CHAPTER 1

General Introduction
1.1. *Aeromonas* species

Members of the genus *Aeromonas* belong to the order *Aeromonadales* which contains the family *Aeromonadaceae*. They are usually oxidase and catalase positive, glucose-fermenting, facultatively anaerobic bacteria whose natural habitat is in the aquatic environment (Janda and Abbott, 2010). *Aeromonas* are Gram negative, coccobacillary to bacillary, rigid, non spore-forming rods with rounded ends and polar or lateral flagella. Cells are 0.3-1.0 x 1.0-3.5 µm in size, and can occur singly, in pairs or rarely in short chains (Martin-Carnahan and Joseph, 2005). Optimum growth of aeromonads is observed within a temperature range of 22-35 ºC; however, for a few species, growth occurs in a temperature range from 0 to 45 ºC (USEPA, 2006). Some species, including most non-motile *A. salmonicida* strains, do not grow at 35 ºC (Martin-Carnahan and Joseph, 2005). *Aeromonas* can tolerate pH range from 4.5 to 9.0, although the optimum pH range is from 5.5 to 9.0 (Isonhood and Drake, 2002). The optimum sodium chloride concentration range for aeromonads is from 0 to 4% (USEPA, 2006).

Aeromonads are increasingly being regarded not only as important pathogens of fish and other cold-blooded organisms, but also as the opportunistic pathogens in both immuno-competent and immuno-compromised humans (Daskalov, 2006). *Aeromonas* species are responsible for intestinal and extra-intestinal infections like septicaemia, cellulitis, wound infections, urinary tract infections, peritonitis, hepatobiliary tract infections, and soft tissue infections in humans (Khajanchi *et al.*, 2010). *Aeromonas* can even cause more severe forms of infections such as haemolytic uremic syndrome (HUS) and necrotizing fasciitis (Igbinosa *et al.*, 2012). Five *Aeromonas* species viz. *A. hydrophila*, *A. caviae*, *A. veronii* (biovars veronii and sobria), *A. jandaei*, and *A. schubertii* are most commonly implicated in human intestinal infections. These five species account for >85% of the clinical *Aeromonas* isolates and are considered major pathogens (Janda...
and Abbott, 2010). Hiransuthikul et al. (2005) reported that *Aeromonas* species, including *A. hydrophila* and *A. sobria*, were the most frequently isolated bacteria from southern Thailand tsunami survivors, exposed to contaminated water. *A. hydrophila* and *A. salmonicida* are important fish pathogens and result in huge economical losses in the fishing industry (Tomas, 2012).

*Aeromonas* have the ability to colonize drinking water distribution systems and produce biofilms that resist disinfection by chlorination and antibiotics, and thus may pose major public health concerns (Edberg et al., 2007). *A. hydrophila* is listed on the first and second Contaminant Candidate List (CCL 1 and CCL 2) of potential water-borne pathogens; and EPA Method 1605 has been validated for detection and enumeration of *A. hydrophila* in drinking water (USEPA, 2006).

### 1.1.1. History and taxonomy

Over 100 years ago, *Aeromonas*-like bacteria were first isolated from water and diseased animals, and first linked to bacteremic “red leg” disease of frogs in 1891. It was later named as *Proteus hydrophilus* by Stanier (1943). Von Graevenitz and Mensch (1968) were the first to report the association of the genus *Aeromonas* with a variety of human infections. Until mid-1970s, based on the growth characteristics and other biochemical features, aeromonads were clustered into two groups: mesophilic *A. hydrophila* group, consisting of motile aeromonads growing well at 35-37 °C and recognized to cause human infections, and psychrophilic *A. salmonicida* group, consisting of non-motile strains with optimum growth temperature of 22-25 °C and causing diseases in fish (USEPA, 2006).

From mid-1970s to 1980s, DNA hybridization studies showed that multiple hybridization groups (HGs) existed within each of the recognized mesophilic species (*A. hydrophila, A. sobria*, and *A. caviae*). However, these newly recognized HGs could not be
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The taxonomy of the genus *Aeromonas* is complex and confusing, and is based upon DNA-DNA hybridization and 16S ribosomal DNA relatedness studies (Figueras et al., 2011). In addition, it has undergone several changes during the last two decades due to continuous addition of an increasing number of novel species, lack of congruity between phenotypic and genotypic characteristics of species, rearrangement of already described strains and species, use of new diagnostic criteria, and discrepancies found in different DNA–DNA hybridization studies (Martinez-Murcia et al., 2011).
According to the recent edition of Bergey’s Manual of Systematic Bacteriology (Martin-Carnahan and Joseph, 2005), the genus *Aeromonas* contains 14 phenospecies (*A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (biovars *sobria* and *veronii*), *A. jandaei*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia* and *A. popoffii*) that correspond to at least 17 DNA hybridization groups (HG) or genomospecies; and two DNA hybridization groups (HG), *Aeromonas* sp. HG11, now included in *A. encheleia* and *Aeromonas* sp. HG13 (formerly *Aeromonas* Group 501) that has been named as *A. diversa* (Martinez-Murcia et al., 2011). Several additional *Aeromonas* species have been recently described, including *A. culicicola*, *A. simiae*, *A. molluscorum*, *A. bivalvium*, *A. aquariorum*, *A. tecta*, *A. piscicola*, *A. fluvialis*, *A. taiwanensis* and *A. sanarellii*, *A. rivuli*, *A. cavernicola* and *A. australiensis* (Beaz-Hidalgo et al., 2013). Moreover, earlier defined species like *A. enteropelogenes* is now considered synonym of *A. trota*; whereas, *A. ichthiosmia* and *A. culicicola* are considered synonyms of *A. veronii*. However, the species *Aeromonas sharmana* sp. nov. is not regarded as a member of the genus *Aeromonas* (Figueras et al., 2011). The controversies related to *Aeromonas* taxonomy have been delineated on the LPSN web page at http://www.bacterio.cict.fr/a/aeromonas.html. The sequencing of more housekeeping genes and multi-locus phylogenetic analysis (MLPA) may contribute to understanding the evolution and taxonomy of the genus *Aeromonas* (Martino et al., 2011).

1.1.2. Clinical infections and treatment

*Aeromonas* are associated with a broad range of infections which include gastroenteritis, wound infections, septicaemia and lung infections (Ghenghesh et al., 2008). Most of the *Aeromonas*-related human intestinal and extra-intestinal infections are caused by *A. hydrophila*, *A. caviae*, *A. veronii* (biovars *veronii* and *sobria*), *A. jandaei*, and
A. schubertii (Janda and Abbott, 2010). Though the role of aeromonads as agents of gastroenteritis remains controversial, several microbiological, epidemiological, and clinical/immunological investigations indicate that some strains of *Aeromonas* are enteric pathogens (USEPA, 2006). Moreover, young children, elderly and immuno-compromised individuals are usually more susceptible to *Aeromonas* infections than immuno-competent individuals (Chopra et al., 2009).

