinity on the growth of *Aeromonas hydrophila* NJ 87

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	86.491	12	7.208	851.535	.000
Within Groups	.220	26	.008		
Total	86.711	38			

Table 3.3(b) Homogeneous Subsets for the effect of salinity on the growth of *Aeromonas hydrophila* NJ 87

Tukey HSD

salinity	N	Subset for alpha = .05					
		1	2	3	4	5	6
6.00	3	.0077					
5.50	3	.0813					
5.00	3	.0833					
4.50	3		.8627				
4.00	3		1.1277				
3.50	3			1.5960			
.00	3				2.7730		
3.00	3				2.7913		
2.50	3					3.3757	
2.00	3						3.6610
1.50	3						3.7670
.50	3						3.8010
1.00	3						3.8110
Sig.		.998	.063	1.000	1.000	1.000	.726

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 4

Arrangement of data used in computation of LD_{50} titre by the method of Reed-Muench

Dilution of Inoculum	Died	Survived	Accumulated values			Percentage mortality
			Mortality			
			Died (a)	Survived (b)	Ratio (a/a+b)	A/(a+b) x 100
Original inoculum	8	0	21	0	21/21	100
10^{-1}	8	0	13	0	13/13	100
10^{-2}	4	4	5	4	5/9	55
10^{-3}	1	7	1	11	1/12	8
10^{-4}	0	8	0	19	0/19	0

LD_{50} of the isolate was found to be $10^{6.1}$cfu/ml and it indicated the virulent nature of the isolate.

Appendix 5.1

Table 5.1(a) Anova for the effect ammonia on the percent survival of *Cyprinus carpio*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1284.722	2	642.361	18.500	.003
Within Groups	208.333	6	34.722		
Total	1493.056	8			

Table 5.1(b) Homogeneous Subsets for the effect of ammonia on the percent survival of *C. carpio*

Duncan

concentration	N	Subset for alpha = .05		
		1	2	3
.41	3	45.8333		
.21	3		58.3333	
.00	3			75.0000
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Appendix 5.2

Table 5.2 (a) Anova for the effect ammonia on the survival rate of bacteria

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	639.049	2	319.525	98.569	.000
Within Groups	19.450	6	3.242		
Total	658.499	8			

Table 5.2 (b) Homogeneous Subsets for the effect of ammonia on the survival rate of bacteria

Duncan

concentration	N	Subset for alpha = .05		
		1	2	3
.00	3	47.3108		
.21	3		56.4258	
.41	3			67.9062
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 5.3

Table 5.3 (a) Anova for the effect ammonia on the pathogen clearance efficiency of blood

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	81312.395	2	40656.197	133.653	.000
Within Groups	1825.147	6	304.191		
Total	83137.542	8			

Table 5.3 (b) Homogeneous Subsets for the effect ammonia on the pathogen clearance efficiency of blood

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
.41	3	-230.8257		
.21	3		-89.0342	
.00	3			.0000
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 5.4

Table 5.4 (a) Anova for the effect ammonia on the total leucocyte count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	401.722	2	200.861	185.410	.000
Within Groups	6.500	6	1.083		
Total	408.222	8			

Table 5.4 (b) Homogeneous Subsets for the effect ammonia on the total leucocyte count

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
.00	3	31.8333		
.21	3		43.3333	
.41	3			47.6667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Table 5.4 (c) Anova for the effect ammonia on the lymphocytes

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	150.889	2	75.444	75.444	.000
Within Groups	6.000	6	1.000		
Total	156.889	8			

Table 5.4 (d) Homogeneous Subsets for the effect ammonia on the lymphocytes

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
.41	3	79.6667		
.21	3		85.3333	
.00	3			89.6667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Table 5.4 (e) Anova for the effect ammonia on the neutrophils

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	150.889	2	75.444	75.444	.000
Within Groups	6.000	6	1.000		
Total	156.889	8			

Table 5.4 (f) Homogeneous Subsets for the effect ammonia on the neutrophils

Duncan

concentration	N	Subset for alpha = .05		
		1	2	3
.00	3	10.3333		
.21	3		14.6667	
.41	3			20.3333
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Appendix 6.1

