A Study on Molecular and Virulence Characterization of *Aeromonas* species Isolated from Different Sources

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(2009-287-D)

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Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir

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MUDASIR ALI RATHER
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Thesis

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Doctor of Philosophy in Veterinary Sciences
(Veterinary Public Health)

2013

Dedicated to:

My Mother

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Division of Veterinary Public Health, Shuhama Campus,
Srinagar-190 006

CERTIFICATE – I

This is to certify that the thesis entitled, **"A Study on Molecular and Virulence Characterization of *Aeromonas* species Isolated from Different Sources"** submitted in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Veterinary Sciences (Veterinary Public Health)**, to the **Faculty of Postgraduate Studies, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Mr. Mudasir Ali Rather (Regd. No. 2009-287-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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CERTIFICATE – II

We, the members of the Advisory Committee of **Mr. Mudasir Ali Rather (Regd. No. 2009-287-D)**, a candidate for the degree of **Doctor of Philosophy in Veterinary Sciences (Veterinary Public Health)** have gone through the manuscript of the thesis entitled, **"A Study on Molecular and Virulence Characterization of *Aeromonas* species Isolated from Different Sources"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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CERTIFICATE – III

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ABSTRACT

The present study describes the prevalence and antibiogram of Aeromonads in foods of animal origin, water and human diarrheal samples. The molecular epidemiology and *in-vitro* and *in-vivo* virulence characterization of the isolated *Aeromonas* spp. was also studied. Of the 609 samples comprising, water (182), fish (172), chicken (57), mutton (83), beef (31) and human diarrheic stools (83) screened for the presence of *Aeromonas* spp., 155 (25.45%) were positive. The prevalence of *Aeromonas* spp. in water, fish, chicken, mutton, beef and human diarrheal samples was 26.37, 39.30, 14.03, 22.89, 19.35 and 7.23%, respectively. The isolates of *Aeromonas* from all sources were identified as *A. hydrophila* (23.87%), *A. caviae* (20.64%), *A. veronii* bv *sobria* (18.06%), *A. salmonicida* (8.39%), *A. popoffii* (6.45%), *A. trota* (5.16%), *A. schubertii* (3.87%), *A. jandaei* (2.58%) and *A. allosaccharophila* (2.58%). Thirteen isolates (8.39%) could be identified to genus level only. A polymerase chain reaction (PCR) used to confirm the *Aeromonas* genus detected 153 of 155 isolates identified as *Aeromonas* phenotypically. The speciation was studied by restriction fragment length polymorphism of PCR amplified segment

of 16S rRNA gene and the expected fragments were generated. Analysis of restriction enzyme digestion pattern indicated that the fragments generated could be used to identify the species of *Aeromonas* barring few exceptions. Molecular characterization of virulence factors encompassed a multiplex PCR for detection of three enterotoxin genes (*act*, *alt* and *ast*) of *Aeromonas* isolates. The *act*, *alt* and *ast* genes were detected in 104 (67.09%), 98 (63.22%) and 11 (7.06%) of isolated Aeromonads, respectively. The gene patterns identified included; *act/alt* (47.09%), *act* (13.54%), *alt* (10.96%), *act/alt/ast* (5.16%) and *act/ast* (1.93%). All the isolates were haemolytic on rabbit blood agar plates while haemolytic activity was shown by 92.25% of the isolates on sheep blood agar. The *in-vivo* virulence characterization of isolates was studied by vascular permeability reaction in rabbit skin which were marked after 12 hr of intradermal injection and ranged between 7 and 19.6 mm in diameter. Strains having enterotoxin genes produced higher VPR zones compared with those not carrying the gene. The molecular epidemiology of isolates was attempted at two different levels by random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR). The RAPD-PCR did not yield enough amplification products; therefore, the genetic variability could not be studied by RAPD-PCR. The ERIC-PCR was however able to type all the isolates and most of the isolates from water were identical to one other and also to the isolates from fish. The two *A. caviae* isolates from human diarrheal samples were identical to *A. caviae* recovered from water, indicating water as the most important source of *Aeromonas* infection. A high degree of resistance was observed against ampicillin (97.42%), ampicillin/cloxacillin (89.03%), polymyxin B (87.74%), amoxycillin (72.90%), roxithromycin (70.32%), erythromycin (61.29%) and streptomycin (54.84%). The isolates were highly sensitive to enrofoxacin (96.77%), ciprofloxacin (94.19%), ofloxacin (92.9%), ceftraixone (90.32%), norfloxacin (85.81%), tetracycline (85.81%), gentamicin (84.52%) and doxycycline (83.23%). The sequence analysis of enterotoxin genes revealed a high similarity of the genes with sequences available in the GenBank.

Key Words: *Aeromonas*, Prevalence, Water, Fish, Raw Meats, Human Diarrhea, RFLP, RAPD-PCR, ERIC-PCR, Multiplex PCR, Sequencing, Haemolysis, Vasopermeabilty Reaction.

Signature of Student

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Place: Shuhama (Mudasir Ali Rather)

Dated: _____

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LIST OF ABBREVIATIONS / ACRONYMS AND SYMBOLS

°C	degree Celsius
dNTPs	Deoxyribonucleotide Triphosphates
DNA	Deoxyribonucleic Acid
ERIC	Enterobacterial Repetitive Intergenic Consensus Sequence
g	Gram
hr	Hour
l	Litre
m	Meter
ml	Milliliter
mm	Millimeter
min	Minute
Mg	Milligrams
MgCl2	Magnesium Chloride
%	Percent
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism

Pmoles	Pico moles
spp.	Species
µg	Microgram
µl	Microlitre
µM	Micromolar
WHO	World Health Organization

Chapter-1

INTRODUCTION

Our health, both physical and mental faculties depends on the food we eat, as is the old proverb “we are what we eat". Access to good quality and safe food has been man's main endeavor from the earliest days of human existence. Food safety implies absence or acceptable and safe levels of pathogens, adulterants, naturally occurring toxins or other substances in food, that make it injurious to human health. Of the many foodborne micro-organisms, one of the emerging pathogens is *Aeromonas*. The factors driving more and more researchers' interest in aeromonal biology include demonstration of the role of *Aeromonas* spp. in a number of human infections, their documentation as potential food and waterborne pathogens and their role as emerging animal pathogens.

The genus *Aeromonas* has gone through turbulent taxonomic changes due to lack of congruity between phenotypic and genotypic characteristics of species. Currently Aeromonads are placed in the family *Aeromonadaceae* under order *Aeromonadales* of the *Gamma Proteobacteria* class (Garitty *et al.*, 2001). The genus comprises a collection of facultatively anaerobic, oxidase and catalase positive, Gram negative, short rods that are resistant to the vibriostatic agent-2,4-diamino-6,7 diisopropylpteridine (Holt *et al.*, 1994). For accurate classification of the genus, multiple methods are employed. Empirically, based on biochemical characterization, 14 phenospecies have been described (Martin-Carnahan and Joseph, 2005) and by extensive DNA-DNA hybridization studies, 17 recognized DNA-homology groups (HGs) are identified (Yanez *et al*., 2003). Molecular techniques have also been used for the identification and speciation of *Aeromonas* spp. based on the restriction fragment length polymorphism (RFLP) profiles of 16S rRNA genes (Martinez-

Murcia *et al*., 1992a; 1992b; Borrell *et al*., 1997). This method is reportedly cheaper and more discriminatory than other methods of classification (Borrell *et al*., 1997).

The *Aeromonas* spp. are autochthonous to the aquatic ecosystems, including ground water, surface waters, marine waters, non chlorinated drinking water, chlorinated drinking water and bottled mineral water (Holmes *et al*., 1996, Abulhamd, 2010). The Safe Drinking Water Act of United States Environmental Protection Agency (EPA) has made it mandatory to screen drinking water for the presence of *Aeromonas hydrophilla*. The organism has been listed on the first and second Contaminant Candidate List (CCL-1 and CCL-2) of potential waterborne pathogens (USEPA, 1998; 2005). The *Aeromonas* spp. are also common contaminants in diverse variety of foods namely fish, sea-foods, raw and cooked meats, poultry, milk and milk products, eggs and vegetables (Palumbo *et al*., 1989; Kirov *et al*., 1990; Hanninen and Siitonen, 1995; Singh *et al*., 1997; Isonhood and Drake, 2002; Sharma and Kumar, 2011; Dallal *et al*., 2012).

Some species of *Aeromonas* are opportunistic pathogens of man, causing a wide variety of extra-intestinal infections and occasionally gastrointestinal diseases as well. The first human case of acute fulminating myositis caused by *Aeromonas* was described in 1954 followed by its isolation from human faeces in 1961 (Lautrop, 1961; Janda and Abott, 1998). The first well documented case linking *Aeromonas* with diarrhea was reported in 1964 (Rosner, 1964). Since then, Aeromonads have been implicated in a number of human ailments like gastroenteritis, septicaemia, wound infections, meningitis, endocarditis, peritonitis, osteomyelitis, septic arthritis, ocular infections, urinary and gynaecologic infections, respiratory tract infections, fatal pneumonia and also in several cases of haemolytic uraemic syndrome (HUS) (Khardori and Fainstein 1988; Krovacek *et al*., 1993; Janda and Abott, 1998; Taher *et al*., 2000; Vasaikar *et al*., 2002; Rodriguez *et al*., 2005).

Aeromonads occur as normal micro-flora of many aquatic and terrestrial animals and are proven diseases causing agents in various cold and warm blooded animals including fishes, amphibia, reptiles, birds and domestic animals (Janda and Duffey, 1988; Cahill, 1990a; Joseph and Carnahan, 1994). Reports of isolation of *Aeromonas* spp. from animal infections include abortion in cows, buffaloes and mares; mastitis in dairy cow, fatal septicaemia in dogs and puppies, ocular infections in birds, gastroenteritis in cats and dogs and septicaemia in grey seal etc. (Wohlgemuth *et al*., 1972; Das and Paranjape, 1987; Misra *et al*., 1993; Andre-fontaine *et al*., 1995; Krovacek *et al*., 1998; Paniagua *et al*., 1998; Kumar *et al*., 2001; Boynukara *et al*., 2002; Malik *et al*., 2002; Zdovc *et al*., 2004).

The versatility of *Aeromonas* spp. in inflicting diseases in man and animals is attributable to an array of virulence factors possessed by the members of the genus (Janda and Abbott, 2010). These virulence factors are broadly divided into structural components, extracellular factors and cell-associated features (Janda, 1991). Number of these virulence factors has been characterized, cloned and sequenced (Abdullah *et al*., 2003). However, their actual role in *Aeromonas* infections is yet to be elucidated completely (Pemberton *et al*., 1997). The degree of virulence may be related to distribution of virulence genes in *Aeromonas* spp. (Albert *et al*., 2000). A number of studies reported the distribution of virulence genes among isolates from different regions of the world (Abdullah *et al*., 2003, Kingombe *et al*., 2010), which have contributed to the understanding of the virulence properties as well as epidemiological aspects of Aeromonads.

The antibiotic era changed the profile of infectious diseases and human demography but the bacterial resistance against these was soon realized and continues with the introduction of new antibiotics every now and then. Among the antibiotic resistant bacteria, the enteropathogens were found to be highly resistant to

antibiotics used for empiric treatment of diarrhea (Neelam *et al*., 2004). The global concern over the indiscriminate use of antimicrobials/antibiotics in animal husbandry and aquaculture and consequent emergence of drug-resistant microbes is increasing every day (WHO, 2000). Aeromonads being emerging pathogens with their increasing refusal to surrender to commonly used antibiotics, thus compound the problem, be it treating human patients or the infected animals.

The virulence markers of *Aeromonas* (aerolysin, haemolysin, enterotoxins etc.) are useful to distinguish between potentially pathogenic and non-pathogenic strains. About 6.5% of diarrheal cases in the southern parts of India have been attributed to *Aeromonas* (Komathi *et al*., 1998), warranting an urgent need for the information on its role as a pathogen in other parts of the country, including J&K, where, the information on the incidence and the phenotypic and genotypic characteristics of Aeromonads is scanty. The fact that the dissemination of the organism may take place via myriad mechanisms necessitates getting into a state of preparedness so that in the event of any *Aeromonas* outbreak, remedial measures are employed at an appropriate time. Based on the above discourse, the present study was proposed with the following objectives:

1. To study the prevalence of *Aeromonas* spp. in foods of animal origin, water and human diarrheal samples.
2. To carry out virulence characterization and study the distribution of virulence genes among isolated Aeromonads.
3. To study genetic diversity and molecular epidemiology of isolates, if any.
4. To study drug resistance pattern of the isolates.

Chapter-2

REVIEW OF LITERATURE

The order *Aeromonadales* comprises a single family of bacteria, *Aeromonadaceae*, where the genus *Aeromonas* resides and *Aeromonas hydrophila* is designated as the type species (Martin-Carnahan and Joseph, 2005). Aeromonads are natural inhabitants of aquatic environment causing diseases in aquatic animals and as an opportunistic pathogen in man.

2.1 Taxonomy

The history of *Aeromonas* is more than a century old, a bacterium resembling motile *Aeromonas* was first isolated from water in 1890 by Zimmerman and was named *Bacillus punctatus* (Zimmerman, 1890). Subsequently, Sanarelli (1891), isolated the organism from frog and linked it to red leg disease of frogs and named it as *Bacillus hydrophilus fuscus*. The current generic name, *Aeromonas* was proposed by Kluyver and Van Niel (1936).

Until late 1970's, Aeromonads were divided into two groups based upon physiological properties and the host range. Motile Aeromonads that grew at 35-37º C and recognized to cause human infections were called *A. hydrophila*. The non-motile Aeromonads that grew at 22-28º C and infected fish were called *A. salmonicida* (Popoff and Veron, 1976). Moreover, owing to similarities it shares with *Vibrio* spp. and *Plesiomonas* spp. the genus was initially placed under the family *Vibrionaceae* (Holt *et al*., 1994). In the course of time, the differences between these three genera and phylogenetic depth within the genus *Aeromonas* assumed more prominence prompting the proposal of a separate family *Aeromonadaceae* (Colwell *et al*., 1986). Following extensive DNA-DNA hybridization studies and 16S-rDNA sequence analysis (Popoff *et al*., 1981; Hickman-Brenner *et al*., 1987; 1988; Kuijper

et al., 1989a; b) the proposal of separate family, *Aeromonadaceae* (Colwell *et al*., 1986) was accepted and the genus *Aeromonas* now resides under the family *Aeromonadaceae*, order *Aeromonadales* of the class gamma proteobacteria (Garitty *et al*., 2001).

The genera of the family *Aeromonadaceae* now include *Aeromonas*, *Oceanimonas*, *Oceanisphaera* and *Tolumonas* (Martin-Carnahan and Joseph, 2005). During the recent past, new species and subspecies of *Aeromonas* have been identified and more will undoubtedly be described. *A. hydrophila* ssp. *dhakensis* (subsp. nov.) (Huys *et al*., 2002a), *A. hydrophila* ssp. *ranae* (subsp. nov.) (Huys *et al*., 2003), *A. culicicola* (sp. nov.) (Pidiyar *et al*., 2002), *A. simiae* (sp. nov.) (Harf-Monteil *et al*., 2004a), and *A. molluscorum* (sp. nov.) (Minana-Galbis *et al*., 2004a) have been proposed as new species and subspecies.

The genus *Aeromonas* consists of straight, coccobacillary to bacillary gram-negative bacteria with rounded ends (Martin-Carnahan and Joseph, 2005). They occur singly, in pairs, and rarely as short chains. Motile strains produce a single polar flagellum, though peritrichous or lateral flagella may be formed on solid media by some species. *Aeromonas* spp. are facultatively anaerobic, catalase positive, oxidase positive, chemoorganotrophic bacteria that exhibit both oxidative and fermentative metabolism on carbohydrates (Carnahan *et al*., 1988).

2.2 Isolation and Identification of *Aeromonas* spp.

Isolation and identification of *Aeromonas* spp. from environmental and clinical samples provides a challenge and multiple methods are required for isolation, identification and speciation of the genus.

2.2.1 Isolation

The optimally temperature range for growth of *Aeromonas* spp. is between

22-35ºC, but growth can occur in a temperature range from 0-45º for some species (Mateos *et al*., 1993). These tolerate a pH range from 4.5 to 9.0, but optimum pH is 5.5 to 9.0 and optimum sodium chloride concentration ranges from 0 to 4% (Isonhood and Drake, 2002). Several culture media for isolation and enumeration of Aeromonads from water samples were compared by Kersters *et al*. (1996a) who concluded that ampicillin dextrin agar (ADA) produced the best results. Water and food samples are usually processed using a multiple tube technique such as the multiple tube fermentation test (MFT) used for coliform analyses but instead alkaline peptone water is employed. Non-turbid water samples may be processed using the membrane filtration (MF) method, where samples are filtered through a membrane with a pore size of 0.45 Fm and the membrane is either placed into APW enrichment broth or placed onto the surface of selective and differential culture media such as ampicillin dextrin agar (ADA) and incubated aerobically at 35º C for 24-48 hr (Kersters *et al*., 1996a).

Maria *et al*. (1988) studied eleven different media for suitability in the selective isolation of *A. hydrophila*. Among these five (inositol-brilliant green-bile salts agar, bile salts-brilliant green agar, Rimler-Shotts agar, xylose-sodium deoxycholate-citrate agar and dextrin-fuchsin-sulfite agar) allowed the growth of several microorganisms. Six media (Modified Rimler-Shotts agar, DNase-toluidine blue-ampicillin agar, Pril-xylose-ampicillin agar, MacConkey-trehalose agar, mA agar and Starch-Bile salt agar) were selected for evaluation as recovery selective media on the basis of their efficiency in isolating of *A. hydrophila* from natural water samples.

Several enrichment and selective media have been evaluated for isolation of aeromomads from foods (Moyer, 1996; Palumbo, 1996). Starch ampicillin agar (SAA) and bile salts inositol brilliant green agar (BIBG) with prior enrichment in

alkaline peptone water (APW) or tryptose broth containing ampicillin (TSB-30) (ampicillin 30 mg/L) are recommended (Moyer, 1996; Palumbo, 1996). After evaluating five different selective media for their effectiveness in the primary isolation of *Aeromonas* spp. from human patients with acute diarrhea and from healthy domestic animals. Sheep blood agar with 30 mg of ampicillin per liter (ASBA30) was found to yield a significantly higher percentage of positive specimens compared to other media (Mishra *et al*., 1987).

2.2.2 Identification

At the phenotypic level, to distinguish each species, it is necessary to integrate the conventional biochemical schemes (Abbott *et al*., 2003) with additional key tests described recently (Beaz-Hidalgo *et al*., 2010).

Phenotypic identification of Aeromonads to the generic level requires biochemical tests which include oxidase, catalase and nitrate production, growth in nutrient broth containing 0, 3% and 6% NaCl, resistance to O/129 (2, 4-diamino 6,7diisopropylpteridine), production of acid from D-trehalose, inability to utilize malonate as the sole carbon source, fermentation of inositol, D-xylose and dulcitol (Ottaviani *et al*., 2006). For species identification a battery of biochemical tests (Abbott *et al*., 2003), including lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase, esculin hydrolysis, lipase and indole production, methyl red, Voges-Proskauer; utilization of citrate, DL-lactate, urocanic acid, acid and gas production from D-glucose, acid from L-arabinose, cellobiose, lactose, glycerol, mannitol, rhamnose, salicin, sorbitol, pyrazinamidase, glucose 1-phosphate and sucrose, hydrolysis of urea, susceptibility to ampicillin (10 μg) and ONPG production (Abbott *et al*., 2003; Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010). For better discrimination of the strains at the phenospecies level, recently proposed supplementary biochemical tests (Beaz-Hidalgo *et al*., 2010) i.e.

growth at 42 °C, acid from melibiose, hydrolysis of starch and gelatin, H_2S from cysteine, ß-haemolysis on sheep blood agar and motility in semisolid agar at 37 °C are used. Using the Abbott *et al*. (2003) scheme, the isolate can be allocated to a species-group and each group includes 3 different phenotypic species. The *A. hydrophila* group includes *A. hydrophila, A. bestiarum* and *A. salmonicida* (both motile and non-motile species), the *A. caviae* group includes *A. caviae, A. media*, and *A. eucrenophila*, and the *A. veronii/sobria* group includes *A. veronii* biovar *sobria, A. jandaei, A. schubertii* and *A. trota*.

The strains presumptively identified as *A. hydrophila* can further be tested to discriminate this species from the new species *A. aquariorum* and *A. piscicola* by interpreting the results of the utilization of L-lactate according to Beaz-Hidalgo *et al*. (2009) and Figueras *et al*. (2009). All strains presumptively identified as *A. caviae* are tested by interpreting the results of lysine decarboxylase, gas from D-glucose and H_2S from cysteine (Figueras *et al*., 2009) to discriminate this species from the recently described new species *A. aquariorum*. Indole production and acid from glycerol are evaluated according to Demarta *et al*. (2008) for all strains presumptively identified as *A. eucrenophila* and *A. encheleia* (discrimination from the new recently described species *A. tecta*). Indole, starch hydrolysis and lysine decarboxylase can be used to discriminate all strains presumptively allocated to the *A. caviae* group from the new species *A. molluscorum* and *A. bivalvium* (Miñana-Galbis *et al*., 2004; 2007). The group of strains presumptively allocated in the *A. veronii/sobria* group are examined for ß-haemolysis (Harf-Monteil *et al*., 2004b), to exclude the new species *A. simiae*. If an isolate could not be definitely placed in one species group or in one of the phenospecies it is considered as “atypical” *Aeromonas* (Janda and Abbott, 2010).

At molecular level, a combination of 16S rRNA-RFLP analysis (Borrell *et al*., 1997; Figueras *et al*., 2000) and sequencing of the housekeeping genes *rpoD* and *gyrB* (Soler *et al*., 2004) is necessary for correct identification of *Aeromonas* strains to the genospecies level. All typical Aeromonads, presumptively identified to the species level or only allocated into a species group should be molecularly confirmed by a multiplex-PCR assay (Janda and Abbott, 2010).

2.3 Distribution of *Aeromonas* spp.

Aeromonads are essentially ubiquitous in the microbial biosphere. They can be isolated from virtually every environmental niche where bacterial ecosystems exist. These include aquatic habitats, fish, foods, domesticated pets, invertebrate species, birds, ticks and insects and natural soils, although extensive investigations on the latter subject are lacking (Janda and Abbott, 2010). The vast panorama of environmental sources from which Aeromonads can be encountered lends itself readily to constant exposure and interactions between the genus *Aeromonas* and humans.

2.3.1 In Water

The natural reservoir for *Aeromonas* spp. is the aquatic environment (Hazen *et al*., 1978; Schubert, 1991; Razzolini *et al*., 2008), wherein they remain associated with various phyto and zooplankton (Chowdhury *et al*., 1990; Dumontet *et al*., 1996). Their detection has been reported from almost all types of waters namely fresh, saline and brackish water (Hazen *et al*., 1978; Sack *et al*., 1987; Araujo *et al*., 1989), chlorinated and unchlorinated drinking water (Lechevallier *et al*., 1982; Burke *et al*., 1984b; Millership and Chattopadhyay, 1985; Kersters *et al*., 1995), ground water (Holmes *et al*., 1996), treated and untreated sewage water (Schubert, 1991), seawater (Echeverria *et al*., 1983; Brandi *et al*., 1996). activated sludge (Neilson, 1978), irrigation water (Miranda and Castillo, 1998), clean river water (Pathak *et al*., 1988),

domestic and industrial waste water (Schubert, 1967; Slade *et al*., 1986), abattoir waste water (Rossi *et al*., 2000), fish farm hatchery tanks (Rhodes *et al*., 2000), untreated hospital effluents (Rhodes *et al*., 2000), reservoirs and drinking fountains (Razzolini *et al*., 2008) and even bottled mineral water (Tsai and Yu, 1997).

Aeromonas spp. can be found in various concentrations in drinking water. Although the significance of Aeromonads in such samples is unknown in relationship to reputed cases of gastroenteritis, the chronic exposure of immunocompromised persons to *Aeromonas* via contaminated waters could potentially lead to an invasive disease, such as septicemia (Leclerc *et al*., 2002). The World Health Organization lists *Aeromonas* in the third edition of *Guidelines for Drinking-Water Quality* (USEPA, 2005). In 1998, the Environmental Protection Agency listed *A. hydrophila* on its "Drinking Water Contaminant Candidate List" (USEPA, 2005). Through the Consumer Confidence Report Rule, public water systems are required to report unregulated contaminants, such as *Aeromonas*, when detected (Edberg *et al*., 2007). *Aeromonas* has also been reported to enter a viable but non-culturable state, similar to other pathogens, including *Vibrio*. The significance of these observations is presently unknown (Mary *et al*., 2002).

Aeromonads have been isolated from chlorinated drinking water supplies around the world (Hazen *et al*., 1978; Burke *et al*., 1984a; van der Kooij, 1988; Fernandez *et al*., 2000; Figueras *et al*., 2005). They occur in distribution system biofilms where they may be protected from disinfection and hence grow in distribution systems (Havelaar *et al*., 1990; Holmes and Nicolls, 1995; van der Kooij *et al*., 1995). Aeromonads have been found at a frequency of 1 to 27% of drinking water supplies examined (Rusin *et al*., 1997). *Aeromonas* spp. have been recovered from mineral water with isolation rates as high as 35.5% and cell concentrations of

greater than 3 $\log_{10}$ CFU/mL (Quevedo-Sarmiento *et al*., 1986; Slade *et al*., 1986; Gonzales *et al*., 1987; Manaia *et al*., 1990; Havelaar *et al*., 1990; Tsai and Yu, 1997; Warburton *et al*., 1998; Croci *et al*., 2001).

The Aeromonads have been recovered from different water sources of India like surface waters of Kolkata (Bhowmik *et al*., 2009), domestic waters of Chennai (Alavandi *et al*., 2001), tap water, well water and water from reservoirs of Manipal (Hande *et al.,* 2009), Narmada river water (Sharma *et al*., 2005) and Sulfur spring in Orissa (Patra *et al*., 2007).

2.3.2 In Foods

A Variety of foods have been shown to harbor Aeromonads (Yadav and Verma, 1998). Ibrahim and MacRae (1991) reported Aeromonads from beef (60%), lamb (58%), pork (74%), and milk (26%). Aeromonads have been recovered from fish and fresh salads (Walker and Brooks, 1993), from lamb (Sierra *et al*., 1995), oysters (Tsai and Chen, 1996), cheese and raw milk (Melas *et al*., 1999) and fish and seafood (Hanninen *et al*., 1997). Szabo *et al*. (2000) isolated *Aeromonas* spp. from 70 of 120 samples of lettuce in Australia. Aeromonads are found in ready to eat foods, including seafoods (Hudson and Avery, 1994; Tsai and Chen, 1996). Glunder and Siegmann (1993) isolated Aeromonads from birds and poultry eggs. Abbey and Etang (1988) reported Aeromonads in 28-29% of snails in Nigeria. Neyts *et al*. (2000b) cultured mesophilic *Aeromonas* spp. in 26% of vegetable samples, 70% of meat and poultry samples and 72% of fish and shrimp samples at numbers from < 2 $\log_{10}$ CFU/g to > 5 $\log_{10}$ CFU/g.

In India Aeromonads have been isolated from fish (13.13%), poultry meat (11.5%), pork (9.85%) and chevon (2.5%) from North-East India, with *A. hydrophila* being the predominant species (92.10%) followed by *A. sobria* (5.26%) and *A. caviae* (2.63%) (Sharma and Kumar, 2011). In Nagpur, *Aeromonas* spp. have

been recovered from commercially available chevon (26%) and freshwater fish (18%). The predominant isolate was *A. hydrophila* (12%), followed by *A. caviae* (8%) and *A. sobria* (6%) (Kolhe *et al*., 2005). Agarwal *et al*. (2000) isolated Aeromonads from fish (22%), snails (6.25%) and quail eggs (18%), buffalo milk (2.8%) and goat meat (8.9%) in India.

Aeromonads have also been detected in soil (Gray and Stickler, 1989), air (Fannin *et al*., 1985) and on the surfaces as biofilms (Bal'a *et al*., 1998).

2.3.3 In Man and Animals

Humans carry *Aeromonas* spp. in their gastrointestinal tract both in the presence and absence of disease. The rates of fecal carriage in asymptomatic persons in developed countries range from 0% to 4% (Millership *et al*., 1983; Agger *et al*., 1985; Svenungsson *et al*., 2000), while the isolation rate from persons with diarrheal illness ranges from 0.8 to 7.4% (Agger *et al*., 1985; Moyer, 1987; Albert *et al*., 2000). In Southeast Asia, asymptomatic carriage rates as high as 27.5% and recovery rates from patients with diarrhea as high as 34% have been reported (Pazzaglia *et al*., 1990). Among western Peace Corp workers in Thailand, Aeromonads were recovered from 8.5% of healthy persons and 30.8% of persons with diarrhea (Echeverria *et al*., 1981).

