DOCTORAL THESIS

Studies on the characterization of mesophilic *Aeromonas* species

isolated from diseased fresh water aquarium fish

using molecular approaches

（罹病淡水観賞魚から単離された中温性エロモナスの分子生物学的特性解析）

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by

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DECLARATION

I, JAGODA SWARNA SUBHANI SAMANTHIKA DE SILVA, hereby declare that the thesis entitled "Studies on the characterization of mesophilic *Aeromonas* species isolated from diseased fresh water aquarium fish using molecular approaches" is an authentic record of the work done by me and that no part therefore has been presented for the award of any degree, diploma or any other similar title.

13th June 2014

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DEDICATION

This thesis is dedicated

to

My parents

who taught me to respect every work that I do, no matter how big or small

My husband

who is my cheerleader in this long journey, for being there for me always through thick and thin

My son

whose love and smile kept me strong through all the hard times

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Contents

Abbreviations

act	:	cytotoxic enterotoxin
aerA	:	aerolysin
alt	:	heat-labile cytotonic enterotoxins
ast	:	heat-stable cytotonic enterotoxins
CARD	:	Comprehensive antibiotic research database
CDS	:	Coding sequences
CLSI	:	Clinical and Laboratory Standard Institute
ERIC-PCR	:	Enterobacterial repetitive intergenic consensus sequence-based PCR
exu	:	DNase
fla	:	flagellin
gcat	:	glycerophospholipid cholesterol acyltransferase
LAMP	:	Loop mediated isothermal amplification
MAD	:	Motile aeromonad disease
MAS	:	Motile Aeromonas septicaemia
MiGAP	:	Microbial Genome Annotation Pipeline
MLST	:	Multilocus sequence typing
RAST	:	Rapid Annotation of microbial genomes using Subsystems Technology
RFLP	:	Restriction fragment length polymorphism
ser	:	serine protease
SMRT	:	Single molecule real time sequencing
SSU	:	Small-Subunit
TSA	:	Trypticase soy agar
UPGMA	:	Unweighted pair group method with arithmetic averages
WGS	:	Whole genome sequencing

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Abstract

Ornamental fish trade is an economically viable, vast global business with a great potential for development in most parts of the world. As the industry expands, the disease incidences and associated costs have become hindrances to the profitable culture of ornamental fish. The supply of high quality, disease free ornamental fish with a high survival rate at the countries of destination is a key challenge to the countries that produce ornamental fish. In this context, a thorough understanding of the pathogens associated with the commercial production of ornamental fish should be considered as the prerequisite to develop more effective diagnostic techniques targeted at the field level pathogen identification that will subsequently heighten the disease control strategies. Through this dissertation we investigated into the aspects of aetiology and diagnosis of motile *Aeromonas* septicaemia (MAS) which is an important disease in fresh water ornamental fish due to its common occurrence and potential adverse impacts. In the present study, a total of 74 phenotypically identified presumptive motile *Aeromonas* isolates recovered from septicaemic freshwater ornamental fish in Sri Lanka were genetically characterized by sequencing of *rpoD* and *gyrB* housekeeping genes. *rpoD/gyrB* phylogeny confirmed only 53 isolates as *Aeromonas*, among which *A. veronii* was the predominant species (79.2%), followed by *A. hydrophila* (7.5%), *A. caviae* (5.7%), *A. jandaei* (1.9%), *A. dhakensis* (3.8%) and *A. trota* (1.9%). The aeromonads confirmed by sequencing were further subjected to 16S rDNA PCR-RFLP which substantiated sequencing results for 83% of isolates. Fingerprinting of *A. veronii* (n = 42) using ERIC-PCR revealed no dominant clones, and the majority were genetically distinct. All isolates were screened by PCR for 7 virulence determinant genes (*aer*, *act*, *ast*, *alt*, *fla*, *ser*, *exu*) and 2 integrase encoding genes (*intI1*, *intI2*). Each isolate contained >3 of the virulence genes tested for, with a heterogeneous distribution. Of the isolates, 77% harboured the *IntI1* gene, while none had *intI2*. *In vitro* antimicrobial susceptibility testing

showed highest resistances towards tetracycline (58.5%) and erythromycin (54.7%). Our results indicate the diverse range of aeromonads that could potentially be associated with motile aeromonad septicaemia in ornamental fish. This is the first isolation of *A. dhakensis* from a septicaemic ornamental fish since its original description from the same host. A Genome of a pathogen should provide insights into evolution and mechanisms involved in mediating the disease. The draft genome of *A.hydrophila* Ae34 isolated from a septicaemic and moribund Koi carp determined in the present study serves as the first genomic information of an ornamental fish borne clinical *Aeromonas* strain. We identified a number of virulence determinants within this genome to which *A.hydrophila* pathogenic potential has classically been linked to. Comparative genomic analysis of this strain with highly virulent *A.hydrophila* ML09-119 strain which was responsible for the recent catfish epidemic outbreak in USA helped us to understand the genetic differences between a highly virulent *A.hydrophila* strain that causes outbreaks spreading over geographical areas and the strain Ae34 which could probably be an opportunistic pathogen responsible for non epidemic, isolated disease incidences. Corroborating the previous studies, we observed the significant contribution of prophages to add plasticity to the *A.hydrophila* genome making them highly diverse in terms of their virulence and adaptability to hosts. Draft genome sequence of this strain together with other sequenced *Aeromonas* genomes provide a better understanding of inter/intraspecies diversity and could be considered as valuable sources to identify molecular markers that are specific to aeromonads. We evaluated the specificity of LAMP primers designed against full length *gcat* sequence of *A. hydrophila* Ae34, to determine its ability to detect aeromonads. A LAMP assay that can successfully amplify *A.hydrophila* and *A.hydrophila* subsp. *dhakensis* was developed and under optimization and further validation. Our final objective is to find species specific markers to identify *A. hydrophila* and *A. veronii*; priority pathogens among fish pathogenic motile aeromonads through a genome informed approach, with the view of incorporating those in a LAMP assay that would detect the presence of the above *Aeromonas* species in the kidney tissues of infected ornamental fish.

List of Publications

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Jagoda S S S De S, Tan E, Arulkanthan A, Kinoshita S, Watabe S, Asakawa S. 2014. Draft genome sequence of *Aeromonas hydrophila* strain Ae34, isolated from a septicemic and moribund koi carp (*Cyprinus carpio koi*), a freshwater aquarium fish. Genome Announc. 2(3):e00572-14. doi:10.1128/genomeA.00572-14.

CHAPTER 1

General Introduction

1.1 BACKGROUND

1.1.1 Ornamental fish industry

1.1.1.1 Growing popularity of ornamental fish

Ornamental fish (aquarium fish) keeping has become one of the most popular hobbies all over the world with millions of enthusiasts (Livengood and Chapman, 2007). Ornamental fish, also known as 'living jewels', make a relaxing sight through their attractive colours and graceful swimming (Cheong, 1996) which is said to have a multitude of health and emotional benefits including a stress relieving ability. Therefore, ornamental fish are becoming increasingly popular as pet animals that are being used both for companionship and decor. The growing interest in keeping ornamental fish has resulted in a significant rise in the number of countries culturing and exporting ornamental fish. Beginning as a small export fishery on very low scale in parts of the Indo-Pacific region during the early 20th century (Steinke *et al*, 2009), the ornamental fish export trade has grown to a multi-million dollar business (Cheong, 1996) over the last fifty years.

This upward trend in the international trade of ornamental fish was a result of the numerous social and economic benefits this industry offers. The value of exports for ornamental fish was reported to have increased by approximately 14% per year since 1985 (Bartley, 2000; FAO, 2014). This resulted in a doubling of the total income generated through ornamental fish exports within a ten year period from US$ 176 in 2000 to US$ 342 in 2010 (Tissera, 2012). At present, over 1 billion ornamental fish comprising more than 4000 freshwater and 1400 marine species are traded globally each year involving more than 100 countries (Whittington and Chong, 2007). Today, ornamental fish industry comprises a rapid growing, diverse and lucrative global business worth of US$900 million (Roberts, 2010).

1.1.1.2 Trade Performance

Being a relatively costly hobby, ornamental fish keeping is primarily practiced in developed, industrialized countries (Oliver, 2001) to which a majority of the imports of tropical ornamental fish are directed. Western Europe, some countries in Asia (Mostly Japan and Singapore) and the USA served as the major import markets for ornamental fish during the last decade. According to FAO 2007 statistical records, Europe imported 53% of the global exports while Asian and American region imported 26% and 18% respectively (Tissera, 2010). In terms of exports, Asia dominates the trade, accounting for 56% of the total global value in 2007 (Tissera, 2010). Among Asian countries, Singapore continues to be the largest exporter of tropical ornamental fish catering to 17% of the world's demand. A number of other countries including Japan, Malaysia, Thailand, Indonesia, China, Israel, Sri Lanka and Phillipines are also involved in the export trade and are specialized in farming fresh water fish (Oliver, 2003; Roberts, 2010). Some countries in the European and American region also contribute substantially to ornamental fish exports. These countries include Czech Republic, Netherlands, France, USA, Columbia and Brazil (Tissera, 2010; Tissera, 2012).

1.1.1.3 Potential as a global business

Ornamental fish production has shown a great promise as an industry that can have a positive impact on the economy of both developed and developing countries. The availability of low cost land and labor, and the conducive climate make the developing countries in the tropics ideal grounds for rearing and breeding of tropical freshwater fish and therefore these countries have

become attractive suppliers of tropical ornamental fish to the world. For them, the potential contribution of ornamental fish industry as a source of employment and as a foreign exchange earner is invaluable to uplift the livelihoods of the poor through efficient utilization of local resources. As an example, Sri Lanka supplies about 2.7% of the world's demand for ornamental fish, generating an annual income of over 8.5 million US$ (EDB Sri Lanka, 2010). Developed countries also have shown a growing interest and an increased involvement in ornamental fish exports as evidenced by the international trade patterns during the past two decades, which indicates the economically appealing nature of this industry. Many of these countries, as a solution to high costs associated with domestic production of ornamental fish, have shown an increasing dependency on using imports to augment their exports. During the period from 1998-2007, Europe and America contributed 29% and 10% of the global export values respectively (Tissera, 2010). Japan, once was a major importer of ornamental fish has shown impressive developments during the last decade as an exporter (Tissera, 2010; Tissera, 2012). Taken together, it is apparent that ornamental fish trade has become an economically viable, vast global business with a great potential for development in most parts of the world.

1.1.2 Fish diseases as a constraint to the ornamental fish culture

Despite the strengths, opportunities and growing interest prevailing for the development of ornamental fish aquaculture, there are certain constraints that hinder the expansion of the industry (Jonathan, 1993). Among those, the diseases are recognized as a primary challenge to the aquaculture production and trade affecting both the economic development and socioeconomic revenue of the sector (Walker and Subasinghe, 1999). Primarily, diseases act as a limiting factor that determines the demand for ornamental fish at the world market since it is a

live product where a clean image/appearance is crucial (Jonathan, 1993). Moreover, farmers experience significant economic losses due to the mortality of fish and the costs associated with therapeutic and prophylactic interventions resulting from disease outbreaks.

About 90–96% of the overall ornamental fish trade, in terms of both value and volume, is represented by freshwater fish species (Roberts, 2010). In contrast to the marine ornamental fish that are derived from wild populations collected from coral reef habitats (Livengood and Chapman, 2007), fresh water ornamental fish are mostly captive bred and farm-raised. During the intensive production of fresh water ornamental fish, it is unavoidable that they are under the constant exposure to many stressors that are inherent to the culture system. Rapid intensification, diversification and commercialization of the ornamental fish trade as a response to cater the growing demands have further aggravated the incidence of diseases. Therefore, the supply of high quality, disease free ornamental fish with a high survival rate in the countries of destination is a key challenge to the countries that produce ornamental fish for international market. In spite of these problems, diseases in ornamental fish remain a less priority and one of the least investigated areas, and their impacts on the industry are often inaccurately known and unappreciated. This could be at least in part, due to the limited investigations in these areas under resource poor settings from where a majority of the ornamental fish exports are originated.

1.1.3 Bacterial diseases of ornamental fish

Bacterial diseases are amongst the most common infectious conditions of ornamental fish. Some bacteria are primary pathogens, while many are opportunistic pathogens. These opportunistic pathogens are natural inhabitants of the aquatic environment and invade the fish by taking the benefit of damaged fish skin or compromise of its immune system (Lewbart, 2001) due to extrinsic stressors or concurrent diseases. Some bacterial pathogens primarily cause a local infection in the surface (skin/gill) while many could cause systemic infections. Systemic infection with bacteria often leads to bacteraemia and septicaemia in fish, which could be fatal unless appropriate measures are taken early in the course. The classical signs indicative of a bacterial toxaemia/septicaemia include diffuse haemorrhages and necrosis of internal organs like spleen and kidney. Further, there may be external signs such as skin ulcers, necrosis or haemorrhages on the body and fins (Noga, 1996). Variety of Gram negative bacteria including *Aeromonas*, *Vibrio*, *Pseudomonas, Edwardsiella, Citrobacter* and *Flavobacterium* are known to cause diseases in fresh water ornamental fish (Lewbart, 2001).

1.1.4 Motile Aeromonas septicaemia (MAS)

1.1.4.1 Aetiology and predisposing factors

Aeromonas species (aeromonads) are free-living Gram negative rod shaped bacteria in the family Aeromonadaceae (Colwell *et al*, 1986) ubiquitous in aquatic environments. The role of aeromonads as causative agents of fish diseases has been known for decades, longer than their comparable role in causing systemic infections in humans (Janda and Abott, 2010). Basically, two groups of aeromonads are involved in fish disease etiologies, namely; mesophilic, motile

aeromonads and psychrophilic, nonmotile aeromonads. The former group (of which *A. hydrophila* was the type strain) consists of motile isolates that grow well at 35 to 37°C and the latter group consists of non motile strains (mainly *A. salmonicida*) that grow well in temperatures of 22 to 25°C (Martin-Carnahan and Joseph, 2005; Janda and Abott, 2010; Beaz-Hidalgo and Figueras, 2012).

Psychrophilic, non motile *A. salmonicida* is an obligatory pathogen in fish and has received an increasing attention as the causative agent of furunculosis particularly in salmonids. In comparison, mesophilic, motile species (comprising mainly of *A. hydrophila*, *A.veronii* and *A.caviae*) often behave as opportunistic pathogens capable of producing infections in weakened, immunosuppressed fish or the fish suffering from other diseases (Camus *et al*, 1998; Roberts, 1993). These mesophilic, motile species have been described as the causative agents of a variety of infections including motile aeromonas septicaemia (MAS) in many fresh water fish species. Ornamental fish reared in captivity are at a greater risk of acquiring this kind of opportunistic infections since they are under the constant exposure to a wide range of stressors induced by intensive culture practices such as fluctuations of water quality, overcrowding, transport, frequent handling, chemical treatments and inadequate nutrition. These factors together with the concurrent diseases (which are relatively common in ornamental fish culture such as parasitic infestations and other bacterial infections) would possibly predispose them to infections caused by motile aeromonads. The release of pathogenic bacteria from stressed, morbid and dead fish into aquarium water shared by other fish exacerbates the risk of disease spread (Smith *et al*, 2012). MAS, also known as motile aeromonad disease (MAD) caused by the mesophilic, motile *Aeromonas* is the most commonly encountered disease in fresh water tropical aquarium fish

(Joseph and Carnahan, 1994; Lewbart, 2001). *A. hydrophila* is the classical pathogenic motile *Aeromonas* species linked to MAS in ornamental fish (Lewbart, 2001). However, *A. veronii, A. sobria,* and *A. caviae* have also been implicated in MAS (Noga, 2000).

The occurrence of this disease in ornamental fish, from the farm level to the hobbyist tank, around the globe has been documented (Gratzek *et al*, 1978; Hettiarachchi and Cheong, 1994; Sreedharan *et al*, 2012) causing significant economic losses through the morbidity, mortality and associated therapeutic and prophylactic costs. *Aeromonas* mediated mass mortality was reported to have caused a loss of 820 tons of goldfish in Indonesia in 2002 resulting in a $37.5 million loss (Janda and Abott, 2010).

1.1.4.2. Characteristics of the disease and clinical signs

MAS is characterized by a wide variety of clinical signs including fin rot, abdominal distension due to ascites, exophthalmia, scale protrusion, dermal ulceration, areas of petechiation and haemorrhages, engorged gill lamellae and sloughing off of skin, fins, and scales (Lewbart, 2001). But, none of these are pathognomonic of MAS since similar signs are produced by a myriad of other fish pathogens. Systemic cases with haemorrhagic septicaemia followed by inflammation and necrosis of internal organs often have a poor prognosis (Lewbart, 2001). Acute septicaemia could lead to death of the fish even before the development of gross signs (Cipriano, 2001).

1.1.4.3 Diagnosis of MAS – Phenotypic Vs. Molecular Identification

The exact aetiological diagnosis of MAS is challenging due to the phenotypic, antigenic and genetic heterogeneity that has been reported to exist within this genus (Cipriano, 2001),

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particularly within the motile group. Phenotypic and biochemical characterization is the regular bacterial identification method used in many ichthyopathological laboratories for the identification of bacterial fish pathogens. However, for *Aeromonas*, the accuracy of species identification through phenotypic and biochemical methods alone is considered unreliable (Beaz-Hidalgo and Figueras, 2012). In the recent years, there have been many reports that showed the poor correlation between genotypic and phenotypic identification (Soler *et al*, 2003; Figueras, 2005; Ormen *et al*, 2005; Beaz-Hidalgo *et al*, 2010). Lack of clear cut phenotypic identification tables to distinguish the ever increasing number of taxa in the genus *Aeromonas* (Abott, 2003) is one of the major challenges in phenotypic identification. Consequently, DNA based molecular tools have widely been used for *Aeromonas* speciation due to their high resolution power and accuracy. Yet, 16S rRNA gene (SSU) sequencing, one of the commonest molecular techniques used in bacterial identification could result in discrepancies in *Aeromonas* species identification (Janda and Abott, 2010) due to the high inter-species similarity of the 16S rRNA gene sequence (Martinez-Murcia *et al*, 2007) and intragenomic heterogeneity manifested by *rrn* nucleotide polymorphisms/ microheterogeneities (Alperi *et al*, 2008; Morandi *et al*, 2005). Phylogenetic analyses based on the 16S rRNA gene indicated that aeromonads are phylogenetically a very tight group of species (Martínez-Murcia *et al*, 1992a). A previously described RFLP method based on the 16S rRNA gene has enabled identification of all *Aeromonas* species described up to the year 2000 (Borrel *et al*, 1997; Figueras *et al*, 2000) and has been used in the identification of fish originated *Aeromonas* strains (Beaz-Hidalgo *et al*, 2010; Kozińska *et al*, 2002; Kozińska, 2007; Nawaz *et al*, 2006). However, the production of unexpected or atypical restriction patterns by some strains (8%) has also been reported (Alperi *et al*, 2008) that limits the differentiation of closely related species. In contrast, molecular phylogenetic analysis based on the sequences of

one or more housekeeping genes have proven to be a useful tools for inferring the taxonomy of the genus *Aeromonas* (Yanez *et al*, 2003; Soler *et al*, 2004; Kupfer *et al*, 2006; Nhung *et al*, 2007; Sepe *et al*, 2008) with *gyrB* and *rpoD*, the first housekeeping genes described for this purpose, found to have high discriminatory power for phylogenetic identification of *Aeromonas*.

In many earlier studies that occupied phenotypic characterization, the diseases of fish caused by mesophilic, motile aeromonads were attributed to one of three phenotypic species namely *A. hydrophila*, *A. caviae* and *A. sobria* (Joseph and Carnahan, 1994; Austin and Austin, 2007). However, over last few decades, with the advent of polyphasic molecular approaches, there have been intensive efforts to refine the methods for identification and classification of *Aeromonas*. As a result, the taxonomy of *Aeromonas* has changed significantly with the addition of newly described species (Beaz-Hidalgo *et al*, 2009) in particular, mesophilic species. The genus is now considered to comprise 25 validated species (Figueras *et al*, 2011). In many earlier studies that occupied conventional and miniaturised biochemical identification systems, *A. hydrophila* received a great deal of attention as the most commonly isolated *Aeromonas* species from diseased fresh water fish (Hettiarachchi and Cheong, 1994; Nielsen *et al*, 2001). However, limitations of identification and taxonomy in the past, may have contributed to the citation of *A. hydrophila* so frequently (Joseph and Carnahan, 1994) because biochemical identification systems erroneously identify up to 70-80% of the strains of *Aeromonas*, as belonging to this species, when in fact they are many different species when identified by molecular methods (Beaz-Hidalgo *et al*, 2010; Figueras, 2005; 2011; Soler *et al*, 2003). This was witnessed by many of the recent studies that occupied molecular identification methods including house keeping gene sequencing, reporting a comparatively higher incidence of other *Aeromonas* species such as

A. veronii (Hu *et al*, 2012, Sreedharan *et al*, 2013). Therefore, it is obvious that the name of the species reported depends largely on the identification technique occupied, hence the techniques such as housekeeping gene sequencing and/or multi locus sequence typing would be more appropriate alternatives to understand the true diversity of aeromonads causing diseases in fish. The newly described species might have an important role in fish pathology that needs to be explored (Beaz-Hidalgo and Figueras, 2012).