Table 6.1 (a) Anova for the effect of nitrite on the percent survival of *C. carpio*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1284.722	2	642.361	18.500	.003
Within Groups	208.333	6	34.722		
Total	1493.056	8			

Table 6.1(b) Homogeneous Subsets for the effect of nitrite on the percent survival of *C. carpio*

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
9.37	3	50.0000		
4.68	3		66.6667	
.00	3			79.1667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Appendix 6.2

Table 6.2 (a) Anova for the effect of nitrite on the survival rate of bacteria

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1453.220	2	726.610	174.037	.000
Within Groups	25.050	6	4.175		
Total	1478.270	8			

Table 6.2 (b) Homogeneous Subsets for the effect of nitrite on the survival rate of bacteria

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
.00	3	46.8555		
4.68	3		61.8917	
9.37	3			77.9754
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Appendix 6.3

Table 6.3 (a) Anova for the effect of nitrite on the pathogen clearance efficiency of blood

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	188926.669	2	94463.334	358.033	.000
Within Groups	1583.038	6	263.840		
Total	190509.707	8			

Table 6.3 (b) Homogeneous Subsets for the effect of nitrite on the pathogen clearance efficiency of blood

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
9.37	3	-354.1172		
4.68	3		-156.7096	
.00	3			.0000
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 6.4

Table 6.4 (a) Anova for the effect of nitrite on the total leucocyte count

Total leucocyte count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	338.722	2	169.361	156.333	.000
Within Groups	6.500	6	1.083		
Total	345.222	8			

Table 6.4 (b) Homogeneous Subsets for the effect of nitrite on the total leucocyte count

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
.00	3	31.1667		
4.68	3		41.3333	
9.37	3			45.8333
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table 6.4 (c) Anova for the effect of nitrite on the lymphocytes

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	124.222	2	62.111	62.111	.000
Within Groups	6.000	6	1.000		
Total	130.222	8			

Table 6.4 (d) Homogeneous Subsets for the effect of nitrite on the lymphocytes

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
9.37	3	81.3333		
4.68	3		84.6667	
.00	3			90.3333
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table 6.4 (e) Anova for the effect of nitrite on the neutrophils

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	124.222	2	62.111	62.111	.000
Within Groups	6.000	6	1.000		
Total	130.222	8			

Table 6.4 (f) Homogeneous Subsets for the effect of nitrite on the neutrophils

Duncan

Concentration	N	Subset for alpha = .05		
		1	2	3
.00	3	9.6667		
4.68	3		15.3333	
9.37	3			18.6667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 7.1

Table 7.1 (a) Anova for the effect of stocking density on the percent survival of *C. carpio*

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1956.501	3	652.167	60.770	.000
Within Groups	85.854	8	10.732		
Total	2042.355	11			

Table 7.1(b) Homogeneous Subsets for the effect of stocking density on the percent survival of *C. carpio*

Duncan

Stocking density	N	Subset for alpha = .05			
		1	2	3	4
30.00	3	38.8889			
24.00	3		51.3889		
18.00	3			64.8148	
12.00	3				72.2222
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 7.2

Table 7.2 (a) Anova for the effect of stocking density on the survival rate of bacteria

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2152.047	3	717.349	321.514	.000
Within Groups	17.849	8	2.231		
Total	2169.897	11			

Table 7.2 (b) Homogeneous Subsets for the effect of stocking density on the survival rate of bacteria

Duncan

Stocking density	N	Subset for alpha = .05			
		1	2	3	4
12.00	3	45.6158			
18.00	3		62.4246		
24.00	3			71.8055	
30.00	3				82.0152
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 7.3

Table 7.3 (a) Anova for the effect of stocking density on the pathogen clearance efficiency of blood

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	261668.539	3	87222.846	102.848	.000
Within Groups	6784.613	8	848.077		
Total	268453.152	11			

Table 7.3 (b) Homogeneous Subsets for the effect of stocking density on the pathogen clearance efficiency of blood

Duncan

Stocking density	N	Subset for alpha = .05			
		1	2	3	4
30.00	3	-370.2089			
24.00	3		-297.9401		
18.00	3			-106.0540	
12.00	3				.0000
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix 7.4