Recovery rates among children with diarrhea vary geographically: 0.62 to 4% in Malaysia (Lee and Puthucheary, 2001; Lee and Puthucheary, 2002), 0.75% in Nigeria (Kehinde *et al*., 2001), 2% in Sweden (Svenungsson *et al*., 2000), 2.3% in Taiwan (Juan *et al*., 2000), 4.8% in Switzerland (Essers *et al*., 2000), and 6.8% in Greece (Maltezou *et al*., 2001). Sinha *et al*. (2004) reported Aeromonads in 6.5% of all patients in India. Chan *et al*. (2003) also reported Aeromonads in 6.9% of adult patients with acute diarrhea in Hong Kong. Reports on isolation of Aeromonads from symptomatic patients range from 0.04% to 21% (Kuijper and Peeters, 1991;

Dumontet *et al*., 2003; Maraki *et al*., 2003). However, isolation rates for human fecal specimens vary widely, as geographical areas, patient populations, food habits, level of sanitation and culture methods influence the recovery rates (Dumontet *et al*., 2003). Pazzaglia *et al*. (1990) reported that 23.1% of newborns in Peru demonstrated transitory gastrointestinal colonization with *Aeromonas* spp. during the first days of life.

Kannan *et al*. (2001) isolated *Aeromonas* spp. from clinical specimens in India which included *A. hydrophila* (59.3%), *A. caviae* (18.7%), *A. veronii* (10.9%), *A. schubertii* (4.6%), *A. jandaei* (3.1%), and *A. trota* (3.1%). Fecal carriage rates of 6.6% of symptomatic 10-year olds have been reported (Komathi *et al*., 1998). Seventeen of 2,565 stool samples (0.66%) were positive for *Aeromonas* spp. (Borchardt *et al*., 2003). The choice of culture methods especially the use of alkaline peptone water or other enrichment methods prior to plating fecal samples markedly affects the detection rate both within and between patient populations.

The distribution of *Aeromonas* spp. in marine ecosystem and retail seafood outlets is well documented and the organism is considered to be the normal flora of a variety of fishes, only to act as an opportunities pathogen under conditions of stress (Dugenci and Candan, 2003; Yogananth *et al*., 2009). *A. hydrophila* has been isolated from diseased turtles, alligators, snakes, and frogs (Gosling, 1996b). Aeromonads have been isolated from feces of wild and pet birds and under stress may cause septicemia in poultry (Saif and Busch, 1974).

Gray (1984) isolated *A. hydrophila* from feces of normal horses (6.4%), pigs (9.6%), sheep (9.0%) and cows (21.1%). The total fecal carriage rate in animals is slightly higher than the fecal carriage rate of normal humans, which is < 1 to 7% for most studies, although some studies report higher rates (Pitaragnsi *et al*., 1982). Populations in animals probably reflect the presence of Aeromonads in their feed and

water.

Figura and Marri (1985) isolated *A. hydrophila* more frequently than *A. caviae* in feces of pigs. Stern *et al*. (1987) isolated Aeromonads from 1 of 32 cows and 3 of 21 turkeys. Gray and Stickler (1989) isolated *A. hydrophila* and *A. caviae* in cow and pig feces respectively. Diet and water sources influenced recovery of Aeromonads from feces of domestic animals. *Aeromonas* spp. have been isolated from feces, bedding, and drinking water of healthy cows and pigs (Hathcock *et al*., 1999). Both healthy and diseased animals shed Aeromonads in feces (Hathcock *et al*., 1999).

Ghenghesh *et al*. (1999a) isolated *Aeromonas* spp. from household pets (dogs and cats) in Libya. The qualitative studies give no estimate of the number of Aeromonads shed in the feces of dogs and cats, and therefore, the public health significance of these findings is unknown.

2.4 Factors Affecting Survival of *Aeromonas* spp.

Environmental survival of bacteria is dependent upon many physical and biological factors. Temperature, pH, ionic strength, sunlight (UV irradiation), moisture, available nutrient, presence of suspended solids, cell-specific protection mechanisms and the presence of toxic substances and predators all interact to determine survival times.

2.4.1 Survival in water and food

Aeromonas spp. have their natural habitat in water and grow over a wide temperature range. Because *Aeromonas* spp. grow between 0º C and 45º C, with a temperature optimum of 22º C to 32º C, there are few environmental habitats where they are not found (Tsai and Yu, 1997; Warburton, 2000; Croci *et al*., 2001). Nutrient availability, temperature and water activity are the most important factors

that determine growth rates. Growth is optimal at 30º C at pH 7 and a water activity of 0.99 (Sautour *et al*., 2003). Imbert and Gancel (2004) studied the effect of temperature downshift on protein synthesis of *A. hydrophila*. While a few proteins were under-expressed, two-dimensional electrophoresis revealed that numerous new proteins appeared with a decrease in temperature and some others were over-expressed. Cold shock proteins distinct from those produced by *E. coli* were recognized. Additional studies are required to elucidate the nature of heat and cold shock proteins produced by Aeromonads.

Aeromonads grew in filtered autoclaved tap water but were inhibited by the presence of heterotrophic bacteria, suggesting that competition for nutrients is a selective factor limiting the growth of Aeromonads (Mary *et al*., 2001). Environmental strains had no competitive advantage over clinical isolates. Survival rates vary by strain, with HG-4 > HG-5B > HG-17 > HG-1. Cell density increased from 1-2 $\log_{10}$ CFU/mL to 6 $\log_{10}$ CFU/mL in 3-5 days in both filtered autoclaved and unfiltered autoclaved tap water. Cells suspended in unfiltered water that had not been autoclaved declined from initial concentrations of 4 $\log_{10}$ CFU/mL to 1 $\log_{10}$ CFU/mL within 14 days. Cells suspended in filtered water that had not been autoclaved showed strain- specific differences in survival with decay rates from 4 to 1 $\log_{10}$ CFU/mL on day 1 (*A. popoffii*), day 3 (*A. media*), day 7 (*A. hydrophila*) and day 14 (*A. caviae*) (Mary *et al*., 2001).

The upper growth limit is approximately $6x10^5$ CFU/mL in nutrient-poor water (Miettinen *et al*., 1997; Tsai and Yu 1997; Kersters *et al*., 1996b; Mary *et al*., 2001). Phosphorous (Miettinen *et al*., 1997) and assimilable organic carbon (AOC) (van der Kooij, 1992) are considered to be the primary factors regulating growth. *A. hydrophila* (HG-1) grows less well than other *Aeromonas* spp. in water poor in nutrients (van der Kooij and Hijnen, 1988; Mary *et al*., 2001).

Studies in low nutrient waters such as bottled mineral water show that *Aeromonas* spp. remain viable for extended periods of time. *A. hydrophila* survives in distilled bottled water between 30-60 days, and for more than 223 days in spring water (Warburton 2000). *A. hydrophila* increased by 1 $\log_{10}$ during the first 24 hr. then decline by 3 $\log_{10}$ CFU in 90 days and 6 $\log_{10}$ CFU in 150 days during a study (Messi *et al*., 2002). Experiments using mixed cultures reduced the survival time of Aeromonads. van der Kooij and Hijnen (1988) have shown that microgram per liter concentrations of amino acids and long chain fatty acids promote growth of Aeromonads in water distribution systems. Aeromonads can also metabolize a wide variety of biopolymers, including proteins, carbohydrates and lipids, which are attacked by Aeromonads wide array of extracellular enzymes.

Photo-oxidation from visible and ultraviolet light has an inimical effect on Aeromonads. *A. salmonicida* populations were reduced by 99.9% after 2 hr of exposure to sunlight (Liltved and Landfald, 2000). Benchokroun *et al*. (2003) examined the effects of solar radiation on *Aeromonas* spp. in an algal pond. Inactivation was enhanced by increasing oxygenation or pH, while addition of catalase or sodium pyruvate prevented die-off, suggesting that damage to cells was through photo-oxidation. *A. sobria* was slightly more resistant to photo-oxidation which may explain its relative presence at high density in waste stabilization ponds.

Aeromonads grow best between pH 7-9 (Vivekanandhan *et al*., 2003). Sautour *et al*. (2003) reported that variation in pH had little effect upon survival over a range of pH 5-9, and this is consistent with the growth range reported by Popoff and Lallier (1984). *Aeromonas* spp. are sensitive to acid conditions below pH 3.5; however, they exhibit an acid stress response in that when they are acclimated at pH 5, the kill time at pH 3.5 is extended. Treatment with protein inhibiting antibiotics prior to exposure to low pH eliminated the acid stress response, suggesting that

protein synthesis is an important part of the acid stress response.

Delamare *et al*. (2000) examined salt tolerance among Aeromonads. All strains challenged were able to grow in the presence of 0.34 M sodium chloride, and all but *A. veronii* biovar *sobria*, *A. sobria* and *A. salmonicida* subspecies *salmonicida* were able to grow in the presence of 0.51 M sodium chloride. *A. hydrophila, A. caviae, A. encheleia, A. trota, A. euchrenophila,* and *A. media* tolerated 0.68 molar salt concentration. *A. caviae* and *A. trota* grew at 0.85 M salt concentration and *A. trota* tolerated 1.02 molar salt concentration.

2.4.2 Survival in Food

Aeromonads have been shown to grow in foods held at refrigerator temperatures (Palumbo and Buchanan 1988; Majeed and MacRae 1991; McMahon 2000). *A. hydrophila* counts increased from 3-5 $\log_{10}$ CFU to 6-7 $\log_{10}$ CFU in ground meat at 7º C over 7 days (Vaid and Garg 2002). Counts reached 8 $\log_{10}$ CFU in one day at 25º C. Background microflora were not inhibitory for Aeromonads. While members of the *Enterobacteriaceae* are inhibited, growth of *A. caviae* was stimulated from 4 $\log_{10}$/g to 8 $\log_{10}$ CFU/g at ambient temperatures and from 5 $\log_{10}$ CFU/g to 7 $\log_{10}$ CFU/g at refrigerator temperature in salad materials packaged under superatmospheric oxygen (Allende *et al*., 2002).

Growth temperature is an important feature in differentiation of clinical and environmental strains. Approximately half of clinical isolates show some growth at 4-5º C, all food isolates grow at this temperature (Knochel and Jeppesen, 1990). While most clinical strains grow at 42º C, only a few isolates from vegetables stored at 5º C grew at elevated temperature. Knochel and Jeppesen (1990) found that some Aeromonads isolated from cold water did not grow at 37º C. The growth temperature range for Aeromonads is from 4 to 44º C, but individual strains

typically have a restricted growth range according to their ecological niche, and growth of a strain at both extremes of the range are rare (Kirov *et al*., 1993b). Aeromonads are considered heat sensitive with respect to other food-borne pathogens.

Aeromonads tolerate high pH well and this feature has been exploited by using alkaline peptone water at pH 8.6 for sample enrichment. Aeromonads grow at pH 5.8 or higher, and may survive at pH 4.6 or higher according to computer modeling using Food Micromodel. Species-specific acid tolerance is known to occur, since *A. caviae* grown on glucose or other simple sugars produces sufficient acetic acid to auto-sterilize a broth culture within 48 hr. in weakly buffered systems. This metabolic activity has been called the suicide phenomenon (Namdari and Cabelli 1989).

Aeromonads do not tolerate high salt concentrations. Knochel and Jeppesen (1990) reported that a few strains tolerated 6% NaCl, but generally Aeromonads do not tolerate concentrations above 5% NaCl. Growth in a competitive environment may not occur at salt concentrations between 3-4% and a few isolates are sensitive to 2% NaCl. Vivekanandhan *et al*. (2003) examined the effects of salt concentration on *A. hydrophila*. Salt concentrations above 2% inhibited growth somewhat. Some growth occurred at 4% NaCl concentration; however, no growth occurred at 5% though cells remained viable. These results may explain the distribution of Aeromonads in seawater and have implication in food preservation.

Modified atmospheres are increasingly being used in food packaging. Pin *et al*. (2004) studied the response of *A. hydrophila* to various combinations of pH, temperature, and CO_2 and O_2 concentrations. The results were used to develop and validate a predictive model for growth and death estimates under modified atmospheres at refrigerator temperature. Reduced oxygen levels do not exert a

detrimental effect on survival and growth of Aeromonads and they may be isolated from vacuum packed foods (Berrang *et al*., 1989). Expression of proteases and hemolysins was inhibited, particularly at low temperature (McMahon, 2000). High amounts of carbon dioxide (94-99%) markedly affected growth and viability (Golden *et al*., 1989). Devlieghere *et al*. (2000) reported that the use of carbon dioxide in the package atmosphere in combination with reduced water activity effectively inhibited growth. Gas-packed cured cooked meat products did not support growth of *Aeromonas* spp. stored at temperature of less than 7 ºC.

Irradiation is effective at doses of 125 to 150 kilorads, which is sufficient to eliminate viable cells from foods. Polyphosphates together with sodium chloride, sorbates, and smoke are inhibitory to Aeromonads (Gram 1991; Palumbo *et al*., 1995). Velazquez *et al*. (2001) studied the antibacterial effects of phosphates, common food additives in meat products. *A. hydrophila* was totally inhibited at concentrations between 0.5 and 3%. 0.5% sodium acid pyrophosphate exhibited the greatest inhibition as it possesses both bactericidal and bacteriolytic properties.

Mary *et al*. (2003) studied the tolerance of *A. hydrophila* to ethanol, sodium chloride, drying and temperature. Starved cells developed increased tolerance to salt and ethanol challenge but not to heat. These findings suggest that less rigorous food processing and preservation methods may not eradicate Aeromonads from foods. Uyttendaele *et al*. (2004) examined the effect of lactic acid, chlorinated water, and thyme essential oil solution for control of *Aeromonas* spp. in fresh vegetables. Lactic acid at 1% or 2%, and 0.5% or 1% thyme essential oil solutions were able to control growth of Aeromonads, while waters with chlorine concentrations of 0.1 to 0.5 mg/L were not.

Usual food preservative processes (pH < 5, sodium chloride > 3.5%,) are sufficient to suppress growth of Aeromonads with the exception of refrigeration, as

refrigeration alone does not suppress growth of Aeromonads (Palumbo *et al*., 1995).

2.5 Infections/Diseases caused by *Aeromonas* spp.

Although the Aeromonads have been discovered a century ago, but it is only recently that their role in multitude of infections was irrefutably proven in humans and animals (Janda and Abbott, 1998). A large majority of infections are caused by *A. hydrophila*, *A. caviae* and *A. veroni* biovar sobria (Janda, 1991; Janda and Abbott, 1998).

2.5.1 In Humans

The first comprehensive review of human infections caused by *Aeromonas* spp. was described by von Graevenitz and Mensch (1968). Many reports followed thereafter (Janda and Abbott, 1996; Chopra and Houston, 1999; Figueras *et al.,* 2005; Kalina, 1977; Janossy and Tarjan, 1980; Agbonlahor *et al.,* 1982). A wide spectrum of infections has been associated with *Aeromonas* spp. which include wound infections, septicemia, lung infections, cellulitis, abscess etc., however, by far the most discussed issue relates to gastroenteritis (Janda, 1991; Janda and Abbott, 2010). Although the role of Aeromonads as agents of gastroenteritis has been questioned by some (Janda and Dufey, 1988), several microbiological, epidemiological clinical and immunological investigations indicate their role of Aeromonads as enteric pathogens (Altwegg and Geiss, 1989; Joseph, 1996). The association is strongest in children under the age of 2 years, adults over 50 years and the immunocompromised (Agger, 1986; Moyer, 1987; San-Joaquin and Pickett, 1988).

Diarrhea due to *Aeromonas* presents with varied clinical manifestations, watery and self-limited diarrhea is common (Ghenghesh *et al*., 1999b). However, some patients may develop fever, abdominal pain and bloody diarrhea. Frank mucus and blood can be seen in more than 25% of stools of children with *Aeromonas*-associated diarrhea and nearly 35% of patients exhibit symptoms of fever and

vomiting (Ghenghesh *et al*., 1999a). *A. caviae* and *A. hydrophila* have been associated with chronic diarrhea lasting up to one year (del Val *et al*., 1990). A syndrome resembling ulcerative colitis has been observed by endoscopy and segmented colitis has also been reported (Farraye *et al*., 1989). Acute self-limiting diarrhea occurs in children, and chronic gastroenteritis or enterocolitis may occur in children and the elderly (Janda and Abbott, 1996; Goldsweig and Pacheco, 2001).

According to Kirov (2001), the majority of Aeromonads associated with gastroenteritis are *A. veronii* biovar sobria (HG-8/10), *A. hydrophila* (HG-1) and *A. caviae* (HG-4), though *A. veronii* biovar veronii (HG-8/10), *A. trota* (HG-13), and *A. jandaei* (HG-9) occur occasionally. Aeromonads have been associated with up to 13% of gastroenteritis cases in the U.S, with O:11 and O:34 being common serotypes (Buchanan, 1984; Thomas *et al*., 1990; Kokka *et al*., 1991b; Merino *et al*., 1993a; Merino *et al*., 1993b).

Besides gastrointestinal disorders, Aeromonads cause soft tissue infections like fatal nosocomial necrotizing fasciitis (Cheng *et al*., 2004), bite wound infections (Raynor *et al*., 1983; Revord *et al*., 1988; Angel *et al*., 2002), burn infections (Kienzle *et al*., 2000). The significance of Aeromonads as a cause of skin and soft-tissue infections was made abundantly clear as a result of the tsunami in Southeast Asia in 2004. Among 777 patients hospitalized for injuries as a result of the tsunami, 515 had skin and soft-tissue infections of which 145 isolates from 305 patients were either *A. hydrophila* or *A. veronii* biovar sobria (Hiransuthikul *et al*., 2005; Lim, 2005).

Aeromonas spp. are also involved in pneumonia and lung abscesses in adults (Goncalves *et al*., 1992; Murata *et al*., 2001), pneumonia in children (Sirinavin *et al*., 1984; Kao *et al*., 2003). meningitis (Parras *et al*., 1993), endocarditis (Brouqui and Raoult, 2001), Osteomyelitis (Janda and Abbott, 1998), hepatobiliary Infections like

cholecystitis and cholangitis (Kumar *et al*., 2000a; Clark and Chenoweth, 2003), peritonitis (Ramos *et al*., 1996), hemolytic uremic syndrome (HUS) (Bogdanovic *et al*., 1991, Robson *et al*., 1992) and Ocular infections (Carta *et al*., 1994; Puri *et al*., 2003; Pinna *et al*., 2004).

Bacteremia resulting from *Aeromonas* infection was reviewed by Ko and Chuang (1995) and Ko *et al*. (2000). *A. hydrophila* sepsis is associated with gastrointestinal disease, liver cirrhosis, diabetes, malignancy, pancreatitis, trauma, cardiac anomalies and respiratory disease (Ko *et al*., 2000). Disseminated infection progresses rapidly and has a high fatality rate (Shiina *et al*., 2004).

2.5.2 In Animals

Aeromonads are also recognized pathogens of cold (Gosling, 1996b) and warm blooded animals (Gray, 1984; Shane and Gifford, 1985). Among cold blooded animals pathogenic potential of Aeromonads was described in amphibians (Shotts *et al*., 1972; Huys *et al*., 2003), reptiles (Marcus, 1971; Shotts *et al*., 1972; Misra *et al*., 1993), fishes (Hazen *et al*., 1978) and snails (Mead, 1969).

In equines they are known to be associated with corneal ulcers (Rebhun *et al*., 1999), diarrhea (Hathcock *et al*., 1999) abortion and infertility problems (Forga-Martel *et al*., 2000; Ulgen *et al*., 2001). The organisms have been isolated from heart blood of aborted fetus and rectal swabs as well (Malik *et al*., 2002). Aeromonads were isolated from bovine mastitis (Ak, 2000; Kumar *et al*., 2001), bovine seminal vesiculitis (Moro *et al*., 1999), gastritis in squirrel monkeys (Khanolkar *et al*., 2000), cases of early abortions in buffalo (Das and Paranjape, 1987), buffalo semen (Ramawamy *et al*., 2002), farm animals (Gray, 1984; Gray *et al*., 1990), racing camel calves (Moore *et al*., 2002), cases of high mortality in rabbits (Paniagua *et al*., 1998), septicaemia in seal (Krovacek *et al*., 1998), normal and diseased, domesticated, pet and wild birds (Oladele *et al*., 1999; Shen and Shen, 2001; Silvanose *et al*., 2001;

Glunder, 2002), wild birds (Oladele *et al*., 1999; Shen and Shen, 2001; Silvanose *et al*., 2001; Glunder, 2002). Aeromonads are reported to cause fatal septicaemia in dogs and puppies (Andre- fontaine *et al*., 1995; Zdovc *et al*., 2004) and have been isolated from dogs and cats frequently (Ghenghesh *et al*., 1999b; Boynukara *et al*., 2002; Ceylan *et al*., 2003).

2.6 Epidemiology and Disease Outbreaks

Aeromonads are ubiquitous in the nature and present multiple opportunities for transmission to humans through food, water, animal contact and direct human contact. Extra- intestinal infections are typically acquired through trauma in an aquatic environment while intestinal infections usually result by ingestion of contaminated food or water.

Aeromonas spp. have been recognized as potential foodborne pathogens since 1984 (Buchanan, 1984). Since then *Aeromonas* spp. has been reviewed as emerging organisms of public health significance present in foods (Kirov, 1993a; Merino *et al*., 1995). The presence of Aeromonads in fish and seafoods lead the U.S. Food and Drug Administration to designate them as new foodborne pathogens (Tsai and Chen, 1996). *A. hydrophila, A. caviae*, and *A. veronii* biovar sobria are commonly found in seafoods (Tsai and Chen, 1996), and may therefore be involved in foodborne outbreaks. *Aeromonas* spp. have been recovered from 93% of fish, 100% of fish eggs, and 16% of shrimp (Hanninen *et al*., 1997). Wang and Silva (1999) reported that 36% of channel catfish samples from 3 processing plants contained *A. hydrophila* and 35.7% contained *A. veronii* biovar sobria. The rates of contamination were higher in summer months when water temperatures are warm (Nishikawa and Kishi, 1988).

Diarrheal diseases have been associated with drinking untreated well water (Holmberg *et al*., 1986). Altwegg *et al*. (1991a) used ribotyping to demonstrate that

shrimp ingestion resulted in gastroenteritis in the first report of foodborne illness attributed to *Aeromonas* spp. Ribotyping was used to demonstrate that a patient with chronic diarrhea carried the same strain for years (Moyer *et al*., 1992a) and ribotyping was used to demonstrate person-to-person transmission of *Aeromonas* spp. between a foster child and a foster parent (Moyer and Larew, 1988). Aquarium water has been suggested as the source of Aeromonads resulting in gastrointestinal infection (San Joaquin *et al*., 1989). Filler *et al*. (2000) reported a case of acute renal failure in a 6-month old infant caused by *A. sobria* acquired from aquarium water. The infection presented with water diarrhea that progressed to bloody diarrhea and hemolytic uremic syndrome.

There are reports of foodborne gastroenteritis by *Aeromonas* spp. around the world including Russia (Kalina, 1977), Hungary (Janossy and Tarjan, 1980), Nigeria(Agbonlahor *et al.*, 1982), Florida(Abeyta *et al.*, 1986), China (Zeng Shan *et al.*,1988), Japan (Kobayashi and Ohnaka, 1989), England (Kirov, 1993a), Switzerland (Altwegg *et al*., 1991a), Japan (Tanaka *et al.*, 1992), U.S (Bottone, 1993), Sweden (Krovacek *et al.*, 1995) and Norway (Granum *et al*.,1998).

2.7 Virulence of *Aeromonas* spp.

Virulence of Aeromonads is multifactorial and incompletely understood (Trower *et al*., 2000). Though the putative virulence factors described can broadly be divided into three categories, structural (pili, flagella, outer membrane proteins, S-Layer proteins, lipopolysaccharide and capsule), cell associated (invasins, adhesins, plasmids and serum resistance) and extracellular (hemolysins, cytotoxin enterotoxin, heat stable cytotonic enterotoxin, heat stable cytotonic enterotoxin, protease, amylase and lipase) factors (Janda, 1991). Since the greatest impact on *Aeromonas* research is imparted by the epidemiological association of these organisms with bacterial gastroenteritis in humans (Janda, 1991), the role of extracellular virulence features are

more investigated compared to other features (Kingombe *et al*., 1999; Sechi *et al*., 2002; Abdullah *et al*., 2003).

Of a number of extracellular factors, enterotoxins are major virulence factors (Abdullah *et al*., 2003). Three different enterotoxins have been described in *Aeromonas* spp., these include, cytotoxic heat-labile enterotoxin (Act), also known as aerolysin/hemolysin (Chopra *et al*., 1993), heat-labile (56ºC) cytotonic enterotoxin (Alt) also known as lipase, extracellular lipase, or phospholipase (Anguita *et al*., 1993; Chopra *et al*., 1996; Sha *et al*., 2002) and the cytotonic heat-stable enterotoxin (Ast) (Chopra *et al*., 1994). The recent isolation of enterotoxigenic Aeromonads from drinking water in the United States (Saavedra *et al*., 2007) reiterates the potential human health hazard of waterborne *Aeromonas* spp. The observation that strains harboring the *alt* and *ast* genes were more prevalent in children with diarrhea than in healthy controls underlines the importance of enterotoxins in the pathogenicity of Aeromonads (Albert *et al*., 2000).

The cytotoxic heat-labile enterotoxin (Act) is known to stimulate pro-inflammatory cytokine and eicosanoid cascades in macrophages in the rat intestinal epithelial cell line ICE-6, leading to tissue damage and fluid secretory response (Chopra *et al*., 2000). There is a good correlation between cytotonic enterotoxins (Alt and Ast) and elongation of Chinese hamster ovary cells and production of c-AMP, which is typical enterotoxic activity (Chopra *et al*., 1994). Previous data show that Act is the major enterotoxin contributing to fluid secretory response, followed by Alt and Ast in *A. hydrophila* (Sha *et al*., 2002). Sinha *et al*. (2004) found that the majority of the *A. veronii* biovar sobria strains contained act. Presence of all three genes is rare (Albert *et al*., 2000; Sinha *et al*., 2004). The presence of these three enterotoxin genes in various combinations may increase or decrease expression of specific enterotoxin genes and mediate the severity of diarrhea (Sha *et al*., 2002).

Act is a single-chain polypeptide 52 kDa in length and has multiple biological functions such as lysis of red blood cells and destruction of cell lines, evoking a fluid secretory response in ligated intestinal loop models and causing lethality in mice (Chopra *et al*., 2000). Act increased cAMP production in macrophages along with PGE2 and promoted fluid secretion in animal models. Act upregulates the production of proinflammatory cytokines and antiapoptotic protein Bcl-2 and activates arachidonic acid metabolism in macrophages and monocytes (Chopra *et al*., 2000). Alt is heat labile at 56º C, it is a single 44 kDa polypeptide chain which elevates cyclic AMP and prostaglandin levels in Chinese hamster ovary and intestinal epithelial cells and caused fluid accumulation in rat ileal loops (Albert *et al*., 2000). Ast is a single 44 kDa polypeptide chain that is heat stable at 56º C, and which evoked fluid secretion in small intestine of rats and cAMP levels in mucosal cells (Chopra *et al*., 1994). McCardell *et al*. (1995) purified an Ast-like toxin, not related to cholera toxin and do not increase cAMP, CGMP or PGE2 levels in CHO cells, but it does cause fluid accumulation in infant mice.

Therefore, a number of studies reported characterization of these factors for their physico-chemical and biological properties as well as characterization of *Aeromonas* isolates for their virulence potential from diverse sources. Over time, analyses of results of these and similar studies have helped associating various phenotypes of Aeromonads epidemiologically in relation to their sources, biotypes and disease processes in human and animals. However, a significant breakthrough in understanding *Aeromonas* biology came with the application of newer molecular tools in *Aeromonas* research.

2.8 Molecular Characterization

Discovery of newer molecular tools to characterize pathogenic microbes enabled *Aeromonas* researchers to describe submicroscopic events in *Aeromonas*

biology more vividly. The major areas where application of molecular techniques imparted profound impacts included *Aeromonas* systematics, virulence, epidemiology and typing of isolates.