1.1.4.4 Multifactorial virulence of motile aeromonads associated with MAS

Establishment of an infection in fish by an aquatic pathogen is the end result of losing the fine balance between the host, pathogen and the environment. Consequently, the severity of MAS is influenced by number of interrelated factors including bacterial virulence, the kind and degree of stress exerted on the population of fish, physiologic condition of the host, and the degree of genetic resistance inherent within specific populations of fish (Cipriano, 2001). It has become clear from a number of studies that the pathogenicity of *Aeromonas* is multifactorial and has been associated with numerous virulence factors, including but not restricted to aerolysin (*aer*), flagella A and flagella B (*fla*), lipase (*lip*), elastase (*ela*), DNases (*exu*), cytotonic heat-labile enterotoxins (*alt*), cytotonic heat-stable enterotoxins (*ast*), cytotoxic heat-labile enterotoxin (*act*), serine protease (*ser*) and glycerophospholipid-cholesterol acyltransferase (*gcat*) (Chacón *et al*, 2003; Sen and Rodgers, 2004; Nam and Joh, 2007; Nawaz *et al*, 2010; Chopra *et al*, 1999; Sha *et al*, 2002). The link between the presence of virulence-related genes/gene combinations and pathogenicity is not clearly established in *Aeromonas* (Roger *et al*, 2012). Therefore, their presence does not necessarily imply causality (Janda and Abott, 2010). Furthermore, motile aeromonads differ interspecifically and intraspecifically in their relative pathogenecity (Cipriano,

2001). However, the detection of the presence of genetic determinants of putative virulence in clinical isolates is important to find out whether those strains could be well differentiated from environmental and commensal strains, and has been occupied by many researchers as a reasonable and rational criterion in determining the potential pathogenicity of an organism (Puthucheary *et al*, 2012). On the other hand, the prevalence of each virulence determining gene in a subset of clinical *Aeromonas* would be helpful to evaluate the use of these genes as targets to develop effective diagnostic and control measures.

1.1.5 Antimicrobial resistance of motile *Aeromonas* species

Resistance of *Aeromonas* species to commonly used antibiotics is an emerging problem in the ornamental fish industry (Dixon and Issvoran, 1992, Dias *et al*, 2012). In a veterinary perspective, treating of ornamental fish diseases caused by antibiotic multi resistant aeromonads would be challenging and could result in possible failures of prophylactic and therapeutic treatments. Antimicrobial drugs that are being applied to the water containing fish for therapeutic purposes can persist in the culture environment for long time and could favor the emergence of resistance strains in the environment (Dias *et al*, 2012). On the other hand, the presence of multi resistant aeromonads in aquarium waters poses some major public health concerns that could be linked with ornamental fish industry (Weir *et al*, 2012) for the reason that *Aeromonas* is an emerging human pathogen responsible for causing intestinal and extraintestinal infections that may range from relatively mild illnesses such as acute gastroenteritis to life-threatening conditions, such as septicemia (Janda and Abott, 2010). Therefore, ornamental fish and their carriage water could act as a reservoir for virulent/pathogenic and antibiotic-resistant strains of *Aeromonas* for which people can be exposed in numerous situations along the production line

from farm to home. This spread is facilitated given the large numbers of ornamental fish that are cultured and the complex and dynamic pathways through which they are shipped internationally (Weir *et al*, 2012; Smith *et al*, 2012). In the literature, reports that describe human illnesses directly related to *Aeromonas* from ornamental fish are limited (Filler *et al*, 2000; Cremonesini and Thomson, 2008), or probably under reported. However, there are frequent reports on antimicrobial resistance of *Aeromonas* strains isolated from ornamental fish and their carriage water to commonly used antibiotics (Trust and Whitby, 1976; Dixon *et al*, 1990; Verner-Jeffreys *et al*, 2009; Cizek *et al*, 2010; John and Hatha, 2012; Sreedharan *et al*, 2012; Dias *et al*, 2012). Moreover, antibiotic resistance genes including cassette-borne resistance genes in class 1 integrons have been described to occur in motile aeromonads (Schmidt *et al*, 2000; Jacobs and Chenia, 2007). In this respect, evaluation of antimicrobial resistance profiles of clinical isolates of *Aeromonas* from fish is a necessary prerequisite to inform veterinarians on the therapeutic value of antimicrobial agents and to make an informed choice within the approved ranges and guidelines. However, to our knowledge, a systematic evaluation of *in vitro* antibiotic resistance profiles, and the presence of antibiotic resistance gene cassettes in clinical isolates of *Aeromonas* from ornamental fish in tropical countries including Sri Lanka has not been attempted despite its common occurrence. The very few available studies are not supported with confirmatory identification of isolates using polyphasic approaches.

1.1.6 Genomic insights into MAS

1.1.6.1 Genome sequencing of motile *Aeromonas* species: *A.hydrophila as an exemplar*

In the recent past, high throughput whole genome sequencing has emerged as a rapid means of understanding the biology, evolution, virulence and diversity of bacterial pathogens. For a

complex genus like *Aeromonas*, genome sequencing could be considered as a promising way to solve many nomenclature and taxonomic issues (Janda and Abott, 2010). The first complete genome of an *Aeromonas* strain, *A. hydrophila* ATCC 7966 was determined in 2006 (Seshadri *et al*, 2006), but it was an isolate recovered from a tin of milk with a fishy odor for which the pathogenicity in fish has not yet been studied. The first complete genome sequence of a fish pathogenic isolate, *A. hydrophila* strain ML09-119 was reported very recently (Tekedar *et al*, 2013). It was a representative of the clonal group of *A. hydrophila* implicated in a disease outbreak in Channel Catfish in West Alabama during 2009. Despite the growing interest and economic importance of global aquarium fish trade, there is a dearth of information on the genome sequence of *A. hydrophila* isolated from diseased tropical ornamental fish. Whole genome sequencing of the clinical isolates of ornamental fish borne *A. hydrophila* would help to reveal what specific genetic features would differentiate a 'fish pathogenic strain' from their 'environmental' counterparts who share the same environmental niche. Moreover, the genome sequencing will enable the revelation of the complete array of virulence factors that could potentially be involved in *A. hydrophila*'s pathogenicity in fish.

1.1.6.2 Comparative genomics of motile *Aeromonas* species

Whole genome sequencing of multiple strains of *Aeromonas* clinical isolates from fish would provide interesting insights into their genetic heterogeneity in terms of virulence and antimicrobial resistance. This information would be of utmost importance to design diagnostic targets with high discriminatory power and to develop control strategies such as vaccines. Comparison of the few available full genome sequences of *Aeromonas* has identified significant horizontal gene transfer as the mechanism that contributes to the genetic heterogeneity observed

among members of genus *Aeromonas* (Seshadri *et al*, 2006). Further, the acquisition of epidemic related genes through horizontal gene transfer was attributed to the emergence of an epidemic clonal group of *A.hydrophila* in epidemic outbreaks in catfish (Hossain *et al*, 2013). In the future, as more and more genomes of fish pathogenic aeromonads are sequenced, we are not far from understanding the genetic mechanisms that shape their adaptation to diverse niches, virulence and defense.

1.1.7 Rapid, sensitive and specific detection of MAS at the field

1.1.7.1 Importance of rapid diagnosis of MAS

Making a prompt and accurate diagnosis is vital for successful treatment and long term prevention of fish diseases. As the clinical signs of MAS are rarely pathognomonic, the diagnosis is accomplished by the isolation of the pathogen from the internal organs and identification using biochemical characterization which is laborious and time consuming. In a typical outbreak of septicaemia in fish, especially in MAS, symptoms occur gradually with an increasing number of fish becoming affected (Andrews *et al*, 1988). To reduce the mortalities and to prevent the spread, it is important to identify the problem as early as possible. Fish in the early stages of infection may respond favorably to antibiotic treatment and environmental modification (Lewbart, 2001). Therefore, time consuming diagnostic methods such as culture and identification of the causative organisms are not of practical value. Molecular methods are rewarding in this respect, but the high cost limits their use in routine diagnosis in fish pathology laboratories with minimum facilities. Therefore, they should be replaced by rapid, simple and accurate diagnostic methods that could be performed at field level. This will enable to spot the early stages of the infection and to apply the appropriate course of treatment as soon as possible.

1.1.7.2 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a molecular diagnostic tool that has extensively been used in identification of aquaculture pathogens (Notomi *et al*, 2000; Biswas and Sakai, 2014). The rapidity, accuracy, and cost-effectiveness of this technique make it a good candidate method for the detection of motile aeromonads that cause MAS in ornamental fish. In the routine ornamental fish disease diagnosis process, fish pathologists or veterinarians do not need to identify an *Aeromonas* isolate from a fish with MAS to the species level if the purpose of diagnosis is to initiate treatment/control measures. Therefore, in a therapeutic and control point of view, rapid detection of *Aeromonas* spp. in the kidney tissues of affected fish could be considered adequate to initiate the treatments. This will enable rapid, onsite and early detection of the disease thereby ensuring that the control measures are adopted on time. On the other hand, this will be helpful to avoid the unnecessary use of antimicrobials on fish diseases by preventing the application of antimicrobial compounds for non bacterial etiologies. However, in an epidemiological point of view, an assay that can detect/ differentially identify major motile fish pathogenic aeromonads such as *A.hydrophila* and *A.veronii* will also be invaluable to understand their true diversity and prevalence.

However, the use of the LAMP technique has rarely been reported for aeromonads, which could partially be due to their ever-changing taxonomy and the genetic heterogeneity. The LAMP assay developed by Kulkarni *et al* in 2009 for the detection of atypical furunculosis caused by *Aeromonas salmonicida* in Atlantic cod, *Gadus morhua* is the first instance in the published literature to use this technique for the detection of an *Aeromonas* fish pathogen (Beaz-Hidalgo

and Figureas, 2012). This assay was based on LAMP primers targeting the *gyrB* gene region described by Beaz-Hidalgo *et al* in 2008 as useful for detecting *A. salmonicida*.

Whole Genome Sequencing (WGS) of bacteria is becoming increasingly and routinely used in research and clinical practice. The bacterial genomes that are being aggregated in the public data bases provide the basis to determine conserved genes and proteins across genera and species, that could be used as targets for phylogenetic identification. As a part of this thesis, we aim to analyse *gcat* gene (that has already been published as an *Aeromonas* genus specific marker by Chacon *et al*, 2002); sequenced from our study isolates and retrived from *Aeromonas* genome sequence data available through public databases, to find a suitable target signature region for the development of LAMP primers targeting aeromonads. We also aim to evaluate (at preliminary level) the specificity of these LAMP primers to identify *Aeromonas* at genus or species level. Our final objective is to develop LAMP assay/s that can detect *A.hydrophila* and *A.veronii* from septicaemic fresh water ornamental fish.

1.2 OBJECTIVES OF THE STUDY

The present study was carried out with the following specific objectives;

- To ascertain the phylogenetic diversity of motile *Aeromonas* species isolated from diseased freshwater ornamental fish and to find out the dominant species and clonal associations
- To gain an understanding about the prevalence of genes that encode major virulence factors among clinical isolates of *Aeromonas*

- To determine their antimicrobial resistance patterns and the prevalence of integrons

- Genome sequencing and comparative genomic analysis of *A.hydrophila* strain Ae34 isolated from fresh water ornamental fish with signs of septicaemia

- To evaluate the suitability of *gcat* gene as a target signature region to detect aeromonads and to evaluate the specificity of LAMP primers designed against *gcat* for genus level identification and/or species identification (*A.hydrophila* and *A.veronii*) of aeromonads with the view of developing a LAMP based diagnostic assay/s for the rapid identification of motile *Aeromonas* isolates from septcaemic fish.

1.3 OUTLINE OF THE THESIS

Chapter 1 of this thesis provides a general background accompanied by a review on motile aeromonas septicameia in tropical fresh water ornamental fish, and the aspects on characterization, antimicrobial resistance, virulence, genome sequencing and rapid diagnostics of motile aeromonads.

Chapter 2 describes the molecular characterization, antimicrobial susceptibility, genetic determinants of virulence of motile aeromonads isolated from fresh water ornamental fish showing signs of septicaemia

Chapter 3 describes the aspects of genome sequencing, assembly, annotation and the comparative genomics of *A. hydrophila* strain Ae 34, a clinical isolate from ornamental fish

Chapter 4 provides an account on preliminary analysis of *gcat* gene of aeromonads in order to use it as a target signature region to design LAMP primers, and evaluation of the specificity of LAMP primers for the detection of Aeromonads at the genus level and/or at the species level (*A.hydrophila*/*A.veronii*). This chapter includes preliminary results of an ongoing experiment.

Chapter 5 provides the conclusions derived from the findings of the present study and the aims achieved with the prospects for future directions

CHAPTER 2

Molecular characterization, antimicrobial susceptibility and virulence determinants of motile aeromonads isolated from freshwater ornamental fish showing signs of septicaemia

The contents of this chapter are published in;

2.1 INTRODUCTION

Motile aeromonad septicaemia (MAS), with mesophilic, motile *Aeromonas* species implicated in its development, is probably the most common bacterial disease in fresh water aquarium fish (Lewbart 2001). It is often an opportunistic infection characterized by non-specific signs such as fin rot, ulceration, haemorrhages, exophthalmia and dropsy. Historically, *Aeromonas hydrophila* has gained much attention as the most common fish pathogenic motile *Aeromonas* species, but other *Aeromonas* species may also have an important role in fish pathology that needs to be explored (Beaz-Hidalgo and Figueras, 2012). Well documented discrepancies in phenotypic identification of *Aeromonas* at species level (Kozinska, 2007; Beaz-Hidalgo *et al*, 2010) may have impacted on the species designations of the fish pathogenic aeromonads identified to date (Austin, 2011) and this favours the use of molecular approaches such as the sequencing *of gyrB* and *rpoD* genes (Yanez *et al*, 2003; Soler *et al*, 2004; Martínez-Murcia *et al*, 2011), that led to increased accuracy of *Aeromonas* species identification. Characterization of the *Aeromonas* strains recovered from diseased fish through sequencing of the housekeeping genes (i.e. *rpoD* and *gyrB*) would help to clarify the diversity of species involved in fish disease.

With the growing interest in aquarium fish trade, a thorough understanding of pathogens associated with the commercial production of aquarium fish seems to be of great importance, not only to develop more effective diagnostic techniques targeted at field level diagnosis, but also to gain insight into their pathogenic and antibiotic resistant mechanisms. Though there are ample number of reports on the occurrence of motile *Aeromonas* in different aquaculture settings, that

of ornamental fish is limited and many of the available studies have focused on the isolates from healthy fish or aquarium water (Dias *et al*, 2012). The presence of motile aeromonads, by itself, is not indicative of disease since they compose part of the normal intestinal microflora of healthy fish (Trust *et al*, 1974). Therefore, it is very important to isolate the bacteria from internal organs of diseased fish with septicaemic signs, specially from the kidney and liver, in an attempt to recover the fish pathogens involved. To date, very few studies have characterized the *Aeromonas* species associated with septicaemia in fresh water ornamental fish using samples obtained from internal organs of moribund fish. To date, the studies that characterised clinical isolates of motile *Aeromonas* from diseased ornamental fish with the identification confirmed through polyphasic molecular approaches are very limited. Moreover, little is known about the genetic determinants of virulence and antimicrobial susceptibility of such isolates.

In this context, the present study has aimed to characterize a collection of *Aeromonas* isolates, from ornamental fish with clinical signs of MAS, at the species level using a polyphasic approach with the view of determining the relative occurrence of different mesophilic *Aeromonas* species as ornamental fish pathogens. This is an area that remains largely unexplored despite the common occurrence of MAS in ornamental fish. We also investigated the antimicrobial susceptibility patterns and the frequency of occurrence of some virulence genes in the study isolates. Molecular fingerprinting of *Aeromonas veronii* which was the predominant species isolated in the present study was also performed using enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) to determine the presence of any dominant clones associated with septicaemia in fish.

2.2 MATERIALS AND METHODS

2.2.1 Sampling of freshwater ornamental fish

Moribund freshwater ornamental fish presenting at least one or more of the clinical signs of septicaemia (focal ulcerations on skin, haemorrhages on skin and fins, fin rot, exophthalmia, abdominal distension and scale protrusion) were collected from commercial aquaria and breeding farms that are located in the northwestern, central and western, north central provinces of Sri Lanka during the period from May 2007 to June 2008. Fish were transported to the laboratory in well-aerated water. Selection of aquaria from which to collect fish was based on the availability of diseased fish at the time of sampling, accessibility and convenience. In addition, septicaemic fish samples submitted for disease investigations to the Center for Aquatic Animal Disease Diagnosis and Research (University of Peradeniya) were also included.

2.2.2 Bacterial isolation and phenotypic characterization

All the diseased fish (Table 2.1) were subjected to a detailed preliminary laboratory examination and humanely euthanised using an overdose of MS 222. The kidneys and/or liver of affected fish were cultured aseptically on trypticase soy agar (TSA; Oxoid) for bacterial isolation and on *Aeromonas* starch DNA agar (Himedia) and Rimler Shotts agar (Himedia) for the preferential selection of aeromonads, and cultures were incubated in duplicate at 28°C and 35°C for 24 h. On few occasions where the fish were not sacrificed for sample collection, swabs from external lesions (ulcers) were used. Cultures in all plates were examined for colony morphology and Gram-staining reactions. Colonies were picked in order to represent all types of colonies in pure cultures and mixed cultures, and were subcultured on TSA. All Gram-negative isolates were

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subjected to a series of classical phenotypic tests including: a cytochrome oxidase test, a motility test, an oxidation fermentation test and a catalase test. Furthermore, the ability to grow at 0% NaCl and the susceptibility to novobicin were tested. Accordingly, the isolates that were Gram negative, cytochrome oxidase positive, motile, fermentative, catalase positive, able to grow at 0% NaCl and resistant to novobicin were presumed to be motile *Aeromonas* and were used in further analysis. In instances where more than 1 fish was sampled from the same disease incidence, only 1 presumptive aeromonad isolate was analysed. Stock cultures were maintained for short periods at room temperature on TSA slants, and, for long-term storage, they were maintained at –20°C in tryptic soy broth medium supplemented with 10% glycerol (v/v). Details of the *Aeromonas* cultures used in this study are included in Table 2.1.

Table 2. 1. Origin, laboratory reference numbers and the phylogenetic identification through *rpoD*/*gyrB* sequencing of *Aeromonas* isolates (n = 53) recovered from diseased freshwater ornamental fish

Sample source	Number of isolates recovered	Lab. ref. no(s).	Phylogenetic identification
Poecillia reticulata **(guppy)**			
Kidney and/or liver	15	Ae 1, Ae 3, Ae 13, Ae 15, Ae 20, Ae 22, Ae 31, Ae 39, Ae 44, Ae 46, Ae 47, Ae 49–Ae 51, Ae 53	15 *A. veronii*
Carassius auratus			

(gold fish)

Kidney	9	Ae 4, Ae 6, Ae 10, Ae 11, Ae 21, Ae 32, Ae 35, Ae 45, Ae 52	3 *A. hydrophila*
			4 *A. veronii*
			1 *A. caviae*
			1 *A. jandaei*
Ulcer	3	Ae 8, Ae 27, Ae 42	2 *A. veronii*
			1 *A. caviae*

Cyprinus carpio (**koi carp**)

Kidney and/or liver	5	Ae 23, Ae 25, Ae 26, Ae 34, Ae 41	*3 A. veronii*
			1 *A. hydrophila*
			1 *A. enteropelogenes*
Ulcer	1	Ae 43	1 *A. dhakensis*

Betta splendens

Kidney and/or liver	5	Ae 2, Ae 19, Ae 29, Ae 33, Ae 38	5 *A. veronii*

Pterophyllum **spp.**

Kidney and/or liver	6	Ae 9, Ae 14, Ae 18, Ae 28, Ae 36, Ae 40	5 *A. veronii*
			1 *A. caviae*

Helostoma temminckii

Kidney and/or liver	3	Ae 5, Ae 17, Ae 30	3 *A. veronii*

Poecilia sphenops

Kidney and/or liver	3	Ae 12, Ae 37, Ae 48	3 *A. veronii*

Osphronemus goramy

Ulcer	1	Ae 24	1 *A. dhakensis*
***Symphysodon* spp.**			
Liver	1	Ae 7	1 *A. veronii*
Xiphophorus helleri			
Liver	1	Ae 16	1 *A. veronii*

2.2.3 DNA extraction and molecular identification

Genomic bacterial DNA was extracted from cultures using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions and stored at −20°C for further use. All presumptive *Aeromonas* isolates were first screened using *Aeromonas*-genus-specific primers (Chacon *et al*, 2002) targeting the glycerophospholipid cholesterol acyltransferase gene (*gcat*) using the reaction and cycling conditions described by Soler *et al* (2002). They were then identified at the species level based on the sequencing analysis of 2 housekeeping genes, *rpoD* and *gyrB*. An approximately 1100 bp fragment of *gyrB* and a 820 bp fragment of *rpoD* were amplified from the template DNA, purified using the FastGene Gel/PCR extraction kit (Nippon Genetics) and sequenced using the BigDye Terminator V3.1 Cycle Sequencing Kit on the 3130*xl* Genetic Analyzer (Applied Biosystems). The primers and conditions used for PCR amplifications and DNA sequencing were those described by Soler *et al* (2004) and Yanez *et al* (2003). Resulting *rpoD* and *gyrB* contigs were assembled separately (DNA baser V.3.5.3, Heracle BioSoft), and a BLAST search was carried out to compare the sequences with those held in the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

The aeromonads confirmed by nucleotide sequencing were re-identified using RFLP (restriction fragment length polymorphism) analysis of the PCR-amplified 16S rRNA gene. The objective of this step was to evaluate the concordance between *rpoD*/*gyrB* gene sequencing and 16S rDNA-RFLP, since the latter can easily be adopted in ordinary fish disease diagnostic laboratories with no access to gene sequencing facilities. Primers, amplification conditions and the endonuclease digestion conditions for 16S rDNA-RFLP were those described by Borrell *et al* (1997) and Figueras *et al* (2000). The 16S rDNA digestion products were electrophoresed in 18% v/v polyacrylamide gels at 155 V for 4 h.