Table 7.4 (a) Anova for the effect of stocking density on the total leucocyte count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1707.167	3	569.056	569.056	.000
Within Groups	8.000	8	1.000		
Total	1715.167	11			

Table 7.4 (b) Homogeneous Subsets for the effect of stocking density on the total leucocyte count

Duncan

Stocking density	N	Subset for alpha = .05			
		1	2	3	4
12.00	3	28.6667			
18.00	3		41.6667		
24.00	3			51.5000	
30.00	3				60.8333
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table 7.4 (c) Anova for the effect of stocking density on the lymphocytes

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	219.000	3	73.000	67.385	.000
Within Groups	8.667	8	1.083		
Total	227.667	11			

Table 7.4 (d) Homogeneous Subsets for the effect of stocking density on the lymphocytes

Duncan

Stocking density	N	Subset for alpha = .05			
		1	2	3	4
30.00	3	77.6667			
24.00	3		82.6667		
18.00	3			85.6667	
12.00	3				89.3333
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Table 7.4 (e) Anova for the effect of stocking density on the neutrophils

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	219.000	3	73.000	67.385	.000
Within Groups	8.667	8	1.083		
Total	227.667	11			

Table 7.4 (f) Homogeneous Subsets for the effect of stocking density on the neutrophils

Duncan

Stocking density	N	Subset for alpha = .05			
		1	2	3	4
12.00	3	10.6667			
18.00	3		14.3333		
24.00	3			17.3333	
30.00	3				22.3333
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

Appendix 8.1

Table 8.1 (a) Student's t test for the effect of probiotic treatment on percent survival of *C. carpio*

Group Statistics

	feed	N	Mean	Std. Deviation	Std. Error Mean
percentsurvival	1.00	3	11.1111	4.81125	2.77778
	2.00	3	63.8889	4.81125	2.77778

Table 8.1 (b) Levene's test for equality of variances

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
percentsurvival	Equal variances assumed	.000	1.000	-13.435	4	.000	-52.77778	3.92837	-63.68468	-41.87087
	Equal variances not assumed			-13.435	4.000	.000	-52.77778	3.92837	-63.68468	-41.87087

Appendix 8.2

Table 8.2 (a) Student's t test for the effect of probiotic treatment on the serum bactericidal efficiency of *C. carpio*

Group Statistics

	feed	N	Mean	Std. Deviation	Std. Error Mean
survivalrateofbacteria	1.00	3	39.1186	2.69181	1.55411
	2.00	3	18.8128	1.47636	.85238

Table 8.2 (b)
Levene's test for equality of variances

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
survivalrateofbacteria	Equal variances assumed	2.315	.203	11.456	4	.000	20.30583	1.77252	15.38453	25.22712
	Equal variances not assumed			11.456	3.103	.001	20.30583	1.77252	14.76974	25.84191

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List of Publications

- Shubhankar Ghosh, Einar RingØ, A. Deborah Gnana Selvam, Mujeeb Rahiman K. M., Naveen Sathyan, **Nifty John**, A. A. M.Hatha (2014). Gut associated Lactic acid bacteria isolated from the estuarine fish *Mugil cephalus*: Molecular diversity and antibacterial activities against pathogens. *International Journal of Aquaculture*, 4(01), 1-11. doi:10.5376/ija.2014.04.0001
- **Nifty John** and A.M. Hatha (2013) Distribution, extracellular virulence factors and drug resistance of motile aeromonads in fresh water ornamental fishes and associated carriage water. *International Journal of Aquaculture*, 3 (17), 92-100.
- **Nifty John** and A.A.M. Hatha (2012) Prevalence, distribution and multiple antibiotic resistance of motile *Aeromonas* in fresh water ornamental fishes. *Indian Journal of Fisheries*, 59 (2), 161-164.