Gastroenteritis is the most common disease associated with aeromonads ranging from a mild self-limiting watery diarrhoea (secretory) to a more severe, invasive *Shigella*-like dysenteric form (Khajanchi et al., 2010). *A. caviae* and *A. veronii* biovar *sobria* are the most common *Aeromonas* species associated with traveler’s diarrhoea (Janda and Abbott, 2010). Aeromonad gastroenteritis usually manifests itself in three ways: an acute self-limiting, watery diarrhoea; dysentery-like mucus and bloody diarrhoea; and chronic diarrhoea (Parker and Shaw, 2011).

Wound infections are the second most common infection among the *Aeromonas*-associated clinical cases (Parker and Shaw, 2011). Severity of wound infections caused by *Aeromonas* varies from mild, topical infections (e.g., cellulitis and funculitis) to serious or life-threatening infections. More than 90% of *Aeromonas* wound infections are community acquired and occur in persons of ≥10 years of age (Janda and Abbott, 2010). *Aeromonas*-associated skin and soft-tissue infections were significantly observed during 2004 Tsunami in Southeast Asia (Hiransuthikul et al., 2005).

A majority of the *Aeromonas*-associated septicaemia occurs in immuno-compromised elderly patients with predisposing medical complications such as impaired hepatobiliary function and malignancy (Igbinosa et al., 2012). It can also occur in other underlying disorders such as trauma, cardiac anomalies, gastrointestinal disorders, anemia, and respiratory problems (Khajanchi et al., 2010). However, in rare occasions, it can occur
in apparently healthy adults having severe aeromonads wound infections. *A. hydrophila*, *A. veronii*, *A. caviae* and *A. jandei*, are the most common cause of septicemia (Janda and Abbott, 2010).

Gastrointestinal infections caused by *Aeromonas* are generally self-limiting, and the patient recovers within a few days. Usually, antibiotic therapy is not specified, except in chronic gastrointestinal cases, immuno-compromised patients and extra-intestinal infections (Hasan, 2006). Rehydration therapy, antibiotic treatment (if required), and nutritional therapy are the most commonly used treatment methods for gastroenteritis caused by *Aeromonas* (Igbinosa et al., 2012). Quinolones, chloramphenical, trimethoprim-sulfamethoxazole, and tetracyclines are the drugs of choice for severe gastrointestinal illness; whereas, sulfonamides, oxytetracycline, and quinolones have been successfully used in aquaculture and veterinary medicine (Janda and Abbott, 2010). However, in cases of extra-intestinal infections such as necrotizing fasciitis, surgical intervention may be required. Only few developed countries have included aeromonads in their standard analysis and necessitate reporting of these organisms from water and foods to authorities (Ghenghesh et al., 2008).

1.1.3. Pathogenesis and virulence factors

The pathogenic mechanisms of *Aeromonas* infections are complex, multifactorial and incompletely understood (Janda and Abbott, 2010). Multiple virulence factors play a pivotal role in the establishment of *Aeromonas* infection. The main virulence factors associated with *Aeromonas* are present in two forms, cell-associated structures and extracellular products. These virulence factors sequentially facilitate *Aeromonas* to colonize, penetrate, establish, replicate, and cause damage in host tissues and to escape the host defense system and proliferate, and thus killing the host (Tomas, 2012).
1.1.3.1. Cell-associated virulence factors

*Capsule:* It is a highly hydrated, outer-most layer of the bacterial cell and made up of polysaccharides (repetitions of monosaccharides, linked within each other by glycosidic bonds). It protects bacteria against phagocytosis, assist in interactions with other bacteria and host tissue, and act as a barrier against hydrophobic toxins. Capsule production has been reported for *A. hydrophila* serotype O: 34 and *A. veronii* bv. *sobria* serotype O:11 when grown in glucose-rich media (Tomas, 2012).

*Outer Membrane Proteins (OMP):* They are known to play a role in adherence of bacteria to host cells. The role of OMPs has been shown in attachment of *Aeromonas* strains to Caco-2, HEp-2, HeLa, Chinese hamster ovary (CHO) and Vero cells (USEPA, 2006).

*Fimbriae/Pili:* These are hair-like appendages on the bacterial surface and composed of oligomeric pilin proteins. In addition to adhesion, they are also involved in other functions, such as phage binding, DNA transfer, biofilm formation, cell aggregation, host cell invasion, and twitching motility (Tomas, 2012). Two morphotypes of pili have been observed in clinical and environmental strains of mesophilic *Aeromonas*; short rigid pili (S/R type) and long wavy flexible pili (L/W type) (USEPA, 2006).

*Lipopolysaccharide (LPS):* It is a surface glycoconjugate present on the outer membrane of the gram-negative bacteria. It consists of lipid A, core oligosaccharide (OS), and O-specific polysaccharide or O antigen (Tomas, 2012). It has a role in adhesion to epithelial cells, resistance to nonimmune serum and virulence (USEPA, 2006).

*S-layers:* They (also known as A-layer in *A. salmonicida*) are a surface protein layer of paracrystalline nature made up of identical protein subunits. It plays a role in pathogenicity by facilitating adhesion, protection against host immune response and phagocytosis, antigenic properties, bacteriophage receptor, and role in colonization. The
role of S-layer was observed in extra-intestinal infection of humans by *Aeromonas* spp. (Gavin *et al.*, 2003).

*Flagella:* It is a thin, threadlike appendage that allows *Aeromonas* to reach target cells where they colonize. *Aeromonas* generally have monotrichous and polar flagella. However, some strains have lateral flagella; while others are nonmotile. Production of flagella is controlled by over 40 genes (Tomas, 2012). Two polar and two lateral flagellins are present in *A. caviae*; whereas, *A. hydrophila* have two polar and only one lateral flagellin. Polar flagella help in adhesions, while lateral flagella are known to play an important role in cell adherence, invasion and biofilm formation (Gavin *et al.*, 2003). Both polar and lateral flagella are enterocyte adhesins that need to be fully functional for optimal biofilm formation on surfaces (Kirov *et al.*, 2004).

### 1.1.3.2. Extracellular virulence factors

Members of the genus *Aeromonas* produce an extensive array of putative virulence factors like exotoxins (aerolysin, haemolysin, cytotoxic and cytotoxic enterotoxins) and extracellular enzymes (proteases, lipases, nucleases, elastase and hydrolytic enzymes) that may play an important role in the development of disease, either in humans or in fish (Igbinosa *et al.*, 2012). The plethora of described virulence factors, dearth of suitable animal models and lack of consistent terminology for these factors among different research groups is the key challenging problem in establishing the role of these virulence factors in *Aeromonas* (Janda and Abbott, 2010).

**Exotoxins**

Enterotoxin production in *Aeromonas* spp. has been classified into two categories: cytotoxic and cytotoxic enterotoxins.
**Cytotoxic enterotoxins:** These toxins are also known as cytolytic enterotoxins, and have haemolytic, cytotoxic and enterotoxic properties. They induce degeneration of crypts and villi of the small intestine (Tomas, 2012).

Aerolysin is a 50-52 kDa heat labile, pore-forming toxin that disrupts cell membrane and produce clear zones of haemolysis on blood agar. It possesses both haemolytic and enterotoxic activity and shares significant homology with the cytotoxic enterotoxin (Act), and two cytotoxic toxins (Alt and Ast) (Janda and Abbott, 2010).

