MOLECULAR AND BIOCHEMICAL STUDIES OF *AEROMONAS* SPP.

By

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As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by **Vandan Nagar** entitled "**Molecular and Biochemical Studies of *Aeromonas* spp.**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications arising from the thesis

Journal

1. "Prevalence, characterization and antibiotic resistance of *Aeromonas* strains from various retail food products in Mumbai, India", Nagar V., Shashidhar R. and Bandekar J. R., *Journal of Food Science*, **2011**, 76(7), M486–M492.
2. "Effectiveness of radiation processing in elimination of *Aeromonas* from food", Nagar V. and Bandekar J. R., *Radiation Physics and Chemistry*, **2011**, 80, 911–916.
3. "Characterization of *Aeromonas* strains isolated from Indian food using *rpoD* gene sequencing and whole cell protein analysis", Nagar V., Shashidhar R. and Bandekar J. R., *World Journal of Microbiology and Biotechnology*, **2013**, 29, 745–752.

Conferences

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2. "Radiation processing for elimination of *Aeromonas* spp. from food", Nagar V. and Bandekar J. R., *NAARRI International Conference: "Isotope Technologies and Applications – New Horizons"*, **2010**, *FT – 9, p. 194-198, Mumbai, Dec. 13-15, 2010*.
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GenBank submission

1. “Partial 16S rRNA gene sequences of 22 different isolates belonging to *Aeromonas* spp.”, Nagar V., Shashidhar R. and Bandekar J. R., **2010**, NCBI (Accession numbers: FJ561050-52, HQ122915-31, HQ413137 and HM002780).
2. “Partial 16S ribosomal RNA gene sequences of 14 different isolates belonging to *Providencia, Comamonas, Pseudomonas, Morganella, Alcaligenes, Delftia, Citrobacter* spp.”, Nagar V., Shashidhar R. and Bandekar J. R., **2010**, NCBI (Accession No. HM055739 – 52).
3. “Partial RNA polymerase, sigma 70 (sigma D) factor gene sequences of 22 different *Aeromonas* isolates” Nagar V., Shashidhar R. and Bandekar J. R., **2011**, NCBI (Accession numbers: JN182265-69, JN412625-30, JN388917-22 and JN544572-76).

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Dedicated to.....

Guruji

&

My Family

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CONTENTS

Synopsis

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SYNOPSIS

Introduction

Aeromonas species are ubiquitous aquatic micro-organisms that are considered as important fish pathogens and opportunistic pathogens in both immuno-competent and immuno-compromised humans. They are found in a variety of environments worldwide, including different water resources and foods of plant and animal origin. *Aeromonas* causes intestinal (gastroenteritis and traveler's diarrhoea) and extra-intestinal infections in humans, and haemorrhagic septicaemia in fish (Beaz-Hidalgo and Figueras, 2013). The major routes of exposure of *Aeromonas* in humans are ingestion of contaminated foods and drinking water, or direct contact with recreational waters (Igbinosa *et al.*, 2012).

Complex taxonomy is the key challenge in establishing an explicit relationship between the genus *Aeromonas* and its pathogenesis in humans. The species level identification of *Aeromonas* based on routine phenotypic characteristics is intricate and confusing (Figueras *et al*., 2011). Many researchers have used molecular methods for the identification of *Aeromonas* species. However, there is a lack of congruity between phenotypic and genotypic methods in the identification of *Aeromonas* strains till species

level, and thus multiple methods are required for the accurate identification (Martinez-Murcia *et al.,* 2011). Virulence of aeromonads is multifactorial, dependent on host susceptibility and less understood (Janda and Abbott, 2010). Many putative virulence factors such as toxins, extra-cellular enzymes and cell-associated structures have been described in *Aeromonas* (Tomas, 2012). *Aeromonas* isolates, belonging to different species, have been sub-typed into different pulsotypes by pulse-field gel electrophoresis (PFGE) (Khajanchi *et al.*, 2010). The generation of genotypic data with PFGE in addition to antibiotic resistance profile and plasmid profile can help in tracing the source of outbreak and proper control of the food production process by hazard analysis at critical control points. Whole cell protein (WCP) profile is another simple and rapid molecular technique used for the characterization of microorganisms below species level (van Belkum *et al.*, 2007).

High prevalence of *Aeromonas* in sprouts, chicken and fish calls for processing them to ensure their safety. Radiation processing has been found effective in the elimination of pathogens from sprouts, poultry meat and sea-food due to its high penetration power (Farkas and Mohácsi-Farkas, 2011).

Aeromonas have the ability to colonize and form biofilms on contact surfaces and drinking water distribution systems, and may pose a threat of contamination in food processing industry. Biofilm formation by an organism depends on its genetic composition and function, nature of the contact surfaces and environmental factors including pH, temperature, medium composition and nutrient components (Xu *et al.*, 2010).

Food-borne pathogens are frequently exposed to a variety of stresses in their natural environment, during food processing and when they are in their host systems. Environmental stresses and food preservation methods are known to induce adaptive responses within the bacterial cell and influence its virulence potential (Wesche *et al.*, 2009).

Objectives of the thesis

- Isolation and identification of *Aeromonas* from various food products (sprouts, chicken, fish) using biochemical and molecular methods (16S rRNA and *rpoD* gene sequencing).
- Detection of virulence genes and extra-cellular enzymes in *Aeromonas* isolates.
- Characterization of *Aeromonas* isolates with respect to antibiotic resistance pattern, and plasmid, WCP and PFGE profiles.
- Determination of radiation sensitivity of *Aeromonas* in different food commodities and optimization of the radicidation dose for ensuring safety.
- Studies on biofilm formation by *Aeromonas* isolates under various food-related stress conditions.
- Expression pattern of general stress-response and virulence genes under various stress conditions

Organization of the thesis

The entire thesis is organized into seven chapters. Chapter 1 provides introduction to the topic and reviews the scientific literature related to the present work. Chapter 2 focuses on the isolation and identification of *Aeromonas* from different food samples using various biochemical and molecular methods. Chapter 3 describes the presence of various virulence genes and extra-cellular enzymes in these isolates. In addition, all the isolates were screened for antibiotic resistance and plasmid profile. These isolates were further characterized below-species level using PFGE and WCP. The effect of gamma-radiation on the survival of *Aeromonas* in different food commodities is discussed in Chapter 4. Chapter 5 describes the effect of stress on the biofilm forming ability of *Aeromonas* strains. The effect of food-processing related stress on the expression of stress-related and virulence genes in *A. hydrophila* is described in Chapter 6. Chapter 7 summarizes the conclusion of the entire thesis work and includes future perspectives.

Chapter 1 [General Introduction]

This is an introductory chapter that reviews the literature related to the prevalence, isolation and identification methods, taxonomy, pathogenesis and clinical manifestations of *Aeromonas*. It highlights the presence and role of different virulence factors in *Aeromonas* pathogenesis. Also, it details the antibiotic susceptibility and plasmid profiles, and the characterization of Aeromonads using various phenotypic and genotypic methods. Special emphasis is given on the effect of food-related stress on the expression of stress-responsive and virulence genes, and biofilm forming ability of *Aeromonas*. The effect of gamma radiation on the survival of *Aeromonas* is explained in detail.

Chapter 2 [Isolation and identification of *Aeromonas*]

This chapter gives details about the prevalence and isolation of *Aeromonas* in sprout, chicken meat and fish samples available in retail outlets in Mumbai, India. In this study, a total of 154 food samples of chicken (14), fresh-water fish (52), marine fish (8), and ready-to-eat mixed sprouts (40) and alfalfa sprouts (40) were obtained from various retail markets of Mumbai. *Aeromonas* were isolated by enrichment in tryptic soya broth (TSB) and followed by streaking onto starch ampicillin agar (SAA) plates (Palumbo *et al.*, 1985). Four hundred and fifty-one presumptive positive isolates were obtained from 154 food samples. Presumptive *Aeromonas* isolates were biochemically identified till species level based on the biochemical tests as described earlier (Abbott *et al.*, 2003; Beaz-Hidalgo *et al.*, 2010; Martin-Carnahan, 2005; Minana-Galbis *et al.*, 2007).

Twenty-two of these 451 presumptive isolates were identified as *Aeromonas* by biochemical tests and validated till species level using 16S rRNA (GenBank accession numbers: FJ561050-52, HQ122915-31, HQ413137 and HM002780) and *rpoD* (GenBank accession number: JN182265-69, JN412625-30, JN388917-22 and JN544572-76) gene sequencing. However, the identification of these isolates based on biochemical tests and

molecular methods showed disparity. For example, the species level identification of only 77.3% (17/22) of these isolates by comprehensive biochemical tests agreed with 16S rRNA gene based identification. The 16S rRNA gene was found to be highly conserved within the genus *Aeromonas* and therefore, showed limited usefulness. Using this approach *Aeromonas* isolates could be identified up to genus level.

Identification of 95.5% (21/22) of these isolates, except A254, by comprehensive biochemical tests agreed with *rpoD* gene based identification. *Aeromonas* isolates belonged to ten different species of *Aeromonas* {*A. salmonicida* (6), *A. veronii* bv. *veronii* (4), *A. caviae* (3), *A. hydrophila* (2), *A. veronii* bv. *sobria* (2), *A. jandaei* (1), *A. trota* (1), *A. sobria* (1), *A. allosaccharophila* (1) and *A. bivalvium* (1)}. Our study showed that *rpoD* gene has approximately 3.5 times more substitution rates as compared to 16S rRNA gene and has higher discriminatory power than 16S rRNA gene to delineate *Aeromonas* strains till species level. We propose that a combination of certain biochemical tests and *rpoD* gene sequencing will be ideal for the simple and accurate identification of *Aeromonas* isolates from food samples up to species level.

Eighteen (11.7%) out of 154 food samples were positive for *Aeromonas* spp. The highest percentages of isolation of *Aeromonas* were from chicken (28.6%) followed by fish (20%) and sprout (2.5%) samples indicating more prevalence in foods of animal origin than of plant origin. Overall, *A. salmonicida* was the most prevalent species, followed by *A. veronii* bv. *veronii* and *A. caviae*. However, *A. caviae* (66.7%) and *A. salmonicida* (35.7%) occurred most frequently in sprouts and fish samples, respectively; whereas, in chicken samples, all the five species (*A. jandaei*, *A. hydrophila*, *A. salmonicida*, *A. veronii* bv. *sobria* and *A. caviae*) were equally prevalent.

Chapter 3 [Characterization of *Aeromonas* isolates]

Section-A: Virulence genes and extracellular enzymes

The production of a broad range of virulence factors by *Aeromonas* species is indicative of its potential to cause severe diseases in humans. Thus, *Aeromonas* isolates were checked for the presence of various virulence factors: cytotoxic enterotoxin (*act*), haemolysin (*hly*), aerolysin (*aer*), elastase (*ahyB*), and lipase (*lip*) using polymerase chain reaction (PCR) method. The production of extra-cellular enzymes *viz*. amylase, gelatinase, lipase, protease and DNase was assessed by observing substrate utilization by *Aeromonas* strains on starch agar, gelatin agar, tributyrin agar, milk agar and DNase agar, respectively; whereas, the haemolytic activity was determined by streaking onto tryptic soya agar (TSA) plates containing 5% defibrinated horse blood.

In the present study, 22.7%, 40.9% and 59.1% of the strains harboured *aer*, *hly* and *act* genes, respectively; whereas, *ahyB* and *lip* genes were present in 54.5% and 31.8% of the isolates. All the five studied virulence genes were present in four *A. salmonicida* (66.7%) isolates. However, all these genes were absent in *A. trota* and *A. allosaccharophila* isolates and one isolate each of *A. caviae* and *A. veronii* bv. *veronii*. The *hly* and *act* genes were present in all the six *A. salmonicida* isolates; whereas, *ahyB* and *aer* genes were present in 83.3% of *A. salmonicida* isolates.

All the isolates were able to produce amylase, gelatinase and DNase enzymes; whereas, lipase and protease production was observed in most (>90%) of these isolates. Majority of the isolates produced clear zones of β-haemolysis on blood agar plates indicating the potential pathogenicity of these strains.

Section-B: Antibiotic resistance pattern and plasmid profile

Though *Aeromonas* have been reported from various clinical and environmental sources in India (Sinha *et al.*, 2004; Vivekanandhan *et al.*, 2005), there is lack of

information about the antibiotic resistance and plasmid profiles of food isolates. Thus, the detailed study was aimed for finding out the resistance profiles of these *Aeromonas* food isolates to 20 different antibiotics. All these isolates were resistant to ampicillin and bacitracin, while majority of these isolates showed higher resistance to cephoxitin (77.3%), ampicillin/sulbactam (72.7%), carbenicillin (68.2%) and piperacillin/tazobactam (59.1%). Moreover, *A. salmonicida* isolates displayed higher levels of resistance to ampicillin, bacitracin, ampicillin/sulbactam and cephoxitin as compared to other *Aeromonas* isolates. On the other hand, all the *Aeromonas* strains were sensitive to gentamicin, third-generation cephalosporins (ceftazidime, cephotaxime, ceftriaxone) and chloramphenicol.

Multiple antibiotic resistance (MAR) index of these *Aeromonas* strains ranged from 0.15 to 0.35. Majority of the fish (92.9%) and chicken (80%) isolates had high MAR index of 0.25 to 0.35 indicating the use of low concentration of antibiotics in feed leading to development of antibiotic resistance. Nearly 77% isolates harboured single and/or multiple plasmids (~ 5 to > 16 kb). Plasmids were present in isolates belonging to nine of the ten identified species, except *A. bivalvium*. However, no clear correlation was observed between the presence of plasmid and antibiotic resistance.

Section-C: Whole-cell protein (WCP) and pulse field gel electrophoresis (PFGE) profiles

Aeromonas isolates from different food samples were analyzed by PFGE after restriction digestion of genomic DNA by *Xba*I enzyme. Overall, PFGE of the *Aeromonas* isolates yielded 13 - 19 well-resolved and reproducible genomic DNA fragments (approximately 48.5 - 436.5 kb). No correlation was observed between PFGE profile and the source of isolation and virulence factors of *Aeromonas* isolates. All the isolates showed different PFGE banding pattern indicating high genetic diversity. Moreover, this is the first

report of PFGE profiles of *Aeromonas* spp. from India and this data can be considered as a reference for any future work regarding epidemiology or genetic diversity of *Aeromonas* spp. in India.

WCP analysis has been used by several researchers to study the diversity of *Aeromonas* strains at and below species level (Maiti *et al.*, 2009). Thus, all the *Aeromonas* isolates were analyzed for WCP analysis by gradient SDS-PAGE (5-18%). The SDS-PAGE exhibited different WCP profiles (22 - 28 polypeptide bands) in the molecular weight region corresponding to ~10 kDa to > 97 kDa, indicating high genetic diversity. The overall protein profiles were very similar among the strains of the same species except for slight variations in the number of bands generated. For majority of the isolates, no clear correlation was observed between the origin of the strains and their protein profiles. However, in the case of *A. hydrophila*, *A. caviae* and *A. veronii* bv. *sobria* species, clustering of strains based on their origin was observed.

Chapter 4 [Radiation sensitivity of *Aeromonas* in different food commodities]

The decimal reduction dose (D_{10} value) for different *Aeromonas* strains, belonging to different species, in saline, inoculated mixed sprouts, chicken and fish samples were determined to assess the sensitivity of *Aeromonas* isolates to gamma radiations. *Aeromonas* cells suspended in 1.2 ml of sterile saline, to cell density of 7-log CFU/ml were irradiated under melting ice conditions for doses of 0, 0.025, 0.05, 0.075, 0.1, 0.125 and 0.15 kGy. The log CFU/ml of surviving *Aeromonas* cells was determined and plotted against radiation doses to determine the D_{10} value of each isolate. All *Aeromonas* isolates were found to be very sensitive to gamma radiation. The D_{10} values of different *Aeromonas* isolates in saline ranged from 0.031 - 0.046 kGy. The maximum D_{10} value was found to be of *A. salmonicida* Y567 (0.046 kGy); whereas, *A. veronii* bv. *veronii* CECT 4257^T (0.031 kGy) was found to be the most sensitive.

A 'cocktail' inoculum of five different *Aeromonas* strains (*A. salmonicida* Y567, *A. caviae* A85, *A. veronii* bv. *veronii* A514A, *A. hydrophila* CECT 839^T and *A. salmonicida* Y47) was prepared to determine the radiation sensitivity of *Aeromonas* in mixed sprouts, chicken and fish samples. *Aeromonas* cocktail was also found to be very sensitive with D_{10} values of 0.081 ± 0.001 kGy, 0.089 ± 0.003 kGy and 0.091 ± 0.003 kGy in mixed sprouts, chicken and fish samples, respectively.

Inoculated pack studies were carried out by inoculating decontaminated mixed sprouts, chicken and fish samples (25 g) with the *Aeromonas* cocktail so as to obtain a count of 1 x 10^5 cells/g. The inoculated samples (10^5 CFU/g of *Aeromonas*), in triplicate, were irradiated at 0 - 4 °C with doses of 0.5, 1, 1.5 and 2 kGy, stored at 4 °C and analyzed for survival and recovery of *Aeromonas* on the 0^{th}, 4^{th}, 8^{th} and 12^{th} day (sprout samples) and on the 0^{th}, 3^{rd}, 5^{th} and 7^{th} day (chicken and fish samples). Enrichment and selective plating were carried out to confirm the complete elimination of the pathogens.

No viable counts were detected in 1, 1.5 and 2 kGy irradiated mixed sprouts, fish and chicken samples on the 0^{th} day when the survival of the pathogens was analyzed immediately after irradiation. However, recovery of the pathogen was observed in 0.5 kGy and 1 kGy treated samples after enrichment in TSB for 24 h followed by selective plating on SAA plates. No such recovery of *Aeromonas* was observed in 1.5 and 2 kGy treated mixed sprout, fish and chicken samples after enrichment and selective plating. Similarly, no recovery of *Aeromonas* was observed in the 1.5 and 2 kGy treated mixed sprouts samples stored at 4 °C up to 12 days and chicken and fish samples up to 7 days, even after enrichment and selective plating. This study demonstrated that a 1.5 kGy dose of irradiation treatment could result in complete elimination of 10^5 CFU/g of *Aeromonas* spp. from mixed sprouts, chicken and fish samples and thereby ensures their safety. The study also revealed the importance of conducting enrichment studies to determine the bactericidal effects of the irradiation process.

Chapter 5 [Biofilm formation by *Aeromonas* under various food-related stress conditions]

Aeromonas may colonize and form biofilms on drinking water distribution systems, food-processing surfaces and food material. Therefore, the study was carried out to evaluate the effect of media composition, temperature, pH, salt concentration and food preservatives on the biofilm formation ability of *Aeromonas* strains using modified crystal-violet assay. The results showed inter-strain variation in biofilm formation by *Aeromonas* under different food-related conditions. Majority of *Aeromonas* strains formed more biofilm in rich medium (TSB) than in minimal medium (M9 media supplemented with 0.4% glucose). Maximum specific biofilm formation (SBF) index in most *Aeromonas* strains was observed at 10 °C as compared to 30 °C and 37 °C. The SBF index of most *Aeromonas* strains decreased with an increase in the concentration of NaCl. Maximum SBF index for most of the *Aeromonas* strains was observed at pH 5 and it reduced with an increase in pH. An increase in SBF index for most of the *Aeromonas* strains was observed with an increase in concentration of added food preservatives (sodium nitrite, sodium benzoate and potassium sorbate).

Essential oils are generally recognized as safe (GRAS) and have demonstrated antimicrobial activity against major food-borne pathogens. The MIC values of different essential oils were determined on *A. hydrophila* CECT 839^T and *A. veronii* CECT 4257^T strains in TSB and M9 minimal media supplemented with 0.4% glucose using microtiter broth micro-dilution method. The MIC values of clove, ajowain, cinnamon, tea-tree and cumin oils were found to be ≤ 2 mg/ml; whereas, for ginger, turmeric, orange, eucalyptus, lemon and lavender oils, MIC values were ≥ 8 mg/ml.

Chapter 6 [Expression pattern of general stress-response and virulence genes in *A. hydrophila* under various stress conditions]

There is lack of information about the impact of various environmental and food-processing related stresses on the expression of general stress-response and virulence genes in *A. hydrophila*. Studies were carried out to determine the effect of various stress events {nutrient replenishment, nutrient deprivation, cold-shock (8 °C), heat-shock (37 °C), acid-shock (pH 4) and alkaline-shock (pH 9)} on the expression of house-keeping (*rpoD* and *gapA*), general stress-response (*uspA* and *rpoS*) and virulence (*aer*) genes in stationary phase *A. hydrophila* CECT 839^T (reference strain) and *A. hydrophila* A331 (food isolate) using real-time PCR (RT-qPCR).

No significant differences ($P > 0.05$) in CFUs of control and all treatment cells were observed in both the *A. hydrophila* strains. Variations in the level of expression of different studied genes under various stress conditions were observed among *A. hydrophila* CECT 839^T and A331 strains indicating heterogeneity within the species. In general, significant induction of housekeeping (*rpoD* and *gapA*), general stress regulators (*uspA* and *rpoS*) and virulence gene (*aer*) was observed following nutrient replenishment and deprivation. Various genes were induced in response to the temperature stress (8 °C or 37 °C). Significant induction of *uspA* gene was observed during acid stress; whereas, alkaline stress showed significant down-regulation of all the studied genes. The above studies showed that *A. hydrophila* strains are highly sensitive and adaptable to environmental stresses and prepare it to survive in the food chain.

Chapter 7 [Summary]

The major findings of the present thesis are summarized in this chapter. The key features described in this chapter are:

1. Sprout, chicken and fish samples marketed in Mumbai and its suburbs were contaminated with *Aeromonas* with higher incidence in samples of animal origin than of plant origin. Therefore, these samples need processing such as radiation treatment prior to consumption.
2. For accurate identification of *Aeromonas* strains till species level *rpoD* gene was found to be a better phylogenetic marker than biochemical tests and 16S rRNA gene analysis. A combination of certain biochemical tests and *rpoD* gene analysis can provide simple, rapid and precise identification of *Aeromonas* strains up to species level.
3. Majority of the *Aeromonas* isolates may be pathogenic since they harbour virulence genes, produce extracellular enzymes and show β-haemolysis.
4. Though majority of these isolates showed presence of plasmids and marked resistance to commonly used β-lactam antibiotics, no clear correlation was observed between the presence of plasmid and antibiotic resistance.
5. High genetic diversity was observed among *Aeromonas* isolates using PFGE and WCP analysis. For majority of the strains, no clear correlation was observed between the origin of the strains and their PFGE and WCP profiles.
6. All *Aeromonas* isolates were very sensitive to gamma radiation and radiation processing with 1.5 kGy is effective in achieving 5-log reductions in *Aeromonas* populations on mixed sprout, chicken and fish samples. No recovery of *Aeromonas* was observed during the storage period even after enrichment and selective plating.
7. Significant strain-dependent variations in the biofilm forming ability under different food-related stresses (media, temperature, pH, NaCl and food preservatives) were observed in different *Aeromonas* strains.

8. *Aeromonas* stains were found to be very sensitive to different essential oils.
9. Differences in the level of expression of different genes under various stress conditions were observed among *A. hydrophila* CECT 839^T and A331 strains indicating genetic heterogeneity within the species.
10. Induction of different stress-response genes under various stress conditions suggests that initial stress events may prepare a cell for surviving in subsequent unfavourable environments.

Future work

Studies on the biofilm forming ability of *Aeromonas* strains under other food-relevant stress (e.g. glucose and ethanol) will provide comprehensive picture to food processors in the prevention of biofilm and consequently will reduce the health risks related to *Aeromonas* biofilm. The effectiveness of essential oils in the inhibition of initial cell attachment, and growth and development of *Aeromonas* biofilm on food or contact surfaces is also worth studying.

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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

separated explicitly from each other due to lack of reliable phenotypic markers. Earlier, genus *Aeromonas* was included in the family *Vibrionaceae*. However, subsequent studies by Colwell *et al.* (1986) using 16S rRNA cataloging, 5S rRNA gene sequence comparisons, and rRNA-DNA hybridization revealed that aeromonads demonstrated an evolutionary divergence that was approximately equidistant from the families *Enterobacteriaceae* and *Vibrionaceae*. Thus, a new family *Aeromonadaceae* was formally proposed which included the single genus, *Aeromonas* with *A. hydrophila* as its type strain. Later on genera *Oceanimonas*, *Oceanisphaera*, and *Tolumonas* (*incertae sedis*) were included in the family *Aeromonadaceae* (Martin-Carnahan and Joseph, 2005). At present, 25 accepted and validated species have been described in the genus *Aeromonas*; however, the taxonomic designation of many species has not been recognized in Bergey's Manual of Systematic Bacteriology (Beaz-Hidalgo and Figueras, 2013). *A. hydrophila* ATCC 7966^{T} was the first member of the genus *Aeromonas* to be completely sequenced (Seshadri *et al.*, 2006). Moreover, the complete genome sequences of five more strains have been determined in the last five years: *A. salmonicida* subsp. *salmonicida* A449, *A. veronii* strain B565, *A. caviae* strain Ae398, *A. aquariorum* strain AAk1 and *A. salmonicida* strain 01-B526 (Beaz-Hidalgo and Figueras, 2013).

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 atleast 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 septicaemia (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, Chineses 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 cytotonic 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 cytotonic 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 cytotonic 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).

Cytotonic enterotoxin: These toxins increase the cyclic adenosine monophosphate (cAMP) levels and prostaglandins in intestinal epithelial cells. Cytotonic 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 pril-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- genomospecies 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.

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* genomospecies 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

integrons (Aravena-Roman *et al.*, 2012). Antibiotic resistance to streptomycin, chloramphenicol, tetracycline, cephalexin, cefoxitin, erythromycin, furazolidone, and sulfathiazole is mediated by plasmids (USEPA, 2006).

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 *Bst*SNI, *Mbo*I 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

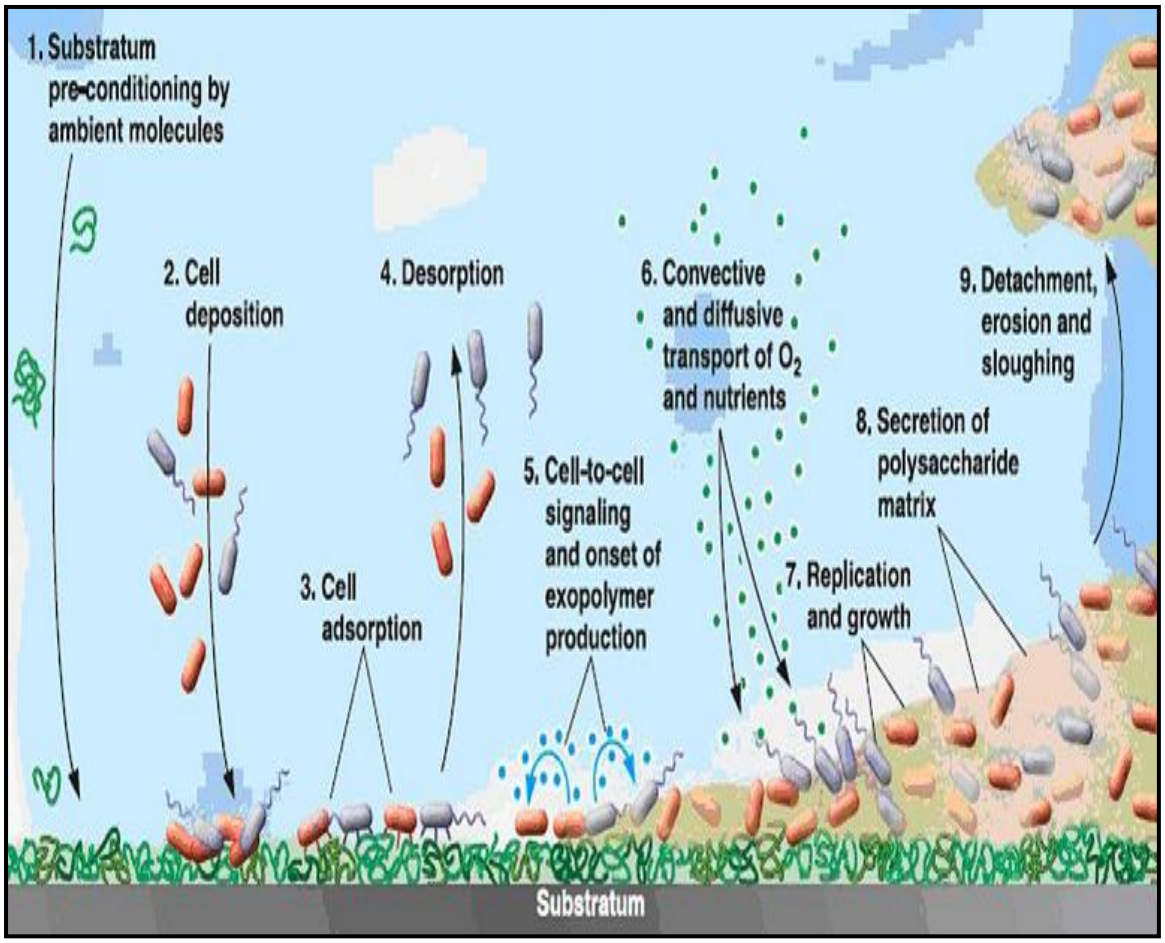


Figure 1.1. Model of biofilm development and processes governing biofilm formation From Simoes *et al.* (2010)

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

(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 (σ^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 (σ^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:

Radiations

$H_2O \rightarrow H_2O^* \rightarrow H^\circ + OH^\circ$

$\downarrow$ Excited molecule free radicals

$H_2O^+ + e^-$

$H_2O^+ + e^- \rightarrow H^+ + OH^\circ + e^-$

$e^- + H_2O \rightarrow H_2O^- \rightarrow OH^- + H^\circ$

$e^- + H^+ \rightarrow H^\circ$

$e^- + H_2O \rightarrow e^-_{aq}$

(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.

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 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

$$\text{Log } N/N_0 = -1/D_{10} \text{ X } D$$

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

CHAPTER 2

Isolation & Identification of *Aeromonas*

2.1. Introduction

The ingestion of contaminated water and food of plant and animal origin have been considered important sources of *Aeromonas* infection (Khajanchi *et al.*, 2010). There are a limited number of reports of food-borne outbreaks caused by *Aeromonas* (USEPA, 2006). However, acute gastroenteritis due to *Aeromonas* has been reported in Pernambuco and Brazil (Guerra *et al.*, 2007; Hofer *et al.*, 2006). Recently, *Aeromonas* have been reported from patients suffering from diarrhoea and other clinical samples (Puthucheary *et al.*, 2012; Senderovich *et al.*, 2012). Thus, the high prevalence of *Aeromonas* species in the food chain should be considered a threat to public health and should not be ignored.

Complex taxonomy is the key challenge in establishing an explicit relationship between the genus *Aeromonas* and pathogenicity in humans. Only a small subset of strains containing putative virulence genes seems to cause infection or diarrhoea (Martino *et al.*, 2011). Precise identification of the concerned pathogen is of great concern from an epidemiological point of view. However, there is a lack of congruity between phenotypic and genotypic characteristics among *Aeromonas* species, and multiple methods are required for the accurate identification. Key difficulties in the phenotypic identification of *Aeromonas* are the use of different methods and conditions for the biochemical tests, high intra-species phenotypic variability, ambiguous results, and requirement of additional cumbersome and time-consuming phenotypic tests (Abbott *et al.*, 2003; Martin-Carnahan and Joseph, 2005).

Commercial identification systems are not very successful in the identification of *Aeromonas* till species level (Lamy *et al.*, 2010b; Soler *et al.*, 2003). Many researchers have used molecular methods for the identification of *Aeromonas* species. The 16S rDNA gene sequences are universally used to understand phylogenetic relationships and species level identification of bacteria (Clarridge III, 2004). However, in case of *Aeromonas*, 16S

rDNA gene has been found to be inefficient in correct identification of closely related *Aeromonas* species (Morandi *et al.*, 2005). Studies have shown that house-keeping genes like *rpoD* and *gyrB* are better molecular markers than the 16S rDNA gene for the study of phylogenetic and taxonomic relationships at the species level in the genus *Aeromonas* (Beaz-Hidalgo *et al.*, 2010; Soler *et al.*, 2004).

In a 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. There is a lack of detailed studies on the prevalence of *Aeromonas* in food samples from India. This chapter evaluates the occurrence of *Aeromonas* spp. in various food products (sprouts, chicken, and fish samples) marketed in Mumbai and its suburb region. All the isolates were identified up to species level using conventional and comprehensive biochemical methods followed by molecular methods such as 16S rRNA and *rpoD* gene sequencing. The extent of agreement between the biochemical schemes and molecular methods (16S rRNA and *rpoD* gene) was analyzed.

2.2. Materials and methods

2.2.1. Sample collection

A total of 154 food samples of chicken, fish and ready-to-eat sprouts were purchased from local retail outlets in Mumbai, India from January 2006 to March 2008. The samples included mixed sprouts {green gram (*Phaseolus aureus*), dew gram (*Phaseolus aconitifolius*), chick pea (*Cicer arietinum*), garden pea (*Pisum sativum*)} (40), alfalfa sprouts (*Medicago sativa*) (40), chicken (14), fresh-water fish {butter cat fish (*Ompok bimaculatus*) (12), scampi (*Macrobrachium rosenbergii*) (2), rohu (*Labeo rohita*) (8), hilsa (*Tenualosa ilisha*) (7), tengan (*Aristichthys nobilis*) (9), catla (*Catla catla*) (7), magur (*Clarias batrachus*) (7)} (52) and marine fish {bombay duck (*Harpadon nehereus*)

(2), pomfret (*Parastromateus nigeri*) (1), prawn (*Penaeus indicus*) (3), anchovies (*Stolephorus commersonii*) (2)} (8). The sprouts were procured from 2 different supermarket outlets; whereas, fish and chicken samples were collected from retail markets of Mumbai. The sprouts and chicken samples were brought directly to laboratory at ambient temperature (26 - 28 °C); whereas, the fish samples were brought in ice. The microbiological analysis was done within one hour.

2.2.2. Chemicals and media

Microbiological media were from Hi-Media Laboratories, Mumbai, India. All polymerase chain reaction (PCR) reagents were obtained from Bangalore Genei Ltd. (Bangalore, India).

2.2.3. Bacterial strains and culture conditions

A. hydrophila CECT 839^{T}and *A. veronii* bv. *veronii* CECT 4257^{T} (kindly supplied by Dr. Valérie Leclère, Université des Sciences et Technologies de Lille USTL, France) were used as controls for the biochemical tests. All *Aeromonas* isolates were grown overnight in 25 ml TSB at 30 °C.

2.2.4. Isolation of *Aeromonas* species

Aeromonas spp. were isolated by pummeling 25 g of sample in 225 ml tryptone soya broth (TSB) in Stomacher 400 Lab Blender (Seward Medical, London, UK) and enriching at 30 °C, 24 h and followed by streaking onto starch ampicillin agar (SAA) (Palumbo *et al.*, 1985) (Figure 2.1). The plates were incubated at 30 °C for 24 h. The SAA plates were examined for typical *Aeromonas* colonial morphology (3 - 5 mm in diameter, yellow to honey coloured). Presumptive *Aeromonas* colonies were streaked on tryptone

soya agar (TSA) plates. Well isolated colonies were kept on TSA slants at 4 °C and stored in TSB with 20% glycerol (v/v) at -80 °C until further use.

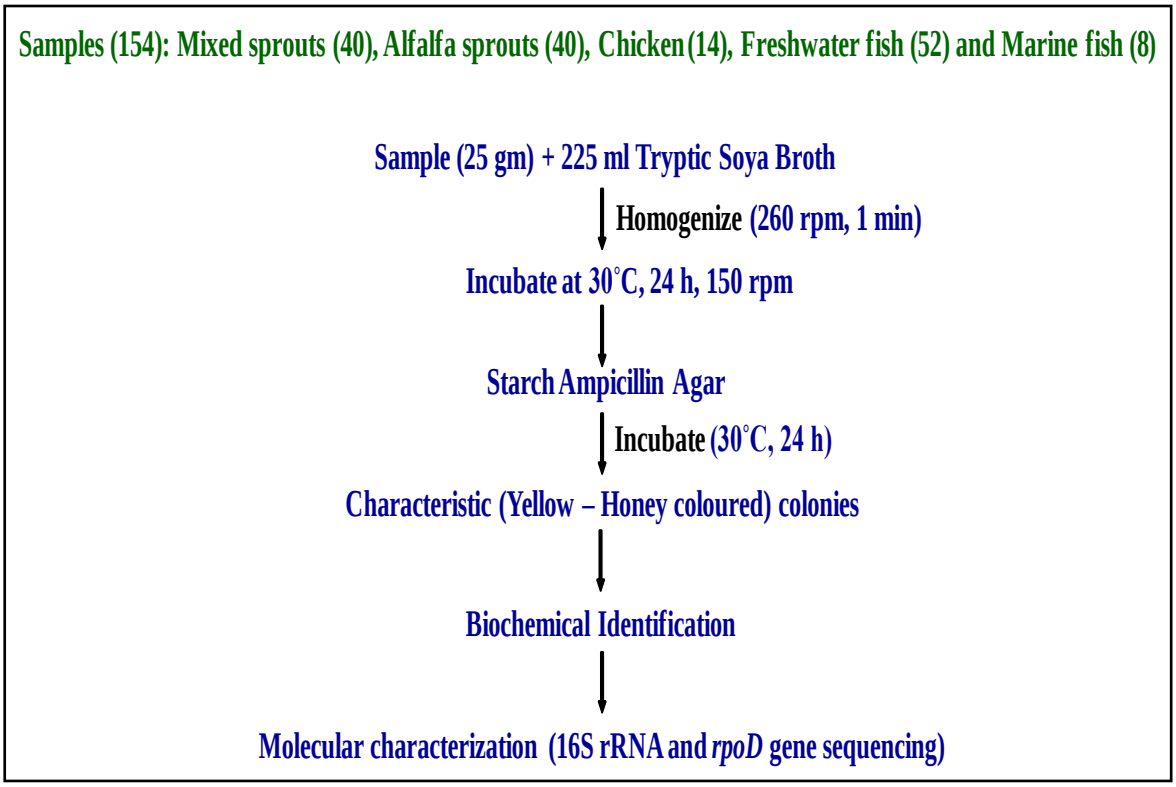


Figure 2.1. Flow chart for screening of *Aeromonas* from sprout, chicken and fish samples

2.2.5. Biochemical identification of *Aeromonas* isolates

Presumptive *Aeromonas* isolates were biochemically identified at a genus level based on the selected tests proposed by Abbott *et al.* (2003). The tests include Gram staining, oxidase, catalase and nitrate reduction reactions, fermentation of D-glucose and trehalose, growth in nutrient broth containing 0% and 3% NaCl, failure to grow in nutrient broth containing 6% NaCl, and resistance to vibriostatic agent O/129.

Assessment of these isolates to the species level within the *Aeromonas* genus was done by the following criteria: Moeller reactions (decarboxylation of lysine (LDC) and ornitine (ODC), and hydrolysis of arginine dihydrolase (ADH), Voges-Proskauer (VP) reaction, esculin hydrolysis, glucose (gas) and L-arabinose tests, indole production, utilization of citrate, urocanic acid and DL-lactate, and acid production from D-sorbitol,

D-mannitol, sucrose, D-mannose, lactose, L-rhamnose, lactulose, glucose-1-phosphate and glucose-6-phosphate. Selected supplementary biochemical tests that have been described lately i.e. acid production from melibiose, salicin and D-mannose, Voges-Proskauer test, and hydrolysis of starch and gelatin were also performed for better discrimination of the strains up to species level (Beaz-Hidalgo *et al.*, 2010; Martin-Carnahan and Joseph, 2005; Minana-Galbis *et al.*, 2007).

2.2.6. Genomic DNA isolation

DNA template was prepared by suspending 2 - 3 colonies of each *Aeromonas* isolate from TSA in 100 µl of sterile distilled water and boiling for 5 min. The tubes were centrifuged at 5,000 x g for 1 min to pellet out the cell debris. The supernatant was stored at -20 °C and used as DNA template for the PCR reactions.

2.2.7. PCR amplification and sequencing of 16S rRNA and *rpoD* genes

All biochemically positive isolates were confirmed to be *Aeromonas* by 16S rRNA gene sequencing. The 16S rRNA gene of *Aeromonas* isolates was amplified using two primers 16S/F (5'-TCATGGCTCAGATTGAACGCT-3') and 16S/R (5'-CCGGGCTTTCACATC TAACTTAT C-3') according to Arora *et al.* (2006). PCR was conducted in the buffer supplied by the manufacturer (Bangalore Genei, India) using 10 pmol of each primer and 0.5U *Taq* polymerase in a 25 µl reaction mixture. The thermal cycling conditions were, 2 min at 94 °C followed by 30 cycles of 1 min at 56 °C, 1 min at 72 °C and 1 min at 94 °C.

A fragment of approximately 820 bp of the *rpoD* gene was amplified by PCR using primers *rpoD*/70F (5'-ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGG NACNGT-3') and *rpoD*/70R (5'-ATAGAAATAACCAGACGTAAGTTNGCYTCNAC

CATYTCYTTYTT-3') (Soler *et al*., 2004), where N = any nucleotide; R = A or G; S = C or G; Y = C or T; M = A or C. A final PCR volume of 25 µl contained buffer supplied by the manufacturer (Bangalore Genei, India), 10 pmol of each primer, 1 µl of genomic DNA and 0.5U *Taq* polymerase. The reaction mixture was subjected to a touch-down PCR regimen of: denaturation at 95 °C for 5 min, followed by 2 cycles at 94 °C for 1 min (denaturation); 63 °C for 1 min (annealing); 72 °C for 1 min (extension); 2 cycles at 94 °C, 1 min; 61 °C, 1 min; 72 °C, 1 min; 2 cycles at 94 °C, 1 min; 59 °C, 1 min; 72 °C, 1 min; and 30 cycles at 94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min as described by Soler *et al.* (2004).

The PCR products were subjected to electrophoresis on 1% agarose gels in 1X Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide (1 mg/ml), visualized with UV. Each PCR product was incubated (37 °C, 15 min) with ExoSAP-IT reagent to remove primers and dNTPs. The mix was further incubated at 80 °C, 15 min to inactivate ExoSAP-IT reagent. Partial sequences of each PCR product were sequenced at MWG-Biotech Pvt. Ltd., Bangalore, India. Newly determined sequences were compared to those available in the GenBank database, using the BLASTN program (www.ncbi.nih.gov/BLAST/), to ascertain their closest relatives. The sequences were submitted to the GenBank database using the web-based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt/).

2.2.8. Molecular identification and phylogenetic data analysis

The 16S rRNA and *rpoD* gene sequences from all *Aeromonas* strains and their corresponding type or reference strains were independently aligned, and DNA sequence similarities were calculated for a continuous stretch of 505 bases (positions 81 - 584 according to *E. coli* numbering, J01695), in case of 16S rRNA gene and 512 bases

(positions 393 - 894 according to *E. coli* numbering, NP_417539.1) for *rpoD* gene. The phylogenetic trees were constructed with the MEGA 5 program package (Tamura *et al.*, 2011) using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method with Kimura two-parameter model.

2.3. Results and Discussion

2.3.1. Occurrence of *Aeromonas* in foods

A total of 154 food samples {mixed sprouts (40), alfalfa sprouts (40), chicken (14), fresh-water fish (52) and marine fish (8)} were analyzed over a period of two years for the occurrence of *Aeromonas*. All the samples analyzed were presumptive positive for *Aeromonas*. Four hundred and fifty-one presumptive positive isolates were obtained from 154 food samples (Table 2.1). However, only twenty-two (4.9%) of these 451 isolates were identified as *Aeromonas* by biochemical tests and confirmed as *Aeromonas* by partial sequencing of 16S rRNA gene. Some of these 451 isolates were found to belong to *Comamonas, Pseudomonas, Alcaligenes, Delftia, Providencia, Morganella* and *Citrobacter* spp. based on partial sequencing of 16S rRNA gene. These sequences were submitted to GenBank at NCBI (Accession No. HM055739 - 52).

Table 2.1 shows the prevalence of *Aeromonas* spp. in different food samples in Mumbai, India. Eighteen (11.7%) out of 154 food samples were positive for *Aeromonas* spp. *Aeromonas* were isolated from 28.6%, 20% and 2.5% of chicken, fish and sprout samples, respectively (Table 2.1). The current study shows that *Aeromonas* are widely distributed in the retail foods from Mumbai with more prevalence in foods of animal origin (high protein content) than of plant origin (low protein content). *Aeromonas* are known to produce a range of proteases, which help in obtaining nutrition from protein rich sources. This is the first report of incidence of *Aeromonas* in sprouts from India.

Xanthopoulos *et al.* (2010) have reported aeromonads from 61.5% of ready-to-eat salads; whereas, only 34% of organic vegetables were found to be contaminated with *Aeromonas* spp. (McMahon and Wilson, 2001). The sprouts screened in the current study were minimally processed and kept at low temperature (< 8 °C). The low occurrence of *Aeromonas* may be attributed to good hygienic practices of the producers and retailers (Nagar and Bandekar, 2009).

Table 2.1. Isolation and identification of *Aeromonas* from different food samples

Food samples	**No. of samples**	**Presumptive positive isolates**	**No. of *Aeromonas* positive samples[a] (%)**	**Confirmed *Aeromonas* isolates[b]**
Alfalfa sprouts	40	55	1 (2.5%)	2
Mixed sprouts	40	65	1 (2.5%)	1
Chicken	14	63	4 (28.6%)	5
Freshwater fish	52	231	10 (19.2%)	12
Marine fish	8	37	2 (25%)	2
Total	154	451	18 (11.7%)	22

[a]Number of positive samples. Figures in parentheses are percentages of positive samples

[b]Number of confirmed *Aeromonas* isolates based on biochemical and molecular methods

Aeromonas were isolated from 25% of marine fish and 19.2% of fresh-water fish samples in the present study (Table 2.1). Since the number of marine fish samples (8) was less as compared to fresh-water fish samples (52) in the present study, it is difficult to

provide conclusive comparison of distribution of *Aeromonas* in the fish samples. Yucel and Balci (2010) have reported more prevalence of *Aeromonas* in marine fish samples than fresh-water fish. *A. hydrophila* were isolated from 33.58% of marine fish and 17.62% of marine prawns in India (Vivekanandhan *et al.*, 2005). Chang *et al.* (2008) found *Aeromonas* in poultry (22.5%), seafood (30%) and aquatic retail (38.9%) samples from Taiwan. Several food- and water-borne outbreaks associated with *Aeromonas* have been reported across the world (Altwegg *et al.*, 1991; Hofer *et al.*, 2006; Krovacek *et al.*, 1995; Taher *et al.*, 2000). There are more than 20 species of *Aeromonas* and majority of them are considered to be pathogenic. Food can be contaminated with *Aeromonas* due to use of contaminated water or secondary contamination during handling, storage, and transportation. *Aeromonas* can survive and grow at storage temperature of $\geq$ 4 °C. Variations in the incidence of *Aeromonas* spp. may be due to the differences in the geographical distribution, origin of the samples, sampling period, methodology of analysis and inconsistent hygienic practices followed during handling.

2.3.2. Identification of isolates based on biochemical tests

Twenty-two *Aeromonas* isolates from sprouts, chicken and fish samples (Table 2.1) were identified up to the species level based on the conventional biochemical characteristics as described by Abbott *et al.* (2003). They belonged to seven different species of *Aeromonas* {*A. salmonicida* (5), *A. veronii* bv. *sobria* (5), *A. hydrophila* (4), *A. caviae* (4), *A. jandaei* (2), *A. trota* (1) and *A. eucrenophila* (1)} (Table 2.3). The species level identification of *Aeromonas* by routine biochemical procedures is found to be indecisive due to the variety of conditions and methodologies employed for the biochemical tests that produce different and/or inconsistent results and absence of an integrated identification scheme. There are many biochemical tests for which many

isolates of the same species are reported to give variable results (Abbott *et al.*, 2003; Martin-Carnahan and Joseph, 2005). There are very few biochemical tests which can be used for the accurate differentiation among *Aeromonas* species. Further, the results of the biochemical testes may vary depending on the time of incubation, age and inoculum of the culture, batch of media etc.

Recently, Ottaviani *et al.* (2011) have stressed the importance of incorporation of newly described supplementary key biochemical tests to conventional biochemical schemes (Abbott *et al.*, 2003) to distinguish each species from its nearest neighbours at the phenotypic level. Therefore, these strains were further characterized using additional biochemical tests as described by Beaz-Hidalgo *et al.* (2010), Martin-Carnahan and Joseph (2005) and Minana-Galbis *et al.* (2007).

Table 2.2. Identification of *Aeromonas* isolates till species level based on comprehensive biochemical tests

Isolate			D-Glucose		Nitrate		Sugar utilization								Zone Inhibition (O/129)		Growth in NaCl					
No.	Oxi	Cat	Ferment	Gas	Reduction	Tre	Xyl	Ado	Dul	Ino	Urease	ONPG	Lipase	Gelatinase	10µg	150µg	0%	3%	6%	Esc	VP	H_2S
A85	+	+	+	-	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-
A90	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	+
A91	+	+	+	-	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-
A329	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	-	+	-
A331	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	-	+
Y47	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	-	+	-
Y113	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	-	+	-
Y324	+	+	+	-	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-
A254	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	-	+	-
A283	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	+	+	-	-	-	-
A501A	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-
A502A	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	-	+	-
A514A	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-
A521	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-
A527	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-
A563	+	+	+	-	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	-	-
A619	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-
Y528	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-
Y556	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	+
Y559	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-
Y567	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-
Y577	+	+	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	-

Isolate No.	ODC	LDC	ADH	Acid Production Ara	Man	Suc	Sor	Mano	Lac	Rha	Cel	Sal	Mel	Citrate	Indole	DL-Lactate	Urocanic acid	G-1-P	G-6-P	Lactulose	Species identification
A 85	-	-	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	-	-	+	***A. caviae***
A90	-	+	+	-	+	+	-	+	-	-	-	+	-	+	+	+	+				***A. hydrophila***
A91	-	-	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	-	-	-	***A. caviae***
A 329	-	+	+	-	+	-	-	+	+	-	-	-	-	+	+						***A. jandaei***
A331	-	+	+	+	+	+	-	+	-	-	-	+	-	+	+	-	-	+	+	-	***A. hydrophila***
Y47	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	+				***A. salmonicida***
Y113	-	+	+	-	+	+	-	+	-	-	+	-	-	+	+						***A. veronii* bv. *sobria***
Y 324	-	-	+	+	+	+	-	-	+	-	-	+	-	+	+	+	+	-	-	-	***A. caviae***
A 254	+	+	+	-	+	+	-	+	-	-	-	-	-	+	+						***A. veronii* bv. *sobria***
A 283	-	+	+	-	+	-	-	+	-	-	+	-		+	+						***A. trota***
A501A	+	+	-	-	+	+	-	+	-	-	+	+	+	+	+	-	-				***A. veronii* bv. *veronii***
A502A	-	+	+	-	+	+	-	+	-	-	-	-	-	+	+						***A. veronii* bv. *sobria***
A514A	+	+	-	-	+	+	-	+	-	-	+	+	-	+	+						***A. veronii* bv. *veronii***
A521	-	+	+	-	+	+	-	+	-	-	+	+	+	-	+	-	-	+	+	-	***A. allosaccharophila***
A 527	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+				***A. salmonicida***
A563	-	+	-	+	+	+	-	-	-	-	+	+	-	+	+	+	+	-	-	-	***A. bivalvium***
A619	+	+	-	-	+	+	-	+	-	-	+	+	-	+	+	+	-				***A. veronii* bv. *veronii***
Y 528	-	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+				***A. salmonicida***
Y 556	-	+	+	-	+	+	+	+	-	-	+	-	-	-	+						***A. sobria***
Y 559	-	+	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+				***A. salmonicida***
Y 567	-	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+				***A. salmonicida***
Y 577	-	+	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+				***A. salmonicida***

Each isolate was identified till species level using comprehensive biochemical scheme (conventional and additional biochemical tests) (Table 2.2). These 22 *Aeromonas* isolates were identified as *A. salmonicida* (6), *A. veronii* bv. *veronii* (3), *A. caviae* (3), *A. hydrophila* (2), *A. veronii* bv. *sobria* (3), *A. jandaei* (1), *A. trota* (1), *A. sobria* (1), *A. allosaccharophila* (1) and *A. bivalvium* (1) based on the comprehensive biochemical tests (Table 2.2). The strain Y47 (*A. veronii* bv. *sobria*) was re-identified as *A. salmonicida*, A283 (*A. veronii* bv. *sobria*) as *A. trota*, A501A (*A. hydrophila*) as *A. veronii* bv. *veronii*, A514A (*A. jandaei*) as *A. veronii* bv. *veronii*, A521 (*A. eucrenophila*) as *A. allosaccharophila*, A563 (*A. caviae*) as *A. bivalvium*, A619 (*A. hydrophila*) as *A. veronii* bv. *veronii* and Y556 (*A. trota*) as *A. sobria* (Table 2.3).