Taxonomic classification of Aeromonads conventionally based on biochemical properties. However, with the application of DNA-DNA hybridization and polymorphism studies of 16S rRNA gene, enough phylogenetic depth within the group was obtained which catapulted them from their previous location under the family *Vibrionaceae* to a new family *Aeromonadaceae* (Colwell *et al*., 1986; Kuijper *et al*., 1989b; Martinez-Murcia *et al*., 1992a; 1992b). Yanez *et al*. (2003) after studying the polymorphism of the housekeeping gene *gyrB,* confirmed the new phylogenetic position of Aeromonads. Again, the identification of individual *Aeromonas* spp. based on standard biochemical fingerprinting have often been pitted with controversy. Moreover, identification of newer species within the genus complicates the problem. However, two studies (Borrell *et al*., 1997; Figuearas *et al*., 2000) came up with a scheme of species identification based on restriction fragment length polymorphism (RFLP) profile of 16S rRNA gene. Yet, the scheme may reportedly lead to possible misidentification of *Aeromonas* spp. (Graf, 1999). A number of studies utilized molecular tools *viz*., cloning, sequencing etc. to characterize virulence properties of Aeromonads and identified the genes encoding various virulence factors eg. aerolysin, haemolysin, enterotoxin etc. (Howard *et al*., 1987; Cahill, 1990b; Hirono *et al*., 1992; Anguita *et al*., 1993; Chopra *et al*., 1993; Wong *et al*., 1998; Xu *et al*., 1998). Findings of these and similar studies spawned a number of studies to characterize *Aeromonas* isolates for distribution of virulence genes (Wang *et al*., 1996; Granum *et al*., 1998; Heuzenroeder *et al*., 1999; Ormen and Ostensvik, 2001; Gonzalez-Rodriguez *et al*., 2002; Rahman *et al*., 2002; Sechi *et al*., 2002; Soler *et al*., 2002; Abdullah *et al*., 2003; Castro-Escarpulli *et al*., 2003; Ullmann *et al*., 2005;). As Albert *et al*. (2000) suggested that variation in distribution

of potential virulence genes among *Aeromonas* isolates might contribute to their degree of virulence, these studies contributed in assessing the virulence potential of Aeromonads from different parts of the world and helped in understanding the epidemiology of *Aeromonas* infections.

A multiplex PCR was developed by Kingombe *et al.* (2010) for the detection of cytotoxic (*act*), heat-labile (*alt*), and heat-stable (*ast*) enterotoxin genes in *Aeromonas* spp. A total of seven gene pattern combinations were encountered, including *act*, *alt*, *act/alt*, *act/alt/ast*, *act/alt*/148-bp amplicon, *alt/ast*, and *alt*/148-bp amplicon. The *alt* gene was detected with 34 reference strains (97%) and occurred singly in 14% of these strains. The frequency of occurrence of the *act/alt*, *act/alt/ast*, and *alt/ast* gene patterns in reference strains was 14 (40%), 2 (6%), and 2 (6%), respectively.

Another area of *Aeromonas* research where recent technologies put a dent is the epidemiological typing of *Aeromonas* isolates. Various techniques viz pulsed field gel electrophoresis (PFGE) of genomic DNA, amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR), repetitive extragenic palindromic (REP) PCR, enterobacterial repetitive intergenic consensus sequence (ERIC) PCR, restriction fragment length polymorphism of 16S-23S intergenic spacer region (16S-23S ISR-RFLP) and amplified ribosomal DNA restriction analysis (ARADA) have been used for typing *Aeromonas* isolates (Huys *et al*., 1996; Oakey *et al*., 1996; Talon *et al*., 1996; Alavandi *et al*., 2001; Ciapini *et al*., 2002; Soler *et al*., 2003; Villari *et al*., 2003; Szczuka and Kaznowski, 2004). However, RAPD-PCR was found to be one of the most useful techniques keeping in view the ease, speed, cost and discriminatory power of the procedure (Szczuk and Kaznowski, 2004).

2.9 Antimicrobial Resistance

One key area that has received little attention is the *in vitro* susceptibility of *Aeromonas* species to chemotherapeutic agents (Janda and Abbott, 1998). Surprisingly, very little has changed in this regard over the intervening years. Only three major studies dealing with the general susceptibility of Aeromonads to various classes and combinations of antimicrobial agents have been published since 1998 and in only two of these investigations have susceptibility data been reported for *Aeromonas* species other than *A. hydrophila*, *A. caviae*, and *A. veronii* bv. sobria (Kampfer *et al*., 1999; Overman and Janda, 1999). Much of the susceptibility information on Aeromonads is based solely upon these three major species associated with human disease, and it is not entirely clear whether those patterns can be extrapolated to other less frequently encountered taxa causing illness (Overman and Janda, 1999). The overall susceptibility profile for the genus *Aeromonas* does not appear to have changed appreciably from what was recorded in studies conducted between the mid-1980s and mid-1990s. Inducible chromosomal β-lactamases are still the major resistance factors for most Aeromonads, although expression of metallo-β-lactamases active against carbapenems is also a concern (Janda, 2001; Zhiyong *et al*., 2002). Although long recognized as a rapid grower, consensus guidelines for the testing of infrequently encountered pathogens, including *Aeromonas* and *Plesiomonas*, have just been published by the Clinical and Laboratory Standards Institute (CLSI) (Jorgensen and Hindler, 2007). CLSI recommends the use of cation adjusted Mueller-Hinton broth for MIC microdilution testing, while Mueller-Hinton agar is recommended for disk diffusion testing (Jorgensen and Hindler, 2007). CLSI document M-45A provides interpretive criteria for disk diffusion and MIC testing for the three primary species plus *A. jandaei* and *A. schubertii* (Clinical and Laboratory Standards Institute, 2006). However, this guideline cautions that most currently

available susceptibility data are based upon studies performed on the three predominant species only.

Several other general conclusions can be drawn regarding the susceptibility patterns of *Aeromonas* species. The use of different methods to assess MICs for Aeromonads does not appear to influence interpretation of susceptibility, for the most part (Kampfer *et al*., 1999). The singular exception to this rule may be in the interpretation of susceptibility status in regards to antifolates (trimethoprim, sulfonamides, trimethoprim-sulfonamide combinations) or certain β-lactamase–inhibitor combinations, including amoxicillin-clavulanic acid (Zhiyong *et al*., 2002). The susceptibility status of *Aeromonas* isolates for therapeutically active drugs also appears to be independent of species designation. Such a conclusion takes into consideration that most *A. trota* strains are susceptible to ampicillin yet use of this β-lactam is contraindicated in regards to treatment of *Aeromonas* infections. While some species-specific susceptibility differences have been found in select studies, these results should be considered preliminary at present (Kampfer *et al*., 1999; Overman and Janda, 1999).

One characteristic of motile Aeromonads, with exception of *A. trota*, *A. media* and *A. eucrenophila*, is universal resistance to ampicillin (Ormen and Ostenvik, 2001). Mounting concerns worldwide over emergence of drug-resistant superbugs have been related to extensive use of antimicrobials in intensive animal husbandry and aquaculture practices (Vivekanandhan *et al*., 2002; WHO, 2000). These concerns are reflected in a number of studies reporting multiple drug resistance in Aeromonads isolated from diverse sources (Motyl *et al*., 1985; Gray *et al*., 1990; Morita *et al*., 1994; Pettibone *et al*., 1996; Son *et al*., 1997; Alavandi *et al*., 2001; Vivekanandhan *et al*., 2002; Radu *et al*., 2003). The problem of multi-drug resistant Aeromonads are more intricate in developing nations like India and other

South east Asian countries (Son *et al*., 1997; Vivekanandhan *et al*., 2002), where regulatory controls of antibiotic usage are often lax (WHO, 2000). Furthermore, there appears to be significant differences in the susceptibilities of Aeromonads to antimicrobial agents based upon origin of isolation (clinical versus environmental), although certainly more studies need to be performed in this area (Kampfer *et al*., 1999).

Chapter-3

MATERIALS AND METHODS

3.1 Bacterial Strains

Three reference strains of *Aeromonas hydrophila* were used in the present study. The *A. hydrophila* strain ATCC 7966 was procured from Hi-Media, Mumbai. The other two standard strains of *A. hydrophila* (MTCC 646 and MTCC 696) were kindly provided by Dr. Jatinder Paul Singh Gill, Prof-cum-Head Department of Veterinary Public Health, College of Veterinary Science and Animal Husbandry, Guru Angad Dev Veterinary and Animal Sciences University-Ludhiana, Punjab. The standard strains of *Bacillus cereus viz* ATCC 14579, NCTC 11143 and NCTC 11145 and *Escherichia coli* MTCC 433 strain available with the division were also used in the study. The isolates were maintained on nutrient agar slants by subculturing fortnightly for purity and viability.

3.2 Sampling

A total of 609 samples comprising human diarrheal, water and food samples were collected for isolation and identification of *Aeromonas* spp. (Table 1).

3.2.1 Water Samples

A total of 182 water samples comprising drinking water (87), water from rivers and streams (38), Dal Lake (12), inlet water source of fish ponds (19) and fish ponds (26) were collected in sterile plastic vials. The samples were processed immediately after collection.

Table-1: Detail of Samples Collected

Sample Collected	Source of Sample	Number of Samples Collected
Water Samples	Drinking Water	87
	Rivers and Streams	38
	Dal Lake	12
	Fish Ponds	26
	Inlet sources to fish ponds	19
Subtotal		**182**
Fish Samples	Retail Fish Markets,	120
	Government Fish Farm	33
	Private Fish Farms	20
Subtotal		**172**
Raw Meats		
Mutton	Retail shops	83
Chicken	Retail shops	57
Beef	Retail Shops	31
Subtotal		**171**
Human Diarrheal Samples	Government Hospitals	47
	Referral Clinics	36
Subtotal		**83**
Grand Total		**609**

3.2.2 Fish

The swabs were taken aseptically from gills of fish (120) from different fish markets of Srinagar City. The samples were also taken from government (33) and private (20) fish farms of Srinagar city and processed immediately after collection.

3.2.3 Raw Meat

One hundred and seventy one raw meat samples comprising chicken (57), mutton (83) and beef (31) were collected from different retail shops in and around Srinagar city. Approximately 30 gm of each of the representative meat sample was collected in a UV irradiated plastic zip lock sachets and brought to laboratory and processed immediately for the isolation and identification of *Aeromonas* spp.

3.2.4 Human Stools

Eighty three stool samples were collected from children less than 6 yrs of age presenting symptoms of diarrhea. The sampling was done prior to initiation of antibiotic therapy, if any. The samples were collected from different government hospitals (Sher-e-Kashmir Institute of Medical Sciences-Srinagar and Government Medical College-Srinagar) and referral clinics of Srinagar City. The stool specimens were collected in sterile plastic vials and processed immediately after collection for isolation of *Aeromonas* spp.

3.3 Isolation and identification of *Aeromonas* spp.

The isolation and identification of *Aeromonas* spp. was done as per the standard microbiological procedures of Havelaar *et al*. (1987) and Janda and Abbott (2010), with suitable modifications.

3.3.1 Isolation.

The food samples (raw meat and fish) were enriched in buffered peptone water (Kingombe *et al*., 2010). The enrichment of water and diarrheal samples was carried in alkaline peptone water (Havelaar *et al*., 1987). Briefly, 5 g of raw meat sample was enriched in 20 ml of buffered peptone water and fish samples were

enriched in 10 ml of buffered peptone water. The water sample (5 ml) was dissolved in 20 ml of alkaline peptone water and the diarrheic samples were dissolved in 10 ml of alkaline peptone water. All the samples were initially enriched at 28^{o}C for 18 hr, after which enrichment was done at 4^{o}C for 4-6 hr. A loopful of enriched broth was inoculated onto two plates of ampicillin dextrin agar (Havelaar *et al*., 1987), one plate was incubated at 28°C and other at 35°C for 18-24 hr. The presumptive isolates of *Aeromonas* spp. were preserved on nutrient agar slants and stored at 4^{o}C till further processing.

3.3.2 Identification and Speciation.

The presumptive isolates were tentatively assigned to genus *Aeromonas* on the basis of oxidase, catalase and nitrate production, resistance to O/129 (2, 4-diamino 6,7diisopropylpteridine), production of acid from D-trehalose, inability to utilize malonate and fermentation of inositol, D-xylose and dulcitol (Ottaviani *et al*., 2006).

For speciation, array of biochemical tests were carried, including hydrolysis of esculin, production of lipase, indole, methyl red and Voges-Proskauer, citrate utilization, production of acid from L-arabinose, cellobiose, lactose, glycerol, mannitol, rhamnose, salicin, sorbitol and sucrose, production of acid and gas from glucose, hydrolysis of urea, susceptibility to ampicillin (10 μg), motility test and lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase tests (Abbott *et al*., 2003; Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010). For better discrimination of the strains at the phenospecies level, recently proposed supplementary biochemical tests (Beaz-Hidalgo *et al*., 2010) i.e. growth at 42°C, acid production from melibiose, hydrolysis of starch and gelatin, H_2S production

from cysteine, β-haemolysis on sheep blood agar and motility in semisolid agar at 37°C were done.

3.4 Molecular Characterization

Molecular characterization of Aeromonads involved identification of genus and species by Polymerase Chain Reaction, study of molecular epidemiology (RAPD, ERIC and RFLP) and *in-vitro* virulence characterization (enterotoxin gene profile) of isolated Aeromonads.

3.4.1 Preparation of DNA template by boiling and snap chilling method.

The cultures were inoculated in 5 ml buffered peptone water and incubated at 28^{o}C for 18 hr. Cells were collected from 1 ml of culture broth by centrifugation at 5000 rpm for 3 minutes. The pellet was washed once with 500 µl sterile double distilled water and resuspended in 100 µl sterile double distilled water. The micro-centrifuge tubes were kept in boiling water bath for 10 min and then in crushed ice for 20 min. The chilled samples were then centrifuged at 6000 rpm for 5 min and the supernatant was collected which served as template.

Alternately the individual colonies from Luria Agar medium were and the individual colony was dissolved in 150 µl sterile double distilled water and boiled for 10 min and then kept in crushed ice for 20 min in microcentrifuge tubes. The tubes were then centrifuged at 6000 rpm for 5 min and the supernatant collected was used as template.

Purity of the extracted DNA was checked by agarose gel electrophoresis and concentration was checked spectrophotometrially. One microlitre of extracted DNA was diluted in 1 ml of sterile double distilled water and the optical density was

measured at 260 nm and 280 nm against sterile double distilled water blank. The purity of the sample was estimated by determining the ratio of absorbance at 260 nm and at 280 nm. For a reasonably pure sample the ratio should be 1.65 to 1.85. The DNA extracted from isolates was stored at -20^{o}C till further use.

3.4.2 Genus specific PCR

The PCR primers used for amplification of DNA fragments containing the 16S±23S intergenic spacers were selected from sequences corresponding to the conserved region of 16S rDNA and the 50 region of 23S rDNA described previously (Kong *et al*., 1999).

3.4.2.1 PCR amplification

Approximately 3 µl of template DNA was used for PCR amplification of the 16S±23S ITS regions. In a final volume of 50 µl, the reaction mixture consisted of 1X PCR buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.2 µM of each primer (forward 5'GGAAACTTCTTGGCGAAAAC3' and Reverse 5'GGTTCTTTTCGCCTTTCCCT3') and 2 U of *Taq* DNA polymerase (MBI-Fermentas). The PCR profile in a Gene Cycler (Applied Biosystems-Singapore) was 35 cycles, of denaturation, annealing and extension at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, respectively. The program also included a preincubation at 94°C for 2 min before the first cycle and incubation at 72°C for 3 min followed by cooling at 4°C after the last cycle. PCR products were analyzed by gel electrophoresis in 1.5% agarose (SRL) stained with ethidium bromide (5 µg/ml). Gene Ruler 100-bp DNA Ladder (MBI Fermentas) was used as a molecular size standard to estimate the size of amplified products.

3.4.3 Speciation by Restriction Fragment Length Polymorphism (RFLP)

The speciation was also done by restriction fragment length polymorphism (RFLP) of PCR-Amplified 16S rRNA gene as described by Borrell *et al*. (1997)

3.4.3.1 Restriction Fragment Length Polymorphism of 16S rRNA gene.

The marked heterogeneity and incongruity between phenotypic and genomic identification of Aeromonads lead to the application of RFLP. Computer analysis of the 16S rRNA gene sequences of *Aeromonas* spp. revealed that restriction fragment length polymorphism of the PCR-amplified 16S rRNA gene can be a good and rapid way of assessing the identities of all known species of *Aeromonas* and also in the epidemiological investigations (Borrell *et al*., 1997; Soler *et al*., 2003).

3.4.3.1.1 Amplification of 16S rRNA gene.

The amplification of 16S rRNA gene was carried as per the method described by Borrell *et al*. (1997) with some modifications. A final volume of 50 µl containing 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (MBI-Fermentas), 2 U of *Taq* DNA polymerase (MBI-Fermentas), 2 µl of template DNA and 10 picomoles of each primer (Reverse and Forward) was used for every reaction (Table 2). PCRs were performed in a thermercycler (Applied Biosystems, Singapore) under the following cyclic conditions: denaturation at 93°C for 3 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min. After the final cycle, extension at 72°C was allowed for 10 min. Negative controls with no template DNA were also included in every set of reactions. The amplified products were checked for amplification in 1% agarose gel, stained with ethidium bromide (5µg/ml) and remaining PCR products were precipitated, dried and resuspended in 25 µl of sterile double distilled water.

3.4.3.1.2 Endonuclease digestions.

Enzymatic digestions were performed as per the method described by Borrell *et al*. (1997) with slight modifications. The amplified product (5 µl) was mixed with 5 U *Alu*I and *Mbo*I enzymes (MBI-fermentas) and 2 µl of the corresponding 10X buffer (buffer Tango for *Alu*I and buffer R for *Mbo*I) in a total volume of 20 µl. *Nar*I and *Hae*III (buffer R for *Hae*III and buffer Tango for *Nar*I) were added to differentiate *A. salmonicida* from *A. encheleia*. The reaction mixture was incubated overnight at 37°C. Aliquots of 10 µl of each restriction reaction mixture were mixed with 2 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), and the mixture was electrophoresed on a 3% agarose gel (SRL) in 1X TAE (Tris Acetate EDTA) buffer. Gels were stained with ethidium bromide (5 µg/ml) and photographed on a UV transilluminator (Cell-Biosciences). The low range gene marker (MBI-Fermentas) was used as a molecular size reference (in base pairs) to detect molecular size of the fragments obtained.

3.4.4 Molecular Epidemiology

The molecular epidemiology and genetic diversity was studied at whole genomic level by random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) and enterobacterial repetitive intergenic consensus sequences polymerase chain reaction (ERIC-PCR) as described elsewhere (Versalovic *et al*., 1991) with needful modifications.

3.4.4.1 Rapid Amplification of Polymorphic DNA (RAPD) PCR

A short random sequence of were designed in the current study for RAPD PCR studies. For RAPD typing the primers used are described in Table 2. RAPD-PCR mixtures had a final reaction volume of 50 µl with slight modifications. The reaction mixture consisted of 5 µl of 10X reaction buffer [750 mM Tris-HCl (pH

8.8), 200 mM $(NH_4)_2$ SO_4, 0.1% Tween 20], a 250 µM concentration of each deoxynucleoside triphosphate (dNTPs), 30 pmol of primer, 3.75 mM $MgCl_2$, 3 µl of template DNA and 2 U of *Taq* polymerase (MBI-Fermentas). The reaction mixture was denatured at 94°C for 1 min and then subjected to 25 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min and 15 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 36°C and extension for 3 min at 72°C with a final extension for 2 min at 72°C.

Aliquots of 10 µl of amplified product was mixed with 2 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) and the mixture was electrophoresed in 1.5% agarose gel in 1X TAE buffer. The gels were stained with ethidium bromide (5 µg/ml) and visualized on a UV light transilluminator. Gene Ruler 100 bp DNA Ladder Plus (MBI-Fermentas) was used as a molecular size standard to estimate the size of amplified products.

3.4.4.2 Enterobacterial Repetitive Intergenic Consensus Sequence (ERIC)-PCR

The ERIC-PCR method utilizes primers complementary to enterobacterial repetitive intergenic consensus (ERIC) sequences of bacterial genomic DNA (Versalovic *et al*., 1991). The reaction mixture consisted of 1X reaction buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2$ SO_4, 0.1% Tween 20], a 250 µM of each dNTPs, 50 pmole of each primer (Table 2), 3.75 mM $MgCl_2$, 5 µl of template DNA and 3 U of *Taq* polymerase (MBI-Fermentas) and reaction volume made up to 25 µl with sterile double distilled water. The reaction mixture was denatured for 7 min at 95°C in a thermocycler (Applied Biosystems) and then subjected to 30 cycles of denaturation for 30 s at 90°C, annealing for 1 min at 52°C, extension for 8 min at 65°C and a final extension for 16 min at 65°C.

Table-2: Primer Sequence used for RFLP, RAPD and ERIC-PCR

PCR	Primer	Sequence (5'→3')	References
16S rRNA for RFLP	FHblC	AGAGTTTGATCATGGCTCAG	Borrell *et al.* (1997)
	RHblC	GGTTACCTTGTTACGACTT	
RAPD-PCR	RAPD 1	CTACGCATGC	This Study
ERIC-PCR	ERIC 1R	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al.*, (1991)
	ERIC 2	AAGTAAGTGACTGGGGTGAGCG	

The amplification products were electrophoresed in 1.5% agarose gel in 1X TAE. Gene Ruler 100-bp DNA Ladder Plus (MBI-Fermentas) was used as a molecular size standard. The gels were stained with ethidium bromide, visualized on a UV light transilluminator. For constructing dendrograms the unweighted pair group method with average linkages (UPGMA) using NTSYSpc, ver. 2.1. software was used.

3.5 Virulence Characterization

The *in-vitro* and *in-vivo* virulence characterization of isolated Aeromonads consisted of molecular detection of enterotoxin genes, analysis of hemolysis on sheep and rabbit blood agar and skin vasopermeabilty reaction in rabbits.

3.5.1 Detection of enterotoxin genes

A multiplex PCR was carried out targeting three *Aeromonas* enterotoxin genes namely cytotoxic enterotoxin (*act*), cytotonic heat labile (*alt*) and cytotonic heat stable enterotoxin (*ast*) genes.

3.5.1.1 Designing of PCR primers for enterotoxin genes.

Novel primers were designed using Primer3 software available on line with national centre for biotechnology information (NCBI) and the specificity was checked by online BLAST (basic local alignment search tool). For the amplification of the heat-labile cytotonic enterotoxin (*alt*) gene (Figueras *et al*., 2005), the design of the unique primers (ALTF and ALTR) was based on the large open reading frame (ORF) sequence (position 339 to 914) of the *A. hydrophila alt* gene (GenBank accession number DQ302128.1). For the amplification of the heat-stable enterotoxin (*ast*) gene (Chopra *et al*., 1994), the primers (ASTF and ASTR) were designed based on ORF1 (position 44 to 259) of the heat-stable cytotonic enterotoxin (*ast*) gene in *A. hydrophila* (GenBank accession number JQ003210.1). For the amplification of cytotoxic enterotoxin (*act*) gene, the design of the unique primers (ACTF and ACTR) was based on ORF1 (position 268 to 1086) of *A. hydrophila* aerolysin gene complete cds (GenBank accession number HQ425626.1). The characteristics of the primers used in this multiplex PCR are described in Table 3. The primers were synthesized by the Operon.

3.5.1.2 PCR Amplifications

The multiplex PCR reaction was performed in a final reaction volume of 50 µl containing 5 µl of template DNA, 1X PCR buffer (10mM Tris-HCl pH 8.3 and 50mM KCl), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 3 U *Taq* DNA Polymerase and 1 µM each primer. PCR reaction was carried out in thermocycler (Applied Biosystems, Singapore) with initial denaturation at 95^{o}C for 5 min followed by 35 cycles of 94^{o}C for 1 min, 60^{o}C for 1 min, 72^{o}C for 1 min and a final extension of 72^{o}C for 10 min. The amplicons were separated on agarose gel (1.5%), applying 5-6 Volts/cm and sizes were estimated using 100 bp DNA ladder (MBI-Fermentas).

Table-3: Primer Sequence of Enterotoxin Genes

Target Gene	Primer	Sequence (5'→3')	Length (bp)	Product size (bp)
act	ACTF	CCGGGCTCGGCGTCCAATAC	20	819
	ACTR	CCAGTTCGGGCGGTTGTCCG	20	
alt	ALTF	GCACGGCGTGACTTCGGTGA	20	576
	ALTR	ACCGCGGTCTTGCAGTTGGG	20	
ast	ASTF	CGCCATCAACAGCTCGCCCA	20	216
	ASTR	CGGGCCTCGTTGAGGAAGCG	20	

3.5.2 Hemolysin Production

The ability to produce hemolysin by the isolates was tested on two different blood agar media as described by Brenden and Janda (1987). Ten microlitre of overnight broth culture was spotted on to tryptic soy agar plates containing 5% defibrinated sheep blood agar or on to 5% defibrinated rabbit blood agar. Plates were incubated at 37^{o}C for 24-72 hr and observed for appearance of zones of hemolysis.

3.5.3 Vasopermeabilty Reaction in Rabbit

The *in-vivo* virulence characterization was studies by skin vasopermeability test in rabbits.

3.5.3.1 Preparation of cell free extract

Cell free supernatants of the isolates were prepared as per the method described by Gray *et al.* (1990) with necessary modifications. The organisms were grown in 5 ml of brain heart infusion (BHI) broth (Hi-Media) fortified with 0.6% (w/v) yeast extract and 3% (w/v) cassamino acid. Following an incubation of 12 h at 37^{o}C, 50 ml of supplemented BHI broth was inoculated with the initial broth culture and incubated in a shaker set at 200 rpm at 37^{o}C. After incubation of 14-16 hr while the growth was in mid-log phase the broth cultures were transfered to 50 ml sterilized tubes and centrifuged at 10000 g for 30 min at 4^{o}C. The supernatants were filtered through 0.22 µm membrane filters (Sartorius, Germany). Sterility of the each preparation was checked by streaking onto nutrient agar and incubating at 37^{o}C for 24 hr. The pH of the filtrates was checked and cell free extracts were stored at -20^{o}C till further use.

3.5.3.2 Vascular permeability reaction:

The toxigenicity of *Aeromonas* enterotoxins was studied by vascular permeability reaction (VPR) in Rabbit skin as per the method described by Ljungh *et al*. (1982) with slight modifications. About 0.05 ml of concentrated enterotoxin from standard and the representative isolates was injected intradermally into clean shaved abdominal area of two adult rabbits. Adjacent to these sites, an equal amount of concentrated enterotoxin obtained from the *Aeromonas* spp. under study was also injected. Filtrate of uninoculated RCB was also injected adjacent to these sites as control. After three hours of inoculation, 2.5 ml of 0.25 percent trypan blue dye in normal saline solution was injected I/V into the ear vein of each of the rabbit. An hour after the administration of the dye, vascular permeability reaction was observed as zones of light and dark blue areas surrounding the point of inoculation. Gross

observations like intensity of color change, presence of oedematous area, necrosis etc. were also recorded at varying time intervals of 3, 6 and 12 hrs post inoculation.

3.6 Sequencing of Enterotoxin Genes

The enterotoxin genes amplified in the current study were sequenced for any possible variability in the sequences of the amplified region.

3.6.1 Purification of PCR product of amplified enterotoxin genes

The enterotoxin genes of *Aeromonas* were amplified in bulk and the PCR products were analyzed on low melting agarose gel (1.5%) containing ethidium bromide (5 µg/ml). The gel slice containing DNA fragment of interest was excised with sterile Baird Parker (BP) blade and placed in 2 ml microcentrifuge tube. The DNA from the gel slice was extracted by two methods which are given here under:

3.6.1.1 Phenol extraction method

The cut fragment was melted at 72ºC and volume was made up to 300 µl with TE pH 8.0. The mix was added with $1/10^{th}$ the volume 5M NaCl and re-melted at 72ºC. Equal volume of tris saturated phenol (which should be at room temperature) was added, vortexed. The vortexed mixture was kept on ice for 1 min and centrifuged at 14000 rpm for 10 min at room temperature. The supernatant after centrifugation was collected and the phenol layer was re-extracted with 100 µl TE (pH 8.0) and aqueous phase was pooled. To the aqueous phase 2 volumes of isopropanol was added and vortexed. The mixture was kept at -20ºC for 1 hr and centrifuged at 12000 rpm for 15 min at 4ºC. The supernatant was discarded without disturbing the pellet. The pellet was washed with 80 per cent alcohol at 10000 rpm for 5 min. The supernatant was carefully discarded and the pellet was dried. Finally the pellet was dissolved in 20 µl of sterile MilliQ water and purified product (5 µl)

was checked on agarose gel (1%) carrying 100bp ladder. Finally the purification and concentration was checked photometrically in Nanodrop.