Aerolysin is one of the best characterized bacterial channel forming toxins. It is synthesized intracellularly in a precursor form, proaerolysin that is converted to aerolysin by proteolytic cleavage of a C-terminal peptide fragment of approximately 40 amino acids. Aerolysin binds to specific surface receptors on the target cell and promotes oligomerisation. It produces a heptamer that inserts into the cell membrane and forms a 1-2 nanometer (nm) channel which results in loss of cell permeability, cell leakage, and eventual cell destruction (USEPA, 2006).

Haemolysin, encoded by the hlyA gene, is a non-channel forming haemolysin sharing significant amino acid sequence homology to the haemolysin of *V. cholera*. The hlyA gene is widely distributed among *Aeromonas* species (Janda and Abbott, 2010).

*Aeromonas* cytotoxic enterotoxin (Act), a 52 kDa single-chain polypeptide from *A. hydrophila* SSU, is a type II secreted pore-forming toxin with haemolytic, cytotoxic, and enterotoxic activities. Act enterotoxin is closely related to aerolysin and causes lethality in mice (Chopra et al., 2009). It leads to tissue damage and high fluid secretion in intestinal epithelial cells due to induction of a pro-inflammatory response in the target cells (Galindo et al., 2006).
Cytotoxic enterotoxin: These toxins increase the cyclic adenosine monophosphate (cAMP) levels and prostaglandins in intestinal epithelial cells. Cytotoxic enterotoxins produced by Aeromonas are classified into two groups: (i) Aeromonas heat-labile (56 °C for 10 min.) toxin (Alt), without cross-reactivity with the choleric anti-toxin, and (ii) Aeromonas heat-stable (100 °C for 30 min.) toxin (Ast) that reacts with the choleric antitoxin. These toxins cause fluid accumulation in ligated ileal loops in animal models and may have a role in causing diarrhoea in humans (Chopra et al., 2009).

Extracellular enzymes

Most aeromonads produce a wide range of extracellular enzymes such as proteases, lipases, amylases, chitinases, nucleases, gelatinases and elastases that may contribute to pathogenesis in some strains (Pemberton et al., 1997). These extracellular enzymes actively degrade a variety of complex protein, polysaccharide, muco-polysaccharide, and lipid-containing molecules.

Proteases: Aeromonas species produce an array of microbial proteases (metalloproteases, serine proteases, and aminopeptidases) that have a role in degradation of complex biological proteins present in serum and connective tissue (Janda and Abbott, 2010). Proteases also help in invasion of host tissue, proteolytic activation of toxins, inactivation of the host complement system, and making nutrients available for cell proliferation (Tomas, 2012).

Lipases: Different kinds of extracellular lipases (lip, lipH3, pla and plc) are secreted by aeromonads. They provide nutrients, alter the host plasma membrane and increase the severity of infection. A. hydrophila and A. salmonicida produce a lipase, glycerophospholipid cholesterol acyltransferase (GCAT) that has lipase or phospholipase activity and digests erythrocyte membranes and lead to their lysis (Pemberton et al., 1997).
Elastase: Elastase is a zinc metalloprotease and an important virulence factor and phenotypic marker in *Aeromonas* (USEPA, 2006).

Nucleases: Though nucleases have been reported in *Aeromonas*, their role in pathogenesis has not been established (Pemberton *et al.*, 1997).

1.1.3.3. Secretion systems

Gram-negative bacteria possess different protein secretion systems to sense the external medium or eukaryotic organisms, and secrete proteins inside the host cell that will help in causing infection (Tomas, 2012). Type II secretion system plays a critical role in secretion of aerolysin as a soluble precursor “pro-aerolysin” in *A. hydrophila* and *A. salmonicida* (USEPA, 2006).

Type III Secretion System (T3SS) is commonly associated with pathogenesis and virulence and consists of a complex multi-component system which transports bacterial effector molecules directly from the bacterial cytoplasm across the inner and outer membrane of the bacterial envelope to either the external medium or directly into the eukaryotic cells (Tomas, 2012). Various researchers have reported the presence of T3SS in *A. salmonicida* and *A. hydrophila* strains AH-1, AH-3 and SSU (USEPA, 2006). A functional type VI secretion system (T6SS) has been identified in a clinical isolate of *A. hydrophila* SSU (Suarez *et al.*, 2008).

1.1.3.4. Siderophores

Siderophores are low-molecular weight, iron-specific ligands produced by microbes during infection that facilitate iron acquisition from the host. They play an important role in the survival of *Aeromonas* within their hosts and establishment of infection (Tomas, 2012). Most of the studied strains of *A. salmonicida*, *A. hydrophila*, *A.*
sobria, and A. caviae synthesize one or more types of siderophores under iron-limiting conditions (Chopra et al., 2009).

1.1.3.5. Quorum sensing

Quorum sensing is a bacterial signaling system that involves the production, release, and subsequent sensing of chemical signaling molecules known as autoinducers (AI) that play a role in sensing bacterial population and regulating gene expression in response to high cell densities (Garde et al., 2010). Quorum sensing is known to regulate various physiological functions such as sporulation, competency for transformation, turning on/off of various virulence factors, conjugation, antibiotic production, and biofilm formation (Khajanchi et al., 2009). Most of the Aeromonas strains, belonging to different species, possess luxRI homologues encoding an acyl-homoserine lactone (acyl-HSL)-dependent transcriptional activator (Jangid et al., 2007). The quorum sensing signal generator and response regulator are known as ahyRI and asaRI in A. hydrophila and A. salmonicida, respectively. Quorum sensing plays a role in biofilm formation, cell division and production of virulence factors in Aeromonas (Beaz-Hidalgo and Figueras, 2013).

Although several animal models have been proposed for Aeromonas virulence studies; mouse model, medicinal leech model and blue gourami model, none of these are able to reproduce gastrointestinal disease to exhibit the Koch-Henle postulates (Tomas, 2012). Several in-vitro cell lines like Vero, HEp-2, INT 407, mouse Y1 adrenal cells, and Caco-2 have been used to study attachment, invasion and toxic effects of Aeromonas virulence factors (USEPA, 2006).

Even though a number of putative virulence factors have been proposed and shown in food strains, the precise role and mechanism of aeromonads in causing diarrhoeal illness
has not been explained. Studies have shown that the infective dose of *Aeromonas* spp. is very large (> 6-8 log$_{10}$ CFU/g) and only selected strains have the ability to produce gastrointestinal disease. However, it is possible that some strains may have a lower infective dose in sensitive sub-populations (USEPA, 2006). The virulence of *Aeromonas* depends on the bacterial strain, route of infection, and the animal used as model organism (Tomas, 2012).

1.1.4. Prevalence

*Aeromonas* are ubiquitous bacteria found in a variety of aquatic environments and food products including fresh grocery produce, seafood, raw meats, packaged ready-to-eat meats, milk and milk products (Igbinosa *et al.*, 2012; Ottaviani *et al.*, 2011). They are also found in the intestinal tract of animals and humans, water sources (chlorinated water, estuarine and marine waters, sewage contaminated waters), and activated sludge (USEPA, 2006). Though different methods of analysis, selective and enrichment media, sampling period, geographical location, and types and sources of commercial products analyzed are used, the collective results from these investigations indicate that aeromonads are common inhabitants of most types of food, regardless of geographic origin (Janda and Abbott, 2010).