Beaz-Hidalgo *et al.* (2010) have reported that some *A. sobria* strains can hydrolyze gelatin and show a positive reaction to ADH and VP tests, contradicting earlier published data by Abbott *et al.* (2003). These observations helped in the correct identification of the isolate Y556 as *A. sobria*. Although biochemical properties for *A. sobria*, *A. allosaccharophila* and *A. veronii* bv. *veronii* were described by Abbott *et al.* (2003), these species were not included in the complex and species level identification due to limited number of available strains. *A. bivalvium* is a recently described species (Minana-Galbis *et al.*, 2007) and was also not included in the biochemical test scheme developed by Abbott *et al.* (2003).

Furthermore, two *A. veronii* bv. *veronii* isolates (A501A and A619) were found to be catalase-negative, and one *A. hydrophila* isolate (A331) to be VP-negative (Table 2.2). Abbott *et al.* (2003) have also reported 10% of the studied *A. veronii* bv. *veronii* isolates to be catalase-negative and around 8% of *A. hydrophila* isolates to be VP-negative. Similarly, in the present study, we observed one *A. veronii* bv. *veronii* isolate (A501A) to be melibiose-positive, one *A. allosaccharophila* (A521) to be salicin- and melibiose-positive,

and one *A. sobria* (Y556) to be sorbitol-positive and citrate utilization-negative (Table 2.2). *Aeromonas* isolates showing atypical biochemical characteristics have been reported by various researchers (Abbott *et al.*, 2003; Ormen *et al.*, 2005). The most common atypical biochemical characteristics observed included fermentation of sugars and urea hydrolysis (Abbott *et al.*, 2003). An increase in phenotypic diversity among *Aeromonas* spp. may be due to the introduction of new taxa with unusual phenotypic characteristics and a rise in *Aeromonas* surveys from environmental sources. The true extent of phenotypic variation for the less well-characterized species remains unknown, and greater phenotypic diversity will be found as more strains of these uncommon genomospecies are identified. Thus, it will become increasingly difficult to identify *Aeromonas* isolates to species level without extensive arrays of biochemical tests (Abbott *et al.*, 2003).

Many reports on *Aeromonas* are based only on biochemical tests, without the use of molecular methods (Akinbowale *et al.*, 2007; McMahon and Wilson, 2001). The results based only on biochemical tests may lead to false positive reports. Food samples contaminated with *Aeromonas* are not acceptable for human consumption and may be rejected based on false positive reports. Therefore, all of our isolates were confirmed as *Aeromonas* using partial 16S rRNA gene sequencing.

Table 2.3. Comparison of phenotypic and genetic identification of 22 *Aeromonas* strains recovered from different food samples

Strain	Origin	Taxonomic identification (species name) based on			
		Biochemical tests[a]	Additional biochemical tests[b]	16S rRNA gene sequences[a]	*rpoD* gene sequences
A 85	Mixed sprouts	*A. caviae*	*A. caviae*	*A. caviae*	*A. caviae*
A90	Alfalfa sprouts	*A. hydrophila*	*A. hydrophila*	*A. trota*	*A. hydrophila*
A91	Alfalfa sprouts	*A. caviae*	*A. caviae*	*A. trota*	*A. caviae*
A 329	Chicken	*A. jandaei*	*A. jandaei*	*A. jandaei*	*A. jandaei*
A331	Chicken	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
Y47	Chicken	*A. veronii* bv. *sobria*	*A. salmonicida*	*A. salmonicida /A. hydrophila*	*A. salmonicida*
Y113	Chicken	*A. veronii* bv. *sobria*	*A. veronii* bv. *sobria*	*A. veronii*	*A. veronii* bv. *sobria*
Y 324	Chicken	*A. caviae*	*A. caviae*	*A. trota /A. caviae*	*A. caviae*
A 254	Tengan	*A. veronii* bv. *sobria*	*A. veronii* bv. *sobria*	*A. veronii*	*A. veronii* bv. *veronii*
A 283	Pomfret	*A. veronii* bv. *sobria*	*A. trota*	*A. caviae*	*A. trota*
A 501A	Butter cat fish	*A. hydrophila*	*A. veronii* bv. *veronii*	*A. veronii*	*A. veronii* bv. *veronii*
A502A	Butter cat fish	*A. veronii* bv. *sobria*	*A. veronii* bv. *sobria*	*A. veronii*	*A. veronii* bv. *sobria*
A514A	Butter cat fish	*A. jandaei*	*A. veronii* bv. *veronii*	*A. trota / A. caviae*	*A. veronii* bv. *veronii*
A521	Butter cat fish	*A. eucrenophila*	*A. allosaccharophila*	*A. allosaccharophila*	*A. allosaccharophila*
A 527	Scampi	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*
A563	Bombay duck	*A. caviae*	*A. bivalvium*	*A. bivalvium / A. popoffi*	*A. bivalvium*
A619	Catla	*A. hydrophila*	*A. veronii* bv. *veronii*	*A. hydrophila / A. trota*	*A. veronii* bv. *veronii*
Y 528	Tengan	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*
Y 556	Butter cat fish	*A. trota*	*A. sobria*	*A. sobria*	*A. sobria*
Y 559	Butter cat fish	*A. salmonicida*	*A. salmonicida*	*A. hydrophila* / *A. salmonicida*	*A. salmonicida*
Y 567	Butter cat fish	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*
Y 577	Tengan	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*

[a]Species level identification based on biochemical tests and partial 16S rRNA gene sequencing
[b]Species level identification based on additional biochemical tests (Beaz-Hidalgo *et al.*, 2010; Martin-Carnahan and Joseph, 2005; Minana-Galbis *et al.*, 2007)

2.3.3. Identification of isolates based on 16S rRNA sequences

Partial 16S rRNA gene analysis was used to confirm the identification based on comprehensive biochemical tests. The 16S rRNA gene sequences were submitted to GenBank at NCBI (Accession numbers: FJ561050 - 52, HQ122915 - 31, HQ413137 and HM002780). However, most of these strains could not be correctly identified till species level using 16S rRNA gene analysis. Disparity was observed between the identification of these isolates based on biochemical tests and 16S rRNA gene sequencing (Table 2.3). Researchers have highlighted considerable number of discrepancies in biochemical and genetic identification of both environmental and clinical *Aeromonas* isolates (Borrell *et al.*, 1997; Soler *et al.*, 2003). The disparity between the biochemical and the genetic identification of *Aeromonas* isolates may be explained by the fact that the biochemical species-identification schemes were developed based on data from the clinical strains. Environmental isolates are often more heterogenic than clinical isolates, and their biochemical profiles are less well known compared to those of clinical isolates (Ormen *et al.*, 2005).

Sequence similarity between all *Aeromonas* strains for the 16S rRNA gene was 94.6 - 100%, corresponding to 0 - 27 nucleotide differences. Mean sequence similarity, an indicator of discriminatory power, was found to be 97.3%. The alignment exhibited a total of 43 variable positions (8.5% of the determined fragment). Analysis of the aligned 16S rRNA gene sequences allowed the construction of the phylogenetic tree (Fig. 2.2). Species level identification of only 59.1% (13/22) of these isolates by biochemical tests (Abbott *et al.*, 2003) agreed with the identification based on 16S rRNA gene sequencing. However, this correlation percentage increased to 77.3% (17/22) on incorporation of additional biochemical tests (Table 2.3). The disparity between biochemical and 16S rRNA gene

identification can be observed in the phylogenetic tree, where the identified strains fail to cluster with their corresponding type strains (Fig 2.2).

The degree of resolution obtained with 16S rRNA gene sequencing was sufficient to correctly identify only *A. salmonicida*, *A. sobria*, *A. bivalvium*, *A. allosaccharophila* and *A. jandaei* isolates up to species level (Fig. 2.2). The 16S rRNA gene was found to be highly conserved within the genus *Aeromonas* and showed limited resolution based on analysis of 1330 bp region (Kupfer *et al.*, 2006). Other researchers (Beaz-Hidalgo *et al.*, 2010; Ormen *et al.*, 2005) have also found environmental *Aeromonas* isolates to be highly heterogeneous in their biochemical properties. Lamy *et al.* (2010a) could correctly identify only 24% of environmental isolates up to species level by biochemical tests. The 16S rRNA gene sequencing was useful to identify *Aeromonas* isolates at the genus level only. Thus, in order to overcome this limitation, *rpoD* gene sequencing of these strains was undertaken to conclusively identify these strains at species level.

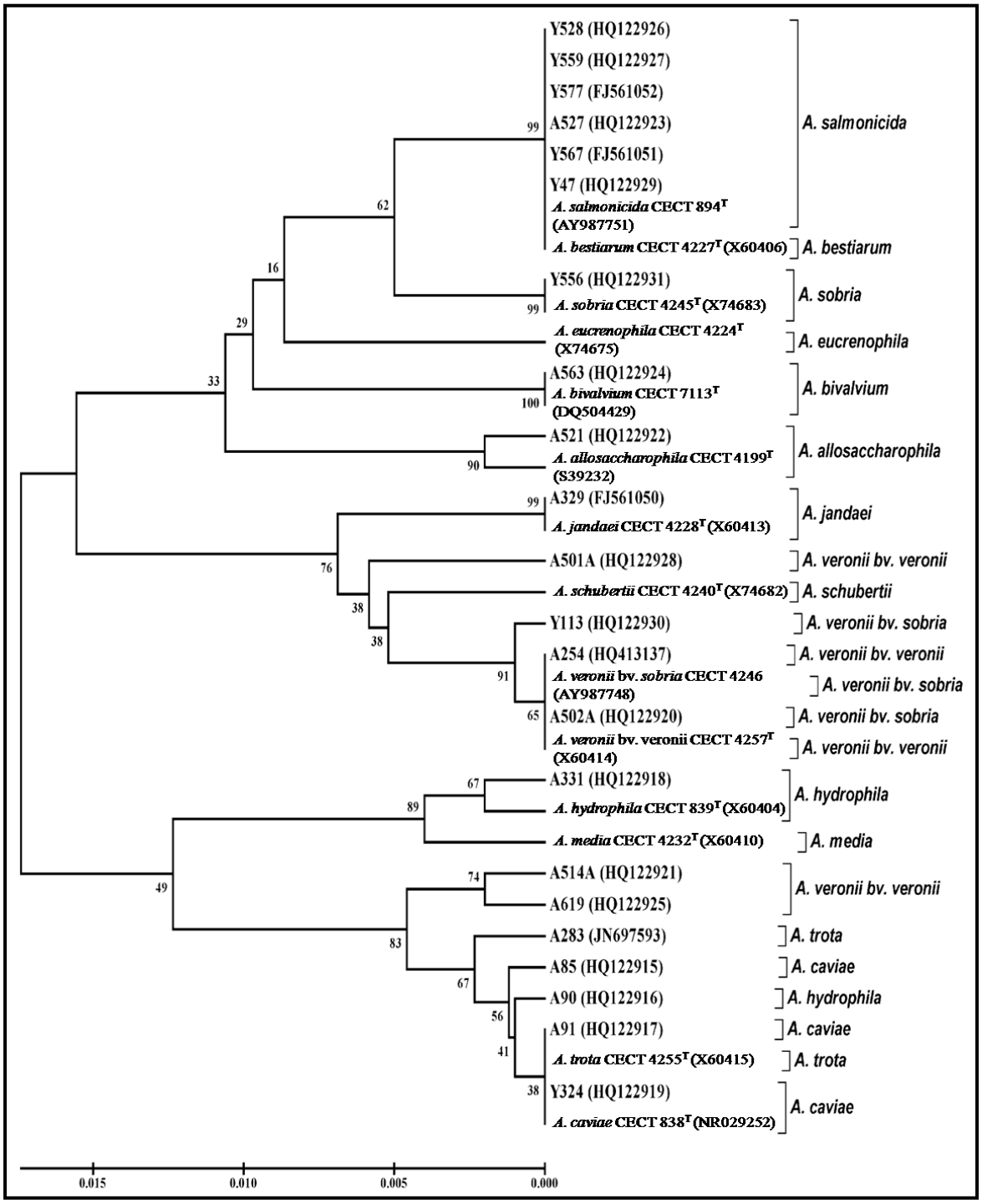


Figure 2.2. Unrooted phylogenetic tree (UPGMA) of *Aeromonas* food isolates and other known *Aeromonas* species based on the 16S rRNA gene sequences. CECT or ATCC numbers indicate the culture collection numbers of the *Aeromonas* reference strains in Spanish Type culture collection and American Type culture collection, respectively. Numbers in the parenthesis represents GenBank accession numbers. Numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates). The bar indicates 0 - 1.5% sequence divergence.

2.3.4. Comparison of identification of isolates based on biochemical tests, 16S rRNA and *rpoD* gene sequences

Partial nucleotide sequences of *rpoD* gene from 22 *Aeromonas* isolates were determined (GenBank accession number: JN182265 - 69, JN412625 - 30, JN388917 - 22 and JN544572 - 76). The sequence similarity between all *Aeromonas* strains was 80.4 - 99.8%, corresponding to 1 - 100 nucleotide differences. Mean sequence similarity was found to be 89.1%. This value is significantly lesser than that of the 16S rRNA gene (97.3 %) of the same isolates and comparable to that of *gyrB* (92.2 %) and *rpoD* (89.3 %) genes in Soler's study (2004). The alignment exhibited a total of 154 variable positions (30.1% of the fragment sequenced), values close to those reported by Soler *et al.* (2004). Deletion of 6 bp in the sequence of *A. salmonicida* CECT 894^T strain (AY169327) was observed as compared to sequences from all other *A. salmonicida* isolates. The observations were confirmed by comparison of sequences from *A. salmonicida* strains from our study with other *A. salmonicida* sequences {190 (FN773330) and 156 (AY169361)}. Deletion of 3 bp was also observed in both A283 and CECT 4255^T sequences (both *A. trota* species) as compared to all other strains.

Identification of 59.1% (13/22) of these isolates by biochemical tests (Abbott *et al.*, 2003) agreed with the identification based on *rpoD* gene sequencing. However, congruity increased to 95.5% (21/22) when additional biochemical tests were incorporated in the phenotypic identification (Table 2.3). The re-identification of all the strains (Y47, A283, A501A, A514A, A521, A563, A619 and Y556) using additional biochemical tests were confirmed by *rpoD* gene sequence analysis (Table 2.3).

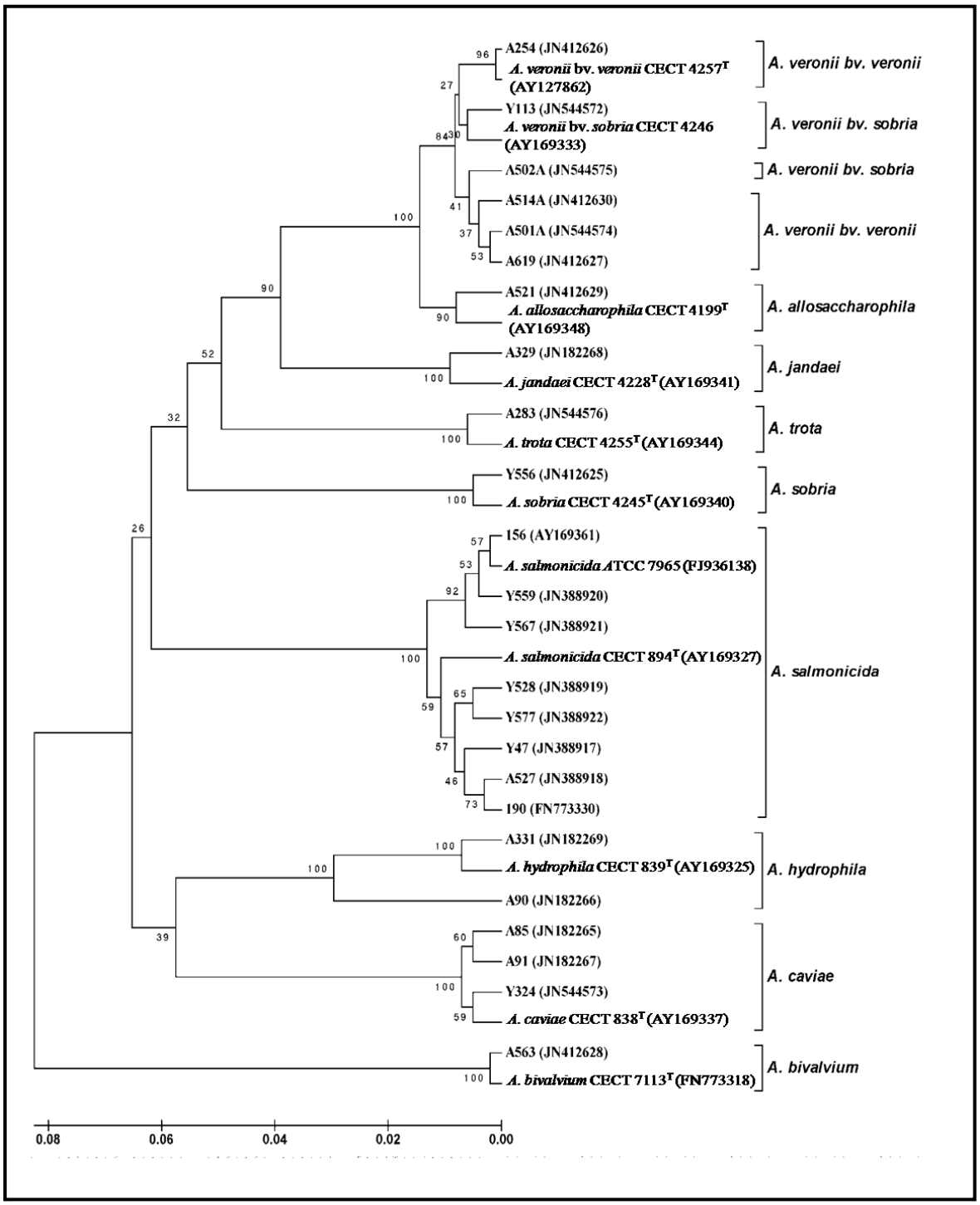
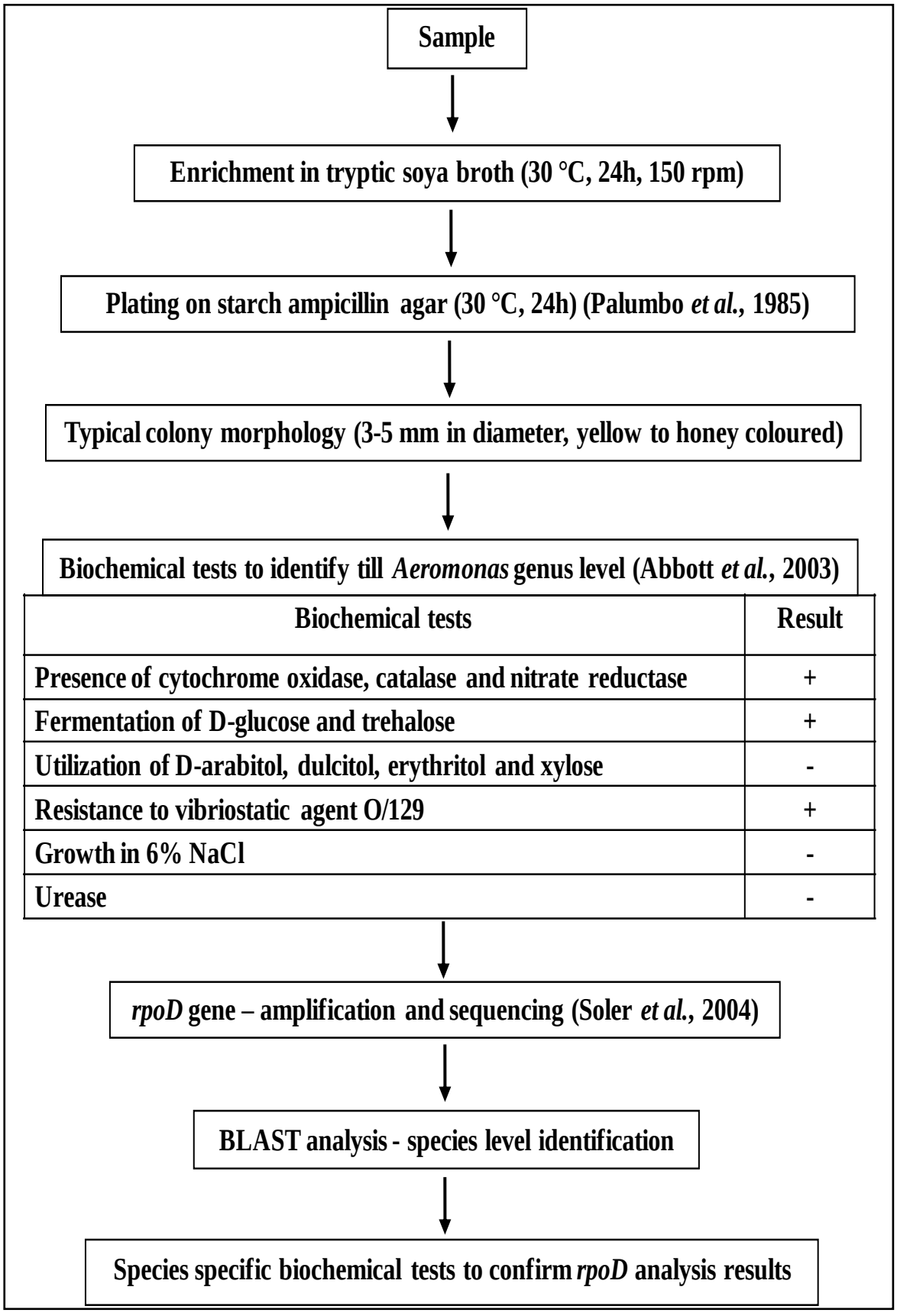


Figure 2.3. Unrooted phylogenetic tree (UPGMA) of *Aeromonas* food isolates and other known *Aeromonas* species based on the *rpoD* gene sequences. CECT or ATCC numbers indicate the culture collection numbers of the *Aeromonas* reference strains in Spanish Type culture collection and American Type culture collection, respectively. Numbers in the parenthesis represents GenBank accession numbers. Numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates). The bar indicates 0 - 8% sequence divergence.

The comparison of 16S rRNA and *rpoD* sequence data indicated that the two genes showed dissimilar substitution rates. The number of variable positions was approximately 3.5 times more in *rpoD* gene as compared to 16S rRNA gene (8.5% for 16S rRNA versus 30.1% for *rpoD*) and the ranges of nucleotide substitutions between all strains were also different (0 - 27 and 1 - 100, for 16S rRNA and *rpoD* genes, respectively). Further, the phylogenetic tree based on *rpoD* gene showed more consistent clustering than that based on 16S rRNA gene between the identified strains and their respective reference strains (Fig 2.2 and 2.3). There was a congruence of 77.3% in the identification of *Aeromonas* strains, based on comprehensive biochemical tests and 16S rRNA gene sequencing. Identification of all the strains, except A254, based on comprehensive biochemical tests matched with *rpoD* gene analysis. The isolate A254 was identified as *A. veronii* bv. *sobria* using comprehensive biochemical tests; whereas, *rpoD* gene sequence analysis identified it as *A. veronii* bv. *veronii*. Multilocus sequence typing (MLST) can be further used for the accurate species level identification of *Aeromonas* strains (Martinez-Murcia *et al.*, 2011; Martino *et al.*, 2011). However, MLST was found to be inefficient to differentiate strains belonging to *A. veronii* group (*A. veronii* bv. *veronii*, *A. veronii* bv. *sobria* and *A. allosaccharophila*) due to high frequency of horizontal gene transfer in this group (Silver *et al.*, 2011). Since the strain A254 grouped with CECT 4257^{T} (*A. veronii* bv. *veronii*) based on the *rpoD* gene sequence analysis (Fig 2.3), it most likely belongs to *A. veronii* bv. *veronii*. Thus, all these 22 isolates were accurately identified up to species level by *rpoD* gene as *A. salmonicida* (6), *A. veronii* bv. *veronii* (4), *A. caviae* (3), *A. hydrophila* (2), *A. veronii* bv. *sobria* (2), *A. jandaei* (1), *A. trota* (1), *A. sobria* (1), *A. allosaccharophila* (1) and *A. bivalvium* (1) (Table 2.3).

The present study confirms that the *rpoD* gene has higher discriminatory power than 16S rRNA gene to delineate *Aeromonas* strains till species level and further validates

the usefulness of *rpoD* gene sequencing for the correct identification of *Aeromonas* strains to the recognized species. In the recent years, *rpoD* gene sequencing has been widely used for the species level identification of *Aeromonas* strains from clinical samples (Puthucheary *et al.*, 2012; Senderovich *et al.*, 2012). They also observed that the identification of *Aeromonas* on the basis of *rpoD* gene sequencing was more accurate than biochemical methods or 16S rRNA gene sequencing. Our study with the food isolates supports the earlier observations that the biochemical tests used to differentiate *Aeromonas* species in different environmental samples is limited and must therefore be regarded only as presumptive. There is a need to integrate the conventional biochemical schemes, additional key tests and molecular methods to correctly identify *Aeromonas* isolates till species level. Based on the earlier reports (Abbott *et al.*, 2003; Beaz-Hidalgo *et al.*, 2010; Soler *et al.*, 2004) and present study, we propose that a combination of certain biochemical tests and *rpoD* gene sequencing (Fig 2.4) would be ideal for the simple and accurate identification of *Aeromonas* isolates from food samples up to species level.



Biochemical tests	Result
Presence of cytochrome oxidase, catalase and nitrate reductase	+
Fermentation of D-glucose and trehalose	+
Utilization of D-arabitol, dulcitol, erythritol and xylose	-
Resistance to vibriostatic agent O/129	+
Growth in 6% NaCl	-
Urease	-

Figure 2.4. Identification scheme for the rapid, convenient and accurate identification of *Aeromonas*

2.3.5. Prevalence of different *Aeromonas* species in food samples

Out of 22 *Aeromonas* isolates, 6 isolates were confirmed as *A. salmonicida,* 4 isolates as *A. veronii* bv. *veronii,* 3 isolates as *A. caviae,* 2 isolates as *A. hydrophila* and *A. veronii* bv. *sobria* each, and 1 isolate each as *A. jandaei*, *A. trota*, *A. sobria*, *A. allosaccharophila* and *A. bivalvium* using biochemical and molecular methods (Table 2.3). Seven and five different *Aeromonas* species were isolated from fish and chicken samples, respectively; whereas, only two *Aeromonas* species were found in the sprout samples (Table 2.4). This indicates higher prevalence and diversity of *Aeromonas* species in food samples of animal origin as compared to plant origin. Human pathogenic species (*A. hydrophila*, *A. veronii* bv. *sobria*, *A. caviae*, *A. jandaei*, *A. trota*, *A. veronii* bv. *veronii* and *A. sobria*) were present in all the categories of the food samples tested (Table 2.4). However, fish pathogens (*A. salmonicida* and *A. allosaccharophila*) were found only in chicken and fresh-water fish samples; whereas, non-pathogenic species *A. bivalvium* was isolated from marine fish.

Overall, *A. salmonicida* was the most prevalent species, followed by *A. veronii* bv. *veronii* and *A. caviae*. *A. salmonicida* is the causative agent of furunculosis in marine and freshwater fish (Toranzo *et al.*, 2005). *A. trota* and *A. bivalvium* were isolated only from marine fish samples; whereas, *A. veronii* bv. *veronii*, *A. sobria* and *A. allosaccharophila* were isolated only from fresh-water fish samples (Table 2.4). *A. trota* has been reported from farm-raised catfish (Nawaz *et al.*, 2006), and food and water samples (Granum *et al.*, 1998). Minana-Galbis *et al.* (2007) isolated *A. bivalvium* from bivalve mollusks; whereas, *A. allosaccharophila* has been recovered from water bodies (Picao *et al.*, 2008). Recently, *A. trota*, *A. sobria*, *A. veronii*, *A. allosaccharophila*, *A. hydrophila*, *A. salmonicida* and *A. caviae* have been isolated from water samples and ornamental fishes (Carvalho *et al.*, 2012; John and Hatha, 2012).

Table 2.4. Prevalence of *Aeromonas* spp. in different food samples in Mumbai, India

Source	*A. hydrophila*[a] (n=2)	*A. veronii* bv. *sobria*[a] (n=2)	*A. caviae*[a] (n=3)	*A. salmonicida*[a] (n=6)	*A. jandaei*[a] (n=1)	*A. trota*[a] (n=1)	*A. allosaccharophila*[a] (n=1)	*A. veronii* bv. *veronii*[a] (n=4)	*A. bivalvium*[a] (n=1)	*A. sobria*[a] (n=1)
Alfalfa sprouts	1	-	1	-	-	-	-			
Mixed sprouts	-	-	1	-	-	-	-			
Chicken	1	1	1	1	1	-	-			
Fresh water fish	-	1	-	5	-	-	1	4		1
Marine fish		-	-			1			1	
Total	2 (9.1%)	2 (9.1%)	3 (13.6%)	6 (27.3%)	1 (4.5%)	1 (4.5%)	1 (4.5%)	4 (18.2%)	1 (4.5%)	1 (4.5%)

[a]Number of strains isolated. Figures in parentheses are relative percentages of isolates of each species

Considering the different sources, *A. caviae* (66.7%) and *A. salmonicida* (35.7%) were the most frequently isolated species from sprouts and fish, respectively; whereas, all the five species (*A. jandaei*, *A. hydrophila*, *A. salmonicida*, *A. veronii* bv. *sobria* and *A. caviae*) were equally distributed in chicken samples. In earlier studies, Xanthopoulos *et al.* (2010) found *A. hydrophila* to be the most frequently isolated species from sprouts and fresh produce; whereas, *A. veronii* bv. *sobria* was the most prevalent *Aeromonas* species in poultry and raw meat (Gobat and Jemmi, 1993). *A. hydrophila* was the most frequently isolated species from seafood in Berlin (Ullmann *et al.*, 2005), and from retail poultry and fish samples in Taiwan (Chang *et al.*, 2008).

2.4. Conclusions

These findings demonstrated that the sprout, chicken and fish samples, marketed in Mumbai, India were contaminated with *Aeromonas*. Foods of animal origin showed higher prevalence and diversity of *Aeromonas* species as compared to plant origin. These data serve as baseline information for developing and monitoring effective risk management strategies associated with consumption of these food products. The present study further confirms that the biochemical tests and 16S rRNA gene analysis are useful in the identification of *Aeromonas* isolates only till genus level. The *rpoD* gene was found to be a better phylogenetic marker than 16S rRNA gene, even at the intra-species level. The present work emphasizes the need of integration of molecular methods such as *rpoD* gene sequence analysis along with comprehensive biochemical scheme for the rapid and accurate identification of *Aeromonas* strains up to species level.

CHAPTER 3

Characterization of *Aeromonas* Isolates

3.1. Introduction

The *Aeromonas* species are widespread in food and water, and most of the isolates from these sources are potentially pathogenic (Garibay *et al.*, 2006). The sprouts, chicken and fish samples obtained from Mumbai and its suburb were contaminated with *Aeromonas* (Chapter 2). Multiple virulence factors play a pivotal role in the establishment of *Aeromonas* infection and the pathogenesis is dependent on host susceptibility (Martin-Carnahan and Joseph, 2005). A wide range of cell-associated and extra-cellular virulence factors that may play an important role in the development of disease, both in humans or in fish, have been detected and studied in several *Aeromonas* spp. (Igbinosa *et al.*, 2012). Most *Aeromonas* produce an array of extra-cellular enzymes that play an important role in pathogenesis (Pemberton *et al.*, 1997).

There is lack of reliable methods for differentiating pathogenic and non-pathogenic *Aeromonas* strains and to predict the ability of a strain to produce gastrointestinal disease in humans (Chopra *et al.*, 2009). Several researchers (Chacon *et al.*, 2003; Sen and Rodgers, 2004) have used PCR based amplification of virulence genes for detecting potentially pathogenic bacteria and differentiation of pathogenic from non-pathogenic strains.

In recent years, antibiotic resistance among *Aeromonas* strains has become a major public health concern in both developed as well as developing countries; especially more serious in developing countries, due to unregulated and uncontrolled use of many antibiotics to treat gastroenteric illness, to treat and prevent fish and chicken diseases, and also as feed additives (Ghenghesh *et al.*, 2008). Most of the information regarding antibiotic susceptibility in *Aeromonas* is based solely upon the most clinically relevant species, i.e., *A. hydrophila*, *A. caviae*, and *A. veronii*bv. *sobria*. It is not clear whether those profiles can be extrapolated to other less frequently encountered taxa causing illness

(Janda and Abbott, 2010). Very few reports (Kampfer *et al.*, 1999; Overman and Janda, 1999) give comprehensive antibiotic susceptibility profiles of the genus *Aeromonas* to different antimicrobial agents. Though antibiotic resistance of *Aeromonas* strains from clinical sources have been studied, very less is known about the antibiotic resistance profiles of food and environmental isolates. Antibiotic resistance is particularly relevant in pathogenic *Aeromonas* species in which, besides the classical resistance to β-lactamic antibiotics, multiple-resistance has been frequently identified. Antibiotic resistance and virulence factors have been correlated with plasmids in *Aeromonas* (Palu *et al.*, 2006).

Pulse field gel electrophoresis (PFGE) is currently considered the "gold-standard" method for subtyping food-borne pathogens due to its sensitivity and discriminatory power (Foley *et al.*, 2009). Also, PFGE is the widely used technique in molecular epidemiological investigation of *Aeromonas* spp. (Pablos *et al.*, 2010). Though *Aeromonas* have been reported from a number of sources in India (Vaseeharan *et al.*, 2005; Vivekanandhan *et al.*, 2002), few studies show their molecular characterization (Sharma *et al.*, 2005), and none report the PFGE profile of the Indian *Aeromonas* isolates. This study uses PFGE to determine the genetic diversity among the *Aeromonas* isolates obtained from sprouts, chicken and fish. Whole cell protein (WCP) analysis using SDS-PAGE is a simple, rapid, inexpensive, reliable and easily applicable molecular technique for the characterization of *Aeromonas* isolates (Maiti *et al.*, 2009).

There is a lack of detailed studies regarding the prevalence of virulence factors, antibiotic resistance and plasmid, WCP and PFGE profiles of *Aeromonas* food isolates in India.

Therefore, the current study was carried out with following objectives:

(i) To assess the presence of various putative virulence genes and extra-cellular enzymes in *Aeromonas* strains obtained from a wide variety of food products in Mumbai, India

(ii) To determine the antibiotic resistance and plasmid profiles of the isolates

(iii) To characterize these strains using PFGE and WCP

3.2. Materials and methods

3.2.1. Bacterial cultures and growth conditions

A collection of 22 strains belonging to 10 different species of genus *Aeromonas* (Chapter 2) were used in the study. All bacterial cultures were maintained in tryptic soya broth (TSB) with 20% of glycerol (v/v) at -80 °C. Working cultures were maintained on tryptic soya agar (TSA) slants at 4 °C for 30 days.

3.2.2. Chemicals and media

Microbiological media were from Hi-Media Laboratories, Mumbai, India. Horse blood used for blood agar was obtained from Haffkine Institute, Pune, India. All polymerase chain reaction (PCR) reagents were obtained from Bangalore Genei Ltd. (Bangalore, India).

3.2.3. Screening for extracellular virulence factors of aeromonads

The enzymes amylase, gelatinase, lipase, protease and DNase were detected on the following media: starch agar medium, gelatin agar medium, tributyrin agar medium, milk agar and DNase agar medium, respectively according to the published methods (Mac Faddin, 1980; Saran *et al.*, 2007; Thomas *et al.*, 2003).

3.2.4. Haemolytic activity

Haemolytic activity of the isolates was determined by streaking onto TSA plates containing 5% defibrinated horse blood cells. The plates were incubated at 37 °C for 24 h and observed for a clear zone of β-haemolysis around colonies (Brender and Janda, 1987).

3.2.5. Detection of virulence and related genes by polymerase chain reaction (PCR)

PCR assays for the amplification of the aerolysin (*aer*), cytotoxic enterotoxin (*act*), haemolysin (*hly*), lipase (*lip*), and elastase (*ahyB*) were performed with the template DNA of the isolates. DNA template of the isolates was prepared as described in section 2.2.6.

PCR amplification of the virulence genes from chromosomal DNA was performed using different set of primers (Table 3.1). The PCR mixture consisted of 2.5 µl of 10X PCR amplification buffer containing 1.5 mM $MgCl_2$, 200 µM (each) of the dNTP, 2 pmole each of primer pairs, 0.75 U of *Taq* polymerase and total volume was made up to 25 µl with double distilled water. A reagent blank contained all the components of the reaction mixture except template DNA, for which sterile distilled water was substituted. The mixture was subjected to PCR cycles in an Eppendorf Mastercycler Gradient (Hamburg, Germany). Different genes were amplified with different program conditions. For aerolysin (*aer*), the program comprised initial denaturation 94 °C for 2 min, followed by 35 cycles each of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplification parameters for *act* and *hly* genes were as follows: 2 min at 94 ºC; followed by 30 cycles each of 1 min at 94 ºC; 1 min at 55 ºC; 1 min at 72 ºC; 10 min at 72 ºC. For *lip* and *ahyB* genes, the amplification conditions were incubation for 2 min at 94 °C, followed by 30 cycles at 94 °C for 1 min, 58 °C for 45 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplicons were detected by resolving 5 µl

of PCR product on 1% agarose gel containing ethidium bromide (50 ng ml^{-1}) for a period of 40 min at 50 V.

Table 3.1. Primers used for PCR detection of putative virulence genes in *Aeromonas* isolates

Target gene	Primer sequence (5'⟶ 3')	Size of PCR amplicons (bp)	Reference
aer/F	GCAGAACCCATCTATCCAG	252	Santos *et al.* (1999)
aer/R	TTTCTCCGGTAACAGGATTG		
act/F	GAGAAGGTGACCACCAAGAACA	232	Kingombe *et al.*
act/R	AACTGACATCGGCCTTGAACTC		(1999)
hly/F	GGCCGGTGGCCCGAAGAACGGG	597	Wong *et al.* (1998)
hly/R	GGCGGCGCCGGACGAGACGGG		
ahyB/F	ACACGGTCAAGGAGATCAAC	540	Sen (2005)
ahyB/R	CGCTGGTGTTGGCCAGCAGG		
lip/F	ATCTTCTCCGACTGGTTCGG	383-389	Sen (2005)
lip/R	CCGTGCCAGGACTGGGTCTT		

3.2.6. Antibiotic susceptibility test

Antibiotic susceptibility test, constituting commonly used antimicrobial agents, was performed on Muller-Hinton agar (MHA) by disc diffusion method as described by National Committee of Clinical Laboratory Standard (NCCLS) (2002). In brief, single colony from TSA plates was inoculated into 10 ml of Mueller Hinton broth (MHB), incubated overnight at 30 °C. A 1:10 dilution of the overnight culture was prepared in MHB. A sterile swab was immersed in the diluted suspension and swabbed over the entire

surface of MHA plates. The plates were held at room temperature for 10 min, and antibiotic discs were dispensed. The plates were incubated at 30 °C and zone of inhibition was measured after 24 h. Testing was done in triplicate and resistance profiles {resistant (R), intermediate (I), or susceptible(S)} were assigned after measuring average zone diameters using NCCLS breakpoints (NCCLS, 2002).

Twenty different antibiotics (Hi-Media, India) used were amikacin (Ak, 30 µg), ampicilllin (A, 10 µg), ampicillin/sulbactam (As, 10/10 µg), aztreonam (Ao, 30 µg), bacitracin (B, 10 units), carbenicillin (Cb, 100 µg), ceftazidime (Ca, 30 µg), ceftriaxone (Ci, 30 µg), cephotaxime (Ce, 30 µg), cephoxitin (Cn, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (Cf, 5 µg), erythromycin (E, 15 µg), gentamicin (G, 10 µg), imipenem (I, 10 µg), kanamycin (K, 30 µg), nalidixic acid (Na, 30 µg), piperacillin/tazobactam (Pt, 100/10 µg), tetracycline (T, 30 µg) and tobramycin (Tb, 10 µg). Strains were considered resistant to the above stated antibiotics when no zones of inhibition were observed or when the zone diameters were less than the manufacturer's recommendations.

Multiple antibiotic resistance (MAR) index: The MAR index, when applied to a single isolate, is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was exposed. It gives an indirect indication of the probable source(s) of organism, is helpful in analyzing health risk, and is used to check the antibiotic resistance (Riaz *et al.*, 2011). MAR index higher than 0.2 identifies organisms that originate from high-risk sources of contamination, where antibiotics are often used (Freeman *et al.*, 1989).

3.2.7. Plasmid DNA extraction and detection

Twenty-two *Aeromonas* isolates were selected for plasmid profile studies. The bacterial cells were grown by inoculating a single colony in 25 ml LB and incubating at 30

°C for 18 h, 150 rpm. Bacterial plasmids from the broth cultures were isolated using alkaline lysis method (Sambrook *et al.*, 1989). Briefly, the culture pellet was suspended in 100 µl of ice-cold alkaline lysis solution I (50 mM Glucose, 25mM Tris-Cl, 10 mM EDTA and 0.1 gm/l RNase H) and 200 µl freshly prepared lysis solution II (1% sodium dodecyl sulphate (w/v) and 0.2 N NaOH (w/v)). The contents were gently mixed by inverting 5-10 times. After incubation on ice bath for 10 min, 250 µl of ice cold (-20 °C) alkaline lysis solution III (3 M Potassium acetate, pH 5.5) was added and gently mixed. After incubation on ice for 10 min, the contents were centrifuged (14,000 x g, 5 min, 4 °C) and the supernatant carefully transferred to a new microfuge tube. Equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 14,000 x g, 5 min, 4 °C. Aqueous phase was separated in a new microfuge tube and equal volume of chilled (-20 °C) isopropanol was added and centrifuged at 14,000 x g, 15 min, 4 °C. The pellet was washed twice with 1 ml chilled (-20 °C) 70% ethanol by centrifugation at 14,000 x g, 15 min, 4 °C. The pellet was air dried for 30 min, dissolved in 20 µl of sterile distilled water and stored at -20 °C refrigerator till further use. The plasmid DNAs were separated by electrophoresis on 0.8% (w/v) agarose gels in 1X Tris-borate-EDTA (TBE) buffer. Supercoiled DNA ladder (Sigma-Aldrich Co., USA) was used as a molecular weight marker.

3.2.8. Whole cell protein profile

For each *Aeromonas* strain, a loopful of culture from TSA slant was suspended in 25 ml of brain heart infusion (BHI) broth and incubated at 30 °C for 18 h, 150 rpm. For WCP analysis, the inocula were prepared by transferring 100 µl of sub-cultured broth into 25 ml of fresh BHI broth, and incubating at 30 °C, 150 rpm for 18 h under uniform conditions (Fig. 3.1). One ml of bacterial cells were harvested and washed twice with

sterile saline solution (0.85% w/v) and cell density was adjusted to uniform OD_{600nm}. The protein content in the samples was determined by Lowry's method, modified by Miller (Lowry *et al.*, 1951) and equal amount of protein was resuspended in 100 µl of 2X SDS gel-loading buffer (4% sodium dodecyl sulfate, 200 mM β-mercaptoethanol, 0.2 % bromophenol blue, 20 % glycerol in 100 mM Tris–HCl at pH 6.8) and incubated at 98 °C for 10 min.

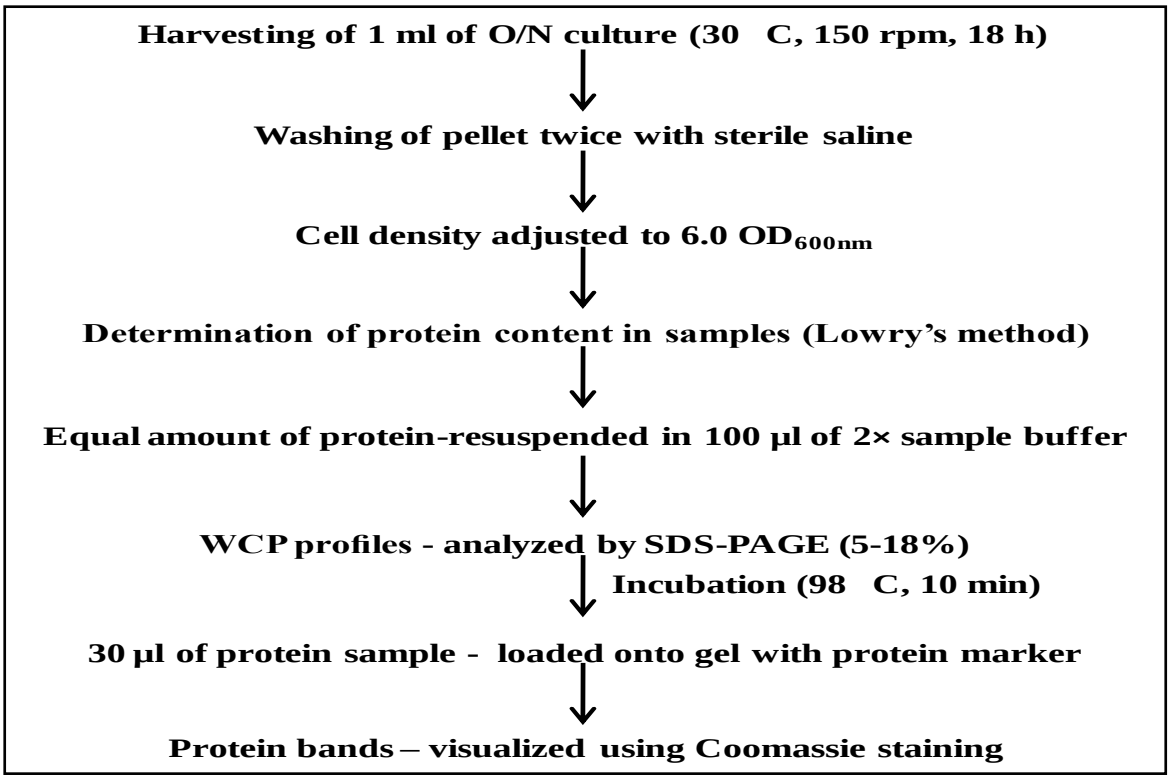


Figure 3.1. Flow chart for whole-cell protein (WCP) analysis

The WCP profiles of different *Aeromonas* spp. were analyzed by SDS-PAGE consisting of 5% stacking and 5 - 18% gradient separating gels in a discontinuous Tris-Gly buffer system (Laemmli, 1970) according to standard protocol (Harlow and Lane, 1988). Thirty microlitres of each protein sample was loaded onto the gel with a reference sample from a protein marker calibration kit (Bangalore Genei, India). The protein bands were visualized by staining the gel with Coomassie brilliant blue R-250 (HiMedia, India).

3.2.9. PFGE for DNA fingerprinting of *Aeromonas* isolates

Intact genomic DNA isolation was carried out using Pulse Net standard protocol (Ribot *et al.*, 2006). Schematic representation of the detailed procedure is given in Figure 3.2. In brief, *Aeromonas* isolates were incubated overnight at 30 °C, 150 rpm in 25 ml Luria broth (LB). Two ml of bacterial cells were harvested and washed twice with cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8) and cell density was adjusted to 1.4 OD_{600nm}. Twenty microlitres of proteinase K (20 mg/ml) was added to 400 µl of cells suspension. 400 µl of 1% PFGE grade agarose (Sigma-Aldrich C., USA) in TE buffer (10 mM Tris,1 mM EDTA, pH 8.0 containing 1% SDS) was added to 400 µl of cells suspension, mixed properly and poured into PFGE plug molds. After solidification, plugs were treated with cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0, 1% sodium lauryl sarcosine, 0.1mg/ml proteinase K) in water bath at 54 °C for 3 h. Single wash with sterile distilled water at 50 °C and subsequent six washes with TE buffer (10mM Tris, 1mM EDTA, pH 8.0) were given to the plugs. Restriction digestion was carried out with 25U of *Xba*I (New England Biolabs, Ipswich, USA) at 37 °C.

PFGE was performed with Gene Navigator System (Amersham biosciences, Sweden) in a 0.8% agarose gel (pulse field certified agarose, Sigma-Aldrich, USA) in 0.5X tris-borate EDTA (TBE) buffer at 9 °C. The electrophoresis was carried out at 150 V with pulse times of 20 s for 12 h and then of 5 - 15 s for 17 h (Talon *et al.*, 1996). Lambda ladder PFGE marker (New England Biolabs) was used as a standard molecular weight marker. The gels were stained in 0.1% ethidium bromide for 30 min and destained in distilled water for 30 min. Strains differing by one band were considered as different pulsed field profiles (PFPs).

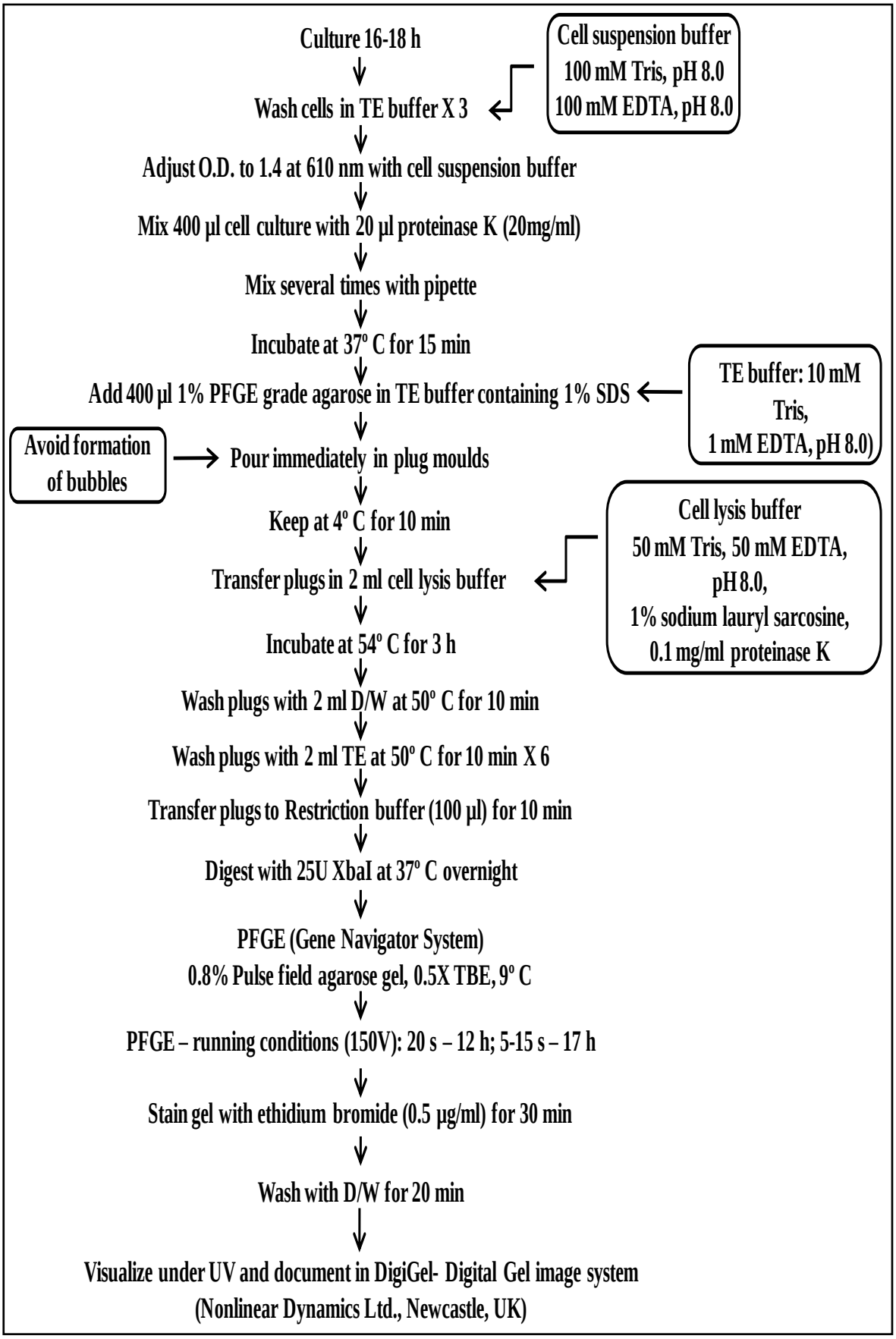


Figure 3.2. Flow chart for pulse-field gel electrophoresis (PFGE)

Studies using various restriction endonucleases have shown that *Xba*I digestion holds the maximum discriminatory power for *Aeromonas* and PFGE of *Xba*I digested *Aeromonas* have been successfully used for the epidemiological analysis (Khajanchi *et al.*, 2010). Therefore, *Xba*I was used in this study for characterization of the *Aeromonas* isolates.