Proceedings of National/International Conferences

- **Nifty John** and A.A.M., Hatha (2013) Extracellular virulence factors and antibiotic resistance of motile aeromonads in fresh water ornamental fish culture system. Proceedings of 25th Kerala Science congress (1), 139-140.
- Subhankar Ghosh, A Deborah Gnana Selvam, K M Mujeeb Rahiman, **Nifty John** and AAM Hatha (2011) Lactic acid bacteria (LAB) from estuarine fish as a probable probiont in different Aquaculture operations. Proceedings of National symposium on Emerging trends in Biotechnology PP.16-24. ISBN no. 978-93-80095-30-1
- Shubhankar Ghosh, **Nifty John** and A.A.M., Hatha (2010) Lactic acid bacteria from the gut of estuarine fish *Mugil cephalus*- a prospective probiont to treat microbial diseases in aquaculture. Proceedings of International conference on the green Path to sustainability prospects and challenges. PP: 251-253. ISBN No: 9788190726993

Paper presentations

- **Nifty John** and A.A.M., Hatha (2013) Extracellular virulence factors and antibiotic resistance of motile aeromonads in fresh water ornamental fish culture system. Paper presented in 25th Kerala Science congress, Thiruvananthapuram.
- **Nifty John**, Shubhankar Ghosh and A.A.M., Hatha (2010) Prevalence, distribution and antibiogram of motile *Aeromonas* in fresh water ornamental fishes, Paper presented in 1st Kerala Women's science congress, Eranakulam.

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Research Report Open Access

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

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

Keywords Ornamental fish; Motile aeromonads; Antibiotic resistance; Diseases

Introduction

Keeping colourful and fancy fishes known as ornamental fishes, aquarium fishes, or live jewels is one of the oldest and most popular hobbies in the world. The growing interest in aquarium fishes has resulted in a steady increase in aquarium fish trade globally. The global scope and scale of the ornamental fish trade and growing popularity of pet fish are strong indicators of the myriad economic and social benefits the pet industry provides. Culture of ornamental fish in the backyards of households requires very little space, skill and time, and has the potential to improve the economic condition of the household. 'Earning a regular income, unlike seasonal work in agriculture, provides further motivation (Shaleesha and Stanley, 2000). Aquaculture is an emerging industrial sector which requires continued research with scientific and technical developments, and innovation. Over one billion ornamental fish comprising more than 4000 freshwater and 1400 marine species are traded internationally each year, making it one of the most important components of the global fish trade. Freshwater species make up 90% of this trade as they are the most popular and widely kept aquarium pets worldwide (Krishnakumar et al., 2009). The trade in ornamental (pet) fish is greater than 1 billion animals per year globally. More than 45 million fish per year are imported into the United Kingdom (UK) alone from a wide range of countries, in particular those in South East Asia (Wittington and Chong, 2007).

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

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

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

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

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

The widespread distribution of these bacteria in the aquatic environment and the stress induced by intensive culture practices predisposes fish to infections. A number of putative virulence factors that may play an important role in the development of disease, have been described in several species of the genus *Aeromonas,* which includes haemolysins, cytotoxins, enterotoxins, proteases, lipases, DNases and adhesins (Sen and Rodgers, 2004). The pathogenesis of *Aeromonas* infections is multifactorial, and no single virulence factor can be unequivocally pinpointed as responsible for particular symptoms or disease stages. Pathological conditions attributed to members of the motile aeromonad complex may include dermal ulceration, tail or fin rot, ocular ulceration, hemorrhagic septicemia and scale protrusion disease. Outbreaks of motile aeromonad septicaemia can reach epidemic proportions in farmed aquatic animals, with high rates of mortality (Liles et al., 2011). The disease problems are treated with antibiotics, but the emergence of antibiotic-resistant bacteria imposes a substantial burden on aquaculture. Antibiotic resistance is particularly relevant in pathogenic *Aeromonas* species in which, besides the classical resistance to β-lactam antibiotics, multiple-resistance has been frequently identified (Goñi-Urriza et al., 2000; Vila et al., 2002; 2003). These bacteria can receive and transfer antibiotic resistance genes to other Gram negative bacteria (Marchandin et al., 2003).