*Aeromonas* have been isolated from different food samples of plant origin such as alfalfa sprouts, asparagus, broccoli, cauliflower, pepper, spinach, celery and lettuce (Stratev *et al.*, 2012). *Aeromonas* were reported 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 (Neyts *et al.*, 2000). Other studies have found aeromonads in fresh and frozen chicken, game birds, raw and pasteurized milk, cheese, baby food, bakery
products, fruits and vegetables, fish, water and soil (Awan et al., 2009; Igbinosa et al., 2012; Yucel and Balci, 2010).

Several incidences of *Aeromonas* associated diarrhoea has been reported from different cities of India (Sinha et al., 2004; Subashkumar et al., 2006). In India, aeromonads have been isolated from various foods of animal origin, fish and prawns (Vivekanandhan et al., 2005), fish, poultry meat, pork and chevon (Sharma and Kumar, 2011), snails, quail eggs, buffalo milk and goat meat (Arora et al., 2006). *Aeromonas* have also been reported from clinical specimens (Sinha et al., 2004) and river water (Sharma et al., 2005) in India.

However, in developing country like India, only a fraction of all food-borne infections are ever diagnosed and officially reported, or can be traced to a certain vehicle and a specific causative agent. India does not have a national surveillance program, particularly for *Aeromonas*-related infections; therefore, incidence values in terms of number of cases for a population do not exist. Availability of such surveillance program will facilitate monitoring outbreaks and even sporadic cases of *Aeromonas*-related gastroenteritis.

**1.2. *Aeromonas*: Isolation, identification and characterization**

**1.2.1. Isolation and enumeration**

Sensitive and specific public health surveillance and diagnostic procedures are required to detect outbreaks of *Aeromonas* species efficiently (Igbinosa et al., 2012). Isolation of *Aeromonas* from food and environmental samples is a challenging procedure due to the presence of competing microflora, and possibility of interference of sample matrix with sample preparation and culture methods. Isolation, enumeration, and culture
and molecular methods based identification of *Aeromonas* spp. from various sources was reviewed by USEPA (2006) and Janda and Abbott (2010).

Selective and differential media are required for the isolation, identification and enumeration of *Aeromonas*. The medium should be selective, differential and recovery of the desired organism should be quantitative. Aeromonads have the ability to grow readily on laboratory culture media, and a large number of selective and differential culture media have been developed for the recovery of *Aeromonas* spp. from the environment, foods and clinical specimens. Broth enrichment methods are frequently used to recover aeromonads from samples where they may be present in low numbers together with larger numbers of other bacteria.

Different culture enrichment media like alkaline peptone water (APW) or tryptone soya broth containing ampicillin (TSB-30) (ampicillin 30 mg/l), starch glutamate ampicillin penicillin (SGAP-10) medium and *Aeromonas* medium (Ryan’s Medium) have been used for selective enrichment of *Aeromonas* from various sources (Igbinosa et al., 2012). Selective media like ampicillin dextrin agar, meso-inositol xylose agar, starch glutamate ampicillin-penicillin agar, tryptose xylose ampicillin agar, xylose ampicillin agar, and prl-ampicillin-dextrin-ethanol agar, MacConkey agar, cefsulodin irgasan novobiocin (CIN) agar, modified bile salts irgasan brilliant green agar (mBIBG medium), *Aeromonas* agar and starch ampicillin agar have been recommended for further isolation and enumeration (Janda and Abbott, 2010). Based on the comparative studies, it was observed that no single medium results in optimum recovery of *Aeromonas*, and various combinations of media and methods are commonly used for the isolation and enumeration of *Aeromonas* (Edberg et al., 2007).
1.2.2. Identification and detection

Presumptive *Aeromonas* isolates can be identified up to species level using biochemical and molecular methods. The presumptive isolates are identified as belonging to genus *Aeromonas* based on few preliminary phenotypic characteristics i.e. Gram staining, motility, oxidase and catalase tests, fermentation of D-glucose and trehalose, nitrate utilization, non-growth in high salt and resistance to vibriostatic agent O/129 (Abbott *et al*., 2003).

1.2.2.1 Biochemical methods

The species level identification of *Aeromonas* based on routine phenotypic characteristics is complex and confusing due to extreme phenotypic diversity among inter- and intra- genospecies of *Aeromonas* (Figueras *et al*., 2011). Various biochemical schemes for the identification of *Aeromonas* based on biochemical characteristics have been proposed (Abbott *et al*., 2003; Carnahan *et al*., 1991; Martin-Carnahan and Joseph, 2005).

Aerokey II, a flexible and dichotomous key based on biochemical tests, was developed by Carnahan *et al.* (1991) for the reliable and accurate identification of most of the clinically significant recognized *Aeromonas* strains. Whole cell fatty acid analysis (FAME) of *Aeromonas* strains by gas-liquid chromatography was shown as a chemotaxonomic marker for the differentiation of the majority of the phenospecies and/or hybridization groups in the genus *Aeromonas* (Huys *et al*., 1995). A monoclonal antibody ELISA test for the rapid detection of *Aeromonas* spp. in human faeces was developed by Delamare *et al.* (2002a). A miniaturized phenotypic identification system with an improved probability matrix was developed by Carson *et al.* (2001) for the convenient identification of *Aeromonas* species in the medical and veterinary laboratories.
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An elaborate scheme, based on biochemical tests, was developed for the identification of nearly every isolate to species levels of *Aeromonas* by Abbott *et al.* (2003) and Martin-Carnahan and Joseph (2005). A culture collection of 193 strains representing 14 different *Aeromonas* genospecies were assessed for 63 phenotypic characteristics by Abbott *et al.* (2003). The observations from this study were used to develop a system for the identification of *Aeromonas* isolates, first to group level, and then to species level within each group. However, the correct identification of atypical environmental isolates is difficult due to continuous addition of an increasing number of novel species, rearrangement of already described strains and species and variable biochemical characteristics (Martinez-Murcia *et al.*, 2011).

Several commercial identification systems (API-20E, API-32GN, Vitek2 system, MicroScan Walk/Away system, ID69-Phoenix system, BBL Crystal Enteric/Nonfermenter system and GN2 microplates-Omnilog system) are available for the identification of *Aeromonas* species. These commercial kits are usually expensive and provide inaccurate identification due to outdated databases and taxonomy, weak algorithms, and exclusion of key substrates required for differentiation of species (Lamy *et al.*, 2010b; Soler *et al.*, 2003).

1.2.2.2. Molecular methods

Application of polymerase chain reaction (PCR) and genetic probes for the direct detection of *Aeromonas* species in a broad range of samples and matrices have increased rapidly in the past two decades. Molecular methods have advantage of the use of stable genotypic characteristics, are simple, reproducible, rapid, specific, more sensitive, culture-independent, and have high throughput and discriminatory power (Prakash *et al.*, 2007).
Martinez-Murcia et al. (1992) used differences in the 16S ribosomal DNA sequences to identify aeromonads up to different genotypes. However, in case of *Aeromonas*, 16S rDNA gene has been found to be inefficient in correct identification of closely related *Aeromonas* species due to its highly conserved gene sequence, presence of 16S rRNA gene polymorphism and some discrepancies between 16S rRNA gene and DNA–DNA hybridization results (Kupfer et al., 2006; Morandi et al., 2005). Borrell et al. (1997) used restriction digestion of PCR-amplified 16S rRNA genes with *Alu*I and *Mbo*I for the identification of ten *Aeromonas* species. They further used *Nar*I and *Hae*III to differentiate *A. salmonicida* from *A. encheleia*. Two additional endonucleases *Alw*NI and *Pst*I were later added to this restriction fragment length polymorphism (RFLP) method to differentiate between *A. salmonicida* and *A. bestiarum* and for the recognition of *A. popoffii* (Figueras et al., 2000).