2.2.4 Phylogenetic analysis

A 667 bp sequence of *rpoD* and a 926 bp sequence of *gyrB* were used in the final analysis (Martinez-Murcia *et al*, 2011). Each nucleotide sequence was determined at least twice to resolve ambiguous areas. These partial gene sequences were aligned (both independently and as a concatenated sequence of 1593 bp) using Clustal W in MEGA Version 5 (Tamura *et al*, 2011) with those from *Aeromonas* reference strains representing all published species to date. Genetic distances and clustering were determined using Kimura's 2-parameter model, and evolutionary trees were constructed by the neighbour-joining method with MEGA 5, with bootstrapping determined for 1000 replicates. The nucleotide sequences determined in this study have been deposited in DDBJ/EMBL/GenBank databases with the following accession numbers: *rpoD* AB828727–AB828779 and *gyrB* AB829112–AB829164 (Table 2.2). The GenBank accession numbers for the *rpoD* and *gyrB* gene sequences of reference strains used in alignments are listed in Table 2.3.

Table 2.2. GenBank accession numbers for the *rpoD*/*gyrB* gene sequences of *Aeromonas* isolates used in the present study

Isolate Ref. No.	GenBank accession numbers for the *rpoD/ gyrB*	Isolate Ref. No.	GenBank accession numbers for the *rpoD/ gyrB*
Ae 1	AB828727/ AB829112	Ae 27	AB828753/ AB829138
Ae 2	AB828728/ AB829113	Ae 28	AB828754/ AB829139
Ae 3	AB828729/ AB829114	Ae 29	AB828755/ AB829140
Ae 4	AB828730/ AB829115	Ae 30	AB828756/ AB829141
Ae 5	AB828731/ AB829116	Ae 31	AB828757/ AB829142
Ae 6	AB828732/ AB829117	Ae 32	AB828758/ AB829143
Ae 7	AB828733/ AB829118	Ae 33	AB828759/ AB829144
Ae 8	AB828734/ AB829119	Ae 34	AB828760/ AB829145
Ae 9	AB828735/ AB829120	Ae 35	AB828761/ AB829146
Ae 10	AB828736/ AB829121	Ae 36	AB828762/ AB829147
Ae 11	AB828737/ AB829122	Ae 37	AB828763/ AB829148
Ae 12	AB828738/ AB829123	Ae 38	AB828764/ AB829149
Ae 13	AB828739/ AB829124	Ae 39	AB828765/ AB829150
Ae 14	AB828740/ AB829125	Ae 40	AB828766/ AB829151
Ae 15	AB828741/ AB829126	Ae 41	AB828767/ AB829152
Ae 16	AB828742 AB829127	Ae 42	AB828768/ AB829153
Ae 17	AB828743/ AB829128	Ae 43	AB828769/ AB829154
Ae 18	AB828744/ AB829129	Ae 44	AB828770/ AB829155
Ae 19	AB828745/ AB829130	Ae 45	AB828771/ AB829156

Ae 20	AB828746/ AB829131	Ae 46	AB828772/ AB829157
Ae 21	AB828747/ AB829132	Ae 47	AB828773/ AB829158
Ae 22	AB828748/ AB829133	Ae 48	AB828774/ AB829159
Ae 23	AB828749/ AB829134	Ae 49	AB828775/ AB829160
Ae 24	AB828750/ AB829135	Ae 50	AB828776/ AB829161
Ae 25	AB828751/ AB829136	Ae 51	AB828777/ AB829162
Ae 26	AB828752/ AB829137	Ae 52	AB828778/ AB829163
		Ae 53	AB828779/ AB829164

Table 2.3 GenBank accession numbers for the *rpoD*/*gyrB* gene sequences of reference strains used in the phylogenetic analysis

Species	GenBank accession number	
	rpoD	*gyrB*
A. allosaccharophila CECT 4199	HQ442825	HQ442733
A. aquariorum CECT 7289	HQ442798	HQ442712
A. bestiarum CECT 4227	HQ442854	HQ442683
A. bivalvium CECT 7113	HQ442817	HQ442703
Aeromonas cavernicola MDC 2508	HQ442864	HQ442702
A. caviae CECT 838	HQ442790	HQ442748
A. diversa CECT 4254	HQ442805	HQ442756
A. encheleia CECT 4253	HQ442777	HQ442651
Aeromonas enteropelogenes CECT 4255	HQ442822	HQ442718
A. eucrenophila CECT 4224	HQ442770	HQ442657
A.fluvialis CECT *7401*	FJ603453	FJ603455
A. hydrophila CECT 839	HQ442791	HQ442746
A. jandaei CECT 4228	HQ442840	HQ442736
A. media CECT 4232	HQ442785	HQ442709
A. molluscorum CECT 5864	HQ442812	HQ442671
A. piscicola CECT 7443	HQ442859	HQ442690
A.popoffii CECT *5176*	HQ442853	HQ442693
A. rivuli DSM 22539 (CECT 7518)	FJ969433	FJ969434
A. salmonicida CECT 894	HQ442843	HQ442680
A. sanarelli CECT 7402	FJ807275	FJ807277
A. schubertii CECT 4240	HQ442809	HQ442755
A. simiae CIP 107798	HQ442811	HQ442758
A. sobria CECT 4245	HQ442867	HQ442698
A. taiwanensis CECT 7403	FJ807271	FJ807272
A. tecta CECT 7082	HQ442762	HQ442662
A. veronii CECT 4257	HQ442833	HQ442728
A. veronii CECT 5761	HQ442831	HQ442729
A. veronii CECT 4246	HQ442829	HQ442724

2.2.5 Molecular fingerprinting of *Aeromonas veronii* using ERIC-PCR

The genetic diversity of *A. veronii* isolates (n = 42) was assessed by ERIC-PCR using the primers ERIC 1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') (Versalovic *et al*, 1991) and the amplification conditions described by Fontes *et al* in 2011. Reactions were carried out 2 times for each isolate to confirm reproducibility. The amplification products were electrophoresed in a 1.2% agarose gel in Tris-borate buffer, and the fragment sizes were analysed using PyElph (Pavel and Vasile, 2012). Banding patterns of each isolate were converted into a binary matrix, based on the presence and absence of the DNA fragments. To construct the dendrogram, levels of similarity between the profiles were calculated using the Dice coefficient, and cluster analysis of similarity matrices was calculated with the unweighted pair group method with arithmetic averages (UPGMA) (Gracia-Vallve *et al*, 1999; Garcia-Vallvé and Puigloo, 2002).

2.2 6 Determination of the susceptibility to antimicrobial agents

Susceptibility of all isolates to 8 different antimicrobial agents was determined by the disk diffusion (Kirby-Bauer) method on Mueller Hinton agar (Oxoid) according to the protocols established by the Clinical and Laboratory Standard Institute (CLSI, 2008). The antibiotics and concentrations tested were as follows: amoxicillin (10 µg), neomycin (10 µg), sulphamethoxasole-trimethoprim (25 µg), chloramphenicol (30 µg), tetracyclin (30 µg), enrofloxacin (0.5 µg), erythromycin (15 µg) and nitrofurantoin (50 µg) (Oxoid). The antimicrobial agents used for susceptibility testing were chosen to cover different antibiotic groups that are used in ornamental

fish aquaculture. Plates were incubated at 28°C for 24 h. Based on the size of the inhibition zones, isolates were characterised as sensitive, intermediate, or resistant.

2.2.7 Detection of virulence-related genes by PCR

All the isolates were screened by PCR for the presence of 7 genomic markers potentially linked to virulence; aerolysin (*aerA*), cytotoxic enterotoxin (*act*), the heat-stable and heat-labile cytotonic enterotoxins (*ast* and *alt*, respectively), serine protease (*ser*), DNase (*exu*) and flagellin (*fla*) using the primers and conditions described by Nawaz *et al* (2010) and Soler *et al* (2002). The sequence of each primer used to amplify the target genes, the expected size of the PCR products and their references are listed in Table 2.4.

Table 2.4. Sequences of PCR primers used to amplify the virulence genes and the integrase genes of *Aeromonas* isolates, expected size of PCR products and their references

Gene	Nucleotide sequence (5¢ to 3¢)	Product size (bp)	Reference
Aerolysin/ hemolysin	aer-f: CCTATGGCCTGAGCGAGAAG aer-r: CCAGTTCCAGTCCCACCACT	431	Soler *et al* (2002)
Serine protease	serine-f: CACCGAAGTATTGGGTCAGG serine-r: GGCTCATGCGTAACTCTGGT	350	Soler *et al* (2002)
DNase	exu-f: (A/G)GACATGCACAACCTCTTCC exu-r: GATTGGTATTGCC(C/T)TGCAA(C/G)	323	Soler *et al* (2002)
Cytotoxic enterotoxin (act)	act F: AGAAGGTGACCACCACCAAGAACA act R: AACTGACATCGGCCTTGAACTC	232	Nawaz *et al* (2010)
Cytotonic enterotoxin (ast)	ast F: TCTCCATGCTTCCCTTCCACT ast R: GTGTAGGGATTGAAGAAGCCG	331	Nawaz *et al* (2010)
Cytotonic enterotoxin (alt)	alt F: TGACCCAGTCCTGGCACGGC alt R: GGTGATCGATCACCACCAGC	442	Nawaz *et al* (2010)
Flagellin	fla F: TCCAACCGTYTGACCTC fla R: GMYTGGTTGCGRATGGT	608	Nawaz *et al* (2010)
Integrase 1	Intl1 F: GGGTCAAGGATCTGGATTTCG Intl1 R: ACATGGGTGTAAATCATCGTC	483	Mazel *et al* (2010)
Integrase 2	Intl2 F: CACGGATATGCGACAAAAAGGT Intl2 R: GTAGCAAACGAGTGACGAAATG	788	Mazel *et al* (2010)

2.2.8 PCR amplification of integrons

To determine whether the *Aeromonas* isolates carry integrons, we used PCR amplification to detect Class 1 and Class 2 integrase genes, *intI1* and *intI2*, respectively (Mazel *et al*, 2000). The primers used for the amplification of these genes and the predicted sizes of the amplification products are listed in Table 2.4.

2.3 RESULTS

2.3.1 Characteristics of diseased fish and phenotypic identification of isolates

A total of 173 moribund freshwater fish (guppy *Poecillia reticulata*, n = 42; goldfish *Carassius auratus*, n = 56; koi carp *Cyprinus carpio*, n = 19; fighter *Betta splendens*, n = 27; kissing gourami *Helostoma temminckii*, n = 9; giant gourami *Osphronemus goramy*, n = 1; platy *Xiphophorus maculatus*, n = 2; angel *Pterophyllum* spp., n = 12; swordtail *Xiphophorus* spp., n = 1; molly *Poecilia sphenops*, n = 3; and discus *Symphysodon* spp., n = 1) originating from 34 aquaria (commercial level, n = 29; small-scale, household-based, n = 5) and ornamental fish breeding farms (n = 7) were examined in this study. All the aquaria/farms from which the diseased fish were collected had many species of fish, and none were single-species farms. Fin rot, haemorrhages, dermal ulceration, ascites, scale protrusion and exophthalmia were the most commonly observed gross signs associated with infections.

Of the bacterial isolates selected from cultures grown on TSA and *Aeromonas*-selective media, a total of 74 isolates were presumptive *Aeromonas*, as identified by the phenotypic methods, while the rest of the isolates consisted of *Citrobacter* spp., *Pseudomonas* spp., *Flavobacterium* spp., *Enterobacter* spp., *Vibrio* spp. and a few other Gram-negative isolates that could not be identified using phenotypic methods (data not shown).

2.3.2 Molecular identification

2.3.2.1 Amplification of *gcat* gene

Out of 74 isolates presumptively identified as *Aeromonas* spp. by phenotypic tests, only 62 isolates (62/74 = 84%) showed amplification of the *gcat* gene, suggesting that 12 isolates do not belong to the genus *Aeromonas*. (However, when the identities of all 74 isolates were confirmed with housekeeping gene sequencing, only 52 *gcat*-positive isolates and one *gcat*-negative isolate were confirmed as aeromonads. Thus, through amplification of the *gcat* gene, correct identification at the genus level occurred in 98% (52/53) of the *Aeromonas* isolates characterised in the present study, corroborating the findings of Chacón *et al* (2002, 2003) and Beaz-Hidalgo *et al* (2010). The rest of the *gcat*-negative isolates were found by nucleotide sequencing to belong to other genera (data not shown). However, a false positive amplification was observed for 10 isolates which were subsequently identified as *Vibrio* spp.

2.3.2.2 Phylogenetic analysis using *rpoD* and *gyrB*

Of the 74 presumptive aeromonads, phylogenetic analysis of the *rpoD* and *gyrB* gene sequences resulted in a definitive identification of 53 isolates (72%) as belonging to *Aeromonas* species. Hence, subsequent investigations were carried out only for those 53 isolates of *Aeromonas* for which speciation was confirmed by *rpoD*/*gyrB* sequencing.

The unrooted neighbour-joining phylogenetic tree, constructed using the concatenated *rpoD* and *gyrB* gene sequences (1593 bp; Fig 2.1) showed a clear clustering of all isolates with the reference strains of the respective species with high bootstrap values. Phylogenetic identification results revealed that *A. veronii* was the predominant species with 42 isolates (79.2%), followed by 4 *A. hydrophila* (7.5%), 3 *A. caviae* (5.7%), 2 *A. dhakensis* (formerly '*A. aquariorum*') (3.8%), 1 *A. jandaei* (1.9%) and 1 *A. trota* (*A. enteropelogenes*) (1.9%) (Table 2.5).

Table 2.5 Comparison of the results obtained through different identification approaches for the clinical isolates of *Aeromonas* from freshwater ornamental fish. gcat: *Aeromonas*-specific glycerophospholipid cholesterol acyl transferase gene

Phenotypic identification	Screening for *gcat*	*rpoD/gyrB* identification	16S rDNA-RFLP identification
74 *Aeromonas* spp.	62 *Aeromonas* spp. (*gcat+*)	41 *A. veronii*	34 *A. veronii*
			7 atypical
		4 *A. hydrophila*	4 *A. hydrophila*
		3 *A. caviae*	3 *A. caviae*
		2 *A. dhakensis*	2 atypical
		1 *A. jandaei*	1 *A. jandaei*
		1 *A. trota*	1 *A. trota*
		10 non-*Aeromonas*	Not done
	12 non-*Aeromonas* (*gcat–*)	1 *A. veronii*	1 *A. veronii*
		11 non-*Aeromonas*	Not done

Figure 2.1

Figure 2.1. Unrooted neighbor-joining phylogenetic tree constructed from the concatenated

partial *gyrB* and *rpoD* gene sequences showing the relationships of 53 *Aeromonas* isolates

isolated in this study with reference strains. Numbers shown next to each node indicate bootstrap values (percentages of 1000 replicates). Colored circles indicate the presence of the virulence factor genes analyzed in this study for each isolate. Colored triangle indicates the presence of integrase 1 gene.

2.3.2 3 Identification by 16S rDNA PCR-RFLP

Out of 53 isolates subjected to 16S rDNA RFLP, 44 isolates (83%) exhibited 'typical' restriction patterns enabling an accurate speciation comparable with identification obtained through housekeeping gene sequencing. The remaining 9 isolates (7 *A. veronii* and 2 *A. dhakensis*) (17%) could not be assigned to a known species by 16S rDNA RFLP. Those 7 *A. veronii* isolates exhibited atypical restriction patterns (i.e. different to the pattern published for this species; Borrell *et al* (1997), Figueras *et al* (2000) (Table 2.5, Fig 2.2). Two *A. dhakensis* isolates shared an atypical pattern that is closely related to *A. caviae*. Figureas *et al* (2009) reported that *A. dhakensis* produce either the *A. caviae* RFLP pattern or a somewhat similar pattern with extra bands.
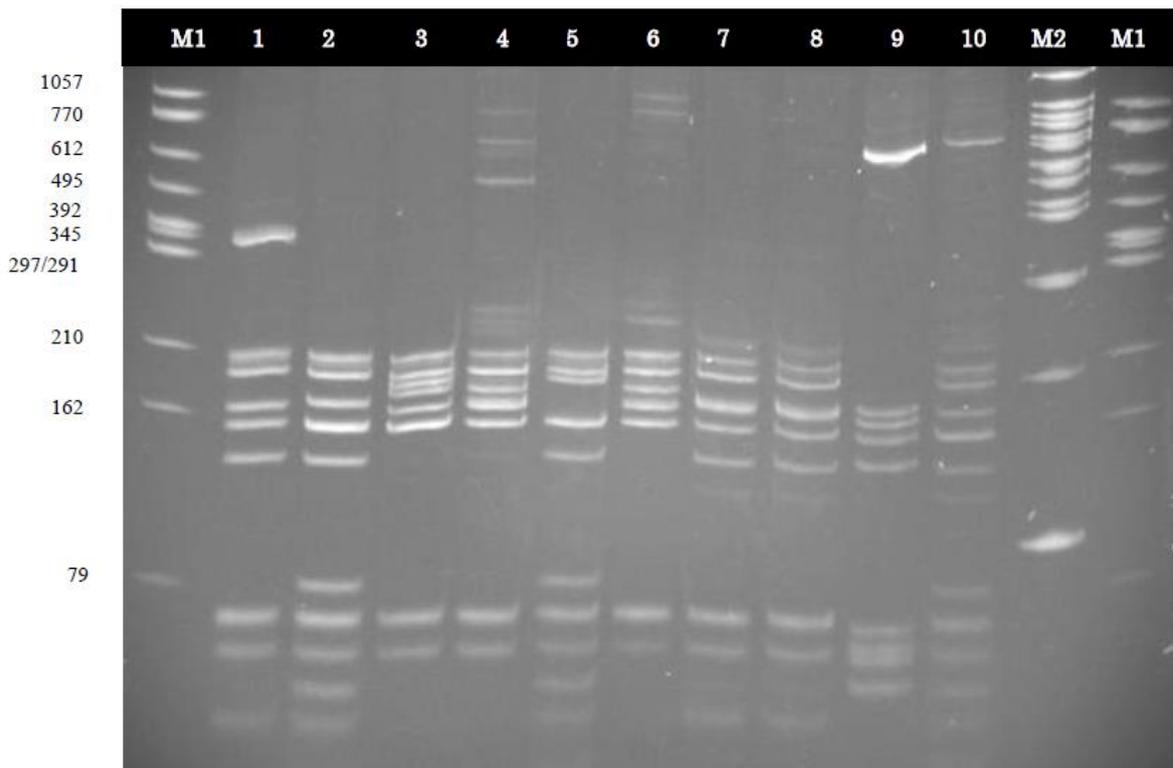
Figure 2.2 Polyacrylamide gel showing 16S rDNA-RFLP patterns (*AluI* and *MboI*). M1: OneSTEP Marker 5 (φX174/*Hinc* II digest) (Wako, Nippongene), Lane 1:typical pattern of *A. hydrophila* (346,207,195,165,157,138,69,66), Lane 2: typical pattern of *A. veronii* (207,195, 174,158,157,138,78,69,66), Lane 3: typical pattern of *A. caviae* (207,195,188,180,165, 158,157, 69,66), Lane 4: atypical pattern observed for *A. dhakensis* , Lane 5: typical pattern of *A. jandaei* (207,195,188, 158,157, 138, 78, 69,66), Lane 6: typical pattern of *A. trota/enteropelogenes* (242,207,195,180, 165,158, 157,69,66), Lane 7-10: atypical patterns of *A.veronii*, M2: Gene Ladder 100 (Wako, Nippongene), Expected fragment sizes (bp) for each species according to Borrell *et al* (1997) are stated within brackets next to each species, fragments <60bp were not considered.