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

1 Results

1.1 Distribution of *Aeromonas* Species

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

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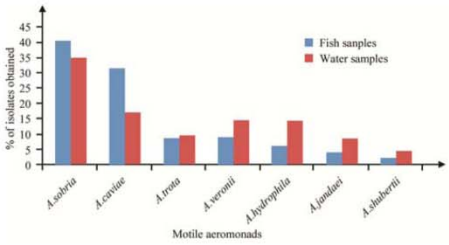

Figure 1 Distribution of *Aeromonas* spp. in fish and water samples

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

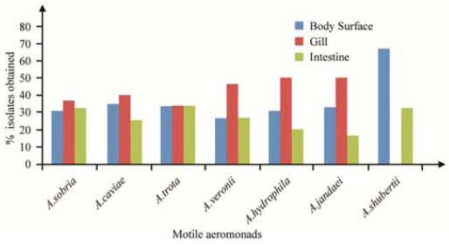

Figure 2 Distribution of *Aeromonas* spp. in the body surface, gill and intestine of fishes

1.2 Extracellular Virulence Factors of *Aeromonas* Species

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

1.3 Antimicrobial Resistance

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

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

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

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

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

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of *A. sobria* and 8% of *A. veronii* from water samples were sensitive to sulfafurazole. Resistance exhibited to other antibiotics by the isolates from fish and water samples are given in Table 3 and Table 4 respectively.

Table 3 Antibiotic resistance of motile aeromonads from fish samples

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

Table 4 Antibiotic resistance of motile aeromonads isolates from water samples

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

2 Discussion

The prevalence and distribution of bacteria belonging to the genus *Aeromonas* in aquatic environments is of great public health concern since *Aeromonas* spp. can cause infections and epizootics in a variety of animals. Motile aeromonads have been recognized as occasional pathogens of cultured fishes and the most common bacteria in freshwater habitats throughout the world. It is the etiological agent for motile aeromonad septicaemia (MAS) in fish. *Aeromonas* has also been frequently isolated from the lesions of epizootic ulcerative syndrome (EUS) fishes (Torres et al., 1990; Subasinghe et al., 1990; Roberts et al., 1990). This disease is a serious threat to the freshwater fish production of Southeast Asian countries. It causes mass mortalities in both cultured and wild fish species every year. The prevalence of different species of *Aeromonas* is likely to vary with geographical locations. In the present study *Aeromonas sobria* was the predominant species isolated from fish samples

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

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

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

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

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

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

There have been conflicting reports on the susceptibility of motile Aeromonads to commonly used antibiotic agents. High level of resistance against amoxycillin, and carbenecillin and 100% sensitivity to chloramphenicol, ciprofloxacin and gentamicin was noticed in the isolates from both fish and water samples. Motile aeromonads isolated from fish and water samples, in India exhibiting 100% sensitivity to, ciprofloxacin and gentamicin and high level of susceptibility to chloramphenicol is reported by Rathore et al. (2005). Complete sensitivity to chloramphenicol, ciprofloxacin and gentamicin by the isolates is reported by Penders and Stobberingh (2008). All the isolates from water samples in the present study were susceptible to nitrofurantoin and only a 3.63% of *A. caviae* isolates from fish samples exhibited resistance to the antibiotic. High level of susceptibility to nitrofurantoin by the isolates is also reported by Castro-Escarpulli et al. (2003) but in contrast to our finding he has reported 100% sensitivity to nalidixic acid and more than 50% resistance to gentamicin and varying degrees of resistance to chloramphenicol and ciprofloxacin.

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

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

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

3 Materials and Methods

3.1 Collection of Samples

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

3.1.1 Isolation and identification of *Aeromonas*

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

The water samples were serially diluted and bacterial isolation was done by spread plate method using Starch Ampicillin Agar plates. The plates were incubated at 37 ℃ for 18-24 h and then flooded with approximately 5 ml of Lugol's iodine solution and amylase positive yellow to honey colored colonies were isolated. The isolated cultures were then purified by repeated streaking on nutrient agar plates. Those strains that were gram negative bacilli, motile, oxidase and catalase positive, glucose fermenting, nitrate reducing, urease negative and which do not grow in 6% NaCl were further tested for arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase activity. Additional tests include acid production from mannitol, sucrose, arabinose, Voges-Proskauer reaction, hydrolysis of esculin and indole production. The isolates were identified to the species level according to Aerokey II (Carnahan et al., 1991).

3.2 Study of Extracellular Virulence Factors

3.2.1 Production of gelatinase

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

3.2.2 Production of caseinase

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

3.2.3 Production of lipase

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

3.2.4 Production of DNase

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

3.2.5 β– Haemolytic assay

Haemolytic activity was determined using blood agar

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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℃ for 24 h.