Various researchers have successfully used the partial sequences of different housekeeping genes: *gyrB*, *rpoD*, *recA*, *rpoB*, *dnaJ* and *cpn60* for the phylogeny and taxonomic identification of *Aeromonas* species (Silver et al., 2011). Studies based on the partial sequences of these house-keeping genes have proved that they are better molecular makers than 16S rDNA gene for assessing phylogeny in the genus *Aeromonas* (Martinez-Murcia et al., 2011). Suitable evidence for some of the recently described new species and correct identification of some of the uncertain strains was provided by the sequence analysis of some of these housekeeping genes (Figueras et al., 2011; Martinez-Murcia et al., 2011). Martinez-Murcia et al. (2011) and Martino et al. (2011) have used multilocus sequence typing (MLST) to study the intra- and inter-species phylogenetic relationships of the genus *Aeromonas*.

Several investigators have also developed PCR based methods targeting aerolysin toxin, extracellular lipase, serine protease, DNAse, glycerophospholipid-cholesterol
acyltransferase (GCAT) and ferric siderophore receptor for the detection of Aeromonas in different food and environmental samples (Chacon et al., 2003; Kingombe et al., 1999; Sen and Rodgers, 2004). Multiplex PCR, a rapid and simple method for the detection of enterotoxin genes in Aeromonas strains was developed (Sen, 2005; Wang et al., 2003). New identification systems such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and MALDI-Time of Flight MS (MALDI-TOF MS) have been used for the rapid and accurate identification of the clinical and environmental Aeromonas isolates (Benagli et al., 2012; Donohue et al., 2007).

Fluorescent in situ hybridization (FISH) and PCR–denaturing gradient gel electrophoresis (DGGE)-based methodologies have been used to assess diversity and dynamics of Aeromonas communities in goldfish and water systems, respectively (Asfie et al., 2003; Calhau et al., 2010). Khushiramani et al. (2009) developed an outer membrane protein-based digoxigenin (DIG)-labeled DNA probe to detect Aeromonas spp. from food/environmental/clinical samples. TaqMan real-time PCR targeting the 16S rRNA and aerA genes has been used for the convenient, rapid, highly sensitive and specific identification of A. hydrophila (Trakhna et al., 2009).

1.2.3. Characterization

Bacterial typing is an important epidemiological tool for tracking source and pathways of outbreaks of infection, examination of virulent strains and infectious diseases (Foley et al., 2009). Typing methods can be broadly classified into two groups i.e. phenotypic methods and genotypic methods.
1.2.3.1. Phenotypic methods

These methods are based on the clustering of organisms according to their similarity in phenotypic characters resulting from the expression of their genotypes. The most commonly used phenotyping methods are described below:

**Biotyping:** It is based on biochemical characteristics that differentiate microorganism to the species level. Generally, this method is easy and inexpensive, and has excellent typeability and variable discriminatory power (van Belkum et al., 2007).

**Phage and bacteriocin typing:** They evaluate the lytic patterns of the isolates that have been exposed to a defined set of bacteriophages or bactericidal toxins (bacteriocins). These methods require technical expertise and prolong efforts, and provide variable discrimination and limited typeability.

**Serotyping:** It is based on the differences of antigens such as lipopolysaccharide, somatic O- and flagella H-antigens or somatic O- and K-antigens. It is a high throughput and widely used method in healthcare and food-related laboratories. Sakazaki and Shimada (1984) developed serotyping of *Aeromonas* based on somatic (O) antigen determinants.

**Protein profile:** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of cellular and extracellular proteins is considered as a rapid, inexpensive and reliable system for characterization of microorganisms. This method has a high discriminatory power, gives reproducible results, and is used for typing of strains for epidemiological assessments (van Belkum et al., 2007).

**Multilocus enzyme electrophoresis (MLEE):** Since metabolic enzymes coded by different alleles of the same gene have small but detectable variations in protein size and charge; MLEE uses this characteristic to detect electrophoretic variants of a set of housekeeping enzymes. This method is highly reproducible and was used to study the
genetic diversity between *A. hydrophila*, *A. bestiarum*, *A. salmonicida* and *A. popoffii* (Minana-Galbis *et al*., 2004).

**Antibiogram-based typing:** This method is based on the antimicrobial susceptibility testing of the strains against different antibiotics, heavy metals, disinfectants and antiseptics. Discriminatory power and usefulness of this method is variable (van Belkum *et al*., 2007).

Antibiotic susceptibility of *Aeromonas* isolates was usually reported based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for the *Enterobacteriaceae* (CLSI, 2006). However, recommendations for the interpretation of MIC values for *Aeromonas* isolates by agar dilution breakpoint method were recently published by Clinical and Laboratory Standards Institute (2011). Antibiotic sensitivity patterns are useful for the selection of antibiotic therapy, and are sometimes used as phenotypic markers for species identification (Overman and Janda, 1999).

*Aeromonas* are typically resistant to penicillins (penicillin, ampicillin, oxacillin, amoxicillin, carbenicillin and ticarcillin). The high resistance is due to the production of inducible chromosomal β-lactamases (Janda and Abbott, 2010). Most of the *Aeromonas* species are characteristically susceptible to aminoglycosides, carbapenems, second and third generation cephalosporins, azithromycin, monobactams, nitrofurans, tetracyclines, chloramphenicol, trimethoprim-sulfamethoxazole, quinolones, piperacillin and azlocillin (Awan *et al*., 2009). The antibiotic sensitivity patterns of *Aeromonas* may vary significantly due to individual species, varying geographic locales and isolation sources, different interpretation methods used or environmental selection pressures (Janda and Abbott, 2010).

Antibiotic resistance in *Aeromonas* spp. is usually chromosomally mediated, but β-lactamases produced by aeromonads may occasionally be encoded by plasmids or
Aeromonads are known to produce one or more unrelated inducible β-lactamases with activity against a wide variety of β-lactam antibiotics, including penicillins, cephalosporins, and extended-spectrum cephalosporins. Three principal classes of β-lactamases are recognized in *Aeromonas* species, namely, a class C cephalosporinase, a class D penicillinase, and a class B metallo-β-lactamase (MBL) (Janda and Abbott, 2010). Oxytetracycline is commonly used in aquaculture, and transfer of large plasmids conferring oxytetracycline resistance among environmental aeromonads and between *Aeromonas* spp. and *E. coli* has been reported (USEPA, 2006).

Recently, several modern methods like Mass spectrometry (MS), Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS, Infrared (IR) or Raman spectroscopy, Gas–liquid chromatography (GLC system) and Fourier-transform (FT)-IR spectrometry/ FT-IR microscopy, proteomics, glycomics and metabolomics have been used to characterize different strains of bacteria (van Belkum *et al.*, 2007).