3.6.1.2 Elution through column

The DNA was purified using AuPreP gel extraction kit (Life Technologies India Pvt. Ltd.). Briefly the procedure is 200 mg of gel slice was placed in 2 ml of micro-centrifuge tube and 0.5 ml of GXE buffer was added to it and incubated at 60ºC in water bath for 10 min until the gel was completely dissolved. The tube was inverted after every min during incubation and the gel mixture was cooled down to room temperature. Gel column was placed on to a collection tube and 0.7 ml of dissolved gel mixture was loaded into the column, then centrifuged at 5000 rpm for 1 min and the flow was discarded, this step was repeated until completion of rest of the gel mixture. The column was washed once with 0.5 ml of WS buffer by centrifugation for 1 min and then again centrifuged for another 1 min at high speed to remove residual ethanol. Now column was placed on a new 2 ml micro-centrifuge tube and 50µl of elution buffer was added on to the centre of membrane and then allowed to stand for 2 min at room temperature. It was then centrifuged for 1 min to elute DNA. Finally the concentration of pure DNA was checked on Nanodrop.

3.6.2 Sequencing of enterotoxin genes (*ast, alt and ast*) gene

After purification the PCR amplicons were sequenced using automated dye-terminator cycle sequencing method with Ampli Taq DNA polymerase in ABI PRIZM 377 DNA sequencer (Perkin-Elmer). The fragments were sequenced at least twice with each primer to reduce possibility of sequencing artifacts. Online similarity searching was performed with BLAST (Basic Local Alignment Search Tool) in GenBank.

3.7 Antimicrobial Sensitivity Test

All the isolates were examined for their antibiotic susceptibility/resistance pattern by disc diffusion technique as described by Bauer *et al*. (1966) against a panel of 20 antibiotics (Table 4). Briefly isolates were grown in nutrient broth at 37^{o}C for 16 hrs. Individual broth cultures were smeared on the Mueller-Hinton (MH) agar plates with the help of sterile cotton swabs. Plates were allowed to dry for few minutes, antibiotic discs (Hi-Media) were placed on the agar surface within 15 minutes of inoculation of plates and were incubated overnight at 37^{o}C. The sensitivity or resistance of an isolate for a particular antibiotic was determined by measuring the diameter of the zone of inhibition of growth. The result was interpreted as sensitive or resistant based on Clinical and Laboratory Standards Institute (CLSI) interpretive standards (Jorgensen and Hindler, 2007).

Table-4: Various Antimicrobial Agents used and their Respective Concentration

S. No.	Name of Antimicrobial agent	Concentration (µg/disc)
1.	Amoxycillin (Am^{30})	30
2.	Ampicillin (A^{10})	10
3.	Ampicillin/Cloxacillin (Ax^{10})	10
4.	Ceftazidime (Ca^{30})	30
5.	Cephalexin (Cp^{30})	30
6.	Ceftriaxone (CTR^{10})	10
7.	Ciprofloxacin (CF^{30})	30
8.	Doxycycline (Do^{30})	30
9.	Enrofloxacin (Ex^{10})	10
10.	Erythromycin (E^{15})	15
11.	Gentamicin (G^{10})	10
12.	Kanamycin (K^{30})	30
13.	Neomycin (N^{30})	30
14.	Norfloxacin (Nx^{10})	10
15.	Ofloxacin (OF^{5})	5
16.	Polymyxin-B (Pb^{100})	100
17.	Roxithromycin (Ro^{30})	30
18.	Streptomycin (S^{10})	10
19.	Sulphadiazine (Sz^{300})	300
20.	Tetracycline (T^{30})	30

Chapter-4

EXPERIMENTAL FINDINGS

The present study was undertaken to study the prevalence and antibiogram of Aeromonads in foods of animal origin, water and human diarrheal samples. The molecular epidemiology and *in-vitro* and *in-vivo* virulence characterization of the isolated *Aeromonas* spp. was also studied.

4.1 Prevalence of *Aeromonas* spp. in samples from different sources.

Of the 609 samples comprising water (182), fish (172), chicken (57), mutton (83), beef (31) and human diarrheic stools (83) screened for the presence of *Aeromonas* spp., 155 samples were positive, with an overall prevalence percentage of 25.45 (Table 5).

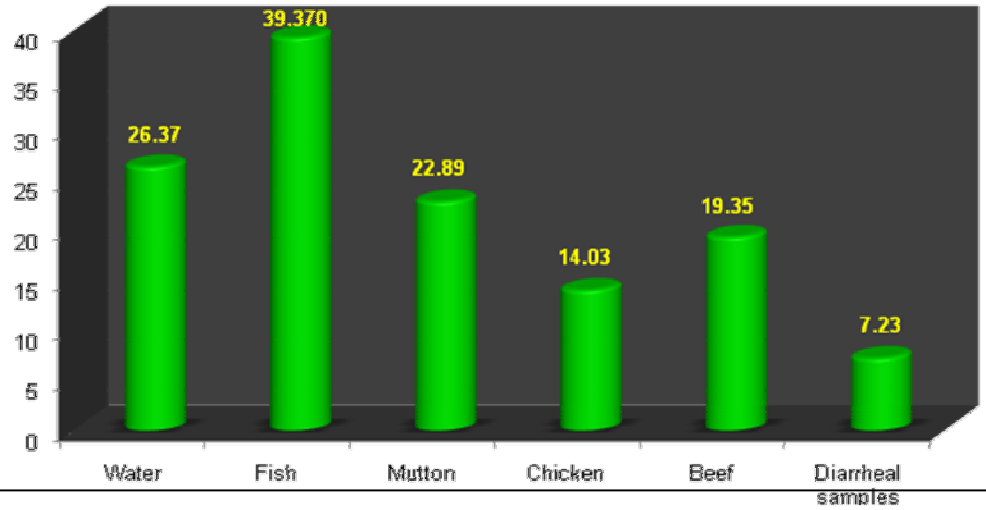
Out of a total of 182 water samples collected from different sources, 48 (26.37%) were positive for the presence of *Aeromonas* spp. (Fig. 1). The organism was also recovered from 12 of the 87 drinking water samples with a prevalence percentage of 13.79. Ten (26.31%) out of 38 water samples from rivers and streams were also found contaminated with *Aeromonas* spp. Likewise, 3 (15.78%) samples from the inlet water source supplying the fish ponds were contaminated with Aeromonads. Water samples from the fish ponds, however, were highly contaminated with *Aeromonas* spp. where 18 (69.23%) out of a total of 26 samples revealed the presence of the organism under study. The results are presented in Table 5.

Aeromonas spp. also contaminated raw meats of all kinds under study and the percentage of contamination for chicken, mutton and beef samples was 14.03, 22.89 and 19.35, respectively. Fish samples collected from retail fish markets were

Table-5: Prevalence of *Aeromonas* spp. in Samples from different Sources.

Nature of the Sample	No. Tested	No. Positive for *Aeromonas* spp.	Per cent Positive for *Aeromonas* spp.
Water Samples			
Drinking Water	87	12	13.79
Rivers and Streams	38	10	26.31
Dal Lake	12	5	41.67
Inlet source to Fish Ponds	19	3	15.78
Fish Ponds	26	18	69.23
Subtotal	**182**	**48**	**26.37**
Fish Samples			
Retail Fish Markets	120	53	44.17
Fish Farms			
Government	33	9	27.27
Private	20	6	30.00
Subtotal	**173**	**68**	**39.30**
Raw Meats			
Mutton	83	19	22.89
Chicken	57	8	14.03
Beef	31	6	19.35
Subtotal	**171**	**33**	**19.29**
Human diarrheal samples			
Government Hospitals	47	4	8.51
Referral Clinics	36	2	5.55
Subtotal	**83**	**6**	**7.23**
Grand Total	**609**	**155**	**25.45**

Fig. 1: Prevalence of *Aeromonas* spp. in different samples



contaminated to an extent of 44.17% whereas, samples from government and private fish farms revealed lesser percentage of contamination of 27.27 and 30.00, respectively. In fish samples the overall percentage prevalence of *Aeromonas* spp. was 39.30.

Of the 83 human diarrheal samples screened, 6 were found positive for the presence of *Aeromonas* spp., with an overall prevalence percentage of 7.22. Prevalence was higher in patients from government hospitals (8.51%) than the referral clinics (5.55%).

4.2. Different *Aeromonas* spp. identified.

The isolates showed typical smooth and yellow colored colonies on Ampicilin Dextrin Agar (Plate 1). Biochemical scheme proposed by Abbott *et al*. (2003), Martin-Carnahan and Joseph (2005) and Janda and Abbott (2010) was used to identify all the isolates of *Aeromonas* recovered from different sources to the species level (Plate 2). *A. hydrophila* (23.87%) was the most common species isolated, followed by *A. caviae* (20.64%), *A. veronii* bv *sobria* (18.06%), *A. salmonicida* (8.39%), *A. popoffi* (6.45%), *A. trota* (5.16%), *A. schubertii* (3.87%), *A. jandaei* (2.58%) and *A. allosaccharophila* (2.58%). However, 13 (8.39%) isolates could not be identified to species level and were identified to generic level only (Table 6, Fig 2).

4.2.1 Water

In all, 48 isolates were recovered from water of which 15 were identified as *A. hydrophila*, 12 as *A. caviae*, 8 as *A. veronii* bv *sobria*, 5 as *A. salmonicida*, 2 each as *A. trota* and *A. popoffii* and 1 isolate as *A. schubertii*. The 3 isolates could, however, be identified to the genus level only. The results are presented in Table 7.

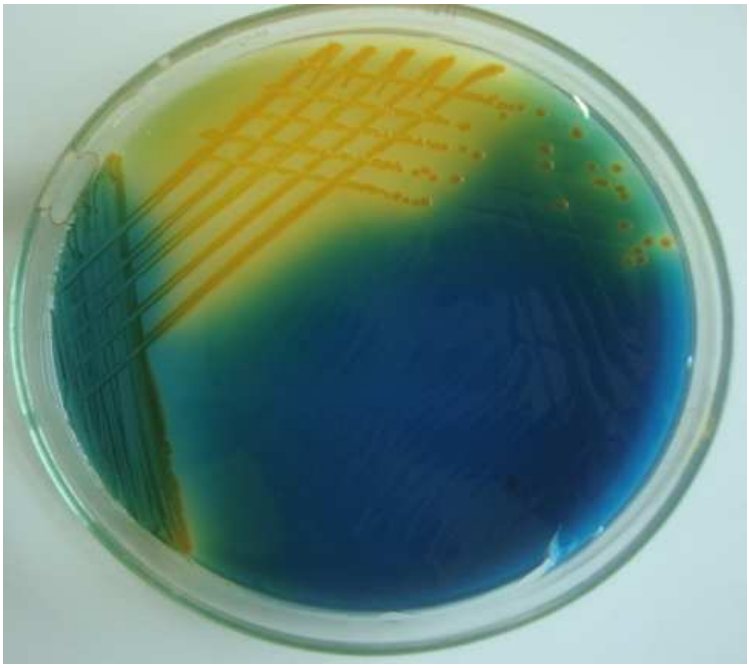


Plate-1 : The colony characteristics of *Aeromonas* spp. on ampicillin dextrin agar

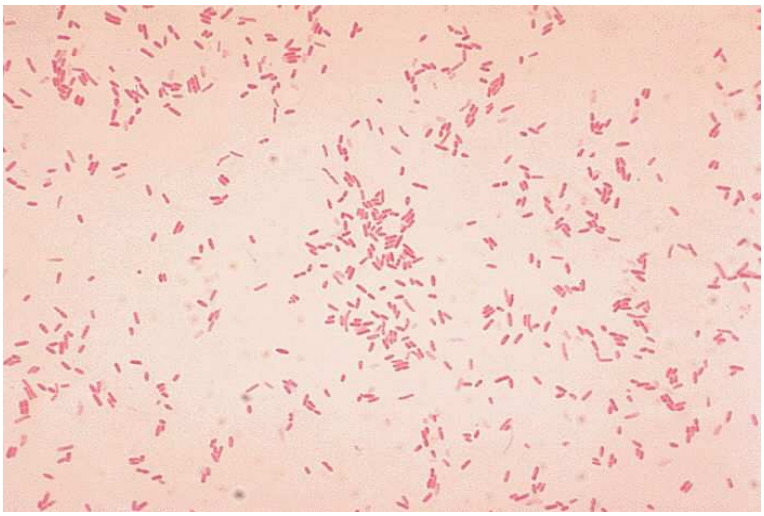


Plate-2 : Gram's Staining of *Aeromonas hydrophila*

Table-6: Distribution of *Aeromonas* spp. in Samples from different Sources.

Species	Water	Fish	Mutton	Chicken	Beef	Stool	Total
A. hydrophila	15	13	3	2	2	2	**37 (23.87)**
A. caviae	12	6	5	3	2	4	**32 (20.64)**
A. veronii bv *sobria*	8	13	4	2	1	Nil	**28 (18.06)**
A. salmonicida	5	8	Nil	Nil	Nil	Nil	**13 (8.39)**
A. popoffii	2	8	Nil	Nil	Nil	Nil	**10 (6.45)**
A. trota	2	4	2	Nil	Nil	Nil	**8 (5.16)**
A. schubertii	1	4	Nil	1	Nil	Nil	**6 (3.87)**
A. jandaei	Nil	3	1	Nil	Nil	Nil	**4 (2.58)**
A. allosaccharophila	Nil	2	2	Nil	Nil	Nil	**4 (2.58)**
Unidentified spp.	3	7	2	Nil	1	Nil	**13 (8.39)**
Total	**48**	**68**	**19**	**8**	**6**	**6**	**155**

Note: The figures in the parentheses indicate the percentages.

In the drinking water samples, the predominant species identified was *A. hydrophila* (6) followed by *A. veronii* bv *sobria* (4), *A. caviae* (1) and *A. trota* (1). Among 10 isolates recovered from rivers and streams, 3 each were identified as *A. hydrophila* and *A. caviae*, and 2 each as *A. veronii* bv *sobria* and *A. salmonicida*.

Fig. 2: Different *Aeromonas* species identified from all sources

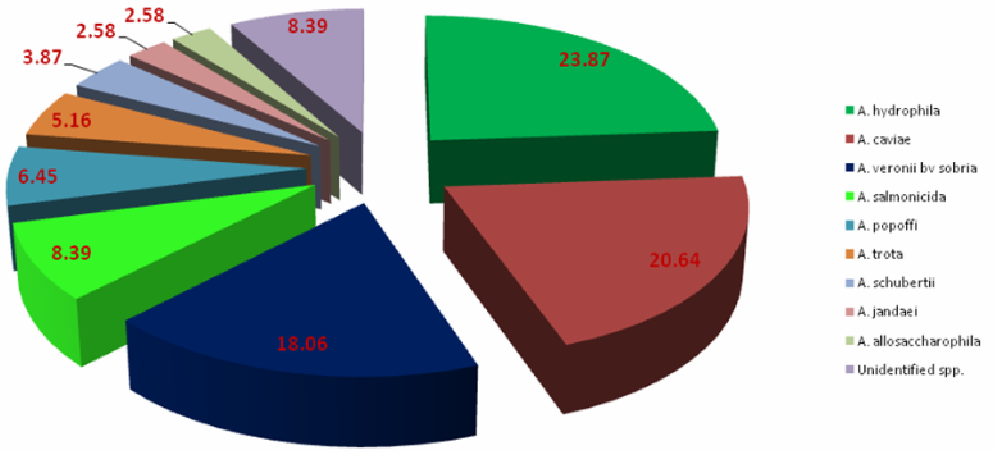


Table-7: Distribution of *Aeromonas* spp. in Water from different Sources.

Source/ Species	Drinking Water	Rivers and Streams	Dal Lake	Inlet Sources of Fish Ponds	Fish Ponds	Total
A. hydrophila	6	3	1	2	3	**15 (31.25)**
A. caviae	1	3	3	Nil	5	**12 (25)**
A. veronii bv *sobria*	4	2	Nil	Nil	2	**8 (16.67)**
A. salmonicida	Nil	2	Nil	1	2	**5 (10.41)**
A. trota	1	Nil	Nil	Nil	1	**2 (4.16)**
A. popoffii	Nil	Nil	Nil	Nil	2	**2 (4.16)**
A. schubertii	Nil	Nil	Nil	Nil	1	**1 (2.08)**
Aeromonas spp.	Nil	Nil	1	Nil	2	**3 (6.25)**
Total	**12**	**10**	**5**	**3**	**18**	**48**

Note: The figures in the parentheses indicate the percentages.

Aeromonas species recovered from Dal Lake were *A. caviae* (3) and *A. hydrophila* (1) while one isolate could not be identified to the species level and was assigned the generic name only. The water source supplying the fish ponds was also contaminated with *Aeromonas* spp., with 3 isolates identified as *A. hydrophila* and 1 as *A. salmonicida.* Samples from fish ponds revealed higher contamination with *A. caviae*

(5) followed by, *A. hydrophila* (3), *A. veronii* bv *sobria* (2). *A. salmonicida* (2), *A. popoffii* (2), *A. trota* (1), *A. schubertii* (1) and unidentified *Aeromonas* spp. (2).

4.2.2 Foods of Animal Origin

A total of 68 isolates could be recovered from fish samples of which the most prevalent species were *A. hydrophila* and *A. veronii* bv *sobria* with a prevalence percentage of 22.05 and 16.17%, respectively. The other species recovered from the fish were *A. popoffii* (11.76%), *A. salmonicida* (11.75%), *A. caviae* (8.82%), *A. trota* (5.88%), *A. schubertii* (5.88%), *A. jandaei* (4.41%). Seven isolates, however, could not be identified (Table 8). Fish samples from retail market showed the highest percentage of prevalence (77.94), whereas samples from government and private fish farms revealed the prevalence percentage of 13.23 and 8.82, respectively (Table 8).

Of the 19 isolates recovered from mutton, 26.31% accounted for *A. caviae*. Other species contaminating mutton included *A. veronii* bv *sobria* (21.05%), *A. hydrophila* (15.78%), *A. trota* (10.52%), *A. allosaccharophila* (10.52%) and *A. jandaei* (5.26%). Two isolates (10.29%), however, could not be identified. In a total of 8 isolates recovered from chicken, 3 (37.50%) belonged to *A. caviae*, *2* each (25.00%) to *A. veronii* bv *sobria* and *A. hydrophila* and 1 (12.50%) to *A. schubertii*. Six isolates from beef samples were identified as *A. caviae* (33.33%), *A. hydrophila* (33.33%) and *A. veronii* bv *sobria* (16.17%). One (16.17%) isolate could only be identified to the generic level (Table 6).

4.2.3 Human Diarrheic stools

Six isolates were recovered from human diarrheal samples of which 4 (66.67%) were identified as *A. caviae* and 2 (33.33%) as *A. hydrophila* (Table 6).

Table-8: Distribution of *Aeromonas* spp. in Fish Samples from different sources.

Species	Fish			Total
	Market	Govt. Farms	Pvt. Farms	
A. hydrophila	10	3	2	**15 (22.05)**
A. veronii bv *sobria*	8	2	1	**11 (16.17)**
A. salmonicida	5	3	Nil	**8 (11.76)**
A. popoffii	8	Nil	Nil	**8 (11.76)**
A. caviae	4	Nil	2	**6 (8.82)**
A. trota	4	Nil	Nil	**4 (5.88)**
A. schubertii	4	Nil	Nil	**4 (5.88)**
A. jandaei	2	Nil	1	**3 (4.41)**
A. allosaccharophila	2	Nil	Nil	**2 (2.94)**
Aeromonas spp.	6	1	Nil	**7 (10.29)**
Grand Total	**53 (77.94)**	**9 (13.23)**	**6 (8.82)**	**68**

Note: The figures in the parentheses indicate the percentages.

4.3 Detection of *Aeromonas* Genus by Molecular Methods

The phenotypic and genotypic characteristics of Aeromonads are incongruous and therefore, for accurate classification of the genus, multiple methods are required. The methods that are frequently employed with high degrees of accuracy are polymerase chain reaction and the restriction fragment length polymorphism.

A pair of primers was used for confirmation of the genus *Aeromonas* (Plate-3) and among a total of 155 *Aeromonas* spp. identified phenotypically, the genus specific PCR detected 153 isolates as *Aeromonas*. The two genotypically unidentified isolates belonged to *A. salmonicida* and *Aeromonas* spp.

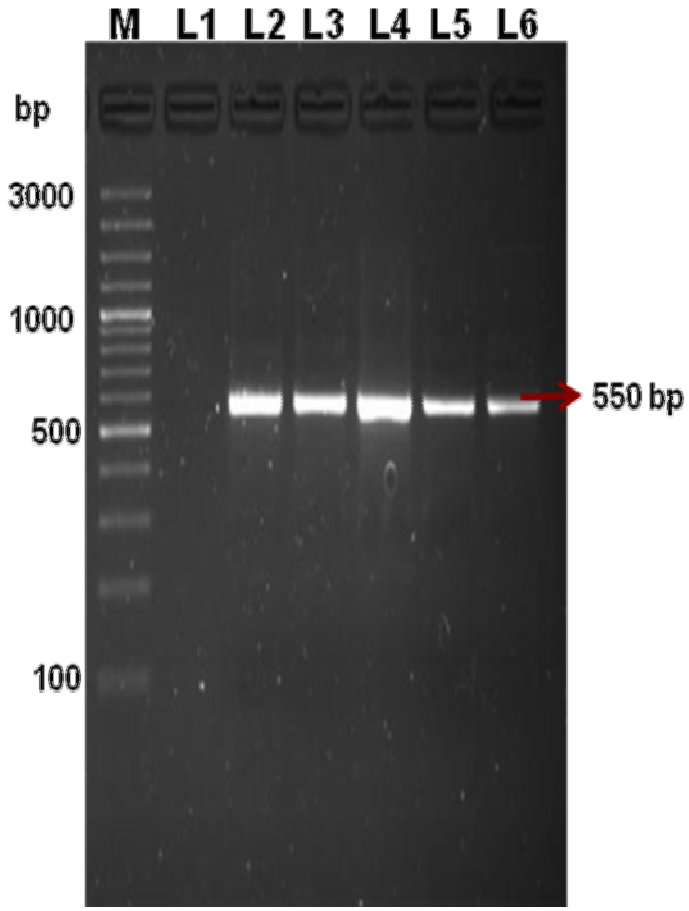


Plate-3 : Agarose gel (1.5%) electrophoresis showing 16±23S rDNA genus specific amplified band of *Aeromonas* spp.

M presents 100 bp plus DNA ladder.

L1, negative control (*B. cereus* ATCC 14579)

L2, *A. hydrophila*; L3, *A. veronii* bv *sobria*; L4, *A. caviae*; L5, *A. jandaei*; L6, *A. schubertii*

Note: bp indicates base pairs

4.4 Restriction Fragment Length Polymorphism (RFLP)

A combination of two enzymes, endonucleases *Alu*I (AGCT) and *Mbo*I (GATC) was used to cleave the amplified 16S rRNA gene sequences (1502 bp) of *Aeromonas* spp. (Plate-4). The resulting DNA fingerprints (ranging from 33 to 346 bp) constituted specific patterns that could be used to identify strains to the level of phylogenetic species (Table 9). The RFLP patterns of the PCR-amplified 16S rRNA genes of some *Aeromonas* species are presented in Plate-5. Only RFLP fragments ranging from 69 to 346 bp were taken into account. *A. salmonicida* was differentiated from *A. encheleia* using enzymes *Nar*I (GGCGCC) and *Hae*III (GGCC). The strains were analyzed by RFLP of PCR-amplified 16S rRNA genes with the selected enzymes *Alu*I and *Mbo*I with those identified by biochemical tests. Majority of the findings from these two different approaches were in agreement, but some discrepancies appeared. Two presumed *A. veronii* bv *sobria* isolates were classified as *A. hydrophila* and *A. caviae* by RFLP analysis. A strain of *A. caviae*, as shown by the 16S rRNA gene restriction patterns was biochemically characterized as *A. hydrophila*. One strain of *A. caviae* was classified by RFLP analysis as *A. media*. The three biochemically unidentified strains presented the RFLP patterns of *A. hydrophila* (2) and *A. caviae* (1).

4.5 Molecular Epidemiology

The molecular epidemiology of the species was studied by Random Amplification of Polymorphic DNA (RAPD) and Enterobacterial Repetitive Intergenic Consensus Sequence (ERIC) PCR.

4.5.1 RAPD PCR

The strains were characterized for the study of genetic relatedness by RAPD PCR, only 1or the maximum of three bands were amplified (Plate 6), therefore

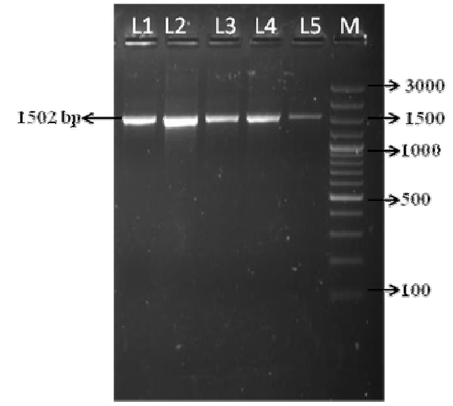


Plate-4 : **Agarose gel (1.5%) electrophoresis showing amplified 16S rRNA gene of *Aeromonas* spp.**
M indicates DNA marker 100 bp plus
L1 presents isolates of *A. hydrophila*; L2-*A. jandaei*; L3-*A. trota*; L4-*A. schubertii*; L5-*A. caviae*

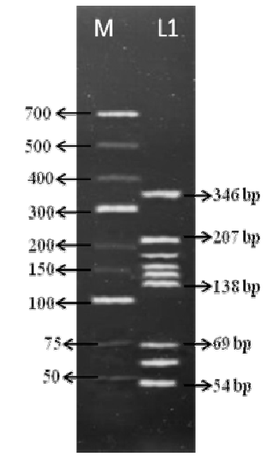


Plate-5 : **Agarose gel (3%) electrophoresis showing RFLP Pattern of *A. hydrophila* by *Alu*I and *Mbo*I enzymes.**
M presents low range molecular marker
L1 Presents RFLP pattern of *A. hydrophila* strain

Table-9 : DNA fragments obtained from the 16S rRNA gene sequence of *Aeromonas* spp. with endonucleases *Alu*I and *Mbo*I

Species	346	242	228	211	207	204	195	188	180	174	172	165	158	157	138	118	78	69	66	54	47	42	40	33
A. hydrophila	+				+		+					+		+	+			+	+	+		+		+
A. veronii bv *sobria*					+		+			+			+	+	+		+	+	+	+	+	+	+	+
A. salmonicida	+				+		+	+				+		+	+	+		+	+	+	+	+		+
A. caviae					+		+	+	+			+	+	+				+	+	+				+
A. trota		+			+		+		+			+	+	+				+	+					+
A. schubertii			+		+		+		+		+			+			+	+	+		+		+	+
A. jandaei					+		+	+					+	+	+		+	+	+	+	+	+	+	+
A. allosaccharophila					+	+	+			+	+			+		+		+		+	+	+		+

enough data was not generated to study the genetic relatedness and therefore the epidemiology of the isolates.

4.5.2 ERIC PCR

The fingerprints of *Aeromonas* spp. by ERIC PCR consisted of 2 to 11 amplification bands, ranging in size from 100 to 3,500 bp (Plate 7). The typing was done to study the epidemiology of the isolates and all strains were typeable by ERIC-PCR. Reproducibility of the DNA patterns from different gels was in the range of 98 to 100%. Among the 37 strains of *A. hydrophila* examined, a high variability was observed (Fig. 3), however, some isolates recovered from water and fish were related (AHW10 and AHF4; AHW6 and AHW12; AHW11, AHW15 and AHF7; AHW8 and AHW13; HAW5 and AHF9). The isolates of *A. hydrophila* from other sources showed less relatedness. The *A. caviae* showed high relatedness (Fig. 4), five closely related clusters were formed by the strains. One cluster was formed by water, fish and mutton isolates (ACW1, ACW4, ACW11, ACF6 and ACM4) and the second by mutton and beef isolates (ACM1, ACM3 and ACB2), third cluster was formed by isolates from fish (ACF2 and ACF5). The clinical isolates from *A. caviae* were identical to isolates recovered from water (ACD2 with ACW2 and ACW9 and ACD4 with ACW10). However, the clinical isolates were not related much each other. While analyzing the relatedness among *A. veronii* bv *sobria* strains from different sources (Fig. 5), it was observed that some isolates recovered from water were related to each other (AVW2, AVW3 and AVW6) and from fish as well (AVF2 and AVF7; AVF5 and AVF8; AVF11 and AVF13). Two isolates of *A. veronii* bv *sobria* recovered from fish were related to water (AVF3 with AVW2, AVW3 and AVW6 and AVF6 with AVW5) and interestingly there was also relatedness among a isolate from beef and chicken (AVC1 and AVB1). There was close relatedness among the isolates of *A. salmonicida* (Fig. 6) recovered from water and fish (ASW2,

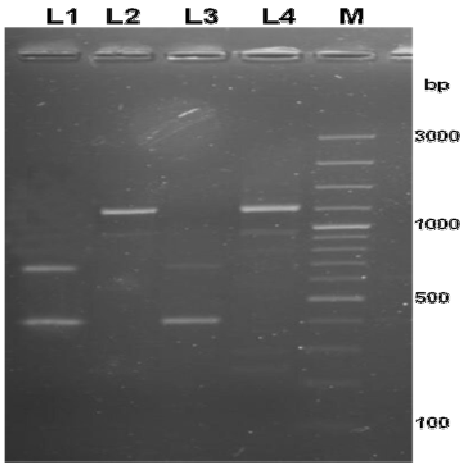


Plate-6 : Agarose gel (1.5%) electrophoresis showing amplified RAPD gene fingerprinting of *A. hydrophila*

M indicates DNA marker 100 bp plus; Lane 1 to 4 indicates RAPD Profile of *A. hydrophila* isolated from water, fish, mutton and chicken, respectively.