3.2.10. Data analysis

The bands of WCP and PFGE profiles were analyzed visually, and the 0 and 1 matrix (binary matrix) of the protein and DNA gels were developed based on the presence or absence of the particular size band on the gel in all the samples. Using the FREETREE software (version 0.9.1.50, Folia Biologica) these matrices were analyzed. The relatedness of the isolates was analyzed using Nei and Li/Dice distance/similarity calculations. The phylogenetic trees based on the WCP and PFGE profiles were constructed using Neighbor-Joining (NJ) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithms, respectively. The output trees were visualized using the Tree View software (version 1.5.2, Roderic D. M.).

3.3. Results and Discussion

3.3.1. Extracellular virulence factors

Presence of hydrolytic enzymes was observed in all the *Aeromonas* isolates (Table 3.2). All these isolates were positive for amylase, DNase and gelatinase production. Amylase, DNase and gelatinase contribute to the virulence of *Aeromonas* species (Gosling, 1996). Emele (2001) has reported amylase activity in all the *A. hydrophila* isolates from different sources (food, clinical and environmental). Fourty percent of *Aeromonas* isolates from river water were positive for amylase activity (Sharma *et al.*,

2005). Castro-Escarpulli *et al.* (2003) have observed DNase activity in all the *Aeromonas* strain from frozen fish. DNase activity has been reported in *Aeromonas* strains from river water (Sharma *et al.*, 2005) and food samples (Yucel and Erdogan, 2010). In an earlier study, 96% of *Aeromonas* spp. isolated from frozen fish showed gelatinase activity (Castro-Escarpulli *et al.*, 2003). Sechi *et al.* (2002) observed that 50% of *A. hydrophila* and 75% of *A. sobria* isolates from patients were positive for gelatinase activity; whereas, none of the environmental strains showed gelatinase activity.

Twenty one (95.5%) isolates were positive for lipase production. The lipase activity was absent in one *A. trota* fish isolate. Lipases are one of the important virulence factors of *Aeromonas* spp. (Janda *et al.*, 1996). Chacon *et al.* (2003) have reported the presence of lipases in both clinical and environmental *Aeromonas* strains. Most of the *A. hydrophila* and *A. caviae* from foods are reported to produce lipase (Scoglio *et al.*, 2001).

Protease production was observed in twenty (90.9%) of these isolates. One *A. caviae* chicken isolate and one *A. veronii* bv. *veronii* fish isolate did not show proteolytic activity. *Aeromonas* species produce a wide variety of microbial proteases like metalloproteases, serine proteases, and aminopeptidases. These proteases play a well established role in pathogenicity by degrading complex biologic proteins present in serum and connective tissue, including albumin, fibrinogen, elastin, and collagen (Janda and Abbott, 2010). Protease activity has been reported in *Aeromonas* isolates from food (69.5%) and environmental (94.3%) samples (Yucel and Erdogan, 2010), and river water (86.7%) (Sharma *et al.*, 2005). Presence of hydrolytic enzymes in most of these *Aeromonas* isolates indicates that these isolates are potentially pathogenic, and consumption of the raw sprout, chicken and fish products sold in Mumbai may lead to food-borne diseases.

Table 3.2. Production of extracellular enzymes and β-haemolysis by *Aeromonas* isolates from various food samples in Mumbai, India

Isolate No.	Isolates	Source	Amylase	DNase	Lipase	Protease	Gelatinase	β-haemolysis
A85	*A. caviae*	Mixed sprouts	+	+	+	+	+	-
A90	*A. hydrophila*	Alfalfa	+	+	+	+	+	-
A91	*A. caviae*	Alfalfa	+	+	+	+	+	-
A329	*A. jandaei*	Chicken	+	+	+	+	+	+
A331	*A. hydrophila*	Chicken	+	+	+	+	+	+
Y47	*A. salmonicida*	Chicken	+	+	+	+	+	+
Y113	*A. veronii* bv. *sobria*	Chicken	+	+	+	+	+	+
Y324	*A. caviae*	Chicken	+	+	+	-	+	-
A254	*A. veronii* bv. *veronii*	Fish	+	+	+	+	+	+
A283	*A. trota*	Fish	+	+	-	+	+	-
A501A	*A. veronii* bv. *veronii*	Fish	+	+	+	-	+	-
A502A	*A. veronii* bv. *sobria*	Fish	+	+	+	+	+	+
A514A	*A. veronii* bv. *veronii*	Fish	+	+	+	+	+	+
A521	*A. allosaccharophila*	Fish	+	+	+	+	+	+
A 527	*A. salmonicida*	Fish	+	+	+	+	+	+
A563	*A. bivalvium*	Fish	+	+	+	+	+	+
A619	*A. veronii* bv. *veronii*	Fish	+	+	+	+	+	+
Y528	*A. salmonicida*	Fish	+	+	+	+	+	+
Y556	*A. sobria*	Fish	+	+	+	+	+	-
Y559	*A. salmonicida*	Fish	+	+	+	+	+	-
Y567	*A. salmonicida*	Fish	+	+	+	+	+	+
Y577	*A. salmonicida*	Fish	+	+	+	+	+	+

3.3.2. Haemolytic activity

Fourteen (63.6%) isolates produced clear zones of β-haemolysis on blood agar plates (Table 3.2). None of the sprout isolates showed β-haemolytic activity; whereas, 80% of poultry and 71.4% of fish isolates were positive for β-haemolysin. The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads. Beta-haemolysin secreted by pathogenic aeromonads induces active chloride secretion in the intestinal epithelium, possibly by channel insertion into the apical membrane and by activation of protein kinase C (Epple *et al.*, 2004). Yucel and Erdogan (2010) observed β-haemolysis in 57% and 60.5% of environmental and food isolates, respectively.

3.3.3. Virulence genes

Several investigators have demonstrated that the virulence of aeromonads is multifactorial and incompletely understood (Janda and Abbott, 2010). One means of determining the potential pathogenicity of *Aeromonas* isolates is to characterize isolates from various sources for the presence of putative virulence factors. Gene disruption experiments have shown that aerolysin (*aerA*), haemolysin (*hlyA*), cytotoxic enterotoxin (*act*), heat-labile toxin (*alt*) and elastase (*ahyB*) are directly involved in pathogenesis in animal models and cell lines (USEPA, 2006).

In the present study, *aer* and *hly* genes were present in seven (31.8%) and nine (40.9%) of the *Aeromonas* strains, respectively (Table 3.3). All the *A. salmonicida* isolates were positive for the *aer* gene; whereas, one (50%) *A. hydrophila* isolate was positive for *aer* gene. Aerolysin is produced by 75% or more of *A. hydrophila* strains and other species, including *A. veronii* bv. *sobria*, *A. caviae*, and *A. trota* (Janda and Abbott, 2010).

In another study, Nawaz *et al.* (2010) have reported the presence of *aerA* gene in 96% of *A. veronii* isolates from catfish.

All the *A. hydrophila* and *A. salmonicida* isolates showed the presence of *hly* gene. The *hlyA* gene is widely dispersed in *Aeromonas* species and is virtually ubiquitous in *A. hydrophila*; it is also found in *A. caviae* (35%), *A. veronii* (12%), *A. trota*, and *A. jandaei* (Wang *et al.*, 2003). Castro-Escarpulli *et al.* (2003) have also reported the presence of aerolysin/haemolysin genes in 98% of the *A. salmonicida* isolates.

Cytotoxic enterotoxin (*act*) gene was present in thirteen (59.1%) of *Aeromonas* isolates. The *act* gene was found in all the *A. salmonicida, A. veronii* bv. *sobria* and *A. sobria* isolates (Table 3.3). Our findings are similar to those reported by Kingombe *et al.* (1999), who showed the presence of *act* gene in food (66%), environmental (58%) and clinical (67%) isolates. In another study, 97% of *A. veronii* isolates from catfish were found to have *act* gene (Nawaz *et al.*, 2010).

The present study indicates that twelve (54.5%) and seven (31.8%) *Aeromonas* isolates contained elastase (*ahyB*) and lipase (*lip*) genes, respectively (Table 3.3). The *ahyB* gene was present in all *A. jandaei* and *A. bivalvium* isolates; whereas, majority of the *A. salmonicida* and *A. caviae* isolates showed the presence of *ahyB* gene. Cascon *et al.* (2000) have shown that elastase is a zinc metalloprotease and an important virulence factor in the pathogenesis of the *Aeromonas*. In earlier studies, Sen and Rodgers (2004) reported the presence of *ahyB* gene in 88% of *Aeromonas* isolates from drinking water; whereas, none of the *A. veronii* isolates from catfish had elastase gene (Nawaz *et al.*, 2010).

Table 3.3. Distribution of putative virulence genes in *Aeromonas* isolates

Virulence genes	**No. (%) of isolates with virulence genes**									
	A. hydrophila	***A. veronii* bv. *sobria***	***A. caviae***	***A. salmonicida***	***A. jandaei***	***A. trota***	***A. allosaccharophila***	***A. veronii* bv. *veronii***	***A. bivalvium***	***A. sobria***
	(n = 2)	(n = 2)	(n = 3)	(n = 6)	(n = 1)	(n = 1)	(n = 1)	(n = 4)	(n = 1)	(n = 1)
aer	1 (50)	-	-	6 (100)	-	-	-	-	-	-
ahyB	1 (50)	1 (50)	2 (66.7)	5 (83.3)	1 (100)	-	-	1 (25)	1 (100)	-
lip	-	-	2 (66.7)	4 (66.7)	-	-	-	-	1 (100)	-
hly	2 (100)	1 (50)	-	6 (100)	-	-	-	-	-	-
act	1 (50)	2 (100)	-	6 (100)	-	-	-	3 (75)	-	1 (100)

The *lip* gene was present in all *A. bivalvium* and majority of the *A. salmonicida* and *A. caviae* isolates. Aeromonads secrete four different kinds of extracellular lipases (*lip, lipH3, pla* and *plc*) which have been identified as potential virulence factors. They actively participate in the alteration of the host plasma membrane and increase the severity of infection (Pemberton *et al.*, 1997). In the previous studies, lipase gene was shown to be present in 97% of *Aeromonas* strains from frozen fish (Castro-Escarpulli *et al.*, 2003) and in 80-85% of *A. veronii* isolates from catfish (Nawaz *et al.*, 2010).

In the present study, all five studied virulence genes were present in four *A. salmonicida* (66.7%) isolates. The *aer, hly* and *act* genes were present in all the six *A. salmonicida* isolates; whereas, *ahyB* gene was present in most (83.3%) of the *A. salmonicida* isolates. High incidence of virulence genes in *A. salmonicida* isolates from aquacultured fish indicates risk to human health on consumption of these fish. On the other hand, all the five virulence genes were absent in one isolate each of *A. caviae, A. trota, A. allosaccharophila* and *A. veronii* bv. *veronii*.

The enterotoxin encoding genes (*aer, hly* and *act*) genes were present in 7.1%, 21.4% and 50% of the human pathogenic (*A. hydrophila*, *A. veronii* bv. *sobria*, *A. caviae*, *A. jandaei*, *A. trota*, *A. veronii* bv. *veronii* and *A. sobria*) isolates (Table 3.3). Majority (85.7%) of the fish pathogens (*A. salmonicida* and *A. allosaccharophila*) had *aer*, *hly* and *act* genes; whereas, none of these genes were present in non-pathogenic *A. bivalvium* isolate. However, *A. bivalvium* isolate had *ahyB* and *lip* genes (Table 3.3).

Despite the presence of plethora of putative and proven virulence factors in *Aeromonas* food and water isolates, the exact role and mechanism of aeromonads in causing diarrhoeal illness has not been elucidated. At present, there is lack of direct relationship between the presence of these virulence factors and the ability of strains to

cause gastroenteritis in humans (Khajanchi *et al.*, 2010). PCR detection of virulence genes may not be sufficient to characterize a strain as potentially virulent, and other factors determine whether or not a strain is pathogenic (Janda and Abbott, 2010). The ability of *Aeromonas* to cause disease in humans is affected by bacterial virulence factors, infectious dose and host immune responses (Galindo *et al.*, 2006). Thus, the public health significance of finding virulence genes in *Aeromonas* isolates from environmental and food samples must be interpreted carefully.

3.3.4. Antibiotic resistance patterns

Majority of studies regarding the general susceptibility of aeromonads to various classes and combinations of antibiotics are related to three major *Aeromonas* species (*A. hydrophila*, *A. caviae*, and *A. veronii* bv. *sobria*) (Janda and Abbott, 2010). Very few reports (Kampfer *et al.*, 1999; Overman and Janda, 1999) give comprehensive antibiotic susceptibility profiles of the genus *Aeromonas* to different antimicrobial agents.

The isolates belonging to the ten identified *Aeromonas* spp. have varying levels of susceptibility/resistance to the different antimicrobial agents (Table 3.4). All *Aeromonas* strains were resistant to ampicillin and bacitracin and majority of the isolates showed higher resistance to cephoxitin (77.3%), ampicillin/sulbactam (72.7%), carbenicillin (68.2%) and piperacillin/tazobactam (59.1%). *Aeromonas* strains from different sources have been reported to have a relatively high resistance to β-lactamic antibiotics. It is usually correlated with production of inducible chromosomal β-lactamases (Aravena-Roman *et al.*, 2012). Our results are in agreement with earlier reports (Guerra *et al.*, 2007) that showed high resistance to ampicillin, carbenicillin, ampicillin/sulbactam and piperacillin/tazobactam. High resistance to carbenicillin was reported in *Aeromonas* strains

from retail fish (Castro-Escarpulli *et al.*, 2003; Radu *et al.*, 2003). Earlier, (Palu *et al.*, 2006) observed 96.2% and 55.1% of *A. hydrophila* and *A. caviae* food strains to be resistant to ampicillin/sulbactam, respectively. In case of cephoxitin, 31% of *A. caviae* food isolates showed resistance, while 11.5% of *A. hydrophila* food strains showed an intermediate level of resistance (Palu *et al.*, 2006). In another study, all *A. hydrophila* and *A. sobria* strains isolated from processed channel catfish were resistant to ampicillin and bacitracin (Wang and Silva, 1999). Vivekanandhan *et al.* (2002) also found high resistance (99%) to bacitracin among *A. hydrophila* strains from fish and prawns.

In the current study, *A. salmonicida* isolates displayed greater levels of resistance as compared to other species. All the *A. salmonicida* isolates were resistant to ampicillin, bacitracin, ampicillin/sulbactam and cephoxitin and majority of these were also resistant to carbenicillin and piperacillin/tazobactam. High resistance to these antibiotics has been reported in *Aeromonas* strains from fish samples and shrimp culture hatcheries and ponds (Radu *et al.*, 2003; Vaseeharan *et al.*, 2005). Castro-Escarpulli *et al.* (2003) also reported that all *A. salmonicida* strains from frozen fish were resistant to ampicillin and carbenicillin.

All *A. hydrophila* isolates were resistant to ampicillin, bacitracin and cephoxitin and showed higher resistance (50%) to carbenicillin, piperacillin/tazobactam and ampicillin/sulbactam (Table 3.4). Wang and Silva (1999) reported all *A. hydrophila* and *A. sobria* isolates from processed channel catfish to be resistant to ampicillin and bacitracin. *A hydrophila* food isolates were found to be resistant to ampicillin/sulbactam (96.2%) and tetracycline (7.7%) (Palu *et al.*, 2006).

Table 3.4. Antibiotic resistance of *Aeromonas* spp. isolated from various food samples in Mumbai, India

Antibiotic (concentration, µg)	*A. hydrophila* (n = 2)			*A. veronii* bv. *sobria* (n = 2)			*A. caviae* (n = 3)			*A. salmonicida* (n = 6)			*A. jandaei* (n = 1)		
	R[b]	I[c]	S[d]	R	I	S	R	I	S	R	I	S	R	I	S
Aztreonam (30)		50	50		100		33.3	33.3	33.3		66.7	33.3		100	
Erythromycin (15)		100			50	50		100			83.3	16.7		100	
Amikacin (30)			100			100			100			100			100
Gentamicin (10)			100			100			100			100			100
Tobramycin (10)			100		50	50			100			100			100
Ciprofloxacin (5)			100		50	50		33.3	66.7			100			100
Ampicilllin (10)	100			100			100			100			100		
Carbenicillin (100)	50	50		100				33.3	66.7	83.3	16.7		100		
Piperacillin/tazobactam (100/10)	50		50	100				33.3	66.7	66.7	16.7	16.7	100		
Ceftazidime (30)			100			100			100			100			100
Bacitracin (10)[a]	100			100			100			100			100		
Cephotaxime (30)			100			100			100			100			100
Tetracycline (30)			100	50		50	33.3		66.7	16.7	16.7	66.7	100		
Kanamycin (30)			100		50	50	33.3		66.7		33.3	66.7			100
Ceftriaxone (30)			100			100			100			100			100
Nalidixic acid (30)			100	50		50	33.3		66.7	16.7		83.3			100
Chloramphenicol (30)			100			100			100			100			100
Ampicillin/sulbactam (10/10)	50	50		100				33.3	66.7	100			100		
Imipenem (10)			100		50	50			100	16.7	16.7	66.7		100	
Cephoxitin (30)	100				100		66.7		33.3	100				100	

[a]Antibiotic concentration in terms of units

Percentage of [b]resistant (R), [c]intermediate resistant (I) and [d]sensitive (S) isolates

Antibiotic (concentration, µg)	*A. trota* (n = 1)			*A. allosaccharophila* (n = 1)			*A. veronii* bv. *veronii* (n = 4)			*A. bivalvium* (n = 1)			*A. sobria* (n = 1)			Resistant strains (%)
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	
Aztreonam (30)		100				100		100		100			100			13.6
Erythromycin (15)			100		100			75	25		100			100		-
Amikacin (30)			100			100			100			100	100			4.5
Gentamicin (10)			100			100			100			100			100	-
Tobramycin (10)			100			100			100			100		100		-
Ciprofloxacin (5)			100			100			100			100			100	-
Ampicilllin (10)	100			100			100			100			100			100
Carbenicillin (100)			100			100	100			100			100			68.2
Piperacillin/tazobactam (100/10)			100			100	100				100		100			59.1
Ceftazidime (30)			100			100			100			100			100	-
Bacitracin (10) [a]	100			100			100			100			100			100
Cephotaxime (30)			100			100			100			100			100	-
Tetracycline (30)			100			100			100			100			100	18.2
Kanamycin (30)		100				100			100			100		100		4.5
Ceftriaxone (30)			100			100			100			100			100	-
Nalidixic acid (30)			100	100					100			100			100	18.2
Chloramphenicol (30)			100			100			100			100			100	-
Ampicillin/sulbactam (10/10)			100	100			100					100	100			72.7
Imipenem (10)			100			100	25	25	50			100			100	9.1
Cephoxitin (30)	100			100			75	25		100				100		77.3

[a]Antibiotic concentration in terms of units

All the *Aeromonas* isolates were sensitive to gentamicin, third-generation cephalosporins (ceftazidime, cephotaxime, ceftriaxone) and chloramphenicol. In earlier studies, all *Aeromonas* strains from food and fish were found to be sensitive to cephotaxime, ceftazidime, gentamicin and chloramphenicol (Akinbowale *et al.*, 2007; Awan *et al.*, 2009; Palu *et al.*, 2006).

In the present study, different *Aeromonas* isolates exhibited varying levels of susceptibility to aztreonam, amikacin, tetracycline, kanamycin, nalidixic acid and imipenem (Table 3.4). Intermediate resistance to different antibiotics has also been reported among *A. hydrophila*, *A. sobria*, *A. salmonicida*, *A. veronii* and *A. caviae* isolates from food, clinical and aquaculture sources (Jacobs and Chenia, 2007; Palu *et al.*, 2006). The variation in sensitivity and resistance patterns of *Aeromonas* may be due to different isolation sources, environmental conditions and variable use of drug from place to place.

Overall, the MAR indices of *Aeromonas* strains ranged from 0.15 to 0.35 (Table 3.5). A total of 92.9% of the fish isolates and 80% of the chicken isolates exhibited resistance to minimum of at least five antibiotics. A total of 81.8% of the strains had MAR index of 0.25 to 0.35; with more fish (92.9%) and chicken (80%) isolates than sprout (33.3%) in this range. Higher MAR indices of fish and chicken isolates indicate that use of low concentration of antibiotics in feed might be responsible for development of antibiotic resistance. Jacobs and Chenia (2007) have reported MAR index of 0.12 to 0.59 in *Aeromonas* spp. isolated from South African aquaculture systems.

Table 3.5. Multiple antibiotic resistance (MAR) index of *Aeromonas* isolates from various food samples

MAR index	Source		
	Sprouts (n=3)	**Chicken (n=5)**	**Fish (n= 14)**
0.05	0	0	0
0.1	0	0	0
0.15	2 (66.7%)	1 (20%)	1 (7.1%)
0.2	0	0	0
0.25	0	1 (20%)	5 (35.7%)
0.3	1 (33.3%)	1 (20%)	4 (28.6%)
0.35	0	2 (40%)	4 (28.6%)

3.3.5. Plasmid DNA profiles

Single and/or multiple plasmids, ranging in size from approximately 5 to > 16 kb, were detected in 77.2% (17/22) of isolates (Fig. 3.3). Earlier researchers have also reported the prevalence of small-sized plasmids in 15% to 94% of *Aeromonas* isolates (Brown *et al.*, 1997; Chaudhury *et al.*, 1996). Radu *et al.* (2003) also found plasmids, ranging in size from 2.3 to 15.7 kb, in 56.7% of the *Aeromonas* isolates from retail fish. In the present study, plasmids were not restricted to a specific *Aeromonas* species, but were present in isolates belonging to nine of the ten identified species (Table 3.6). However, plasmid was absent in one *A. bivalvium* isolate. Plasmids were detected in all the *A. salmonicida*, *A. hydrophila*, *A. jandaei*, *A. allosaccharophila*, *A. veronii* bv. *sobria*, *A. trota* and *A. sobria* isolates, and 50% and 33.3% of *A. veronii* bv. *veronii* and *A. caviae*, respectively. Radu *et al.* (2003) reported plasmids in 50%, 56.3% and 60% of *A. caviae*, *A. veronii* and *A.*

hydrophila strains, respectively. In the present study, all the chicken isolates had plasmids; whereas, the plasmids were present in only 78.6% and 33.3% of fish and sprout isolates, respectively. More than one plasmid was present in 8 isolates. No clear correlation between the presence of plasmid and antibiotic resistance was observed. Radu *et al.* (2003) have also suggested that the antibiotic resistance in *Aeromonas* may be of chromosomal origin.

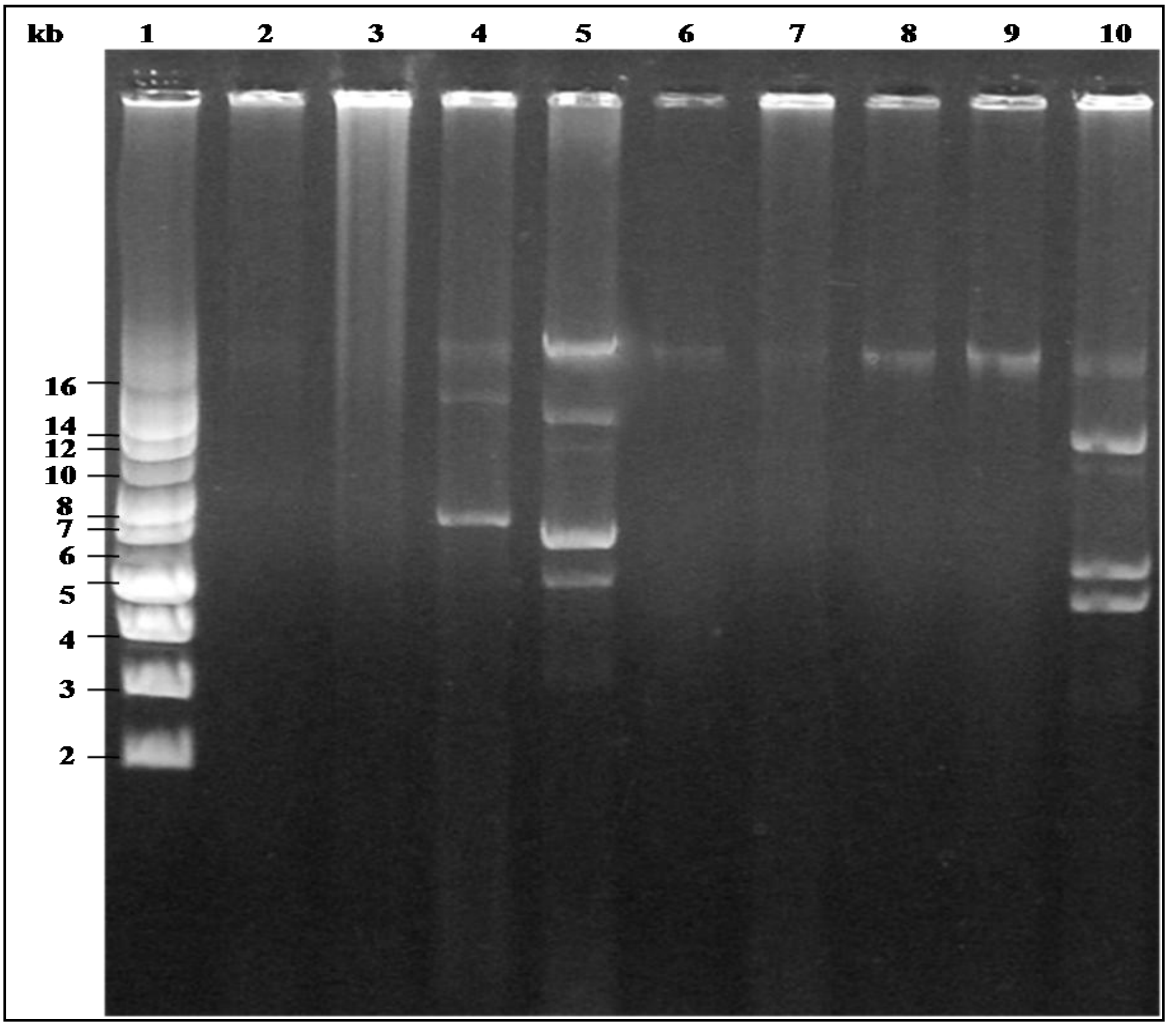


Figure 3.3. Plasmid profiles of *Aeromonas* isolates from various food samples. Lane 1: Supercoiled DNA Ladder, Lane 2: *A. caviae* A85, Lane 3: *A. caviae* A91, Lane 4: *A. trota* A283, Lane 5: *A. hydrophila* A331, Lane 6: *A. veronii* bv. *veronii* A514A, Lane 7: *A. caviae* Y324, Lane 8: *A. salmonicida* Y528, Lane 9: *A. sobria* Y556, Lane 10: *A. salmonicida* Y47

Table 3.6. Plasmid profile of *Aeromonas* isolates from sprout, chicken and fish samples

Isolate No.	Species Identification	Plasmid no. (size in kb)
A85	*A. caviae*	0
A90	*A. hydrophila*	1 (14)
A91	*A. caviae*	0
A329	*A. jandaei*	5 (5, 5.5, 6.5, 8, 16)
A331	*A. hydrophila*	4 (5.5, 7, 14, >16)
Y47	*A. salmonicida*	4 (5.5, 6.5, 14, >16)
Y113	*A. veronii* bv. *sobria*	2 (10, 16)
Y324	*A. caviae*	1 (>16)
A254	*A. veronii* bv. *veronii*	1 (14)
A283	*A. trota*	3 (8, 14/15, >16)
A501A	*A. veronii* bv. *veronii*	0
A502A	*A. veronii* bv. *sobria*	1 (14)
A514A	*A. veronii* bv. *veronii*	1 (> 16)
A521	*A. allosaccharophila*	3 (5.5, 6.5, 14)
A527	*A. salmonicida*	1 (14)
A563	*A. bivalvium*	0
A619	*A. veronii* bv. *veronii*	0
Y528	*A. salmonicida*	1 (> 16)
Y556	*A. sobria*	1 (> 16)
Y559	*A. salmonicida*	3 (9, 14, 16)
Y567	*A. salmonicida*	1 (14)
Y577	*A. salmonicida*	2 (5.5, 14)

3.3.6. WCP profile analysis

The WCP profiles of 22 *Aeromonas* isolates yielded 22 to 28 polypeptide bands ranging from ~10 kDa to > 97 kDa and were reproducible (Fig 3.4). All the strains were typeable and showed unique banding patterns indicating a high level of diversity among

Aeromonas strains. Protein bands of 97, 68, 64, 45, 40, 29, 28, 26, 25, 18, 16, 14, 12 and 10 kDa were detected in all the *Aeromonas* isolates. Protein bands of 64, 45, and 25 kDa appeared as major bands in all the strains. Major variation in the banding pattern was observed in two main regions, 29 to 45 kDa and 66 to 97 kDa.

Figure 3.5 shows the dendrogram, of strains belonging to different *Aeromonas* species, produced after numerical analysis of the WCP profiles using the Nei and Li/Dice distance/similarity calculations and Neighbor-joining (NJ) tree-construction algorithm. The overall protein profiles were very similar among the strains of the same species except for slight variations in the number of bands generated. For majority of the isolates, no clear correlation was observed between the origin of the strains and their protein profiles. However, in the case of *A. hydrophila*, *A. caviae* and *A. veronii* bv. *sobria* species, clustering of strains based on their origin was observed (Fig 3.5 (a), (b) and (c)). *A. caviae* strains, A85 and A91, both isolated from sprout samples clustered together, while Y324 (chicken isolate) diverged into a separate clade. *A. hydrophila* isolates, A331 (chicken) and A90 (sprout), and *A. veronii* bv. *sobria* strains, A502A (fish) and Y113 (chicken) segregated into separate clades based on their WCP profiles. It was also observed that *Aeromonas* strains from different sources also shared similar WCP profiles. Strains Y559, Y567 and Y528 belonging to *A. salmonicida* and isolated from *Ompok bimaculatus* and *Aristichthys nobilis* fish samples, respectively, showed similar protein profiles. Similarly, *A. caviae* strains A85 (mixed sprouts) and A91 (alfalfa sprouts) and *A. veronii* bv. *veronii* strains A254 (*Aristichthys nobilis*) and A501A (*Ompok bimaculatus*) also showed similar WCP profiles. The strains contaminating these samples may be from common source or origin.

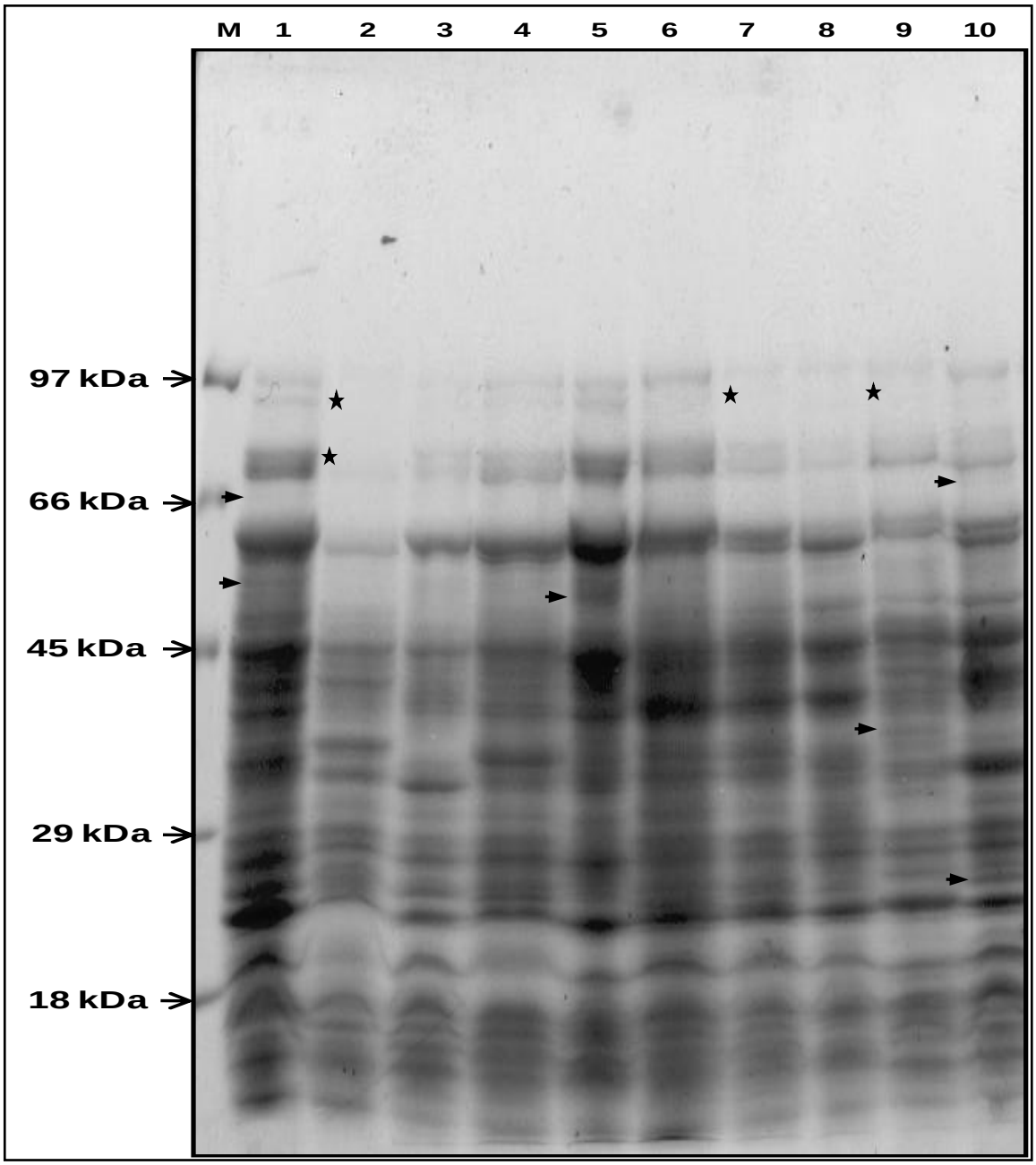


Figure 3.4. WCP profiling of *Aeromonas* spp. on 5-18% gradient SDS-PAGE. M: PMW-M Protein Marker (GeNei™, Bangalore, India); Lane 1: A283 (*A. trota*), Lane 2: A329 (*A. jandaei*), Lane 3: A331 (*A. hydrophila*), Lane 4: A521 (*A. allosaccharophila*), Lane 5: A563 (*A. bivalvium*), Lane 6: A619 (*A. veronii* bv. *veronii*), Lane 7: Y113 (*A. veronii* bv. *sobria*), Lane 8: Y324 (*A. caviae*), Lane 9: Y556 (*A. sobria*), Lane 10: Y567 (*A. salmonicida*). "*" indicates the absence of band in the lane; while " ➔ " indicates the presence of extra band in the lane

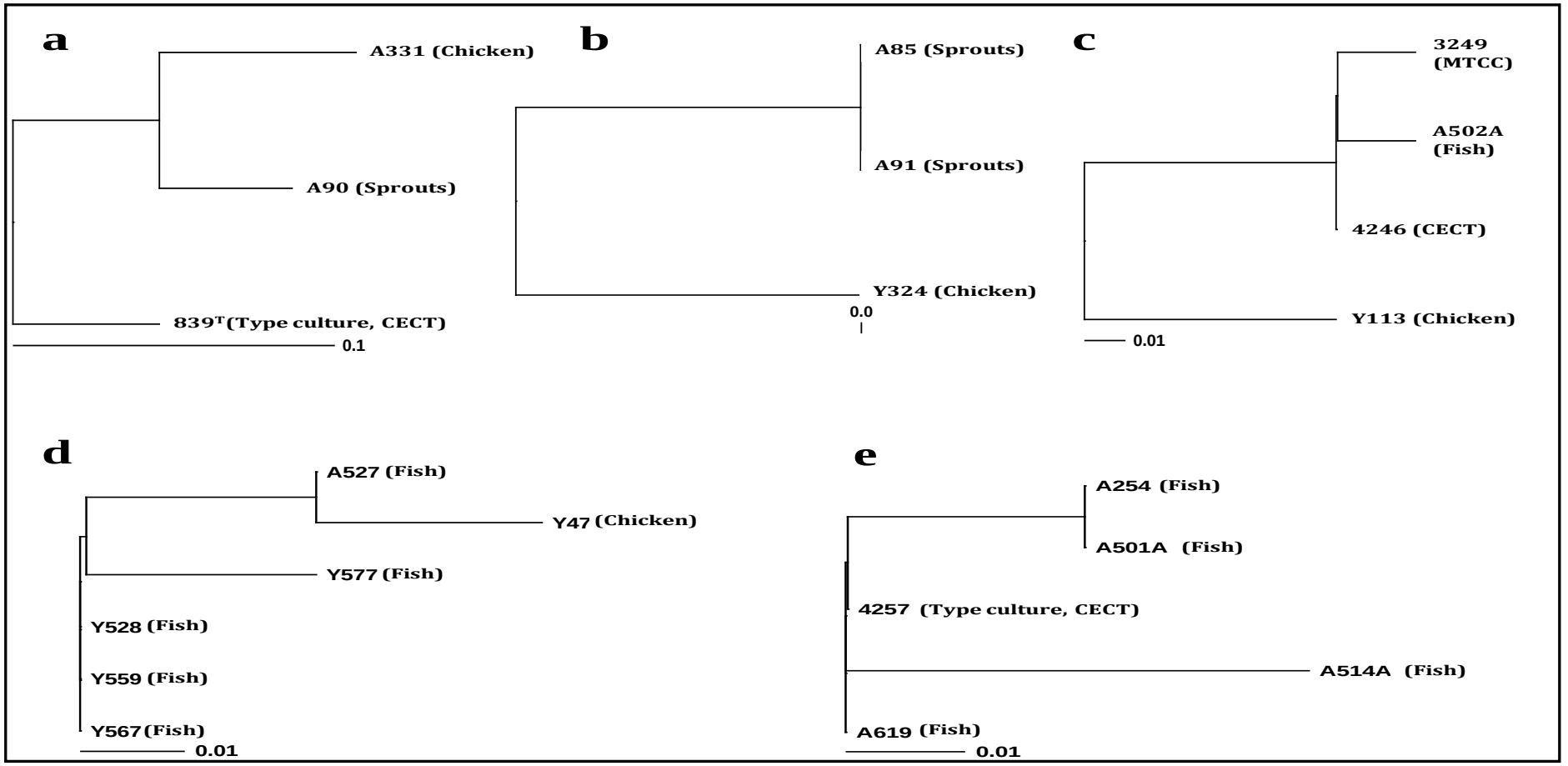


Figure 3.5. Dendrogram of protein similarity of (a) *A. hydrophila*, (b) *A. caviae*, (c) *A. veronii* bv. *sobria*, (d) *A. salmonicida* and (e) *A. veronii* bv. *veronii* strains determined by the gradient SDS-PAGE protein pattern analysis using Nei and Li/Dice similarity matrix and Neighbor-joining (NJ) tree-construction method. Source of the isolate is indicated in the parenthesis. CECT and MTCC indicate the Spanish Type Culture Collection, Valencia, Spain and Microbial Type Culture Collection, Chandigarh, India, respectively.

Several researchers have used WCP analysis to study the diversity of *Aeromonas* strains at and below species level (Delamare *et al.*, 2002b). Maiti *et al.* (2009) and Szczuka and Kaznowski (2007) have successfully used WCP profiling of *Aeromonas* isolates and observed high level of diversity among *Aeromonas* strains. The genus *Aeromonas* is phenotypically heterogeneous with its members exhibiting an extremely wide range of nutritional requirements (carbohydrate metabolism), growth conditions, metabolic diversity and DNA base composition (Janda and Abbott, 2010).

3.3.7. PFGE profile

Digestion of genomic DNA with rare cutting endonucleases and separation of the fragments by PFGE has been widely used in recent years for typing of various bacterial species (Foley *et al.*, 2009). PFGE is currently the method for the subtyping of sporadic or epidemic *Aeromonas* isolates. Twenty two isolates of *Aeromonas* spp., including 6 isolates of *A. salmonicida* from chicken and fish samples, 4 isolates of *A. veronii* bv. *veronii* from fish samples, 3 isolates of *A. caviae* from sprouts and chicken samples, 2 isolates of *A. hydrophila* from sprouts and chicken samples, 2 isolates of *A. veronii* bv. *sobria* from chicken and fish samples, and 1 isolate each of *A. allosaccharophila*, *A. jandaei*, *A. bivalvium*, *A. sobria* and *A. trota* were typed by using macro-restriction analysis using *Xba*I and PFGE (Fig. 3.6).

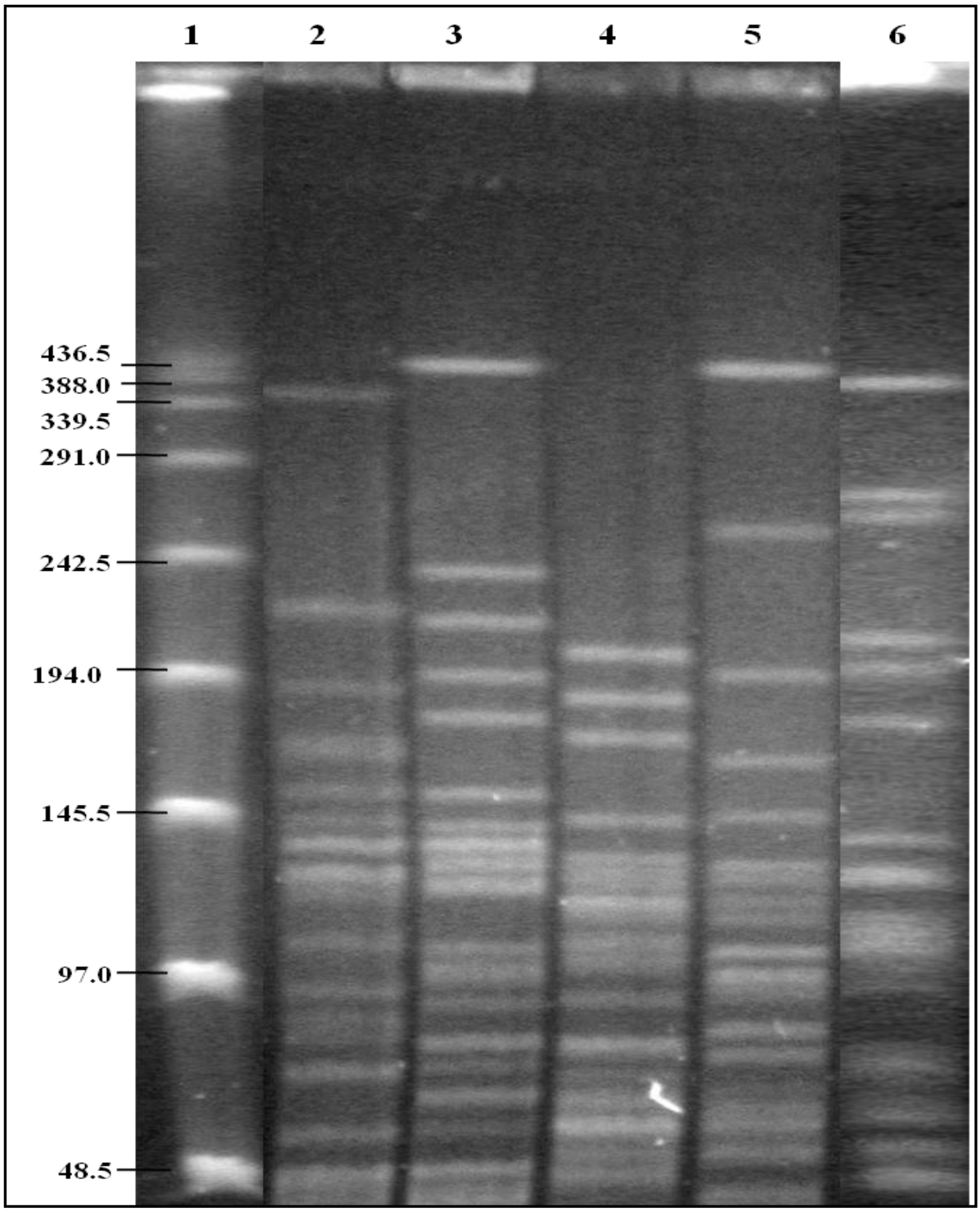


Figure 3.6. Pulse-field gel electrophoresis of *Xba*I-digested DNA from *Aeromonas* isolates. Lane1: Lambda PFGE marker (New England Biolabs), Lane 2: *A. hydrophila* A90, Lane 3: *A. hydrophila* A331, Lane 4: *A. veronii* bv. *veronii* A501A, Lane 5: *A. veronii* bv. *veronii* A619, Lane 6: *A. hydrophila* CECT 839^{T}.

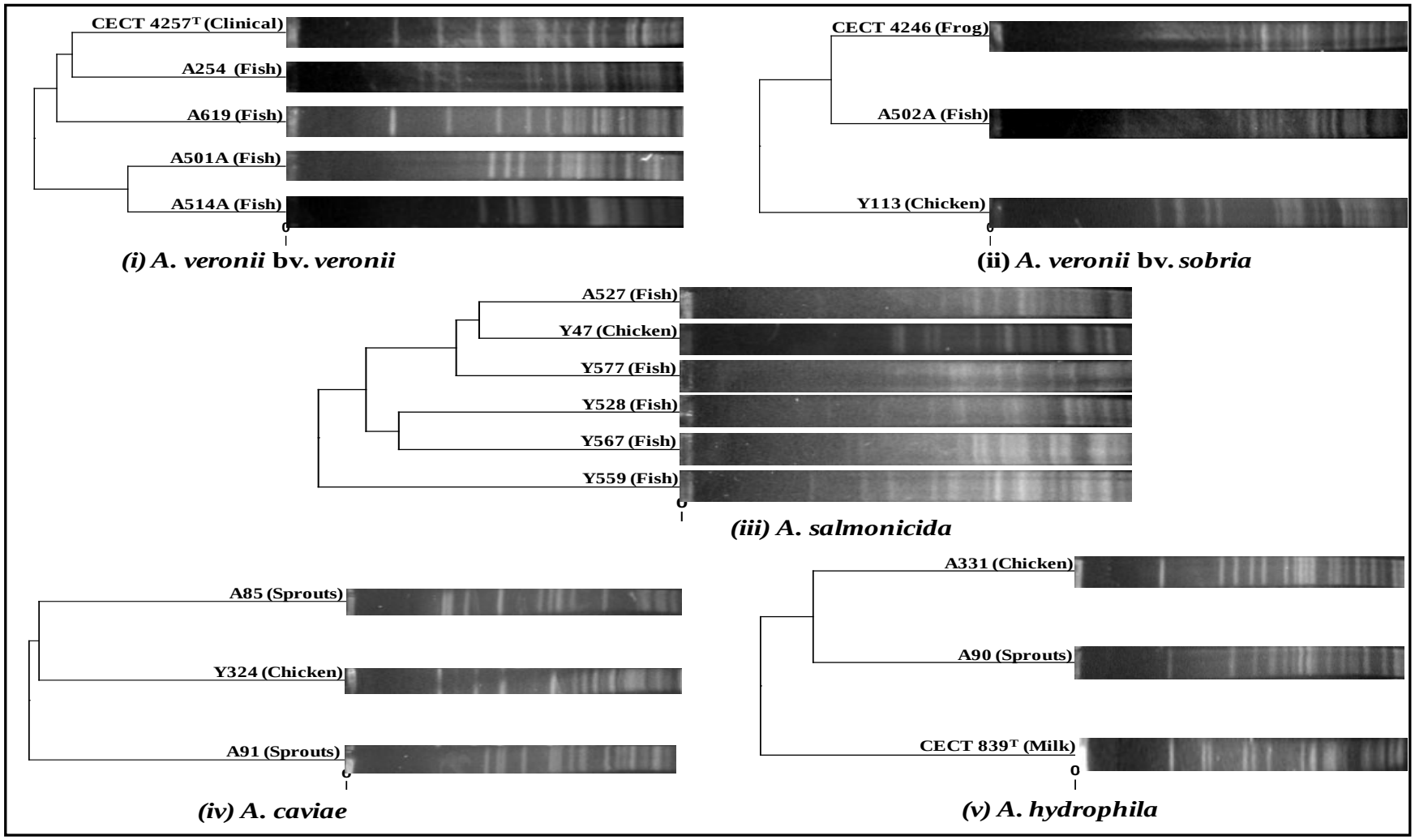


Figure 3.7. Dendrogram and PFGE profiles of *Aeromonas* isolates (i) *A. veronii* bv. *veronii*, (ii) *A. veronii* bv. *sobria*, (iii) *A. salmonicida*, (iv) *A. caviae*, and (v) *A. hydrophila* from various retail food products in Mumbai, India.

PFGE of the six *A. salmonicida* isolates yielded 13 - 17 well-resolved genomic DNA fragments (approximately 48.5 - 388 kb) {Fig. 3.7(iii)}. Restriction analysis of four *A. veronii* bv. *veronii*, three *A. caviae*, two *A. hydrophila* and two *A. veronii* bv. *sobria* isolates resulted in 13 - 18 fragments (48.5 - 436.5 kb), 14 - 16 fragments (48.5 - 388 kb), 16 - 19 fragments (48.5 - 436.5 kb) and 16 - 18 fragments (48.5 - < 388 kb), respectively {Fig. 3.7(i)-(v)}. Our results confirm that PFGE is a highly efficient technique for characterization of *Aeromonas* strains.

All the isolates showed different PFGE banding pattern indicating high genetic diversity. This finding is in accordance with previous studies of *Aeromonas* strains isolated from different geographical locations (Bonadonna *et al.*, 2002; Pablos *et al.*, 2010). In earlier studies, Villari *et al.* (2000) found 24 PFGE patterns among 27 *A. hydrophila* strains and 20 PFGE patterns among 23 *A. caviae* isolates from ready-to-eat foods. Fifty-eight distinct PFGE patterns were observed in 65 *Aeromonas* isolates from stool and groundwater samples by Borchardt *et al.* (2003). In the present study, PFGE profile does not show any correlation with the source of isolation and virulence factors of *Aeromonas* isolates. However, studying the relationship between hybridization groups (HG), occurrence and virulence factors may give novel information regarding *Aeromonas* epidemiology.

In the present study, the typeability and reproducibility of PFGE were best possible as reproducible PFGE patterns were obtained for all strains obtained from different foods. Talon *et al.* (1996) have also reported that variation in PFGE patterns of *Aeromonas* strains is not due to unstable genome. They showed that PFGE patterns do not change during frozen storage and long-term laboratory culture. Moreover, this is the first report of PFGE profiles of *Aeromonas* spp. from India. Therefore, in future, this data can be

considered as a reference for any work regarding epidemiology or genetic diversity of *Aeromonas* spp. in India.

3.4. Conclusions

The study revealed that the majority of the *Aeromonas* isolates from food were positive for putative virulence factors and thus might be potentially pathogenic. Marked resistance to commonly used β-lactam antibiotics and high genetic diversity using PFGE and WCP was observed in the isolates. Though WCP profile has less discriminatory power, it can be used to characterize *Aeromonas* isolates below species level. The occurrence of these bacteria in food should be regarded as an important threat to public health. Although *Aeromonas* is eliminated by adequate cooking, it can contaminate the food due to undercooking or cross-contamination.