### 1.2.3.2. Genotypic methods

These methods analyze the variation in the genetic material of bacterial strains with respect to their composition, overall structure, or precise nucleotide sequence. Different genotyping methods are grouped based on the basic principle behind the technique: (i) Hybridization-mediated methods (ribotyping, array hybridization), (ii) Fragment-based methods {Plasmid typing, RFLP, PFGE, PCR fingerprinting: amplified ribosomal DNA restriction analysis (ARDRA), AFLP, RAPD, multilocus variable number tandem repeat
(VNTR) analysis (MLVA), (iii) Sequence-based methods (single-locus sequence typing (SLST), multi-locus sequence typing (MLST) and SNP genotyping) (Foley et al., 2009).

**Plasmid analysis:** It is a simple method to determine the number, size and/or restriction endonuclease digestion profiles of plasmids in bacterial isolates. Plasmid and antibiotic resistance profile of the *Aeromonas* strains are frequently determined together in clinical microbiology laboratories to assess the reason for antibiotic resistance (Palu et al., 2006). The typeability and discriminatory power of this method are variable and depend on the bacterial species. Earlier studies have shown the prevalence of plasmids in *Aeromonas* isolates from various sources (Brown et al., 1997; Chaudhury et al., 1996; Radu et al., 2003).

**Restriction fragment length polymorphism (RFLP):** RFLP is a technique that detects a difference in restriction endonucleases profiles after digestion of DNA samples with specific restriction endonucleases. Differences in fragment length result from base substitutions, additions, deletions or sequence rearrangements within RE recognition sequences. It is a rapid and reproducible method used to determine the differences among microorganism strains. Clinically important *Aeromonas* spp. were identified till species level by RFLP of 16S rDNA using BstSNI, MboI and *Pvu*II restriction endonucleases (Ghatak et al., 2007).

**Randomly amplified polymorphic DNA (RAPD):** The method is based on the PCR amplification of random genomic DNA segments with small random arbitrary primers under low stringency primer annealing conditions. It is a rapid, sensitive, and inexpensive method for subtyping of different strains, gene mapping studies and plant genetic analysis. RAPD has been successfully used for typing of *Aeromonas* isolates from clinical and environmental sources (Maiti et al., 2009).
Amplified restriction fragment length polymorphism (AFLP): AFLP is a PCR-based DNA fingerprinting technique that involves restriction digestion of total genomic DNA with two restriction enzymes, usually a ‘rare’ and a ‘frequent cutter’. The resultant DNA fragments are ligated to oligonucleotide adapters, and a subset of DNA fragments are amplified using primers containing adapter defined sequences which do not require prior knowledge of nucleotide sequence. Polymorphisms are detected by differences in the banding patterns of the amplified fragments and are assessed as the presence or absence of bands. AFLP is a highly reproducible and high-throughput molecular typing method used for the genotyping of different bacterial species and strains. Huys et al. (1996) have found AFLP to be a valuable high-resolution genotypic tool for the classification of *Aeromonas* species.

Multilocus sequence typing (MLST): It is a generic typing method to evaluate the DNA sequence variation among the alleles (usually five to ten) of housekeeping genes. DNA sequences (approx. 450-500 bp) of internal fragments of each housekeeping gene are amplified, sequenced and concatenated to obtain a unique allelic sequence. The matrix of pair-wise differences between these allelic profiles is used to construct a dendrogram that shows the relatedness among the isolates. MLST is a universal, robust and portable method for determining allelic profile or sequence type (ST) that can be exchanged between laboratories. It is a reproducible, scalable and automated technique that can be used for evolutionary and population studies of a wide range of bacteria, regardless of their diversity, population structure, or evolution (Foley et al., 2009). A number of *Aeromonas* isolates have been characterized using MLST with putative virulence factors (*ast, ahh1, act, asa1, eno, ascV, and aexT*) and housekeeping gene fragments (*gyrB, rpoD, recA, dnaJ, gyrA, dnaX, and atpD*) by (Martino et al. (2011) and Martinez-Murcia et al. (2011), respectively. The results showed that the genus *Aeromonas* comprises of a number of well
separated clusters of strains, and taxonomical structure based on MLPA may clarify some previously reported contentious identification and provide support to the recently described species (Martinez-Murcia et al., 2011).

**DNA-DNA hybridization:** It is a technique to determine the genetic relationship between two species by measuring the degree of genetic similarity between their whole-genome sequences. This technique is usually considered as the ‘gold standard’ for the description of new bacterial species; however, it is a technically-challenging, labourious and time-consuming procedure that requires labeled or large amounts of DNA (Foley et al., 2009). DNA-DNA hybridization has been widely used for the description and validation of novel Aeromonas species (Janda and Abbott, 2010).

**Pulse field gel electrophoresis (PFGE) profile:** PFGE is a ‘gold standard’ and most widely used agarose-gel electrophoresis method for characterization and epidemiological studies of pathogenic organisms. The large DNA fragments (approximately 20 and 600 kb) are generated by restriction digestion of the intact chromosome with ‘rare cutters’ restriction endonucleases. These fragments are separated in agarose gels by periodic alternation of the angle of the constant low electric field’s direction. Smaller DNA pieces will move more freely and re-orient to the new field more quickly than the larger ones, and therefore will migrate further down the gel creating a characteristic pattern of a particular species or strain of bacteria that differentiates between two bacterial strains. PFGE has remarkable discriminatory power and reproducibility, and has been used for the differentiation of various food-borne pathogens such as E. coli, Salmonella and Aeromonas (Foley et al., 2009; Pablos et al., 2010).

PFGE has been widely used for determining the genetic relatedness between Aeromonas isolates from various sources (water, environment and clinical specimens) (Bonadonna et al., 2002; Pablos et al., 2010).
1.3. Stress induced responses in food-borne pathogens towards food environments

Food-borne pathogens are frequently exposed to a variety of environmental stresses in their natural environment, food industry and host systems. Environmental stress and food preservation methods are known to induce adaptive responses within the bacterial cell. These stresses can be broadly classified into three categories: physical (drying, heat, high hydrostatic pressure, low temperature, pulsed white light, gamma, UV and X-ray radiations), chemical (chemical sanitizers, oxidative treatments, pH and preservatives) and nutritional (Wesche et al., 2009). Most microorganisms are able to tolerate and adapt to small changes in the environmental factors by either being compliant to the stress conditions and developing appropriate mechanisms for survival or attempting to resist the stress (Battesti et al., 2011).

1.3.1. Microbial biofilm

_Aeromonas_ can exist in either a free-living, planktonic phase, or in a sessile community attached to a substrate, known as a biofilm. Bacterial biofilms are complex communities of viable and nonviable microorganisms, enclosed by hydrated polyanionic extracellular polymeric substances (EPS), anchored to biotic or abiotic surfaces and formed by one or more microbial species (Kolter and Greenberg, 2006). EPS may contain polysaccharides, proteins, phospholipids, teichoic and nucleic acids, and other polymeric substances hydrated to 85-95% water (Flemming and Wingender, 2010). Microorganisms growing in biofilm have various advantages: concentration of nutrients for growth; protection from antimicrobials (antibiotics and sanitizers) and adverse environmental stresses such as acidification, temperature changes, desiccation, and UV exposure; sequestration of metals and toxins; proximity to progeny and other bacteria, thus
facilitating higher rates of DNA transfer; and resistance to phagocytosis and other host immune mechanisms (Chmielewski and Frank, 2003).