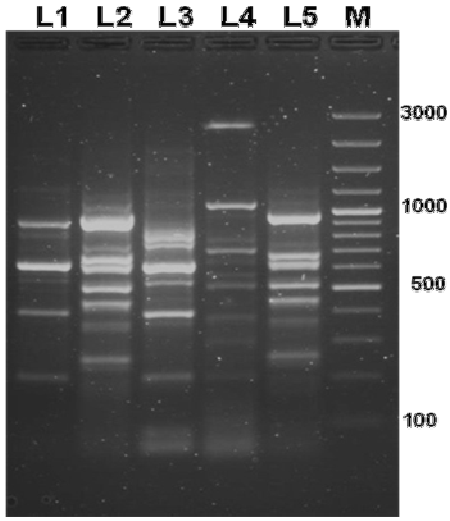
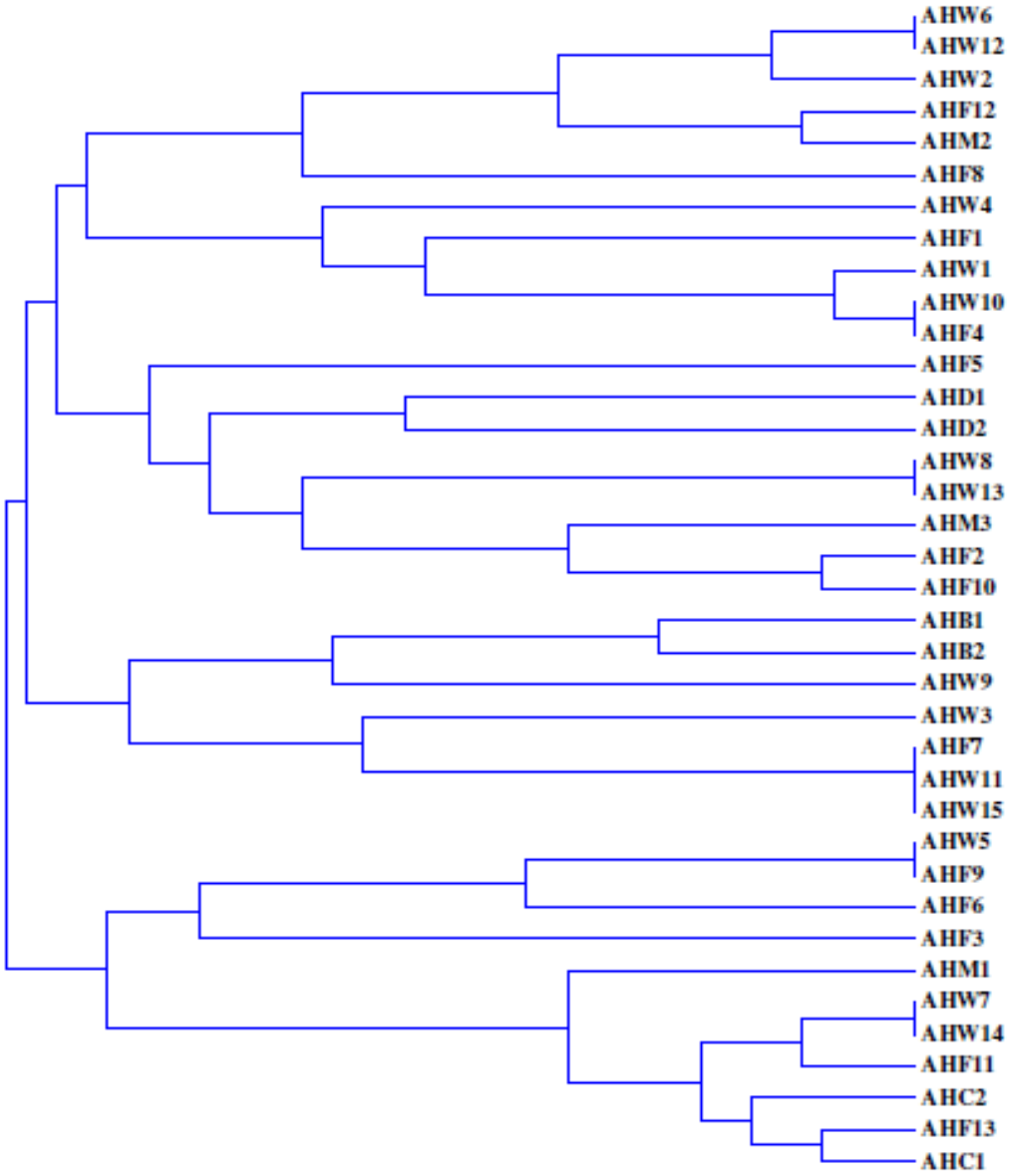


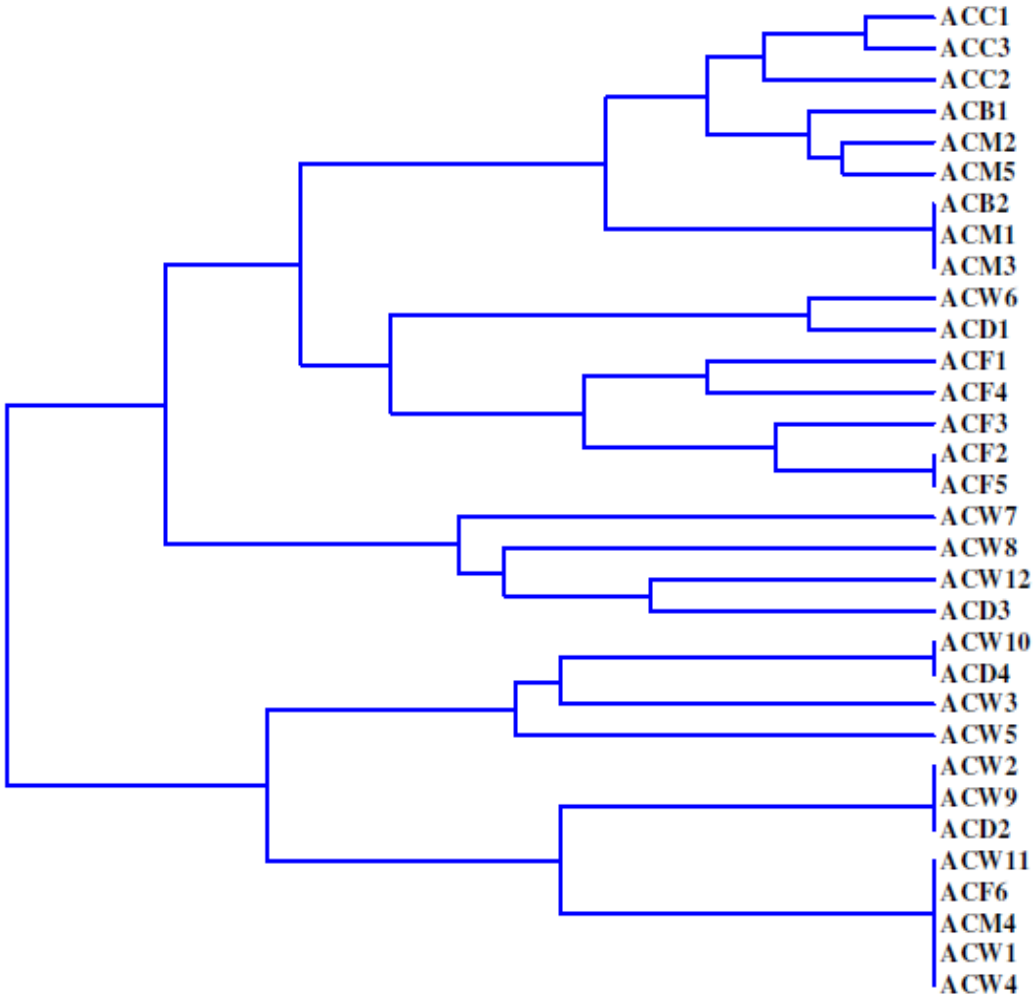
Plate-7 : Agarose gel (2%) electrophoresis showing amplified ERIC gene fingerprinting of *A. caviae*

M indicates DNA marker 100 bp plus; Lane 2 and 5 presents ERIC Profile of *A. caviae* isolated from water and human diarrheal samples; Lane 1 presents *A. caviae* isolated from water; Lane 3 presents *A. caviae* isolated from mutton; Lane 4 presents *A. caviae* isolated from chicken



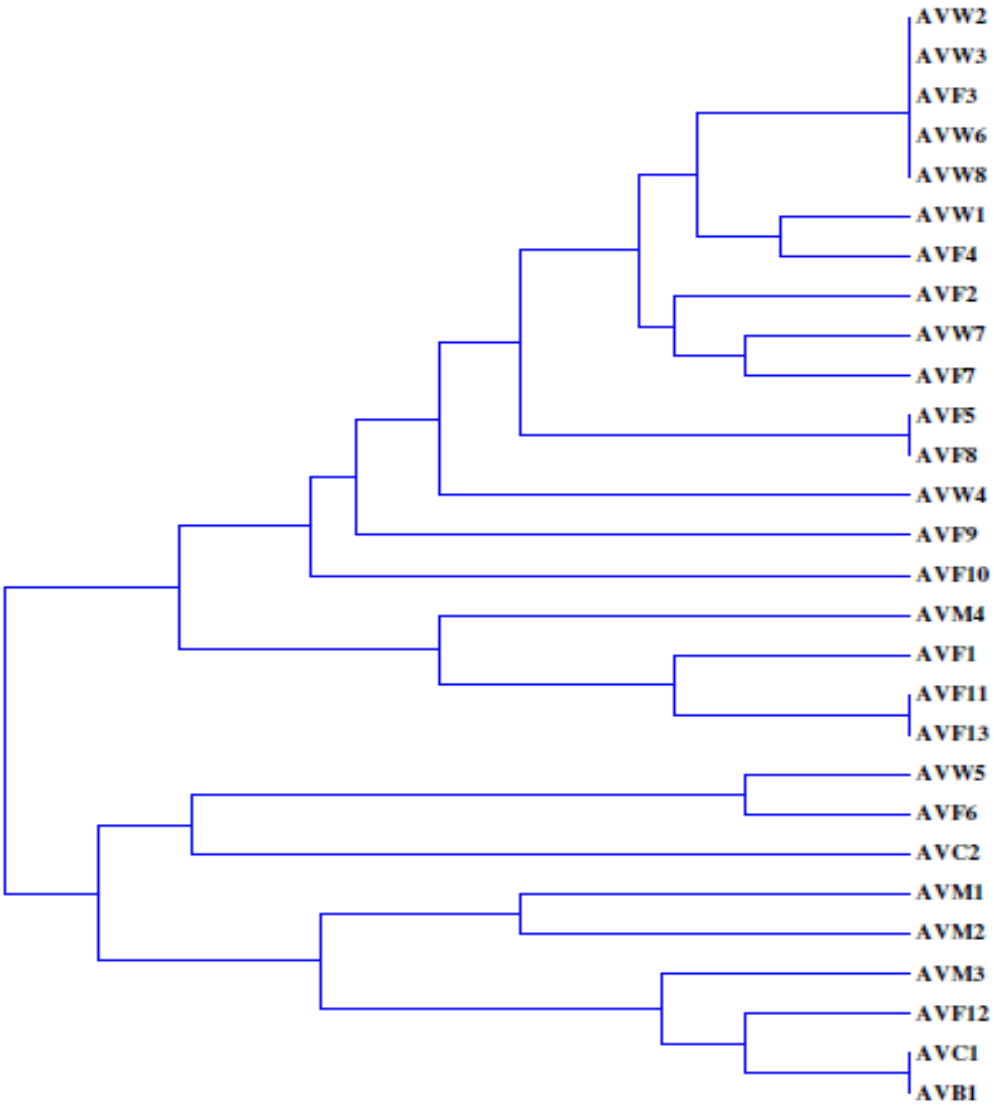
AHW= *A. hydrophila* from water; AHF= *A. hydrophila* from fish; AHM= *A. hydrophila* from mutton; AHB= *A. hydrophila* from beef; AHC= *A. hydrophila* from chicken and AHD= *A. hydrophila* from human diarrheal sample.

Fig. 3 : Dendrogram showing genetic relatedness among *A. hydrophila* strains (37) determined by ERIC-PCR fingerprinting.



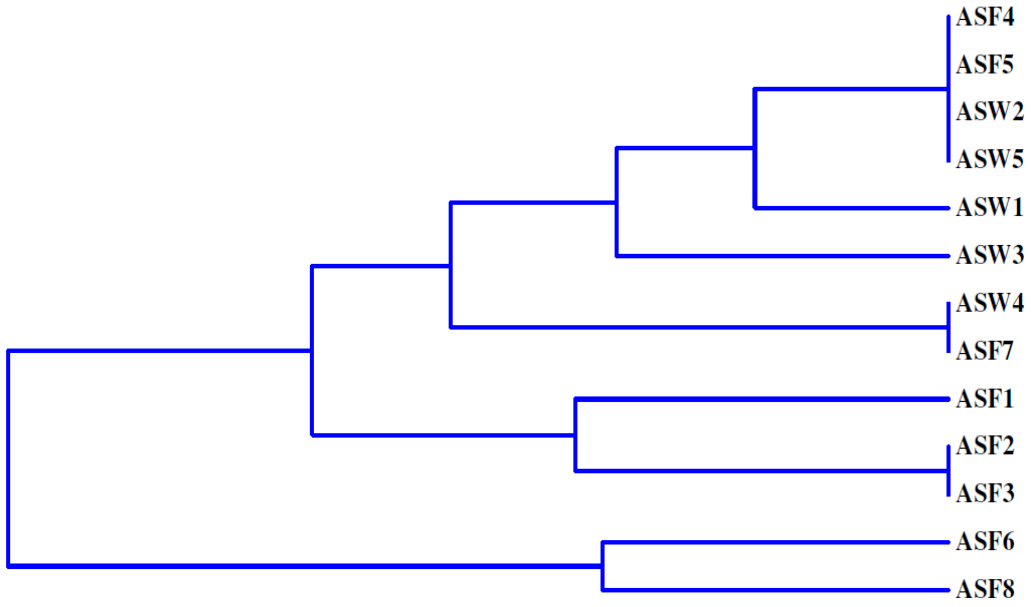
ACW = *A. caviae* from water; ACF = *A. caviae* from fish; ACM= *A. caviae* from mutton; ACB = *A. caviae* from beef; ACC= *A. caviae* from chicken and ACD= *A. caviae* from human diarrheal sample.

Fig. 4 : Dendrogram showing genetic relatedness among *A. caviae* strains (32) determined by ERIC-PCR fingerprinting.



AVW = *A. veronii* bv *sobria* from water; AVF = *A. veronii* bv *sobria* from fish; AVM= *A. veronii* bv *sobria* from mutton; AVB= *A. veronii* bv *sobria* from beef and AVC= *A. veronii* bv *sobria* from chicken

Fig. 5 : Dendrogram showing genetic relatedness among *A. veronii* bv *sobria* strains (28) determined by ERIC-PCR fingerprinting.



ASW= *A. salmonicida* from water; ASF= *A. salmonicida* from Fish

Fig. 6 : Dendrogram showing genetic relatedness among *A. salmonicida* strains (13) determined by ERIC-PCR fingerprinting

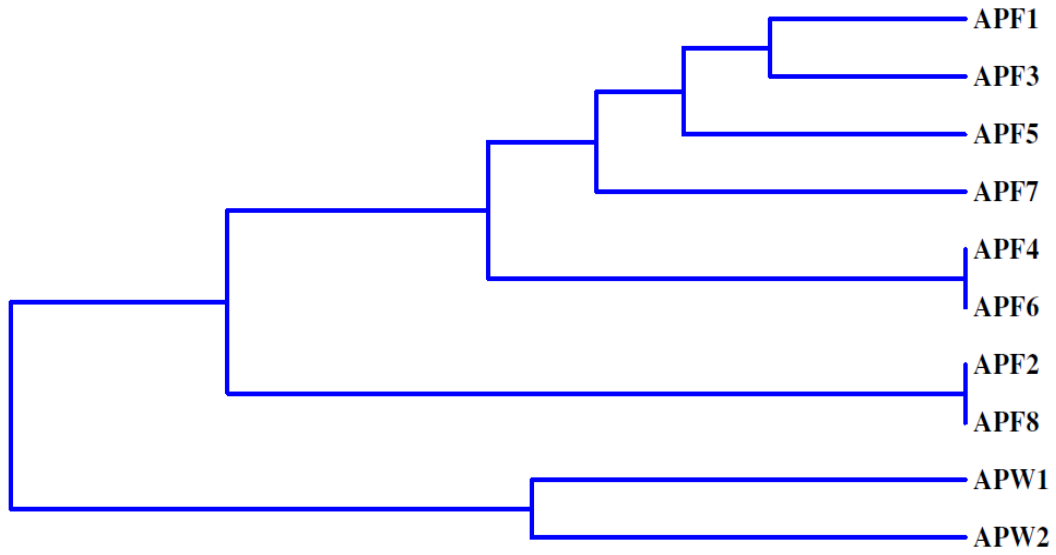
ASW5, ASF4 and ASF5; ASW4 and ASF7). The two isolates from fish were identical (ASF2 and ASF3). The *A. popoffii* isolates (Fig. 7) from water formed close cluster and the identical fish isolates were APF4 and APF6 and APF2 and APF8. The other *A. popoffii* isolates formed close cluster but relatedness was between 70 to 85%. The *A. trota* strains showed less relatedness amongst each other (Fig. 8), however, the two isolates from fish (ATF1 and ATF3) were identical showing same DNA profile. Two clusters were formed by *A. schubertii* isolates (Fig. 9) and the isolates from fish (AShF2 and AShF3) were identical. Among the four isolates of *A. jandaei*, isolate from mutton and fish (AJM1 and AJF3) showed 75% similarity (Fig.10). The four isolates of *A. allosaccharophila* (Fig. 11) were less related and separate cluster was formed by isolates from fish and isolates from water. The two isolates of *A. allosaccharophila* from mutton (AAM1 and AAM2) showed 75% of relatedness. The 13 unidentified *Aeromonas* spp. (Fig. 12) exhibited a high genetic variability, the isolates from mutton, however, showed good similarity (89%). The unidentified isolates from fish formed distinct cluster when compared to isolates from water, beef and mutton.

4.6 Virulence Characterization

4.6.1 Enterotoxin gene profile

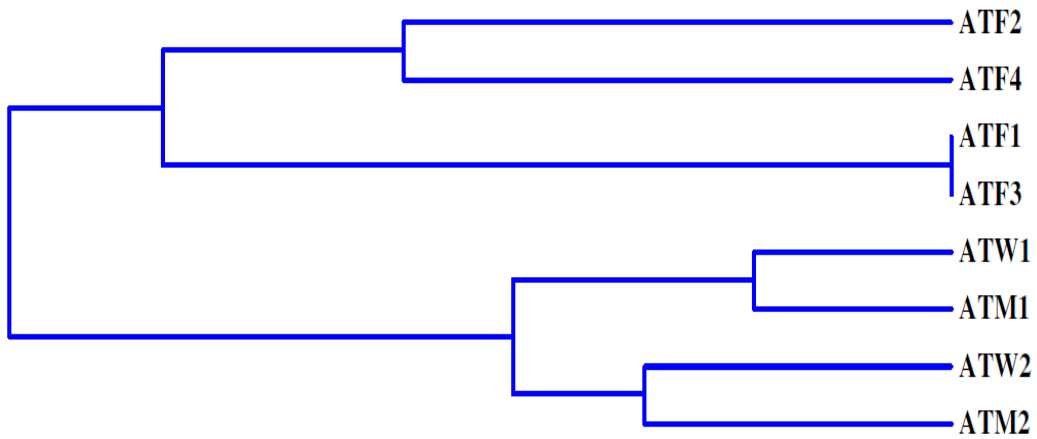
4.6.1.1 Detection of enterotoxin genes (*act*, *alt* and *ast*) in Aeromonads by Multiplex PCR

All the 155 isolates of *Aeromonas* spp. from different sources were screened for the presence of three enterotoxin genes encoding cytotoxic enterotoxin (*act*), cytotonic heat labile enterotoxin (*alt*) and cytotonic heat stable enterotoxin (*ast*) using the novel multiplex PCR assay. Under the assigned multiplex PCR condition, three fragments of enterotoxin genes were amplified having the predicted size of 819, 576 and 216 bp fragments for *act*, *alt* and *ast* genes, respectively (Plate-8). Five enterotoxin gene patterns, including *act*, *alt*, *act/alt*, *act/alt/ast*, and *act/ast* (Table 10,



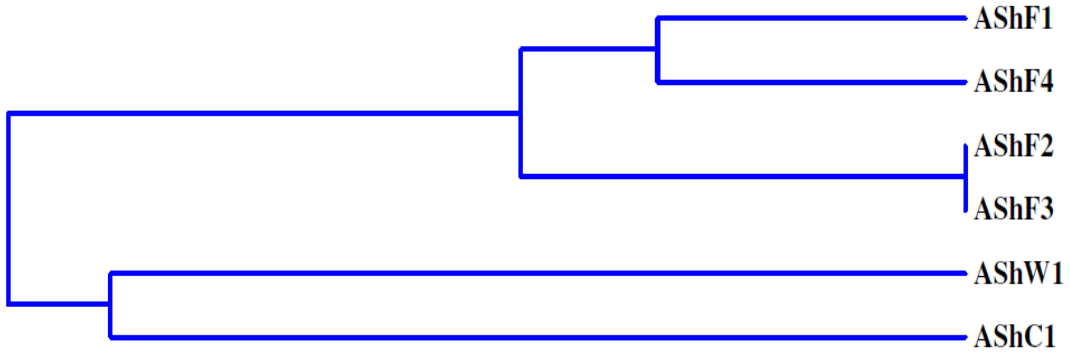
APW= *A. popoffi* from water; APF= *A. popoffi* from Fish

Fig. 7 : Dendrogram showing genetic relatedness among *A. popoffii* strains (10) determined by ERIC-PCR fingerprinting



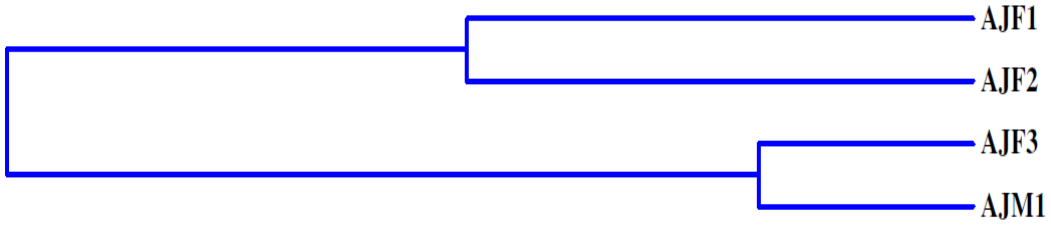
ATW= *A. trota* from water; ATF= *A. trota* from Fish; ATM= *A. trota* from mutton

Fig. 8 : Dendrogram showing genetic relatedness among *A. trota* strains (8) determined by ERIC-PCR fingerprinting



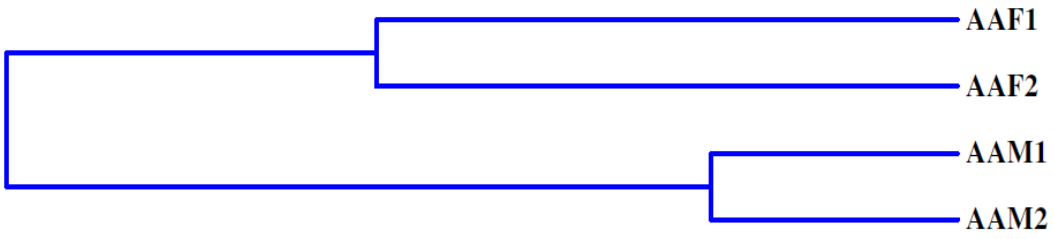
AShW = *A. schubertii* from water; AShF= *A. schubertii* from fish; AShC= *A. schubertii* from chicken

Fig. 9 : Dendrogram showing genetic relatedness among *A. schubertii* strains (6) determined by ERIC-PCR fingerprinting



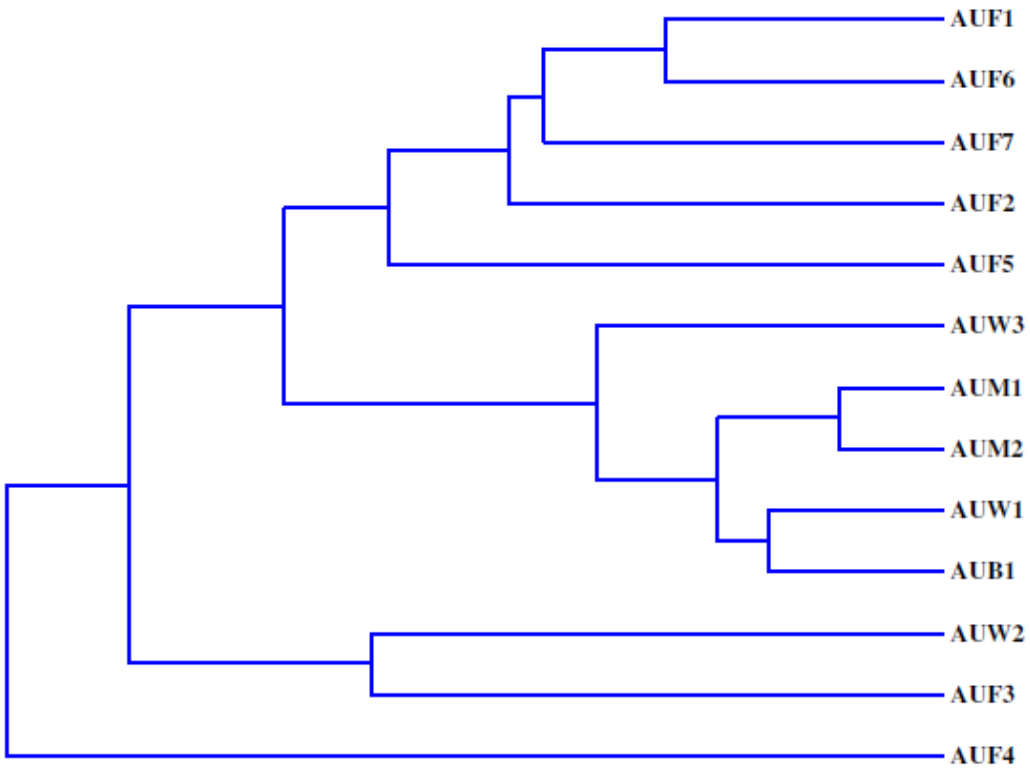
AJF= *A. jandaei* from fish; AJM= *A. jandaei* from mutton

Fig. 10 : Dendrogram showing genetic relatedness among *A. jandaei* strains (4) determined by ERIC-PCR fingerprinting



AAF= *A. allosaccharophila* from fish; AAM= *A. allosaccharophila* from mutton

Fig. 11 : Dendrogram showing genetic relatedness among *A. allosaccharophila* strains (4) determined by ERIC-PCR fingerprinting



AUW= unidentified from water; AUF= unidentified from Fish; AUM= unidentified from mutton; AUB= unidentified from beef.