3.3 Antimicrobial Susceptibility Test

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

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

Authors' contributions

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

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Prevalence, distribution and drug resistance of motile aeromonads in freshwater ornamental fishes

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ABSTRACT

The objective of the present study was to assess the prevalence of various motile aeromonads in freshwater ornamental fishes and to elucidate the antibiogram and beta hemolytic activity among the isolates. A total of 120 ornamental fish samples were screened and analyzed for *Aeromonas* spp. Motile aeromonads were isolated from 37.5% of the ornamental fish samples. Various species of motile aeromonads such as *Aeromonas caviae, Aeromonas hydrophila, Aeromonas jandaei, Aeromonas schubertii, Aeromonas sobria, Aeromonas trota* and *Aeromonas veronii* were detected. All the isolates were sensitive to ceftazidime, chloramphenicol, ciprofloxacin and gentamicin. Multiple antibiotic resistance was observed in 58% of the isolates.

Keywords: Diseases, Multiple antibiotic resistance, Motile aeromonads, Ornamental fish

Introduction

The ever-increasing demand for aquarium fishes gradually paved the way towards global trade of ornamental fishes. Substantial international trade takes place within the ornamental fish industry today. Diseases in aquaculture systems are recognized as an important limiting factor to production and trade. Bacterial organisms may be the primary cause of disease, or they may be secondary invaders. The majority of bacterial fish pathogens are natural inhabitants of the aquatic environment. Infections caused by motile members of the genus *Aeromonas,* are amongst the most common and troublesome diseases diagnosed in cultured warm water fishes and have been referred to by various names, including motile aeromonad septicemia (MAS), motile aeromonad infection (MAI), hemorrhagic septicemia, red pest, and red sore. *Aeromonas* bacteria causing these infections are called aeromonads (Camus *et al*., 1998). The widespread distribution of these bacteria in the aquatic environment and the stress induced by intensive culture practices predisposes fish to infections.

The disease problems are treated with antibiotics, the indiscriminate use of which can result in the rapid spread of multi-drug resistant pathogens across the system. This fact along with the financial crisis caused by the mortality of ornamental fishes makes the study of different geographical isolates of aeromonads important. Kerala has immense potential for developing the ornamental fish industry and the development of the ornamental fish industry would bring in economic growth to the state. There are a number of issues that continue to challenge the ornamental fish industry, of which disease is a major risk factor (Citarasu *et al*., 2011). Hence the present study was taken up to investigate the level of prevalence of motile aeromonads among freshwater ornamental fishes collected from aquarists in Cochin area.

Materials and methods

Collection of samples

Ornamental fish samples were collected from aquarium vendors in Cochin over a period of two years and transported to the laboratory in sterile polythene bags. The samples were then analysed for aeromonads within 4 h of collection.

Isolation and identification of Aeromonas spp.

Bacteria were isolated from different parts of the body (body surface, gill and intestine) of the fishes. The body surface and the gill surface of the fishes were swabbed with separate, sterile cotton swabs. Using a pair of scissors, an incision was made near the vent of the fish facilitating the swabbing of intestine. The swabs were then transferred to alkaline peptone water (APW pH 8.4) which was used as the enrichment medium. After incubation at 37 °C for 18 h, a loopful of APW was streaked on starch ampicillin agar (Ampicillin 10 mg l^{-1}) for selective isolation and incubated at 37 °C for 18-24 h (Palumbo *et al.,* 1985). The plates were 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. Those strains that were gram negative bacilli, motile, oxidase and catalase positive, glucose fermenting, nitrate reducing, urease negative and which do not grow at 6% NaCl were further tested for arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase activity. Additional tests performed include acid production from mannitol, sucrose, arabinose, Voges –Proskauer reaction, hydrolysis of esculin and indole production. The isolates were identified to the species level using Aerokey II (Carnahan *et al*., 1991).

Antimicrobial susceptibility test

Susceptibility to antimicrobial agents was performed for the identified *Aeromonas* spp. by the disc diffusion method (Bauer *et al*., 1966). MAR index (Multiple Antibiotic Resistance) values were calculated for all the isolates according to Krumperman (1983).