CHAPTER 4

Radiation Sensitivity of *Aeromonas* in Different Food Commodities

4.1. Introduction

Aeromonas inhabit a wide range of ecosystems and can infect humans via consumption of contaminated food or water (Khajanchi *et al.*, 2010). In recent years, there is an increase in appreciation and demand among health-conscious consumers for the ready-to-cook/eat minimally processed fresh produce of plant origin, and seafood and meat products in both developed and developing countries (Rajkowski, 2008). Being psychrophilic in nature, *Aeromonas* are capable of surviving and multiplying at low temperatures (2 to 10 °C) applied to the minimally processed food products (Mano *et al.*, 2000). Since the ingestion of contaminated water or food leads to gastrointestinal infections, the high prevalence of *Aeromonas* species in the food chain should be considered a threat to public health (Garibay *et al.*, 2006).

A high percentage of sprouts, chicken and fish samples marketed in Mumbai and its suburbs were found to be contaminated with *Aeromonas* (Chapter 2). Therefore, these food products marketed in Mumbai and its suburbs are not safe for raw consumption. There is a need for processing of these food products to ensure their microbial safety and prevent food-borne illness by *Aeromonas* spp.

Several physical and chemical treatment methods have been found to be ineffective in complete elimination of the food-borne pathogens under experimental conditions (Bari *et al.*, 2005). Radiation processing, a cold process, has high penetration power and has been shown to ensure 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).

The objectives of the present study were:

(i) To determine the D_{10}-values of gamma radiation for *Aeromonas* isolates in saline.

(ii) To determine the radiation dose for a 5-log elimination of *Aeromonas* from sprouts, chicken and fish samples.

(iii) To study the survival and recovery, if any, of *Aeromonas* in inoculated and radiation-treated sprout, chicken and fish samples, during storage at 4 °C for 12 days (sprouts) and 7 days (chicken and fish).

4.2. Materials and methods

4.2.1. Bacterial strains

A. hydrophila CECT 839^T and *A. veronii* bv. *veronii* CECT 4257^T were kindly supplied by Dr. Valérie Leclère, Université des Sciences et Technologies de Lille USTL, Villeneuve d'Ascq cedex, France. *A. hydrophila* A90 and *A. caviae* A85 were isolated from alfalfa and mixed sprouts, respectively. *A. hydrophila* A331 and *A. salmonicida* Y47 were obtained from chicken samples; whereas, *A. veronii* bv. *veronii* (A514A and A619), and *A. salmonicida* (A527, Y528, Y559 and Y567) strains were isolated from fish samples. All *Aeromonas* cultures were maintained at 4 °C on tryptic soy agar (TSA) slants.

4.2.2. Chemicals and media

Microbiological media were from Hi-Media Laboratories, Mumbai, India.

4.2.3. Decimal reduction dose (D_{10}) in saline

Aeromonas strains were inoculated in 25 ml of tryptic soya broth (TSB) and grown at 30 °C, 150 rpm for 18 - 20 h (≈ 10^9 CFU/ml). *Aeromonas* cells were harvested and centrifuged to obtain pellet which was washed twice with sterile saline to remove media components. The cells were resuspended in 1.5 ml of sterile saline and further diluted to obtain a cell density of 10^7 CFU/ml. Twelve-hundred microlitres of this suspension was

transferred into five 1.5 ml microfuge tubes. The tubes were placed in ice and irradiated to doses of 0, 0.05, 0.1, 0.15 and 0.2 kGy at 0 - 4 °C in a cobalt-60 irradiator (Gamma cell 220, Atomic Energy of Canada Ltd., Ontario, Canada) at a dose rate of 5.4 Gy/min. After irradiation, total viable count (TVC) was determined by pour plating appropriately diluted aliquots with TSA. The plates were incubated at 30 °C for 24 h and the colonies were counted. Each experiment included three samples per dose and was repeated thrice. The average number of surviving viable cells (CFU/ ml) in the saline was plotted against the radiation dose. The slopes of the individual survivor curves were calculated by linear regression using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA). The D_{10}-value was calculated by taking the negative reciprocal of the survival curve slope.

4.2.4. Preparation of inocula

For the inoculated pack studies, *A. caviae* A85, *A. veronii* bv. *veronii* A514A, *A. salmonicida* Y47, *A. hydrophila* CECT 839^T and *A. salmonicida* Y567 were cultured separately in 25 ml TSB at 30 °C on a rotary shaker at 150 rpm for 18 - 20 h. Two ml culture of each strain was pelleted by centrifugation (8,000 x g, 2 min) and resuspended in 2 ml saline. Equal volumes (2 ml) of cell suspensions of five strains were pooled to produce approximately equal populations of each strain. The 'cocktail' inoculum containing approximately 10^9 CFU/ml was used to inoculate sprout, chicken and fish samples.

4.2.5. Sample preparation

Fifty grams each of mung (green gram {*Phaseolus aureus*}), matki (dew gram {*Phaseolus aconitifolius*}), chana (chick pea {*Cicer arietinum*}) and vatana (garden pea {*Pisum sativum*}) seeds were mixed and soaked in tap water for 10 h, and then sprouting

was carried out for 24 h at room temperature (28 °C). The mixed sprouts were used for the radiation sensitivity and inoculated pack studies.

Boneless chicken and Rohu (*Labeo rohita*) fish fillets, used for the inoculated pack studies, were procured from the super-market in Mumbai, India.

4.2.6. Irradiation of samples

Twenty-five grams each of mixed sprout, fish fillets and chicken meat were packed separately in low density polyethylene bags (LDPE) of 35 µm thickness (ACE packaging Ltd., Mumbai, India) using heat sealer (Sevana, Mumbai, India) and kept in ice. Radiation processing for the decontamination of packed samples was carried at 0 - 4 °C in a cobalt-60 irradiator (Gamma cell 5000, Board of Radioisotope and Technology, Mumbai, India) at a dose rate of 74 Gy/min. Mixed sprouts were exposed to 8 kGy and chicken or fish samples to 15 kGy dose for the elimination of native microbial flora.

Dose rate of all the radiation sources was measured using the Fricke method (Sehested, 1970). Variations in doses absorbed by experimental samples were minimized by placement within a uniform area of the radiation field.

4.2.7. Determination of D_{10} values in mixed sprouts, chicken and fish samples

D_{10} value of *Aeromonas* cocktail was determined in mixed sprouts, chicken and fish samples. Conditions necessary to get a desired number of cells (10^5 CFU/g) of *Aeromonas* attached to mixed sprouts were standardized. Mixed sprout samples (350 g) were dipped in sterile tap water (3 L) containing 10^6 CFU/ml of *Aeromonas* cocktail for 3 min and dried on sterile blotting paper to remove excess water under aseptic conditions. Samples were then packed in LDPE bags (25 g each) and sealed using heat sealer (Sevana,

Mumbai, India). Sprout samples inoculated with *Aeromonas* were exposed to a radiation dose of 0.05, 0.1, 0.15 and 0.2 kGy.

Radiation decontaminated chicken and fish samples (25 g), in triplicates, were inoculated with the *Aeromonas* cocktail so as to obtain a count of 1 x 10^5 cells/g of the meat. The inoculated packs were irradiated at 0 - 4 °C with doses of 0.05, 0.1, 0.15 and 0.2 kGy.

After irradiation, the mixed sprouts, chicken and fish samples were aseptically homogenized for 1 min in a sterile stomacher bag (Stomacher lab blender, model 400, Seward, London, UK) containing 225 ml of sterile saline. Serial dilutions of the homogenate were prepared and appropriate dilutions were used to determine viable counts with TSA. The plates were incubated at 30 °C for 24 h and CFU values were determined. Each study included three samples per dose and was repeated thrice. The average number of surviving viable cells (CFU/g) in the samples was plotted against the radiation dose. The slopes of the individual survivor curves were calculated by linear regression using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA). The D_{10} value was calculated by taking the negative reciprocal of the survival curve slope.

4.2.8. Determination of the dose required to eliminate 5-log CFU/g of the inoculated cells

The decontaminated mixed sprouts, chicken and fish samples (25 g) were inoculated with *Aeromonas* cocktail as described in section 4.2.7. The inoculated samples (10^5 CFU/g of *Aeromonas*) in triplicate were irradiated at 0.5, 1, 1.5 and 2 kGy in a cobalt-60 irradiator (Gamma cell 5000, Board of Radioisotope and Technology, Mumbai, India), and the surviving population was determined by plating of serial dilutions with TSA after an incubation of 24 h at 30 °C. Enrichment (TSB) and selective plating (starch ampicillin

agar, SAA) were carried out to confirm the complete elimination of these pathogens. Each experiment was repeated three times.

4.2.9. Storage studies on gamma-irradiated mixed sprout, chicken and fish samples inoculated with *Aeromonas*

Decontaminated mixed sprout, chicken and fish samples were inoculated with 10^5 CFU/g of *Aeromonas* cocktail as described in section 4.2.7. The inoculated packs were irradiated in melting ice conditions with doses of 0.5, 1, 1.5 and 2 kGy in a cobalt-60 irradiator (Gamma cell 5000, Board of Radioisotope and Technology, Mumbai, India) and stored at 4 °C. The sprout samples were screened for the presence of *Aeromonas* on the 0^{th}, 4^{th}, 8^{th} and 12^{th} day, while chicken and fish samples on the 0^{th}, 3^{rd}, 5^{th} and 7^{th} day. Enrichment and selective plating were carried out to confirm the complete elimination of the pathogens. Each experiment was repeated three times.

4.2.10. Statistical analysis

All the data for D_{10}-values of *Aeromonas* strains in saline and food samples were analyzed statistically using Origin 6.1 software version 6.1052 B232 (OriginLab Corporation, Northampton, MA, USA). Significant differences in D_{10} values between different isolates were analyzed by one-way ANOVA.

4.3. Results and Discussion

4.3.1. D_{10}-values of *Aeromonas* isolates in saline

All *Aeromonas* isolates were found to be very sensitive to gamma radiation. The D_{10} values of different *Aeromonas* isolates in saline ranged from 0.031 to 0.046 kGy (Fig. 4.1). *A. veronii* bv. *veronii* CECT 4257^T was found to be the most sensitive with D_{10} value

of 0.031 kGy. *A. salmonicida* Y567 was the most resistant with D_{10} value of 0.046 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 different *A. hydrophila* isolates (CECT 839^T, A90 and A331) from different sources were compared (Fig. 4.1). There was significant difference ($P < 0.05$) in the radiation sensitivity of *A. hydrophila* isolates (0.032 - 0.040 kGy). Among different *A. hydrophila* isolates, the maximum D_{10} value was found to be for CECT 839^T (0.040 kGy) and lowest D_{10} value for A331 (0.032 kGy).

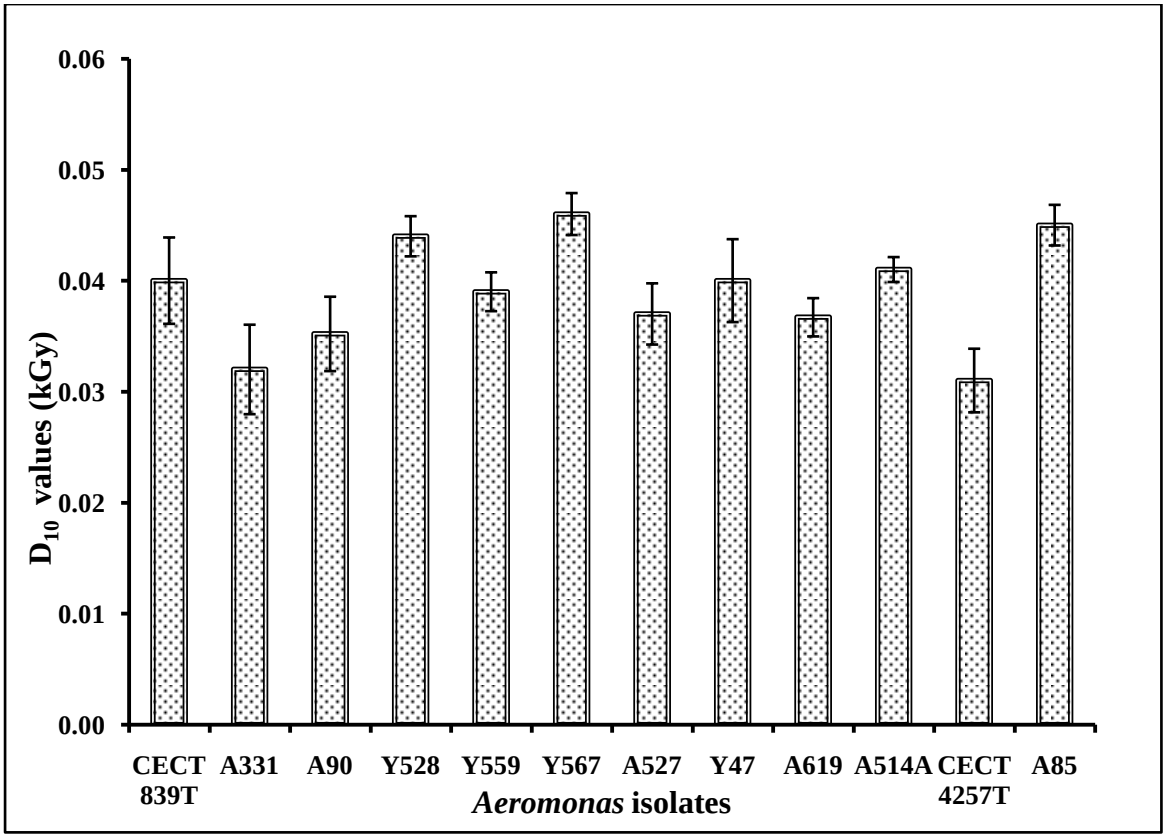


Figure 4.1. D_{10} values of gamma radiation for *Aeromonas* isolates irradiated in saline. *A. hydrophila* (CECT 839^T, A331 and A90), *A. salmonicida* (Y528, Y559, Y567, A527 and Y47), *A. veronii* bv. *veronii* (A619, A514A and CECT 4257^T) and *A. caviae* A85.

The D_{10} values of different *A. salmonicida* and *A. veronii* bv. *veronii* isolates in saline ranged from 0.037 to 0.046 kGy and 0.031 to 0.041 kGy, respectively (Fig. 4.1).

Significant difference ($P < 0.05$) in the radiation sensitivity of different *A. salmonicida* and *A. veronii* bv. *veronii* strains in saline was also observed. Y567 was the most resistant among *A. salmonicida* isolates with D_{10} value of 0.046 kGy. The maximum D_{10} value was observed for A514A (0.041 kGy) among *A. veronii* bv. *veronii* isolates.

Rashid *et al.* (1992) reported 0.05 kGy as the D_{10} value for *A. hydrophila* in 0.067 M phosphate buffer containing 1% (w/v) NaCl. The D_{10} values of all *Aeromonas* strains from local foods ranged from 0.109–0.253 kGy in nutrient broth and increased when some strains were irradiated in ground meat at room temperature (Rashad and AbdelKareem, 1995). The lower D_{10} values observed in the present study may be due to the different *Aeromonas* strains studied. Moreover, this is the first report of radiation sensitivity of *Aeromonas* isolates from various Indian food samples.

A 'cocktail' inoculum of five different *Aeromonas* cultures (*A. salmonicida* Y567, *A. caviae* A85, *A. veronii* bv. *veronii* A514A, *A. hydrophila* CECT 839^T and *A. salmonicida* Y47) was prepared to determine the radiation sensitivity of *Aeromonas* in mixed sprouts, chicken and fish samples.

4.3.2. D_{10}-values of *Aeromonas* 'cocktail' in sprouts, chicken and fish samples

To decontaminate the mixed sprouts, chicken and fish samples, they were irradiated at different doses and aerobic plate counts were determined. The treatment with 8 kGy dose was found sufficient for the destruction of the native flora from mixed sprouts; whereas, chicken and fish samples required a dose of 15 kGy. Similar high doses were reported for decontamination of different types of sprouts (Saroj *et al.*, 2006) and fishery products (Arvanitoyannis *et al.*, 2009).

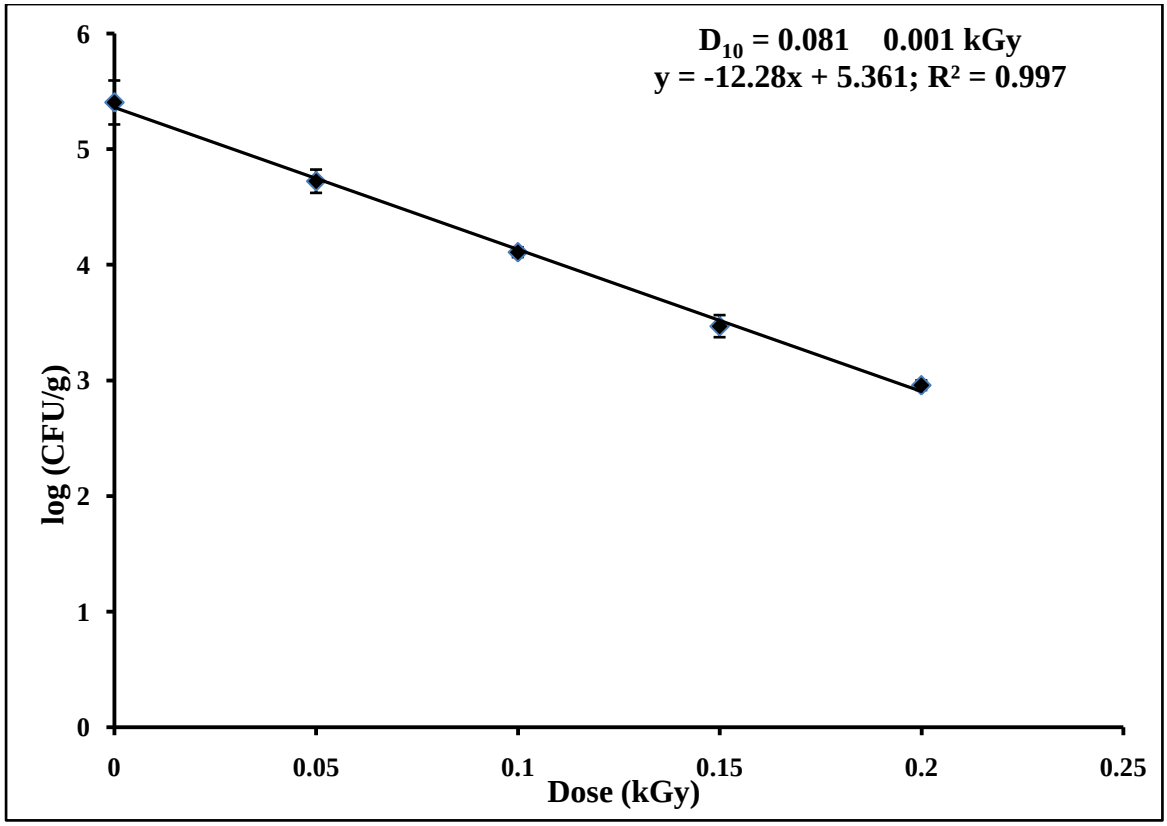


Figure 4.2. Survival of a mixture of *A. salmonicida* Y567, *A. caviae* A85, *A. veronii* bv. *veronii* A514A, *A. hydrophila* CECT 839^T and *A. salmonicida* Y47 on mixed sprouts after gamma radiation. D_{10} = 0.081 ± 0.001 kGy, y = -12.28x + 5.361, r^2 = 0.997. Each symbol represents plate counts at each dose. Average values of three experiments are plotted along with standard deviation.

Aeromonas cocktail was also found to be very sensitive in mixed sprouts, chicken and fish samples. The D_{10} values of *Aeromonas* cocktail in mixed sprouts, chicken and fish samples were 0.081 ± 0.001 kGy, 0.089 ± 0.003 kGy and 0.091 ± 0.003 kGy, respectively (Fig. 4.2, 4.3 and 4.4). Stecchini *et al.* (1995) have reported the D_{10} values of *A. hydrophila* in minced poultry meat, using electron beam, to be 0.12 and 0.14 kGy in air and under vacuum, respectively. The D_{10} values of *A. hydrophila* in ground blue-fish and ground beef at 2 °C were found to be 0.14 to 0.19 kGy, respectively (Palumbo *et al.*, 1986). The lower D_{10} values observed in the present study may be due to the different *Aeromonas* strains used, intrinsic properties of the food products or irradiation conditions.

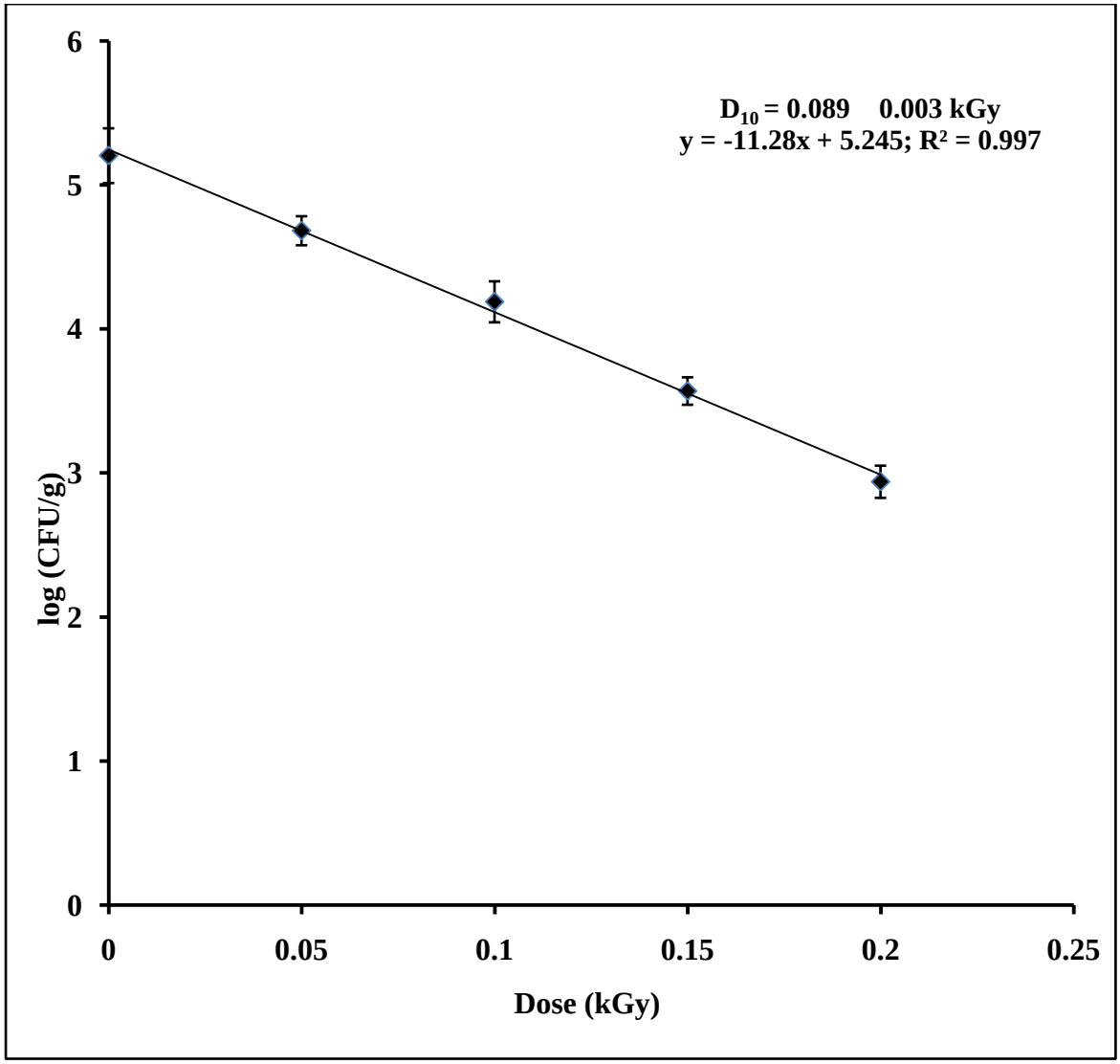


Figure 4.3. Survival of a mixture of *A. salmonicida* Y567, *A. caviae* A85, *A. veronii* bv. *veronii* A514A, *A. hydrophila* CECT 839^T and *A. salmonicida* Y47 on chicken sample after gamma radiation. D_{10} = 0.0089 ± 0.003 kGy, y = -11.28x + 5.245, r^2 = 0.997. Each symbol represents plate counts at each dose. Average values of three experiments are plotted along with standard deviation.

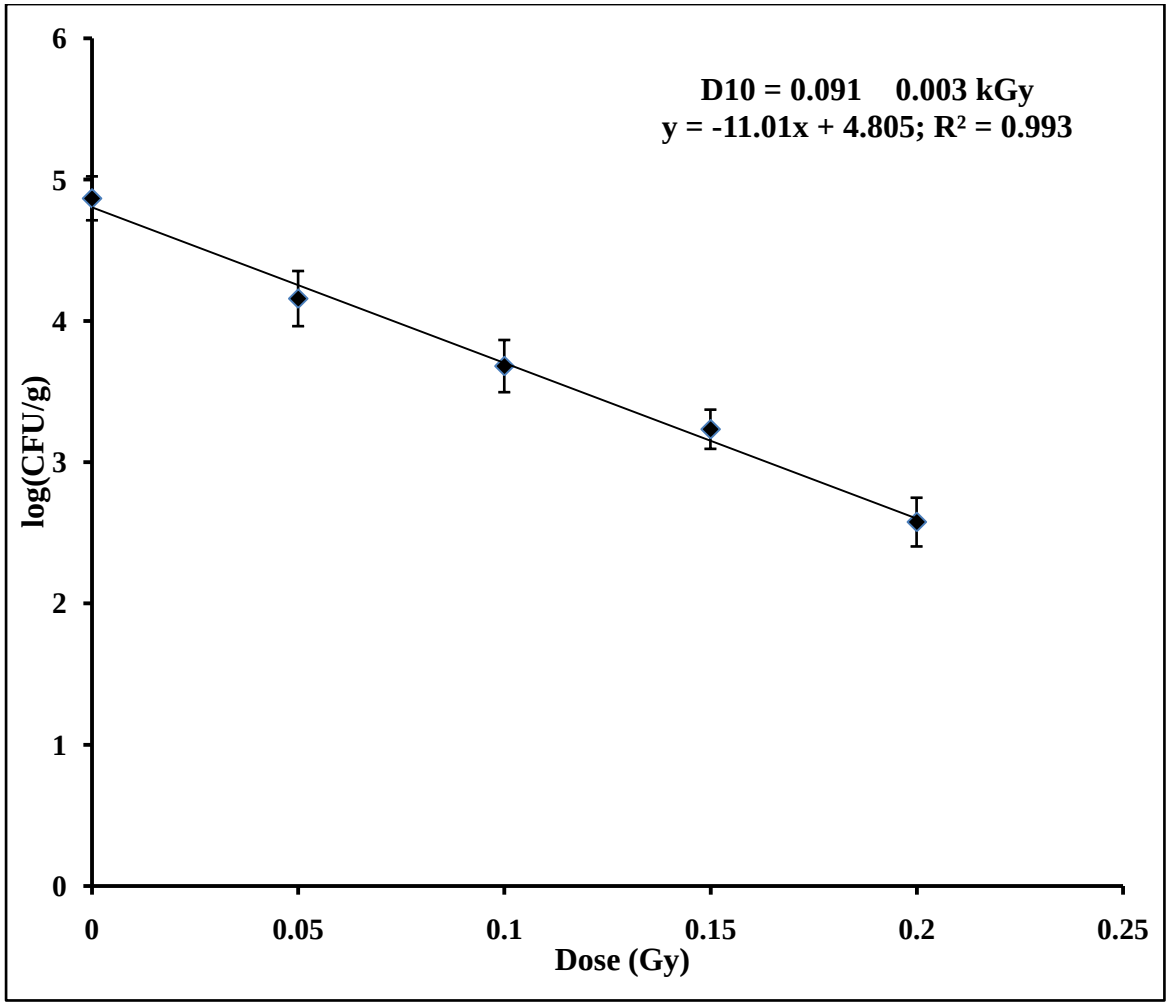


Figure 4.4. Survival of a mixture of *A. salmonicida* Y567, *A. caviae* A85, *A. veronii* bv. *veronii* A514A, *A. hydrophila* CECT 839^T and *A. salmonicida* Y47 on fish sample after gamma radiation. D_{10} = 0.091 ± 0.003 kGy, y = -11.01x + 4.805, r^2 = 0.993. Each symbol represents plate counts at each dose. Average values of three experiments are plotted along with standard deviation.

A number of factors such as water activity, composition of food material, irradiation temperature, presence of oxygen etc. affect the D_{10} values of bacteria in food (Mendonca, 2002). Some of the constituents, such as proteins, in a complex food system, are thought to compete with cells for interaction with radiolytic free radicals. Thus, they reduce the net effect of radiation damage and make organisms more radiation resistant (Urbain, 1986). To date, there are no reports on radiation sensitivity of *Aeromonas* in raw

sprouts. To the best of our knowledge, this is the first report of radiation sensitivity of *Aeromonas* in sprouts.

4.3.3. Radiation dose for 5-log elimination of *Aeromonas* from sprouts, chicken and fish samples

Table 4.1 (A) shows the effect of irradiation on the survival of *Aeromonas* cocktail inoculated in mixed sprouts. Inoculation of mixed sprouts with the cocktail of *Aeromonas* achieved a level of 2.9 X 10^5 CFU/g. No significant difference ($P > 0.05$) was observed in the levels of *Aeromonas*, recovered from mixed sprouts after attachment, in different experiments. Treatment with 1 kGy and above doses could eliminate 5-log CFU/g of *Aeromonas* spp. from mixed sprouts, when the survival of the pathogens was analyzed immediately after irradiation. However, recovery of the pathogen was observed in 0.5 kGy treated samples after enrichment in TSB for 24 h followed by selective plating on SA agar plates on all the studied days. This could be due to repair of radiation-induced damage during enrichment. In 1 kGy treated mixed sprouts, recovery of *Aeromonas* was observed in only one sample out of three replicates on the 0^{th} day samples. A dose of 1 kGy may not have resulted in complete removal of *Aeromonas* from few packets which can result in revival during 0^{th} day. However, no such recovery was observed in all the three replicates of 1 kGy treated mixed sprouts during storage on 4^{th}, 8^{th} and 12^{th} day. 1 kGy dose may have resulted in complete elimination of *Aeromonas* in few samples which resulted in no revival during storage. This is a probabilistic phenomenon. The presence of *Aeromonas* in any of the stored samples after enrichment shows ineffectiveness of 1 kGy dose for complete elimination of *Aeromonas* in the sample.

Table 4.1. Effect of irradiation on the growth of *Aeromonas* cocktail inoculated in (A) mixed sprouts, (B) fish, and (C) chicken samples during storage at 4 °C

A. Mixed sprouts

Days **Dose (kGy)**	**0th day**		**4th day**		**8th day**		**12th day**	
	log CFU/g	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**
0	5.46 ± 0.18	+	5.79 ± 0.24	+	6.23 ± 0.42	+	6.91 ± 0.38	+
0.5	0.32 ± 0.04	+	0.84 ± 0.09	+	1.04 ± 0.14	+	1.54 ± 0.10	+
1	NVC[a]	+	NVC[a]	-	NVC[a]	-	NVC[a]	-
1.5	NVC[a]	-	NVC[a]	-	NVC[a]	-	NVC[a]	-
2	NVC[a]	-	NVC[a]	-	NVC[a]	-	NVC[a]	-

B. Fish

Days **Dose (kGy)**	**0th day**		**3rd Day**		**5th day**		**7th day**	
	log CFU/g	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**
0	5.24 ± 0.23	+	5.52 ± 0.61	+	6.24 ± 0.54	+	6.64 ± 0.49	+
0.5	0.62 ± 0.09	+	1.08 ± 0.06	+	1.41 ± 0.16	+	1.76 ± 0.19	+
1	NVC[a]	+	NVC[a]	-	NVC[a]	-	NVC[a]	-
1.5	NVC[a]	-	NVC[a]	-	NVC[a]	-	NVC[a]	-
2	NVC[a]	-	NVC[a]	-	NVC[a]	-	NVC[a]	-

C. Chicken

Days Dose (kGy)	**0th day**		**3rd Day**		**5th day**		**7th day**	
	log CFU/g	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**	**log CFU/g**	***Aeromonas*[b]**
0	5.45 ± 0.61	+	5.69 ± 0.43	+	6.20 ± 0.52	+	6.79 ± 0.47	+
0.5	0.31 ± 0.08	+	0.48 ± 0.07	+	0.69 ± 0.09	+	0.90 ± 0.05	+
1	NVC[a]	+	NVC[a]	+	NVC[a]	+	NVC[a]	-
1.5	NVC[a]	-	NVC[a]	-	NVC[a]	-	NVC[a]	-
2	NVC[a]	-	NVC[a]	-	NVC[a]	-	NVC[a]	-

[a] NVC, no viable counts detected

[b] Recovery of *Aeromonas* was checked by enrichment and selective plating

Sprout samples were enriched prior to plating on selective media to check the resuscitation of *Aeromonas* cells that might have been injured during the irradiation process. No such recovery of *Aeromonas* was observed in 1.5 and 2 kGy treated mixed sprout samples after enrichment and selective plating.

Aeromonas counts of control mixed sprout samples increased from 5.46 log CFU/g to 6.91 log CFU/g during storage at 4 °C till 12th day. No viable counts were detected in 1.5 and 2 kGy irradiated sprout samples, during storage, even after enrichment and selective plating. Thus, radiation treatment of mixed sprout samples with 1.5 kGy is sufficient to eliminate 10^5 CFU/g of *Aeromonas* spp. This is the first report on the effectiveness of irradiation to eliminate *Aeromonas* from mixed sprouts, which are commonly used in India.

Though irradiation is recognized as an effective intervention technology for the purpose of pathogen reduction, the radiation dose utilized for pathogen reduction should not significantly impact the product quality factors such as taste and aroma if the process is to be used commercially (Sommers *et al.*, 2004). Earlier studies have shown that radiation treatment of different sprouts with a dose of 2-kGy does not adversely affect their textural, nutritional, and organoleptic qualities (Bari *et al.*, 2004; Hajare *et al.*, 2007).

The results of *Aeromonas* counts in the fish and chicken samples are given in Table 4.1 (B) and 4.1 (C), respectively. The initial cell counts in the inoculated fish and chicken samples were found to be 5.24 log CFU/g and 5.45 log CFU/g, respectively. No viable counts were detected in 1, 1.5 and 2 kGy irradiated fish and chicken samples on the 0^{th} day when the survival of the pathogens was analyzed immediately after irradiation. However, 0.5 kGy and 1 kGy treated samples showed recovery of the pathogen after enrichment and selective plating; whereas, no such recovery was observed in 1.5 and 2 kGy treated fish and chicken samples.

An increase in *Aeromonas* counts was observed in the control (non-irradiated) fish and chicken samples from 5.24 log CFU/g to 6.64 log CFU/g and 5.45 log CFU/g to 6.79 log CFU/g, respectively during storage at 4 °C for 7 days. No viable counts were detected in 1.5 and 2 kGy irradiated fish and chicken samples, during storage, even after enrichment and selective plating. Thus, a dose of 1.5 kGy could completely eliminate 5-log CFU/g of inoculated *Aeromonas* from fish and chicken samples. Ozbas *et al.* (1996) suggested that a dose of 0.75 kGy in combination with conventional cooking procedure is sufficient to destroy approximately 10^4 CFU/g of *Aeromonas hydrophila* in meatball. Though a radiation dose of 1.5 kGy has been recommended for the elimination of *A. hydrophila* in fresh fish, sea food, red meat and poultry (Palumbo *et al.*, 1986), this study did not check for the actual elimination of 10^5 CFU/g of *Aeromonas* from food products

using inoculated pack studies. The earlier studies also did not check for resuscitation of *Aeromonas* after enrichment and selective plating. This is also the first report of storage studies of control and irradiated fish samples inoculated with *Aeromonas*.

Several researchers have shown that a radiation dose of less than 2 kGy does not adversely affect microbiological, chemical, and sensory acceptability of both raw and cooked chicken (Balamatsia *et al.*, 2006). (Kanatt *et al.*, 2010) have shown that 2.5 kGy irradiation, in combination with chilled storage, improves the safety and extends the shelf-life of ready-to-cook meat products that are sold in Indian supermarkets.

Gamma irradiation at low doses (1-3 kGy) has been accepted by several countries for extension of shelf life of marine and freshwater fishery products (Venugopal *et al.*, 1999). Several studies have shown that radiation at a low dose (up to 3 kGy), in combination with chilled storage, can be used to preserve the fishery products without adversely affecting their biochemical, textural and sensory attributes (Mbarki *et al.*, 2008; Moini *et al.*, 2009).

Aeromonas were detected in chicken (28.6%), fish (20%) and sprout (2.5%) samples marketed in Mumbai (Chapter 2). Usually, the cell number of *Aeromonas*, if present in different food products, is 10^2-10^5 CFU/g (Gobat and Jemmi, 1993; Pin *et al.*, 1994). Though, the infectious dose of *Aeromonas* range from 10^3- 10^9 CFU/g (Yucel and Erdogan, 2010), they can survive and grow to higher numbers in food products stored at low temperature. It is also possible that some *Aeromonas* strains may have a lower infective dose in sensitive sub-populations like children and immune-compromised persons (USEPA, 2006). Thus, radiation treatment of sprout, chicken and fish products with 1.5 kGy will be sufficient to eliminate 10^5 CFU/g of *Aeromonas* and thereby ensuring their safety.

4.4. Conclusions

The results from this study showed that all *Aeromonas* isolates were very sensitive to gamma radiation. A radiation dose of 1.5 kGy is effective in achieving 5 log reductions in *Aeromonas* populations on mixed sprout, chicken and fish samples. The study also revealed the importance of conducting enrichment studies to determine the bactericidal effects of the irradiation process. This is the first report of radiation sensitivity and inoculated pack studies of *Aeromonas* isolates from Indian food samples, irradiated in different food products. The radiation doses selected for partial or full elimination of other pathogens with higher D_{10}-values (*Salmonella*, *L. monocytogenes*, etc.) could provide complete elimination of *Aeromonas* as an ancillary benefit.

CHAPTER 5

Biofilm Formation by *Aeromonas* under Various Food-Related Stress Conditions

5.1. Introduction

Aeromonas have the ability to form biofilms on surfaces such as glass, polystyrene plastic surfaces and stainless steel (Kirov *et al.*, 2004; Santos *et al.*, 2011). These materials are possible food contact surfaces in the food processing industry. Thus, the biofilms formed on these surfaces may become a continuous source of cross contamination during food processing and pose a threat to food industry and public health (Chmielewski and Frank, 2003). In addition to forming biofilms on a variety of food processing surfaces, *Aeromonas* can also attach, colonize and form biofilms on green-leafy vegetables (cabbage and lettuce) and intestinal cell lines (Elhariry, 2011; Kirov *et al.*, 2004). The ability of *A. hydrophila* to form biofilm on food samples varies according to the type of food, source of isolation, hydrophobicity of bacterial cells and substratum, and environmental conditions (Khajanchi *et al.*, 2009).

General predictions regarding the biofilm formation by an organism on a particular material cannot be made as the biofilm forming ability depends on the genetic composition and regulation of the bacterium, substratum and bacterial cell's properties and environmental factors (Xu *et al.*, 2011). The characteristics of attachment surface play an important role in the biofilm formation process (Chmielewski and Frank, 2003).

Essential oils (EOs) have been a part of the human diet for hundreds of years and thus have been generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (Adams and Taylor, 2010). Various essential oils have been traditionally used by people for various purposes in different parts of the world (Prabuseenivasan *et al.*, 2006). Individual essential oil components have been demonstrated to possess antimicrobial activity against major food-borne pathogens (Bakkali *et al.*, 2008). Due to the presence of diverse antimicrobial components, essential oils are very active against a wide variety of

microorganisms, including gram-negative and gram-positive bacteria (Tajkarimi *et al.*, 2010). Benzoates, nitrites, sulphites, propionates, and sorbates are widely used GRAS food additives in the food industry (Smid and Gorris, 2007). However, less attention has been given to the effect of essential oils and food preservatives on bacterial biofilms, which are more resistant to therapeutic intervention including antibiotics.

There is a need to understand the characteristics of biofilm formation under food processing-related stress conditions. It will help food processors and regulators to prevent biofilm formation and reduce the health risks related to biofilm-forming food-borne pathogens. Thus, the aim of this study was to evaluate the impact of media, temperature, pH and salt on biofilm production in different species of *Aeromonas*. Further, the effects of food-relevant concentrations of the commonly used food preservatives on *Aeromonas* biofilm formation were studied. The minimum inhibitory concentration (MIC) of different EOs against *Aeromonas* species was also determined.

5.2. Materials and methods

5.2.1. Bacterial strains and culture conditions

Ten different strains (*A. hydrophila*, CECT 839^T, AH1N, A331 and MTCC 2654; *A. sobria* MTCC 1608; *A. veronii* bv. *veronii* CECT 4257^T and MTCC 3249; *A. veronii* bv. *sobria* CECT 4246; *A. bivalvium* CECT 7112 and *A. caviae* A85) (Table 5.1), belonging to six species of *Aeromonas*, were evaluated for their biofilm forming ability under the effect of media composition, pH, salinity and temperature and food preservatives. *A. hydrophila* CECT 839^T, *A. veronii* bv. *veronii* CECT 4257^T, *A. veronii* bv. *sobria* CECT 4246 and *A. bivalvium* CECT 7112 were kindly provided by Dr. Valérie Leclère, Université des Sciences et Technologies de Lille USTL, Villeneuve d'Ascqcedex;

MTCC 2654, MTCC 1608, MTCC 3249 were obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India); *A. hydrophila* AH1N was a kind gift from Prof. P. Williams, University of Nottingham, Nottingham, UK; whereas, A331 and A85 were isolated from the chicken and sprout samples, respectively (Chapter 2).

Table 5.1. *Aeromonas* species used in the study

Strain	Species	Origin	Reference
CECT 839^T	*A. hydrophila*	Tin of milk	Stanier (1943)
AH1N	*A. hydrophila*	Environment	Lynch *et al.* (2002)
A331	*A. hydrophila*	Chicken	Chapter 2
MTCC 2654	*A. hydrophila*	Unknown	Conroy (1961)
MTCC 1608	*A. sobria*	Activated sludge	Popoff and Veron (1976)
CECT 4257^T	*A. veronii* bv. *veronii*	Clinical specimen	Hickman-Brenner *et al.* (1987)
MTCC 3249	*A. veronii* bv. *veronii*	Insect gut	Pidiyar *et al.* (2002)
CECT 4246	*A. veronii* bv. *sobria*	Frog	Popoff and Veron (1976)
CECT 7112	*A. bivalvium*	Razor shells	Minana-Galbis *et al.* (2007)
A85	*A. caviae*	Sprouts	Chapter 2

The cultures were revived from glycerol stock cultures (TSB with 20% glycerol stored at -80 °C) and sub-cultured onto sterile tryptic soya agar (TSA) plates and incubated at 30 °C for 24 h. Following incubation on TSA plates, a loopful of cultures were inoculated into sterile tryptic soya broth (TSB) and grown for 16 - 18 h at 30 °C, 150 rpm. For each strain, 1 ml of overnight grown culture was further inoculated in 25 ml TSB broth

separately and grown for 18 h at 30 °C, 150 rpm prior to experiments. The overnight cultures were standardized to yield a count ≈ 2 x 10^8 CFU/ml before biofilm formation. This was achieved by diluting the overnight cultures with TSB to obtain an absorbance (OD_{600nm}) of 0.2 using a spectrophotometer (Eppendorf Bio-photometer plus, Wesseling-Berzdorf, Germany).

5.2.2. Essential oils

Nutmeg, ajowain, basil, black pepper, cumin, ginger, clove, turmeric, cinnamon oils (Konark Herbal & Health Care Ltd, India), eucalyptus and orange oils (Hi-Media, India), and peppermint, citronella, lemon, lavender and tea-tree oils (Soulflower Co. Ltd., Bangkok, Thailand) were used in this study. These oils were selected based on the literature survey, commercial importance and their use in traditional medicine. Quality of the oils was ascertained to be more than 98% pure. Solubility of all essential oil was tested before application. Dilution was made after a known volume of each oil was diluted by adding methanol and stored at 5 ºC till used.

5.2.3. Biofilm formation

The effect of different media, pH, temperature, salt concentration and food preservatives on biofilm formation was studied by culturing the organism in 96-well flat-bottomed microtiter plates (Falcon, BD Biosciences, NJ, USA). The individual well containing 180 µl of tested medium (adjusted to defined pH or salt concentration, with/without food preservatives) was inoculated with 20 µl of overnight grown culture (30 °C, 150 rpm, 16 h), standardized to an optical density of 0.2 (approximately 2.0 x 10^8 CFU/ml) using a spectrophotometer (Eppendorf Bio-photometer plus, Wesseling-

Berzdorf, Germany). Wells containing only medium were used as blank. Plates were then incubated at defined temperature for the defined time period (24 h or 48 h) under stationary conditions.

5.2.4. Influence of growth medium on biofilm formation

The effect of different growth media on the biofilm formation was studied by inoculating *Aeromonas* strains into different media (TSB and M9 minimal media supplemented with 0.4% glucose). TSB and M9 minimal media supplemented with 0.4% glucose were poured into the wells of microtiter plates as described in section 5.2.3 for subsequent quantification of biofilm formation.

5.2.5. Influence of incubation temperature, NaCl, pH on biofilm formation

The effect of incubation temperature on biofilm formation of selected strains was studied at three different temperatures, i.e. 10 °C, 30 °C and 37 °C. The effect of pH and salt on the biofilm formation of selected strains was studied at 30 °C. The following pH values were tested: 5.0, 6.0, 7.0, 8.0 and 9.0 in TSB. The pH was adjusted by the addition of 1N HCl or 1N NaOH. The different concentrations of NaCl tested were 1, 2 and 3% (w/v) in addition to 0.5% NaCl that TSB contains. The control for the pH studies was TSB with pH 7.3; whereas, control for the sodium chloride studies the control was TSB already containing 0.5% NaCl.

5.2.6. Influence of food preservatives on the biofilm formation

The effect of food preservatives on the biofilm formation was studied using sodium benzoate (Hi-Media, India) (0.025, 0.05, 0.1, 0.2 and 0.4 % w/v), potassium sorbate (Hi-

Media, India) (0.025, 0.05, 0.1, 0.2 and 0.4 % w/v), and sodium nitrite (Hi-Media, India) (0.03, 0.06, 0.12 and 0.24 % w/v). The control for the food preservative studies was TSB containing no additional food preservatives.

5.2.7. Determination of minimal inhibitory concentration (MIC)

The antibacterial activity of essential oils was tested on *Aeromonas hydrophila* CECT 839^T and *A. veronii* bv. *veronii* CECT 4257^T strains. MIC values of different essential oils for *A. hydrophila* CECT 839^T and *A. veronii* bv. *veronii* CECT 4257^T in TSB and M9 minimal media were determined using microtiter broth micro-dilution method (Wiegand *et al.*, 2008) with the following modification; a final concentration of 0.1% (v/v) Tween-20 (Sigma) was incorporated into the broth medium to enhance oil solubility. A series of two fold dilution of each oil (0.0625 - 8 mg/ml) in TSB were prepared in 96-well plate (200 µl per well). Ciprofloxacin (0.0039065 - 0.0625 mg/ml) were used as the positive control for the MIC determination. A diluted bacterial suspension was added to each well to give a final concentration of 1 - 5 x 10^5 colony-forming units (CFU)/ml. Wells without bacteria were used as a negative growth control. The plate was incubated for 24 h at 30 °C and growth was assessed turbidometrically (OD_{600nm}). The MIC was defined as the lowest concentration of EO inhibiting detectable growth of organism based on optical density in the microtiter plate.

5.2.8. Inhibition of biofilm formation by essential oils

The effect of three EOs (clove, ajowain and cinnamon), with MIC values $\leq$ 1 mg/ml, on the inhibition of initial cell attachment was studied. The methanol extracts of these EOs at a concentration of 1 mg/ml were tested for their potential anti-adhesion

properties. The concentration of 1 mg/ml was chosen based on the previous study (Rios and Recio, 2005) which reported that only extracts exhibiting MIC values < 1 mg/ml are noteworthy. One hundred and eighty microlitres of TSB containing essential oils was added to the microtiter plates to yield a final concentration of 1 mg/ml in each well. One hundred and eighty microlitres of sterile TSB and ciprofloxacin (0.007813 mg/ml) (MIC value) were used as negative and positive controls, respectively. Twenty microlitres of the standardized culture (2.0 x 10^8 CFU/ml) were pipetted into the wells. The plates were sterile sealed with sealing tape and incubated at 30 °C for 8 h without shaking to allow cell attachment and biofilm development. Following incubation, the modified crystal violet assay was performed to assess biofilm biomass.

5.2.9. Assessment of biofilm biomass (crystal-violet staining assay)

The biofilm-forming ability was indirectly assessed using the modified crystal-violet (CV) assay (O'Toole *et al.*, 1999). The microtiter plate based crystal violet (CV) staining assay used in this study is a commonly used, high-throughput, simple and rapid experimental setup which provides reproducible results and allows for a large number of strains and conditions to be studied simultaneously (Lianou and Koutsoumanis, 2012).

Following incubation, the total cell mass was measured as absorbance at 600 nm (G) by micro-plate reader (µQuant, Biotek, USA).The content of the wells were poured off and the wells were washed three times with sterile distilled water to remove loosely attached or planktonic cells. The plates were air-dried and then oven-dried at 50 °C for 30 min. Following drying, the cells were fixed with 200 µl of 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 10 min. The wells were again washed three times with distilled water and oven-dried at 50 °C for 30 min. The wells were stained with

200 µl of 0.2% crystal violet (in methanol) and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilled water to remove unabsorbed stain. The plates were air-dried for 30 min, and then 200 µl of ethanol-acetone mix (80 - 20%) (v/v) was added to each well to destain the biofilm and the concentration of crystal violet was determined by measuring optical density at 570 nm using a micro-plate reader (µQuant, Biotek, USA). All tests were carried out three times and the mean values were determined.

5.2.10. Biofilm quantification

Different mathematical formulae have been used by various researchers for the quantification of biofilm formation. Naves *et al.* (2008) have reported significant differences in biofilm quantification based on three different methods. Among three different methods, the specific biofilm formation (SBF) index was found to be the most appropriate as it also considers bacterial growth rate (OD_{600nm}) and provides less differences in categorization of bacterial biofilm. Thus, SBF index was used for the quantification of biofilm in the present study.

The specific biofilm formation (SBF) index was determined by normalizing biofilm accumulation (OD_{570nm}) with respect to growth (OD_{600nm}). SBF was determined by using the following formula: SBF = (B - NC)/G, where B is the OD_{570nm} of the attached and stained bacteria, NC is the OD_{570nm} of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values), G is the OD_{600nm} of cell growth in broth (Niu and Gilbert, 2004).

The biofilm formation (OD_{570nm}) and growth (OD_{600nm}) of each isolate under different conditions were determined and the SBF index was calculated. The SBF index was plotted against different conditions for each isolate using Microsoft Excel (2007).

The degree of biofilm production (SBF) was classified into three categories according to Jahid *et al.* (2013): no biofilm (SBF < 0.1), weak (0.1 ≤ SBF ≤ 0.5), moderate (0.5 ≤ SBF ≤ 1), or strong (SBF > 1).

5.3. Results and Discussion

5.3.1. Effect of growth medium on biofilm formation

The biofilm formation by 10 different *Aeromonas* strains, belonging to six different species, was estimated in two different media: TSB (24 h) and M9 supplemented with 0.4% glucose (48 h). The incubation times for TSB and M9 minimal media were selected based on the findings from the preliminary experimentations exhibiting inconsistent growth (OD_{600nm}) and biofilm formation (OD_{570nm}) in M9 minimal medium prior to 48 h at 30 °C.

The results revealed that all tested *Aeromonas* strains were able to produce biofilm in both TSB and M9 medium (Fig. 5.1). TSB was chosen as one of the nutrient-rich laboratory media most frequently used in the previous studies evaluating the biofilm formation by *Aeromonas* (Gavin *et al.*, 2003); whereas, M9 minimal media supplemented with 0.4% glucose was chosen as the media often used to mimic food industry conditions, given that these do not ordinarily provide as much nutrients comparable with rich laboratory media (Stepanovic *et al.*, 2004).