2.3.2.4 Molecular fingerprinting of *Aeromonas veronii* by ERIC-PCR

ERIC-PCR fingerprints of *Aeromonas* isolates (Figure 2.3) consisted of 2 to 12 fragments ranging from 125 to 5015 bp. The dendrogram obtained from the ERIC-PCR analysis (Figure 2.3) revealed 7 clusters at the 90% similarity level. Isolates within these clusters were considered to be genetically related. Six of these clusters were 2-isolate clusters and had 100% similarity. In the remaining cluster, 3 isolates grouped with a similarity level of 100% and grouped with a similarity of 90.9% with the fourth isolate. The ERIC patterns of all remaining isolates (n = 26) were diverse and hence considered as genetically distinct and unrelated, with similarities below 90%. Among these genetically unrelated members, the highest similarity between 2 isolates was 83.3%, as indicated by the Dice coefficient and observed for the isolates Ae 13 and Ae 46, which were both isolated from the same fish species (guppy).

It was of interest to see that isolates with identical ERIC-PCR patterns (100% similarity) were isolated from different species of fish, indicating that genetically related isolates could infect different species of fish.
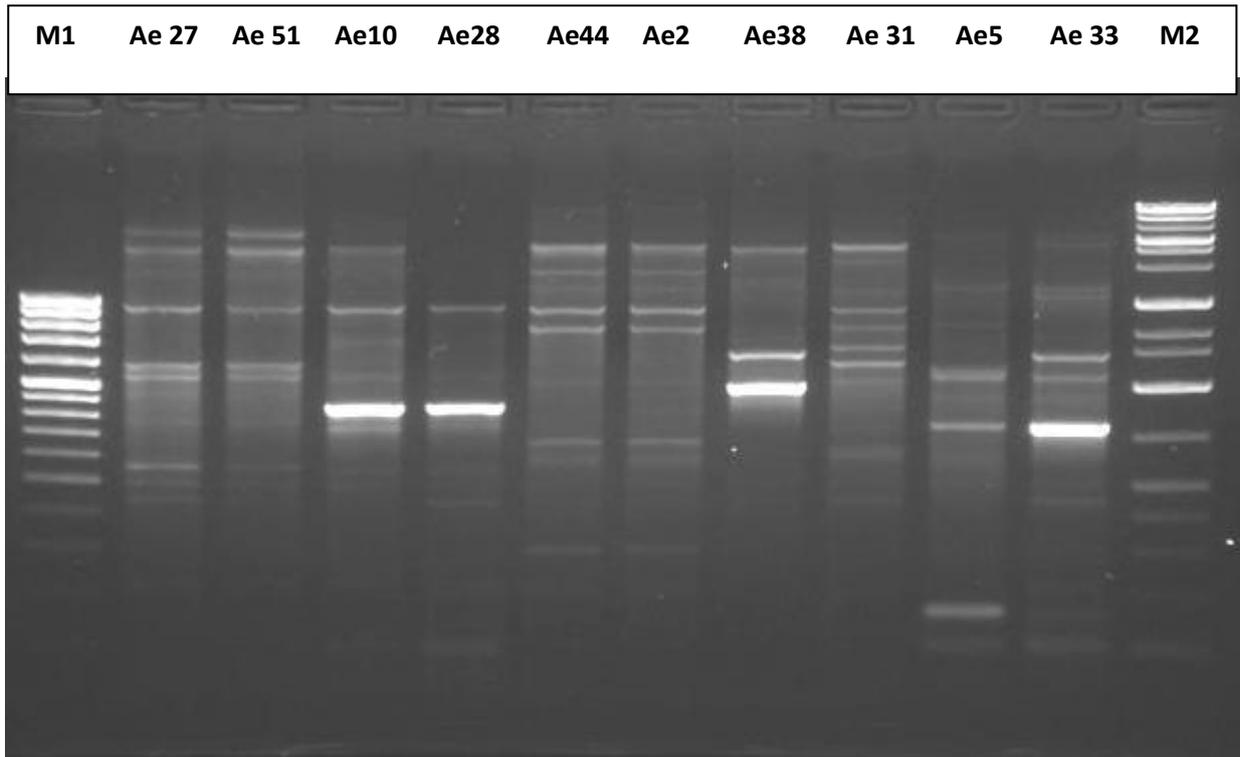
Figure 2.3 ERIC-PCR fingerprints of some of the *Aeromonas* isolates used in the present study. The numbers at the top (Line 2-11) are the isolate numbers listed in table 2.1. Lane M1: OneSTEP Ladder 100 (Wako, Nippongene), Lane M2: Gene Ladder Wide 1 (Wako, Nippongene)
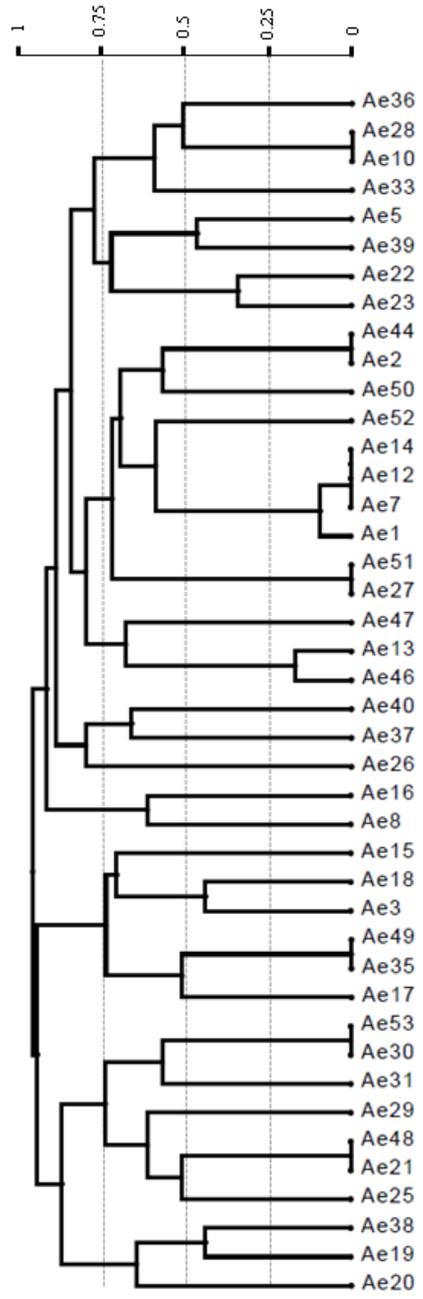
Figure 2.4 Dendrogram illustrating the relatedness of 42 isolates of *Aeromonas veronii* ERIC-PCR fingerprint patterns. The scale shows the genetic distance as calculated by UPGMA cluster method based on Dice coeeficient

2.3.3 Antimicrobial susceptibility testing

The susceptibility levels of the 53 *Aeromonas* isolates against 8 antimicrobial agents are shown in Table 2.6. Besides the classical resistance of aeromonads to amoxicillin (beta lactam antibiotics) (98.1%), the highest resistances encountered were 58.5% to tetracycline and 54.7% to erythromycin. In contrast, the majority of isolates were susceptible to enrofloxacin (84.9%), followed by chloramphenicol (81.1%), neomycin (77.3%) and sulphamethoxasole-trimethoprim (71.7%). Multi-resistance to the tested antibiotics was found in 26 isolates (49%).

Table 2.6. Numbers of *Aeromonas* isolates (n = 53) susceptible to antimicrobial agents. Percent of isolates in parentheses

Antimicrobial agent	Susceptibility level		
	Susceptible	Intermediate	Resistant
Amoxicillin	1 (1.9)	0 (0)	52 (98.1)
Neomycin	41(77.3)	7 (13.2)	5 (9.4)
Trimethoprim-sulfamethoxazole	38 (71.7)	1 (1.9)	14 (26.4)
Chloramphenicol	43 (81.1)	6 (11.3)	4 (7.5)
Tetracycline	15 (28.3)	7 (13.2)	31 (58.5)
Enrofloxacin	45 (84.9)	4 (7.5)	4 (7.5)
Erythromycin	4 (7.5)	20 (37.7)	29 (54.7)
Nitrofurantoin	34 (64.1)	7 (13.2)	12 (22.6)

2.3.4 Detection of virulence-related genes and integrons

The distribution of the 7 virulence genes among motile *Aeromonas* isolates is shown in Table 2.7. All the isolates harboured at least 3 of the virulence genes tested, while all the virulence genes were present in 6/53 (11%) of isolates that included 5 isolates of *A. veronii* and 1 isolate of *A. dhakensis*. Overall, the genomic marker for flagellin (*fla*, 100%) was the most prevalent, followed by that for DNase (*exu*, 98%), aerolysin/hemolysin (*aerA*, 94%), cytotoxic enterotoxin (*act*, 83%), serine protease (*ser*, 62%), heat-labile cytotoxic enterotoxin (*alt*, 49%) and heat-stable cytotoxic enterotoxin (*ast*, 38%) in the isolates analysed. The most common combination of putative virulence genes was $aer^+ser^+fla^+exu^+act^+alt^-ast^-$, which was present in 19% (10/53) of isolates. Results of the amplification of integrase genes revealed that *IntI1*-carrying bacteria corresponded to 77% (41) of the isolates, whereas the *intI2* gene was not detected in any of the isolates.

Table 2.7. Distribution of putative virulence genes in 53 *Aeromonas* isolates recovered from ornamental fish

Aeromonas species	Numbers (%) of the isolates with the respective virulence genes						
	aerolysin (*aer*A)	cytotoxic enterotoxin (*act*)	heat-labile cytotonic enterotoxin (*alt*)	heat-stable cytotonic enterotoxin (*ast*)	serine protease (*ser*)	DNase (*exu*)	flagellin (*fla*)
Aeromonas veronii (n=42)	42 (100)	36 (86)	22 (52)	14 (33)	27 (64)	41 (98)	42 (100)
Aeromons hydrophila (n=4)	2 (50)	3 (75)	2 (50)	4 (100)	3 (75)	4 (100)	4 (100)
Aeromonas caviae (n=3)	2 (67)	2 (67)	1 (33)	1 (33)	0 (0)	3 (100)	3 (100)
Aeromonas dhakensis (n=2)	2 (100)	2 (100)	1 (50)	1 (50)	2 (100)	2 (100)	2 (100)
Aeromonas jandaei (n=1)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)
Aeromonas trota (n=1)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)
Total (n=53)	50 (94)	44 (83)	26 (49)	20 (38)	33 (62)	52 (98)	53 (100)

2.4 DISCUSSION

Isolation of 6 different species of motile aeromonads from 10 diverse species of ornamental fish during the current study adds more evidence for the wide host range and geographical distribution of this bacterium. Motile aeromonads often act as secondary pathogens in fish; therefore, their isolation from septicaemic fish is not so surprising in view of the intensive culture practices in ornamental fish farming that might favour opportunistic infections. The role of other bacteria such as *Citrobacter* spp., *Pseudomonas* spp., *Flavobacterium* spp., *Enterobacter* spp., or *Vibrio* spp., isolated either as sole pathogens or co-pathogens with aeromonads, should not be overlooked since a certain role of these genera as fish pathogens has been recognized (Lewbart, 2001; Musa *et al*, 2008). Moreover, mixed infections are probably not uncommon in ornamental fish that live together with highly diverse microbial communities. However, incidences of mixed infections were not examined in the current study, which focused preferentially on aeromonads and characterised only a single isolate from each infection incidence.

Aeromonas hydrophila is the most cited motile *Aeromonas* species classically linked to MAS in freshwater fish (Hettiarachchi and Cheong, 1994; Nielsen *et al*, 2001; Austin, 2011). In contrast, we found *A. veronii* (79.2%) to be the predominant species which is in agreement with others who reported the dominance of *A. veronii* among fish pathogenic aeromonad isolates following molecular identification (Sreedharan *et al*, 2011, 2013; Hu *et al*, 2012; Yi *et al*, 2013). This difference could possibly be related to the host species selected and the geographical location, but it may have been due, at least in part, to the methods of bacterial identification employed. In a study done to investigate the causative organisms behind incidences of bacterial disease (n = 23) among a number of different species of ornamental fish in Sri Lanka, Hettiarachchi and Cheong (1994) found *A. hydrophila* to be the most

dominant species, associated with 18 incidences (78.26%). In contrast, Sreedharan *et al* (2013) investigated 3 incidences of disease (1 each in gourami, goldfish and oscar) in Kerala, India, and isolated *A. veronii* as the causative organism in all 3 cases. A comparatively higher occurrence of *A. veronii* over *A. hydrophila* has been reported among aeromonads isolated from diseased eels in the Republic of Korea (Yi *et al*, 2013) and diseased freshwater aquaculture fish from China (Hu *et al*, 2012).

The identification of aeromonads is fraught with numerous difficulties due to the phenotypic, serological and genotypic heterogeneity existing within the genus. Discrepancies in identification of *Aeromonas* resulting from poor correlation between phenotypic and genetic identification schemes have been well documented (Kozińska, 2007; Beaz-Hidalgo *et al*, 2010). This problem also occurred in the present study, where identification using specific but a limited number of phenotypic properties resulted in the apparent misidentification of 21 isolates as aeromonads. *gcat*, a gene that is used to identify *Aeromonas* at the genus level (Chacón *et al*, 2002), was useful in the majority (98%) of isolates, with slight deviations in the rest. *gcat* is often considered a virulence-related gene but aeromonads for which the *gcat* gene cannot be amplified have been reported (Nawaz *et al*, 2010) as we observed for 1 of our isolates. This could be a result of the possible mismatch of primers, as noted by Chacón *et al* (2002). However, false positive amplification of this gene resulted in the misidentification of 10 isolates as belonging to the genus *Aeromonas* though they actually belonged to the genus *Vibrio*. This could have been due to the low annealing temperature (56°C) during amplification. Weak amplification of the same size fragment at low annealing temperatures was observed in some *Vibrio* isolates by Chacón *et al* (2002); this problem was overcome by increasing the temperature to 65°C.

The rpoD and gyrB genes have already been used successfully as accurate, unequivocal molecular chronometers for identification of the genus Aeromonas (Yáñez *et al*, 2003; Soler *et al*, 2004; Martínez-Murcia *et al*, 2011). Combined analysis of the rpoD and gyrB genes improved resolution and enabled unambiguous speciation of all isolates used in the present study. In comparison, 16S rRNA PCR-RFLP (Borrell *et al*, 1997; Figueras *et al*, 2000), while being discriminatory for the majority of isolates (83%), produced either atypical restriction patterns or patterns that were very similar to those of other species in the rest of the isolates. This could be due to the high sequence similarity and the occurrence of microheterogeneities in the 16S rRNA genes (Alperi *et al*, 2008). Closely related species (species that have an identical or almost identical 16S rRNA gene sequence) produce the same RFLP pattern, as in the case of *A. piscicola* which has the same pattern as *A. salmonicida* and *A. bestiarum* (Beaz-Hidalgo *et al*, 2010) and *A. dhakensis* which has the same pattern as *A. caviae* (Figueras *et al*, 2009). According to these observations, a need for the incorporation of polyphasic molecular approaches in precise species identification of aeromonads becomes clearly evident. While the exact taxonomic position of fish-pathogenic *Aeromonas* species might not be of interest to all fish pathologists, its use in epidemiological studies and in recognising new pathogenic species and subspecies should not be overlooked. However, for ordinary fish disease diagnostic laboratories, with no access to gene sequencing, other techniques such as those mentioned above are still of use, even though the known limitations could result in misidentification/underestimation of different pathogenic species.

It is also noteworthy to mention that *A. jandaei* and *A. trota* have rarely been isolated from ornamental fish (John and Hatha, 2012), and little information is available regarding their association with clinical disease in ornamental fish. To the best of our knowledge, this is the first time that *Aeromonas dhakensis* sp. nov. comb nov. (formerly known as *A. aquariorum*)

has been recovered from clinically diseased ornamental fish since the original description of this species from the aquarium water and skin of ornamental fish (Martínez-Murcia *et al*, 2008; Beaz-Hidalgo *et al*, 2013). Indeed, it is the first time this species has been isolated in Sri Lanka. Isolation of this seemingly globally distributed species (Aravena-Román *et al*, 2011; Yi *et al*, 2013) from diseased ornamental fish provides additional evidence that a diverse range of motile aeromonads could potentially be associated with septicaemia in ornamental fish. The occurrence and pathogenesis of these rarely isolated motile aeromonads in tropical aquarium fish are not well understood and deserve further study.

ERIC-PCR, a low cost, rapid, reproducible strain typing method with high discriminatory power (Soler *et al*, 2003) revealed the intraspecific diversity that exists within the *A. veronii* isolated in the present study. The majority of isolates were genetically distinct (62% of the isolates had similarities below 90%), with no dominant clones of *A. veronii* associated with MAS in the fish population investigated. This kind of high genetic diversity had been observed among clinical and environmental isolates of aeromonads from different sources typed by the above technique (Davin-Regli *et al*, 1998; Szczuka and Kaznowski, 2004).

High levels of antimicrobial resistance in bacteria isolated from ornamental fish and their environment is not a novel observation (Verner-Jeffreys *et al*, 2009; Cízek *et al*, 2010; Dias *et al*, 2012). In agreement, surprisingly numerous multi-antibiotic-resistant bacteria (49%) were observed among our isolates, apart from their classical resistance to beta lactam antibiotics (Janda and Abbott, 2010). Tolerance to tetracycline and erythromycin was particularly widespread (>50%), a finding that was in common with other comparable investigations of motile aeromonads from ornamental fish (Dias *et al*, 2012; Sreedharan *et al*,

2012). While tolerance of these antibiotics has likely resulted from their use in the aquarium fish industry, resistance can also arise from gene mutations or by acquisition of transferable genetic elements such as integrons (Jacobs and Chenia, 2007). The observed levels of multi-resistance could be attributed to the horizontal spread of resistance genes, which is further supported by the presence of Class 1 integrons in 77% of the isolates.

Screening for the presence of virulence genes as a method to evaluate the potential virulence of aeromonads could be speculative, since virulence is a complex process, and empirical testing with a disease challenge is often necessary for conclusive results. However, expression of the putative virulence-associated factors in *Aeromonas* appears to be affected by environmental conditions (Tso and Dooley, 1995; Merino *et al*, 1998), making the detection of true virulent strains difficult. Nevertheless, screening for virulence genes has been used in many studies as a rational approach for evaluating the genetic potential of aeromonads to express virulence factors (Puthucheary *et al*, 2012). In agreement with previous studies (Nawaz *et al*, 2010; Hu *et al*, 2012; Yi *et al*, 2013), we found high heterogeneity in the distribution of toxin genes among the tested isolates, with a heterogeneous distribution forming 18 different virulence gene combinations. High prevalences of *gcat*, *exu*, *fla*, *act* and *aerA* genes are consistent with the results of Yi *et al* (2013) and Nawas *et al* (2010) from clinical isolates of fish.

In conclusion, the present study highlights the diversity of mesophilic *Aeromonas* species that could potentially be associated with MAS in ornamental fish.

CHAPTER 3

Genome sequencing and comparative genomic analysis of *Aeromonas hydrophila* strain Ae34 isolated from fresh water ornamental fish with signs of septicaemia

3.1 INTRODUCTION

Aeromonas hydrophila, a mesophilic member of the genus *Aeromonas*, is widespread in aquatic environments and has increasingly been implicated in infections in a wide range of host species including fish, amphibians, reptiles and humans (Janda and Abott, 2010). It has long been considered as an important and major pathogen of many species of fresh water fish (Austin and Adams, 1996). In the recent past, *A. hydrophila* has received increasing attention due to its role as an emerging pathogen in humans causing intestinal and extraintestinal infections that range from relatively mild illnesses to life-threatening conditions (Janda and Abott, 2010; Parker and Shaw, 2011).

A. hydrophila is primarily a free living organism that inhabits fresh water (and estuarine waters to a lesser extent) (Janda and Abott, 2010) and a commensal in the skin and gastrointestinal tracts of healthy fish. Under favorable conditions, this bacterium acts as an opportunistic pathogen (secondary invader) in stressed or immunocompromised fish causing diverse pathological conditions that are collectively known as motile aeromonad septicaemia /haemorrhagic septicaemia. However, there have been instances where this pathogen acted as a primary pathogen (Hazen *et al*, 1978; Griffin *et al*, 2013). Haemorrhagic septicaemia caused by *A.hydrophila* is characterized by dropsy, fin rot, ulcers and haemorrhages on skin, exophthalmia and scale protrusion and primarily affects a variety of fresh water fish including channel catfish (*Ictalurus punctatus*), carp (*Cyprinus carpio*), gizzard shad (*Dorosoma cepedianum*), striped bass (*Morone saxatilis*), largemouth bass (*Micropterus salmoides*), tilapia, minnows, baitfish and many species of tropical aquarium fish (Cipriano, 2001; Hettiarachchi and Cheong, 1994). This infection progresses rapidly and could reach epizootic proportions with high rates of mortality (Joseph and Carnahan, 1994) causing

severe economic losses to the aquaculture industry. In the recent past, an epidemic outbreak caused by a highly virulent strain of *Aeromonas hydrophila* in market size channel catfish *Ictalurus punctatus*, in 48 catfish farms in west Alabama, USA led to an estimated loss of more than 3 million pounds (Pridgeon and Klesius, 2011; Griffin *et al*, 2013).