Beta haemolytic assay

Haemolytic activity was determined using blood agar medium containing 5% human blood. Pure cultures of bacterial isolates were spot inoculated onto blood agar plates and â haemolytic activity was recorded as clear zones around the colony after incubation at 37 °C for 24 h.

Results and discussion

Motile *Aeromonas* were isolated from 37.5% of the ornamental fish samples. The 45 aeromonad isolates obtained from the samples were identified to different phenospecies level such as *Aeromonas caviae, A. hydrophila, A. jandaei, A. schubertii, A. sobria, A. trota* and *A. veronii* (Table1). All the isolates were gram negative, motile, oxidase positive, catalase positive, amylase positive, nitrate reductase positive and glucose fermenting with acid production.

Species of the genus *Aeromonas* have long been recognized as pathogens of fish, amphibians and reptiles (Austin and Adams, 1996). Citarasu *et al.* (2011) reports *A. hydrophila* as the dominant microbiota among other pathogens during the massive outbreak of bacterial infection in the ornamental fish hatchery. The prevalence of different species of *Aeromonas* is likely to vary with geographical locations. In terms of prevalence and abundance, the most predominant species was found to be *A. caviae* during the present study, followed by the other species. Araujo *et al*. (1990) reports *A. caviae* to be the predominant species in water. In a study from a coastal bay in Japan, Nakano *et al*. (1990) found that *A. caviae* was the most abundant of the mesophilic aeromonads, while *A. sobria* predominated in brackishwaters. Hatha *et al*. (2005) reported *A. hydrophila* to be the predominant species in the intestine of farm raised freshwater fish followed by *A. caviae* and *A. sobria.*

Table 1. Prevalence of motile *Aeromonas* spp. among freshwater ornamental fishes

Ornamental fish species	Common name	% of samples positive for *Aeromonas* spp.				% of samples positive for motile *Aeromonas* spp.
		A. caviae	*A. sobria*	*A. hydrophila*	Other *Aeromons* spp.	
Carassius auratus	Gold fish	11.1	5.5	2.8	11.1	30.5
Cyprinus carpio	Carp	5	10	0	15	30
Poecilia sphenops	White molly	0	0	0	0	0
Datnioides polota	Tiger fish	20	0	10	10	40
Poecilia sphenops	Black molly	12.5	12.5	0	50	75
Poecilia latipinna	Balloon molly	0	0	0	60	60
Xiphophorus helleri	Red sword tail	40	0	20	40	100
Gymnocorymbus ternetzi	Widow tetra	50	0	0	0	50
Puntius conchonius	Rosy barb	33.3	0	0	33.3	66.6
Carassius auratus	Black moor	0	0	0	33.3	33.3
Pterygoplichthys pardalis	Sucker	50	0	50	0	100
Labeo gonius	White shark	0	0	0	0	50
Astronotus ocellatus	Oscar	0	0	0	100	100
Poecilia reticulata	Guppy	0	0	0	0	0
Poecilia latipinna	Red molly	0	0	0	0	0
Colisa chuna	Honey gourami	0	0	0	0	0

There have been conflicting reports on the susceptibility of motile aeromonads to commonly used antibiotic agents. The aeromonads have been regarded as universally resistant to penicillins (penicillin, ampicillin, carbenecillin and ticarcillin) for quite a long time. For this reason, ampicillin has been generally incorporated in the culture media for selective isolation of the aeromonads. All the strains isolated exhibited sensitivity to ceftazidime, chloramphenicol, ciprofloxacin and gentamicin. Only 2.2% of the isolates exhibited resistance to Trimethoprim. All the isolates of *A. hydrophila* and *A. jandaei* were resistant to cephalothin and 50% of *A. caviae* were also resistant to this antibiotic. Except *A. jandaei,* members of all the species exhibited some degree of resistance to streptomycin. Resistance to nalidixic acid was exhibited by all the species, although in variable degree. Overall prevalence of antibiotic resistance among motile aeromonads is given in Table 2. Our results are in tune with the previous observations with regard to trimethoprim, gentamicin, chloramphenicol and ciprofloxacin (Kudinha *et al*., 2004; Penders and Stobberingh, 2008). Though all the isolates were sensitive to gentamicin, ciprofloxacin and chloramphenicol as previously observed (Vasaikar *et al.*, 2002; Kashedikar and Chhabra, 2010), nearly 50% isolates were resistant to nalidixic acid. Resistance to nalidixic acid was considerably high when compared to previous results (Huddleston *et al*., 2006), indicating the use of this antibiotic by the farmers in the region.