The influence of growth medium composition on the biofilm formation by *Aeromonas* strains is not clearly noticeable as no significant effect of the growth medium

composition on biofilm formation was observed in 50% of *Aeromonas* strains (Fig. 5.1). No significant difference was observed in the biofilm formation by five *Aeromonas* strains (*A. hydrophila* AH1N, *A. hydrophila* MTCC 2654, *A. veronii* bv. *veronii* MTCC 3249, *A. caviae* A85 and *A. bivalvium* CECT 7112) in TSB and M9 medium. However, three *Aeromonas* strains (*A. hydrophila* CECT 839^T, *A. sobria* MTCC 1608 and *A. veronii* bv. *veronii* CECT 4257^T) showed significantly more biofilm formation in M9 medium than in TSB; whereas, two *Aeromonas* strains (*A. hydrophila* A331 and *A. veronii* bv. *sobria* CECT 4246) formed significantly more biofilm in TSB than in M9 medium. Overall, more biofilm was formed in rich media (TSB) than in minimal media (M9) for majority of the strains. However, moderate to strong biofilm was formed by three *Aeromonas* strains (*A. hydrophila* CECT 839^T, *A. sobria* MTCC 1608 and *A. veronii* bv. *veronii* CECT 4257^T) only in M9 minimal media; whereas, none of the strains could form moderate to strong biofilm in TSB medium (Fig. 5.1).

Poor correlation between biofilm formations by various *Aeromonas* strains in different media suggests that various *Aeromonas* strains respond very differently to the different media composition. Hood and Zottola (1997) studied biofilm formation by *Salmonella Typhimurium*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Pseudomonas fragi* and *Pseudomonas fluorescens* in different media (tryptic soy broth, diluted TSB, 1% reconstituted skim milk and diluted meat juice) and observed that the medium which induced highest biofilm formation was different for each studied microorganism. The biofilm formation by all *Salmonella* and *L. monocytogenes* isolates was not influenced to the same extent by the medium composition (Stepanovic *et al.*, 2004).

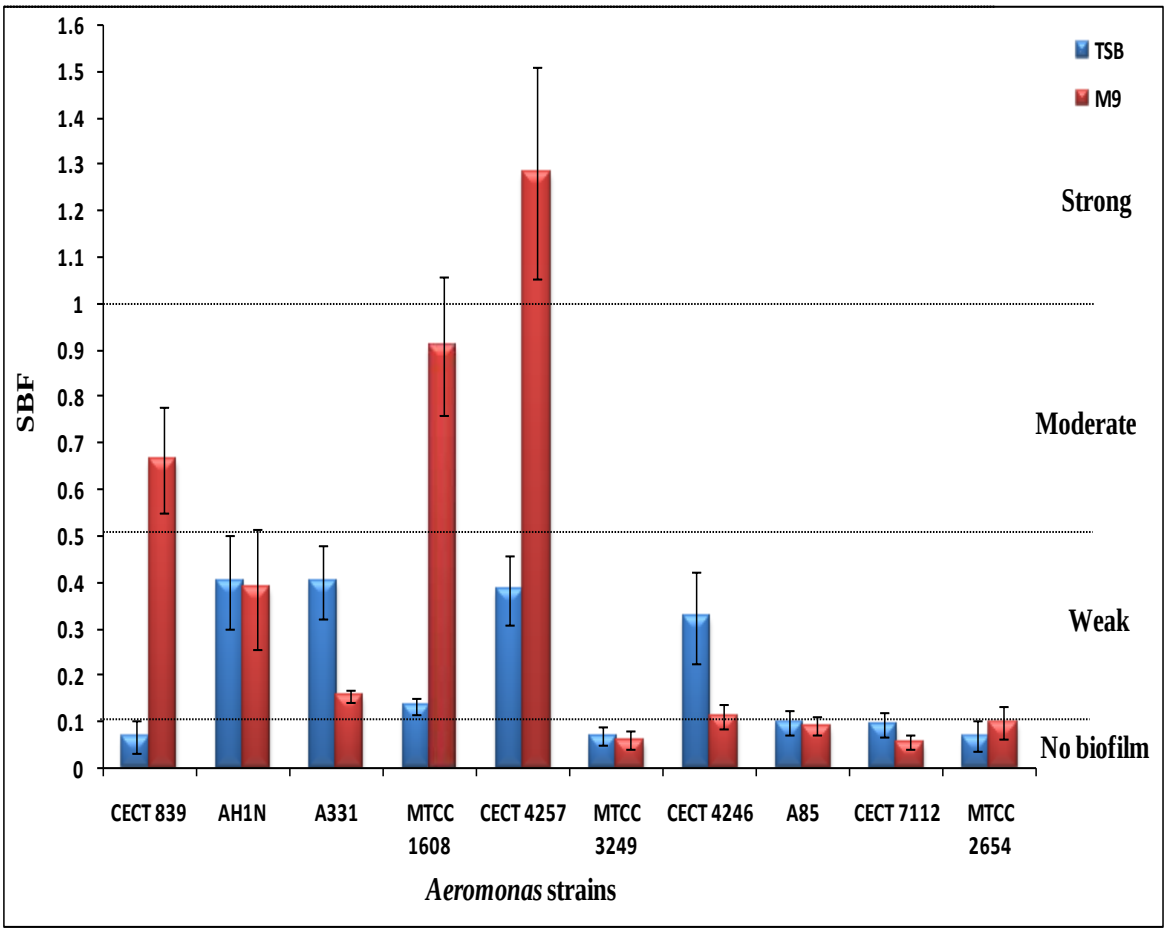


Figure 5.1. Biofilm formation by different *Aeromonas* strains in TSB and M9 minimal media at 30 °C

Biofilm formation is a complex process regulated by diverse factors including the growth medium; however, this process is still poorly understood (Harvey *et al.*, 2007). Therefore, reasons for the influence of nutritional content of growth medium on the biofilm formation can only be speculated. The variations in the published results may be due to differences in media, contact surfaces, quantification methods, sample sizes and strains. Further investigation needs to be done to explain the difference in biofilm production by *Aeromonas* isolates in TSB and M9 minimal media.

The present study showed considerable variation in the *in vitro* biofilm forming capacity (SBF index) among different *Aeromonas* strains belonging to the same species

(Fig. 5.1). *A. hydrophila* strains CECT 839^T and MTCC 2654 produced more biofilm in M9 minimal media; whereas, other *A. hydrophila* strains AH1N and A331 produced more biofilm in TSB. In the similar manner, significant differences were observed among SBF index of *A. veronii* bv. *veronii* strains CECT 4257^T and MTCC 3249. These variations may be due to differences in genetic composition of the strains. Various researchers have reported considerable strain variability in the biofilm production for food-borne pathogens *L. monocytogenes* (Nilsson *et al.*, 2011), *Salmonella enterica* (Lianou and Koutsoumanis, 2012) and *Escherichia coli* (Skyberg *et al.*, 2007).

No clear correlation was observed between the source of *Aeromonas* strains (from humans, animal, environment or food) and their biofilm forming ability. Jahid *et al.* (2013) have reported that the SBF values for different *A. hydrophila* are strain specific and have no correlation with origin of the isolates. Our results are in agreement with various studies (Nilsson *et al.*, 2011; Stepanovic *et al.*, 2004) that have shown no clear correlation between the source/origin of isolates and their biofilm forming ability.

Grouping of *Aeromonas* strains based on the differences in biofilm formation was possible (Fig. 5.1). The variations clearly indicate that different patterns of biofilm formation exist among *Aeromonas* strains. Five *Aeromonas* strains (*A. hydrophila* AH1N, *A. hydrophila* A331, *A. sobria* MTCC 1608, *A. veronii* bv. *veronii* CECT 4257^T and *A. veronii* bv. *sobria* CECT 4246) formed weak biofilm in TSB; whereas, no significant biofilm formation was observed in five strains (*A. hydrophila* CECT 839^T, *A. hydrophila* MTCC 2654, *A. veronii* bv. *veronii* MTCC 3249, *A. bivalvium* CECT 7112 and *A. caviae* A85). However, these *Aeromonas* strains formed strong (*A. veronii* bv. *veronii* CECT 4257^T), moderate (*A. hydrophila* CECT 839^T and *A. sobria* MTCC 1608), weak (*A. hydrophila* AH1N, *A. hydrophila* A331 and *A. veronii* bv. *sobria* CECT 4246) and no

significant biofilm (*A. veronii* bv. *veronii* MTCC 3249, *A. caviae* A85, *A. bivalvium* CECT 7112 and *A. hydrophila* MTCC 2654) in M9 minimal media supplemented with 0.4% glucose.

All *Aeromonas* strains formed either weak or insignificant biofilm in TSB. None of these strains were able to form moderate to strong biofilm in TSB. However, these strains formed different types of biofilm (insignificant, weak, moderate and strong) in M9 minimal media. Four *Aeromonas* strains (*A. veronii* bv. *veronii* MTCC 3249, *A. caviae* A85, *A. bivalvium* CECT 7112 and *A. hydrophila* MTCC 2654) formed insignificant biofilm in both TSB and M9 minimal media indicating that these strains may be genetically inefficient to form moderate to strong biofilm. The present study showed variation in the biofilm forming abilities of the strains belonging to the same species in both TSB and M9 minimal media. Our results are in agreement with earlier studies which showed high inter-strain variation in biofilm formation by *Listeria monocytogenes* (Nilsson *et al.*, 2011), *Salmonella* (Lianou and Koutsoumanis, 2012) and *Escherichia coli* (Skyberg *et al.*, 2007). Equal percentage (33.3%) of *A. hydrophila* isolates formed strong, moderate and weak biofilm in nutrient broth (Jahid *et al.*, 2013). The diversity indicates that the conclusions regarding biofilm formation from a single strain or using single media should be carefully interpreted.

Only few *Aeromonas* strains (*A. hydrophila* CECT 839^{T}, *A. hydrophila* A331, *A. sobria* MTCC 1608, *A. veronii* bv. *veronii* CECT 4257^{T} and *A. veronii* bv. *sobria* CECT 4246) were significantly influenced by media composition in the present study (Fig. 5.1). Significant influence of nutrient content of the medium on the quantity of biofilm produced by most of the *Salmonella* and *L. monocytogenes* strains was observed (Stepanovic *et al.*, 2004). Cell surface hydrophobicity which regulates the mechanisms of

cell adhesion to the surface is reported to vary with different growth media thus leading to variable biofilm formation potential in media with varied nutrient level along with various associated factors (Pagedar *et al.*, 2010). Avian *E. coli* isolates were found to be highly variable in their ability to form biofilms in three different media and an isolate's ability to form a biofilm in one type of medium was not necessarily indicative of its capability to form a biofilm in another (Skyberg *et al.*, 2007). The present study revealed that different species of *Aeromonas* have the ability to form biofilm on plastic surfaces, which are frequently used nowadays in food-processing industry. These results confirmed the earlier studies which showed that *Aeromonas* are able to form biofilm on plastic surfaces (Santos *et al.*, 2011).

In the present study, four *Aeromonas* strains (*A. veronii* bv. *veronii* MTCC 3249, *A. bivalvium* CECT 7112, *A. caviae* A85 and *A. hydrophila* MTCC 2654) did not form significant biofilm in either TSB or M9 minimal media. Thus, these four strains were not studied for their biofilm forming abilities under different stress conditions. Among remaining isolates, three strains (*A. hydrophila* CECT 839^T, *A. sobria* MTCC 1608 and *A. veronii* bv. *veronii* CECT 4257^T) formed significantly more biofilm in M9 minimal medium than TSB; whereas, two strains (*A. hydrophila* A331 and *A. veronii* bv. *sobria* CECT 4246) formed significantly more biofilm in TSB than M9 minimal medium. No significant influence of medium composition on biofilm formation was observed for the *A. hydrophila* AH1N strain. Thus, three strains (*A. hydrophila* CECT 839^T, *A. sobria* MTCC 1608 and *A. veronii* bv. *veronii* CECT 4257^T), forming more biofilm in M9 minimal medium, were grown in M9 minimal medium supplemented with 0.4% glucose for 48 h to study their biofilm forming ability under different stress conditions: temperature (10 °C, 30 °C and 37 °C), pH (5, 6, 7, 8 and 9), additional NaCl (1%, 2% and 3%), food

preservative {potassium sorbate and sodium benzoate (0 to 0.4%) and sodium nitrite (0 to 0.24%)}. However, these strains showed insignificant growth (OD_{600nm}) in M9 minimal medium under different stress conditions (data not shown). Out of different conditions studied, the growth (OD_{600nm}) of these isolates was < 0.05 at temperature 10 °C; pH 5, 8 and 9; 2% and above concentration of additional NaCl; 0.1% and above concentration potassium sorbate and sodium benzoate; 0.06% and above concentration of sodium nitrite.

The prolonged incubation of these plates for 96 h to obtain significant growth (OD_{600nm}: >0.1) lead to evaporation and drying of the microtiter plate which gave variable and erroneous results. It was difficult to evaluate the biofilm formation under these stress conditions using M9 minimal medium. Thus, further studies on the effect of temperature, salt, pH and food preservatives on biofilm formation by these *Aeromonas* strains (*A. sobria* MTCC 1608, *A. veronii* bv. *veronii* CECT 4257^T, *A. hydrophila* A331, *A. veronii* bv. *sobria* CECT 4246 and *A. hydrophila* AH1N) was done using TSB.

5.3.2. Effect of incubation temperature on biofilm formation

Temperature is one of the important environmental factors that affect the bacterial growth, attachment and biofilm formation. Generally, growth of bacterial population is maximal at optimum temperatures; whereas, sub-optimal temperatures reduce bacterial growth efficiency. This is due to a reduction in bacterial enzyme reaction rates. Different temperatures used in the present study were selected based on their importance: 10 °C is generally used during transportation and storage of food; 30 °C is the optimum temperature for growth of *Aeromonas* species and 37 °C is the temperature of human body (host).

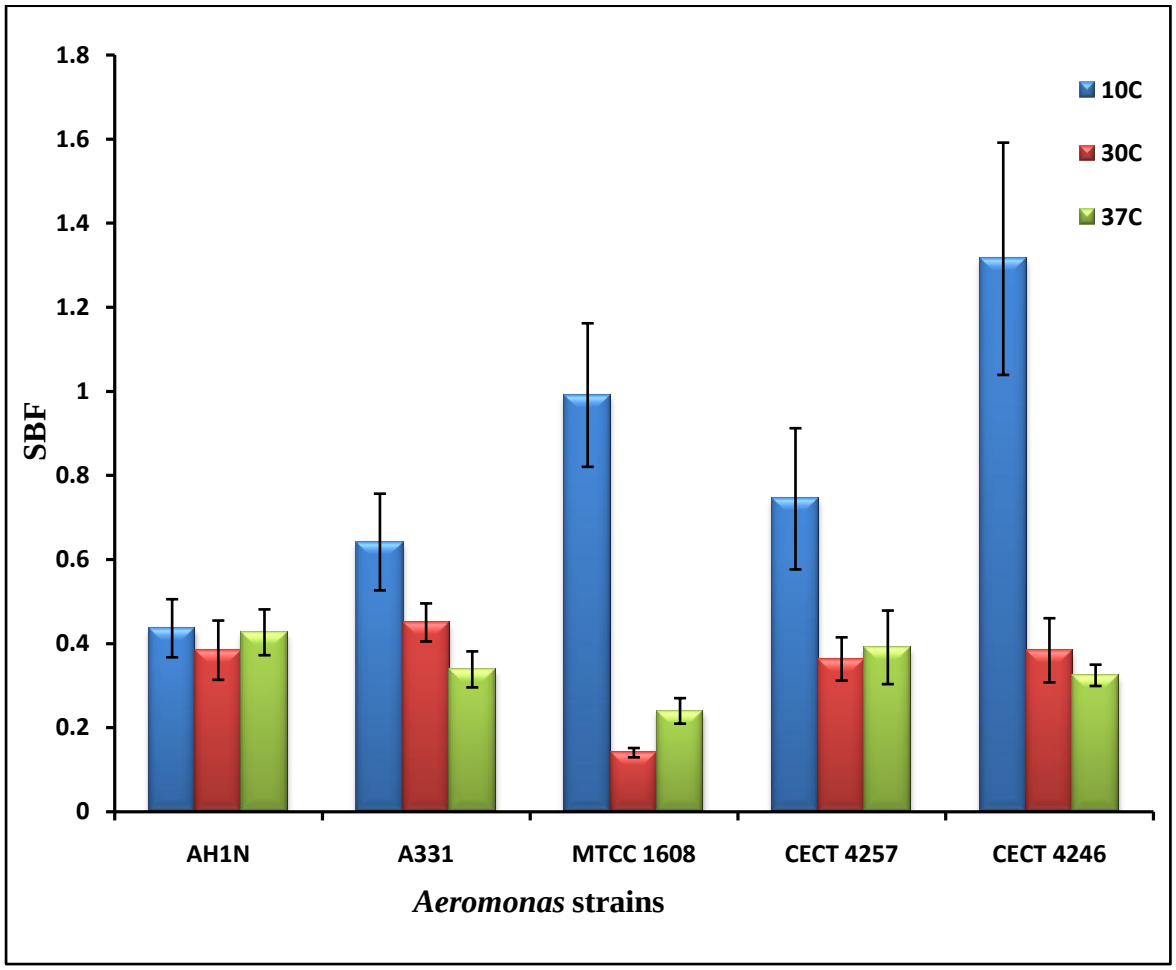


Figure 5.2. Biofilm formation by *Aeromonas* strains in TSB medium at different temperatures (10 °C, 30 °C and 37 °C)

For most of the *Aeromonas* isolates, more SBF index was observed at 10 °C than 30 °C or 37 °C (Fig. 5.2). Though less amount of biofilm (OD_{570nm}) was formed at 10 °C as compared to other temperatures, reduced bacterial growth (OD_{600nm}) at this temperature resulted in higher SBF index values. The mechanisms behind increased biofilm formation at sub-optimal temperatures are not known (Rode *et al.*, 2007). However, the temperature that is optimum for cell growth might not be optimum for cell adhesion because temperature affects the physical properties of the compounds present within and surrounding the cells (Garrett *et al.*, 2008). Chavant *et al.* (2002) have shown that the hydrophilic surface properties of *L. monocytogenes* are affected by temperature. The low

temperatures may increase the hydrophilic properties of the cells and reduce cellular movement and thus alter the bacteria's ability to adhere to hydrophobic materials like polystyrene (Rode *et al.*, 2007). Our results agree with earlier observations (Schonewille *et al.*, 2012) where higher biofilm was formed at low temperature (20 °C) as compared to the optimal temperature (37 °C) in different serotypes of *Salmonella enterica*, derived from the poultry farm environment.

The effect of temperature on attachment has been reported in only few microorganisms and the mechanism is still unknown. Hostacka *et al.* (2010) have reported reduction in biofilm formation by different organisms (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Vibrio cholerae* non-O1 and O1) at 37 °C as compared to 30 °C. However, significantly higher biofilm production was observed in *Stenotrophomonas maltophilia* at optimum temperature (32 °C) in comparison to 18 or 37 °C (Di *et al.*, 2007). The lower biofilm production of the tested bacteria at 18 °C was explained as a consequence of lower bacterial growth at this temperature.

Majority of researchers have calculated biofilm formation under different stress conditions using difference in optical density of stained attached bacteria and stained bacteria-free medium. They have not considered growth (OD_{600nm}) of the bacteria under the studied conditions. Bacteria may grow at different rates under different conditions, and this affects their biofilm forming ability. Naves *et al.* (2008) have suggested that bacterial growth rate (OD_{600nm}) should also be used for the calculation of specific biofilm formation (SBF) index, which gives better information regarding biofilm forming ability of the isolate. However, the comparison of biofilm forming ability based on two different calculations is complex and difficult.

5.3.3. Effect of sodium chloride on biofilm formation

Sodium chloride is another factor that may play an important role in bacterial cell attachment and biofilm development. Addition of NaCl to TSB medium influenced the biofilm formation, and variation in the biofilm forming capacity among *Aeromonas* species was observed under these conditions (Fig. 5.3). For most of the isolates, maximum SBF values were observed in control (0.5% NaCl) condition and the biofilm forming ability reduced with an increase in the concentration of additional NaCl from 1% to 3%. The optimum sodium chloride concentration range for most of the *Aeromonas* species is 0 to 4% (Delamare *et al.*, 2000). For most of the isolates, no significant effect on the growth (OD_{600nm}) of bacteria was observed with addition of NaCl till 2%; however, reduction in growth was observed with addition of 3% NaCl. Simultaneous reduction in biofilm formation (OD_{570nm}) and bacterial growth (OD_{600nm}) due to additional 3% sodium chloride concentration resulted in reduction in SBF index.

There was a reduction in biofilm formation (OD_{570nm}) with an increase in sodium chloride concentration for the isolate A331. However, there was simultaneous reduction in growth (OD_{600nm}) that resulted in an increase in SBF index at 3% additional sodium chloride concentration. On the other hand, the biofilm formation (OD_{570nm}) reduced significantly with additional 1% sodium chloride for the isolate CECT 4257^{T}; whereas, there was no significant reduction in growth (OD_{600nm}) resulting in a decrease in SBF index. These observations provide supportive evidence for using SBF over other methodology for biofilm quantification as it normalizes the variation in growth due to different stress conditions and isolates. Since it is a well known fact that there is poor correlation between growth (OD_{600nm}) and biofilm formation (OD_{570nm}), SBF will give more realistic picture of influence of different parameters on the biofilm formation.

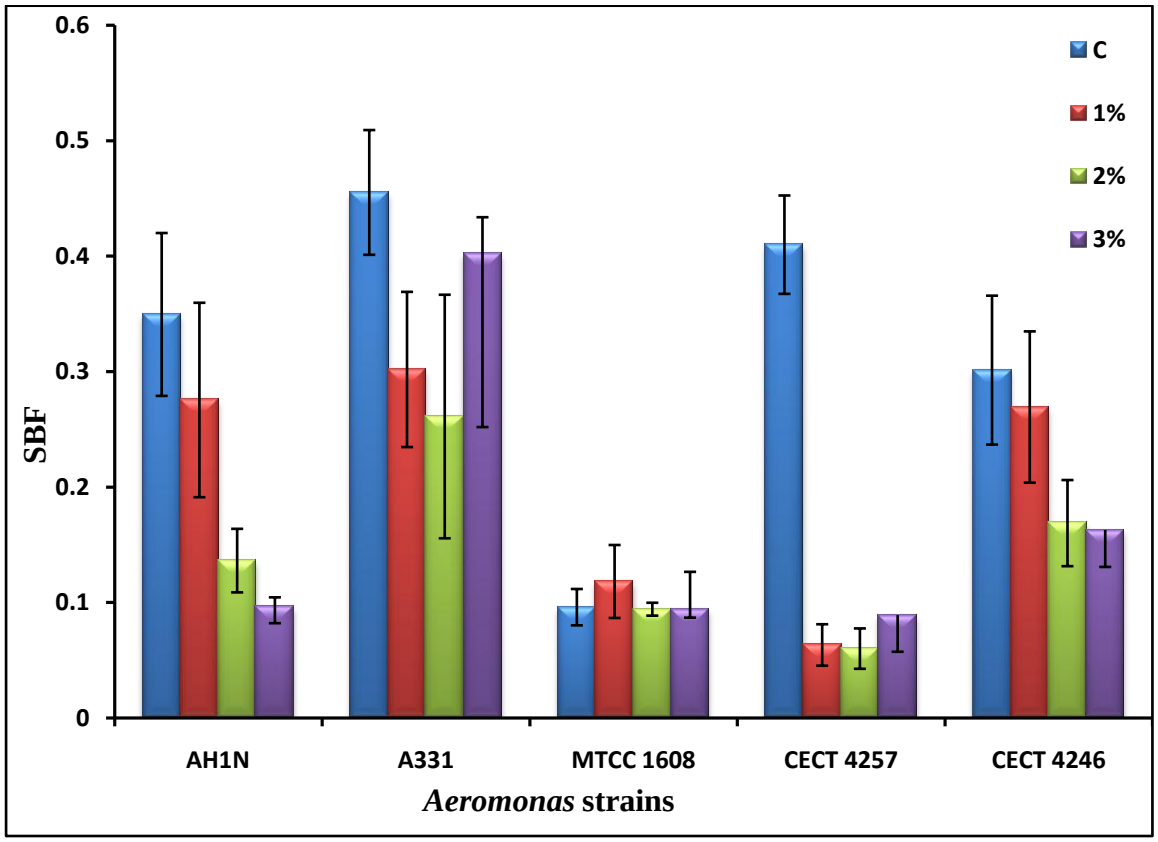


Figure 5.3. Biofilm formation by *Aeromonas* strains in TSB media containing additional different concentration of NaCl (0 to 3%)

Tang *et al.* (2012) have also reported reduction in biofilm formation by *Staphyloccoccus aureus* with increase in NaCl concentration. A reduction in hydrophobicity and increase in auto aggregation was observed in *L. monocytogenes*, *S. aureus*, *Shigella boydii*, and *Salmonella Typhimurium* strains with an increase in NaCl concentrations (0% to 10%) which highly correlated with the biofilm formation of these pathogens (Xu *et al.*, 2010).

5.3.4. Effect of pH on biofilm formation

Changes in pH can have a marked effect on bacterial growth and extreme pH is frequently exploited in the control of micro-organisms in food environments. Bacteria are

able to adapt to changes in internal and external pH by adjusting the activity and synthesis of proteins associated with many different cellular processes, including cell adhesion. Production of these proteins may lead to enhanced or reduced cell adhesion ability. In addition, production of extracellular polysaccharides which play an important role in anchorage and immobilizing bacterial cells on the surface is dependent on environmental pH.

Differences in the biofilm forming capacity of *Aeromonas* isolates were observed under different pH conditions (Fig. 5.4). Maximum SBF values were observed at pH 5 for most of the strains. However, among different pH range (5 to 9), minimum growth ($OD_{600\ nm}$) was observed at pH 5 for all the isolates. *Aeromonas* can tolerate pH range from 4.5 to 9.0, but the optimum pH range is from 5.5 to 9.0 (Isonhood and Drake, 2002). Increased biofilm production in stressful environments may be a form of survival response and has mainly been attributed to stress induced physiological adjustment in the cells (e.g., production of surface binding structures and EPS) resulting in an increased ability of the organism to attach to surfaces (Nilsson *et al.*, 2011). The chemical and physical parameters of the attachment surface may also be altered due to low pH (Folsom *et al.*, 2006). Nilsson *et al.* (2011) have also reported increased biofilm production in *L. monocytogenes* under the most acidic or most alkaline growth conditions.

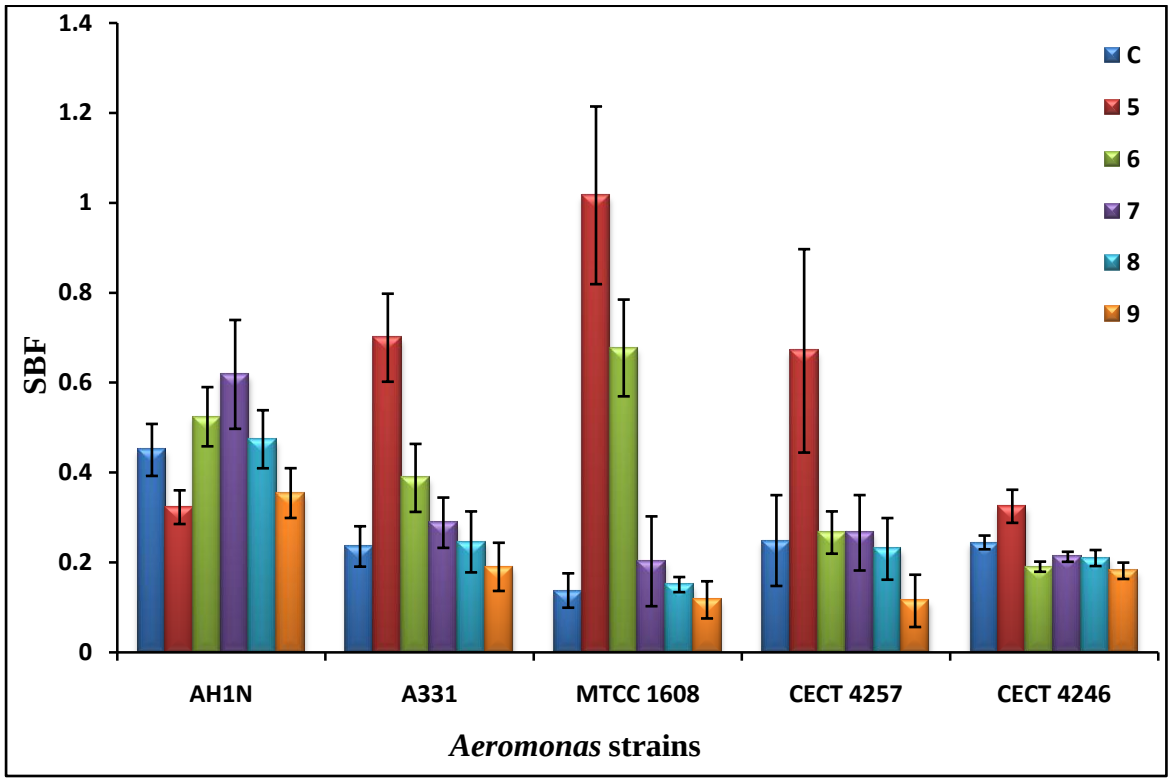


Figure 5.4. Biofilm formation by *Aeromonas* strains in TSB media with pH adjusted to different values (5 to 9)

Variations in the biofilm formation pattern by different isolates under various pH values were observed. *A. sobria* MTCC 1608 produced maximum biofilm at pH 5 and SBF index reduced with increase in pH from 5 to 9. In agreement with observations made in other studies (Reisner *et al.*, 2006; Schonewille *et al.*, 2012), the data presented here indicate that the effects of environmental conditions on biofilm formation may be strain-specific. The variability in biofilm formation by *Salmonella enterica* strains due to pH was found to be much greater than that caused by NaCl or temperature, and appeared to increase as the environmental conditions became less favorable for the organism (Lianou and Koutsoumanis, 2012).

5.3.5. Effect of food preservatives on biofilm formation

Food preservatives that are generally recognized as safe (GRAS) compounds are commonly added to food products to prevent the growth of food spoilage microbes in the food industry (Smid and Gorris, 2007). The effect of different concentration of food additives (sodium nitrite, sodium benzoate and potassium sorbate) on biofilm formation by *Aeromonas* strains was studied (Fig 5.5. A-C).

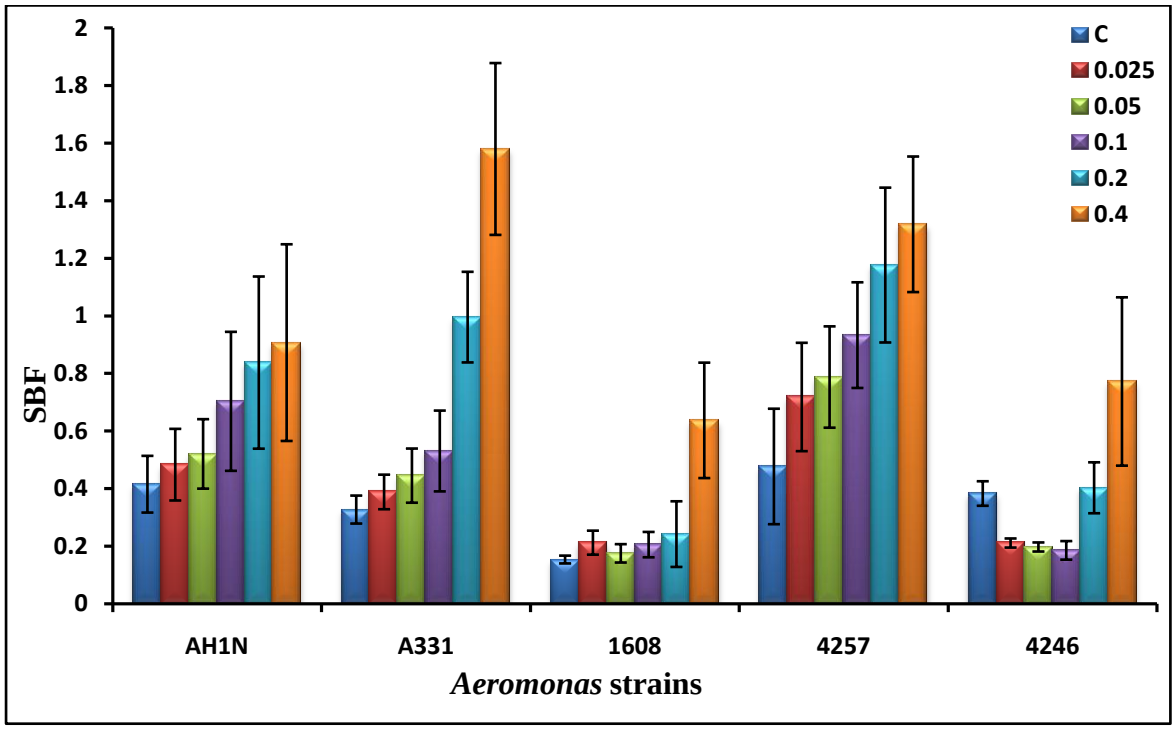


Figure 5.5.A. Biofilm formation by *Aeromonas* strains in TSB medium containing different concentration of potassium sorbate (0 to 0.4%)

An increase in the SBF index was observed with an increase in the concentration of potassium sorbate from 0.025% to 0.4% for most of the *Aeromonas* strains (Fig. 5.5.A). A reduction in biofilm (OD_{570nm}) was observed with an increase in concentration of potassium sorbate from 0.025% to 0.4%. However, significant reduction in the bacterial growth (OD_{600nm}) was simultaneously observed with an increase in concentration of potassium sorbate. Thus, overall it resulted in an increase in SBF index which indicates

that an increase in concentration of potassium sorbate resulted in more biofilm production per unit number of attached cells.

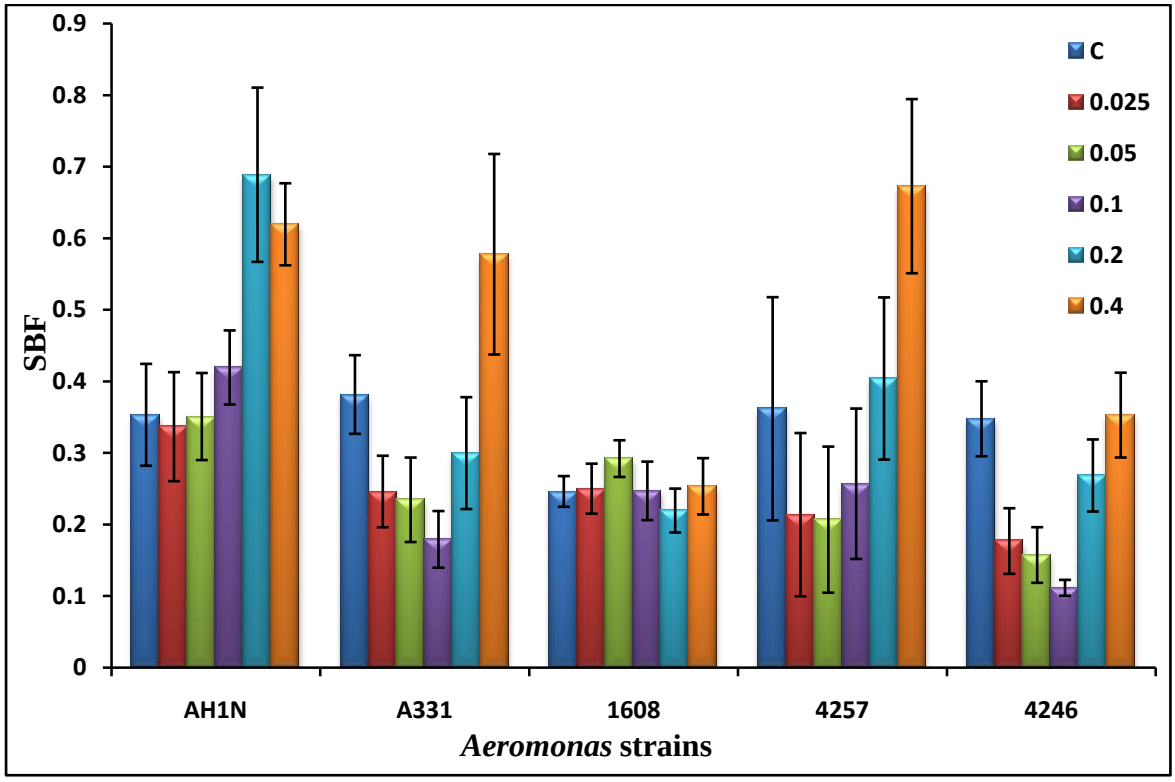


Figure 5.5.B. Biofilm formation by *Aeromonas* strains in TSB medium containing different concentration of sodium benzoate (0 to 0.4%)

The variability in biofilm formation by *Aeromonas* strains due to different concentration of sodium benzoate was observed. A decrease in the SBF index for most of the *Aeromonas* isolates was observed with an increase in sodium benzoate concentration from 0% to 0.1% (Fig. 5.5.B). A reduction in biofilm (OD_{570nm}) and growth (OD_{600nm}) was observed with an increase in sodium benzoate from 0% to 0.1% resulting in reduction of SBF index. However, the SBF index increased with an increase in sodium benzoate concentration from 0.1% to 0.4%. Insignificant reduction in biofilm (OD_{570nm}) and simultaneously significant reduction in growth (OD_{600nm}) was observed with an increase in sodium benzoate concentration from 0.1% to 0.4% resulting in increase in SBF index.

Strain-dependent variations in the biofilm forming ability of *Aeromonas* strains were observed under different concentrations of sodium nitrite. An increase in the SBF index was observed with an increase in sodium nitrite concentration from 0.03% to 0.24% (Fig. 5.5.C) for *A. hydrophila* strains A331 and AH1N. On the other hand, the SBF index of CECT 4257^{T} and CECT 4246 strains reduced with an increase in sodium nitrite concentration.

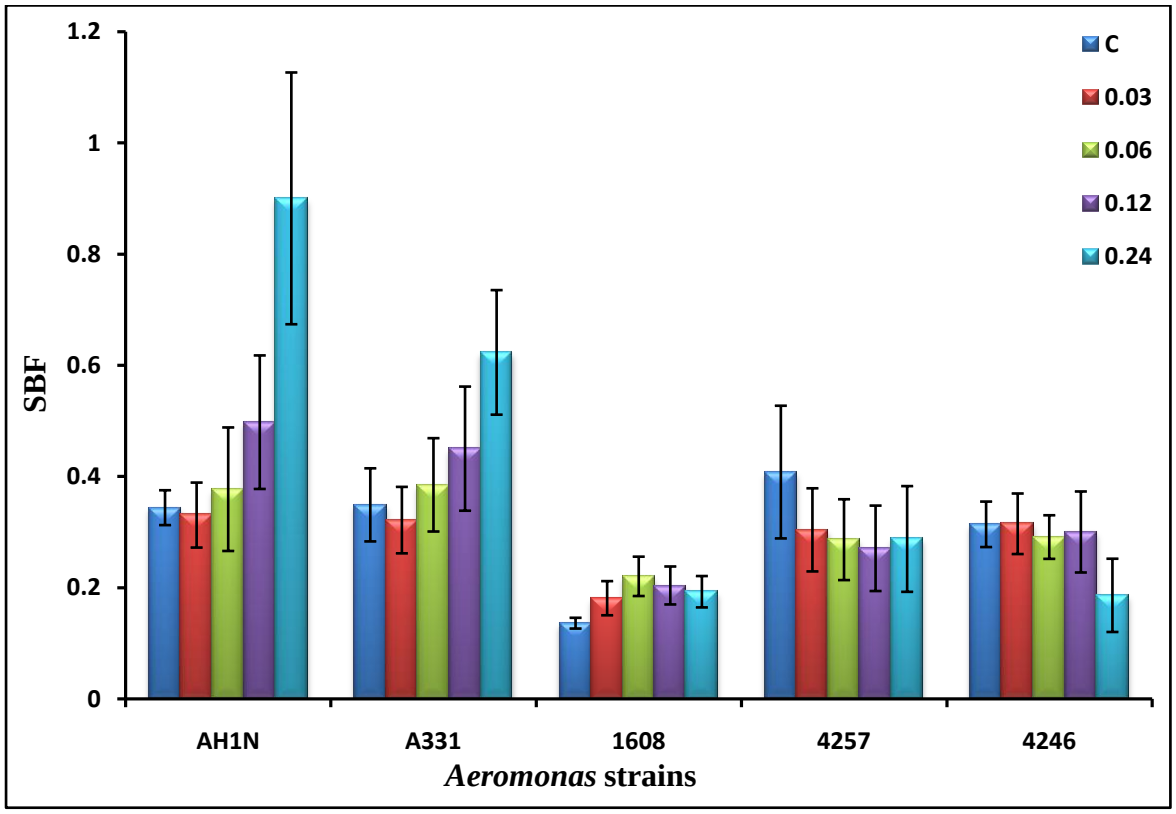


Figure 5.5.C. Biofilm formation by *Aeromonas* strains in TSB medium containing different concentration of sodium nitrite (0 to 0.24%)

The inhibitory mechanisms for sodium nitrite are not well known. Nitrite is reported to have dual effect on the cells. On one side, it can have a growth-promoting role; on the other side, high concentrations of nitrite and nitrite-derived compounds can be toxic due to the production of nitrous acid, which has strong bactericidal activity (Schlag *et al.*,

2007). The effectiveness depends on several factors including residual nitrite level, pH, salt concentration, reductants present and iron content (Sindelar and Milkowski, 2012).

Inhibition of biofilm formation by *Streptococcus mutans* using different food preservatives (sodium nitrite, sodium benzoate and potassium sorbate) has been observed (Al-Ahmad *et al.*, 2008). Inhibition of staphylococcal biofilm formation by sodium nitrite was observed by (Schlag *et al.* (2007). The food preservatives tested in this study are typical additives found in widely consumed processed food products in order to prolong their use. The food-relevant concentrations of 0.1% (w/v) for sodium benzoate and potassium sorbate and 0.06% (w/v) for sodium nitrite can be studied for biofilm inhibition in food systems. The study revealed that growth and biofilm formation by *Aeromonas* strains is inhibited by preservative concentrations similar to those typically used in food.

5.3.6. Minimum inhibitory concentrations (MIC) of different essential oils for *Aeromonas*

Essential oils are potential sources of novel antimicrobial compounds that inhibit bacterial growth and have variable effectiveness (Sandasi *et al.*, 2010). The MIC values of different essential oils for *Aeromonas* strains were determined using microtiter broth micro-dilution method. The MIC values for different essential oils against *A. hydrophila* CECT 839^T and *A. veronii* bv. *veronii* CECT 4257^T in M9 minimal media supplemented with 0.4% glucose and TSB ranged from 0.125 to > 8.0 mg/ml (Table 5.2). Both *Aeromonas* species were found to be very sensitive to different essential oils. Our results are in agreement with Burt (2004) who has reported *A. hydrophila* to be the most sensitive to antimicrobials amongst gram-negative species. Species-dependent differences in MIC values were not observed for most of the essential oils. In both M9 minimal media and

TSB, the MIC values of clove, ajowain, cinnamon, tea-tree and cumin oils were found to be ≤ 2 mg/ml (Table 5.2). Hoque *et al.* (2008) have reported the MIC values of clove and cinnamon oil against *A. hydrophila* to be 0.5 - 0.8 mg/ml and 1.25 mg/ml, respectively. Both garlic (75%) and nutmeg (100%) extracts showed antibacterial activity against *A. hydrophila*; however, ginger (100%) extract did not show any growth inhibition (Indu *et al.*, 2006).

An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Tajkarimi *et al.*, 2010). Extensive loss of cell contents from bacterial cells or the exit of critical molecules and ions will lead to death (Tiwari *et al.*, 2009). Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but that there are several targets in the cell (Bakkali *et al.*, 2008).

Out of 21 different plant essential oils tested, cinnamon oil (MIC values - 0.8 to 3.2 mg/ml) and clove oil (MIC values - 1.6 to 6.4 mg/ml) were found to be most effective against six different pathogens (*S. aureus*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *E. coli*.) (Prabuseenivasan *et al.*, 2006). Ajowain oil showed significant antibacterial activities against *S. aureus*, *E. coli* and *S. Typhimurium* (Goudarzi *et al.*, 2011). The MIC values of tea-tree oil against *E. coli* were in the range of 0.08 - 2% v/v (Carson *et al.*, 2006) and clinical *S. aureus* isolates in the range 0.125 - 0.5% v/v (Kwiecinski *et al.*, 2009).

Table 5.2. Minimum inhibitory concentration (MIC) of selected essential oils for *A. hydrophila* CECT 839^T and *A. veronii* bv. *veronii* CECT 4257^T

S. No.	Essential Oil	Botanical name (Family)	Media	MIC values (mg/ml)	
				***A. hydrophila* CECT 839^T**	***A. veronii* bv. *veronii* CECT 4257^T**
1	Cinnamon	*Cinnamomum zeylanicum* (*Lauraceae*)	M9	0.125	0.125
			TSB	0.125	0.125
2	Clove	*Eugenia caryophyllus* (*Myrtaceae*)	M9	0.125	0.125
			TSB	0.5	1
3	Ajowain	*Carum copticum* (*Apiaceae*)	M9	1	1
			TSB	0.25	0.25
4	Tea-tree	*Melaleuca alternifolia* (*Myrtaceae*)	M9	0.5	1
			TSB	2	2
5	Cumin	*Cuminum cyminum* (*Umbelliferae*)	M9	1	2
			TSB	1	1
6	Basil	*Ocimum sanctum* (*Labiatae*)	M9	1	1
			TSB	2	4
7	Citronella	*Cymbopogon winterianus* (*Poaceae*)	M9	2	4
			TSB	1	1
8	Peppermint	*Mentha piperita* (*Labiatae*)	M9	2	4
			TSB	2	2
9	Nutmeg	*Myristica fragrans* (*Myristicaceae*)	M9	2	2
			TSB	8	8
10	Black Pepper	*Piper nigrum* (*Piperaceae*)	M9	4	4
			TSB	>8	>8
11	Lemon	*Citrus limon* (*Rutaceae*)	M9	8	>8
			TSB	8	>8
12	Ginger	*Zingiber officinale* (*Zingiberaceae*)	M9	8	8
			TSB	>8	>8
13	Turmeric	*Curcuma longa* (*Zingiberaceae*)	M9	>8	>8
			TSB	>8	>8
14	Orange	*Citrus sinensis* (*Rutaceae*)	M9	>8	>8
			TSB	>8	>8
15	Eucalyptus	*Eucalyptus globulus* (*Myrtaceae*)	M9	8	8
			TSB	>8	>8
16	Lavender	*Lavandula angustifolia* (*Labiatae*)	M9	>8	>8
			TSB	>8	>8
	Ciprofloxacin		M9	0.007813	0.007813
			TSB	0.007813	0.007813

Generally, the EOs possessing the strongest antibacterial properties against food-borne pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol. It seems reasonable that their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (Burt, 2004). The major antibacterial compounds present in different essential oils are: cinnamaldehyde (cinnamon oil), eugenol and isoeugenol (clove oil), thymol and carvacrol (ajowain oil), carvone and carvacrol (cumin oil) and terpinen-4-ol and terpineol (tea-tree oil) (Hyldgaard *et al.*, 2012).

The antibacterial activity of clove oil may be attributed to eugenol which disrupts cell membrane and inactivates enzymes and genetic material. Cinnamaldehyde has been shown to penetrate the cell membrane due to its lipophilicity of terpenoids and phenyl propanoids and reach the inner part of the cell and impair bacterial enzyme system (Bakkali *et al.*, 2008). Thymol present in ajowain oil might induce its antimicrobial action by perturbation of the lipid fraction of the microorganism plasma membrane, resulting in alterations of the membrane permeability and leakage of intracellular materials (Goudarzi *et al.*, 2011).

The MIC values for ginger, turmeric, orange, eucalyptus, lemon and lavender oils were $\geq$ 8 mg/ml in both TSB and M9 minimal media (Table 5.2). However, the MIC values of nutmeg and basil oils were found to be $\leq$ 2 mg/ml in M9 minimal media; whereas, MIC values were 8 and $\leq$ 4 mg/ml in TSB media, respectively. These results indicate that media components present in TSB may interact with essential oils, reduce its antimicrobial activity and thus increase the MIC values. Haraszthy *et al.* (2006) have also observed media and method-dependent variations in minimal inhibitory concentrations of

anti-plaque agents on oral bacteria. Since *Aeromonas* species are sensitive to clove, cinnamon and ajowain in both rich and nutrient-limited media, these essential oils with MIC values ≤ 1 mg/ml can be further used to inhibit the growth or attachment of *Aeromonas* to food or contact surfaces.

5.3.7. Effect of essential oils on biofilm formation

The effect of three essential oils (clove, ajowain and cinnamon) at a concentration of 1 mg/ml on the inhibition of cell attachment and biofilm growth of *Aeromonas* (*A. hydrophila* CECT 839^T and *A. veronii* bv. *veronii* CECT 4257^T) strains was studied using crystal-violet (CV) assay. The growth (OD_{600nm}) and biofilm formation (OD_{570nm}) for both the strains was significantly inhibited by these essential oils after incubation for 8 h. The results indicate the potential of these essential oils in inhibition of growth and cell attachment of *Aeromonas* strains on the microtiter plates. The attachment of cell to the surface is the initial stage in biofilm formation following surface conditioning which creates a favourable environment for the bacterial attachment. Surface conditioning is achieved by the adsorption of substances that include nutrients, organic and inorganic molecules that are important for the growth of the cells, which in turn promotes cell adhesion (Jahid and Ha, 2012). The pre-treatment of the surface with essential oils may produce an unfavourable film that promotes cell detachment and thereby reducing the surface adhesion. However, earlier reports have shown that the inhibition of cell attachment to a substrate is easier to achieve than inhibiting the growth of an already established biofilm (Sandasi *et al.*, 2010). Thus, further studies on the effect of these essential oils on the pre-formed *Aeromonas* biofilm on the food or contact surfaces need to be done to ensure their application in the food industry.

5.4. Conclusions

The present study showed highly diverse and complex patterns of biofilm formation by ten *Aeromonas* strains exposed to a range of media, temperature, pH, NaCl and food preservatives. Significant strain-dependent variations in the biofilm forming ability under different food-related stresses were observed. Thus, screening of several *Aeromonas* isolates should be done before drawing general conclusions regarding biofilm formation. The study also demonstrated the antibacterial activity of different essential oils against *Aeromonas* species. These EOs are considered as safe food additives and are promising antibacterial agents. However, the anti-biofilm ability and the practicality of using of these essential oils in the food-processing environment needs to be further studied.

CHAPTER 6

Expression Pattern of General Stress-Response and Virulence Genes in *A. hydrophila* under Various Stress Conditions

6.1. Introduction

Aeromonas hydrophila possesses intrinsic characteristics such as resistance to antibiotics and chlorine, ability to survive at low temperature, ability to form biofilm on food surfaces, and production of virulence factors like aerolysin that contribute to its significance as a food-borne pathogen (Elhariry, 2011). A large array of genes involved in metabolic fitness and virulence make *A. hydrophila* adaptive to diverse environmental conditions and hosts. This is why it is generally referred as "jack-of-all-trades" (Seshadri *et al.*, 2006). The control of *A. hydrophila* during production and storage of food products is one of the vital steps in public protection against its infection. In addition, the environmental conditions that *A. hydrophila* may encounter in foods could influence its virulence potential. As the exposure of bacteria to various stresses often leads to increased virulence (Wesche *et al.*, 2009), there is a need to understand the influence of different stresses on the expression of virulence and stress responsive genes in *A. hydrophila*.

Though there are few reports regarding the influence of environmental factors like temperature, salinity and pH on the survival and pathogenicity of *A. hydrophila* (Pianetti *et al.*, 2008; Vivekanandhan *et al.*, 2003), a knowledge void exists regarding the impact of intervention strategies, food matrices, and processing environment–related conditions on the expression of general stress-response and virulence genes in *A. hydrophila*. Till date, there are no reports regarding the effect of stress on the expression of stress-response and virulence genes in *A. hydrophila*.