Aquarium raised tropical ornamental fish are generally considered more prone to stress borne opportunistic infections as they are constantly exposed to various stressors that accompany intensive management practices in commercial ornamental fish production. *A. hydrophila* has been linked to many fish kills and isolated cases of mortalities in Goldfish, Koi carps and many other species of tropical fresh water aquarium fish due to its isolation from disease incidences as the sole pathogen or a co pathogen (Hettiarachchi and Cheong, 1994; Citarasu *et al*, 2011; Cızek *et al*, 2010; John and Hatha, 2012). However, the actual incidence of *A. hydrophila* associated septicaemia in ornamental fish might have been underrepresented in the published literature due to the limited investigations in to the aetiological diagnosis of ornamental fish pathogens in resource limited settings of developing countries where majority of the aquarium fish is being produced.

The adverse impacts of *Aeromonas hydrophila* on aquarium fish industry are numerous. Sudden and unexpected fish mortalities, growth retardations in survived fish, loss of market value and therapeutic and/or prophylactic interventions result in enormous economic losses to the farmers. Moreover, *A.hydrophila* is a zoonotic bacterium and hence its presence in aquaria and aquarium fish carriage water poses a public health risk. Besides, *Aeromonas hydrophila* is a pathogen having the ability to produce resistant phenotypes to many antimicrobials, which is not only an emerging challenge in treating affected fish but also a risk to the public exposed to *A.hydrophila* through ornamental fish or aquarium water.

In the recent past, high throughput whole genome sequencing has emerged as a rapid means of understanding the biology, evolution, virulence and diversity of bacterial pathogens. The number of genomes of fish pathogenic bacteria available to date is a good indication of the increasing trend of using of this technology in aquatic animal health field (Sudheesh *et al*, 2012). Among Aeromonads, *Aeromonas hydrophila* ATCC 7966 (a well-characterized type strain for this species) was the first genome sequence determined (Seshadri *et a*l, 2006), but it was an isolate recovered from a tin of milk with a fishy odor. This strain was determined to be among the most pathogenic strains in mouse lethality experiments (Janda and Kokka, 1991); however its pathogenicity in fish has not yet been studied. Until 2013, none of the *A. hydrophila* isolates obtained from diseased fish has been subjected to whole genome sequencing. Han *et al* in 2013 reported a draft genome sequence of *A. hydrophila* strain SNUFPC-A8 isolated from the kidney of a moribund cherry salmon (*Oncorhynchus masou masou*). The first complete genome sequence of a fish pathogenic isolate, *A. hydrophila* strain ML09-119 was reported very recently (Tekedar *et al*, 2013). It was a representative of the clonal group of *A. hydrophila* implicated in a disease outbreak in Channel Catfish in West Alabama during 2009. Acquisition of novel genetic elements through lateral genetic transfer has been involved as the molecular basis of the emergence of this pathogenic clonal group of *A. hydrophila*. This was a finding based on next-generation sequencing and comparative analysis of the epidemic isolates of *A. hydrophila* genomes with reference isolates of non epidemic origin, which demonstrated that the former isolates carry unique epidemic associated regions that are missing in reference isolates (Hossain *et al*, 2013).

The pathogenesis of *A. hydrophila* is multifactorial (Zhang *et al*, 2000) as it encodes for many genetic determinants of virulence. While many bacterial genes and potential virulence factors must be involved in the complicated process of *Aeromonas* virulence, only a few have

been studied in any great detail (Janda and Abott, 2010). Among those, some determinants such as pili, hemolysin, serine protease, metalloprotease, cytotoxic enterotoxin, S-layer, outer-membrane protein (Omp), multidrug-resistance protein and histone-like protein (HU-2) and the type III secretion system (Li *et al*, 2011; Zhang *et al*, 2000) have been shown to be important in causing disease in fish. However, there is no agreed consensus on differentiating fish pathogenic strains from environmental or 'non pathogenic' strains based on their virulence gene profile. Among virulent members, heterogeneity of virulence genes has also been reported (Zhang *et al*, 2000). Expression of virulence factors has been reported to depend on the external triggers such as temperature (González-Serrano, 2002). In this context, genome sequencing of multiple strains of fish pathogenic *A.hydrophila* would be a promising approach that will enable the revelation of the complete array of virulence factors that could potentially be involved in *A. hydrophila*'s pathogenicity in fish. This will also add evidences to the acquisition of virulence determinants through phage mediated lateral gene transfer.

Despite the growing interest and economic importance of global aquarium fish trade, there is a dearth of information on the genome sequence of *A. hydrophila* isolated from tropical ornamental fish. A Genome of a pathogen should provide an insight into evolution and mechanisms involved in mediating the disease. Whole genome sequencing of the clinical isolates of ornamental fish borne *A. hydrophila* would help to reveal what specific genetic features would differentiate a 'fish pathogenic strain' from their 'commensal' and 'environmental' counterparts who share the same environmental niche. Further, in time to come, whole genome sequencing of many clinical, free living environmental and commensal isolates would provide insights into the molecular mechanisms behind the opportunistic pathogenic behavior of this pathogen and the factors that determine the pathogenic, free

living and commensal lifestyles. Besides, it would be interesting to understand the genetic background of metabolic versatility that enable this bacterium to exist in virtually every environmental niche where bacterial ecosystems exist and infect a broad host range (Janda and Abott, 2010).

In the long run, sequenced whole genomes would provide the basis to make genome informed decisions in the development of rapid diagnostics, vaccines and therapeutics for the diagnosis and control of motile aeromonas septicaemia in ornamental fish. In this context, we sequenced the genome of *Aeromonas hydrophila* Ae34, isolated from a moribund Koi carp (*Ciprinus carpio koi*) with signs of haemorrhagic septicaemia using high throughput next generation sequencing to gain insight into the biology and evolution of this important opportunistic pathogen of aquarium fish.

3.2 MATERIALS AND METHODS

3.2.1 Isolation and identification of the isolate

During an investigation of septicaemia causing bacterial pathogens in tropical fresh water ornamental fish in Sri Lanka, *Aeromonas hydrophila* strain Ae34 was isolated from the kidney of a moribund Koi carp (*Ciprinus carpio koi*) with signs of haemorrhagic septicaemia. The fish was collected from a commercial aquarium and represented an isolated case of septicaemia. This particular isolate was selected for the whole genome sequencing since it was isolated as the sole agent responsible for causing septicaemia and found to be multidrug-resistant. The identity was confirmed as *A. hydrophila* through phenotypic identification and the sequence analysis of *gyrB* and *rpoD* genes (Jagoda *et al*, 2014).

3.2.2   Extraction of DNA for genome sequencing

Genomic DNA was extracted by using DNeasy Blood and Tissue kit (Qiagen) from an overnight culture of *A. hydrophila* Ae34 grown on Trypticase soy agar according to the manufacturer's instructions. The quality of the genomic DNA was checked by gel electrophoresis and the concentration was estimated using a Nanophotometer (IMPLEN).

3.2.3   Genome sequencing on the Ion Personal Genome Machine (PGM) system

Genome sequencing was performed using the Ion PGM Sequencer (Life Technologies) using 200 bp reads chemistry (Ion PGM 200 sequencing kit) with Ion 318 Chip. The sequencing protocol included the flowing steps. First, the genomic DNA was fragmented and ligated to adapters. Then, the adapter ligated libraries were clonally amplified into beads. Template bearing beads were enriched and the enriched libraries were sequenced using Ion Torrent PGM sequencer according to manufacturer's instructions.

A genomic library was prepared using 100 ng of genomic DNA using the Ion Plus fragment library kit according to the protocol (Part No: 4471989 Rev.D, 05/2012). Size selection was done with E-Gel SizeSelect 2% Agarose gel. Size distribution of the library was assessed with Bioanalyzer 2100 (Agilent technologies). Template preparation was carried out with the Ion PGM 200 Xpress Template kit (Catalogue no: 4474280). Sample was loaded onto an Ion 318 chip and subsequently sequenced with the Personal Genome machine according to the Ion Sequencing 200 kit user guide. Sequencing was performed in two consecutive times.

3.2.4 Sequence data trimming, filtering, assembly and annotation

The total number of reads generated in the first time sequencing was 323 Mbp with an average read length of 193 bp. Second time sequencing generated 427 Mbp with an average read length of 199 bp. The average coverage in two consecutive sequencing was 69x and 91x respectively. In total, the generated sequencing output consisted of 750 Mb of DNA sequences, corresponding to about 160x coverage of the Ae34 genome, using the 4.7-Mb size of the genome of *A. hydrophila* ATCC 7966 as the reference genome. The reads were subjected to quality trimming using the CLC Genomics Workbench v 6.5 before assembly. After quality trimming thereby discarding the reads with an average quality score below Q20, a total of 1,484,357 reads (with an average length of 275 bp) were selected as quality reads to be used in the subsequent assembly.

The reads were *de novo* assembled using MIRA v3.9.5 (Chevreux *et al*, 1999) and CLC genomic workbench v 6.5. We also used the reference guided assembly option of the CLC genomic workbench v 6.5. using as references the *A. hydrophila* ATCC 7966[T] (CP000462.1) genome and *A. hydrophila* ML09-119 (CP005966.1) genome. We used initial MIRA assembly (n=2935) as the backbone assembly and CLC assembly as a supporting assembly to countercheck MIRA assembly. We attempted to join the contigs of MIRA assembly using the CLC Microbial Genome Finishing Module, after aligning them to the genomes of *A. hydrophila* ML09-119 (CP005966.1) and *A. hydrophila* ATCC 7966[T] (CP000462.1). This process generated 59 consensus contigs [731 to 297,409 nucleotides]. Then, we *De novo* assembled all unmapped reads followed by mapping them back to consensus contigs and joining them whenever possible. This resulted in a draft genome assembly consisting of 28 contigs with a total size of 4,705,099 nt. CONTIGuator is a software tool for contigs mapping

over a reference genome which allows the visualization of a map of contigs, underlining loss and/or gain of genetic elements (Galardini *et al*, 2011). We used CONTIGuator to map the draft genome contigs against the reference genomes of *A. hydrophila* ML09-119 (CP005966.1) and *A. hydrophila* ATCC 7966^T (CP000462.1).

The draft genome was annotated using three open source prokaryotic genome annotation tools, namely; RAST *(*Rapid Annotation of microbial genomes using Subsystems Technology) (Aziz *et al*, 2008)*,* MiGAP (Microbial Genome Annotation Pipeline ver 2.18 **http://www.migap.org/index.php/en**) (Sugawara *et al*, 2009) and Bacterial Annotation System (BASys) (Van Domselaar *et al*, 2005). At the same time, we annotated the two reference genomes above using the RAST annotation tool in order to compare them with the draft genome in terms of the differences in features/coding sequences. The comprehensive antibiotic research database (CARD; http://arpcard.mcmaster.ca) (McArthur *et al*, 2013) was used to identify putative antibiotic resistance genes. A phage search tool, PHAST (Zhou *et al*, 2011) was used to detect the presence of prophage sequences within Ae34 draft genome if any. Using the same tool, we compared the presence of phages in 06 other *Aeromonas hydrophila* strains (Table 3.7) isolated from different sources (both fish borne and non fish borne) available to date in GenBank to determine if there is any relationship between the presence of phage DNA and fish pathogenicity.

3.2.5 Ordering contigs against reference genomes and comparative genomics

We used Mauve multiple genome alignment tool to order contigs and to inspect assembly statistics. Using the contigs ordered via Mauve, we generated a multiple alignment of the *A. hydrophila* Ae34 draft genome with the type strain *A. hydrophila* ATCC 7966^T and *A.*

hydrophila ML09-119 from the recent epidemic outbreak in Catfish. We inspected the genes that are annotated in the ornamental fish septicaemia causing strain but missing in the type strain.

3.3 RESULTS

The summary statistics of the sequencing libraries (of two consecutive sequencing) before and after trimming is shown in table 3.1. Assembly and subsequent manual contig joining using CLC microbial genome finishing module resulted in a draft genome of 28 contigs (mean size, 168,039 bp, maximum length, 762,403 bp). The total size of the draft genome (4,705,099 nt) and G+C content (61.6%) were in good agreement with the respective figures for the published *A. hydrophila* genomes (4.5 to 5.0 Mb and 60.8 to 62%). The draft genome sequence has been deposited in the DDBJ/EMBL/GenBank databases under the accession no. BAXY01000001 to BAXY01000028.

Table 3.1 Summary statistics of the sequencing libraries of two consecutive sequencing before and after trimming

Library Summary		
	First time	Second time
Total Number of Bases [Mbp]	322.77	426.89
Longest Read [bp]	382	488
Mean Length [bp]	192	199
Final Library reads	1,680,302	2,149,286
Coverage	69	91
Trim summary		
	First time	Second time
Number of reads	1,680,302	2,149,286
Average length	192.1	198.6
Number of reads after trim	514,208	970,149
Trimming parameters	<250,290<	<250,310<
% trimmed	30.6	45.14
Average length after trimming	269.4	277.5

Mapping of draft genome contigs to the two reference genomes using CONTIGuator (Fig. 3.1) revealed that a higher nucleotide percentage of draft genome was mapped to *A. hydrophila* ML09-119 (CP005966.1) compared to that with *A. hydrophila* ATCC 7966^{T} (CP000462.1) genome (Table 3.2).

Table 3.2 Statistics of contig mapping using CONTIGuator bacterial genome finishing tool when draft genome contigs were mapped to two selected *A.hydrophila* reference genomes

Category	*A. hydrophila* ATCC 7966[T] (4744448 bp)		*A. hydrophila* ML09-119 (5024500 bp)	
	Number	**bp**	**Number**	**bp**
Input contigs	28	4705099	28	4705099
Mapped contigs	26	4673076 (99.31%)	27	4703853 (99.97%)
UnMapped contigs	2	32023	1	1246
UnMapped: Short contigs	0	0	0	0
UnMapped: Poor coverage	2	32023	1	1246
UnMapped: Borderline coverage	0	0	0	0
UnMapped: More than one replicon	0	0	0	0
UnMapped: Duplicated hits	0	0	0	0
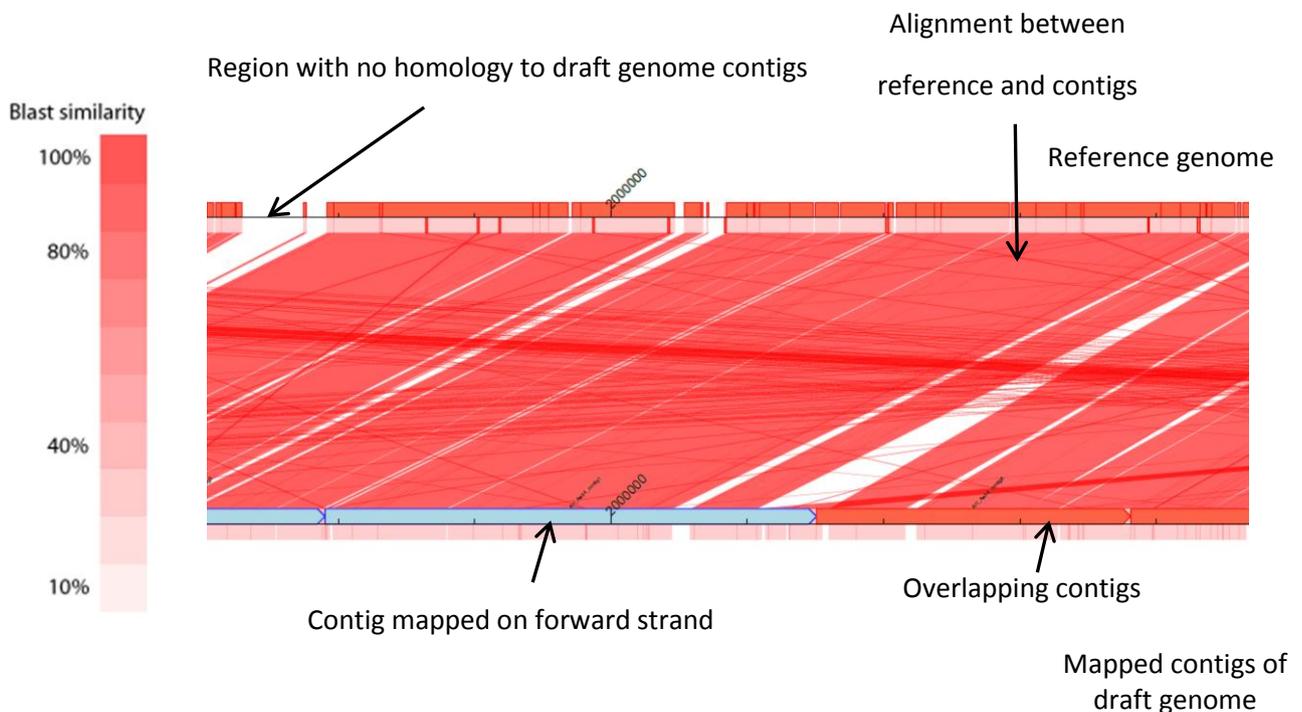
Figure 3.1

A legend visualizing the positions of the draft genome contigs over the reference genome *A. hydrophila* ML09-119 (the region corresponding to 1.7 Mb - 2.5Mb of reference genome magnified). BLAST similarity colour intensity index adapted from CONTIGuator web server http://bazzigroup.dbe.unifi.it/contiguator/legend.html (Galardini *et al*, 2011).

Annotation of the genome using RAST identified 4256 protein coding sequences. A total of 117 tRNAs and 31 rRNAs were predicted by using tRNAscan-SE 1.23 (Lowe AND Eddy, 1997) and RNAmmer 1.2 (Lagesen *et al*, 2007). In contrast, for the same draft genome, MiGAP predicted 4429 coding sequences and BASys annotated 5913 genes. However, the predicted numbers of tRNAs and rRNAs by these annotation pipelines were the same. Of all CDS annotated by RAST, 54% had RAST subsystem coverage. RAST predicted numerous genes encoding for virulence and defence, of which 67 are related to resistance to antibiotics/toxic compounds. These include multidrug resistance efflux pumps [20], beta-lactamases [2], multiple antibiotic resistance locus [1], lysozyme inhibitors [1] and genes encoding fluoroquinolone resistance [4]. A comparison of the results of RAST annotation for the *A. hydrophila* Ae34 draft genome and the two reference genomes with respect to the number of annotated features in each RAST subsystem is given in table 3.3 and table 3.4.

Table 3.3

RAST Subsystem feature counts for the *A. hydrophila* Ae 34 draft genome and two reference genomes

Subsystem Feature Counts	*A. hydrophila* strain Ae34	A. *hydrophila* ML09-119	A. *hydrophila* ATCC7966
Cofactors, Vitamins, Prosthetic Groups, Pigments	282	295	293
Cell Wall and Capsule	183	191	191
Virulence, Disease and Defense	89	89	96
Potassium metabolism	38	38	39
Miscellaneous	27	27	27
Phages, Prophages, Transposable elements, Plasmids	18	24	3
Membrane Transport	165	183	200
Iron acquisition and metabolism	52	56	52
RNA Metabolism	223	224	221
Nucleosides and Nucleotides	128	127	126
Protein Metabolism	247	265	273
Cell Division and Cell Cycle	42	44	43
Motility and Chemotaxis	179	127	131
Regulation and Cell signaling	99	102	99
Secondary Metabolism	6	6	6
DNA Metabolism	112	127	112
Regulons	9	9	9
Fatty Acids, Lipids, and Isoprenoids	138	136	139
Nitrogen Metabolism	46	45	45
Dormancy and Sporulation	2	3	2
Respiration	165	174	172
Stress Response	153	159	151

Metabolism of Aromatic Compounds	11	11	11
Amino Acids and Derivatives	425	437	425
Sulfur Metabolism	36	37	42
Phosphorus Metabolism	44	44	44
Carbohydrates	437	468	465

Table 3.4 Genome characteristics of *A. hydrophila* strain Ae34 and two other *A. hydrophila* complete genomes as determined by the RAST annotation pipeline

Organism	No. of contigs	No. of bp	No. of CDSs	No. of RNAs	No. of tRNAs	No. of rRNAs	No. of rRNA operons	G+C content (%)
A. hydrophila Ae34	28	4,705,099	4256	148	117	31	10	61.6
A. hydrophila ML09-119	1	5,024,500	4520	143	112	31	10	60.8
A. hydrophila ATCC 7966T	1	4,744,448	4286	158	128	30	10	61.5

The preliminary annotation of putative antimicrobial resistance genes in the draft genome using the resistance gene identifier version II of the comprehensive antibiotic research database (CARD) revealed that the strain Ae34 encodes genetic determinants for the resistance against several classes of antibiotics (Table 3.5, Figure 3.2). This resistance annotation is based on BLASTP hits to curated protein sequences present in CARD (McArthur *et al*, 2013). This strain has initially shown multi resistance for several antimicrobials including penicillins, erythromycin, oxytetracycline, chloramphenicol and intermediate resistance to enrofloxacin.