Fig. 12 : Dendrogram showing genetic relatedness among unidentified *Aeromonas* spp. (13) determined by ERIC-PCR fingerprinting

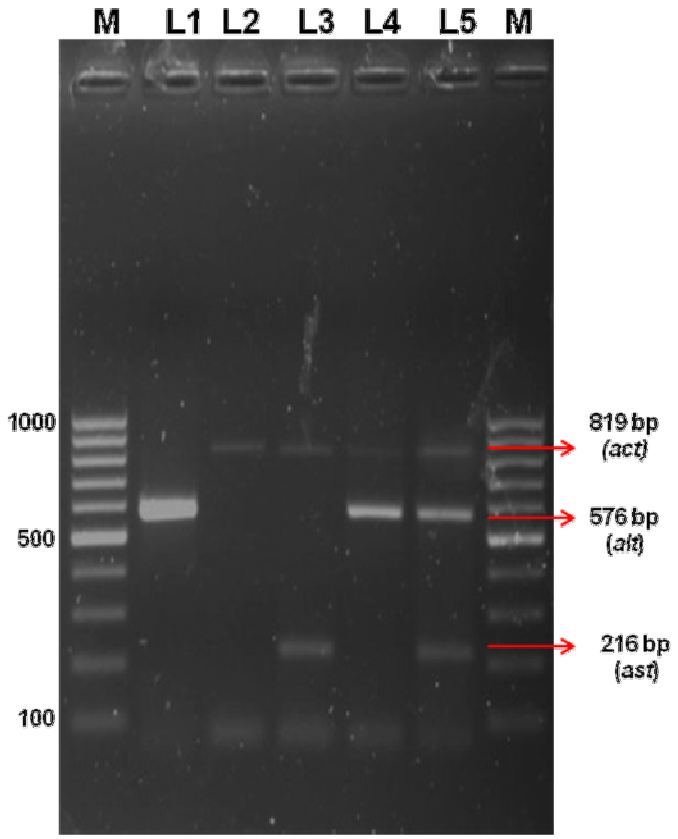


Plate-8 : Agarose gel (1.5%) electrophoresis showing five different enterotoxin gene patterns of *Aeromonas* spp.

M Presents 100 bp DNA ladder
L1 indicates presence of *alt* gene in *Aeromonas* spp.
L2 indicates presence of *act* gene in *A. veronii* bv *sobria*
L3 indicates presence of *act* and *ast* genes in *A. caviae*
L4 indicates presence of two enterotoxin genes (*act/alt*) in *A. hydrophila*
L5 shows presence of all the three enterotoxin genes (*ast/alt/ast*) in *A. hydrophila*

Fig. 13), were identified. The *act* gene was detected in 104 (67.09%) isolates. In 21 (13.54%) isolates, *act* gene occurred alone whereas in 8 (5.16%) isolates, it appeared in combination with the *alt* and *ast* genes, in 73 (47.09%), it was present with *alt* gene and in 3(1.93%), it occurred with *ast* gene. The *alt* gene was detected in 98 (63.22%) isolates, either alone (10.96%) or in combination with the *act*/*ast* genes (5.16%) and with *alt* in 73 (47.09%) isolates. The *ast* was the least prevalent gene (7.06%) and always expressed itself in combination with other enterotoxin genes (with *alt*/*act* in 8 isolates and with *act* in 3 isolates).

Of the 155 isolates of Aeromonads, 122 (78.70%) carried one or more enterotoxin genes. Among 37 isolates of *A. hydrophila*, one or more enterotoxin genes were detected in 35 (94.59%) isolates. The *act* (86.48%) and *alt* (83.78%) were prominent genes in *A. hydrophila*, whereas, *ast* gene occurred in only 2 (5.40%) isolates (Fig. 14). In *A. caviae*, 30 (93.75%) isolates carried one or more enterotoxin genes but 2 isolates were devoid of any genes. The *alt* (90.25%) gene was more prevalent in *A. caviae* and *act* and *ast* genes were detected in 25 (78.12%) and 5 (15.62%) isolates, respectively. Of the 28 isolates of *A. veronii* bv *sobria*, 24 (85.71%) were positive for one or the other enterotoxin genes, with *act* (85.71%) being the predominant enterotoxin gene followed by *alt* (67.85%) and *ast* (7.14%) genes. In *A. salmonicida*, *A. popoffii*, *A. trota*, *A. schubertii*, *A. jandaei*, *A. allosaccharophila* and *Aeromonas* spp. one or more enterotoxin genes were detected in 30.69, 40.00, 62.50, 16.67, 75.00, 50.00 and 92.30% isolates, respectively. The *act* gene was detected in 30.77%, 30.00%, 50.00%, 16.67%, 50.00%, 25.00% and 61.54% of *A. salmonicida*, *A. popoffii*, *A. trota*, *A. schubertii*, *A. jandaei*, *A. allosaccharophila* and *Aeromonas* spp., respectively. The *alt* gene was present in *A. salmonicida* (15.38%), *A. popoffii* (10%), *A. trota* (37.5%), *A. jandaei* (50%), *A. allosaccharophila* (50%) and *Aeromonas* spp. (69.23%). The *ast* gene was absent in most of the species (*A. salmonicida*, *A. popoffii*, *A. schubertii*, *A. jandaei*,

Table-10: Enterotoxin Gene Pattern and Profile of *Aeromonas* spp.

Species	No. of Isolates	Enterotoxin Gene Pattern						Enterotoxin Gene Profile		
		act/alt/ast	*act/alt*	*act/ast*	*act*	*alt*	None	*act* (%)	*alt* (%)	*ast* (%)
A. hydrophila	**37**	2	26	-	4	3	2	32 (86.48)	31 (83.78)	2 (5.40)
A. caviae	**32**	4	21	1	-	4	2	25 (78.12)	29 (90.25)	5 (15.62)
A. veronii bv *sobria*	**28**	1	16	1	6	2	2	24 (85.71)	19 (67.85)	2 (7.14)
A. salmonicida	**13**	-	2	-	2	-	9	4 (30.77)	2 (15.38)	-
A. popoffii	**10**	-	-	-	3	1	6	3 (30.00)	1 (10.00)	-
A. trota	**8**	1	1	-	2	1	3	4 (50.00)	3 (37.50)	1 (12.50)
A. schubertii	**6**	-	-	-	1	-	5	1 (16.67)	-	-
A. jandaei	**4**	-	1	-	1	1	1	2 (50.00)	2 (50.00)	-
A. allosaccharophila	**4**		1	-	0	1	2	1 (25.00)	2 (50.00)	-
Aeromonas spp.	**13**	-	5	1	2	4	1	8 (61.54)	9 (69.23)	1 (7.70)
Total	**155**	**8 (5.16)**	**73 (47.09)**	**3 (1.93)**	**21 (13.54)**	**17 (10.96)**	**33 (21.90)**	**104 (67.09)**	**98 (63.22)**	**11 (7.06)**

Note: The figures in the parentheses indicate the percentages.

Fig. 13: Occurrence of different enterotoxin gene patterns in *Aeromonas* spp.

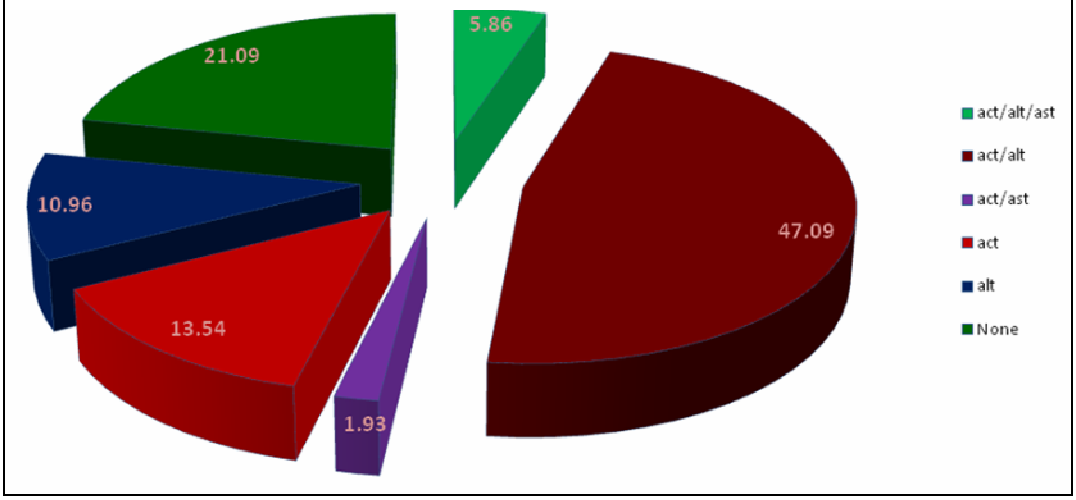
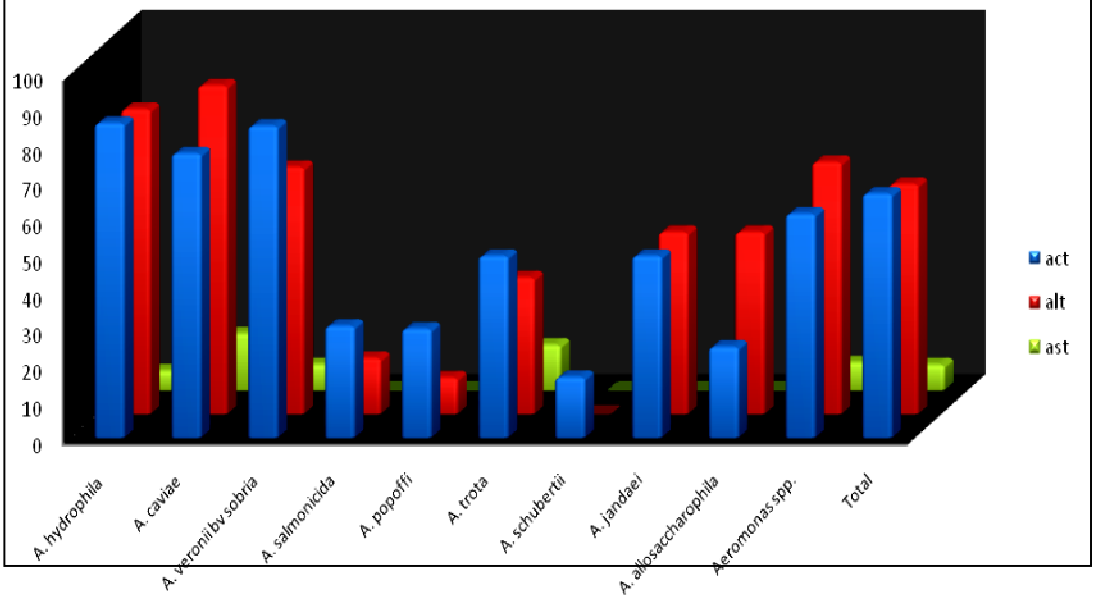


Fig. 14: Enterotoxin gene profile in different species of *Aeromonas*



A. allosaccharophila and *Aeromonas* spp., respectively. The *alt* gene was present in *A. salmonicida* (15.38%), *A. popoffii* (10%), *A. trota* (37.5%), *A. jandaei* (50%), *A. allosaccharophila* (50%) and *Aeromonas* spp. (69.23%). The *ast* gene was absent in most of the species (*A. salmonicida, A. popoffii, A. schubertii, A. jandaei, A. allosaccharophila*) but *A. trota* (12.5%) and *Aeromonas* spp. (7.7%) carried the *ast* enterotoxin gene.

4.6.1.2 Distribution of Enterotoxin genes in Aeromonads from Different sources

The number of *A. hydrophila* strains carrying enterotoxin genes recovered from water, fish, mutton, chicken, beef and human diarrhea were 14 (93.33%), 12 (86.15%), 3 (100%), 2 (100%), 2 (100%) and 2 (100%), respectively, (Table 11). In *A. hydrophila*, *act* (32) and *alt* (31) were predominant enterotoxin genes recovered from all the sources. The only two *ast* gene positive strains of *A. hydrophila* were recovered from mutton and human diarrheal sample. Among the *A. caviae* strains isolated from water, fish, mutton, chicken, beef and human diarrhea; 11 (91.67%), 6 (100%), 4 (80%), 3 (100%), 2 (100%) and 4 (100%) were, respectively, positive for one or more enterotoxin genes. Among the 30 (93.75%) enterotoxin gene positive strains of *A. caviae*; *act, alt* and *ast* genes occurred in 25 (80.64%), 29 (93.54%) and 5 (16.1%) strains, respectively. The enterotoxin gene positive *A. veronii* bv *sobria* strains were present in 7 (26.92%), 12 (38.46%), 4 (15.38%), 2 (7.69%) and 1 (3.84%) isolates. The *ast* (92.30%) and *alt* (73.07%) genes were prevalent and *ast* was detected in 2 (7.69%) isolates of *A. veronii* bv *sobria*. The experimental results revealed that 20% strains of *A. salmonicida* from water harboured one or more enterotoxin genes. Likewise, 50% strains of *A. trota* and 67.67% of *Aeromonas* spp., from water were also positive for one or more enterotoxin genes. In samples from fish, the enterotoxin genes were present in strains of *A. salmonicida* (3), *A. popoffii* (4), *A. trota* (3), *A. schubertii* (1), *A. jandaei* (3), *A. allosaccharophila* (1) and *Aeromonas* spp. (7). Three strains each of *A. trota* and *A. jandaei*, one of *A.*

Table-11: Distribution of Enterotoxin Genes in *Aeromonas* spp. from different sources.

Species	Source	No. of Isolates	No. of Enterotoxin Gene Positive Isolates	*Enterotoxin Genes*		
				act	*alt*	*ast*
A. hydrophila	Water	15	14 (93.33)	12	11	-
	Fish	13	12 (92.30)	11	11	-
	Mutton	3	3 (100)	3	3	1
	Chicken	2	2 (100)	2	2	-
	Beef	2	2 (100)	2	2	-
	Diarrheal	2	2 (100)	2	2	1
Subtotal		**37**	**35 (94.59)**	**32**	**31**	**2**
A. caviae	Water	12	11 (91.67)	9	11	2
	Fish	6	6 (100)	4	5	1
	Mutton	5	4 (80)	4	4	-
	Chicken	3	3 (100)	2	3	-
	Beef	2	2 (100)	2	2	-
	Diarrheal	4	4 (100)	4	4	2
Subtotal		**32**	**30 (93.75)**	**25**	**29**	**5**
A. veronii bv *sobria*	Water	8	7 (87.5)	7	6	2
	Fish	13	12 (92.3)	11	8	-
	Mutton	4	4 (100)	3	3	-
	Chicken	2	2 (100)	2	1	-
	Beef	1	1 (100)	1	1	-
Subtotal		**28**	**26 (92.85)**	**24**	**19**	**2**
A. salmonicida	Water	5	1 (20)	1	1	-
	Fish	8	3 (37.5)	3	1	-
Subtotal		**13**	**4 (30.69)**	**4**	**2**	**-**
A. popoffii	Water	2	-	-	-	-
	Fish	8	4 (50)	3	1	-
Subtotal		**10**	**4 (40)**	**3**	**1**	**-**
A. trota	Water	2	1 (50)	1	1	-
	Fish	4	3 (75)	2	2	1
	Mutton	2	1 (50)	1	-	-
Subtotal		**8**	**5 (62.5)**	**4**	**3**	**1**
A. schubertii	Water	1	-	-	-	-
	Fish	4	1 (25)	1	-	-
	Chicken	1	-	-	-	-
Subtotal		**6**	**1 (16.67)**	**1**	**-**	**-**
A. jandaei	Fish	3	3 (100)	2	1	-
	Mutton	1	-	-	1	-
Subtotal		**4**	**3 (75)**	**2**	**2**	**-**
A. allosaccharophila	Fish	2	1 (50)	1	1	-
	Mutton	2	1 (50)	-	1	-
Subtotal		**4**	**2 (50)**	**1**	**2**	**-**
Aeromonas spp.	Water	3	2 (67.67)	2	2	-
	Fish	7	7 (100)	4	5	1
	Mutton	2	2 (100)	1	1	-
	Beef	1	1 (100)	1	1	-
Subtotal		**13**	**12 (92.3)**	**8**	**9**	**1**
Grand Total		**155**	**122 (78.70)**	**104**	**98**	**11**

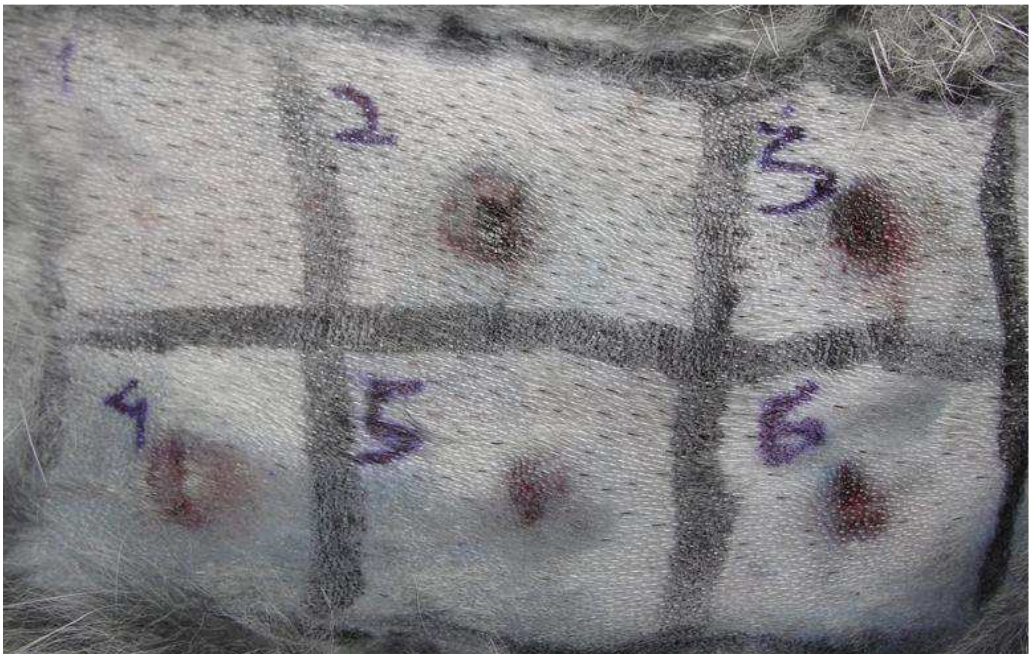


Plate-9 : **Picture showing vasopermeability reaction in rabbit skin**

The box-1 indicates the control in which sterile BHIB was injected

The box-2 indicates VPR of ATCC 7966 *A. hydrophila* strain

The box-3 indicates VPR of *A. hydrophila* strain showing presence of all the three enterotoxin genes.

The box-4 indicates VPR of *A. hydrophila* strain showing presence of *act/alt* enterotoxin genes.

The box-5 indicates VPR of *A. hydrophila* of strain carrying no enterotoxin gene.

The box-6 indicates VPR of *A. hydrophila* strain carrying *act* enterotoxin gene.

allosaccharophila and 7 strains of *Aeromonas* spp. from samples of mutton and also one strain of *Aeromonas* spp. from beef revealed one or more enterotoxin genes.

4.6.2 Haemolysis on Rabbit and Sheep Blood Agar

All the isolates of *Aeromonas* spp. exhibited haemolytic activity on 5% Rabbit Blood Trypticase Soya Agar plates, while only 92.25% of the isolates caused haemolysis on 5% Sheep Blood Trypticase Soya Agar. The haemolytic activity on sheep blood agar plates was demonstrated by 93.75% (45) of the isolates from water, 88.23% (60) from fish, 94.73% (18) from mutton and all of chicken, beef and human diarrheic stools (Table 12). All the strains of *Aeromonas* spp. caused uniform hemolytic activity on rabbit blood agar plates whereas 91.89% of *A. hydrophila*, 96.85% of *A. caviae*, 76.92% of *A. salmonicida*, 90% of *A. popoffii*, 87.50% of *A. trota*, 83.33% of *A. schubertii*, 75% of *A. allosaccharophila*, 92.32% of *Aeromonas* spp. and all the isolates of *A. veronii* bv *sobria* and *A. jandaei* caused haemolysis on sheep blood agar plates.

4.6.3 Vascular Permeability Reaction in Rabbit Skin

The enterotoxigenicity of the representative species of *Aeromonas viz*. *A. hydrophila*, *A. caviae* and *A. veronii* bv *sobria* (isolated more often) was studied. The isolates showing different enterotoxin gene pattern for each species was selected for entetotoxgenic assay. The field isolates were compared with the standard ATCC 7966 strain, with respect to their biological effects in rabbit skin. The VPRs were pronounced in rabbit skin and the inflammatory changes could easily be measured. VPR zones were pronounced following intradermal inoculation of *Aeromonas* enterotoxin by 12 hours and ranged between 7 and 19.6 mm in diameter, with an overall average of 10.91 mm (Plate-9). The *A. hydrophila* showed an average VPR zone of 12.36 mm and the average area of necrosis was 5.46 mm. Among the *A. hydrophila* strains the highest VPR zone (18.5 mm) was shown by ATCC strain followed by a strain isolated from water (16.5 mm), both these showed the presence of all the three enterotoxins (*ast/alt/ast*).

Table-12: Haemolytic Activity of *Aeromonas* spp. on Rabbit and Sheep Blood Trypticase Soya Agar

Species	Source	No. of Isolates	Haemolysis on Blood Agar	
			Rabbit (%)	Sheep (%)
A. hydrophila	Water	15	15 (100)	13 (86.67)
	Fish	13	13 (100)	12 (92.31)
	Mutton	3	3 (100)	3 (100)
	Chicken	2	2 (100)	2 (100)
	Beef	2	2 (100)	2 (100)
	Diarrheal	2	2 (100)	2 (100)
Subtotal		**37**	**37**(100)	**34 (91.89)**
A. caviae	Water	12	12 (100)	12 (100)
	Fish	6	6 (100)	5 (83.33)
	Mutton	5	5 (100)	5(100)
	Chicken	3	3 (100)	3 (100)
	Beef	2	2 (100)	2 (100)
	Diarrheal	4	4 (100)	4 (100)
Subtotal		**32**	**32 (100)**	**31 (96.85)**
A. veronii bv *sobria*	Water	8	8 (100)	8 (100)
	Fish	13	13 (100)	13 (100)
	Mutton	4	4 (100)	4 (100)
	Chicken	2	2 (100)	2 (100)
	Beef	1	1 (100)	1 (100)
Subtotal		**28**	**28 (100)**	**28 (100)**
A. salmonicida	Water	5	5 (100)	4 (80)
	Fish	8	8 (100)	6 (75)
Subtotal		**13**	**13 (100)**	**10 (76.92)**
A. popoffii	Water	2	2 (100)	2 (100)
	Fish	8	8 (100)	7 (87.5)
Subtotal		**10**	**10 (100)**	**9 (90)**
A. trota	Water	2	2 (100)	2 (100)
	Fish	4	4 (100)	3 (75)
	Mutton	2	2 (100)	2 (100)
Subtotal		**8**	**8 (100)**	**7 (87.50)**
A. schubertii	Water	1	1 (100)	1 (100)
	Fish	4	4 (100)	3 (75)
	Chicken	1	1 (100)	1 (100)
Subtotal		**6**	**6 (100)**	**5 (83.33)**
A. jandaei	Fish	3	3 (100)	3 (100)
	Mutton	1	1 (100)	1 (100)
Subtotal		**4**	**4 (100)**	**4 (100)**
A. allosaccharophila	Fish	2	2 (100)	2 (100)
	Mutton	2	2 (100)	1 (50)
Subtotal		**4**	**4 (100)**	**3 (75)**
Aeromonas spp.	Water	3	3 (100)	3 (100)
	Fish	7	7 (100)	6 (85.75)
	Mutton	2	2 (100)	2 (100)
	Beef	1	1 (100)	1 (100)
Subtotal		**13**	**13 (100)**	**12 (92.32)**
Grand Total		**155**	**155 (100)**	**143 (92.25)**

Note: The figures in the parentheses indicate the percentages.

The least VPR zone was shown by *A. hydrophila* strain (6.1 mm) recovered from water with absence of all the there enterotoxin genes. The average VPR zone and average necrotic area shown by *A. caviae* was 11.28 mm and 5.62 mm, repectively (Table 13).

Table-13: Vasopermeabilty Reaction of *Aeromonas* spp. in Rabbit.

S. No.	Species	Enterotoxin genes present	VPR Zone (mm)	Necrosis (mm)
1.	*A. hydrophila* ATCC 7966	*ast/alt/ast*	18.5	7.6
2.	*A. hydrophila*	*ast/alt/ast*	16.5	7.3
3.	*A. hydrophila*	*act/alt*	11	5.2
4.	*A. hydrophila*	*act*	9.7	4.2
5.	*A. hydrophila*	*None*	6.1	3.0
	Average		**12.36**	**5.46**
6.	*A. caviae*	*act/alt/ast*	19.6	7.1
7.	*A. caviae*	*act/alt*	9.8	6.5
8.	*A. caviae*	*act/ast*	11.7	6.8
9.	*A. caviae*	*act*	8.3	4.4
10.	*A. caviae*	*alt*	7	3.3
	Average		**11.28**	**5.62**
11.	*A. veronii* bv *sobria*	*act/alt/ast*	9	5.3
12.	*A. veronii* bv *sobria*	*act/ast*	9.5	5.8
13.	*A. veronii* bv *sobria*	*act*	8.2	3.4
14.	*A. veronii* bv *sobria*	*act*	7.9	5.3
	Average		**8.65**	**4.95**
Overall Mean			**10.91**	**5.37**

Note: The figures in the parentheses indicate the percentages.

The *A. caviae* strain recovered from clinical case (diarrheal sample) showed highest VPR zone (19.6 mm) when compared with other isolates. The clinical isolate of *A. caviae* showed the presence of all the three enterotoxin genes (*ast/alt/ast*). *A. veronii* bv *sobria* produced an average VPR and necrotic zones of 8.65 mm and 4.95 mm, respectively. The strain of *A. veronii* bv *sobria* carrying the genes *ast/alt* showed higher VPR zone (9.5 mm) followed by a strain (9 mm) carrying all the three genes (*ast/alt/ast*). Across all the isolates the lowest VPR zones were shown by the isolates carrying *alt* gene. The controls in which sterile brain heart infusion broth was injected showed no inflammatory reaction even after 48 hr of intradermal injection.

4.7 Antibiogram of *Aeromonas* spp.

All the strains of *Aeromonas* spp. isolated from different sources were subjected to antibiotic sensitivity test against a panel of 20 antimicrobial agents. Based on the Clinical and Laboratory Standards Institute (CLSI) interpretive standards for *Aeromonas* spp., the isolates were found sensitive to enrofoxacin (96.77%), ciprofloxacin (94.19%), ofloxacin (92.9%), ceftraixone (90.32%), tetracycline (85.81%), norfloxacin (85.81%), gentamicin (84.52%) and doxycycline (83.23%) in order (Table 14). The highest resistance was noted against ampicillin (97.42%). The antibiotics to which most of the isolates presented resistance included ampicillin/cloxacillin (89.03%), polymyxin B (87.74%), amoxycillin (72.9%), roxithromycin (70.32%), erythromycin (61.29%) and streptomycin (54.84%). Resistance of less than 50% was noted against, neomycin (45.16%), kanamycin (38.71%), sulphadiazime (32.9%), ceftazidime (32.26%) and cephalexin (43.23%). The isolates were found to be highly susceptible to ofloxacin, norfloxacin, ciprofloxacin, enrofloxacin and ceftriaxone irrespective of source from which they were isolated. The isolates from water samples showed no resistance against ofloxacin, ciprofloxacin and enrofloxacin. The isolates recovered from the

Table-14: Antimicrobial resistance/sensitivity Pattern of *Aeromonas* spp.