Most of the earlier studies on other food-borne pathogens have reported effect of various stresses on the stress responsive and virulence genes of the exponentially growing cells. These logarithmically growing cells are physiologically distinct from cells found in

natural and production environments (Navarro Llorens *et al.*, 2010). The cells in the food environment are generally in stressed conditions similar to that of stationary phase-like state. The microorganism encounters various stress conditions like nutrient replenishment, nutrient starvation, acid shock, alkaline shock, cold shock and heat shock during various stages of food production, processing and preservation, and in natural and host environments.

A number of studies have reported the expression of various housekeeping, stress response and virulence genes of different bacterial species under diverse stress conditions. These include the genes known to be involved in housekeeping functions, like the genes encoding primary sigma factor (*rpoD*) which keep essential genes and pathways operational, and glyceraldehyde-3-phosphate dehydrogenase (*gapA*), general stress regulatory genes *rpoS* and *uspA* and virulence genes. The aim of this study was to determine the impact of various food-related stress events (nutrient replenishment, nutrient starvation, pH 4 and pH 9, temperature 8 °C and 37 °C) on the expression of housekeeping (*rpoD* and *gapA*), general stress response (*rpoS* and *uspA*) and virulence (*aer*) genes in stationary phase *A. hydrophila* CECT 839^T (reference and sequenced strain) and *A. hydrophila* A331 (food isolate) using quantitative real-time PCR (RT-qPCR). The expression of different genes in two different *A. hydrophila* strains, CECT 839^T (milk isolate) and A331 (chicken isolate), under various stress conditions was compared.

6.2. Materials and methods

6.2.1. Bacterial strains and growth conditions

For the gene expression studies, two strains of *A. hydrophila* were used: the type strain CECT 839^T (milk isolate; obtained from Dr. Valérie Leclère, Université des

Sciences et Technologies de Lille USTL, Villeneuve d'Ascq cedex, France) and other strain A331 (chicken isolate; identified as *A. hydrophila* by 16S rRNA and *rpoD* gene sequencing) (Chapter 2). Stocks of *A. hydrophila* strains were maintained in tryptic soy broth (TSB; Hi-Media, India) containing 20% glycerol at -80 °C. Cultures were also maintained at 4 °C on tryptic soy agar (TSA; Hi-Media, India) plates.

6.2.2. Stress treatment

The impact of six treatments (acid/ alkaline shock, cold/ heat stress, and nutrient deprivation/ replenishment) was assessed in stationary phase of both the *A. hydrophila* strains (CECT 839^T and A331) (Figure 6.1). In brief, a loopful of culture from TSA slant was grown for 18 h (30 °C, 150 rpm) in 25 ml TSB broth. For each biological replicate of both the strains, a 1:100 dilution in 100 ml of TSB in a 250-ml flask was incubated for 18 h at 30 °C, 150 rpm separately. Both the cultures were divided into seven groups of 5-ml aliquots each representing one control and six treatments (nutrition replenishment, nutrition deprivation, cold-shock, heat-shock, acid-shock and alkaline-shock). All the tubes were centrifuged (22 °C for 10 minutes at 12,000 x g) and resuspended in the spent media adjusted to different conditions.

Nutritionally replenished (NR) and deprived (ND) cells were resuspended in 5 ml of pre-tempered (30 °C) fresh TSB (pH 7.2) and 0.85% saline, respectively. For acid and alkaline shocks, cells were resuspended in 5 ml of spent TSB media adjusted to pH 4 using 1N HCl and pH 9 using 1N NaOH, respectively. For cold shock and heat stress, cells were resuspended in 5 ml of pre-tempered spent TSB media (temperature adjusted to 8 °C and 37 °C), respectively. All the cells exposed to different treatments were incubated for 30 minutes under defined conditions on shaker incubator at 150 rpm.

Control cells were resuspended in the original 5 ml of spent TSB maintained at 30 °C. Post-incubation, samples were taken for the lethality assessment and RNA isolation.

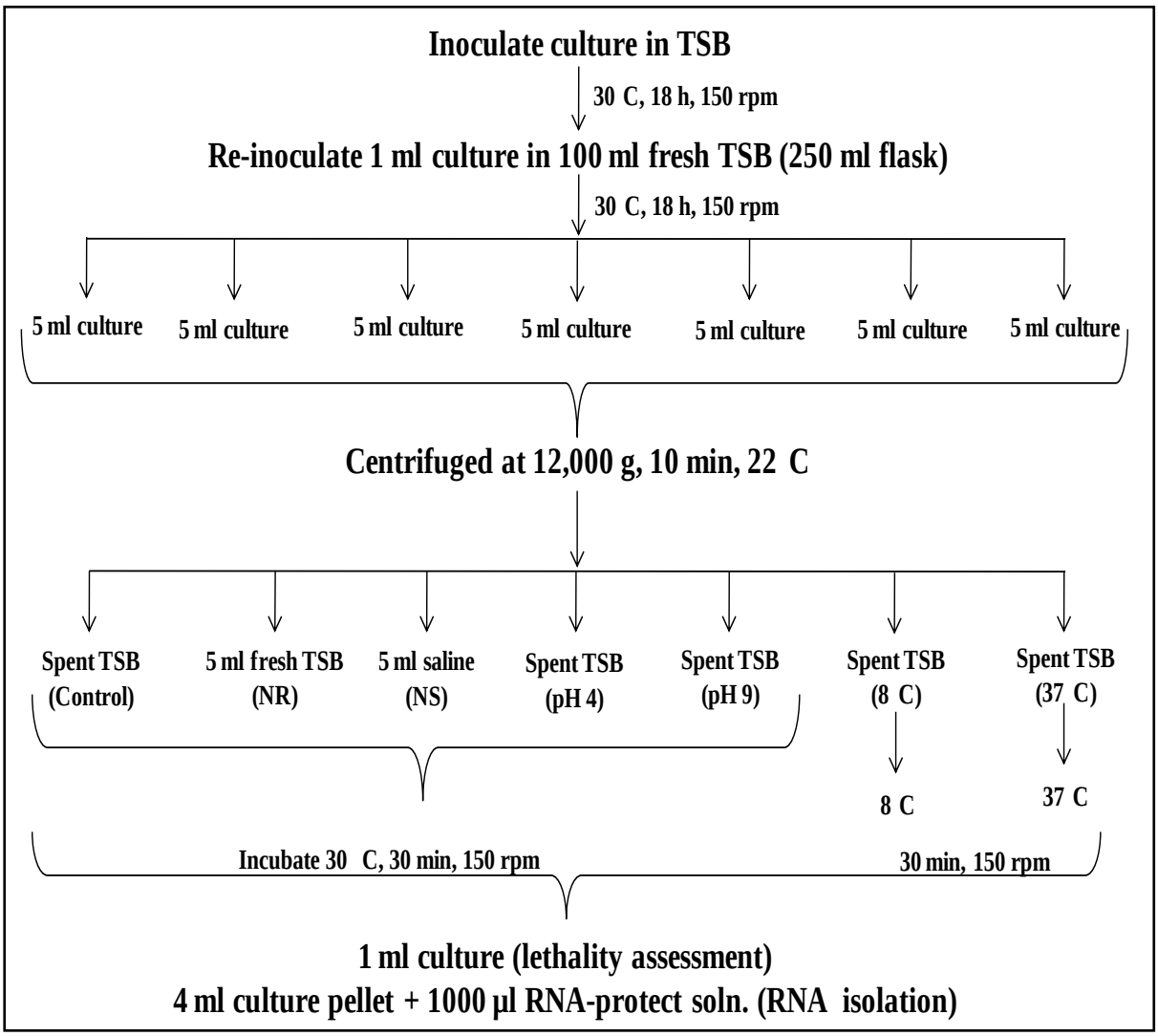


Figure 6.1. Flow chart for stress treatment and lethality assessment

6.2.3. Lethality assessment

For lethality assessment, 1 ml of culture was taken out at 0 min from control, and 30 min from the control and six treatment conditions, serially diluted, and pour-plated onto TSA. Enumeration of colony forming units (CFU) was performed after incubation at 30 °C for 24 h and comparisons were made between control and treatment samples.

6.2.4. RNA isolation

Four ml of culture from control and treatment samples was transferred to microcentrifuge tubes and centrifuged at 10,000 x g for 5 min. To stabilize RNA transcripts, cell pellets were resuspended in 1 ml of RNA protect bacteria reagent (Qiagen, GmbH, Hilden, Germany). All samples were stored at -80 °C until RNA extraction was carried out. Total RNA was isolated using Triazol method (Tri-Reagent-RT, Molecular Research Center Inc., Cincinnati, Ohio, USA) according to the manufacturer's instructions. Briefly, cells were lysed by suspending in 10 mM Tris-Cl-1 mM EDTA buffer (pH 8.0 containing lysozyme 2 mg/ml) and then thawed in liquid nitrogen. One ml Tri-reagent-RT was added to the sample and vortexed for 10 min. Fifty microlitres of bromoanisole was added to the mixture, incubated at room temperature for 10 min and centrifuged at 10,000 x g for 10 min at 4 °C. The upper aqueous phase was mixed with equal volume of isopropanol and the samples were centrifuged at 12,500 x g for 15 min at 4 °C. The RNA pellet was washed twice with 800 µl of chilled 70% ethanol, and then air-dried, and the RNA was resuspended in RNase-free milliQ water and stored at -80 °C until used. RNA quality and quantity was determined for individual preparations using non-denaturing agarose gel electrophoresis and by spectrophotometric analysis (Eppendorf, Germany) at OD_{260nm} and ratio of $OD_{260:280nm}$ and $OD_{260:230nm}$. The RNA samples were treated with RNase-free DNase I (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions to remove any contaminating genomic DNA. Complete removal of genomic DNA from the DNase-treated samples was checked by performing real-time PCR.

6.2.5. cDNA preparation

About 1µg of DNase-treated total RNA was subjected to cDNA synthesis using DyNAmo^{TM} cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the supplier's directions. The reverse transcription reaction was performed sequentially for 10 min at 25 °C, for 60 min at 37 °C, and for 5 min at 85 °C. All the cDNA preparations were stored at -20 °C.

6.2.6. Primer design

Complete sequences for the genes of interest were obtained from the PubMed database (accession number NC_008570.1). The gene-specific primers for the housekeeping, general stress response and virulence genes (Table 6.1) were designed using integrated DNA technologies Primer Quest software (www.idtdna.com/site). The amplicon sizes ranged between 110 to 190 bp. Melting temperatures of the primers were designed for 60 °C, with a melting temperature difference of less than 2 °C for each primer pair. Primers were obtained from Metabion International (Germany) (Table 6.2).

Table 6.1. Selected *A. hydrophila* genes related to ancillary housekeeping functions, stress response and virulence for quantitative real-time PCR analysis

Functional category	**Genes**
Housekeeping genes	*gapA*, *rpoD*, 16S rRNA
General stress-response & global regulators	*rpoS*, *uspA*
Virulence genes	*aer*

Table 6.2. Primers for quantitative real-time PCR analysis of various housekeeping, stress-response and virulence genes in *A. hydrophila* CECT 839^{T} and A331 following various food-environment related stresses

Gene	**Sequence (5' ⟶ 3')**
16S rRNA	Forward: TGGCCTTGACATGTCTGGAATCCT
16S rRNA	Reverse: ACCATTACGTGCTGGCAACAAAGG
gapA	Forward: TGCTCAAGGTGATGCAGGACAAGT
gapA	Reverse: AGTTGTCGATGAGGTTCTGGTCGT
rpoD	Forward: AACAAGCTCAACCGTATCTCCCGT
rpoD	Reverse: TGGATATGGGCTCTTTGGCGATCT
rpoS	Forward: TCCGATGAACTGATGGCACCTGAA
rpoS	Reverse: GCAAATTGGACTCGATCATGCGCT
uspA	Forward: CGCTCAAATCACCACAGATCACCA
uspA	Reverse: TATCGACATCGACAACGTGCAGGA
aerA	Forward: TTGCATACAACCTGGACCCTGACA
aerA	Reverse: TCTTGGACCAGTTGGTGGCAGTAT

6.2.7. Quantitative real-time PCR (RT-qPCR) analysis

The SYBR green RT-qPCR assay was performed using amplification master cycler ep *realplex* (Eppendorf, Germany) and DyNAmo Flash SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) at 94 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 10 s, annealing at 56 °C for 10 s and extension at 72 °C for 20 s. Two microlitres of the template was amplified in a 20 µl reaction volume containing primers at a final concentration of 0.5 µM, 10 µl of 2X DyNAmo Flash SYBR Green

Master Mix, and water. Following amplification, threshold cycle (C_T) values for each target genes over different stress conditions for each *A. hydrophila* strain were determined and a melting curve analysis of PCR products was performed to ensure the specificity of the PCR. The relative expression ratios of the target genes over different stress conditions for each *A. hydrophila* strain were calculated according to the Pfaffl method using 16S rRNA levels as the reference (Pfaffl, 2001). As 16S rRNA gene was not affected by various stress treatments, it was selected as the calibrating gene. All treatments were compared to control cDNA derived from stationary phase cells. The relative expression ratios were calculated using the REST-MCS version 2 software (http://www.gene-quantification.de/rest-mcs.html). The RT-qPCR was performed with three biological and three technical replicates. Only genes with a relative signal $\log_2$ ratio value above 1.0 or below −1.0 were considered to be significant.

6.3. Results and Discussion

6.3.1. Effect of stress on survival of *A. hydrophila* cells

The effect of different stresses on the survival of *A. hydrophila* CECT 839^T and A331 was studied. Cell counts of the control CECT 839^T and A331 strains were 3.1 x 10^9 and 4.2 x 10^9 CFU/ml, respectively at 0 min. No significant differences ($P > 0.05$) in CFUs were observed between control and all treatment cells for both the strains after 30 min (Fig. 6.2). Results indicate that the viability of both *A. hydrophila* CECT 839^T and A331 cells were not significantly affected by the treatment conditions and the cells were under sub-lethal stress. Similar observations were reported in CFUs of control and treatment (nutritional replenished, acid-shocked and cold-shocked) cells of *E. coli* O157:H7 (Allen *et al.*, 2008).

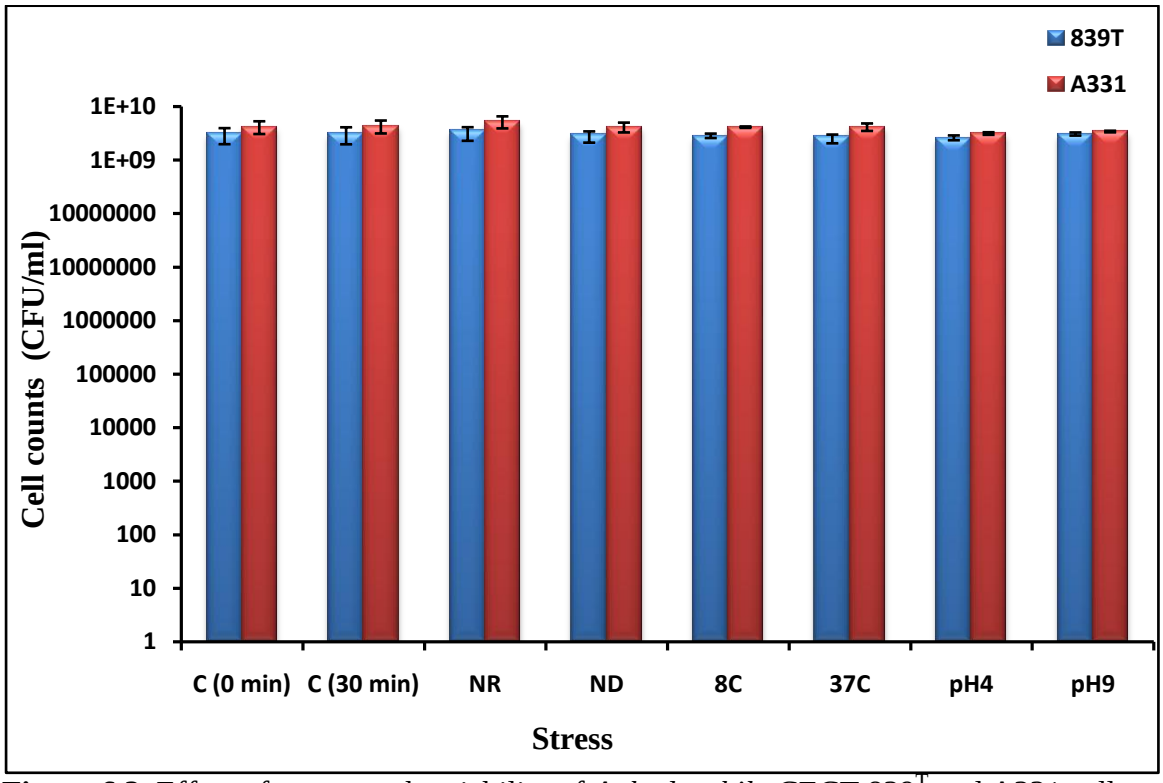


Figure 6.2. Effect of stress on the viability of *A. hydrophila* CECT 839^T and A331 cells

6.3.2. Gene expression after nutrient replenishment for 30 min

The housekeeping genes (*rpoD* and *gapA*) were significantly up-regulated by 6.2- and 1.3-fold in *A. hydrophila* CECT 839^T and 5.5- and 1.2-fold in *A. hydrophila* A331, respectively on resuspension of 18 h old *A. hydrophila* cells into fresh TSB (Fig. 6.3). RpoD (σ^{70}) is a 70 kDa "housekeeping" or primary sigma factor that recognizes promoters of the growth-related and housekeeping genes expressed in the exponential phase of bacterial growth and transcribes most genes in the growing cells. Glyceraldehyde-3-phosphate dehydrogenase (*gapA*) is a housekeeping gene that catalyzes the conversion of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate during glycolysis. The increased expressions of these genes show that the cells are actively growing after nutrient replenishment. *A. hydrophila* strains may be experiencing the ‘‘feast’’ aspect of bacterial life on resuspension of the stationary phase cells into fresh TSB. Similar trend was

observed in *E. coli* O157:H7 following nutrient replenishment with significant up-regulation of 52% genes, including genes involved in DNA replication and maintenance (*dnaE, hupB, mutH, mutL* and *mutS*) and housekeeping genes (*dnaA, rpoA, tufA, gapA* and *narH*) involved in protein synthesis and carbon and amino acid metabolism (Allen *et al.*, 2008).

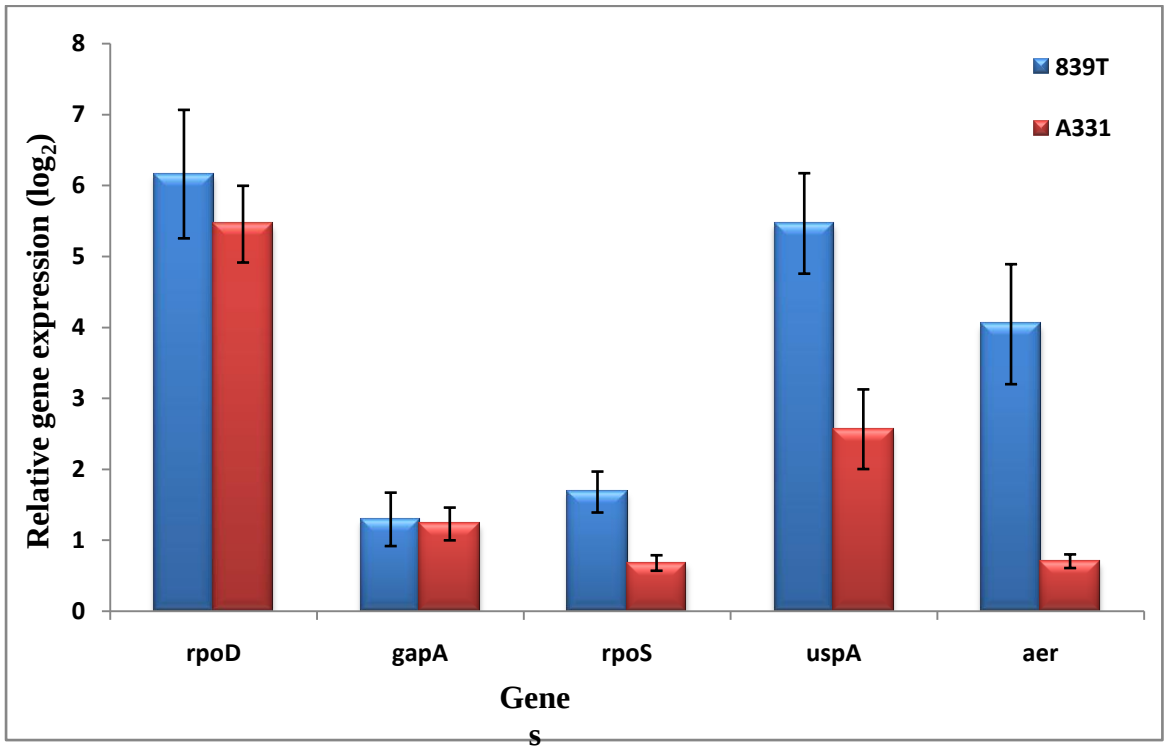


Figure 6.3. Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839^T and A331 in response to nutrient replenishment for 30 min

RpoS (σ^S), a 38 kDa alternative sigma factor, widely present in many gram-negative bacteria, is a general stress response regulator and regulates different stationary phase and stress response genes. Significant induction of *rpoS* gene was observed in CECT 839^T (1.7-fold); whereas, it was marginally expressed in A331 (0.7-fold) (Fig. 6.3) indicating that different strains of *A. hydrophila* may have different abilities to cope with the stress. Though it is well documented that *rpoS* plays an important role in stationary

phase, its role in exponential phase cells has been shown lately (Dong *et al.*, 2008). RpoS is likely to play a fine-tuning regulatory role in early exponential phase to adjust gene expression in the preparation for any potential stress, distinct from its role of active protection for cell survival in stationary phase cells (Battesti *et al.*, 2011). It is possible that stationary phase *A. hydrophila* cells may undergo considerable physiological reprogramming dominated initially by stress response induction to adapt to a nutrient rich environment.

The expression of *uspA* gene in CECT 839^T and A331 was significantly up-regulated by 5.5- and 2.6-fold, respectively on resuspension of 18 h old *A. hydrophila* cells into fresh TSB (Fig. 6.3). UspA, a member of universal stress proteins (Usps), is reported to be highly expressed in response to a large number of stresses like heat, substrate starvation for glucose or phosphate, upon entry to stationary phase in rich medium, exposure to antimicrobial agents and oxidative stress (Kvint *et al.*, 2003). Allen *et al.* (2008) have also observed induction of *uspA* gene by 2.8-fold on resuspension of stationary phase *E. coli* O157:H7 cells into fresh TSB.

Aerolysin (*aer*) is a major virulence factor of *Aeromonas* and possesses both haemolytic and enterotoxic activity. The *aer* gene was up-regulated by 4.0- and 0.7-fold in *A. hydrophila* CECT 839^T and A331, respectively (Fig. 6.3) demonstrating that the ability to produce aerolysin may vary in different strains of *A. hydrophila*. Expression of *aer* gene was significantly induced in *A. hydrophila* CECT 839^T (milk isolate) as compared to A331 (chicken isolate) following nutrient replenishment. It is possible that media stimulation resulting from fresh TSB or the induced physiological switch from stationary phase to exponential growth may contribute to induction of virulence genes. Similar significant inductions of virulence (*stx*1 and *hly*) genes have been reported in *E. coli* O157:H7

following media stimulation (Allen *et al.*, 2008). Up-regulation of numerous genes associated with attachment, virulence, oxidative stress, antimicrobial resistance and DNA repair was observed in *E. coli* O157:H7 on exposure to carbohydrates rich lysates of lettuce leaves (Kyle *et al.*, 2010).

6.3.3. Gene expression after nutrient deprivation for 30 min

Significant up-regulation of *gapA* gene by 1.9- and 1.6-fold was observed in *A. hydrophila* CECT 839^T and A331, respectively. The *rpoD* gene was significantly up-regulated by 2.0- fold in CECT 839^T; whereas, it was slightly induced in A331 (0.8-fold) (Fig. 6.4). There are no reports on the expression of these genes under nutrient deprivation in *Aeromonas* species. However, our results contradict the observation of Allen *et al.* (2010), who showed attenuation of house-keeping genes (*dnaA*, *rpoA*, *tufA*) in *E. coli* O157:H7 cells subjected to nutrient deprivation.

Significant up-regulation of *rpoS* gene by 2.3- and 1.5-fold was observed in CECT 839^T and A331, respectively on resuspension of 18 h cells into normal saline (Fig. 6.4). RpoS controls up to 10% of the *E. coli* genes and prepare the cell for survival in stress conditions (Weber *et al.*, 2005). Induction of *rpoS* gene by 1.2-fold was observed in microarray analysis of *E. coli* O157:H7 cells subjected to nutrient deprivation (Allen *et al.*, 2010). A mild induction of *rpoS* gene may have a significant impact on survival of the microorganism in the food chain (Vasudevan and Venkitanarayanan, 2006).

The *uspA* gene was up-regulated by 3.3- and 2.2-fold in *A. hydrophila* CECT 839^T and A331, respectively (Fig. 6.4). Synthesis of UspA (*uspA*) and its paralogs: UspC (*yecG*), UspD (*yiiT*), UspE (*ydaA*), and UspG (*ybdQ*) is reported to be induced by

starvation for glucose or phosphate and upon entry to stationary phase in rich medium (Siegele, 2005).

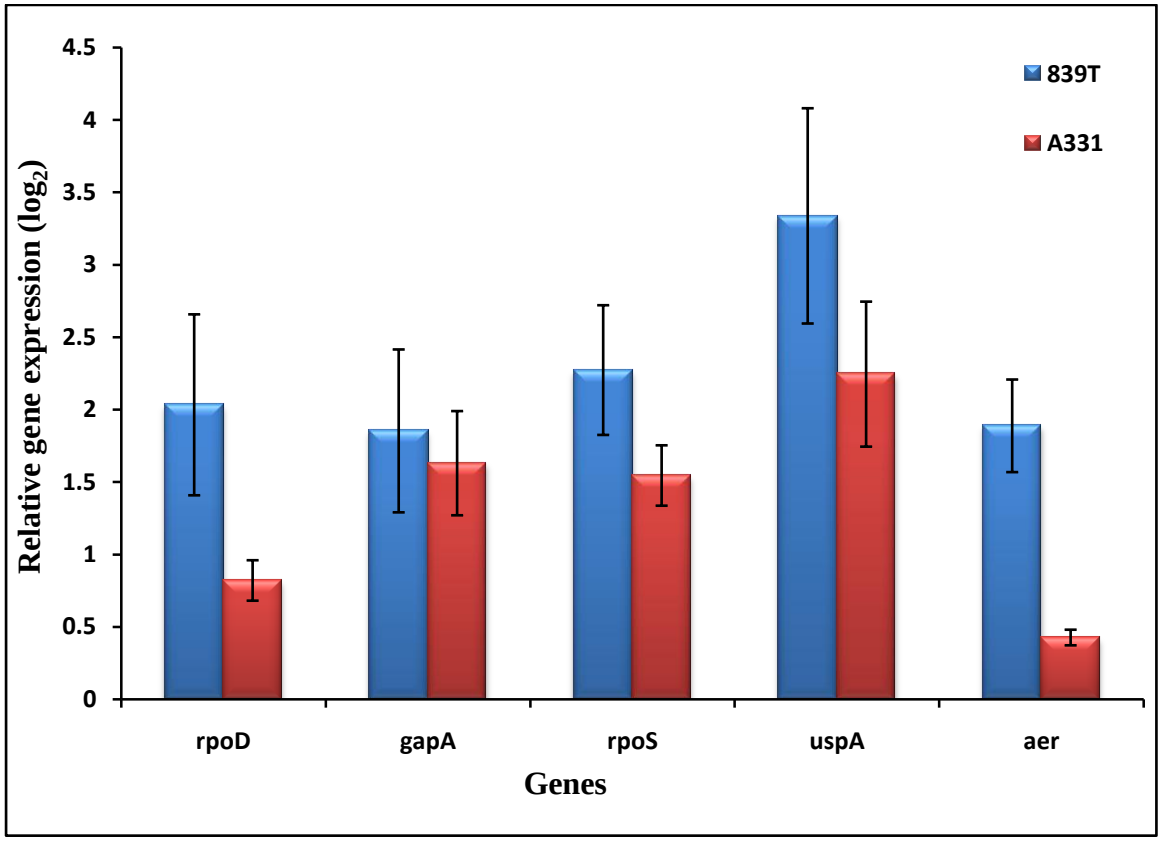


Figure 6.4. Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839^T and A331 in response to nutrient deprivation for 30 min

The expression of *aer* gene was significantly induced in CECT 839^T (1.9-fold), whereas, it was marginally expressed in A331 (0.4-fold) after nutrient deprivation for 30 min (Fig. 6.4). Strain-dependent variation in the expression of aerolysin was observed in *A. hydrophila* following nutrient deprivation. There are no reports of expression of aerolysin under nutrient deprivation in *Aeromonas* species. However, induction of Shiga toxin (*stx*) due to starvation has been reported in *E. coli* O157:H7 (Leenanon *et al.*, 2003). Allen *et al.* (2010) have reported the induction of virulence genes (*hlyB*, *katP* and *stx2b*) in *E. coli* O157:H7 following nutrient deprivation.

Overall, the expression of *rpoD, uspA* and *aer* genes was more in both the *A. hydrophila* strains under nutrient replenishment condition as compared to nutrient deprivation; whereas higher expressions of *gapA* and *rpoS* genes were observed in nutrient deprivation as compared to nutrient replenishment.

6.3.4. Gene expression after cold shock (8 °C) for 30 min

A. hydrophila may be exposed to cold shock and long-term refrigeration during prolonged storage of foods. Thus, the impact of cold shock on *A. hydrophila* stress and virulence physiology was studied. Significant induction of housekeeping (*rpoD* and *gapA*) genes was observed in *A. hydrophila* CECT 839^T and A331 cells following cold-shock (8 °C) for 30 min (Fig. 6.5). In only cold stress, the induction of *rpoD* gene was more in A331 as compared to CECT 839^T; whereas, in all other studied stresses higher induction of *rpoD* gene was observed in CECT 839^T. Allen *et al.* (2008) observed induction of housekeeping gene *dnaA* in *E. coli* O157:H7 cells subjected to cold shock; whereas, *gapA* and *tufA* genes were attenuated.

The *rpoS* gene was significantly induced in *A. hydrophila* CECT 839^T (1.9-fold); whereas, it was marginally induced in A331 (0.9-fold) (Fig. 6.5). The secondary structure of the *rpoS* transcript may be altered due to the decreased temperature and thus resulting in a stabilized molecule that leads to increased levels of σ^S (Hengge-Aronis, 2002). Our results agree with Allen *et al.* (2008) who observed up-regulation of *rpoS* gene by 6.2-fold in *E. coli* O157:H7 cells following cold shock (7.5 °C) for 15 min. The *uspA* gene was insignificantly induced in both *A. hydrophila* CECT 839^T (0.9-fold) and A331 (0.5-fold) following cold shock. Nystrom and Neidhardt (1993) have also reported that synthesis of UspA is not induced in *E. coli* due to cold shock.

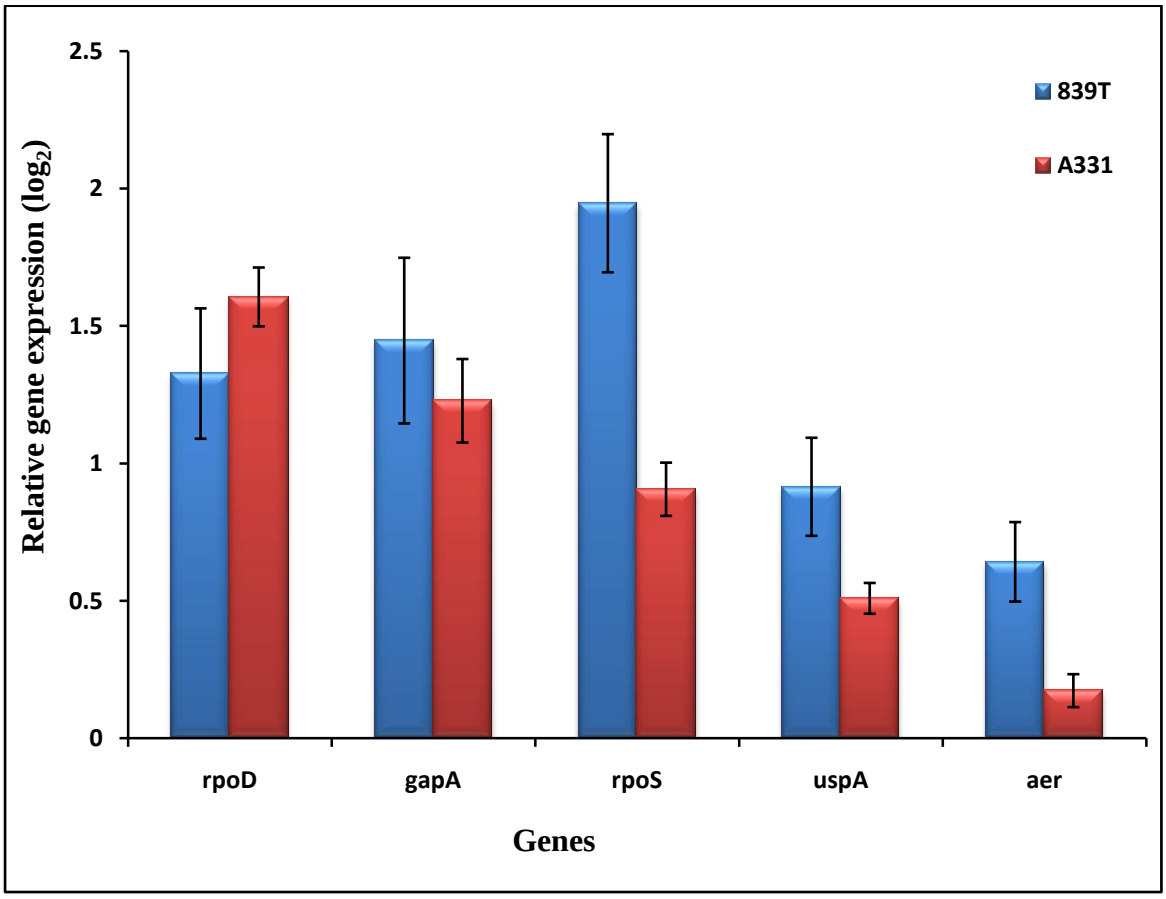


Figure 6.5. Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839^T and A331 in response to cold shock (8 °C) for 30 min

In the present study, no significant expression of *aer* gene was observed in *A. hydrophila* CECT 839^T (0.6-fold) and A331 (0.2-fold) under cold shock at 8 °C for 30 min (Fig. 6.5). This is the first study regarding the expression of various genes following cold shock in *Aeromonas* species. Inter-strain variability on virulence (*aer*) and stress response (*rpoS* and *uspA*) gene transcription levels was observed indicating that the virulence and stress gene transcriptions of the two strains are not equally affected by the cold shock. Similar inter-strain variation in the expression of virulence and stress response genes has been reported in *C. jejuni* following cold shock (Poli *et al.*, 2012).

6.3.5. Gene expression after heat shock (37 °C) for 30 min

A. hydrophila is exposed to variations in temperature during its survival in food processing conditions and when inside the human body. Many virulence genes in the food-borne pathogens are regulated by the shift in temperature. Hence, the gene expression profile of housekeeping, stress response and virulence genes in *A. hydrophila* CECT 839^T and A331 cells subjected to heat shock (37 °C) for 30 min was studied. The housekeeping (*rpoD* and *gapA*) genes were marginally induced in *A. hydrophila* CECT 839^T and A331 with exception of 1.1-fold induction of *gapA* gene in CECT 839^T (Fig. 6.6). Significant induction of various genes involved in the uptake and utilization of amino acids, carbohydrates, and iron was observed in *E. coli* K-12 at human body temperature (37 °C) compared to 23 °C (White-Ziegler *et al.*, 2007).

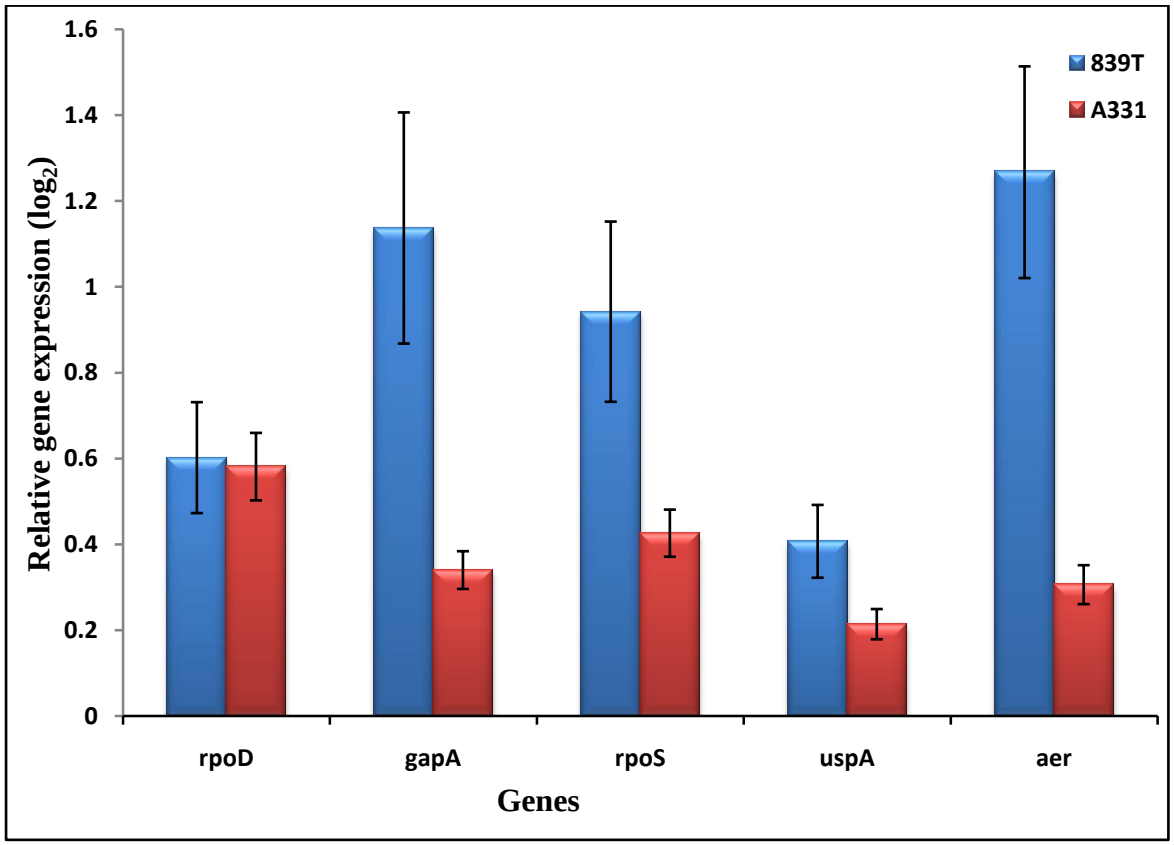


Figure 6.6. Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839^T and A331 in response to heat stress (37 °C) for 30 min

No significant induction of *rpoS* and *uspA* genes in *A. hydrophila* CECT 839^T and A331 cells was observed in response to heat shock (37 °C) for 30 min (Fig. 6.6). However, significant up-regulation of *uspA* gene was observed in *Salmonella* Typhimurium following temperature upshift from 30 °C to 37 °C indicating that growth at and transition to higher temperatures may be stressful to the organism (Liu *et al.*, 2007).

Significant induction of *aer* gene in CECT 839^T (1.3-fold) was observed following heat shock (37 °C) for 30 min; whereas, it was marginally induced in A331 (0.2-fold) (Fig. 6.6). Strain dependent variation in the expression of virulence and stress response genes was observed in *A. hydrophila* strains following temperature up-shift.

6.3.6. Gene expression after acid shock (pH 4) for 30 min

Food-borne pathogens encounter organic and inorganic acids in foods or in the gastrointestinal tract and cells of the host. The housekeeping genes (*gapA* and *rpoD*) were marginally induced in *A. hydrophila* CECT 839^T and A331 with exception of 1.3-fold induction of *gapA* gene in CECT 839^T (Fig. 6.7) indicating that minimum level of RNA transcription and carbon metabolism is occurring in *A. hydrophila* strains following acid shock.

No significant induction of *rpoS* gene was observed in *A. hydrophila* CECT 839^T and A331 following acid shock (pH 4) for 30 min (Fig. 6.7). Lee et al. (1995) reported the role of RpoS in survival of virulent *Salmonella* Typhimurium in acid stress. Marginal induction of *rpoS* gene (0.6-fold) was observed in *Shewanella oneidensis* following acid shock (Leaphart *et al.*, 2006).

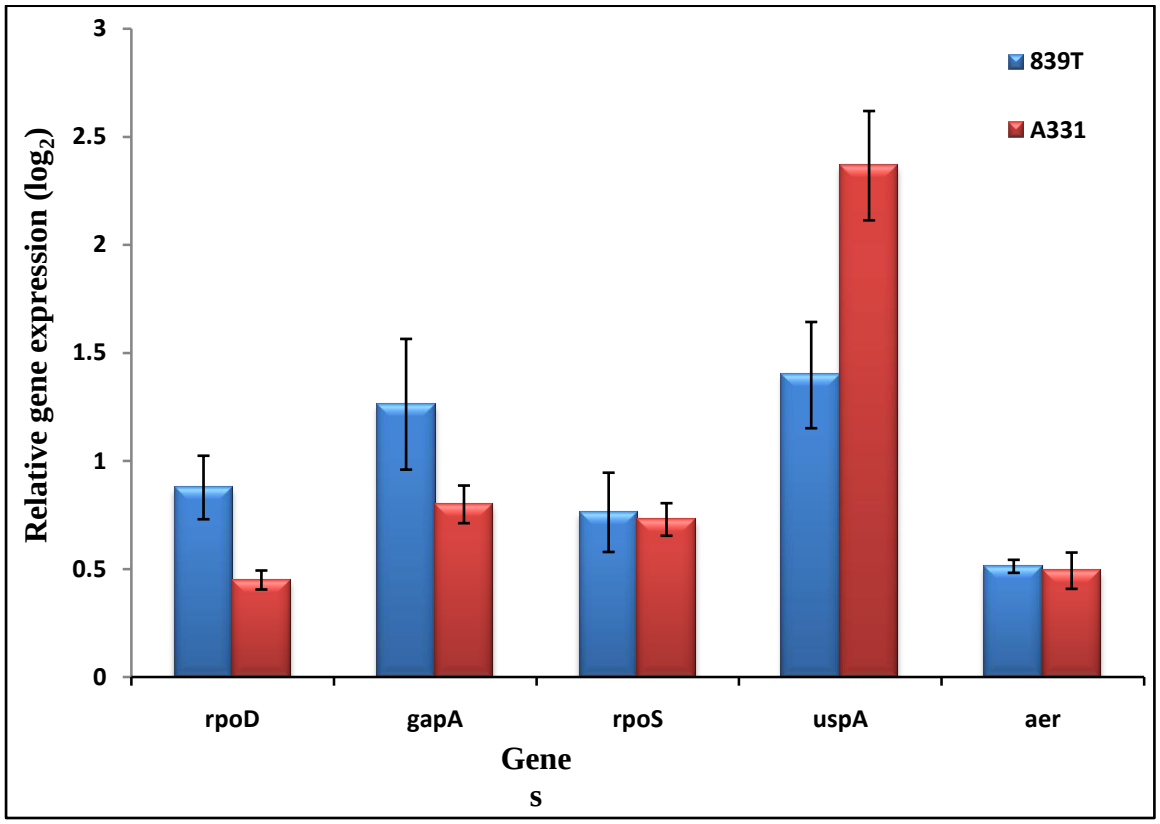


Figure 6.7. Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839^T and A331in response to acid stress (pH 4) for 30 min

The acid-resistance response is complex and involves many regulators. Moreover, RpoS-dependent genes have been shown to play a major role in acid resistance (Battesti *et al.*, 2011). Though the pathway of induction of RpoS in cells exposed to low pH is not fully understood, stabilization of RpoS by the two-component PhoPQ system may play an important role in its induction (Bougdour *et al.*, 2008). Induction of 48 acid shock proteins, including inter-lapping proteins RpoS, Fur, PhoP and OmpR, were observed in *S.* Typhimurium stationary phase cells during acid adaptation and shock. These proteins, which are under the control of multiple, overlapping regulatory systems, protect the cell against acid and perhaps other environmental stresses (Wesche *et al.*, 2009). Significant induction of *uspA* gene was observed in *A. hydrophila* CECT 839^T (1.4-fold) and A331

(2.4-fold) in response to acid shock (pH 4) for 30 min (Fig. 6.7). The synthesis of UspA protein is induced in *E. coli* in response to growth inhibition caused by various factors, including acidic shock (Liu *et al.*, 2007). As compared to other stresses, only in acid stress, the induction of *uspA* gene was more in A331 than CECT 839^T. A protein belonging to universal stress protein family was induced by 0.2-fold in *S. oneidensis* subjected to acid shock (pH 4) for 30 min (Leaphart *et al.*, 2006).

Insignificant induction of *aer* gene was observed in *A. hydrophila* CECT 839^T and A331 following acid shock (pH 4) for 30 min (Fig. 6.7). House *et al.* (2009) have also reported no significant change in the Shiga toxin production (both secreted and periplasmic extracts) in *E. coli* O157:H7 following acute acid stress treatment.

6.3.7. Gene expression after alkaline shock (pH 9) for 30 min

Food-borne pathogens are exposed to alkaline conditions in natural environments, human body (pancreatic duct just below the pylorus) and food industry. Many detergents and chemical sanitizers, such as caustic soda (NaOH) and ammonium compounds, are used to clean food processing facilities and food contact surfaces. Studies of bacterial response to alkaline pH have been less extensive to date compared with those of acidic pH.

The expression of housekeeping genes (*rpoD* and *gapA*) was down-regulated by 0.5 to 4.2 fold in *A. hydrophila* CECT 839^T and A331 subjected to alkaline shock (pH 9) for 30 min (Fig. 6.8). Marginal attenuation (-0.752) of *gapA* gene has been reported in *E. coli* K-12 following alkaline stress (pH 8.7) (Maurer *et al.*, 2005). Global regulators and stress response genes (*rpoS* and *uspA*) were insignificantly attenuated in CECT 839^T and A331 following alkaline stress (Fig. 6.8) indicating insignificant/no role of these genes in adaptation to alkaline stress in *A. hydrophila*.

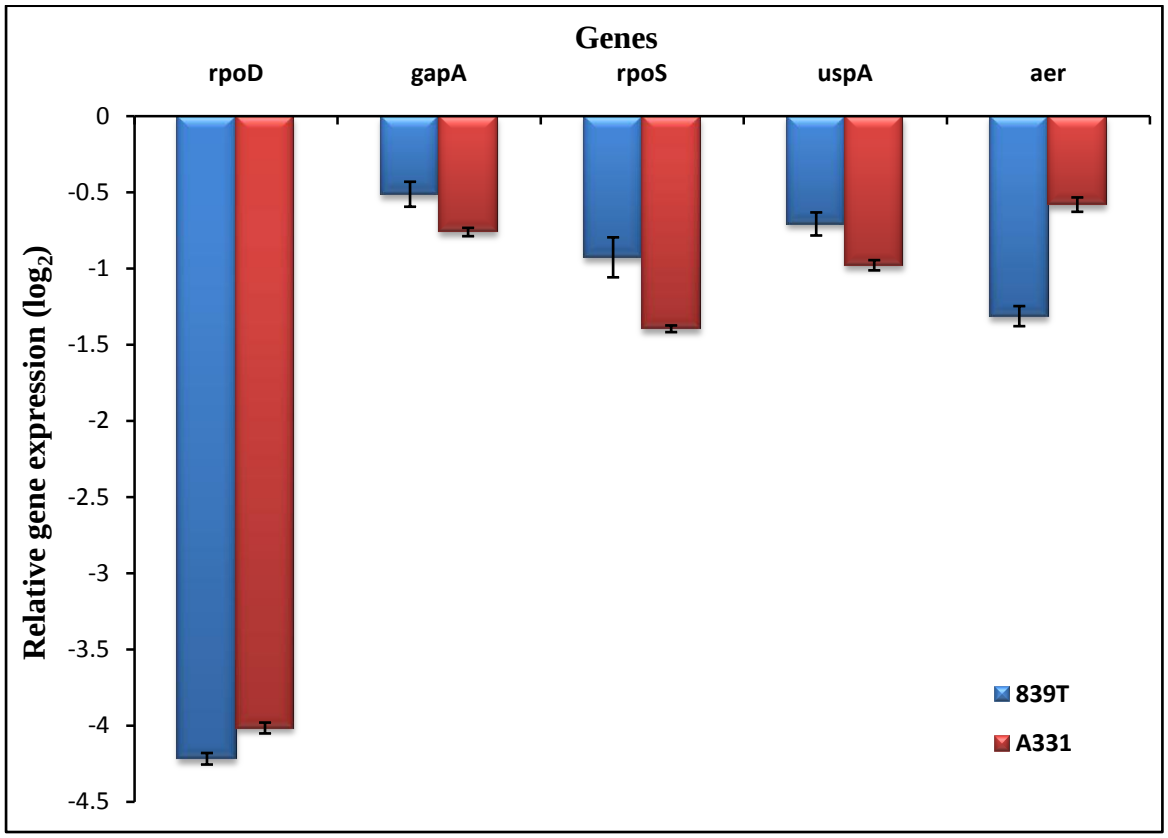


Figure 6.8. Gene expression analysis of *rpoS*, *aer*, *uspA*, *rpoD* and *gapA* genes in *A. hydrophila* CECT 839^T and A331 in response to alkaline stress (pH 9) for 30 min

There are no reports on the expression of these studied genes under alkaline stress in *Aeromonas* species. Leaphart *et al.* (2006) have also reported insignificant down-regulation of *rpoS* and universal stress protein in *S. oneidensis* following alkaline stress for 30 min. Similar to *E. coli*, *S. oneidensis* appears to modulate its transcriptome in response to an alkaline environment primarily by affecting the expression of genes involved in central intermediary metabolism (particularly assimilative sulfur metabolism), sulfate transport, and Na^+/H^+ antiporter systems (Leaphart *et al.*, 2006). Attenuation of *aer* gene by 1.3- and 0.6-fold was observed in *A. hydrophila* CECT 839^T and A331, respectively (Fig. 6.8).

Overall, the trend of expression of all the studied genes under all the stress conditions in both *A. hydrophila* 839^T and A331 strains remained the same. However, inter-strain variability in the virulence and stress response gene transcription levels was observed among *A. hydrophila* 839^T and A331 strains. More pronounced up-regulation or down-regulation for most of the genes was observed in *A. hydrophila* 839^T as compared to A331 for all the stress conditions. These variations indicate the physiological differences among these strains in encountering various stresses. These observations are consistent with previous report where strain-dependent differences in general stress-response and virulence gene expression was observed in *Enterococcus faecalis* (Lenz *et al.*, 2010).

6.4. Conclusions

Quantitative real time-PCR analysis was successfully used to examine the effect of food-environment related stresses on the expression of housekeeping, stress-response regulator and aerolysin genes in *A. hydrophila* CECT 839^T and A331. Strain-dependent differences in the level of expression of different genes under various stress conditions were observed among *A. hydrophila* CECT 839^T and A331 strains indicating genetic heterogeneity within the species. The study also indicated that different strains of *A. hydrophila* have different abilities to cope with stress and induce aerolysin production. Overall, nutrient replenishment and deprivation significantly induced the expression of housekeeping (*rpoD* and *gapA*), general stress regulators (*uspA* and *rpoS*) and virulence gene (*aer*) indicating their importance in regulating the survival and virulence of *A. hydrophila* under these stress conditions. RpoS was significantly induced in cold shock indicating its role in cold shock adaptation. Significant induction of *aer* gene in *A. hydrophila* CECT 839^T during an increase in temperature (37 °C) signify that this strain

may be potentially pathogenic to humans. Acid stress significantly induced the expression of *uspA* gene, which may regulate various genes required for its survival. However, alkaline stress showed significant down-regulation of all the studied genes. Induction of different stress regulators under various stress conditions suggests that initial stress events may prepare a cell for surviving and sustaining any subsequent stress in a better manner. To the best of our knowledge, this is the first study to investigate the gene expression of virulence and stress response genes in *A. hydrophila* strains as affected by food environmental parameters. However, further studies are required to better understand these adaptive responses in the context of pathogen persistence and virulence in the food.