Table 3.5 Different antimicrobial resistance genes that are found to be present in the *A.hydrophila* Ae34 draft genome as annotated by CARD, their definitions and the antibiotics to which resistance is conferred by each gene

Antibiotic Resistance Ontology	Definition	Antibiotics to which resistance is conferred to
cmeB	Inner membrane transporter the Cme ABC multidrug efflux complex	cefotaxime fusidic acid erythromycin
macB	A subunit of efflux pump	erythromycin
chloramphenicol acetyltransferase (CAT)	Inactivates chloramphenicol	chloramphenicol
mexH	MexH is the membrane fusion protein of the efflux complex MexGHI-OpmD	norfloxacin acriflavin
patA	ABC transporter fluoroquinolone resistance	ciprofloxacin norfloxacin
Cfr 23S ribosomal RNA methyltransferase	The product of cfr catalyzes methylation of the 23S rRNA subunit at A2503, conferring resistance to many drugs that target protein synthesis	lincosamide macrolide
tet35	tetracycline efflux pump	tetracycline
FOX-2	beta-lactamase	FOX beta-lactamase
CMY-9	beta-lactamase	CMY beta-lactamase
quinolone resistance protein (Qnr)	Pentapeptide repeat proteins that mimic DNA and protect the cell from the activity of fluoroquinolone antibiotics	ciprofloxacin gatifloxacin levofloxacin moxifloxacin nalidixic acid norfloxacin
Multiple peptide resistance factor (mprF)	MprF is a integral membrane protein that modifies the negatively-charged phosphatidylglycerol on the membrane surface of both Gram-positive and Gram-negative bacteria.	defensin
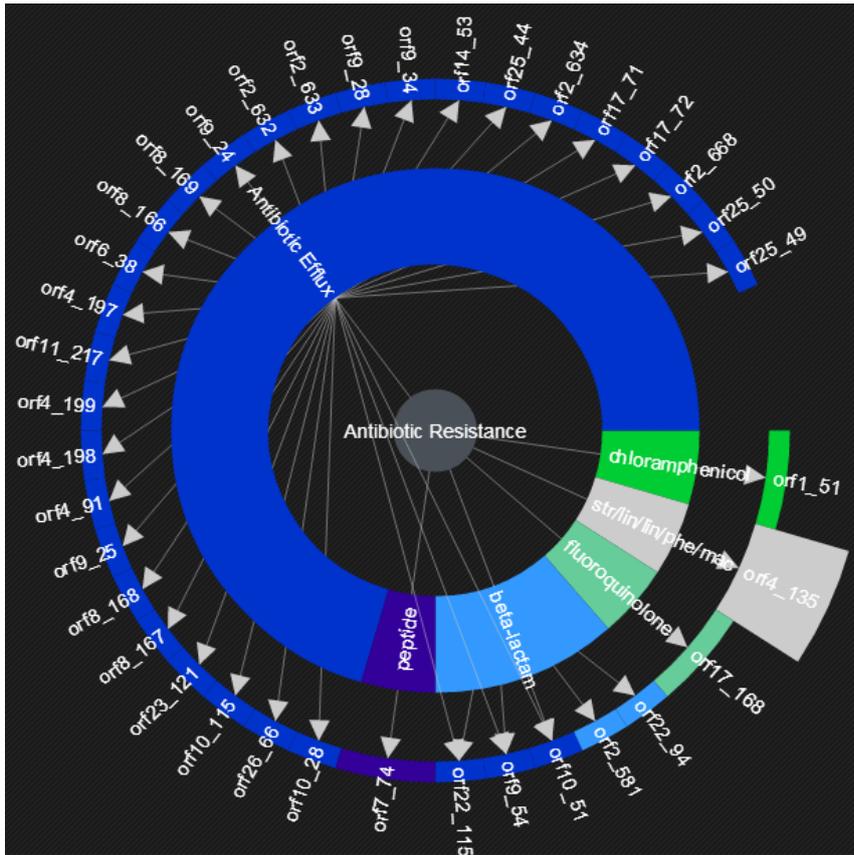
Figure 3.2 Resistance wheel generated by the CARD (comprehensive antibiotic research database) for *A. hydrophila* strain Ae34 showing the resistance classes and individual resistance genes

PHAST detected two intact prophages (Table 3.6, Figure 3.3) showing 77% and 69% identity respectively to *phi*O18P, a bacteriophage from *A. media* (Beilstein and Dreiseikelmann, 2008). Analysis of the numbers of phages in different strains of *A.hydrophila* sequenced to date showed that there is an observable relationship with the occurrence of phages and their isolation source (Table 3.6). All the fish disease related genome sequences of *A.hydrophila* found to harbour phage DNA, while the number is comparatively low or absent in environmental strains.

Table 3.6 Details of the two prophages detected by the phage search tool, PHAST in the draft genome of *A.hydrophila* Ae34

Region	Region Length	Completeness	#CDS	Region Position	Possible phage	GC%
1	40.5Kb	intact	48	311318-351881	PHAGE_Aeromo_phiO18 P_NC_009542	60.44%
2	30Kb	intact	41	4673908-4703963	PHAGE_Aeromo_phiO18 P_NC_009542	60.09%

Table 3.7 The details of the phages present in different strains of *Aeromonas hydrophila* genomes as determined by PHAST

Name of the strain	Source	Number of Intact Phages	Number of incomplete and/or questionable phages
A.hydrophila Ae34	Diseased fish, Sri Lanka	2	0
A.hydrophila ML 09-119	Cat fish disease outbreak, USA	2	2
Aeromonas hydrophila SNUFPC-A8	Moribund cherry salmon, South Korea	2	0
Aeromonas hydrophila AL09-71	(highly virulent strain) Cat fish disease outbreak, USA (Pridgeon *et al*, 2014)	2	2
A.hydrophila pc104A	Soil of a Catfish Pond, USA, 1,000-fold-less virulent than *A. hydrophila* AL09-71 (Pridgeon *et al*, 2014)	2	2
A.hydrophila 4AK4	Used for industrial production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) (Gao *et al*, 2013)	1	0
A.hydrophila ATCC 7966	Tin of milk with fishy odour	0	0
Aeromonas hydrophila YL17	A Quorum Sensing Strain Isolated From Compost	0	0
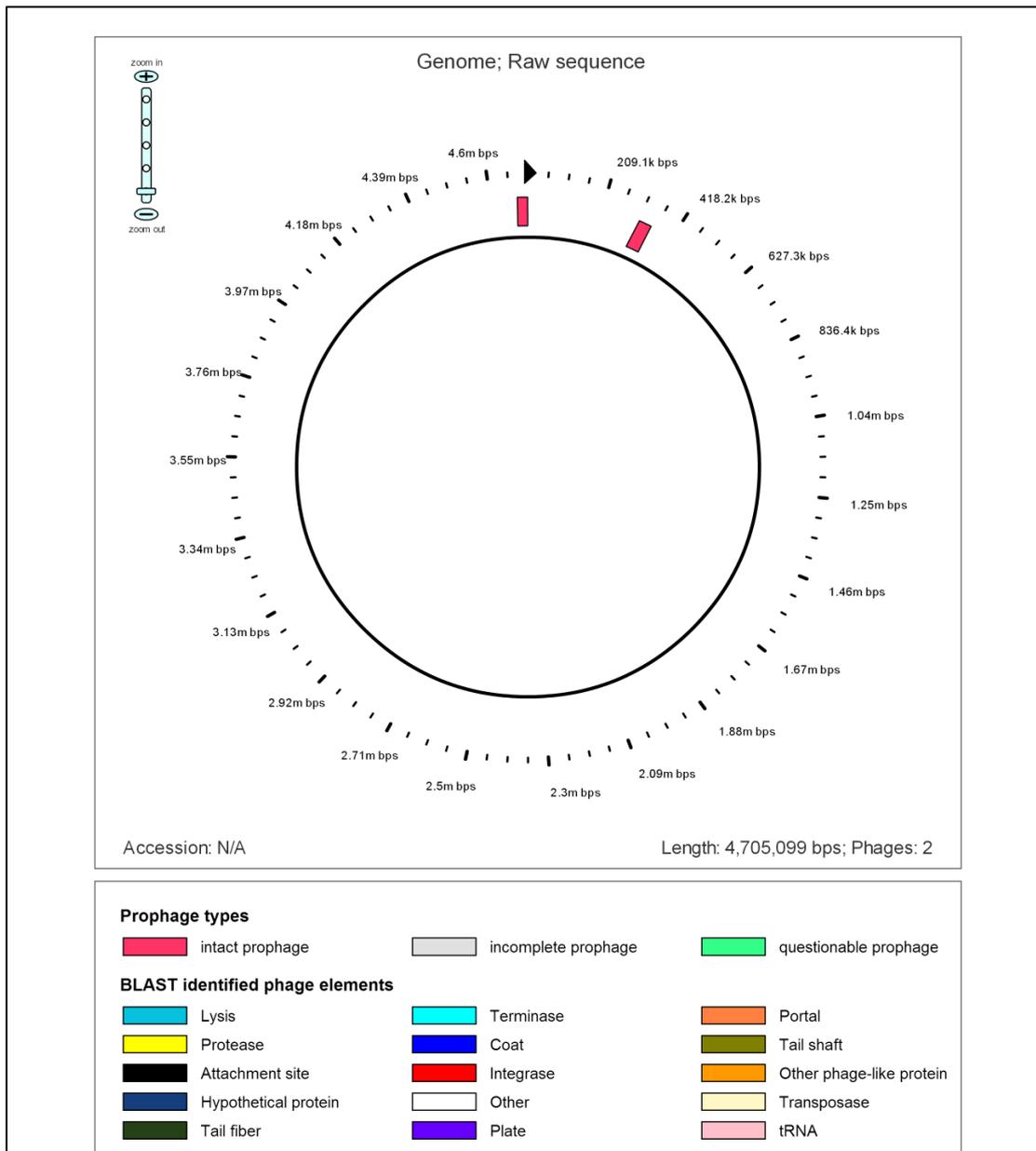
Figure 3.3 Genome map showing the positions of two intact phages within the draft genome of *A. hydrophila* Ae34

Function based comparison of RAST annotation results of each reference genome (ML09-119 and ATCC 7966; with that of strain Ae34 draft genome helped to understand what genetic features are unique to strain Ae34 (Figure3.4) We also analysed what features distinguish fish pathogenic strains (Ae34 and ML09-119) and environmental type strain ATCC 7966. Similarly, we found that the outbreak related genome features that are present in ML09-119 are generally absent in Ae34 which supports the role of *A.hydrophila* strain Ae34 as an opportunistic pathogen (Table 3.8). However, some of those predicted genes that have homology to putative virulence factors and are present within virulent *A.hydrophila* ML09-119. are also annotated in Ae34. Examples for these include PrfC protein, probable oxidoreductase, outer membrane protein A.
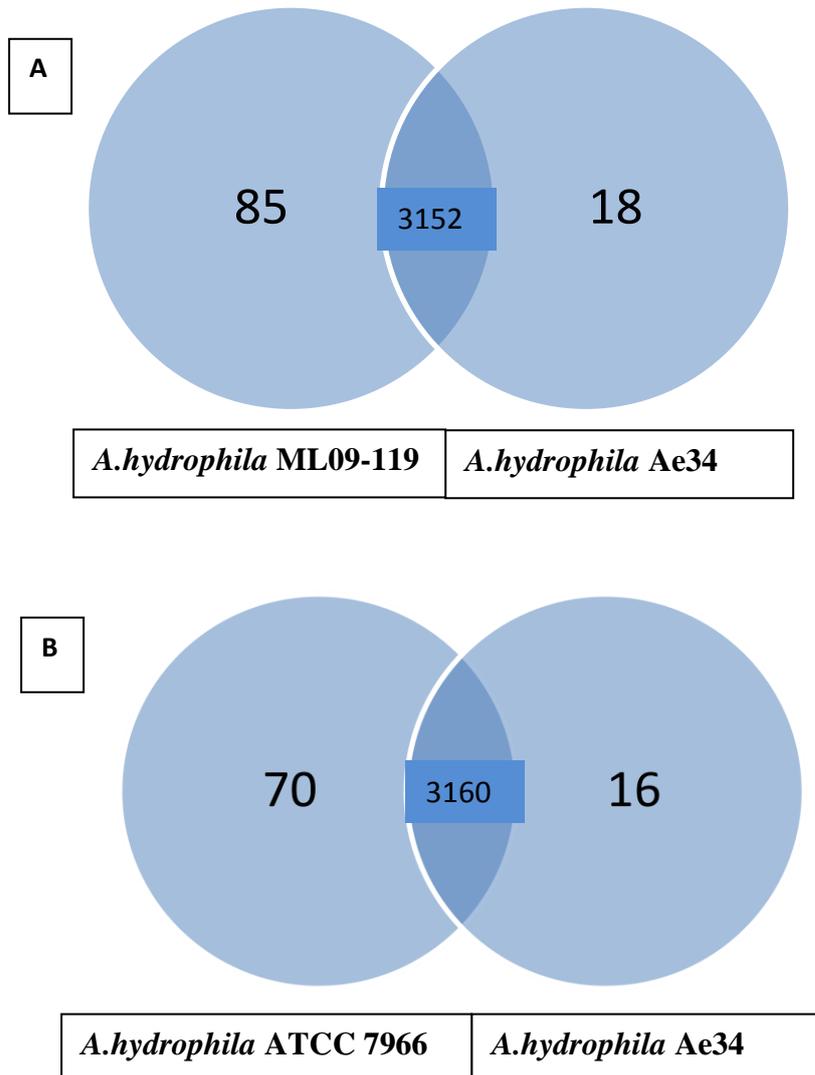
Figure 3.4

A diagram showing the distribution of protein CDSs inferred from the genomes of *A. hydrophila Ae34* in relation to *A.hydrophila* strains ML09-119 *and* ATCC 7966 reference genome is indicated as predicted by the RAST annotation pipeline.

A. Numbers of genes unique to and common to *A. hydrophila* Ae34 in relation to *A.hydrophila* strain ML09-119.

B. Numbers of genes unique to and common to *A. hydrophila* Ae34 *in relation to A.hydrophila* strain ATCC 7966.

Table 3.8 Comparative analysis of RAST annotated CDS data to show the unique regions in

A.hydrophila Ae34 and two reference genomes

Unique regions/features present in *A.hydrophila* Ae34 compared to two reference genomes	Unique regions/features present in epidemic outbreak strain ML09-119 compared to *A.hydrophila* Ae34	Unique regions/features present in *A.hydrophila* Ae34 compared to epidemic outbreak strain ML09-119
Alanine biosyntheis	Protein secretion system, Type II- Widespread colonization island	D-gluconate and ketogluconates metabolism
Capsular and extracellular polysaccharides- Rhamnose containing glycans	Type IV pilus	DNA repair- DNA-cytosine methyltransferase
LOS core oligosaccharide biosynthesis -Beta-1,3-glucosyltransferase	Outer membrane receptor proteins, mostly Fe transport	DNA repair -Error-prone, lesion bypass DNA polymerase V (UmuC)
DNA repair- Very-short-patch mismatch repair endonuclease (G-T specific)	Persister Cells	Type I secretion system for aggregation
External and Outer Membrane Nucleases- Extracellular deoxyribonuclease Xds	RTX toxin cluster	Flagellum- Flagellar biosynthesis protein FliS, Flagellin protein FlaG
Phage packaging machinery- Phage terminase, large subunit	KDO2-Lipid A biosynthesis	N-linked Glycosylation in Bacteria -Glycosyltransferase PglI
Invasion and intracellular resistance- Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	Sialic Acid Metabolism	tRNA modification Bacteria-tRNA dimethylallyltransferase
Arsenic resistance- Arsenical pump-driving ATPase	Inositol catabolism	Aresenic resistance- Arsenical resistance operon trans-acting repressor ArsD
	Lactate utilization	Multidrug Resistance Efflux Pumps- Multidrug and toxin extrusion (MATE) family efflux pump YdhE/NorM
	Mannose Metabolism	
	Sucrose, Fructose utilization	
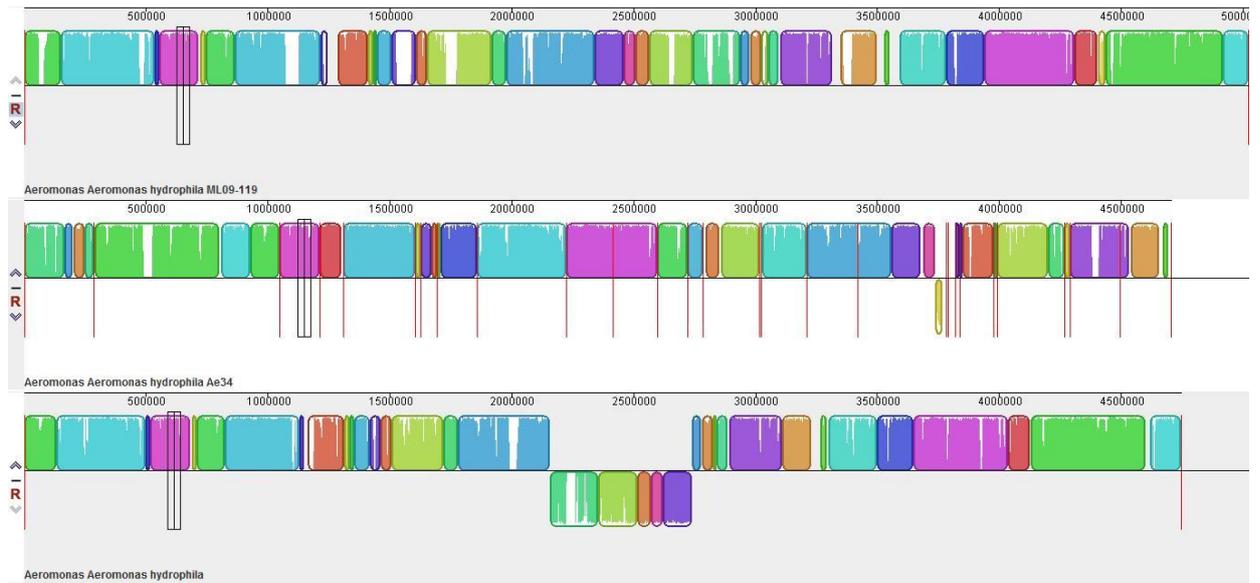
Figure 3.5

Ordeing contigs of *A.hydrophila* Ae34 using Mauve multiple genome alignment tool against the two reference genomes
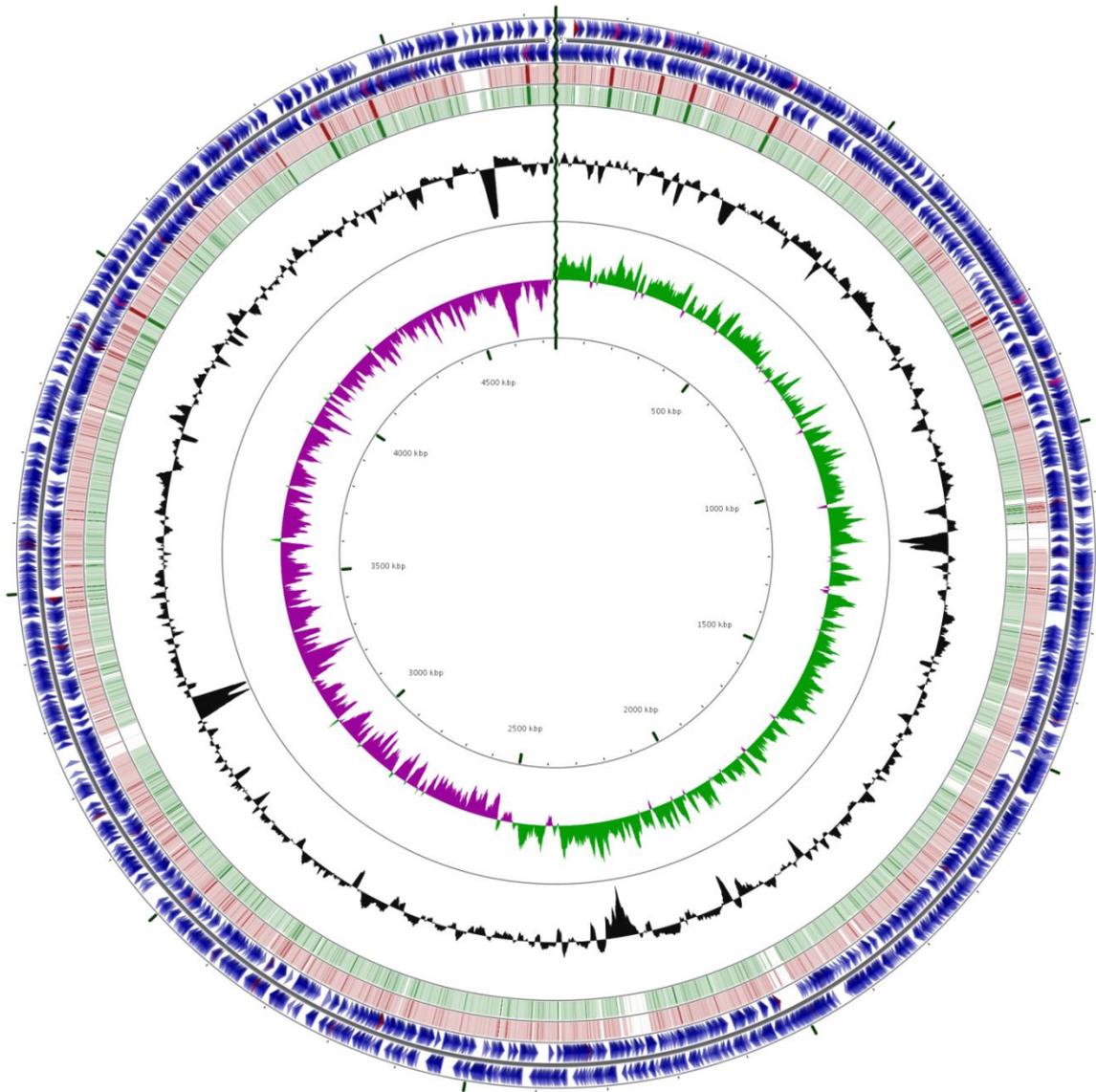
Figure 3.6 Graphical map developed by CGView, circular genome viewer visualizing the sequence feature information of Ae34 compared to the BLAST analysis results of two reference genomes; ML09-119-BLAST 1, ATCC7966-BLAST 2

3.4 DISCUSSION

The draft genome of *A.hydrophila* Ae34 isolated from a septicaemic and moribund Koi carp determined in the present study serves as the first genomic information of an ornamental fish borne clinical *Aeromonas* strain. *A.hydrophila* Ae34 draft genome contains 10 rRNA operons (≈5000 bp each), equal to the genome of *A. hydrophila* type strain ATCC 7966. These repetitive regions together with the regions with phage DNA resulted in difficulties in the process of genome finishing using short reads of Ion Torrent PGM. The relatively high numbers of tRNAs and rRNAs present in this genome may be correlated with its ability to rapidly adapt to changing environmental conditions (Jumas-Bilak *et al*, 1998; Seshadri *et al*, 2006). Through annotation of this draft genome using several open source annotation tools, we identified the presence of a number of virulence determinants to which *A.hydrophila* pathogenic potential has classically been linked to. These include, elastase, putative hemolysins, serine proteases, enterotoxin, proteases, flagellin, metalloprotease, hyaluronidase, fimbrillin, Type-IV pilus, ferric uptake regulators, enolases, collagenase, lipases, Exotoxin A and motility factors such as flagellum.

