Synopsis
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 (Figuera *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 (Martínez-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 pulsortypes 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).
Synopsis

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 extracellular 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 extracellular 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<sup>T</sup> 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 $1 \times 10^5$ 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<sup>th</sup>, 4<sup>th</sup>, 8<sup>th</sup> and 12<sup>th</sup> day (sprout samples) and on the 0<sup>th</sup>, 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> 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<sup>th</sup> 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.

**References**


**Publications in Refereed Journal:**

a. Published:


b. Other Publications:

**Symposia:**

Synopsis


GenBank submission:


Signature of Student: 

Date: 01/05/2013