CHAPTER 7

Summary

7.1. Summary

The major findings of the present thesis are summarized as below:

1. The findings demonstrated that the sprout, chicken and fish samples marketed in Mumbai and its suburbs were contaminated with *Aeromonas* with higher incidence in samples of animal origin than of plant origin. Therefore, these samples need processing such as radiation treatment prior to consumption.
2. The biochemical tests and 16S rRNA gene analysis were useful in the identification of *Aeromonas* food isolates only till genus level. The *rpoD* gene was found to be a better phylogenetic marker than 16S rRNA gene, even at the intra-species level.
3. A combination of certain biochemical tests and *rpoD* gene analysis can provide simple, rapid and precise identification of *Aeromonas* strains up to species level.
4. Majority of the *Aeromonas* strains harboured virulence genes, produced extracellular enzymes and showed β-haemolysis and thus maybe potentially pathogenic.
5. Marked resistance to commonly used β-lactam antibiotics and presence of plasmids was observed in the majority of the isolates. However, no clear correlation was observed between the presence of plasmid and antibiotic resistance.
6. High genetic diversity was observed among *Aeromonas* isolates based on PFGE and WCP analysis. For majority of the strains, no clear correlation was observed between the origin of the strains and their PFGE and WCP profiles.
7. All *Aeromonas* isolates were very sensitive to gamma radiation and radiation processing with 1.5 kGy was effective in achieving 5-log reductions in *Aeromonas* populations on mixed sprout, chicken and fish samples. No recovery of *Aeromonas* was observed during the storage period even after enrichment and selective plating.
8. Significant strain-dependent variations in the biofilm forming ability under different food-related stresses (media, temperature, pH, NaCl and food preservatives) were observed in different *Aeromonas* strains.

9. *Aeromonas* stains were found to be very sensitive to different essential oils.
10. Differences in the level of expression of different genes under various stress conditions were observed among *A. hydrophila* CECT 839^T and A331 strains indicating genetic heterogeneity within the species.
11. Induction of different stress regulators under various stress conditions suggested that initial stress events may prepare a cell for surviving subsequent stress events.

7.2. Future work

The present study shows that *Aeromonas* strains are sensitive (MIC values ≤ 1 mg/ml) to clove, ajowain and cinnamon oils in both rich and nutrient-limited media. Thus, the effectiveness of these essential oils in the inhibition of pre-formed *Aeromonas* biofilm need to be further studied. The information regarding the biofilm forming ability of *Aeromonas* strains under glucose and ethanol stress will help food processors and regulators to prevent biofilm formation and reduce the health risks related to biofilm.

Ionizing radiation can serve as an effective antimicrobial agent. Determining the relative irradiation susceptibilities of planktonic versus biofilm-associated cells of *Aeromonas* can help in designing the method to reduce or eliminate *Aeromonas* from food products. *In-vitro* cell invasion studies of *Aeromonas* in animal cell lines will further give more information regarding the pathogenicity potential of these isolates. A rapid detection method using real-time PCR will help in evaluating a large number of food samples for the presence of *Aeromonas* and will ensure the safety of consumers.

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Publications

Prevalence, Characterization, and Antimicrobial Resistance of *Aeromonas* Strains from Various Retail Food Products in Mumbai, India

Vandan Nagar, Ravindranath Shashidhar, and Jayant R. Bandekar

Abstract: A total of 154 food samples (chicken, fish, and ready-to-eat sprouts) from various retail outlets in Mumbai, India, were analyzed for the presence of *Aeromonas* spp. over a period of 2 y (January 2006 to March 2008). Twenty-two *Aeromonas* isolates belonging to 7 different species were isolated from 18 (11.7%) food samples. The highest percentages of isolation were from chicken (28.6%) followed by fish (20%) and sprout (2.5%) samples. *Aeromonas caviae*, *A. veronii* bv. *sobria*, and *A. salmonicida* were the most frequently isolated species from sprouts, chicken, and fish samples, respectively. The genes encoding for putative virulence factors, cytotoxic enterotoxin (*act*), hemolysin (*hly*), aerolysin (*aer*), elastase (*ahyB*), and lipase (*lip*) were detected using polymerase chain reaction method in 59.1%, 40.9%, 22.7%, 54.5%, and 31.8% of the strains, respectively. The isolated *Aeromonas* strains were found to be positive for virulence factors, that is, amylase, DNase, gelatinase, protease, and lipase production. More than 60% isolates were also positive for β-hemolytic activity. All these food isolates were found to be resistant to ampicillin and bacitracin, and sensitive to gentamicin, 3rd-generation cephalosporins (ceftazidime, cephotaxime, ceftriaxone), and chloramphenicol. Seventeen (77.2%) isolates harbored single and/or multiple plasmids (approximately 5 to >16 kb). The *Xba*I digestion patterns of chromosomal DNA of these isolates, using pulsed field gel electrophoresis, showed high genetic diversity among these isolates. Our results demonstrate the presence of various *Aeromonas* spp. with virulence potential and antimicrobial resistance in different food products marketed in Mumbai, India. The potential health risks posed by consumption of these raw or undercooked food products should not be underestimated.

Keywords: *Aeromonas*, antibiotic resistance, food, PFGE, virulence genes

Introduction

The genus *Aeromonas* is regarded not only as an important pathogen of fish but also as the opportunistic pathogens in both immunocompetent and immunocompromised humans (Janda and Abbott 2010). In humans, *Aeromonas* species are responsible for intestinal and extra-intestinal infections (Khajanchi and others 2010). Five *Aeromonas* spp., *A. hydrophila*, *A. caviae*, *A. veronii* (biovars *veronii* and *sobria*), *A. jandaei*, and *A. schubertii* are most commonly implicated in human intestinal infections (Janda and Abbott 2010). *Aeromonas* inhabit a wide range of ecosystems (Garibay and others 2006). Aeromonads can infect humans via consumption of contaminated food or water (Khajanchi and others 2010). The pathogenesis of *Aeromonas* infections is multifactorial and incompletely understood (Janda and Abbott 2010). A wide range of putative virulence factors that may play an important role in the development of disease have been studied in several *Aeromonas* spp. (Yücel and Erdogan 2010). Acute gastroenteritis due to *Aeromonas* has been reported in Pernambuco and Brazil (Hofer and others 2006; Guerra and others 2007). Thus, the high prevalence of *Aeromonas* species in the food chain should be considered a threat to public health.

Though antimicrobial resistance of *Aeromonas* strains from clinical sources have been studied, very little is known about the antibiotic resistance profiles of food and environmental isolates. Antibiotic resistance is particularly relevant in pathogenic *Aeromonas* species in which, besides the classical resistance to β-lactamic antibiotics, multiple-resistance has been frequently identified (Palu and others 2006). Pulsed field gel electrophoresis (PFGE) profiling is the widely used technique in molecular epidemiological investigation of *Aeromonas* due to its sensitivity and discriminatory power (Bonadonna and others 2002).

Though *Aeromonas* have been reported from a number of sources in India (Vivekanandhan and others 2002; Vaseeharan and others 2005), few studies show their genotypic characterization (Sharma and others 2005), and none report the PFGE profile of the Indian *Aeromonas* isolates. Therefore, the current study was carried out with following objectives: (i) to determine the prevalence of *Aeromonas* in a wide variety of food products in Mumbai, and to characterize the isolates by phenotypic and molecular criteria, (ii) to assess the presence of various putative virulence factors, and (iii) to determine the antimicrobial susceptibility, plasmid, and PFGE profiles of the isolates.

Materials and Methods

Sample collection

A total of 154 food samples of chicken, fish, and ready-to-eat sprouts were purchased from local retail outlets in Mumbai, India from January 2006 to March 2008. The samples included

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mixed sprouts (Green gram [*Phaseolus aureus*], Dew gram [*Phaseolus aconitifolius*], Chick pea [*Cicer arietinum*], and Garden pea [*Pisum sativum*]) (40), alfalfa sprouts (*Medicago sativa*) (40), chicken (14), freshwater fish (52), and marine fish (8). The sprouts were procured from 2 different supermarket outlets; whereas, fish and chicken samples were collected from retail markets of Mumbai. The sprouts and chicken samples were brought directly to laboratory at ambient temperature (26 to 28 °C); whereas, the fish samples were brought in ice. The microbiological analysis was done within 1 h.

Chemicals and media

Microbiological media used in the study were from Hi-Media Laboratories, Mumbai, India. Horse blood used for blood agar was from Haffkine Inst., Pune, India. All polymerase chain reaction (PCR) reagents were obtained from Bangalore Genei Ltd. (Bangalore, India).

Aeromonas isolation and identification

Aeromonas spp. were isolated by pummeling 25 g of sample in 225 mL Tryptone Soya Broth (TSB) in Stomacher 400 Lab Blender (Seward Medical, London, U.K.) and enriching at 30 °C for 24 h followed by streaking on Starch Ampicillin Agar (SAA) (Palumbo and others 1985). The plates were incubated at 30 °C for 24 h. The SAA plates were examined for typical *Aeromonas* colonial morphology. Presumptive *Aeromonas* isolates were stored in 20% glycerol in TSB at –70 °C until further use. Presumptive *Aeromonas* isolates were identified to the species level based on the selected biochemical tests proposed by Abbott and others (2003). *Aeromonas hydrophila* CECT 839^T, *A. salmonicida* CECT 894^T, *A. sobria* CECT 4245^T, and *A. veronii* CECT 4257^T (kindly supplied by Dr. Valérie Leclère, Université des Sciences et Technologies de Lille USTL, France) were used as controls for the biochemical tests. All *Aeromonas* isolates were grown overnight in 25 mL Luria broth at 30 °C.

Genetic identification

All biochemically positive isolates were confirmed to be *Aeromonas* by 16S rRNA gene sequencing using primers and amplification conditions as described by Arora and others (2006). PCR product was sequenced at MWG-Biotech Pvt. Ltd., Bangalore, India. The 16S rRNA gene sequence identity was determined using the BLASTN at the Natl. Center for Biotechnology Information website (www.ncbi.nih.gov/BLAST/). The sequences were deposited in the GenBank database using the web-based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt/).

Screening for extracellular virulence factors of Aeromonads

Amylase, gelatinase, lipase, protease, and DNase were detected on the starch agar, gelatin agar, tributyrin agar, milk agar, and DNase agar, respectively according to published methods (Mac Faddin 1980; Thomas and others 2003; Saran and others 2007).

Hemolytic activity

Hemolytic activity of the isolates was determined by streaking onto tryptone soya agar plates containing 5% defibrinated horse blood cells. The plates were incubated at 37 °C for 24 h and observed for a clear zone of β-hemolysis around the colonies (Brender and Janda 1987).

Detection of virulence and related genes by PCR

PCR amplification of the virulence genes (aerolysin [*aer*], cytotoxic enterotoxin [*act*], hemolysin [*hly*], lipase [*lip*], and elastase [*ahyB*]) from chromosomal DNA was performed using different set of primers and amplification conditions as described in Table 1.

Antimicrobial susceptibility testing

Antimicrobial susceptibility test, constituting commonly used antimicrobial agents, was performed on Muller-Hinton Agar by disc diffusion method as described by National Committee of Clinical Laboratory Standard (NCCLS) (2002). Testing was done in triplicate and resistance profiles (resistant [R], intermediate [I], or susceptible [S]) were assigned after measuring average zone diameters using NCCLS breakpoints (NCCLS 2002).

Twenty different antibiotics (Hi-Media Laboratories) used were amikacin (30 μg), ampicilllin (10 μg), ampicillin/sulbactam (10/10 μg), aztreonam (30 μg), bacitracin (10 units), carbenicillin (100 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cephotaxime (30 μg), cephoxitin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), piperacillin/tazobactam (100/10 μg), tetracycline (30 μg), and tobramycin (10 μg).

Multiple antibiotic resistance (MAR) index: the MAR index, when applied to a single isolate, is defined as a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was exposed.

Plasmid DNA extraction and detection

Plasmid DNA was extracted from the cultures using alkaline lysis method (Sambrook and others 1989) and analyzed on 0.8% (w/v) agarose gel along with supercoiled DNA ladder (Sigma-Aldrich Co., St. Louis, Mo., U.S.A.).

Table 1–Primers used for PCR detection of putative virulence genes in *Aeromonas* isolates from food.

Target gene	Primer sequence (5′ ⟶ 3′)	Size of PCR amplicons (bp)	Reference
aer F	GCAGAACCCATCTATCCAG	252	Santos and others (1999)
aer R	TTTCTCCGGTAACAGGATTG		
act F	GAGAAGGTGACCACCAAGAACA	232	Kingombe and others (1999)
act R	AACTGACATCGGCCTTGAACTC		
hly F	GGCCGGTGGCCCGAAGATACGGG	597	Wong and others (1998)
hly R	GGCGGCGCCGGACGAGACGGG		
ahyB F	ACACGGTCAAGGAGATCAAC	540	Sen (2005)
ahyB R	CGCTGGTGTTGGCCAGCAGG		
lip F	ATCTTCTCCGACTGGTTCGG	383 to 389	Sen (2005)
lip R	CCGTGCCAGGACTGGGTCTT		

PFGE for DNA fingerprinting of *Aeromonas* isolates

Intact genomic DNA isolation was carried out using Pulse Net standard protocol (Ribot and others 2006). Restriction digestion was carried out with 25U of *Xba*I (New England Biolabs, Ipswich, Mass., U.S.A.) at 37 °C. PFGE was performed with Gene Navigator System (Amersham Biosciences, Uppsala, Sweden) in a 0.8% agarose gel (pulse field certified agarose, Sigma-Aldrich). The electrophoresis was carried out at 150 V with pulse times of 20 sec for 12 h and then of 5 to 15 sec for 17 h (Talon and others 1996) using lambda ladder PFGE marker (New England Biolabs). The gels were stained in ethidium bromide. Strains differing by one band were considered as different pulsed field profiles.

Results and Discussion

Aeromonas spp. from various food samples

Eighteen (11.7%) of 154 food samples were positive for *Aeromonas* spp. *Aeromonas* were isolated from 28.6%, 20%, and 2.5% of chicken, fish, and sprout samples, respectively (Table 2). The current study shows that *Aeromonas* are widely distributed in the retail foods from Mumbai with more prevalence in foods of animal origin than of plant origin. Moreover, this is the first report of incidence of *Aeromonas* in sprouts from India. Xanthopoulos and others (2010) have reported aeromonads from 61.5% of ready-to-eat salads; whereas, only 34% of organic vegetables were found to be contaminated with *Aeromonas* spp. (McMahon and Wilson 2001). The sprouts screened in the current study were minimally processed and kept at low temperature (<8 °C). The low occurrence of *Aeromonas* may be attributed to good hygienic practices of the producers and retailers (Nagar and Bandekar 2009).

In the present study, somewhat higher incidence of *Aeromonas* was observed in marine fish (25%) as compared to freshwater fish (19.2%) samples. Yücel and Balci (2010) have also reported more prevalence of *Aeromonas* in marine fish samples than freshwater fish. Seven different species of *Aeromonas* were isolated from the fish samples. Presence of *Aeromonas* in fish samples may be due to contaminated water or secondary contamination during handling, storage, and transportation. Chang and others (2008) found *Aeromonas* in poultry (22.5%), seafood (30%), and aquatic retail (38.9%) samples from Taiwan. Variations in the incidence of *Aeromonas* spp. may be due to the differences in the geographical distribution, origin of the samples, sampling period, methodology of analysis, and inconsistent hygienic practices followed during handling.

Four hundred and fifty-one presumptive positive isolates were isolated from 154 food samples. Twenty-two of these 451 isolates were identified as *Aeromonas* by biochemical tests and confirmed as *Aeromonas* by molecular typing. The 16S rRNA gene sequences were submitted to GenBank at NCBI (accession numbers: FJ561050–52, HQ122915–31, HQ413137, and HM002780). Researchers have highlighted considerable number of discrepancies in biochemical and genetic identification of both environmental and clinical *Aeromonas* isolates (Soler and others 2003). Based on DNA–DNA hybridization studies, several researchers have also classified *Aeromonas* into hybridization groups (HGs) (Martin-Carnahan and Joseph 2005; Saavedra and others 2006). Many reports on *Aeromonas* are based only on biochemical tests, without the use of molecular methods (McMahon and Wilson 2001; Akinbowale and others 2007). Therefore, we confirmed all isolates by 16S rRNA gene sequencing. The results based only on biochemical tests may lead to false positive reports.

Based on the biochemical characteristics (Abbott and others 2003), *Aeromonas* isolates were identified upto the species level. They belonged to 7 different species of *Aeromonas* (*A. salmonicida*, *A. veronii* bv. *sobria*, *A. hydrophila*, *A. caviae*, *A. jandaei*, *A. trota*, and *A. eucrenophila*) (Table 2). Overall, *A. salmonicida* and *A. veronii* bv. *sobria* were the most prevalent species, followed by *A. hydrophila* and *A. caviae*. *Aeromonas hydrophila* and *A. caviae* were isolated from all types of food samples analyzed, indicating that these species are widely distributed in the study area (Table 2); whereas, *A. salmonicida*, *A. trota*, and *A. eucrenophila* were isolated only from freshwater fish samples. Considering the different sources, *A. caviae* (66.7%), *A. veronii* bv. *sobria* (40%), and *A. salmonicida* (35.7%) were the most frequently isolated species from sprouts, chicken, and fish, respectively.

Extracellular virulence factors

All the isolates were positive for amylase, DNase, and gelatinase production. DNase activity has been observed in *Aeromonas* strains

Table 2–Prevalence of *Aeromonas* spp. in different food samples in Mumbai, India.

Source	Nr. of samples	Nr. of *Aeromonas* positive samples (%)	*A. hydrophila*[a]	*A. veronii bv. sobria*[a]	*A. caviae*[a]	*A. salmonicida*[a]	*A. jandaei*[a]	*A. trota*[a]	*A. eucrenophila*[a]
Alfalfa sprouts	40	1(2.5%)	1	–	1	–	–	–	–
Mixed sprouts	40	1(2.5%)	–	–	1	–	–	–	–
Chicken	14	4(28.6%)	1	2	1	–	1	–	–
Freshwater fish	52	10(19.2%)	2	2	–	5	1	1	1
Marine fish	8	2(25%)		1	1				
Total	154	18(11.7%)	4	5	4	5	2	1	1

[a]Number of strains isolated.

Table 3–Distribution of putative virulence genes in *Aeromonas* isolates.

Virulence genes	Nr. (%) of isolates with virulence genes						
	A. hydrophila ($n = 4$)	*A. veronii bv. sobria* ($n = 5$)	*A. caviae* ($n = 4$)	*A. salmonicida* ($n = 5$)	*A. jandaei* ($n = 2$)	*A. trota* ($n = 1$)	*A. eucrenophila* ($n = 1$)
aer	–	1 (20)	–	4(80)	–	–	–
ahyB	2 (50)	1 (20)	3 (75)	5(100)	1 (50)	–	–
lip	–	–	3 (75)	4(80)	–	–	–
hly	2 (50)	2 (40)	–	5(100)	–	–	–
act	3 (75)	4 (80)	–	5(100)	–	1 (50)	–

Table 4–Percentage antimicrobial resistance of *Aeromonas* spp. isolated from various food samples in Mumbai, India.

	A. hydrophila (*n* = 4)			*A. veronii bv. sobria* (*n* = 5)			*A. caviae* (*n* = 4)			*A. salmonicida* (*n* = 5)			*A. jandaei* (*n* = 2)			*A. trota* (*n* = 1)			*A. eucrenophila* (*n* = 1)			
Antibiotic (concentration, μg)	**R**[b]	**I**[c]	**S**[d]	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**Resistant strains (%)**
Aztreonam (30)		75	25		100		50	25	25		60	40		100		100					100	13.6
Erythromycin (15)		100			60	40		100			80	20		50	50		100			100		–
Amikacin (30)			100			100			100			100			100	100					100	4.5
Gentamicin (10)			100			100			100			100			100			100			100	–
Tobramycin (10)			100		20	80			100			100			100		100				100	–
Ciprofloxacin (5)			100		20	80		25	75			100			100			100			100	–
Ampicillin (10)	100			100			100			100			100			100			100			100
Carbenicillin (100)	75	25		60	20	20	25	25	50	100			100			100					100	68.2
Piperacillin/tazobactam (100/10)	75		25	60		40		50	50	80	20		100			100					100	59.1
Ceftazidime (30)			100			100			100			100			100			100			100	–
Bacitracin (10)[a]	100			100			100			100			100			100			100			100
Cephotaxime (30)			100			100			100			100			100			100			100	–
Tetracycline (30)			100	20	20	60	25		75	20		80	50		50			100			100	18.2
Kanamycin (30)			100		60	40	25		75		20	80			100		100				100	4.5
Ceftriaxone (30)			100			100			100			100			100			100			100	–
Nalidixic acid (30)			100	40		60	25		75			100			100			100	100			18.2
Chloramphenicol (30)			100			100			100			100			100			100			100	–
Ampicillin/sulbactam (10/10)	75	25		80		20		25	75	100			100			100			100			72.7
Imipenem (10)	25		75		20	80			100	20	20	60		100				100			100	9.1
Cephoxitin (30)	100			60	40		75		25	100			50	50			100		100			77.3

[a]Antibiotic concentration in terms of units.
[b]Percentage of resistant (R) isolates.
[c]Percentage of intermediate resistant (I) isolates.
[d]Percentage of sensitive (S) isolates.

from frozen fish (Castro-Escarpulli and others 2003) and food samples (Yücel and Erdogan 2010). Emele (2001) has reported amylase activity in all the *A. hydrophila* isolates from different sources (food, clinical, and environmental). In an earlier study, 96% of *Aeromonas* spp. isolated from frozen fish showed gelatinase activity (Castro-Escarpulli and others 2003).

Twenty-one (95.5%) and 20 (90.9%) isolates were positive for lipase and protease production, respectively. However, lipase activity was absent in one *A. veronii* bv. *sobria* fish isolate. Scoglio and others (2001) have reported the presence of lipases in most of the *A. hydrophila* and *A. caviae* from foods. One *A. caviae* chicken isolate and one *A. hydrophila* fish isolate showed absence of proteolytic activity. Yücel and Erdogan (2010) have reported protease activity in *Aeromonas* isolates from food (69.5%) and environmental (94.3%) samples.

Hemolytic activity

In the present study, 14 (63.6%) isolates produced clear zones of β-hemolysis on blood agar plates. None of the sprout isolates showed β-hemolytic activity; whereas, 80% of poultry and 71.4% of fish isolates were positive for β-hemolysin. Yücel and Erdogan (2010) observed β-hemolysis in 57% and 60.5% of environmental and food isolates, respectively.

Virulence genes (*act*, *aerA*, *hly*, *lip*, *ahyB*)

Aerolysin (*aer*) and hemolysin (*hly*) genes were present in 22.7% and 40.9% of the strains, respectively (Table 3). Nawaz and others (2010) have reported the presence of aerolysin gene (*aerA*) from 96% of *A. veronii* isolates from catfish. The variation in the prevalence of *aer* gene in *Aeromonas* spp. may be due to geographical location (Albert and others 2000). *hlyA* is widely dispersed in *Aeromonas* species (Wang and others 2003). In the present study, *aer* and *hly* genes were present in 80% and 100% of the *A. salmonicida* isolates, respectively. Castro-Escarpulli and others (2003) have also reported the presence of aerolysin/hemolysin genes in 98% of the *A. salmonicida* isolates.

Cytotoxic enterotoxin (*act*) gene was present in 59.1% of *Aeromonas* isolates. *act* gene was found in all the *A. salmonicida* and more than 75% of *A. hydrophila* and *A. veronii* bv. *sobria* isolates (Table 3). Our findings are similar to those reported by Kingombe and others (1999), who showed the presence of *act* gene in food (66%), environmental (58%), and clinical (67%) isolates.

The present study indicates that 54.5% and 31.8% of the isolated strains contained elastase (*ahyB*) and lipase (*lip*) genes, respectively (Table 3). *ahyB* and *lip* genes were present in more than 75% of *A. salmonicida* and *A. caviae* isolates. In earlier studies, Sen and Rodgers (2004) reported the presence of *ahyB* gene in 88% of drinking water *Aeromonas* isolates; whereas, it was absent in all *A. veronii* isolates from catfish (Nawaz and others 2010). In the previous studies, lipase gene was shown to be present in 97% of *Aeromonas* strains from frozen fish (Castro-Escarpulli and others 2003) and in 80% to 85% of *A. veronii* isolates from catfish (Nawaz and others 2010).

In the present study, all 5 studied virulence genes were present in 4 *A. salmonicida* (80%) isolates. *ahyB*, *hly*, and *act* genes were present in all the 5 *A. salmonicida* isolates. High incidence of virulence genes in *A. salmonicida* isolates from aquacultured fish indicates risk to human health on consumption of these fish. On the other hand, all the 5 virulence genes were absent in one isolate each of *A. caviae*, *A. jandaei*, *A. eucrenophila*, and *A. veronii* bv. *sobria*.

Despite the presence of plethora of putative and proven virulence factors in *Aeromonas* food and water isolates, the exact role and mechanism of aeromonads in causing diarrhoeal illness has not been elucidated. At present, there is lack of direct relationship between the presence of these virulence factors and the ability of strains to cause gastroenteritis in humans (Khajanchi and others 2010). The ability of *Aeromonas* to cause disease in humans is affected by bacterial virulence factors, infectious dose, and host immune responses (Galindo and others 2006). Thus, the public health significance of finding virulence genes in *Aeromonas* isolates from environmental and food samples must be interpreted carefully.

Antimicrobial resistance patterns

The isolates belonging to the 7 identified *Aeromonas* spp. have varying levels of susceptibility/resistance to the different antimicrobial agents. *Aeromonas salmonicida* isolates displayed greater levels of resistance as compared to other species (Table 4).

All *Aeromonas* strains were resistant to ampicillin and bacitracin and majority of the isolates showed higher resistance to cephoxitin (77.3%), ampicillin/sulbactam (72.7%), carbenicillin (68.2%), and piperacillin/tazobactam (59.1%). All 5 *A. salmonicida* isolates were resistant to ampicillin, bacitracin, cephoxitin, ampicillin/sulbactam, and carbenicillin. High resistance to these antibiotics has been reported in *Aeromonas* strains from fish samples and shrimp culture hatcheries and ponds (Radu and others 2003; Vaseeharan and others 2005). The high resistance is due to the production of inducible chromosomal β-lactamases (Janda and Abbott 2010).

In the current study, all *A. hydrophila* isolates were resistant to ampicillin, bacitracin, and cephoxitin and showed higher resistance (75%) to carbenicillin, piperacillin/tazobactam, and ampicillin/sulbactam. All *A. hydrophila* and *A. sobria* strains isolated from processed channel catfish were resistant to ampicillin and bacitracin (Wang and Silva 1999). One *A. trota* showed resistance to aztreonam, amikacin, carbenicillin, piperacillin/tazobactam, and ampicillin/sulbactam.

All the *Aeromonas* isolates were sensitive to gentamicin, 3rd-generation cephalosporins (ceftazidime, cephotaxime, ceftriaxone), and chloramphenicol. All *Aeromonas* strains from food and fish were found to be sensitive to cefotaxime, ceftazidime, gentamicin, and chloramphenicol (Palu and others 2006; Akinbowale and others 2007).

In the present study, different *Aeromonas* isolates showed intermediate resistance to aztreonam, erythromycin, tobramycin, ciprofloxacin, carbenicillin, piperacillin/tazobactam, tetracycline, kanamycin, ampicillin/sulbactam, imipenem, and cephoxitin (Table 4). Intermediate resistance to different antibiotics has also been reported among *A. hydrophila*, *A. sobria*, *A. salmonicida*, *A. veronii*, and *A. caviae* isolates from food, clinical, and aquaculture sources (Palu and others 2006; Jacobs and Chenia 2007).

Very few reports (Kampfer and others 1999; Overman and Janda 1999) give comprehensive antimicrobial susceptibility profiles of

Table 5–The percentage occurrence of multiple antibiotic resistance (MAR) index of *Aeromonas* strains from various food samples.

	Source		
MAR index	Sprouts ($n = 3$)	Chicken ($n = 5$)	Fish ($n = 14$)
0.05	0	0	0
0.1	0	0	0
0.15	2 (66.7%)	1 (20%)	1 (7.1%)
0.2	0	0	0
0.25	0	1 (20%)	5 (35.7%)
0.3	1 (33.3%)	1 (20%)	4 (28.6%)
0.35	0	2 (40%)	4 (28.6%)

the genus *Aeromonas* to different antimicrobial agents. The variation in sensitivity and resistance patterns of *Aeromonas* may be due to different isolation sources, environmental conditions, and variable use of drug from place to place.

Overall, the MAR indices ranged from 0.15 to 0.35 (Table 5). A total of 92.9% of the fish isolates and 80% of the chicken isolates exhibited resistance to minimum of at least 5 antibiotics. A total of 81.8% of the strains had MAR index of 0.25 to 0.35, with more fish (92.9%) and chicken (80%) isolates than sprout (33.3%) in this range. In the present study, higher MAR indices of fish and chicken isolates indicate that use of low concentration of antibiotics in feed might be responsible for development of antibiotic resistance.

Plasmid DNA profiles

Single and/or multiple plasmids, ranging in size from approximately 5 to >16 kb, were detected in 77.2% (17/22) of isolates. Earlier researchers have also reported the prevalence of small-sized plasmids in 15% to 94% of *Aeromonas* isolates (Chaudhury and others 1996; Brown and others 1997). In the present study, plasmids were not restricted to a specific *Aeromonas* species, but were present in isolates belonging to each of the 7 identified species. Plasmids were detected in all the *A. salmonicida*, *A. veronii*, *A. jandaei*, *A. eucrenophila*, and *A. trota* isolates and 50% and 25% of *A. hydrophila* and *A. caviae*, respectively. Radu and others (2003) reported plasmids in 50%, 56.3%, and 60% of *A. caviae*, *A. veronii*, and *A. hydrophila* strains, respectively. In the present study, all the chicken isolates had plasmids; whereas, the plasmids were present in only 78.6% and 33.3% of fish and sprout isolates, respectively. More than one plasmid was present in 8 isolates. No clear correlation between the presence of plasmid and antibiotic resistance was observed. Radu and others (2003) have also suggested that the antibiotic resistance in *Aeromonas* may be of chromosomal origin.

PFGE profile

PFGE of the 4 *Xba*I digested *A. hydrophila* isolates yielded 14 to 19 well-resolved genomic DNA fragments (approximately 48.5 to 436.5 kb) (Figure 1A). Restriction analysis of 5 *A. salmonicida*, 5 *A. veronii* bv. *Sobria*, and 4 *A. caviae* isolates resulted in 12 to 16 fragments (48.5 to 339.5 kb), 15 to 18 fragments (48.5 to <339.5 kb), and 14 to 17 fragments (48.5 to 388 kb), respectively (Figure 1B to D). All the isolates showed different PFGE banding pattern indicating high genetic diversity. This finding is in accordance with the previous studies of PFGE profiles of *Aeromonas* strains from different geographical locations (Bonadonna and others 2002; Villari and others 2003). In the present study, PFGE profile does not show any correlation with the source of isolation and virulence factors of *Aeromonas* isolates. However, studying the relationship between HGs, occurrence, and virulence factors may give novel information regarding *Aeromonas* epidemiology.

PFGE is currently the method for the subtyping of sporadic or epidemic *Aeromonas* isolates. Studies using various restriction endonucleases have shown that *Xba*I digestion holds the maximum discriminatory power for *Aeromonas* and thus has been successfully used for epidemiological analysis (Bonadonna and others 2002; Khajanchi and others 2010). Therefore, *Xba*I was used in this study for characterization of the *Aeromonas* isolates. Our results confirm that PFGE is a highly efficient technique for characterization of *Aeromonas* strains.

In the present study, the typeability and reproducibility of PFGE were best possible as reproducible PFGE patterns were obtained for all strains obtained from different foods. Moreover, this is the first report of PFGE profiles of *Aeromonas* spp. from India. Therefore, in future, this data can be considered as a reference for any work regarding epidemiology or genetic diversity of *Aeromonas* spp. in India.

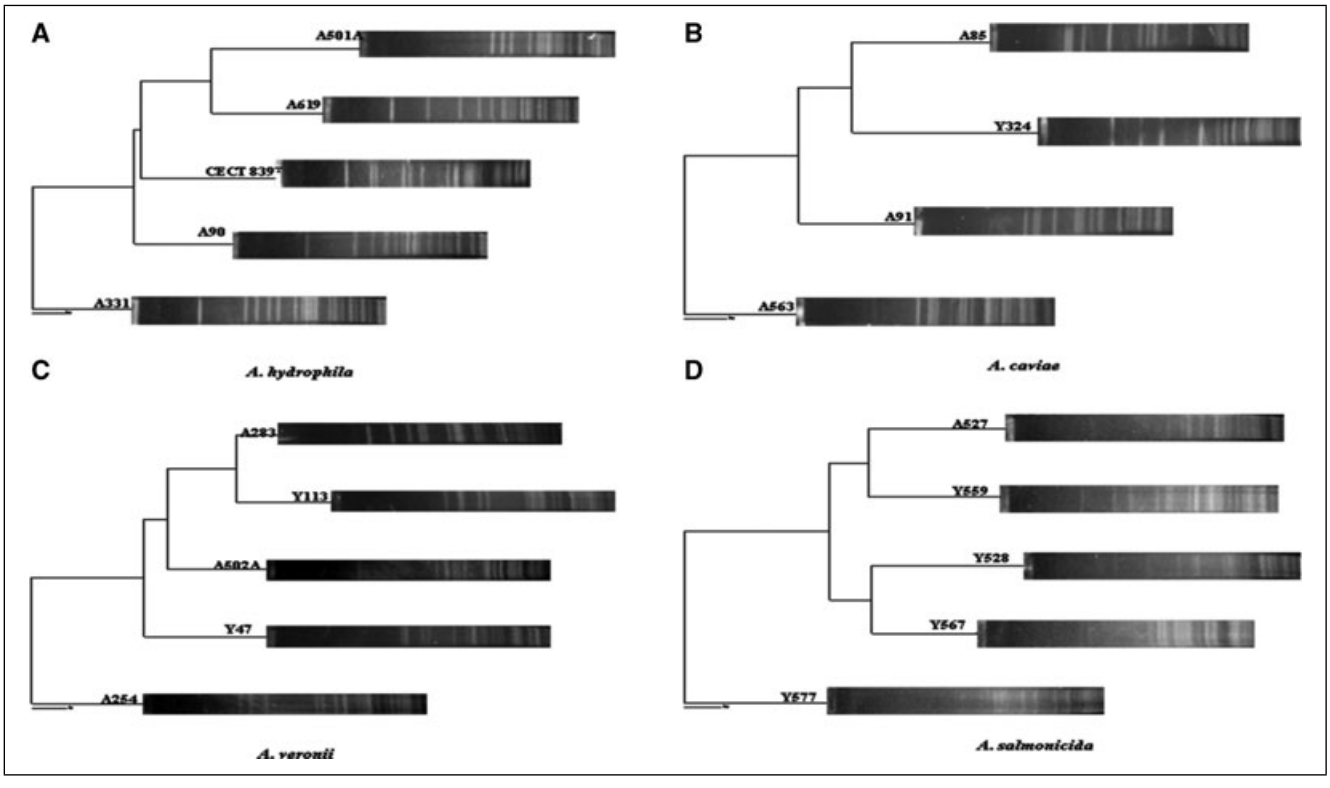

Figure 1–Dendogram and PFGE profiles of *Aeromonas* isolates (A) *A. hydrophila*, (B) *A. caviae*, (C) *A. veronii* bv. *sobria*, and (D) *A. salmonicida*, from various retail food products in Mumbai, India.

Conclusions

Our findings demonstrated that the sprout, chicken, and fish samples, intended for human consumption, in Mumbai, India were contaminated with *Aeromonas* with higher incidence in samples of animal origin than of plant origin. Seven different *Aeromonas* species were isolated from fish samples; whereas, only 2 species were found in sprout samples. The majority of the aeromonads had the presence of putative virulence factors and thus may be potentially pathogenic. Marked resistance to commonly used β-lactam antibiotics and high genetic diversity using PFGE was observed in the isolates. The occurrence of these bacteria in food should be regarded as an important threat to public health. Although *Aeromonas* is eliminated by adequate cooking, it can contaminate the food due to undercooking or cross-contamination. This is the first comprehensive study of the biochemical and molecular characteristics of *Aeromonas* spp. from food products in India.

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Effectiveness of radiation processing in elimination of *Aeromonas* from food

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ABSTRACT

Genus *Aeromonas* has emerged as an important human pathogen because it causes a variety of diseases including gastroenteritis and extra-intestinal infections. Contaminated water, sprouts, vegetables, seafood and food of animal origin have been considered to be the important sources of *Aeromonas* infection. In the present study, radiation sensitivity of indigenous strains of *Aeromonas* spp. from different food samples was evaluated. The decimal reduction dose (D_{10}) values of different *Aeromonas* isolates in saline at 0–4 °C were in the range of 0.031–0.046 kGy. The mixed sprouts, chicken and fish samples were inoculated with a cocktail of five most resistant isolates (*A. salmonicida* Y567, *A. caviae* A85, *A. jandaei* A514A, *A. hydrophila* CECT 839^T and *A. veronii* Y47) and exposed to γ radiation to study the effectiveness of radiation treatment in elimination of *Aeromonas*. D_{10} values of *Aeromonas* cocktail in mixed sprouts, chicken and fish samples were found to be 0.081 ± 0.001, 0.089 ± 0.003 and 0.091 ± 0.003 kGy, respectively. Radiation treatment with a 1.5 kGy dose resulted in complete elimination of 10^5 CFU/g of *Aeromonas* spp. from mixed sprouts, chicken and fish samples. No recovery of *Aeromonas* was observed in the 1.5 kGy treated samples stored at 4 °C up to 12 (mixed sprouts) and 7 days (chicken and fish samples), even after enrichment and selective plating. This study demonstrates that a 1.5 kGy dose of irradiation treatment could result in complete elimination of 10^5 CFU/g of *Aeromonas* spp. from mixed sprouts, chicken and fish samples.

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1. Introduction

Aeromonas species have been implicated as causative agents of a number of diseases in both immunocompetent and immunocompromised humans and lower vertebrates, including amphibians, reptiles and fish (Daskalov, 2006; Janda and Abbott, 2010). In human beings, *A. hydrophila*, *A. caviae*, *A. veronii* (biovars *veronii* and *sobria*), *A. jandaei*, *A. trota* and *A. schubertii* have been associated with gastroenteritis and extra-intestinal infections like septicemia, cellulitis, wound infections, urinary tract infections and soft tissue infection (Khajanchi et al., 2010). These species account for >85% of the clinical *Aeromonas* isolates and are considered major pathogens (Sen and Rodgers, 2004). *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. veronii* biovar *sobria* have also been implicated with broad spectrum of fish diseases (Faisal et al., 2007).

Aeromonas are largely isolated from aquatic ecosystems and various food products of animal and plant origin (Isonhood and Drake, 2002; Palu et al., 2006). Being psychrophilic in nature, *Aeromonas* spp. are capable of surviving and multiplying at low temperatures (2–10 °C) applied to the minimally processed food products (Mano et al., 2000). *Aeromonas* are active spoilers of minimally processed plant produce, fish and meat (Jacxsens et al., 1999; Janda and Abbott, 1998) and can produce virulence factors even at these low temperatures (Mateos et al., 1993; Merino et al., 1995). *Aeromonas* have the ability to colonize and form biofilms on food surfaces, drinking water distribution systems and may pose a threat of contamination in food processing industry (Hasan, 2006). Since the ingestion of contaminated water or food leads to gastrointestinal infections, the high prevalence of *Aeromonas* species in the food chain should be considered a threat to public health (Alavandi and Ananthan, 2003; Garibay et al., 2006).

In recent years, there is an increase in appreciation and demand among health-conscious consumers for the ready-to-cook/eat minimally processed fresh produce of plant origin, and seafood and meat products in both developed and developing countries (Jaquette et al., 1996; Lee et al., 2005; Rajkowski 2008). Rohu (*Labeo rohita*), the most important freshwater fish of India, is a natural inhabitant of rivers and freshwater lakes in and around south and south-east Asia. Green gram, dew gram, chick pea and garden pea are the most common sprouts consumed in India.

Several physical and chemical treatment methods have been found to be ineffective in complete elimination of the food-borne pathogens under experimental conditions (Bari et al., 2005;

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Thayer et al., 2003). Radiation processing, a cold process, has been shown to be an effective method for eliminating food-borne pathogens in sprouts (Saroj et al., 2006; Bari et al., 2004) and flesh products such as meat and fish (Meng and Doyle, 2002). Gamma radiation has high penetration power and can inactivate pathogens that may have gained entry in tissues of sprouts or flesh. Irradiation ensures the microbiological safety without compromising the sensory and nutritional properties of meat and poultry (AbuTarboush et al., 1997; Hashim et al., 1995) and fresh plant produce (Hajare et al., 2006; Thayer et al., 2003).

Although *Aeromonas* have been reported in sprouts (Callister and Agger, 1987; Hora et al., 2005), there are no studies on the use of ionizing radiation to reduce *Aeromonas* level on sprouts. Studies from our laboratory have shown that the sprouts, chicken and fish samples procured from Mumbai markets are contaminated with *Aeromonas* (unpublished data). Thus, processing of these food products is necessary to prevent food-borne illness by *Aeromonas* spp.

The objectives of the present study were (i) to determine the D_{10} values of gamma radiation for *Aeromonas* isolates in saline, (ii) to determine the radiation dose for a 5-log elimination of *Aeromonas* from sprouts, chicken and fish samples and (iii) to study the survival and recovery, if any, of *Aeromonas* in inoculated and radiation-treated sprout, chicken and fish samples during storage at 4 °C.

2. Materials and methods

2.1. Bacterial strains

A. hydrophila CECT 839^T and *A. veronii* CECT 4257^T were kindly supplied by Dr. Valérie Leclère, Université des Sciences et Technologies de Lille USTL, Villeneuve d'Ascq cedex, France. *A. hydrophila* A90 and *A. caviae* A85 were isolated from alfalfa and mixed sprouts, respectively. *A. hydrophila* A331 and *A. veronii* Y47 were obtained from chicken samples, whereas *A. hydrophila* A619, *A. jandaei* A514A and *A. salmonicida* A527, Y528, Y559 and Y567 were isolated from fish samples. All *Aeromonas* cultures were maintained at 4 °C on Tryptic Soy Agar (TSA) slants.

2.2. Chemicals and media

Microbiological media were from Hi-Media Laboratories, Mumbai, India.

2.3. Decimal reduction dose (D_{10}) in saline

Aeromonas strains were inoculated in 25 ml of Tryptic Soya Broth (TSB) and incubated at 30 °C at 150 rpm. 18–20 h ($\approx 10^9$ CFU/ml) grown *Aeromonas* cells were harvested and centrifuged to obtain pellet, which was washed twice with sterile saline to remove media components. The cells were resuspended in 1.5 ml of sterile saline and further diluted to obtain a cell density of 10^7 CFU/ml. 1.2 ml of this suspension was transferred into five 1.5 ml microfuge tubes. The tubes were placed in ice and irradiated to doses of 0, 0.05, 0.1, 0.15 and 0.2 kGy at 0–4 °C in a cobalt-60 irradiator (Gamma cell 220, Atomic Energy of Canada Ltd., Ontario, Canada) at a dose rate of 5.4 Gy/min. After irradiation, total viable count (TVC) was determined by pour plating using appropriately diluted aliquots with TSA. The plates were incubated at 30 °C for 24 h and the colonies were counted. Each experiment included three samples per dose and was repeated thrice. The average number of surviving viable cells (CFU/ml) in the saline was plotted against the radiation dose. The slopes of the individual survivor curves were calculated by linear regression using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA). The D_{10} value was calculated by taking the negative reciprocal of the survival curve slope.

2.4. Preparation of inocula

For the inoculated pack studies, *A. caviae* A85, *A. jandaei* A514A, *A. veronii* Y47, *A. hydrophila* CECT 839^T and *A. salmonicida* Y567 were cultured separately in 25 ml TSB at 30 °C, 150 rpm for 18–20 h. 2 ml culture of each strain was pelleted by centrifugation (8000 rpm, 2 min) and resuspended in 2 ml saline. Equal volumes (2 ml) of cell suspensions of five strains were pooled to produce approximately equal populations of each strain. The 'cocktail' inoculum containing approximately 10^9 CFU/ml was used to inoculate sprout, chicken and fish samples.

2.5. Sample preparation

50 g each of mung (green gram {*Phaseolus aureus*}), matki (dew gram {*Phaseolus aconitifolius*}), chana (chick pea {*Cicer arietinum*}) and vatana (garden pea {*Pisum sativum*}) seeds were mixed and soaked in tap water for 10 h, and then sprouting was carried out for 24 h at room temperature (28 °C). The mixed sprouts were used for the radiation sensitivity and inoculated pack studies.

Boneless chicken and Rohu (*Labeo rohita*) fish fillets were procured from the local market.

2.6. Irradiation of samples

25 g each of mixed sprout, fish fillets and chicken meat were packed separately in low density polyethylene bags (LDPE) of 35 μm thickness (ACE packaging Ltd., Mumbai, India) using heat sealer (Sevana, Mumbai, India) and kept in ice. Radiation processing for the decontamination of packed samples was carried at 0–4 °C in a cobalt-60 irradiator (Gamma cell 5000, Board of Radioisotope and Technology, Mumbai, India) at a dose rate of 74 Gy/min. Mixed sprouts were exposed to 6 kGy and chicken or fish samples to 10 kGy dose for the elimination of native microbial flora.

Dose rate of all the radiation sources was measured using the Fricke method (Sehested, 1970). Variations in doses absorbed by experimental samples were minimized by placement within a uniform area of the radiation field.

2.7. Determination of D_{10} values in mixed sprouts, chicken and fish samples

D_{10} value of *Aeromonas* cocktail was determined in mixed sprouts, chicken and fish samples. Conditions necessary to obtain a desired number of cells (10^5 CFU/g) of *Aeromonas* attached to mixed sprouts were standardized. Mixed sprout samples (350 g) were dipped in sterile tap water (3 L) containing 10^6 CFU/ml of *Aeromonas* cocktail for 3 min and dried on sterile blotting paper to remove excess water under aseptic conditions. Samples were then packed in LDPE bags (25 g each) and sealed using heat sealer (Sevana, Mumbai, India). Sprout samples inoculated with *Aeromonas* were exposed to the radiation doses of 0.05, 0.1, 0.15 and 0.2 kGy.

Radiation decontaminated chicken and fish samples (25 g), in triplicates, were inoculated with the *Aeromonas* cocktail so as to obtain a count of 1×10^5 cells/g of the meat. The inoculated packs were irradiated at 0–4 °C with doses of 0.05, 0.1, 0.15 and 0.2 kGy.

After irradiation, the mixed sprouts, chicken and fish samples were aseptically homogenized for 1 min in a sterile stomacher bag (Stomacher lab blender, model 400, Seward, London, UK) containing 225 ml of sterile saline. Serial dilutions of the

homogenate were prepared and appropriate dilutions were used to determine viable counts with TSA. The plates were incubated at 30 °C for 24 h and CFU values were determined. Each study included three samples per dose and was repeated thrice. The average number of surviving viable cells (CFU/g) in the samples was plotted against the radiation dose. The slopes of the individual survivor curves were calculated by linear regression using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA). The i_{10} value was calculated by taking the negative reciprocal of the survival curve slope.

2.8. Determination of the dose required to eliminate 5-log CFU/g of the inoculated cells

The decontaminated mixed sprouts, chicken and fish samples (25 g) were inoculated with *Aeromonas* cocktail, as described in Section 2.7. The inoculated samples (10^5 CFU/g of *Aeromonas*) in triplicate were irradiated at 0.5, 1, 1.5 and 2 kGy in a cobalt-60 irradiator (Gamma cell 5000, Board of Radioisotope and Technology, Mumbai, India), and the surviving population was determined by plating the serial dilutions with TSA after an incubation of 24 h at 30 °C. Enrichment (TSB) and selective plating (Starch Ampicillin Agar, SAA) were carried out to confirm the complete elimination of these pathogens. Each experiment was repeated three times.

2.9. Storage studies of gamma-irradiated mixed sprout, chicken and fish samples inoculated with Aeromonas

Decontaminated mixed sprout, chicken and fish samples were inoculated with 10^5 CFU/g of *Aeromonas* cocktail, as described in Section 2.7. The inoculated packs were irradiated in melting ice conditions with doses of 0.5, 1, 1.5 and 2 kGy in a cobalt-60 irradiator (Gamma cell 5000, Board of Radioisotope and Technology, Mumbai, India) and stored at 4 °C. The sprout samples were screened for the presence of *Aeromonas* on the 0th, 4th, 8th and 12th day, while chicken and fish samples on the 0th, 3rd, 5th and 7th day. Enrichment and selective plating were carried out to confirm the complete elimination of the pathogens. Each experiment was repeated three times.

2.10. Statistical analysis

All the data for D_{10} values of *Aeromonas* strains in saline and food samples were analyzed statistically using Origin 6.1 software version 6.1052 B232 (OriginLab Corporation, Northampton, MA, USA). Significant differences in D_{10} values between different isolates were analyzed by one-way ANOVA.

3. Results and discussion

3.1. D_{10} values of Aeromonas isolates in saline

All *Aeromonas* isolates were found to be very sensitive to gamma radiation. The D_{10} values of different *Aeromonas* isolates in saline ranged from 0.031 to 0.046 kGy (Fig. 1). *A. veronii* CECT 4257^T was found to be most sensitive with D_{10} value of 0.031 kGy. The maximum D_{10} value was found to be of *A. salmonicida* Y567 (0.046 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 different *A. hydrophila* isolates (CECT 839^T, A90, A331 and A619) from different sources were compared (Fig. 1). There was significant difference ($P < 0.05$) in the radiation sensitivity of different *A. hydrophila* isolates. Among different

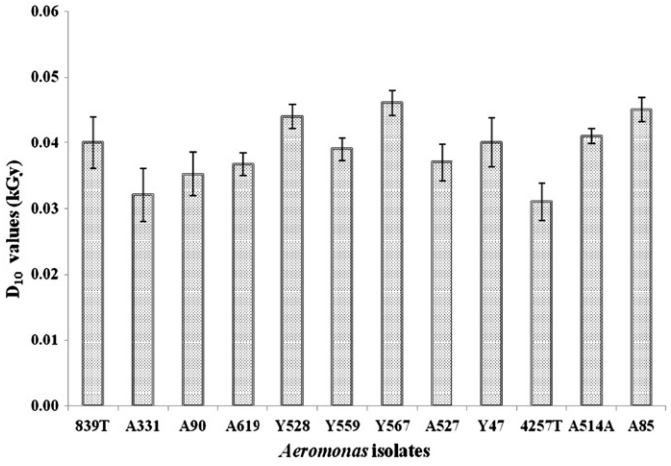

Fig. 1. D_{10} values of gamma radiation for *Aeromonas* isolates irradiated in saline *A. hydrophila* (CECT 839^T, A90, A331 and A619), *A. salmonicida* (Y528, Y559, Y567 and A527), *A. veronii* (Y47 and CECT 4257^T), *A. jandaei* A514A and *A. caviae* A85.

A. hydrophila isolates, the maximum D_{10} value was found to be for CECT 839^T (0.040 kGy).

The D_{10} values of different *A. salmonicida* and *A. veronii* isolates in saline ranged from 0.037 to 0.046 kGy and from 0.031 to 0.040 kGy, respectively (Fig. 1). Significant difference ($P < 0.05$) in the radiation sensitivity of different *A. salmonicida* and *A. veronii* in saline was also observed. Y567 was the most resistant one among *A. salmonicida* isolates with D_{10} value of 0.046 kGy. The maximum D_{10} value was observed for Y47 (0.040 kGy) among *A. veronii* isolates.