Biofilms have been reported from diverse locations and are a major concern in medical devices, industrial water system, and food processing industries (Simoes et al., 2010). Various food-borne pathogenic or spoilage microorganisms have the ability to attach and grow on food surfaces, equipment and processing environments to form biofilms. These may lead to mechanical blockages, reduction of heat transfer and efficiency and increase in corrosion rate of surfaces (Jahid and Ha, 2012).

**Characteristics and structure of biofilms**

The structure of biofilm is not simple and uniform in time or space, but rather more complex and differentiated. Microscale biofilm research has demonstrated that biofilms are structurally heterogeneous, fluid and constantly fluctuate over time and space (Srey et al., 2013). Biofilms are dynamic and adaptive and they respond to their environmental conditions. The architecture of biofilms is influenced by many factors, including hydrodynamic conditions, concentration of nutrients, bacterial motility and intercellular communication as well as exopolysaccharides and proteins. Biofilms found in many clinical, industrial, and natural environments are frequently mixed species (Flemming and Wingender, 2010).

**Process of biofilm development**

Biofilm development is a stepwise, dynamic, highly complex and regulated process. Various physical, chemical, genetic, and biological processes are involved in the final maturation of biofilms (Srey et al., 2013). The development of biofilms can be seen as a five-stage process: (i) initial reversible adsorption of cells to the solid surface, (ii)
irreversible attachment through production of quorum sensing molecules and EPS, (iii)
microcolony formation, (iv) colonization or maturation steps, and finally (v) dispersal

Figure 1.1. Model of biofilm development and processes governing biofilm formation
From Simoes et al. (2010)

Factors affecting microbial attachment to abiotic surfaces:

1. Topography of adhesion surfaces (texture, hydrophobicity, charge)
2. Contact time
3. Bulk fluid characteristics (flow velocity, pH, temperature)
4. Substratum preconditioning
5. Bacterial cell surface properties (hydrophobicity, appendages, EPS, signaling molecules)
The structure, function and composition of the biofilm development is determined by both cell-surface and cell-cell interactions (Kolter and Greenberg, 2006). Such interactions are affected by the chemical and physical environment to which the bacterial cells and the surface are exposed, and occur under complex regulatory network (Jahid and Ha, 2012). Biofilm formation by an organism depends on its genetic composition and regulation, substratum and bacterial cell’s properties and environmental factors including pH, temperature, medium composition and nutrient components Xu et al. (2011). The roles of various environmental factors, relevant to the food industry, on the biofilm formation ability of different food-borne pathogens have been studied (Stepanovic et al., 2004).

Aeromonads are known to colonize and form biofilms in water distribution and food processing systems, and gastrointestinal tract of host (Elhariry, 2011; Kirov et al., 2004). Biofilm formation on foods and food contact surfaces is the major reason for contamination, cross contamination and post-processing contamination of the final food product leading to food spoilage, product rejection, economic losses and food-borne diseases (Srey et al., 2013). The ability to attach and form biofilm on different surfaces varies among different species of microorganisms (Jahid and Ha, 2012).

Bacteria present in biofilms are inherently more robust in nature in their ability to withstand chemical and physical stresses and are more resistant to host defenses compared to planktonic cells (Chmielewski and Frank, 2003). Elimination of biofilms from food processing facilities is a big challenge due to the production of EPS materials, and the difficulties associated with cleaning complex processing equipment and processing environments (Jahid and Ha, 2012). Some of the methods traditionally used to control and remove biofilm formation include mechanical and manual cleaning using alkali and acid cleaners, chemical cleaning and sanitation with sanitizers/disinfectants, and application of
hot water (Chmielewski and Frank, 2003). However, numerous concerns have been raised over the safety of synthetic chemicals especially in the food processing industry and this has resulted in the preferential use of natural products as biocides (Jahid and Ha, 2012). Currently, there is no single known technique/strategy for the complete prevention or control of the biofilm formation (Srey et al., 2013). However, new control strategies are constantly emerging with main emphasis on the use of biosolutions (enzymes, phages, interspecies interactions, and antimicrobial molecules from microbial and plant origin) (Simoes et al., 2010).

Essential oils (EOs) are volatile, natural complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. They are usually obtained by steam or hydro-distillation of various plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (Bakkali et al., 2008). They are well-known antimicrobial agents that can be used to control food spoilage and food-borne pathogenic bacteria (Tajkarimi et al., 2010). Studies on the use of essential oils in the food system have increased with an objective to reduce the need for antibiotics and to control microbial contamination in food. They are also used for increasing the shelf life of food products by elimination of undesirable pathogens or delay in microbial spoilage.

1.3.2. Gene expression

In recent years, there is an increase in appreciation and demand among health-conscious consumers for the ready-to-eat foods. This has led to the changes in food processing, storage and distribution conditions. Earlier studies have shown that the sub-lethal stress can provide resistance to that stress or other stresses via cross-protection in the microorganism. Many “stress-hardened” pathogens either retain or exhibit enhanced virulence in foods; thus, making their detection crucial to safeguard the food supply.
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Molecular and Biochemical Studies of Aeromonas spp. (Wesche et al., 2009). These stresses often induce the expression of specific sigma factors which regulate the production of factors involved in stress protection as well as virulence of pathogenic bacteria (Dong et al., 2008).

Sigma factors are proteins that regulate transcription in bacteria and can be activated in response to different environmental conditions. RpoD ($\sigma^D$), is a "housekeeping" or primary sigma factor that transcribes most genes in growing cells and makes the necessary proteins to keep the cell alive. An alternative sigma subunit of RNA polymerase, RpoS ($\sigma^S$) regulates the general stress response in most gram-negative bacteria. rpoS gene is transcribed in the late exponential phase, and RpoS is the primary regulator of stationary phase genes. It controls the expression of numerous genes that fall into various functional categories: stress resistance, cell morphology, metabolism, virulence and lysis (Battesti et al., 2011). RpoS can mediate virulence either directly by controlling expression of virulence factors or indirectly by stimulating the general adaptation response to enhance survival of pathogens in hostile host environments. RpoS is required for resistance to many stresses in bacteria; however, the effect of RpoS on virulence is variable, differing even in closely related species (Dong and Schellhorn, 2010).

Generally, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is used as an accurate method to determine changes in gene expression, when a moderate number of genes in small number of samples need to be studied. Real-time PCR, also known as quantitative real-time PCR (qRT-PCR) is a sensitive and specific method used predominantly to detect, identify and quantify either pathogens or beneficial populations in food, and to study the expression of stress-response and virulence genes under environmental stress. RT-qPCR is often described as a “gold standard”, and has become the method of choice for the quantification of mRNA (Postollec et al., 2011).
1.4. Effect of gamma radiation on *Aeromonas* survival

The presence of spoilage and pathogenic microorganisms in food products is a major concern for the food processing industry, the administration, and consumers. Several physical and chemical treatment methods have been tried for the complete elimination of the food-borne pathogens, but they have been found to be ineffective under experimental conditions (Bari *et al*., 2005). Heat and chemical treatment methods tend to change the properties of the food in an undesirable manner (Hajare *et al*., 2007).