Antibiotic	Resistance	Sensitivity	Intermediate Susceptibility
Gentamicin	17 (10.97)	131 (84.52)	7 (4.52)
Kanamycin	60 (38.71)	80 (51.61)	15 (9.68)
Sulphadiazime	51 (32.9)	89 (57.42)	15 (9.68)
Ceftazidime	50 (32.26)	95 (61.29)	10 (6.45)
Ofloxacin	8 (5.16)	144 (92.9)	3 (1.94)
Tetracycline	16 (10.32)	133 (85.81)	6 (3.87)
Neomycin	70 (45.16)	75 (48.39)	10 (6.45)
Roxithromycin	109 (70.32)	37 (23.87)	9 (5.81)
Ciprofloxacin	5 (3.23)	146 (94.19)	4 (2.58)
Streptomycin	85 (54.84)	52 (33.55)	18 (11.61)
Ampicillin	151 (97.42)	0 (0)	4 (2.58)
Amoxycillin	113 (72.9)	38 (24.52)	4 (2.58)
Norfloxacin	16 (10.32)	133 (85.81)	6 (3.87)
Doxycycline	13 (8.39)	129 (83.23)	13 (8.39)
Enrofloxacin	0	150 (96.77)	5 (3.23)
Ceftriaxone	11 (7.1)	140 (90.32)	4 (2.58)
Erythromycin	95 (61.29)	42 (27.1)	18 (11.61)
Polymyxin-B	136 (87.74)	11 (7.1)	8 (5.16)
Ampicillin/Cloxacillin	138 (89.03)	16 (10.32)	1 (0.65)
Cephalexin	67 (43.23)	84 (54.19)	4 (2.58)

Note: The figures in the parentheses indicate the percentages.

water showed a high (more than 80%) susceptibility against gentamicin, tetracycline, norfloxacin and cefatriaxone and in contrast a high resistance was noted against amoxycillin, ampicillin, polymyxin B, ampicillin/cloxacillin. Among the isolates obtained from fish a comparatively higher resistance was noted than isolates from water, against the antibiotics like gentamicin, neomycin, kanamycin, ceftriaxone and the only antibiotic against which no resistance was noted was enrofloxacin. Compared to isolates from water and fish a higher resistance was recorded for the isolates recovered from raw meats with resistance noted for all the antibiotics, except for enrofloxacin. More than 50% resistance was noted against roxithromycin, streptomycin, ampicillin, amoxycillin, erythromycin, polymyxin B and ampicillin/cloxacillin. The highly effective antibiotic against the isolates from raw meats was enrofloxacin with no resistance noted against it. The resistance pattern was highest among the isolates of human diarrheal samples in comparison to the isolates recovered from all other sources. More than 50% resistance was noted against kanamycin, sulphadiazime, neomycin, roxithromycin, streptomycin, ampicillin, amoxycillin, doxycycline, erythromycin, polymyxin-B, ampicillin/cloxacillin and cephalexin. Whereas, the most effective antibiotic was found to be enrofloxacin with all isolates susceptible against it (Table 15). There was a uniform resistance pattern among different species of *Aeromonas* (Table 16).

Table-15: Atibiogram of *Aeromonas* spp. from various sources

S. No.	Antibiotic	Water (N=48)			Fish (N=68)			Raw Meats (Mutton, Chicken, Beef) (N=33)			Humans (N=6)		
		R	S	I	R	S	I	R	S	I	R	S	I
1	Gentamicin	2 (4.17)	44 (91.67)	2 (4.17)	6 (8.82)	58 (85.29)	4 (5.88)	7 (21.21)	25 (75.76)	1 (3.03)	2 (33.33)	4 (66.67)	-
2	Kanamycin	15 (31.25)	28 (58.33)	5 (10.42)	26 (38.24)	35 (51.47)	7 (10.29)	16 (48.48)	14 (42.42)	3 (9.09)	3 (50.00)	3 (50.00)	-
3	Sulphadiazine	13 (27.08)	31 (64.58)	4 (8.33)	22 (32.35)	39 (57.35)	7 (10.29)	12 (36.36)	17 (51.52)	4 (12.12)	4 (66.67)	2 (33.33)	-
4	Ceftazidime	16 (33.33)	28 (58.33)	4 (8.33)	22 (32.35)	41 (60.29)	5 (7.35)	10 (30.30)	22 (66.67)	1 (3.03)	2 (33.33)	4 (66.67)	-
5	Ofloxacin	-	47 (97.92)	1 (2.08)	1 (1.47)	65 (95.59)	2 (2.94)	5 (15.15)	28 (84.85)	-	2 (33.33)	4 (66.67)	-
6	Tetracycline	4 (8.33)	42 (87.50)	2 (4.17)	6 (8.82)	59 (86.76)	3 (4.41)	4 (12.12)	28 (84.85)	1 (3.03)	2 (33.33)	4 (66.67)	-
7	Neomycin	19 (39.58)	26 (54.17)	3 (6.25)	31 (45.59)	32 (47.06)	5 (7.35)	16 (48.48)	16 (48.48)	1 (3.03)	4 (66.67)	1 (16.67)	1 (16.67)
8	Roxithromycin	34 (70.83)	12 (25.00)	2 (4.17)	47 (69.12)	17 (25.00)	5 (5.88)	24 (72.73)	7 (21.21)	2 (6.06)	4 (66.67)	1 (16.67)	1 (16.67)
9	Ciprofloxacin	-	47 (97.92)	1 (2.08)	2 (2.94)	65 (95.59)	1 (1.47)	3 (9.09)	29 (87.88)	1 (3.03)	-	5 (83.33)	1 (16.67)
10	Streptomycin	25 (52.08)	18 (37.50)	8 (10.42)	39 (57.35)	25 (36.76)	4 (11.76)	18 (54.55)	11 (33.33)	4 (12.12)	3 (50.00)	2 (33.33)	1 (16.67)

Table-15: contd…

S. No.	Antibiotic	Water (N=48)			Fish (N=68)			Raw Meats (Mutton, Chicken, Beef) (N=33)			Humans (N=6)		
		R	S	I	R	S	I	R	S	I	R	S	I
11	Ampicillin	47 (97.92)	-	1 (2.08)	67 (98.53)	-	1 (1.47)	31 (93.94)	-	2 (6.06)	6 (100)	-	-
12	Amoxycillin	34 (70.83)	13 (27.08)	1 (2.08)	51 (75)	16 (23.53)	1 (1.47)	24 (72.73)	8 (24.24)	1 (3.03)	4 (66.67)	1 (16.67)	1 (16.67)
13	Norfloxacin	4 (8.33)	42 (87.50)	1 (4.17)	6 (8.82)	59 (86.76)	3 (4.41)	4 (12.12)	28 (84.85)	1 (3.03)	2 (33.33)	4 (66.67)	-
14	Doxycycline	3 (6.25)	41 (85.42)	4 (8.33)	2 (2.94)	59 (86.76)	7 (10.29)	4 (12.12)	27 (81.82)	2 (6.06)	4 (66.67)	2 (33.33)	-
15	Enrofloxacin	-	47 (97.92)	1 (2.08)	-	65 (95.59)	3 (4.41)	-	32 (96.97)	1 (3.03)	-	6 (100)	-
16	Ceftriaxone	1 (2.08)	46 (95.83)	1 (2.08)	4 (5.88)	63 (92.65)	1 (1.47)	4 (12.12)	28 (84.85)	1 (3.03)	2 (33.33)	3 (50)	1 (16.67)
17	Erythromycin	28 (58.33)	13 (27.08)	7 (14.58)	41 (60.29)	18 (26.47)	9 (13.24)	22 (66.67)	9 (27.27)	2 (6.06)	4 (66.67)	2 (33.33)	-
18	Polymyxin-B	41 (85.42)	4 (8.33)	3 (6.25)	61 (89.71)	3 (4.41)	4 (5.88)	29 (87.88)	3 (9.09)	1 (3.03)	5 (83.33)	1 (16.67)	-
19	Ampicillin/Cloxacillin	44 (91.67)	3 (6.25)	1 (2.08)	60 (88.24)	8 (11.76)	-	29 (87.88)	4 (12.12)	-	5 (83.33)	1 (16.67)	-
20	Cephalexin	20 (41.67)	27 (56.25)	1 (2.08)	29 (42.65)	37 (54.41)	2 (2.94)	15 (45.45)	17 (51.52)	1 (3.03)	3 (50)	3 (50)	-

Table-16: Antibiogram (%) of different species of *Aeromonas*

Antibiotic	*A. hydrophila*		*A. caviae*		*A. veronii* bv *sobria*		*A. salmonicida*		*A. popoffii*		*A. trota*		*A. schubertii*		*A. jandaei*		*A. allosaccharophila*		*Aeromonas* spp.	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
G	10.8	83.8	9.4	84.4	10.7	85.7	7.7	92.3	10.0	90.0	0	87.5	16.7	66.7	25.0	75.0	25.0	75.0	15.4	84.6
K	40.5	48.6	40.6	50	39.3	50	23.1	69.2	40.0	50.0	37.5	50	33.3	50	50.0	50.0	50.0	50.0	38.5	53.8
Sz	35.1	54.1	34.4	53.1	35.7	53.6	15.4	76.9	30.0	60.0	37.5	50	33.3	66.7	25.0	75.0	25.0	75.0	38.5	53.8
Ca	35.1	59.5	34.4	59.4	35.7	57.1	23.1	69.2	30.0	70.0	25	62.5	33.3	66.7	25.0	75.0	25.0	50.0	30.8	61.5
OF	2.7	94.6	3.1	93.8	3.6	92.9	0	100	0.0	100	12.5	87.5	16.7	83.3	25.0	75.0	25.0	75.0	7.7	92.3
T	8.1	86.5	9.4	84.4	10.7	85.7	7.7	92.3	10.0	90.0	12.5	87.5	16.7	83.3	25.0	75.0	25.0	75.0	7.7	84.6
N	45.9	45.9	46.9	46.9	46.4	46.4	23.1	61.5	50.0	50.0	50	50	50	50	50.0	50.0	50.0	50.0	46.2	46.2
RO	70.3	21.6	71.9	25	71.4	25	61.5	30.8	70.0	20.0	62.5	25	83.3	16.7	75.0	25.0	75.0	25.0	69.2	23.1
CF	2.7	94.6	3.1	93.8	3.6	92.9	0	100	10.0	90.0	0	87.5	0	100	0.0	100	0.0	100	7.7	92.3
S	54.1	32.4	56.3	31.3	53.6	35.7	53.8	38.5	50.0	40.0	50	37.5	50	33.3	75.0	25.0	75.0	25.0	53.8	30.8

Note: Gentamicin (G) Kanamycin (K) Sulphadiazime (Sz) Ceftazidime (Ca) Ofloxacin (OF) tetracycline (T) Neomycin (N) Roxithromycin (Ro) Ciprofloxacin (CF) and Streptomycin (S): R indicates resistance and S indicates susceptibility

Table-16: contd..

Anti-biotic	*A. hydrophila*		*A. caviae*		*A. veronii* bv *sobria*		*A. salmonicida*		*A. popoffii*		*A. trota*		*A. schubertii*		*A. jandaei*		*A. allosaccharophila*		*Aeromonas* spp.	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
A	100	0	96.9	0	96.4	0	92.3	0	100	0.0	100	0	100	0.0	100	0.0	100	0.0	92.3	0.0
AM	75.7	21.6	75	21.9	75.0	21.4	76.9	23.1	70.0	30.0	0	87.5	83.3	16.7	75.0	25.0	100	0.0	84.6	15.4
Nx	10.8	86.5	9.4	87.5	10.7	85.7	7.7	92.3	10.0	90.0	12.5	75	0.0	83.3	0.0	75.0	25.0	75.0	15.4	84.6
DO	5.4	86.5	6.3	84.4	10.7	82.1	0	92.3	10.0	80.0	12.5	75	16.7	83.3	25.0	75.0	0.0	75.0	15.4	76.9
Ex	0	97.3	0.0	96.9	0	96.4	0	92.3	0.0	100	0	100	0.0	100	0.0	100	0.0	100	0.0	92.3
CTR	5.4	91.9	6.3	90.6	3.6	92.9	7.7	84.6	10.0	90.0	0	100	16.7	83.3	25.0	75.0	25.0	75.0	7.7	92.3
E	59.5	27	59.4	28.1	60.7	25	61.5	30.8	60.0	30.0	62.5	25	66.7	33.3	75.0	25.0	75.0	25.0	61.5	23.1
PB	89.2	5.4	84.4	9.4	89.3	7.1	92.3	7.7	90.0	10.0	87.5	0	83.3	16.7	75.0	0.0	75.0	0.0	92.3	7.7
Ax	89.2	10.8	90.6	9.4	89.3	10.7	92.3	7.7	90.0	10.0	62.5	25	100	0.0	100	0.0	75.0	25.0	92.3	7.7
CP	45.9	51.4	46.9	53.1	42.9	53.6	38.5	61.5	40.0	60.0	25	62.5	50.0	50.0	50.0	50.0	25.0	50.0	46.2	53.8

Note: Ampicillin (A), Amoxycillin (Am), Norfloxacin (Nx), Doxycycline (Do), Enrofloxacin (Ex), Ceftriaxone (CTR), Erythromycin (E), Polymyxin (Pb), Ampicillin/Cloxacillin (Ax) and Cephalexin (Cp); R indicates resistance and S indicates susceptibility

4.9 Nucleotide sequence analysis of enterotoxin genes (*alt, act* and *ast*).

The length of cytotoxic heat-labile enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*) and cytotonic heat-stable enterotoxin (*ast*) genes was 819, 576 and 216 bp, respectively. The amplified PCR products of all the three enterotoxin genes obtained from *A. hydrophila* strain recovered from water were sequenced (Appendix-II). The nucleotide sequence that was readable were 706 bp for *act*, 504 bp for *alt* and 205 bp for *ast* genes of *A. hydrophila* strain. From *A. caviae* only *alt* gene fragment could be sequenced with 519 bp. The sequences of all the fragments were highly similar (more than 90%) when compared with other sequences of *A. hydrophila* [*A. hydrophila* (HQ425626.1), *A. hydrophila* (DQ408263.1), *A. hydrophila* (GU229024.1), *A. hydrophila* (DQ186611.1), *A. hydrophila* (DQ302122.1), *A. hydrophila* (GU-169709.1), *A. hydrophila* (AB237174.1) etc.] in GenBank.

Chapter-5

DISCUSSION

Although the history of Aeromonads is more than a century old (Zimmerman, 1890), yet their role in a number of human illnesses was recognized only during past three decades (Janda and Abbott, 1998). In humans, Aeromonads are not only capable of inflicting gastrointestinal disorders but can also be involved in a wide range of extra-intestinal infections (Janda and Abott, 1998).

The organisms are widely distributed in the aquatic environment (Holmes *et al*., 1996) and are found in a variety of foods including raw meats, fish, packaged ready-to-eat meats, sea foods etc. (Palumbo, 1996). A number of human illnesses are, therefore, implicated to food borne nature of *Aeromonas* spp. (Wadstrom and Ljungh, 1991).

In the present study, 609 samples of water, fish, chicken, mutton, beef and human diarrheic stools were screened for the presence of Aeromonads, of which 155 were found positive, with an overall percentage of prevalence of 25.45. Being essentially ubiquitous in the microbial biosphere, Aeromonads are expected in virtually every environmental niche where bacterial ecosystems exist but their role in human and animal diseases depend on many environmental and host factors besides virulence of the species itself.

The natural reservoir for *Aeromonas* spp. is the aquatic environment (Hazen *et al*., 1978; Schubert, 1991; Razzolini *et al*., 2008) and as such their detection has been reported from almost all types of waters namely fresh, saline and brackish water (Hazen *et al*., 1978; Sack *et al*., 1987; Araujo *et al*., 1989), chlorinated and un-chlorinated drinking water (Millership and Chattopadhyay, 1985; Kersters *et al*.,

1995), ground water (Holmes *et al*., 1996), treated and untreated sewage water (Schubert, 1991), seawater (Brandi *et al*., 1996), clean river water (Pathak *et al*., 1988), fish farm hatchery tanks (Rhodes *et al*., 2000), reservoirs and drinking fountains (Razzolini *et al*., 2008) and even bottled mineral water (Tsai and Yu, 1997).

In the present investigation as well, 26.37% of water samples from different sources were found contaminated with *Aeromonas* spp. The organism was recovered from drinking water samples with a prevalence percentage of 13.79. Presence of these organisms in drinking water depicts their resistance to the conventional water treatment methods and needs more elaborative studies for better understanding of the resistance pattern of Aeromonads. The organisms have been found at a frequency of 1 to 27% of drinking water supplies by other workers as well (Rusin *et al*., 1997) which substantiates the findings of the present investigation. Reports of their recovery from mineral water with isolation rates as high as 35.5% and cell concentrations of greater than 3 $\log_{10}$ CFU/mL are also documented (Quevedo-Sarmiento *et al*., 1986; Slade *et al*., 1986; Gonzales *et al*., 1987; Havelaar *et al*., 1990; Manaia *et al*., 1990; Tsai and Yu, 1997; Warburton *et al*., 1998; and Croci *et al*., 2001). This is another area which needs attention since mineral water plants are generally designed to provide zero bacteria packaged water to the consumers and have the required facilities to render the water bacteria free before packaging. Not much is known about the role of Aeromonads in human gastrointestinal infections as primary agents of the disease but their detection in human stools suggests their role at least as secondary invaders. The other possibility may be that the organisms cause a self limiting mild type of gastroenteritis, possibly trying to adapt to new host system. Even in the absence of their documented role in human gastroenteritis, their presence in high numbers in drinking water may have serious consequences in infants, old people and the immune-compromised persons where it may

potentially lead to an invasive disease, such as septicemia (Leclerc *et al*., 2002). The contamination of drinking water with Aeromonads could be due to their presence in water sources supplying the treatment plants as the organism has been recovered from these sources more frequently (Sharma *et al*., 2005; Bhowmik *et al*., 2009; Hande *et al*., 2009). In our study, ten of the 38 samples from rivers and streams were found contaminated with *Aeromonas* spp. Although the percentage of contamination of inlet water source supplying the fish ponds was less (15.78%), yet the samples taken from the fish ponds revealed a higher percentage of prevalence of 69.23. This could be due to continuous excretion of Aeromonads by fishes kept under closed rearing conditions as the organisms are considered as normal micro-flora of many aquatic animals and are proven disease agents in various cold blooded animals including fishes, amphibia and reptiles (Janda and Duffey, 1988; Cahill, 1990a). Reports on the higher prevalence in fish ponds are also on record (Rhodes *et al*., 2000).

Raw meats of all kinds were contaminated by *Aeromonas* spp. with percentage of contamination for chicken, mutton and beef samples as 14.03, 22.89 and 19.35, respectively. A higher percentage of prevalence of Aeromonads was reported by Ibrahim and MacRae (1991). In their study, 60.00% samples of beef, 58% of lamb and 74% of pork were contaminated with Aeromonads. The distribution of Aeromonads may not be uniform and, as such, varies from place to place and area to area. The housing system, transportation of animals before slaughter, slaughtering methods and the sanitary measures taken from farm to kitchen, all contribute to the prevalence of food-borne pathogens in meat and meat products. Our findings are in agreement with the findings of Sharma and Kumar (2011) who reported a prevalence percentage of 13.13 in fish, 11.5 in poultry meat, 9.85 in pork and 2.5 in chevon from North-East India. Contrary to these findings, a

higher percentage of prevalence (26%) was recorded in commercially available chevon from Nagpur (Kolhe *et al*., 2005). Agarwal *et al*. (2000) isolated Aeromonads from 22% of fish and 8.9% of goat meat samples in India, indicating geographical variation in occurrence of *Aeromonas* spp.. Their lower prevalence in meat and meat products is therefore, justifiable. In the present study, fish samples from retail fish markets were highly contaminated (44.17%) whereas samples from government and private fish farms revealed a lesser percentage of contamination of 27.27 and 30.00, respectively. The higher percentage recorded in samples from retail markets might be due to the incubation time in the absence of proper storage facilities during marketing. Overall percentage of prevalence of *Aeromonas* spp. in fish recorded in the present study (39.30) was far more than the findings of other workers (Kolhe *et al*., 2005; Sharma and Kumar, 2011) which is a matter of concern for public safety needing an immediate attention of the Public Health Authorities.

Because of their presence in all types of waters, Aeromonads are expected in any food suitable for human consumption. The organisms have been isolated from ready-to-eat foods (Hudson and Avery, 1994; Tsai and Chen, 1996), from birds and poultry eggs (Glunder and Siegmann, 1993), and also from vegetables (Neyts *et al*., 2000b). Because of its ubiquitous nature, *Aeromonas* spp. is harbored by humans in their gastrointestinal tracts both in the presence and absence of the disease. The rates of fecal carriage in asymptomatic persons in developed countries range from 0% to 4% (Millership *et al*., 1983; Agger *et al*., 1985; Quan *et al*., 1986; Svenungsson *et al*., 2000), while the isolation rate from persons with diarrheal illness ranges from 0.8 to 7.4% (Agger *et al*., 1985; Moyer, 1987; Albert *et al*., 2000). In the present study, 6 of the 83 human diarrheal samples screened were found positive for the presence of *Aeromonas* spp., with an overall percentage prevalence of 7.22, which is similar to the findings of these workers. However, prevalence was higher in patients

from government hospitals (8.51%) than in the referral clinics (5.55%). In Southeast Asia, asymptomatic carriage rates as high as 27.5% and recovery rates from patients with diarrhea as high as 34% have been reported (Pazzaglia *et al*., 1990).

The taxonomy of the genus *Aeromonas* was codified with publication of the Second Edition of *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010) and new species continue to be described. Multiple methods are required for accurate classification of the genus and based on biochemical characterization and DNA hybridization techniques, 14 phenospecies (Martin-Carnahan and Joseph, 2005) and 17 DNA-homology groups (HGs), respectively, have been identified (Yanez *et al*., 2003). Molecular technique like restriction fragment length polymorphism (RFLP) profiles of 16S rRNA genes (Martinez-Murcia *et al*., 1992a; 1992b; Borrell *et al*., 1997) is also used for speciation of the genus.

The isolates of *Aeromonas* spp. produced typical colony characteristics (circular, smooth edged yellow colored) on Ampicillin Dextrin Agar. Similar characteristics have been reported by (Kersters *et al*., 1996a and Kingombe *et al*., 2010). The biochemical characteristics expressed by the isolates were in total agreement with the previous reports (Abbott *et al*., 2003, Martin-Carnahan and Joseph, 2005; Ottaviani *et al*., 2006; Janda and Abbott, 2010; Kingombe *et al*., 2010). The species isolated from all sources comprised *A. hydrophila* (23.87%), *A. caviae* (20.64%), *A. veronii* bv *sobria* (18.06%), *A. salmonicida* (8.39%), *A. popoffii* (6.54%), *A. trota* (5.16%), *A. schubertii* (3.87%), *A. jandaei* (2.58%), *A. allosaccharophila* (2.58%). Thirteen (8.39%) isolates could not be identified to the species level.

The most common isolated species from water was *A. hydrophila* (31.25%) although other species were also recovered with higher percentages viz. *A. caviae* (25%) *A. veronii* bv *sobria* (16.67%) and *A. salmonicida* (10.41%). Reports on contamination of water by these species in different geographic situations are documented with more frequency (Havelaar *et al*., 1992; Chauret *et al*., 2001; Sen and Rodgers, 2004; Razzolini *et al*., 2008). The other less prevalent species of *Aeromonas* isolated from water include *A. trota, A. allosaccharophila, A. popoffii* and *A. schubertii* (Ivanova *et al*., 2001; Soler *et al*., 2002). In accordance with the earlier reports (Ivanova *et al*., 2001), lesser percentage of contamination of water samples was recorded with A. *trota, A. popoffii* and *A. schubertii*.

Water and fish appear to be responsible for sustenance of *Aeromonas* spp. in nature although raw meats, in the present study also revealed a good number of isolates (33) which were less in comparison to the fish (68). The prevalent species in both fish and raw meats were *A. hydrophila*, *A. veronii* bv *sobria* and *A. caviae*. Similar reports are published on their predominance in fish and raw meats (Okrend *et al*., 1987; Fricker and Thompsett, 1989; Radu *et al*., 2003).

The prevalence of different species of *Aeromonas* is found to vary with geographical locations. In our study, an overall prevalence of *Aeromonas* spp. in human diarrheic cases was 7.23%. *A. caviae* (66.67%) appeared to be a more frequent visitor of human gastrointestinal tracts than *A. hydrophila* (33.33%). *A. hydrophila* and *A. veronii* bv *sobria* have been reported to be dominant species of *Aeromonas* causing diarrhea in Australia and Thailand (Altwegg and Geiss, 1989). In Bangladesh, *A. trota* has been isolated from a large number of diarrheal patients (Albert *et al*., 2000). A study in southern India revealed *A. hydrophila* as the predominant species causing diarrhea (Komathi *et al*., 1998). In Europe and America, majority of the cases of human diarrhea has been attributed to *A. caviae*

(Altwegg and Geiss, 1989). *A. caviae* was also found to be the predominant species in Kolkata (Sinha *et al*., 2004). Variation in geographical distribution may, therefore, reflect the tentativeness of *Aeromonas* taxonomy and as unified identification keys were not followed, the possibility of misidentification of species cannot be excluded.

A lack of congruity exists between phenotypic and genotypic characteristics of Aeromonads, therefore, for accurate classification of the genus, multiple methods are required. The most popularly described are polymerase chain reaction and the restriction fragment length polymorphism. Although certain biochemical tests allowed some improvements (Abbott *et al.*, 1992; Carnahan *et al*., 1991), phenotypic identification of the species appeared to be difficult (Borrell *et al*., 1997). Therefore, to validate the data, PCR was performed to identify the isolated Aeromonads that were phenotypically confirmed as *Aeromonas* spp. to the generic level. Among a total of 155 *Aeromonas* spp. identified phenotypically, the genus specific PCR detected 153 as *Aeromonas* spp. The two genotypically unidentified isolates belonged to *A. salmonicida* and *Aeromonas* spp. The discrepancies could be due to mutation in the said isolates or the difference in their genetic make-up so as to render the PCR unable to identify the species.

Identification by PCR with 16S rRNA gene targeted oligonucleotides has been evaluated for *A. sobria*, *A. schubertii*, and *A. jandaei* (Ash *et al.*, 1993a, 1993b) and for *A. hydrophila* and *A. veronii* (Dorsch *et al.*, 1994). However, some species had identical sequences in this selected target region and therefore could not be distinguished. The first attempt to identify Aeromonads to genotype relied upon differences in 16S ribosomal DNA sequences (Martinez-Murcia *et al*., 1992a), and several investigators developed probes for detection of various *Aeromonas* spp. (Demarta *et al*., 1999; Khan *et al*., 1999). *Aeromonas* taxonomy based upon 16S ribosomal DNA is complex and investigators have examined alternative means to

sort out the genotypic maze. Borrell *et al*. (1997) were able to identify ten species using endonucleases *Alu*I and *Mbo*I, but needed to add *Nar*I and *Hae*III to differentiate *A. salmonicida* from *A. encheleia*. Using the method as described by Borrell *et al*. (1997), most of the findings from the two different approaches i.e. phenotypic and RFLP characterization were in agreement, barring some discrepancies. This could be due to the genetic heterogeneity as a result of cross-over in ribosomal sequences or genetic recombinations obscuring phylogenetic relationships in Aeromonads (Morandi *et al*., 2005).

The infection caused by Aeromonads is attributed to a number of virulence factors possessed by them (Cahill, 1990b). These virulence factors are important in determining the role of *Aeromonas* spp. in human disease processes (Sinha *et al*., 2004). As a result, a significant quantum of research endeavours in aeromonal biology targeted at characterization of the role played by these virulence factors namely cytotoxic and cytotonic enterotoxins, haemolysin, aerolysins, etc. and large number of reports are available regarding distribution of theses virulence genes among Aeromonads in developed countries (Janda, 1991; Gray *et al*., 1990; Granum *et al*., 1998; Heuzenroeder *et al*., 1999; Abdullah *et al*., 2003; Kingombe *et al*., 2004; 2010). However, scanty information is available in India in general and in J&K in particular regarding the occurrence of these virulence factors in *Aeromonas* spp. of either human origin or food sources.

The exotoxins are major virulence factors of *Aeromonas* spp. that include a cytotoxic heat-labile enterotoxin (Act), also known as aerolysin/hemolysin (Chopra *et al.*, 1993); a cytotonic heat-labile enterotoxin (Alt), also known as lipase, extracellular lipase, or phospholipase (Anguita *et al.*, 1993; Chopra *et al.*, 1996; Sha *et al.*, 2002); and a cytotonic heat-stable enterotoxin (Ast) (Chopra *et al.*, 1994). These toxins are encoded by the genes *act, alt* and *ast* (Sen and Rodgers, 2004). In

our study, all the strains were screened by multiplex PCR for different enterotoxin genes. Among 155 *Aeromonas* spp., 122 (78.70%) carried one or more enterotoxin genes. In the PCR assay, *Aeromonas* strains were found with different virulence gene combinations. The dominant combination of enterotoxin genes was *act* (67.09%) and *alt* (63.22%) while *ast* (7.06%) presented least occurrence.

The *act* gene showed highest occurrence in *A. hydrophila* (86.48%). There are reports on occurrence of *act* gene in 65% of the *A. hydrophila* isolates originating from clinical, food and environmental samples (Abdullah *et al.,* 2003; Kingombe *et al.*, 1999; Wu *et al.,* 2007) and 83% of the food-borne isolates (Kingombe *et al*., 2010). The *act* gene was detected in 85.71% *A.veronii* bv *sobria* isolates, which is in contradiction to other studies (Kingombe *et al.,* 1999; Abdullah *et al.*, 2003; Wu *et al.*, 2007). The observed discrepancies can be due to different sources of isolation. The *act* gene showed comparatively less occurrence in *A. salmonicida* (30.77%), *A. popoffii* (30%), *A. trota* (50%), *A. schubertii* (16.67%), *A. jandaei* (50%), *A. allosaccharophila* (25%) and *Aeromonas* spp. (61.54%). Lesser occurrence of the enterotoxin genes has also been reported previously (Sinha *et al* 2004; Kingombe *et al.*, 2010).