In the present study, the genome sequences of *A.hydrophila* ATCC7966 and ML09-119 provided an excellent basis for comparative genomic analysis. By analysing the draft genome of *A. hydrophila* strain Ae34 alongside those of type strain ATCC7966 and a highly virulent fish clinical isolate ML09-119, we identified some genomic features that are unique to strain Ae34 that could probably play a role in opportunistic fish pathogenicity of this strain. These include Mycobacterium virulence operon, Phage terminase, Arsenic resistance, Phage packaging machinery, Capsular and extracellular polysaccharides, LOS core oligosaccharide biosynthesis and Alanine biosynthesis. On the other hand, strain Ae34 genome lacks many of the epidemic associated unique genetic regions (Hossain *et al*, 2013) present in highly

virulent fish pathogenic strain ML09-119 such as a complete inositol utilization pathway and pathogenicity islands unique to outbreak strains. This explains the fact that the field strains such as Ae34 are opportunistic pathogens that are responsible for non epidemic, isolated disease incidences while clonal, highly virulent strains result in massive outbreaks spreading over geographical areas. Interestingly, strain Ae34 genome contains more motility and chemotaxis genes compared to both reference genomes (Table 3.3). Enhanced motility using lateral and polar flagella is often an indication of virulence in *A.hydrophila*. However, the absence of type III secretion system in both fish pathogenic strains (Ae34 and ML09-119) was a striking feature since type III secretion system has been shown to play a crucial role in *Aeromonas hydrophila* 's interactions with its host (Vilches *et al,* 2009). It is reported that many Gram-negative pathogens utilize the type III secretion system (T3SS) to inject virulence determinants into the cytosol of host cells (Yu *et al*, 2004; Hueck, 1998). As pointed out by Sheshadri *et al* in 2006, T3SS may be complemented by the presence of the other secretion systems. While all three genomes lack Type III and type V protein secretion systems, all of them bear Type I , Type IV and Type VII (Chaperone/Usher pathway, CU) secretion systems. Interestingly, Type VI was only present in ATCC 7966 but not in two fish pathogenic strains Ae34 and ML. Understanding of the complete array of virulence genes required for the pathogenicity in fish could only be accomplished by virulence genes comparisons among the genomes of many fish pathogenic strains. This would eventually help to estimate the virulence potential of a given isolate by screening the virulence genes and to detect novel virulence factors through sequence analysis alone (Grim *et al*, 2013).

Prophages contribute significantly to the evolution of their bacterial hosts (Casjens, 2003). The two intact phages present in the Ae34 draft genome encodes 48 and 41 coding sequences respectively among which 17 and 16 are hypothetical proteins that should probably have

important implications for virulence in this strain. In a recent analysis by Hossain *et al*, 2013, it was reported that epidemic clonal isolates of *A.hydrophila* from the cat fish epidemic outbreak in USA bear unique phage DNA sequences that are absent in other fish pathogenic genomes from non outbreak incidences. This makes a good example of the way of emergence of highly virulent strains through acquisition of foreign genetic elements by means of prophages. By analyzing the prophage sequences in all *A.hydrophila* complete genomes available to date we realized that strains that have been pathogenic for either in fish, mouse or humans have more likeliness to bear phages than their environmental counterparts (Table 3.7). It is noteworthy to mention that all fish pathogenic strains carry two or more phages while *A.hydrophila* strains isolated from soil does not bear phage sequences. The only exception was *A.hydrophila* ATCC7966 which is reported as one of the most pathogenic species to mice. However, PHAST phage search tool did not detect any intact or incomplete phages within this genome. However, according to Sheshadri *et al*, 2006, this genome contains a large phage region marked by atypical trinucleotide composition and a cluster of pilus accessory genes. Given these observations it is evident that the prophages add plasticity to the *A.hydrophila* genomes making them highly diverse in terms of their virulence and adaptability to hosts.

The presence of an array of antimicrobial resistance genes and multidrug resistance efflux pumps well explain the multi resistance nature phenotypically expressed by this organism. Tetracycline is an antimicrobial drug that is widely used in aquaculture. Tet35 (tetracycline efflux pump) and TetR (tetracycline transcriptional regulator) could be identified as the genetic mechanism underlying the tetracycline resistance of this isolate. TetR family regulators are reported to be involved in the transcriptional control of multidrug efflux pumps, pathways for the biosynthesis of antibiotics, response to osmotic stress and toxic

chemicals, control of catabolic pathways, differentiation processes, and pathogenicity (Ramos *et al*, 2005).

Draft genome sequence of this strain together with other sequenced *A.hydrophila* genomes provide a better understanding of intraspecies diversity and could be considered as valuable sources to identify molecular markers that are specific to this particular species. These molecular markers could be helpful in the development of rational diagnostic tools and more efficient control strategies in aquaculture. Furthermore, these markers are equally useful in population structure analysis of this important bacterial species and in epidemiological surveys to monitor disease occurrence. The available sequences also provide valuable baseline data for the strategies such as multilocus sequence typing (MLST).

The analysis of common and unique genes based on RAST analysis showed that the number of genes that are unique to strain Ae34 (18 genes compared to ML09-119 and 16 genes compared to ATCC7966) were comparatively low compared to the number of unique genes in two reference genomes used (85 and 70 respectively; Figure 3.4). The reason behind this could be the missing genes due to the gaps present in Ae34 draft genome and the broken genes during assembly process. On the other hand, one can argue that the gene loss is an adaptive mechanism of virulence in this particular strain as observed among other pathogenic bacteria. However, in order to ascertain the frequency of core and accessory genes in environmental and clinical *Aeromonas* isolates it is necessary to carry out more extensive genomic investigations using complete genomes (Grim *et al*, 2013).

Next generation sequencing is considered as a cost effective method of generating bacterial whole genome sequences. However, short read output of second generation sequencing

results in certain limitations that impede the assembly of genomes like *A.hydrophila* with high plascticity; with multiple, heterogenous rRNA operons, prophages and genomic islands into a single contig. In order to achieve the complete genome sequence of this strain, we sequenced it using single molecule real time (SMRT) sequencing of Pacific BioSciences. The error correction and assembly is underway. In detail analysis of this genome would follow the assembly of SMRT data.

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CHAPTER 4

Identification of target signature regions to develop a loop mediated isothermal amplification (LAMP) assay for the rapid and sensitive detection of *Aeromonas* spp. causing septicaemia in diseased ornamental fish

4.1 INTRODUCTION

Making a prompt and accurate diagnosis is vital for successful treatment and long term prevention of fish diseases. As the clinical signs of motile *Aeromonas* septicaemia are rarely pathognomonic, the diagnosis is accomplished by the isolation of the pathogen from the internal organs and identification using biochemical characterization (Plumb, 1999; Noga, 1996). This process is laborious, time consuming and needs microbiology expertise. In a typical outbreak of septicaemia in fish, especially in motile *Aeromonas* septicaemia, symptoms occur gradually with an increasing number of fish becoming affected (Andrews *et al*, 1988). To reduce the mortalities and to prevent the spread, it is important to identify the problem as early as possible which will allow the farmers, aquaculturists and fish health professionals to adopt corrective measures (both therapeutic interventions and environmental manipulations) within the shortest possible time.

In most bacterial septicaemia cases encountered in the intensive fish farming, application of antimicrobial compounds is the most effective way to control the disease before spreading in to an outbreak. Fish in the early stages of infection may respond favorably to antibiotic treatment and environmental modification (Lewbart, 2001). However, the antimicrobial treatment should be started well before the fish stop feeding due to septicaemia since the large scale antimicrobial application to fish is often done through in feed medication. Considering all these facts, it is clear that the early diagnosis is the primary step in treatment and prevention of fish bacterial septicaemia. During the early stages of infection, there might not be any external signs associated with disease, and the responsible bacterial pathogen may be present in low numbers in internal organs. Therefore, culture based identification is of little practical value for early diagnosis of motile *Aeromonas* septicaemia.

Development of immunological test systems using polyclonal and monoclonal antibodies to detect/diagnose motile *Aeromonas* species has been hampered by the presence of large number of serotypes and the antigenic sharing among different species. As a result, immunological tests developed for aeromonads are often reported to be non specific due to high antigenic cross reactivity (Leblanc *et al*, 1981; Noga, 1996; Toranzo *et al*, 1986; Faude and Hofle, 1997; Chang and Nandapalan, 1989).

Different molecular diagnostic methods to identify *Aeromonas* spp. (both clinical and environmental) at the genus level and species level have been reported. These include PCR probes, 16S rDNA-RFLP, real time-PCR, housekeeping gene sequencing and Multilocus Sequence Typing (MLST) (Beaz-Hidalgo and Figueras, 2012). Molecular methods are rewarding for *Aeromonas* detection in aquaculture, but the high cost limits their use in routine diagnosis in fish pathology laboratories with minimum facilities. Many on-farm laboratories are not usually equipped with sophisticated molecular equipments (Savan *et al*, 2005). Therefore, rapid, simple and accurate diagnostic methods that could be performed at the field are more appropriate to detect *Aeromonas* infections in ornamental fish aquaculture.

LAMP is an auto-cycling strand displacement DNA synthesis performed under isothermal conditions (Notomi *et al*, 2000). A LAMP reaction requires a minimum of four specially designed primers that recognize a total of six distinct sequences on the target DNA (Notomi *et al*, 2000). The set of four specific primers: a forward inner primer (FIP), a backward inner primer (BIP), and two outer primers (F3 and B3) recognize a total of six distinct nucleotide sequences on the target gene (Notomi *et al*, 2000). By adding an additional set of two primers, LAMP reaction time can be reduced by half (Nagamine *et al*, 2002; Biswas and Sakai, 2014). This methodology is widely accepted as a rapid, economical, easy to perform

and specific method of identifying pathogens and has been used successfully for the fish and shellfish disease diagnosis at the field level. The advantages and the potential application of this technique in detection of pathogens associated with aquaculture are discussed in extensive reviews done by Savan *et al* in 2005 and Biswas and Sakai in 2014, giving examples of such pathogens detected by LAMP assays. The rapidity, accuracy, and cost-effectiveness of LAMP technique make it a good candidate method for the detection of motile aeromonads that cause MAS in ornamental fish. However, the use of the LAMP technique has rarely been reported for aeromonads, which could partially be due to their ever-changing taxonomy and the genetic heterogeneity.

It is understood by the present study (Chapter 2) and the studies by others (Sreedharan *et al*, 2013) that the opportunistic motile aeromonads causing septicaemia in ornamental fish are genetically distinct and belong to many different species. In the routine ornamental fish disease diagnosis process, fish pathologists or veterinarians may not need to identify the causative motile aeromonad to the species level if the purpose of diagnosis is to initiate treatment/control measures. Therefore, in a therapeutic and control point of view, rapid detection of *Aeromonas* species in the kidney tissues of affected fish would be adequate enough to initiate treatment/control options. However, identification of pathogenic *Aeromonas* at the species level, at least the clinically significant species, is invaluable to understand the true etiological involvement of different *Aeromonas* species in MAS.

Two *Aeromonas* genus specific probes have been developed to detect the members of *Aeromonas*, one using glycerophospholipid-cholesterol acyltransferase (*gcat*) gene (Chacon *et al*, 2002) and the other using an outer membrane protein (ompA) gene (Khushiramani *et al*, 2008). These probes have reportedly been able to detect most of the members of the genus

Aeromonas. Neither of these probes is commercially available (Janda and Abott, 2010). However, the universal presence of these target genes in all members of *Aeromonas* spp. should be evaluated using multiple strains. The presence of *gcat* gene has been verified in many strains by multiple researchers and is reported to be present in >98% of aeromonads, which was also proved using the present study strains (Chapter 2). However, there are conflicting reports on its absence in few strains (Guerra *et al*, 2007; Nawaz *et al*, 2010). Unlike in many other bacteria, unique virulence genes conserved among all strains in a given species of motile aeromonads have not yet been reported. Thus, many probes developed for *Aeromonas* have a very narrow spectrum. Therefore, housekeeping genes are increasingly been used for species discrimination of *Aeromonas*. These geneus specific probes/assays are very important to prevent the misidentification at the genus level. Strains identified correctly to the genus level should then be screened through suitable species specific markers for correct species identification.

Utilizing bacterial genomes to develop diagnostic markers for the identification and discrimination of pathogens is becoming an emerging and promising approach in diagnostic microbiology. Ever increasing numbers of bacterial whole genomes and draft genomes serve as powerful resources to determine conserved genes and proteins across genera and species through genome comparisons, which could be used as targets for bacterial identification. While complete genomes provide superior quality of comparison over the other genomes, the draft genomes are also worth using since they contain most of the genomic information. Hence, the publicly available genomes of *Aeromonas*, either complete or draft, would be a valuable source in finding suitable diagnostic markers for *Aeromonas* identification at the genus level or even at the species level given that genomes of different strains of a species are available.

The objective of this experiment is to identify suitable genus specific markers for *Aeromonas,* and species specific markers for *A. hydrophila* and *A. veronii*; priority pathogens among motile aeromonads in fresh water ornamental fish, with a view of using those markers in a LAMP assay to detect the presence of *Aeromonas* in the kidney tissues of infected ornamental fish. This chapter describes the preliminary results of the analysis and specificity evaluation of *gcat* gene for the identification of *Aeromonas* spp. by evaluating the ability of different sets of LAMP primers designed against this gene to detect field isolates.

4.2 MATERIALS AND METHODS

4.2.1 Creating an *Aeromonas* genome sequence collection through available data

The genome information of all publicly available complete genomes of *Aeromonas* was retrieved from public databases and a comprehensive genome library was made in CLC genomics work bench.

4.2.2 Comparative sequence analysis of glycerophospholipid cholesterol acyltransferase (*gcat*) gene

We amplified a 232 bp fragment of the glycerophospholipid cholesterol acyltransferase (*gcat*) gene in 15 isolates of *Aeromonas* (from our collection representing all *Aeromonas* species associated with septicaemia in ornamental fish) according to the published protocols (Chacon *et al*, 2002; Soler *et al*, 2002) (described in Chapter 2) and sequenced using the same primers to observe conservation level of this gene across the genus. These partial *gcat* sequences were aligned with CLUSTALW. We mapped these partial *gcat* sequences to the *Aeromonas* genomes in the in house database to locate the respective *gcat* sequence in each genome, and included those sequences in the alignment. *A.hydrophila* showed the highest number of strain

representation and therefore *gcat* sequences of *A.hydrophila* were aligned separately to observe the conservation level within the species. *A.hydrophila* Ae34 *gcat* fullength sequence (Figure 4.3) was used to design LAMP primers.

4.2.3 Designing of LAMP primers

LAMP primers targeting the above *gcat* consensus sequence were designed using Primer Explorer v. 4 software (http://primerexplorer.jp/lamp) according to the manual instructions. All primers were synthesized by Eiken Genomics Ltd, Japan.

4.2.3.1 LAMP reaction conditions

LAMP assay was carried out in a total of 25 µl volume containing 40 pmol each of the inner primers (FIP and BIP), 5 pmol each of the outer primers (F3 and B3), 12.5 µl of 2x reaction mix (Loopamp DNA amplification kit, Eiken Chemical Co. Ltd), 1 µl of Bst DNA polymerase (Loopamp DNA amplification kit, Eiken Chemical Co. Ltd), 4.5 µl of distilled water, 1 µl of fluorescent detection reagent (Eiken Chemical Co. Ltd) and DNA template. Incubation was carried out at 65°C for 60 min, and the reaction was terminated at 95°C for 2 min. Colour change from yellow to green was observed visually. For comparison, the LAMP-amplified products were also analysed by 1.5% agarose gel electrophoresis.

4.2.4 Specificity of the LAMP assay

The specificity of different LAMP primer sets designed against *gcat* gene was evaluated (on a preliminary basis) on 9 *Aeromonas* strains and 4 non target pathogen strains (other genera) isolated from the septicaemic ornamental fish in the present study (Table 4.1). DNA templates isolated from the above bacterial cultures were subjected to LAMP amplification.

4.3 RESULTS

4.3.1 Creating a comprehensive *Aeromonas* genome library

A total of eleven *Aeromonas* complete genomes were downloaded and stored in CLC genomics workbench bench (06 *A.hydrophila*, 1 *A.media*, 1 *A.veronii*, 1 *A.salmonicida*, 1 *A.aquariorum*, 1 *A.caviae*). We also included the draft genomes of *A.hydrophila* strain Ae34, *A.hydrophila* strain SNUFPC-A8, *Aeromonas diversa* type strain CECT 4254, *Aeromonas molluscorum* 848, *A. salmonicida* 01-B526.

4.3.2 Comparative sequence analysis of glycerophospholipid cholesterol acyltransferase (*gcat*)

Full length (1166 bp) and partial (235 bp) *gcat* sequences were aligned using CLUSTAL Omega. We observed a higher degree of conservation among *gcat* partial/fulllength sequences of *A.hydrophila* strains (Figure 4.1) as opposed to the partial/fullength alignment of the *gcat* gene from multiple *Aeromonas* species (Figure 4.2). This suggests that the 237 bp partial *gcat* sequence is a good candidate for the identification of *A.hydrophila* in a LAMP assay.

```
A.h-X07279      CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGACTACGAGGTCACCCAGTTCCT      60
A.h-A25         CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
A.aquar_Ae43    CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
A.h-ATCC        CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
A.h-Ae34        CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
A.h-ML          CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
A.h-AL09-71     CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
A.h-pc104A      CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
A.h-BSK-10      CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTCCT      60
                *********************************** ******* **********

A.h-X07279      GCAAAAAGACAGCTTCAAGCCGGACGATCTGGTGATCCTCTGGGTCGGCGCCAACGACTA     120
A.h-A25         GCAAAAGGACAGCTTCAAACCCGATGATCTGGTGATCCTCTGGGTCGGTGCCAACGATTA     120
A.aquar_Ae43    GCAAAAGGACAGCTTCAAACCCGATGATCTGGTGATCCTCTGGGTCGGTGCCAACGATTA     120
A.h-ATCC        GCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGATTA     120
A.h-Ae34        GCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGATTA     120
A.h-ML          GCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGATTA     120
A.h-AL09-71     GCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGATTA     120
```

```
A.h-pc104A       GCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGATTA    120
A.h-BSK-10       GCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGATTA    120
                 ****** *********** ** ** ********************** ******* **


A.h-X07279       TCTGGCCTATGGCTGGAACACAGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.h-A25          CCTGGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.aquar_Ae43     CCTGGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.h-ATCC         CCTGGCCTATGGCTGGAACACTGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.h-Ae34         CCTGGCCTATGGCTGGAACACGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.h-ML           CCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.h-AL09-71      CCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.h-pc104A       CCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
A.h-BSK-10       CCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGCGA    180
                 ****************** ** ********************************


A.h-X07279       TGCGGCCAACCGCATGGTGCTGAACGGCGCCAAGGAGATACTGCTGTTCAACCT-    234
A.h-A25          TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATACTGCTGNTCAACCT-    234
A.aquar_Ae43     TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATACTGCTGNTCAACCT-    234
A.h-ATCC         TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG    235
A.h-Ae34         TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG    235
A.h-ML           TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG    235
A.h-AL09-71      TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG    235
A.h-pc104A       TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG    235
A.h-BSK-10       TGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCT-    234
                 ****************** ************* **** *** ** *******
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Figure 4.1 multiple sequence alignment of partial *gcat* sequences of 8 strains of *A.hydrophila* and one strain of *A.hydrophila* subsp. *dhakensis* (*A.aquariourum*) using CLUSTAL. Sequences in which the names are bolded are from study isolates while the rest is from fully sequenced genomes from GenBank.