Use of ionizing radiation for decontamination of food material is the most versatile treatments among other decontamination methods. Gamma radiation has high penetration power and can inactivate pathogens that may have gained entry in tissues of food material. Irradiation of food material is techno-commercially feasible, safe, environmentally clean, and energy efficient process. Irradiation is particularly valuable as an end-product decontamination procedure (Farkas and Mohácsi-Farkas, 2011). Radiation processing, a cold process, ensures the microbiological safety without compromising the sensory and nutritional properties of meat and poultry (Chouliara *et al*., 2008) and fresh plant produce (Hajare *et al*., 2007).

Ionizing radiations are high-energy radiations and as the name implies, the exposure to these radiations causes ionization and structural changes in exposed molecules. In case of living organisms, exposure to radiations may cause structural and functional changes in important macromolecules (DNA, RNA, and proteins), thereby leading to cell death.

**Mechanism**

The action of radiation could be direct, i.e. biomolecule absorbs radiation and is damaged, or indirect i.e. radiation absorbed by other molecules such as water and the free
radicals then produce react with biomolecules. Radiolysis of water is particularly important in case of food irradiation, where free radicals generated due to water radiolysis are harmful to the bacterial cells (Alpen, 1997).

The action of radiation on water molecules is represented as follows:

\[
\begin{align*}
\text{Radiations} \\
\text{H}_2\text{O} & \rightarrow \text{H}_2\text{O}^+ \rightarrow \text{H}^0 + \text{OH}^0 \\
\downarrow \quad \text{Excited molecule} \quad \text{free radicals} \\
\text{H}_2\text{O}^+ + e^- & \rightarrow \text{H}^+ + \text{OH}^0 + e^- \\
e^- + \text{H}_2\text{O} & \rightarrow \text{H}_2\text{O}^- \rightarrow \text{OH}^- + \text{H}^0 \\
e^- + \text{H}^+ & \rightarrow \text{H}^0 \\
e^- + \text{H}_2\text{O} & \rightarrow e^-_{\text{aq}}
\end{align*}
\]

(Solvated electron)

The damage to DNA is of various types- single strand and double strand breaks, alteration of purine and pyrimidine bases or interchain or intrachain bond formation. The prokaryotic and the eukaryotic cells posses various DNA repair mechanisms such as direct rejoining of broken ends, excision repair and post replication repair but the double strand breaks are important because most of the microorganisms cannot repair these damages and the cell cannot replicate, thus killing the microorganisms (Alpen, 1997). In addition to effects on the genetic material, radiation has direct or indirect effects on other components of the cell such as membranes and enzymes.
Chapter 1

Decimal reduction dose ($D_{10}$)

When a population of microorganisms is irradiated with a low dose, only a few of the cells will be damaged or killed. With increasing radiation dose the number of surviving organisms decrease exponentially. Different species and different strains of the same species require different doses to reach the same degree of inactivation. In order to characterize organisms by their radiation sensitivity, the decimal reduction dose ($D_{10}$) is used. $D_{10}$ is the dose required to kill 90% of the population. A pure culture of bacteria is exposed to increasing doses of ionizing radiation and the treated cells are plated on appropriate medium and the number of colonies produced was counted. If $N_0 =$ initial population, $N =$ population after dose D and $D_{10} =$ dose needed to reduce population by a factor of 10, then

$$\log \frac{N}{N_0} = \frac{-1}{D_{10}} \times D$$

When ionizing radiations penetrate into a medium (for instance, irradiated food) all or a part of the radiant energy is absorbed by the medium. This is called the absorbed dose. The unit in which the absorbed dose is measured is Gray (Gy).

- 1 Gy = 1J/kg
- 1kGy = 1000 Gy

The $D_{10}$ values are expressed in Gray or kilo Gray. The radiation treatments meant for reducing microbes in foods to safe levels have been assigned specific terminologies by a group of international experts, each type of treatment serving definite objectives and involving the application of a defined range of radiation dose.

Radappertization: Dose sufficient to reduce the number and activity of viable microorganisms to such an extent that very few, if any, are detectable in the treated food. The required dose is usually in the range of 25 - 45 kGy.
**Radicidation:** The application of radiation dose sufficient to reduce the number of viable specific non-spore forming pathogenic bacteria that none are detectable in the treated food. The required dose is in the range of 2 to 8 kGy.

**Radurization:** The application of radiation dose sufficient to enhance keeping quality of food by causing a substantial decrease in the number of viable specific spoilage microorganisms. The required dose is in the range of 1-2.5 kGy.

*Aeromonas* are known to be more sensitive to gamma radiation than other food-borne pathogens like *Salmonella*, *Campylobacter* and *Listeria* (Monk et al., 1995). The D$_{10}$ values of five *A. hydrophila* ranged from 0.14 to 0.22 kGy at 2±1°C, in growth broth, phosphate buffer, ground blue-fish or ground beef (Palumbo et al., 1986). A radiation dose of 0.75 kGy in combination with conventional cooking procedure was found to be sufficient to destroy approximately $10^4$ CFU/g of *A. hydrophila* in meatball (Ozbas et al., 1996). Though radiation processing has been recommended for the elimination of *A. hydrophila* in fresh fish, sea food, red meat and poultry (Palumbo et al., 1986), there is a lack of studies regarding radiation sensitivity of *Aeromonas* in sprouts. Moreover, radiation sensitivity of different *Aeromonas* species in saline, poultry and fish samples has not been studied.

### 1.5. Scope of thesis

The aim of the present thesis is to evaluate the incidence of *Aeromonas* species in food products (sprouts, fish and poultry meat), and to identify these isolates up to species level using biochemical and molecular methods. These *Aeromonas* isolates will be further
analyzed for genetic relatedness using PFGE and whole cell protein profiles. Occurrence of virulence genes and production of β-haemolysin and extra-cellular enzymes will be further studied in these *Aeromonas* food isolates. The correlation between the antibiotic resistance and plasmid profiles of these isolates will be determined. The radiation sensitivity of the isolates in different food commodities will be evaluated, and the radicidation dose for ensuring safety will be optimized.

The ability of different *Aeromonas* strains to form biofilm under different food-related stress conditions (media, pH, temperature, salt, food preservatives and essential oils) will be studied. The effect of various food-related stress conditions on the expression pattern of different stress-response and virulence genes in *A. hydrophila* will be studied.

Objectives of the present work are:

1. Isolation and biochemical characterization of *Aeromonas* from various food products (sprouts, chicken and fish)
2. Molecular characterization of *Aeromonas* isolates using 16S rRNA and rpoD gene sequencing
3. Detection of virulence genes in *Aeromonas* isolates
4. Characterization of *Aeromonas* isolates with respect to antibiotic resistance pattern, plasmid, PFGE and protein profiles
5. Determination of radiation sensitivity of *Aeromonas* with different food commodities and optimizing the dose for ensuring safety
6. Study on biofilm formation by *Aeromonas* strains under different food-related stress (media, pH, temperature, salt, food preservatives and essential oils)
7. Expression pattern of stress-response and virulence genes in *A. hydrophila* under various stress condition