Table 2. Overall prevalence of antibiotic resistance among motile aeromonads from freshwater ornamental fishes

Antibiotic	% of strains showing resistance (total n=45)
Amoxycillin (30 mcg)	100
Carbenicillin (100 mcg)	33.3
Cefpodoxime (10 mcg)	8.8
Ceftazidime (30 mcg)	0
Cephalothin (30 mcg)	37.7
Chloramphenicol (10 mcg)	0
Ciprofloxacin (5 mcg)	0
Gentamicin (10 mcg)	0
Nalidixic acid (30 mcg)	48.8
Streptomycin (10 mcg)	44.4
Tetracycline (30 mcg)	15.5
Trimethoprim (5 mcg)	2.2

Further concern is the increasing incidence of multidrug resistance amongst *Aeromonas* spp. that has been observed worldwide (Ottoviani, 2006; Matyar *et al.,* 2007). Fifty eight percentage of *Aeromonas* isolates in this study exhibited Multiple Antibiotic Resistance. Twenty five percent of *A. hydrophila* isolates were resistant to six of the antibiotics tested. The MAR index values of the aeromonad isolates ranged from 0.08 to 0.5. The MAR index values of the isolates and the resistance profile of antibiotics is given in Table 3. High MAR index exhibited by more than half of the isolates in the present study points to the indiscriminate use of antibiotics in aquaculture systems. High incidence of multiple antibiotic resistance has been reported in aquatic environments and fish isolates by Hatha *et al.* (2005). More than 50% of the strains of *A. hydrophila* and *A. caviae* isolated from freshwater fishes of Kolkata, India exhibited MAR (Abraham, 2011).

Table 3. MAR index and resistance profile of motile aeromonads from freshwater ornamental fishes

MAR index	Resistance profile	Number of isolates showing similar pattern
0.25	AX,NA,S	5
0.25	AX,CB,S	2
0.25	AX,CB,CH	1
0.25	AX,S,TR	1
0.25	AX,CEP,CH	2
0.25	AX, CH,NA	1
0.33	AX, CB,NA,S	2
0.33	AX, CB,NA, CH	1
0.33	AX, CB,CH,S	1
0.33	AX, CB,CH,T	1
0.33	AX,CEP,CH, T	1
0.33	AX,CB,CH,NA	1
0.33	AX,CH,NA,T	1
0.33	AX, CH,NA,S	1
0.33	AX, CH,S,T	1
0.33	AX,CB,S,T	1
0.33	AX, CB,CH, T	1
0.42	AX,CB,CEP,CH,NA	1
0.5	AX,CB,CH,NA,S,T	1

AX– Amoxycillin, NA – Nalidixic acid, S – Streptomycin, CB – Carbenicillin, CEP – Cefpodoxime, CH – Cephalothin, T – Tetracycline

Production of haemolysin has been reported as a virulence factor in motile aeromonads. Varying levels of beta hemolytic activity among *A. hydrophila* has been reported (Thayumanavan *et al*., 2003; Hatha *et al*., 2005). While all the *A. hydrophila* strains in this study were beta hemolytic, 85.7% of *A. caviae* and 80% of *A. sobria* exhibited β haemolytic activity.

Ornamental fish trade is growing at rapid pace in Kerala, with many small scale investors involved in breeding, rearing and marketing of popular ornamental fishes such as those analyzed in the present study. Very often these fishes are kept in crowded conditions at retail vendor level which is highly stressful to the fishes making them more prone to diseases by opportunistic and obligate

pathogens. The results of the present study reveal the prevalence of motile aeromonads at varying levels in these fishes. The multidrug resistant nature of the motile aeromonads make it difficult for successful disease control through antibiotics. The fish keepers at farm/ retail vendor level should be aware of the stress reduction procedures so as to prevent massive death due to multiple drug resistant fish pathogens.

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