Rashid et al. (1992) reported 0.05 kGy as the D_{10} value for *A. hydrophila* in 0.067 M phosphate buffer containing 1% (w/v) NaCl. The D_{10} values of all *Aeromonas* strains from local foods ranged from 0.109 to 0.253 kGy in nutrient broth and increased when some strains were irradiated in ground meat at room temperature (Rashad and AbdelKareem, 1995). The lower D_{10} values observed in the present study may be due to the different *Aeromonas* strains studied. Moreover, this is the first report of radiation sensitivity of *Aeromonas* isolates from various Indian food samples.

The most resistant *Aeromonas* isolates, one from each of the five different *Aeromonas* species (*A. salmonicida* Y567, *A. caviae* A85, *A. jandaei* A514A, *A. hydrophila* CECT 839^T and *A. veronii* Y47), were selected to make a 'cocktail' inoculum of cultures to determine the radiation sensitivity of *Aeromonas* in mixed sprouts, chicken and fish samples.

3.2. D_{10} values of Aeromonas 'cocktail' in sprouts, chicken and fish samples

To decontaminate the mixed sprouts, chicken and fish samples, they were irradiated at different doses and aerobic plate counts were determined. The treatment with a 6 kGy dose was found sufficient for the destruction of the native flora from mixed sprouts, whereas, chicken and fish samples required a dose of 10 kGy (data not shown). Similar high doses were reported for decontamination of different types of sprouts (Rajkowski and Thayer, 2000; Saroj et al., 2006) and fishery products (Arvanitoyannis et al., 2009; Rashid et al., 1992).

Aeromonas cocktail was also found to be very sensitive in mixed sprouts, chicken and fish samples. The D_{10} values of *Aeromonas* cocktail in mixed sprouts, chicken and fish samples were found to be 0.081 ± 0.001, 0.089 ± 0.003 and 0.091 ± 0.003 kGy, respectively (Figs. 2–4). Stecchini et al. (1995) have reported the D_{10} values of *A. hydrophila* in minced poultry meat, using electron beam, to be 0.12 and 0.14 kGy in air and under vacuum, respectively. The D_{10} values of *A. hydrophila* in ground

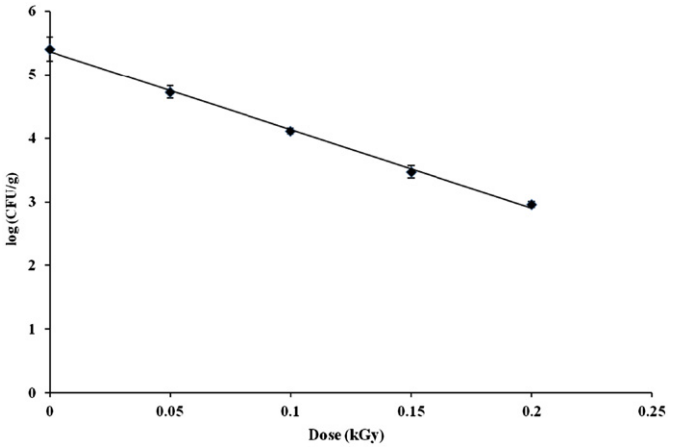

Fig. 2. Survival of a mixture of *A. salmonicida* Y567, *A. caviae* A85, *A. jandaei* A514A, *A. hydrophila* CECT 839^T and *A. veronii* Y47 on mixed sprouts after gamma radiation. $D_{10}=0.081\pm0.001$ kGy, $y=-12.28x+5.361$, $r^2=0.997$. Each symbol represents plate counts at each dose. Average values of three experiments are plotted along with standard deviation.

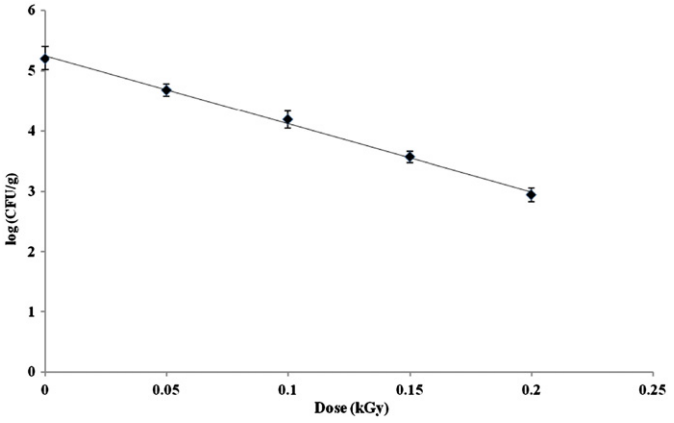

Fig. 3. Survival of a mixture of *A. salmonicida* Y567, *A. caviae* A85, *A. jandaei* A514A, *A. hydrophila* CECT 839^T and *A. veronii* Y47 on chicken sample after gamma radiation. $D_{10}=0.0089\pm0.003$ kGy, $y=-11.28x+5.245$, $r^2=0.997$. Each symbol represents plate counts at each dose. Average values of three experiments are plotted along with standard deviation.

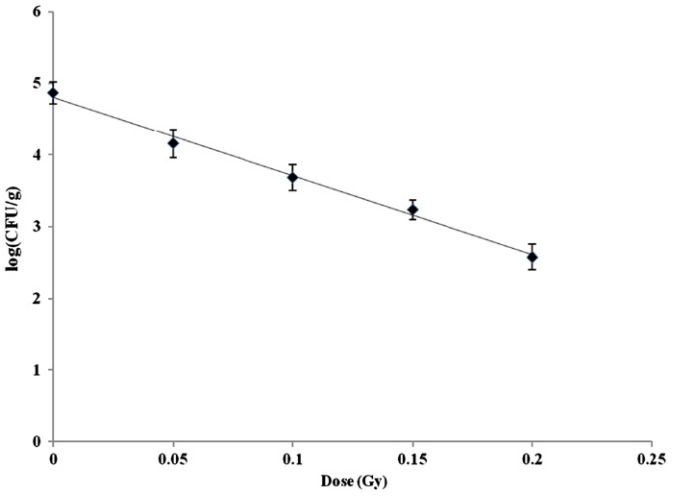

Fig. 4. Survival of a mixture of *A. salmonicida* Y567, *A. caviae* A85, *A. jandaei* A514A, *A. hydrophila* CECT 839^T and *A. veronii* Y47 on fish sample after gamma radiation. $D_{10}=0.091\pm0.003$ kGy, $y=-11.01x+4.805$, $r^2=0.993$. Each symbol represents plate counts at each dose. Average values of three experiments are plotted along with standard deviation.

blue-fish and ground beef at 2 °C were found to be 0.14–0.19 kGy (Palumbo et al., 1986). The lower D_{10} values observed in the present study may be due to the different *Aeromonas* strains used, intrinsic properties of the food products or irradiation conditions. A number of factors such as water activity, food material's composition, irradiation temperature, presence of oxygen, etc. affect the D_{10} values of bacteria in food (Mendonca, 2002). Some of the constituents, such as proteins, in a complex food system, are thought to compete with cells for interaction with radiolytic free radicals. Thus, they reduce the net effect of radiation damage and make organisms more radiation resistant (Urbain, 1986). To date, there are no reports on radiation sensitivity of *Aeromonas* on raw sprouts. To the best of our knowledge, this is the first report of radiation sensitivity of *Aeromonas* in sprouts.

3.3. Radiation dose for 5-log elimination of Aeromonas from sprouts, chicken and fish samples

Table 1(a) shows the effect of irradiation on the survival of *Aeromonas* cocktail inoculated in mixed sprouts. Inoculation of mixed sprouts with the cocktail of *Aeromonas* achieved a level of 2.9×10^5 CFU/g. No significant difference ($P>0.05$) was observed in the levels of *Aeromonas*, recovered from mixed sprouts after attachment, in different experiments. Treatment with 1 kGy and above doses could eliminate 5-log CFU/g of *Aeromonas* spp. from mixed sprouts, when the survival of the pathogens was analyzed immediately after irradiation. However, recovery of the pathogen was observed in 0.5 and 1 kGy treated samples after enrichment in TSB for 24 h followed by selective plating on SA agar plates. This could be due to repair of radiation-induced damage during enrichment. Sprout samples were enriched prior to plating on selective media to check the resuscitation of *Aeromonas* cells that might have been injured during the irradiation process. No such recovery of *Aeromonas* was observed in 1.5 and 2 kGy treated mixed sprout samples after enrichment and selective plating.

Aeromonas counts of control samples increased from 5.46 to 6.91log CFU/g during storage at 4 °C till 12th day. No viable counts were detected in 1.5 and 2 kGy irradiated sprout samples during storage, even after enrichment and selective plating. Thus, radiation treatment of mixed sprout samples with 1.5 kGy is sufficient to eliminate 10^5 CFU/g of *Aeromonas* spp. This is the first report on the effectiveness of irradiation to eliminate *Aeromonas* from mixed sprouts, which are commonly used in India.

Though irradiation is recognized as an effective intervention technology for the purpose of pathogen reduction, the radiation dose utilized for pathogen reduction should not significantly impact the product quality factors such as taste and aroma if the process is to be used commercially (Sommers et al., 2004). Earlier studies have shown that radiation treatment of different sprouts with a dose of 2 kGy does not adversely affect their textural, nutritional and organoleptic qualities (Bari et al., 2004; Hajare et al., 2007; Rajkowski et al., 2003).

The results of *Aeromonas* counts in the fish and chicken samples are given in Table 1(b) and (c), respectively. The initial cell counts in the inoculated fish and chicken samples were found to be 5.24 and 5.45log CFU/g, respectively. No viable counts were detected in 1, 1.5 and 2 kGy irradiated fish and chicken samples on the 0th day when the survival of the pathogens was analyzed immediately after irradiation. However, 0.5 and 1 kGy treated samples showed recovery of the pathogen after enrichment and selective plating, whereas, no such recovery was observed in 1.5 and 2 kGy treated fish and chicken samples. Our observation is in agreement with the findings of Lamuka et al. (1992), who have reported that the radiation-induced injured cells can be resuscitated when placed under favorable growth conditions.

Table 1
Effect of irradiation on the growth of *Aeromonas* inoculated in (a) mixed sprouts, (b) fish and (c) chicken samples during storage at 4 °C.

(A) Mixed sprouts Days dose (kGy)	0th day		4th day		8th day		12th day	
	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]
0	5.46 ± 0.18	+	5.79 ± 0.24	+	6.23 ± 0.42	+	6.91 ± 0.38	+
0.5	0.32 ± 0.04	+	0.84 ± 0.09	+	1.04 ± 0.14	+	1.54 ± 0.10	+
1	NVC[b]	+	NVC[b]	−	NVC[b]	−	NVC[b]	−
1.5	NVC[b]	−	NVC[b]	−	NVC[b]	−	NVC[b]	−
2	NVC[b]	−	NVC[b]	−	NVC[b]	−	NVC[b]	−
(B) Fish Days dose (kGy)	0th day		3rd Day		5th day		7th day	
	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]
0	5.24 ± 0.23	+	5.52 ± 0.61	+	6.24 ± 0.54	+	6.64 ± 0.49	+
0.5	0.62 ± 0.09	+	1.08 ± 0.06	+	1.41 ± 0.16	+	1.76 ± 0.19	+
1	NVC[b]	+	NVC[b]	−	NVC[b]	−	NVC[b]	−
1.5	NVC[b]	−	NVC[b]	−	NVC[b]	−	NVC[b]	−
2	NVC[b]	−	NVC[b]	−	NVC[b]	−	NVC[b]	−
(C) Chicken Days dose (kGy)	0th day		3rd Day		5th day		7th day	
	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]	log CFU/g	*Aeromonas*[a]
0	5.45 ± 0.61	+	5.69 ± 0.43	+	6.20 ± 0.52	+	6.79 ± 0.47	+
0.5	0.31 ± 0.08	+	0.48 ± 0.07	+	0.69 ± 0.09	+	0.90 ± 0.05	+
1	NVC[b]	+	NVC[b]	+	NVC[b]	+	NVC[b]	−
1.5	NVC[b]	−	NVC[b]	−	NVC[b]	−	NVC[b]	−
2	NVC[b]	−	NVC[b]	−	NVC[b]	−	NVC[b]	−

[a] Recovery of *Aeromonas* was checked by enrichment and selective plating.
[b] NVC, no viable counts detected.

An increase in *Aeromonas* counts was observed in the control (non-irradiated) fish and chicken samples from 5.24 to 6.64log CFU/g and from 5.45 to 6.79log CFU/g, respectively, during storage at 4 °C for 7 days. No viable counts were detected in 1.5 and 2 kGy irradiated fish and chicken samples during storage, even after enrichment and selective plating. Thus, a dose of 1.5 kGy could completely eliminate 5-log CFU/g of inoculated *Aeromonas* from fish and chicken samples. Özbas et al. (1996) suggested that a dose of 0.75 kGy in combination with conventional cooking procedure is sufficient to destroy approximately 10^4 CFU/g of *Aeromonas hydrophila* in meatball. Though a radiation dose of 1.5 kGy has been recommended for the elimination of *A. hydrophila* in fresh fish, seafood, red meat and poultry (Palumbo et al., 1986), this study did not check for the actual elimination of 10^5 CFU/g of *Aeromonas* from food products using inoculated pack studies. The earlier studies also did not check for resuscitation of *Aeromonas* after enrichment and selective plating. This is also the first report of storage studies of control and irradiated fish samples inoculated with *Aeromonas*.

Several researchers have shown that a radiation dose of less than 2 kGy does not adversely affect microbiological, chemical and sensory acceptability of both raw and cooked chicken (AbuTarboush et al., 1997; Balamatsia et al., 2006). Recently, Kanatt et al. (2010) have shown that 2.5 kGy irradiation, in combination with chilled storage, improves the safety and extends the shelf life of ready-to-cook meat products that are sold in Indian supermarkets.

Gamma irradiation at low doses (1–3 kGy) has been accepted by several countries for extension of shelf life of marine and freshwater fishery products (Venugopal et al., 1999). Recently several researchers have shown that radiation at a low dose (up to 3 kGy), in combination with chilled storage, can be used to preserve the fishery products without adversely affecting their biochemical, textural and sensory attributes (Mbarki et al., 2008, 2009; Moini et al., 2009).

Aeromonas were detected in chicken (28.6%), fish (20%) and sprout (2.5%) samples marketed in Mumbai (unpublished data). Usually, the cell number of *Aeromonas*, if present in different food products, is 10^2–10^5 CFU/g (Gobat and Jemmi, 1993; Pin et al., 1994). Though, the infectious dose of *Aeromonas* range from 10^3 to 10^9 CFU/g (Yücel and Erdogan, 2010), they can survive and grow to higher numbers in food products stored at low temperature. It is also possible that some *Aeromonas* strains may have a lower infective dose in sensitive subpopulations like children and immune-compromised people (Hasan, 2006). Thus, radiation treatment of sprout, chicken and fish products with 1.5 kGy will be sufficient to eliminate 10^5 CFU/g of *Aeromonas*, thereby ensuring their safety.

4. Conclusions

The results from this study showed that all *Aeromonas* isolates were very sensitive to gamma radiation. A radiation dose of 1.5 kGy is effective in achieving 5-log reductions in *Aeromonas* populations in mixed sprout, chicken and fish samples. The study also revealed the importance of conducting enrichment studies to determine the bactericidal effects of the irradiation process. This is the first report of radiation sensitivity and inoculated pack studies of Indian *Aeromonas* isolates, irradiated in different food products. The radiation doses selected for partial or full elimination of other pathogens with higher D_{10} values (*Salmonella*, *L. monocytogenes*, etc.) could provide near-complete elimination of *Aeromonas* as an ancillary benefit.

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A. sobria CECT 4245^T and *A. veronii* CECT 4257^T. Technical help from Kalpana Bagade of Food Technology Division, BARC is acknowledged.

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SHORT COMMUNICATION

Characterization of *Aeromonas* strains isolated from Indian foods using *rpoD* gene sequencing and whole cell protein analysis

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Abstract *Aeromonas* are responsible for causing gastroenteritis and extra-intestinal infections in humans. Twenty-two *Aeromonas* strains isolated from different food sources were re-identified up to species level using *rpoD* gene sequence analysis. Biochemical tests and 16S rRNA gene sequencing were insufficient to identify *Aeromonas* till species level. However, incorporation of additional biochemical tests lead to correct identification of 95.5 % strains up to species level. The 16S rRNA gene sequencing was useful to identify *Aeromonas* isolates at the genus level only. Sequences of the *rpoD* gene showed greater discriminatory power than 16S rRNA gene and provided conclusive discrimination of the strains for which the phenotypic species identification was uncertain. All these 22 strains were accurately identified up to species level by *rpoD* gene as *A. salmonicida* (6), *A. veronii* bv. *veronii* (4), *A. caviae* (3), *A. hydrophila* (2), *A. veronii* bv. *sobria* (2), *A. jandaei* (1), *A. trota* (1), *A. sobria* (1), *A. allosaccharophila* (1) and *A. bivalvium* (1). All these strains were also characterized using whole cell protein (WCP) analysis by gradient SDS-PAGE and showed different whole cell protein (WCP) profile [22–28 polypeptide bands (~10 to >97 kDa)], indicating high genetic diversity. The present work emphasizes the use of molecular methods such as *rpoD* gene sequencing along with comprehensive biochemical tests for the rapid and accurate identification of *Aeromonas* isolates till species level. The WCP profile can be subsequently used to characterize *Aeromonas* isolates below species level.

Keywords *Aeromonas* · *rpoD* gene · Whole cell protein analysis · Sprouts · Chicken · Fish

Introduction

Members of the genus *Aeromonas* are regarded as opportunistic as well as primary pathogens of both immunocompetent and immuno-compromised humans, and aquatic and terrestrial animals (Nagar and Bandekar 2011). Complex taxonomy is the key challenge in establishing an explicit relationship between the genus *Aeromonas* and pathogenicity in humans. Only a small subset of strains containing putative virulence genes seems to cause infection or diarrhea (Martino et al. 2011). Precise identification of the concerned pathogen is of great concern from an epidemiological point of view.

The taxonomy of the genus *Aeromonas* is complex and has undergone several changes during the last two decades due to continuous addition of an increasing number of novel species, rearrangement of already described strains and species, and discrepancies found in different DNA–DNA hybridization studies (Martinez-Murcia et al. 2011). Key difficulties in the phenotypic identification of *Aeromonas* are the use of different methods and conditions for the biochemical tests, high intra-species phenotypic variability, ambiguous results, requirement of additional cumbersome and time-consuming phenotypic tests (Abbott et al. 2003; Martin-Carnahan and Joseph 2005). Commercial identification systems are also not very successful in the identification of *Aeromonas* species up to species level (Lamy et al. 2010). Thus, many researchers have used

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molecular methods for the identification of *Aeromonas* species. The 16S rRNA gene sequences are universally used to understand phylogenetic relationships and species identification of bacteria (Clarridge III 2004). However, in case of *Aeromonas*, 16S rRNA gene has been found to be inefficient in species level identification due to its highly conserved gene sequence (Morandi et al. 2005). Studies have shown that house-keeping genes like *rpoD* and *gyrB* are better molecular markers than the 16S rRNA gene for the study of phylogenetic and taxonomic relationships at the species level in the genus *Aeromonas* (Beaz-Hidalgo et al. 2010; Soler et al. 2004). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell protein (WCP) has been widely used for typing strains within particular bacterial species (Szczuka and Kaznowski 2007). It is a simple, rapid, inexpensive, reliable and easily applicable system for characterization of *Aeromonas* isolates (Maiti et al. 2009).

In the present study, 22 *Aeromonas* strains, isolated from different food samples and biochemically identified to different species (Nagar et al. 2011), were re-assessed till species level using additional key tests recently described (Beaz-Hidalgo et al. 2010; Martin-Carnahan and Joseph 2005; Minana-Galbis et al. 2007), along with conventional biochemical schemes (Abbott et al. 2003) and partial sequencing of the *rpoD* gene. The extent of agreement between the conventional biochemical schemes and molecular methods (16S rRNA and *rpoD* gene) was analyzed and these isolates were identified up to species level. The WCP profiles of *Aeromonas* isolates, from different food samples, were also determined using gradient SDS-PAGE.

Materials and methods

Bacterial strains and growth conditions

A collection of 22 *Aeromonas* strains belonging to the 7 different species (Nagar et al. 2011) were analyzed (Table 1). *A. hydrophila* CECT 839^T, *A. veronii* CECT 4257^T and *A. veronii* CECT 4246 were kindly supplied by Dr. Valérie Leclère, Université des Sciences et Technologies de Lille USTL, France. *A. veronii* bv. *sobria* MTCC 3249 was obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India). All bacterial cultures were maintained in tryptic soya broth (TSB) (HiMedia, India) with 20 % of glycerol (v/v) at −80 °C. Working cultures were maintained on tryptic soya agar (TSA) (HiMedia, India) slants and plates at 4 °C. The cultures from TSA plate were retrieved in brain heart infusion (BHI) broth (HiMedia) by incubating for 18 h at 30 °C, 150 rpm and used for the study.

Re-evaluation of *Aeromonas* strains by additional biochemical tests

In the previous study (Nagar et al. 2011), species level identification of twenty-two *Aeromonas* isolates was done using biochemical tests described by Abbott et al. (2003). Selected supplementary biochemical tests that have recently been described (Beaz-Hidalgo et al. 2010; Martin-Carnahan and Joseph 2005; Minana-Galbis et al. 2007) were performed for better discrimination of the strains up to species level: i.e. acid production from melibiose, salicin and D-mannose, Voges–Proskauer test, and hydrolysis of starch and gelatin.

PCR amplification and sequencing of *rpoD* gene

DNA template was prepared by suspending 2–3 colonies of each *Aeromonas* isolate from TSA plate in 100 µl of sterile distilled water and boiling for 5 min. The tubes were centrifuged at 6,000 rpm for 1 min to pellet out the cell debris. The supernatant was stored at −20 °C and used as DNA template for the PCR reactions. A fragment of approximately 820 bp of the *rpoD* gene was amplified by PCR using primers and amplification conditions as described by Soler et al. (2004). Amplified products were purified using the GenElute PCR Clean-up kit (Sigma-Aldrich, St Louis, MO, USA). Partial sequences of each PCR product were sequenced at MWG-Biotech Pvt. Ltd., Bangalore, India. Newly determined sequences were compared to those available in the GenBank database, using the BLASTN program (www.ncbi.nih.gov/BLAST/), to ascertain their closest relatives. The sequences were submitted to the GenBank database using the web-based data submission tool, BankIt (http://www.ncbi.nlm.nih.gov/BankIt/).

Molecular identification and phylogenetic data analysis

The nucleotide sequences of 16S rRNA gene of these 22 strains were retrieved from the GenBank (Accession numbers: FJ561050-52, HQ122915-31, HQ413137 and JN697593). The 16S rRNA and *rpoD* gene sequences from all *Aeromonas* strains and their corresponding type or reference strains were independently aligned, and DNA sequence similarities were calculated for a continuous stretch of 505 bases (positions 81–584 according to *Escherichia coli* numbering, J01695), in case of 16S rRNA gene and 512 bases (positions 393–894 according to *E. coli* numbering, NP_417539.1) for *rpoD* gene. The phylogenetic trees were constructed with the MEGA 5 program package (Tamura et al. 2011) using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method with Kimura two-parameter model.

Table 1 Comparison of phenotypic and genetic identification of 22 *Aeromonas* strains recovered from different food samples

Strain	Origin	Taxonomic identification (species name) based on			
		Biochemical tests[a]	Additional biochemical tests[b]	16S rRNA gene sequences[a]	*rpoD* gene sequences
A 85	Mixed sprouts	*A. caviae*	*A. caviae*	*A. caviae*	*A. caviae*
A90	Alfalfa sprouts	*A. hydrophila*	*A. hydrophila*	*A. trota*	*A. hydrophila*
A91	Alfalfa sprouts	*A. caviae*	*A. caviae*	*A. trota*	*A. caviae*
A 329	Chicken	*A. jandaei*	*A. jandaei*	*A. jandaei*	*A. jandaei*
A331	Chicken	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
Y47	Chicken	*A. veronii* bv. *sobria*	*A. salmonicida*	*A. salmonicida/A. hydrophila*	*A. salmonicida*
Y113	Chicken	*A. veronii* bv. *sobria*	*A. veronii* bv. *sobria*	*A. veronii*	*A. veronii* bv. *sobria*
Y 324	Chicken	*A. caviae*	*A. caviae*	*A. trota/A. caviae*	*A. caviae*
A 254	*Aristichthys nobilis*	*A. veronii* bv. *sobria*	*A. veronii* bv. *sobria*	*A. veronii*	*A. veronii* bv. *veronii*
A 283	*Parastromateus niger*	*A. veronii* bv. *sobria*	*A. trota*	*A. caviae*	*A. trota*
A 501A	*Ompok bimaculatus*	*A. hydrophila*	*A. veronii* bv. *veronii*	*A. veronii*	*A. veronii* bv. *veronii*
A502A	*Ompok bimaculatus*	*A. veronii* bv. *sobria*	*A. veronii* bv. *sobria*	*A. veronii*	*A. veronii* bv. *sobria*
A514A	*Ompok bimaculatus*	*A. jandaei*	*A. veronii* bv. *veronii*	*A. trota/A. caviae*	*A. veronii* bv. *veronii*
A521	*Ompok bimaculatus*	*A. eucrenophila*	*A. allosaccharophila*	*A. allosaccharophila*	*A. allosaccharophila*
A 527	*Macrobrachium rosenbergii*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*
A563	*Harpadon nehereus*	*A. caviae*	*A. bivalvium*	*A. bivalvium/A. popoffi*	*A. bivalvium*
A619	*Catla catla*	*A. hydrophila*	*A. veronii* bv. *veronii*	*A. hydrophila/A. trota*	*A. veronii* bv. *veronii*
Y 528	*Aristichthys nobilis*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*
Y 556	*Ompok bimaculatus*	*A. trota*	*A. sobria*	*A. sobria*	*A. sobria*
Y 559	*Ompok bimaculatus*	*A. salmonicida*	*A. salmonicida*	*A. hydrophila/A. salmonicida*	*A. salmonicida*
Y 567	*Ompok bimaculatus*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*
Y 577	*Aristichthys nobilis*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*	*A. salmonicida*

[a] Species level identification based on biochemical tests and partial 16S rRNA gene sequencing (Nagar et al. 2011)

[b] Species level identification based on additional biochemical tests (Beaz-Hidalgo et al. 2010; Martin-Carnahan and Joseph 2005; Minana-Galbis et al. 2007)

Whole cell protein profiling

The WCP profiles of different *Aeromonas* strains were determined according to SDS-PAGE protocol described by Maiti et al. (2009) with modification of 5 % stacking and 5–18 % gradient separating gels. The bands were analyzed visually and the 0 and 1 matrix (binary matrix) of the protein gel was developed based on the presence or absence of the particular size band on the gel in all the samples. The matrix was analyzed using the FREETREE software (version 0.9.1.50, Folia Biologica). The relatedness of the isolates was analyzed using Nei and Li/Dice distance/similarity calculations and Neighbor-joining as the tree-construction algorithm. The output tree was visualized using the Tree View software (version 1.5.2, Roderic D. M.).

Results and discussion

Identification of isolates based on biochemical tests and 16S rRNA sequences

In the previous study, twenty-two *Aeromonas* strains were identified from 451 presumptive positive isolates using biochemical tests (Abbott et al. 2003) and confirmed as *Aeromonas* genus by 16S rRNA partial gene sequencing (Table 1) (Nagar et al. 2011). However, disparity was observed between the identification of these isolates based on biochemical tests and 16S rRNA gene. Recently, Ottaviani et al. (2011) have stressed the importance of incorporation of newly described supplementary key biochemical tests to conventional biochemical schemes (Abbott et al. 2003) to distinguish each species from its nearest neighbours at the phenotypic level. Therefore, these strains were further characterized using additional biochemical tests (Beaz-Hidalgo et al. 2010; Martin-Carnahan and Joseph 2005; Minana-Galbis et al. 2007).

Based on the additional biochemical tests, 22 of earlier phenotypically identified *Aeromonas* isolates were re-identified as *A. salmonicida* (6), *A. veronii* bv. *veronii* (3), *A. caviae* (3), *A. hydrophila* (2), *A. veronii* bv. *sobria* (3), *A. jandaei* (1), *A. trota* (1), *A. sobria* (1), *A. allosaccharophila* (1) and *A. bivalvium* (1) (Table 1). The strain Y47 (*A. veronii* bv. *sobria*) was re-identified as *A. salmonicida*, A283 (*A. veronii* bv. *sobria*) as *A. trota*, A501A (*A. hydrophila*) as *A. veronii* bv. *veronii*, A514A (*A. jandaei*) as *A. veronii* bv. *veronii*, A521 (*A. eucrenophila*) as *A. allosaccharophila*, A563 (*A. caviae*) as *A. bivalvium*, A619 (*A.*

hydrophila) as *A. veronii* bv. *veronii* and Y556 (*A. trota*) as *A. sobria*. Beaz-Hidalgo et al. (2010) reported that some *A. sobria* strains can hydrolyze gelatin and show a positive reaction to ADH and VP tests, contradicting earlier published data by Abbott et al. (2003). These observations helped in the correct identification of isolate Y556 as *A. sobria*. Further, partial 16S rRNA gene analysis was used to confirm the identification based on additional biochemical tests. However, most of these strains could not be correctly identified till species level using 16S rRNA gene analysis (Table 1).

Sequence similarity between all *Aeromonas* strains for the 16S rRNA gene was 94.6–100 %, corresponding to 0–27 nucleotide differences. Mean sequence similarity, an indicator of discriminatory power, was found to be 97.3 %. The alignment exhibited a total of 43 variable positions (8.5 % of the determined fragment). Analysis of the aligned 16S rRNA gene sequences allowed the construction of the phylogenetic tree (Fig. 1a). Species level identification of only 59.1 % (13/22) of these isolates by biochemical tests (Abbott et al. 2003) agreed with the identification based on 16S rRNA gene sequencing. However, this correlation percentage increased to 77.3 % (17/22) on incorporation of additional biochemical tests (Table 1). The disparity between biochemical and 16S rRNA gene identification can be observed in the phylogenetic tree, where the identified strains fail to cluster with their corresponding type strains (Fig 1a). The 16S rRNA gene sequences were successful in identification of *A. salmonicida*, *A. sobria*, *A. bivalvium*, *A. allosaccharophila* and *A. jandaei* isolates up to species level (Fig. 1a). However, the degree of resolution obtained with 16S rRNA gene sequencing was not sufficient to correctly identify all the *Aeromonas* strains till species level (Table 1). The 16S rRNA gene was found to be highly conserved within the genus *Aeromonas* and showed limited resolution based on analysis of 1,330 bp region (Kupfer et al. 2006). Other researchers (Beaz-Hidalgo et al. 2010; Ormen et al. 2005) have also found environmental *Aeromonas* isolates to be highly heterogeneous in their biochemical properties. Lamy et al. (2010) could correctly identify only 24 % of environmental isolates up to species level by biochemical tests. The 16S rRNA gene sequencing was useful to identify *Aeromonas* isolates at the genus level only. Thus, in order to overcome this limitation, *rpoD* gene sequencing of these strains was undertaken to conclusively identify these strains at species level.

Identification of isolates based on biochemical tests and *rpoD* gene sequences

Partial nucleotide sequences of *rpoD* gene from 22 *Aeromonas* strains were determined (GenBank accession number: JN182265-69, JN412625-30, JN388917-22 and JN544572-76). The sequence similarity between all *Aeromonas* strains was 80.4–99.8 %, corresponding to 1–100 nucleotide differences. Mean sequence similarity was found to be 89.1 %. This value is significantly lesser than that of the 16S rRNA gene (97.3 %) of the same strains and comparable to that of *gyrB* (92.2 %) and *rpoD* (89.3 %) genes as reported by Soler et al. (2004). The alignment exhibited a total of 154 variable positions (30.1 % of the fragment sequenced), values close to those reported by Soler et al. (2004). Deletion of 6 bp in the sequence of *A. salmonicida* CECT 894^T strain (AY169327) was observed as compared to sequences from all other *A. salmonicida* isolates. The observations were confirmed by comparison of sequences from *A. salmonicida* strains from our study with other *A. salmonicida* sequences [190 (FN773330) and 156 (AY169361)]. Deletion of 3 bp was also observed in both A283 and CECT 4255^T sequences (both *A. trota* species) as compared to all other strains.

Identification of 59.1 % (13/22) of these isolates by biochemical tests (Abbott et al. 2003) agreed with the identification based on *rpoD* gene sequencing. However, congruity increased to 95.5 % (21/22) when additional biochemical tests were incorporated in the phenotypic identification (Table 1). The re-identification of all the strains (Y47, A283, A501A, A514A, A521, A563, A619 and Y556) using additional biochemical tests were confirmed by *rpoD* gene sequence analysis (Table 1).

The comparison of 16S rRNA and *rpoD* sequence data indicated that the two genes showed dissimilar substitution rates. The number of variable positions was approximately 3.5 times more in *rpoD* gene as compared to 16S rRNA gene (8.5 % for 16S rRNA vs. 30.1 % for *rpoD*) and the ranges of nucleotide substitutions between all strains were also different (0–27 and 1–100, for 16S rRNA and *rpoD* genes, respectively). Further, the phylogenetic tree based on *rpoD* gene showed more consistent clustering than that based on 16S rRNA gene between the identified strains and their respective reference strains (Fig 1). There was a congruence of 77.3 % in the identification of *Aeromonas* strains, based on comprehensive biochemical tests and 16S rRNA gene sequencing. However, identification of all the strains, except A254, based on comprehensive biochemical tests matched with *rpoD* gene analysis. The isolate A254 was identified as *A. veronii* bv. *sobria* using comprehensive biochemical tests; whereas, *rpoD* gene sequence analysis identified it as *A. veronii* bv. *veronii*. Multilocus sequence typing (MLST) can be further used for the accurate species level identification of *Aeromonas* strains (Martinez-Murcia et al. 2011; Martino et al. 2011). However, MLST was found to be inefficient to differentiate strains belonging to *A. veronii* group (*A. veronii* bv. *veronii*, *A. veronii* bv. *sobria* and *A. allosaccharophila*) due to high frequency of horizontal gene transfer in this group (Silver et al. 2011).

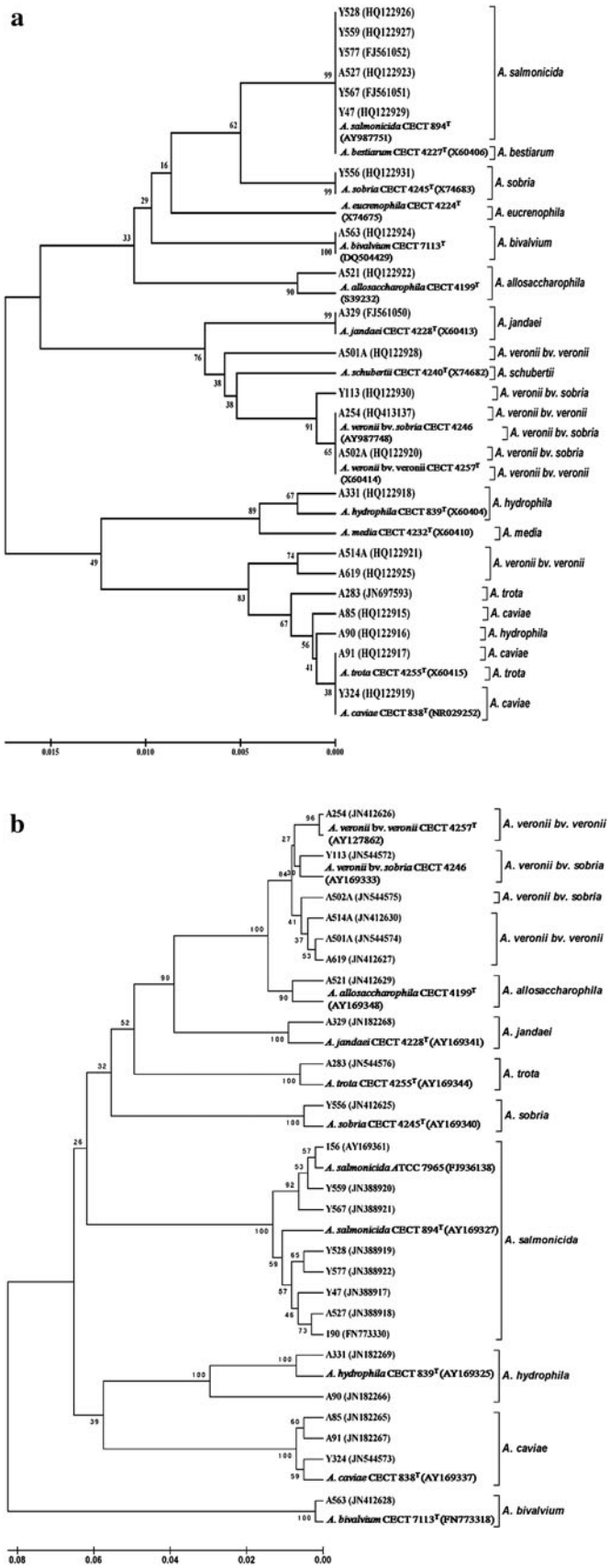

Fig. 1 Unrooted phylogenetic tree (UPGMA) of *Aeromonas* food isolates and other known *Aeromonas* species based on the **a** 16S gene and **b** *rpoD* gene sequences. CECT and ATCC *numbers* indicate the Spanish Type culture collection and American Type culture collection numbers of the *Aeromonas* reference strains, respectively. All the reference strains are shown in the *bold font*. *Numbers* in the *parenthesis* represent the GenBank accession numbers. *Numbers* shown next to each node indicate bootstrap values (percentage of 1,000 replicates). The *bar* indicates **a** 0–1.5 % sequence divergence, **b** 0–8 % sequence divergence

Our results further confirm that the *rpoD* gene has higher discriminatory power than 16S rRNA gene to delineate *Aeromonas* strains till species level. In the recent years, *rpoD* gene sequencing has been widely used for the species level identification of *Aeromonas* strains from clinical samples (Puthucheary et al. 2012; Senderovich et al. 2012). They also observed that the identification of *Aeromonas* on the basis of *rpoD* gene sequencing was more accurate than biochemical methods or 16S rRNA gene sequencing. The disparity between the biochemical and the genetic identification of the environmental isolates may be explained by the fact that the biochemical species-diagnostic scheme was developed based on data from clinical strains. Environmental isolates are often more heterogenic than clinical isolates, and their biochemical profiles are less well-known compared to those of clinical isolates (Ormen et al. 2005). Based on the earlier reports (Abbott et al. 2003; Beaz-Hidalgo et al. 2010; Soler et al. 2004) and present study, we propose that a combination of certain biochemical tests and *rpoD* gene sequencing (Supplementary Fig 1) would be ideal for the simple and accurate identification of *Aeromonas* isolates from food samples up to species level.

WCP profile analysis

The WCP profiles of 22 *Aeromonas* isolates yielded 22–28 polypeptide bands ranging from ~10 to >97 kDa and were reproducible (Fig 2). All the strains were typeable and showed unique banding patterns indicating a high level of diversity among *Aeromonas* strains. Protein bands of 97, 68, 64, 45, 40, 29, 28, 26, 25, 18, 16, 14, 12 and 10 kDa were detected in all the *Aeromonas* isolates. Protein bands of 64, 45, and 25 kDa appeared as major bands in all the strains. Major variation in the banding pattern was observed in two main regions, 29–45 and 66–97 kDa.

Figure 3 shows the dendogram, of strains belonging to different *Aeromonas* species, produced after numerical analysis of the WCP profiles using the Nei and Li/Dice distance/similarity calculations and Neighbor-joining tree-construction algorithm. The overall protein profiles were very similar among the strains of the same species except for slight variations in the number of bands generated. For majority of the isolates, no clear correlation was observed between the origin of the strains and their protein profiles. However, in the case of *A. hydrophila*, *A. caviae* and *A. veronii* bv. *sobria* species, clustering of strains based on their origin was observed (Fig 3a–c). *A. caviae* strains, A85 and A91, both isolated from sprout samples clustered together, while Y324 (chicken isolate) diverged into a separate clade. *A. hydrophila* isolates, A331 (chicken) and A90 (sprout), and *A. veronii* bv. *sobria* strains, A502A (fish) and Y113 (chicken) segregated into separate clades based on their WCP profiles. It was also observed that

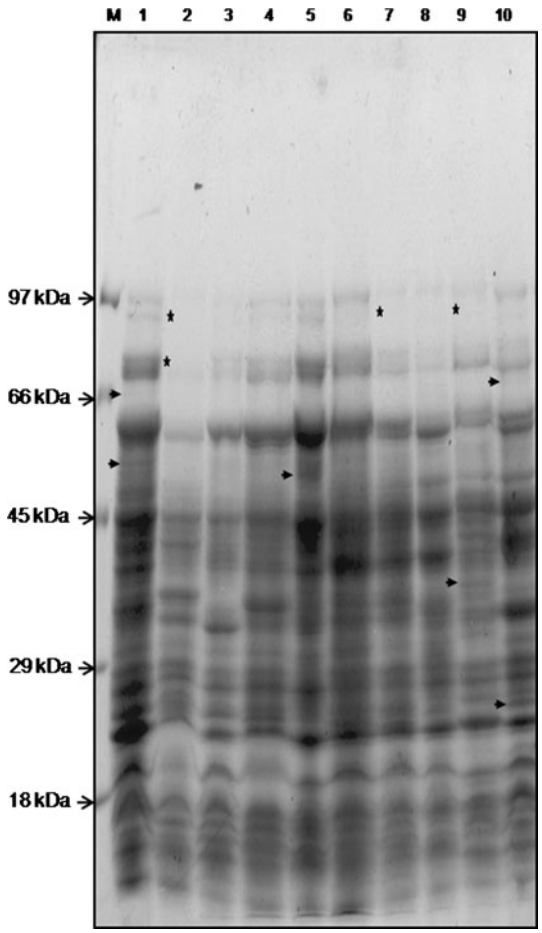

Fig. 2 WCP profiling of *Aeromonas* spp. on 5–18 % gradient SDS-PAGE. M: PMW-M Protein Marker (GeNei™, Bangalore, India); *Lane 1* A283 (*A. trota*), *Lane 2* A329 (*A. jandaei*), *Lane 3* A331 (*A. hydrophila*), *Lane 4* A521 (*A. allosaccharophila*), *Lane 5* A563 (*A. bivalvium*), *Lane 6* A619 (*A. veronii* bv. *veronii*), *Lane 7* Y113 (*A. veronii* bv. *sobria*), *Lane 8* Y324 (*A. caviae*), *Lane 9* Y556 (*A. sobria*), *Lane 10* Y567 (*A. salmonicida*). *Asterisk* indicates the absence of band in the lane; while *arrow* indicates the presence of extra band in the lane

Aeromonas strains from different sources also shared similar WCP profiles. Strains Y559, Y567 and Y528 belonging to *A. salmonicida* and isolated from *Ompok bimaculatus* and *Aristichthys nobilis* fish samples, respectively, showed similar protein profiles. Similarly, *A. caviae* strains A85 (mixed sprouts) and A91 (alfalfa sprouts) and *A. veronii* bv. *veronii* strains A254 (*Aristichthys nobilis*) and A501A (*Ompok bimaculatus*) also showed similar WCP profiles. The strains contaminating these samples may be from common source or origin.

Several researchers have used WCP analysis to study the diversity of *Aeromonas* strains at and below species level (Delamare et al. 2002). Maiti et al. (2009) and Szczuka and Kaznowski (2007) have successfully used WCP profiling of *Aeromonas* isolates and observed high level of diversity among *Aeromonas* strains. The genus *Aeromonas* is

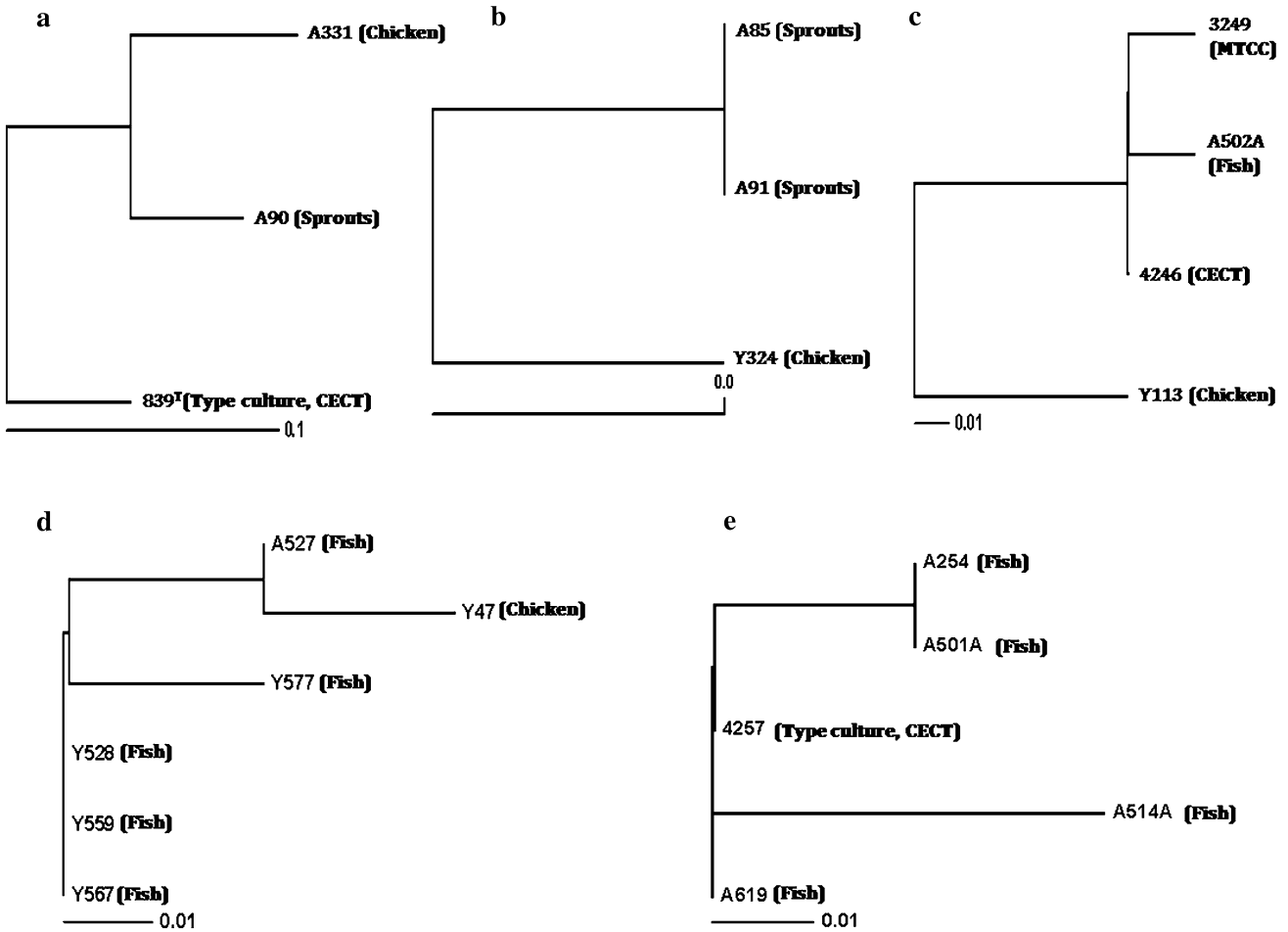

Fig. 3 Dendogram of protein similarity of **a** *A. hydrophila*, **b** *A. caviae*, **c** *A. veronii* bv. *sobria*, **d** *A. salmonicida* and **e** *A. veronii* bv. *veronii* strains determined by the gradient SDS-PAGE protein pattern analysis using Nei and Li/Dice similarity matrix and Neighbor-joining tree-construction method. Source of the isolate is indicated in the *parenthesis*. CECT and MTCC indicate the Spanish Type culture collection and Microbial Type culture collection, India, respectively

phenotypically heterogeneous with its members exhibiting an extremely wide range of nutritional requirements (carbohydrate metabolism), growth conditions, metabolic diversity and DNA base composition (Janda and Abbott 2010).

Conclusion

The biochemical tests and 16S rRNA gene analysis were useful in the identification of *Aeromonas* food isolates only till genus level. The *rpoD* gene was found to be a better phylogenetic marker than 16S rRNA gene, even at the intra-species level. There is a need to integrate the comprehensive biochemical scheme with *rpoD* gene sequence analysis for the explicit identification of *Aeromonas* strains up to species level. Though WCP profile has less discriminatory power, it can be subsequently used to characterize *Aeromonas* isolates below species level.

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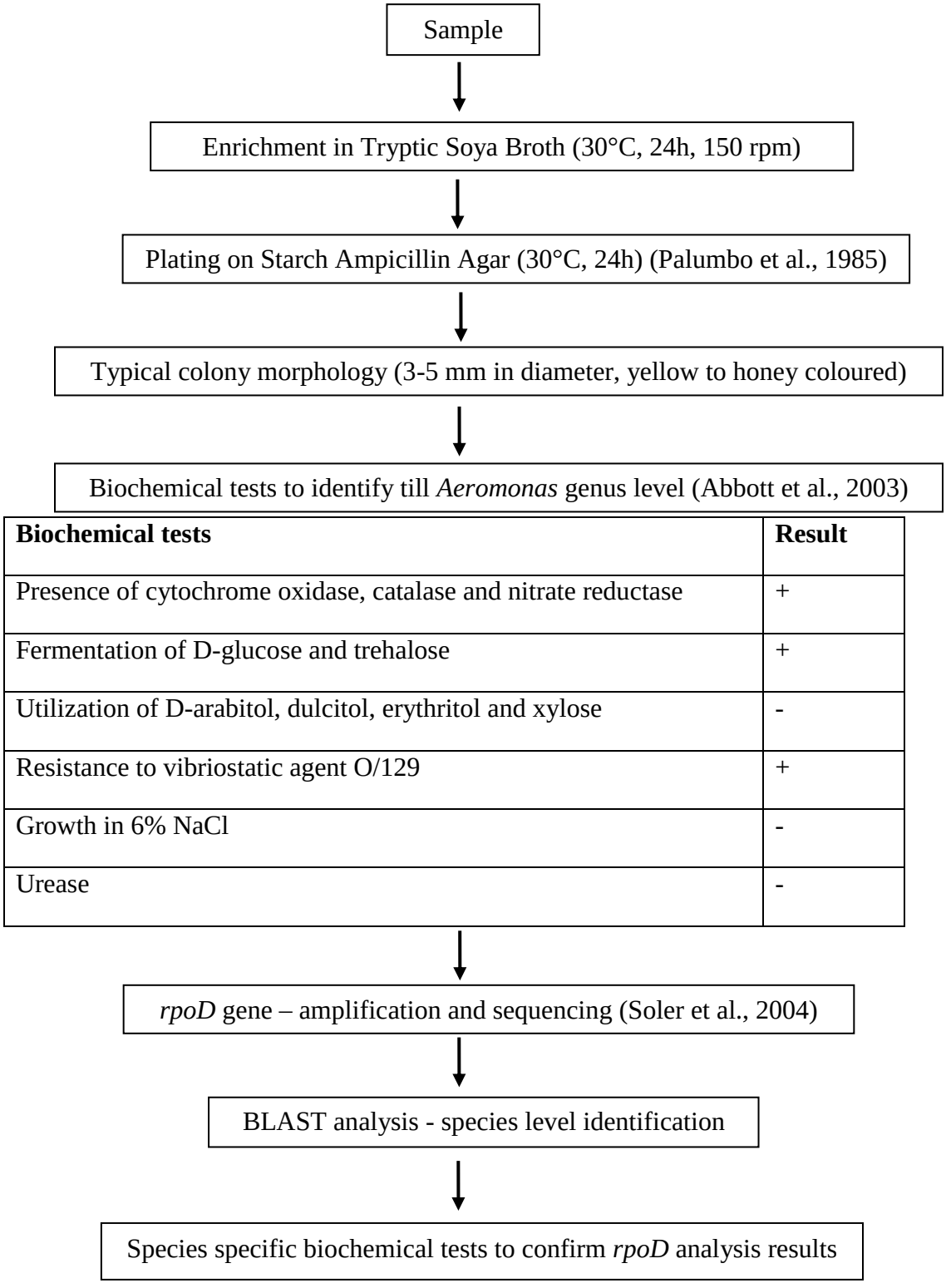

Fig. S1 Identification scheme for the rapid, convenient and accurate identification of *Aeromonas*