In conformation to the previous reports (Chacon *et al.,* 2003), the *alt* gene occurred commonly (63.22%) in *Aeromonas* spp. in the present study. The *alt* gene showed highest occurrence in *A. caviae* (90.25%) followed by *A. hydrophila* (83.78%) and *A. veronii* bv *sobria* (67.85%). The results are in concurrence with the findings of other workers (Sinha *et al*., 2004) with *alt* occurring more often in *A. caviae* (97.3%) followed by *A. hydrophila* (88.6%) and *A. veronii* bv *sobria* (48%). The slight difference could be due to different sources of isolation. In *A. salmonicida* (15.38%), *A. popoffii* (10%), *A. trota* (37.5%), *A. jandaei* (50%), *A. allosaccharophila* (50%) and *Aeromonas* spp. (69.23%) the *alt* occurred less

frequently as has been reported by Sinha *et al*. (2004). The *alt* gene occurred frequently in combination with *act* gene (47.09%), rather than alone or in combination with other enterotoxin genes. The observed high frequency of occurrence of the *alt* gene and its combination with the *act* gene in *Aeromonas* spp. concurs with earlier findings (Abdullah *et al.,* 2003; Saavedra *et al.*, 2007). The *ast* gene was detected in *A. hydrophila* (5.4%), *A. caviae* (15.62%), *A. veronii* bv *sobria* (7.14%), *A. trota* (12.5%) and *Aeromonas* spp. (7.7%). Sinha *et al*. (2004) detected *ast* gene in *A. caviae* (1.4%), *A. veronii* bv *sobria* (8%) and *A. trota* (10%).

Among various virulence factors, haemolysin is the most prominent factor (Janda, 1991). Isolates in the present study were examined for their haemolytic properties on TSA plates containing 5% defibrinated rabbit or sheep blood. Haemolysis was observed in 100% and 93.75% of the isolates on RRBC and SRBC, respectively. Haemolytic properties in sheep blood agar was observed in 91.89% of *A. hydrophila*, 96.85% of *A. caviae*, 76.92% of *A. salmonicida*, 90% of *A. popoffii*, 87.50% of *A. trota*, 83.33% of *A. schubertii*, 75% of *A. allosaccharophila*, 92.32% of *Aeromonas* spp. and all the isolates of *A. veronii* bv *sobria* and *A. jandaei*. These results, therefore, indicate increased sensitivity of RRBC compared to SRBC towards *Aeromonas* haemolysin. Similar differences between RRBC and SRBC have also been reported by Santos *et al*. (1999). High prevalence of haemolytic properties is common among Aeromonads from different sources and our results are in concert with earlier reports (Gray *et al*., 1990; Singh and Sanyal, 1992; Krovacek *et al*., 1994; Kuhn *et al*., 1997a; 1997b).

The *in-vivo* virulence characterization was studied by vascular permeability reaction in rabbit skin. The presence of enterotoxin genes in Aeromonads can to some extent determine pathogenic nature of organism (Sinha *et al*., 2004). Therefore, the representative isolates with different entertotoxin gene pattern were used to study

the *in-vivo* virulence. More severe VPR was produced by the isolates carrying all the enterotoxin genes. The higher VPR zones were recorded in the isolates carrying *act* and *ast* genes. There is a good correlation between the cytotonic enterotoxins (*alt* and *ast*) and elongation of Chinese hamster ovary cells and production of c-AMP, which is typical enterotoxic activity (Chopra *et al*., 1994). It is evident from a previous study (Sha *et al*., 2002) that Act is the major enterotoxin contributing to fluid secretory response, followed by Alt and Ast enterotoxins in *A. hydrophila*. The presence of three enterotoxins in various combinations in different *Aeromonas* strains could increase or decrease expression of the specific enterotoxin gene and thus dictate the severity of diarrhea (Sha *et al*., 2002). The *act* gene is known to stimulate proinflammatory cytokine and eicosanoid cascades in macrophages in the rat intestinal epithelial cell line ICE-6, leading to tissue damage and fluid secretary response (Chopra *et al*., 2000). The *ast* positive strains may not only induce an infection but also causes intoxication due to the presence of the heat stable enterotoxin, even though the contaminated food vehicle was heat treated (Kingombe *et al*., 2010).

Several studies have provided strong evidence that some bacterial epidemic clones could circulate in an infected population for several years and may be responsible for outbreaks in future (Poh *et al.,* 1996; Caugant, 1998; Robinson *et al.*, 1998). RAPD results of our study did not yield enough genetic diversity. But in this study, only one primer was used and that primer might not have amplified the genes. Sporadic studies are available on the relationship between clones of the pathogenic *Aeromonas* spp. from clinical human cases and the environmental and food sources responsible for transmission of Aeromonads. The data indicated that the clinical isolates from human cases formed a group of unrelated strains and, therefore, children were not presumably infected with clonally related strains. Similar

observations have also been published by some workers previously (Moyer *et al.*, 1992; Szczuka and Kaznowski, 2004). The strains from water and fish were closely related to each other compared to isolates from all other sources. Since most of the water samples were collected from streams, inlet sources to fish ponds and the ponds themselves, the isolates recovered from fish and the water might have a common origin or developed a closed relationship in the aquatic environment. Moreover, there is hardly any stream or a water body in Kashmir valley which does not harbor fishes as part of the ecosystem. The strains isolated from clinical cases were related to water as was shown by dendrogram, suggesting the possibility of waterborne *Aeromonas* infection in humans. These findings are agreement with the findings of various authors (Martinetti *et al.,* 1993; Krovacek *et al.,* 1994; Pin *et al.,* 1995; Talon *et al.,* 1996; Kuhn *et al.,* 1997a; 1997b; Demarta *et al.,* 2000).

Global concern over the use and abuse of antimicrobials and subsequent emergence of drug resistant microbes is increasing. Wide spread use of antibiotics for treating bacterial diseases and sub-therapeutic doses of antibiotics in animal husbandry and aquaculture are chiefly responsible for emergence of antibiotic resistance (Vivekanandhan *et al*., 2002). The situation is more serious in developing countries including India where regulatory control over usage of antibiotics is less stringent with extensive use of antibiotics in animal husbandry and aquaculture (WHO, 2000; Vivekanandhan *et al*., 2002). The next step in the “drug-resistance” menace is the transfer of resistance to susceptible microbial population leading to compromising of the clinical efficacy of antimicrobial agents (Salyers and Whitt, 2002). *Aeromonas* spp. are known to be able to transfer antibiotic resistance horizotally to other pathogens (Bruun and Scmidt, 2003).

In the present study, attempts were made to document antibiotic susceptibility/resistance pattern of *Aeromonas* isolates from different sources. All

isolates were tested against a panel of 20 antimicrobial agents. A high sensitivity of *Aeromonas* spp. was observed against enrofloxacin (96.77%), ciprofloxacin (94.19%), ofloxacin (92.9%), ceftriaxone (90.32%), norfloxacin (85.81%), tetracycline (85.81%), gentamicin (84.52%) and doxycycline (83.23%). These results are in concurrence with the reports of Soliman (1999), Chandrakanth *et al*. (2000), Yucel and Ctak (2003), Emekdas *et al*. (2006), Kaskhedikar and Chhabra (2010) and Dallal *et al*. (2012) who reported inhibition of most of the *Aeromonas* spp. by cephalosporins like cephotaxime, aminoglycosides, ciprofloxacin, chloramphenicol, tetracycline and nitrofurantoin.

In contrast to this, a high degree of resistance was noted against ampicillin (97.42%) with most of the isolates showing no inhibitory signs on the agar plates. The results are in agreement with previous reports of 100% resistance against ampicillin by other workers (Goni-Urriza *et al*., 2000a; 2000b; Subashkumar *et al*., 2006; Meiyanti *et al*., 2010). The inherent resistance of Aeromonads against ampicillin has been used to the advantage of its cultivation in the selective media (ADA) which contains a considerable amount of the antibiotic to suppress other commonly occurring bacterial species. The other antibiotics against which high resistance was noted were roxithromycin (70.32%), amoxycillin (72.9%), penicillin G (87.74%) and ampicillin/cloxacillin (89.03%). High resistance of *Aeromonas* spp. against antibiotics like penicillin, amoxycillin, amoxicillin/sulbactam have been published previously as well (Guerra *et al*., 2007; Ghenghesh *et al*., 2008).

An intermediate resistance was noted against kanamycin (38.71%), sulphadiazime (32.9%), ceftazidime (32.26%), neomycin (45.16%), streptomycin (54.84%), erythromycin (61.29%) and cephalexin (43.23%). The findings are in agreement with previous reports (Goni-Urriza *et al*. 2000a; 2000b; Sinha *et al*., 2004; Subashkumar *et al*., 2006).

Interestingly, most of the *A. trota* isolates expressed resistance towards ampicillin in the present study. This is in contrast with earlier findings (Overman and Janda, 1999). Some workers have also observed previously the susceptibility of *A. trota* against ampicillin (Carnaham *et al*., 1991). Therefore, incorporation of this antibiotic in the selective medium may inhibit *A. trota* rendering the studies erroneous. were unlikely to be recovered in the present study as the isolation medium contained ampicillin (10 μg/ml). Moreover, in antibiotic sensitivity testing they showed resistance to 10 μg/ml of ampicillin. However, in light of the facts that minimum inhibitory concentration for 90% of *A. trota* organisms being 8 μg/ml (Overman and Janda, 1999) it may be postulated that *A. trota* isolates might have acquired some degree of resistance to ampicillin.

The similarity in sequences between the enterotoxin genes of *Aeromonas* spp. in present study with those from other sequences available in Genbank indicates the potential pathogenic nature of Aeromonads and genetic similarity of the enterotoxin genes.

Chapter-6

SUMMARY AND CONCLUSION

Of the total 609 samples of water, food and human origin screened for the presence of *Aeromonas* spp., 155 (25.45%) samples were positive. The prevalence in water, fish, raw meats and human diarrheal samples was 26.37, 39.30, 19.29 and 7.23%, respectively. Fish samples from retail market were highly contaminated (77.94%) compared to samples from government (13.23%) and private (8.82%) fish farms. A total of 33 *Aeromonas* spp. were obtained from raw meats of which 19 (22.89%) were from mutton, 8 (14.03%) were from chicken and 6 (19.35%) were from beef.

Irrespective of the source the species that could be recovered were *A. hydrophila* (23.87%), *A. caviae* (20.64%), *A. veronii* bv *sobria* (18.06%), *A. salmonicida* (8.39%), *A. popoffii* (6.45%), *A. trota* (5.16%), *A. schubertii* (3.87%), *A. jandaei* (2.58%) and *A. allosaccharophila* (2.58%). Thirteen isolates (8.39%) were identified to genus level. The 48 isolates recovered from water were identified as *A. hydrophila* (31.25%), *A. caviae* (25.00%), *A. veronii* bv *sobria* (16.67%), *A. salmonicida* (10.41%), *A. trota* and *A. popoffii* (4.16% each), *A. schubertii* (2.08%) and *Aeromonas* spp. (6.25%). A total of 68 isolates were isolated from fish samples of which the most prevalent species was *A. hydrophila* (22.05%) followed by *A. veronii* bv *sobria* (16.17%), *A. popoffii* (11.76%), *A. salmonicida* (11.75%), *A. caviae* (8.82%), *A. trota* (5.88%), *A. schubertii* (5.88%), *A. jandaei* (4.41%) and *Aeromonas* spp. (10.29%). The 19 isolates recovered from mutton, of which 5, 4, 3, 2, 2 and 1 were identified as *A. caviae*, *A. veronii* bv *sobria*, *A. hydrophila*, *A. trota*, *A. allosaccharophila* and *A. jandaei*, respectively. In chicken samples the isolates were identified as, *A. caviae*, (3), *A. veronii* bv *sobria* (2), *A. hydrophila* (2) and *A. schubertii* (1). Six isolates from beef samples were assigned to *A. caviae* (2), *A.*

hydrophila (2) and *A. veronii* bv *sobria* (1). The isolates from human diarrheal samples were assigned to *A. caviae* (4) and *A. hydrophila* (2).

Of the 155 phenotypically identified *Aeromonas* spp., the genus specific PCR confirmed 153 isolates as *Aeromonas*. The two unidentified isolates were phenotypically identified as *A. salmonicida* and *Aeromonas* spp. Restriction fragment length polymorphism studies of *Aeromonas* isolates were based on variations in 16S rRNA gene of isolates identified from RE pattern obtained by digestion with selected enzymes. PCR amplified segment of 16S rRNA gene, on digestion with various enzymes (*Alu*I, *Mbo*I, *Nar*I and *Hae*III) yielded RE pattern as expected. Analysis of RE digestion pattern indicated that patterns can be used to identify the species of *Aeromonas* barring few exceptions.

The typing of the Aeromonads isolated from different sources was studied by RAPD and ERIC PCR. There was variability among most of *A. hydrophila* strains, but some isolates recovered from water were identical to strains isolated from fish. There was a high relatedness among some strains of *A. veronii* bv *sobria* isolated from water and some strains isolated from fish were also related. Two isolates of *A. veronii* bv *sobria* recovered from fish were related to water and there was also relatedness among one isolate each recovered from beef and chicken. There was close relatedness among the isolates of *A. salmonicida* recovered from water and fish. The *A. popoffii* isolates from water formed close cluster and close cluster was also formed by fish isolates. The *A. trota* strains showed less relatedness amongst each other. Two clusters were formed by *A. schubertii* isolates and two isolates from fish were identical. The four isolates of *A. allosaccharophila* were less related and separate cluster was formed by isolates from fish and isolates from water. There was a high variability among the 13 unidentified *Aeromonas* spp. However, isolates from fish showed distinct cluster compared to isolates from water, beef and mutton.

To ascertain pathogenic potential of *Aeromonas* isolates characterization of virulence factors was carried out at molecular and phenotypic level. Molecular characterization of virulence factors in this study encompassed PCR based detection of three enterotoxin genes (*act*, *alt* and *ast*) among *Aeromonas* isolates. The *act*, *alt* and *ast* genes were detected in 104 (67.09%), 98 (63.22%) and 11 (7.06%) isolates, respectively. The enterotoxin gene pattern in the isolates was either *act*, *alt*, *act/alt*, *act/alt/ast* or *act/ast*. The *act/alt* pattern was present more often (47.09%) followed by *act* (13.54%), *alt* (10.96%), *act/alt/ast* (5.16%) and *act/ast* (1.93%).

The haemolytic property was tested on blood agar plates with 5% sheep blood agar or 5% rabbit blood agar. Results of this test revealed 100% of isolates to be haemolytic on rabbit blood agar plates while 92.25% of isolates caused haemolysis of sheep blood agar. This indicated a higher sensitivity of rabbit blood agar towards aeromonal haemolysin.

Enterotoxigenic potential of isolates was tested by vascular permeability reaction in rabbits. The VPR zones were pronounced after 12 hours of intradermal injection of *Aeromonas* enterotoxin and ranged between 7 and 19.6 mm in diameter, with overall average zone of VPR as 10.91 mm. The strains with presence of enterotoxin genes showed higher VPR zones compared with isolates not carrying any enterotoxin gene. Among the isolates, the average VPR zones for *A. hydrophila*, *A. caviae* and *A. veronii* bv *sobria* was 12.36 mm, 11.28 mm and 8.65 mm, respectively.

To characterize *Aeromonas* spp. with regard to their antibiotic sensitivity/resistance profiles, all isolates were tested against 20 antimicrobial agents by standard disc diffusion technique. Results of this study revealed uniform resistance against antibiotics like ampicillin, ampicillin/cloxacillin, polymyxin B,

amoxicillin, roxythromycin, erythromycin and streptomycin. The isolates were highly sensitive to enrofoxacin, ciprofloxacin, ofloxacin, ceftraixone, tetracycline, norfloxacin, gentamicin and doxycycline in order. An intermediate resistance was noted against kanamycin, neomycin, sulphadiazime, ceftazidime and cephalexin.
The sequence analysis by online BLAST programme indicated that the sequences of *alt*, *ast,* and *act* genes were identical to other isolates.

The following **conclusions** were drawn from current study:

- This investigation recognized a considerable prevalence of Aeromonads in humans, drinking water and food samples.
- The predominant species recovered from all sources were *A. hydrophila, A. caviae* and *A. veronii* bv *sobria*
- The predominant species recovered from human diarrheal cases was *A. caviae*.
- The molecular methods like RFLP can validate the identification procedures of *Aeromonas*.
- ERIC PCR is useful method for distinguishing *Aeromonas* spp. and for epidemiological investigation.
- The RAPD method used in the current study for analysis for typing of *Aeromonas* strains may not be recommended because the strains could not be typed by this method.
- A multiplex PCR has been developed, to detect all the three enterotoxin genes in *Aeromonas* spp. simultaneously.
- Predominant enterotoxin genes in Aeromonads were *act* and *alt* and *ast* was the least prevalent enterotoxin gene.

- The dominant gene pattern was *act*/*alt*.
- The virulence potential of isolates can be better studied by haemolytic activity on sheep blood agar.
- The *in-vivo* virulence study in rabbit was directly related to the presence of enterotoxin genes.
- The flouroquinole group were found highly effective antibiotics against Aeromonads among which enrofloxacin was the most effective antibiotic.
- The gene sequences of the three enterotoxin genes were quite similar to other sequences available in the GenBank.

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APPENDIX-I

1. PHOSPHATE BUFFERED SALINE (PBS)

Ingredients	
Sodium chloride	8.00g
Disodium hydrogen phosphate	1.44g
Potassium dihyrogen phosphate	0.24g
Potassium chloride	0.20g
Distilled water	1000.00 ml

2. OXIDASE REAGENT

Ingredients	
N,N,N,N Tetramethyl-P-Phenylenediamine dihydrochloride	1.0 g
Distilled water	10.0 ml

The reagent was prepared fresh every time and used without sterilization.

3. AESCULIN AGAR

Ingredients	
Aesculin	1.0 g
Agar	40.0 g
Ferric citrate	0.5g
Peptone water	1000.0 ml

4. α- NAPTHOL SOLUTION

Ingredients	
α- napthol	5.0 g
Ethanol	100.0 ml

5. AMPICILLIN DEXTRIN AGAR

Ingredients	
Tryptone	5.0 g
Dextrin	10.0g
Yeast extract	2.0g
Sodium chloride	3.0g
Potassium chloride	2.0g
Magnesium sulphate	0.2g
Iron (iii)chloride	0.1g
Bromothymol blue	0.08g
Agar	15.0g
Distilled water	1000.0ml

pH :8.5±0.1
After autoclaving cool to 45-50°C and add ampicillin to a final concentration of 10 µg/ml.

6. VOGES-PROSKAUER TEST

GLUCOSE-PHOSPHATE BROTH (VP test medium)

Ingredients	
Peptone	0.5 g
Dipotassium hydrogen orthophosphate	5.0 g
Distilled water	1000.0 ml
Glucose	5.0 g

pH: 7.5
Sterilize at 115^0C for 10 min.

7. POTASSIUM HYDROXIDE (40%)

Ingredients	
Potassium hydroxide (40%)	40.0 g
Distilled water	100.0 ml

It was sterilized by filtration.

8. BROTH SUGARS

Ingredients	
Peptone	10.0 g
Meat/Beef extract	3.0 g
Sodium chloride	5.0 g
Distilled water	1000.0 ml
Bromothymol blue solution	12.0 ml

pH: 7.1± 0.1

After autoclaving aseptically add filter sterilized 1% appropriate carbohydrate. Mix well and distribute in sterile test tubes. Add inverted Durham's tube and remove any trapped gas. Steam for 30 min.

9. BROMOTHYMOL BLUE INDICATOR SOLUTION

Ingredients	
Bromothymol blue	100 mg
Sodium hydroxide (0.1N)	2.5 ml
Distilled water	47.5 ml

10. NUTRIENT AGAR

Ingredients	
Peptic digest of animal tissue	5 g/l
Beef extract	1.5 g/l
Yeast extract	1.5 g/l
Sodium chloride	5 g/l
Agar	15 g/l

Suspend 28.0 g in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min.

11. DECARBOXYLASE MEDIUM

Ingredients	
Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Distilled water	1000 ml
Bromocresol purple (0.2%- Soln.)	10 ml

Dissolve the solids in water and adjust pH to 6.7. Add indicator solution and divide base in 4 equal volumes. Sterilize by autoclaving and add following:

1st flask L. arginine hydrochloride 0.5%
2nd flask L. Lysine hydrochloride 0.5%.
3rd flask L. ornithine hydrochloride 0.5%.
4th flask No addition (Control)

Readjust pH to 6.7, if necessary. Dispense in 2 ml volumes in small tubes and sterilize at 115^0C for 10 min.

12. TRYPTONE WATER

Ingredients	
Tryptone	10.0 g
Sodium chloride	5.0 g
Distilled water	1000.0 ml

pH: 7.2$\pm$ 0.2

13. KOVAC'S REAGENT FOR INDOLE

Ingredients	
p- dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Conc. HCl	25.0 ml%

Dissolve the aldehyde in the alcohol by gently warming in a water bath at about 50-55^0C. Cool and add the acid. Protect from light and store at 4^0C.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. Tris –EDTA (TE) Buffer (pH 8.0)

Ingredients	
Tris HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

2. TRIS -Acetate EDTA (TAE) buffer (50x)

Ingredients	
Tris base	242 g
Glacial Acetic acid	57.1ml
EDTA (0.5M; pH 8.0)	100 ml
DW to make	1000 ml

3. ETHIDIUM BROMIDE

Ingredients	
Ethidium bromide	10 mg
DW	1ml

4. LOADING DYE (6X)

Ingredients	
Orange G	0%
Xylene cyanol	0%
Bromophenol blue	0%
Glycerol	60%
EDTA	60mM

Stored at 4℃

APPENDIX-II

1. **Gene Sequence of cytotoxic enterotoxin (*act*) gene of *Aeromonas hydrophila* strain JKAHM3, partial cds**

ACCTTGTCAGCTCTGGATATTCCAGACGGTGACGAAGTGGACGTGCAGTGG
CGACTGGTACACGACAGCGCGAATTTTATCAAGCCAACCAGCTATCTGGCG
CATTATCTCGGTTATGCCTGGGTGGGTGGCAATCACAGCCAATATGTCGGT
GAAGACATGGACGTGACCCGTGATGGCGATGGCTGGGTGATCCGTGGCAAC
AATGACGGCGGTTGCGAGGGGTATCGTTGTGGCGAGAAGACGGCCATCAAG
GTCAGCAATTTTGCGTACAACCTGGACCCTGACAGCTTCAAACATGGTGAT
GTGACCCAGTCTGATCGCCAGCTGGTCAAGACGGTGGTGGGCTGGGCGATC
AACGACAGCGACACCCCGCAATCCGGCTATGATGTCACCCTGCGTTACGAT
ACCGCCACCAACTGGTCCAAGACCAATACCTATGGCCTGAGCGAGAAGGTG
ACCACCAAGAACAAGTTCAAGTGGCCACTGGTAGGGGAAACCGAACTCTCC
ATCGAGATTGTGGCCAACCAGTCCTGGGCCTCCCAGAACGGGGGATCTACC
ACCACCTCCCTGTCGCAATCCGTGCGGCCGACGGTGCCGGCCCGCTCCAAG
ATCCCGGTGAAGATCGAGCTCTACAAGGCTGACATCTCCTATCCCTATGAA
TTCAAAGCCGATGTCAGCTATGACCTGACCCTGAGCGGCTTCC

Protein Sequence

TLSALDIPDGDEVDVQWRLVHDSANFIKPTSYLAHYLGYAWVGGNHSQYVG
EDMDVTRDGDGWVIRGNNDGGCEGYRCGEKTAIKVSNFAYNLDPDSFKHGD
VTQSDRQLVKTVVGWAINDSDTPQSGYDVTLRYDTATNWSKTNTYGLSEKV
TTKNKFKWPLVGETELSIEIVANQSWASQNGGSTTTSLSQSVRPTVPARSK
IPVKIELYKADISYPYEFKADVSYDLTLSGF

2. **Gene Sequence of cytotonic heat labile enterotoxin (*alt*) gene of *Aeromonas hydrophila* strain JKAHM3, partial cds**

ATGACCCAGTCCTGGCACGGCGCCATCCCCAGCCTGTACGCCATCGCCA
ATGCCCTGAAAGCGTCCGACAGCGAAGTGATCGCCGGGCTGGTGGGCGC
GGGTGTGGACCCGGCCCTGCTGGCGACGCTGATTGCCGATCCGACCCGT
CAGGCCGAGCTGCTGGCCGAGGCGAGCAAGCTGATCGGGGTGACCCTCA
CCTCCGGCGGCAAGCCGCTGGATGCCGAGCAGAACATCGGCCGCTTCAA
CCCGCTGCCCATGCTGGAAGAGGTGCAGTCGGTGCCGATGCGGGTCTTC
GCCAAGGATGCCCTCAACACCATCACCGACGTGATCATCTACCAGCACG
GCGTGACTTCGGTGAAAGAGAACGCCTATGCGCTGGCGCTGGGCCAGAT
CTATGCCGGCATGCAGGCAGGCAAGAAGGTGGCGCTGGTGGTGATCGAT
CACC

Protein Sequence

MTQSWHGAIPSLYAIANALKASDSEVIAGLVGAGVDPALLATLIADPTR
QAELLAEASKLIGVTLTSGGKPLDAEQNIGRFNPLPMLEEVQSVPMRVF
AKDALNTITDVIIYQHGVTSVKENAYALALGQIYAGMQAGKKVALVVID
H

3. **Gene Sequence of cytotonic heat stable enterotoxin (*ast*) gene of *Aeromonas hydrophila* strain JKAHM3, partial cds**

CGCCATCAACAGCTCGCCCATCGTCAGCGACAGCTTCTTCATTACCCCCCA
GAATCCACTGGTCAATACCCGGGCTTACGAGGGTGGCGTCAGCCAGCTGAT
CCCGCTCAAGCTGCCGCTCGCCCAGGGCAAGCCGCTCAGCTATCGCACCTA
TGTCGGCACCTTTGGCGAGGGTCAGCTCAGGCGCGACTTCAACCGCTTCCT
C

Protein Sequence

RHQQLAHRQRQLLHYPPESTGQYPGLRGWRQPADPAQAAARPGQAAQLSHL
CRHLWRGSAQARLQPLP

4. **Gene Sequence of cytotonic heat labile enterotoxin (*alt*) gene of *Aeromonas caviae* strain JKACD4, partial cds**

TGCCGGCATGCAGGCAGGCAAGAAGGTGGCGCTGGTGGTGATCGATCACCC
GCTGCACGGTGAGCGTGGCTTCGCTCTGAGTGGCAGCATGGCGACCGTGAC
CACCTCCGACAACCCGACCCCGTATCTGAACCTGAGCTACCTGACAGTGGC
CCGCGACAACCTGAAACAGAGCGTGGCGGATCTGCTGGGCCTGCGTCTGGC
GGTCGGTCTGGCCAATGCCAAGGGCGCCATCGGGACGCCCGGCAACCTCAA
GGTGCACTTCCTGGGTCACTCCCTGGGGGCCATTTCGGGTACCAACCTGCT
GGCGGTCGCCAACCAGACCCTGGGCAACGCGCAGGGGGATGCCCTGTTCAA
GTTCGACACCGGTGGTCTGGCCATGCCGGGTGGCGGCATAGCGCCGCTGCT
GCTGAACTCGCCGACCTTCGGCCCGACCATCAAGATGGGCGTGCTGACCAG
CGGCAGTGCCGAGCTGAAAGCGGGCTTCACCGCCTACGCGCCCAACTGCAA
GACCGCGGT

Protein Sequence

CRHAGRQEGGAGGDRSPAAR.AWLRSEWQHGDRDHLRQPDPVSEPELPDSG
PRQPETERGGSAGPASGGRSGQCQGRHRDARQPQGALPGSLPGGHFGYQPA
GGRQPDPGQRAGGCPVQVRHRWSGHAGWRHSAAAAELADLRPDHQDGRADQ
RQCRAESGLHRLRAQLQDRG

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner **Prof. J.P.S. Gill, Director School of Public Health and Zoonoses, of Veterinary Public Health, Birsa Agricultural University, Jarkhand** during Viva-voce examination held on 24th January 2013 have been incorporated in the manuscript entitled **"A Study on Molecular and Virulence Characterization of *Aeromonas* species Isolated from Different Sources"** submitted by **Mr. Mudasir Ali Rather (Regd. No. 2009-287-D).**

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