```
A.h-X07279       --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGACTACGAGGTCACCCAGTTC    58
A.h-ATCC         --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    58
A.h-Ae34         --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    58
A.h-ML09-119     --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    58
A.h-AL09-71      --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    58
A.h-pc104A       --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    58
A.h-BSK-10       --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    58
A.cav-Ae6        TCTCCTGGAATTCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    60
A.cav-Ae9        -TCTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    59
A.cav-Ae42       -TCTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    59
A.h-Ae11         --CTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    58
A.aqua-Ae24      -TCTCCTGGAATCCCAAGTATCAGGTCATCAACAACCTGGATTACGAGGTGACCCAGTTC    59
A.jand-Ae32      -TCTCCTGGAATCCCAAGTATCAGGTGATCAATAACCTGGAGTACGAGATCAAACAGTTC    59
A.entero-Ae41    -TCTCCTGGAANCCCAAGTATCAGGTGATCAACAACCTCGACTACGAGATCGATCAGTTC    59
A.vero-Ae26      -TCTCCTGGAATCCCAAGTATCAGGTGATCAACAACCTCGATTACGAGATCGGTCAGTTC    59
A.vero-Ae31      --CTCCTGGAATCCCAAGTATCAGGTGATCAATAATCTCGATTACGAGATCGATCAGTTC    58
A.vero-Ae3       -TCTCCTGGAATCCCAAGTATCAGGTGATCAACAACCTCGATTACGAGATCGATCAGTTC    59
A.vero_Ae37      --CTCCTGGAATCCCAAGTATCAGGTGATCAACAACCTCGATTACGAGATCGATCAGTTC    58
A.vero-Ae35      -TCTCCTGGAATCCCAAGTATCAGGTGATCAACAACCTCGATTACGAGATTGATCAGTTC    59
A.vero-Ae36      --CTCCTGGAATCCCAAGTATCAGGTGATCAACAACCTCGATTACGAGATTGATCAGTTC    58
                 *  * *  ************* ***** ** ** ** ****** *    ******


A.h-X07279       CTGCAAAAAGACAGCTTCAAGCCGGACGATCTGGTGATCCTCTGGGTCGGCGCCAACGAC    118
A.h-ATCC         CTGCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGAT    118
A.h-Ae34         CTGCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGAT    118
A.h-ML09-119     CTGCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGAT    118
A.h-AL09-71      CTGCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGAT    118
A.h-pc104A       CTGCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGAT    118
A.h-BSK-10       CTGCAAAAGGACAGCTTCAAGCCCGATGATCTGGTGATCCTCTGGGTCGGCGCCAACGAT    118
A.cav-Ae6        CTGCAAAAGGACAGCTTCAAGCCCGACGATCTGGTGATCCTCTGGGTCGGTGCCAACGAC    120
A.cav-Ae9        CTGCAAAAGGACAGCTTCAAGCCCGACGATCTGGTGATCCTCTGGGTCGGTGCCAACGAC    119
A.cav-Ae42       CTGCAAAAGGACAGCTTCAAGCCCGACGATCTGGTGATCCTCTGGGTCGGTGCCAACGAC    119
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A.h-Ae11          CTGCAAAAGGACAGCTTCAAACCCGATGATCTGGTGATCCTCTGGGTCGGTGCCAACGAT       118
A.aqua-Ae24       CTGCAAAAGGACAGCTTCAAACCCGATGATCTGGTGATCCTCTGGGTCGGTGCCAACGAT       119
A.jand-Ae32       CTGAAAAAGACAAGTTCCGCCCCGATGATCTGGTGGTGATTTGGGTGGGAGCCAACGAT       119
A.entero-Ae41     CTGGCGAAGGACAGCTTGCGTCCCGACGATCTGGTGGTGATCTGGGTGGGGGCCAACGAC       119
A.vero-Ae26       CTGAAAAGGACAAGTTCCGCCCCGATGATCTGGTGGTGATCTGGGTCGGAGCCAACGAC       119
A.vero-Ae31       CTGAAAAAGGACAAGTTCCGCCCCGATGATCTGGTGGTGATCTGGGTCGGTGCCAACGAC       118
A.vero-Ae3        CTGAAAAAGGACAAGTTCCGTCCCGATGATCTGGTGGTGATCTGGGTCGGTGCCAACGAC       119
A.vero_Ae37       CTGAAAAGGACAAGTTCCATCCCGATGATCTGGTGGTGATCTGGGTCGGTGCCAACGAC       118
A.vero-Ae35       CTGAAAAAGGACAAGTTCCGTCCCGATGATCTGGTGGTGATCTGGGTCGGTGCCAACGAC       119
A.vero-Ae36       CTGAAAAAGGACAAGTTCCGCCCCGATGATCTGGTGGTGATCTGGGTCGGTGCCAATGAC       118
                  ***     ** ****  **      ** ** ********* *  * ***** ** ***** **


A.h-X07279        TATCTGGCCTATGGCTGGAACACAGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.h-ATCC          TACCTGGCCTATGGCTGGAACACTGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.h-Ae34          TACCTGGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.h-ML09-119      TACCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.h-AL09-71       TACCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.h-pc104A        TACCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.h-BSK-10        TACCTGGCCTATGGCTGGAACACCGAACAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.cav-Ae6         TACCTTGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGATGCCATCAGC       180
A.cav-Ae9         TACCTTGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGATGCCATCAGC       179
A.cav-Ae42        TACCTGGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       179
A.h-Ae11          TACCTGGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       178
A.aqua-Ae24       TACCTGGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTGCGCGACGCCATCAGC       179
A.jand-Ae32       TATCTGGCCTATGGCTGGAACAAGGAGAAGGATGCAGATCGGGTGGTAGCCACCATTCAG       179
A.entero-Ae41     TATCTGGCTTATGGCTGGAATCAGGAGAAGGATGCCGATCGGGTGATCGAGACCATCCGC       179
A.vero-Ae26       TATCTGGCCTATGGCTGGAACACCGAGCGGGATGCGGATCGGGTCATCGATACCATCCGG       179
A.vero-Ae31       TATCTGGCCTATGGCTGGAACACCGAGCGGGATGCGGATCGGGTGATCGATACCATCCGG       178
A.vero-Ae3        TATCTGGCCTATGGCTGGAACACCGAGCGGGATGCGGATAGGGTGATCGATACCATCCGG       179
A.vero_Ae37       TATCTGGCCTATGGCTGGAACACCGAGCGGGATGCGGATCGGGTGATCGATACCATCCGG       178
A.vero-Ae35       TATCTGGCTTATGGCTGGAACACCGAGCGGGATGCGGATCGGGTGATCGATACCATCCGG       179
A.vero-Ae36       TATCTGGCTTATGGCTGGAACACCGAGCGGGATGCGGATCGGGTGATCGATACCATCCGG       178
                  ** ** ** **********        **    ****** * ****    *    ****


A.h-X07279        GATGCGGCCAACCGCATGGTGCTGAACGGCGCCAAGGAGATACTGCTGTTCAACCT-       234
A.h-ATCC          GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG      235
A.h-Ae34          GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG      235
A.h-ML09-119      GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG      235
A.h-AL09-71       GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG      235
A.h-pc104A        GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCTG      235
A.h-BSK-10        GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATTCTGTTGTTCAACCT-      234
A.cav-Ae6         GATGCGGCCAACCGCATGGTATTGAACGGTGCCAGGCAGATACTGCTGNTCAACCT-      236
A.cav-Ae9         GATGCGGCCAACCGCATGGTATTGAACGGTGCCAGGCAGATACTGCTGNTCAACCT-      235
A.cav-Ae42        GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATACTGCTGTTCAACCT-      235
A.h-Ae11          GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATACTGCTGNTCAACCT-      234
A.aqua-Ae24       GATGCGGCCAACCGCATGGTATTGAACGGCGCCAAGCAGATACTGCTGNTCAACCT-      235
A.jand-Ae32       GCGGCAGCGAATAATCTGGTTTTTAAATGGCGCCAAACAGATACTGCTGTTCAACCTG      236
A.entero-Ae41     CTGGCATCCAACCGACTGGTGCTCAACGGGGCGCAGCAGATACTGCTGNTCAACCT-      235
A.vero-Ae26       CTGGCATCCAACCGGTTGGTACTCAACGGTGTGCAGCAGATACTGCTGNTCAACCT-      235
A.vero-Ae31       CTGGCATCCAACCGGTTGGTGCTCAACGGCGCGCAGCAGATACTGCTGNTCAACCT-      234
A.vero-Ae3        CTGGCATCCAACCGGTTGGTGCTCAACGGCGCGCAGCAGATACTGCTGNTCAACCT-      235
A.vero_Ae37       CTGGCATCCAACCGGTTGGTGCTCAACGGCGCGCAGCAGATACTGCTGTTCAACCT-      234
A.vero-Ae35       CTGGCATCCAACCGGTTGGTGCTCAACGGCGCGCAGCAGATACTGCTGTTCAACCT-      235
A.vero-Ae36       CTGGCATCCAACCGGTTGGTGCTCAACGGCGCGCAGCAGATACTGCTGTTCAACCT-      234
                  **  * **      ****  * ** ** *       **** *** ** *******
```

Figure 4.2 Multiple sequence alignment of partial *gcat* sequences of different species of *Aeromonas* (*A.hydrophila*, *A.caviae*, *A.aquariorum*, *A.jandaei*, *A.enteropelogenes*, *A.veronii*) Sequences in which the names are bolded are from study isolates while the rest is from fully sequenced genomes from GenBank.

4.3.3 Designing of LAMP primers

Using the 1166 bp full length *gcat* sequence of *A.hydrophila* Ae34, we designed 5 sets of LAMP primers using LAMP primer designing software Primer Explorer V4.

Figure 4.3 Nucleotide sequence of *A.hydrophila* Ae34 *gcat* gene used to design primers for LAMP. The colour code indicates the locations of inner and outer primers (F3 and B3) of each primer set labelled from 1-5. The yellow highlighted area represents the *gcat* sequence for which a DNA probe was designed by Chacon *et al* in 2002.

AATCCATACCGCATACTGCGAAATTAAATTCGGCTAGATACCCTGCAAGATGGCAAAACAGCTTCCCAGCACC

3-F3

TTT**GCCTATCGAGGAACAGTCTG**CAGGCACCACCTGCTTCCAACCAATGAGAACAACAAGATGAAAAAATGG

1-F3

TTTGTGTGTTTATTGGGACTCTTCGCGCTGACAGTTC**AGGCAGCCGACAGTCG**CCCCGCCTTCTCCCGGATCGT

2-F3　　　**3-B3**

GATGTTTGGCGATAGCCTCTCCGACACCGGCAAGATGTACAGCAAGATGC**GCGGTTATCTCC***CCTCCAGCCCT*

1-B3

*CCCTACT*ATCAGGGCCGCTTCTCCAACGGGCCGGTCTGGCTGGAGCAGCT*GACCAAGCAGTTCCCGG*GGCTG

ACCATCGCCAACGAGGCGGAAGGGGGCGCCACCGCAGTGGCCTACAACAAGAT<mark>CTCCTGGAATCCCAAGTAT</mark>

4-F3　　　　　　　　　**2-B3**

<mark>CAGGTCATCAACAACCT**GGATTACGAGGTGACCCA***GTTCCTGCAAAAGGACAGC*TTCAAGCCCGATGATCTG</mark>

5-F3

<mark>GTGATCCTCTGGGT**CGGCGCCAACGATTACCT**GGCCTATGGCTGGAACACCGAGCAGGATGCCAAGCGGGTG</mark>

4-B3

<mark>CGCGACGCCATCAGCGATGCGGCCAACCGCATGGTATT*GAACGGCGCCAAGCAG*ATTCTGTTGTTCAACCTG</mark>

5-B3

<mark>CC</mark>GGATCTGGGCCAGAACCCCTCGGCCCGCAGC*CAGAAGGTGGTCGAGGCG*GCCAGCCATGTCTCCGCCTAC

CACAACCAGCTGCTGCTGAACCTGGCACGCCAGCTGGCCCCCACCGGCATCGTCAAGCTGTTCGAGATCGACA

AGCAGTTCGCCGAGATGCTGCGCGAGCCGCAGAACTTCGGCCTGAGCGACACCGAGAACGCCTGCTACGGCG

GCGGCTATGTGTGGAAGCCGTTTGCCTCCCGCAGTGCAGCCAGCGTCGCCAGCGACAGCAGCCTCTCCGCCTT

CAACCCGCAGGAGCGGCTCGCCATCGCTGGCAACCCGCTGCTGGCCCAGGCCGTTGCCAGCCCCATGGTGCG

GCGCAGCGCCAGCACCCTCGACTGCGAAGGCAAGATGTTCTGGGATCAGGTGCACCCCACCACCGTCGTGCA

CGCCGCCCTGAGCGAGCGGGCCGGCGCCTTCATCGAGGCTCAATACGAGTTCCTCGCCCACTGATGAAAAAG

GGCCGACG

4.3.4 Specificity of the LAMP assay

Evaluation of LAMP primer sets using the bacterial strains stated in Table 4.1 showed that the primer set 2 is highly specific and efficient in amplifying all *A.hydrophila* and all *A.aquariorum* but not the other *Aeromonas* species and related genera tested.

Table 4.1 Bacterial isolates used in this study, and their specificity results with the second set of LAMP primers

Species	Number of	Laboratory	*gcat*
A.hydrophila	3	Ae45, Ae34, Ae11	+
A.hydrophila subsp. dhakensis	1	Ae43	+
A.veronii	1	Ae2	_
A.caviae	2	Ae6, Ae 9	_
A.jandaei	1	Ae32	_
Aeromonas enteropelogenes	1	Ae41	_
Citrobacter freundii	2	Q, A41	_
Enterobacter spp.	1	A1	_
Pseudomonas spp.	1	R10	_

With this primer set, a visually detectable fluorescence (Figure 4.4) and the characteristic ladder-like pattern of LAMP amplicons in agarose gels were observed with *A.hydrophila* and *A.aquariorum* whereas no LAMP amplicons or visually detectable fluorescence were detected in other *Aeromonas* species and related genera tested (Figure 4.4). The results of the visual LAMP assay agreed with those of the gel electrophoresis. Optimization of this LAMP assay and specificity testing of other primer sets are in progress.
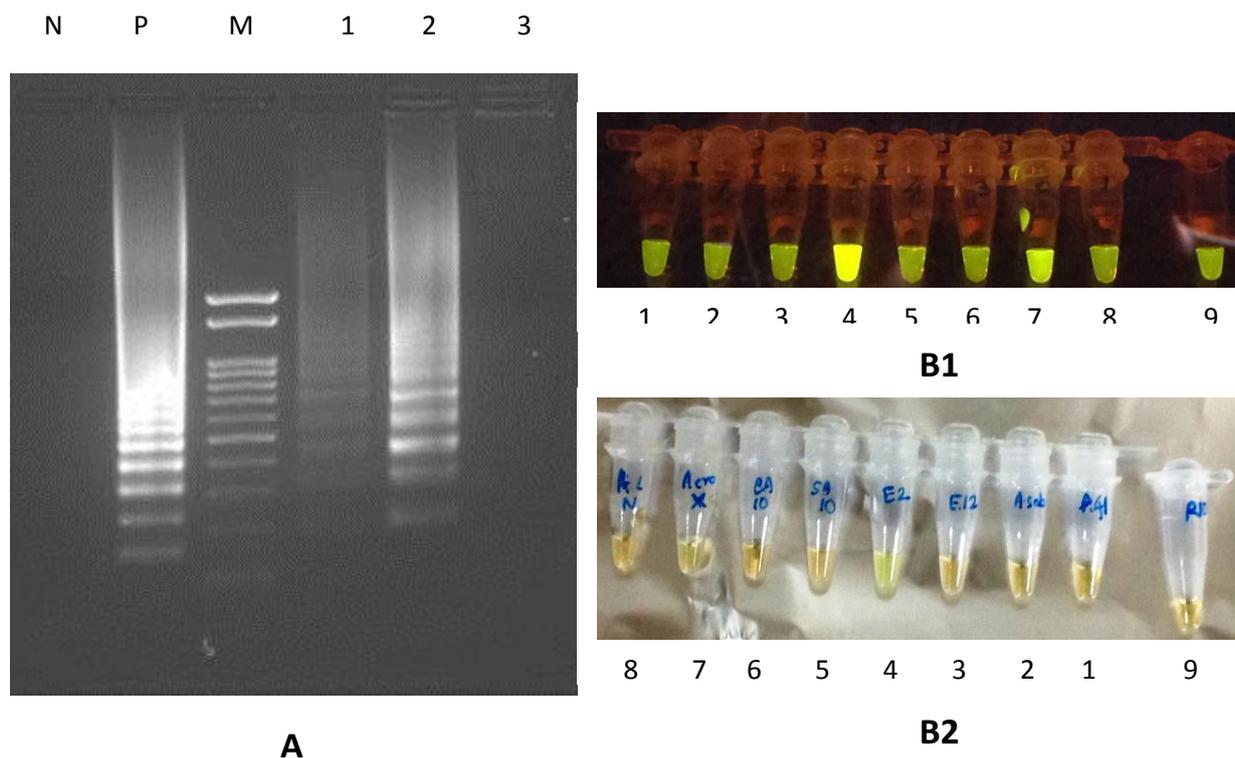
Figure 4.4 Detection of LAMP amplicons:

A. Agarose gel electrophoresis of LAMP-amplified products: N, negative (no template) control, P, Positive control, M, GL100 DNA ladder; 1 and 2, amplified *A.hydrophila* LAMP products showing a ladder-like pattern, 3, *A.caviae*

B. Visual detection of LAMP products under UV light (B1) and natural light (B2); 1, *Citrobacter fruendii*; 2, *A.enteropelogenes*; 3, *A.jandaei*; 4, *A.aquariourum*; 5 and 6, *A.caviae*; 7, *A.hydrophila*; 8, *A.veronii*; 9, *Pseudomonas* spp.

4.4 DISCUSSION

In this chapter, we discuss a part of an ongoing experiment to develop a LAMP based diagnostic assay to identify motile aeromonads causing septicaemia in fresh water ornamental fish (in particular, *A.hydrophila* and *A.veronii*). Many LAMP assays developed to date to detect bacterial pathogens have used either virulence genes or housekeeping genes that are unique or conserved in the target species. Properly optimized LAMP assay would identify

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these target DNA sequences with high accuracy through hybridization of four primers against six distinct sequences in the target DNA. To expedite the identification of suitable target sequences for the LAMP primer designing, we started with the evaluation of gene sequences that have been published previously as suitable markers to identify *Aeromonas* at the genus or species level. One such gene is *gcat* (Chacon *et al*, 2002), a lipase that is been considred as an important virulence factor.

Analysis of *gcat* sequences from our isolates as well as from published genome sequence data, supported the fact that *gcat* is highly conserved in *A.hydrophila* compared to its level of conservation within the genus *Aeromonas*. By incorporating the *gcat* sequences retrieved through genome data, we were able to increase the number of sequences used in the analysis, both the target and non target species. This way, freely available genome information could effectively be used for the diagnostic marker identification and validation. In due course, we hope to analyse whole genomes through opensource softwares such as Gegenees (Agren *et al*, 2012) to identify suitable target signature sequences through fragmented all in all comparison of all *Aeromonas* published genomes. However, non-availability of complete genome sequences of all *Aeromonas* species identified to date is a limitation in this approach. As the number of strains representing a given species increases, easier it is to predict the conserved regions among them. This was evident in *A.hydrophila* full length *gcat* alignment since the other species were represented by less number of strains.

In this preliminary experiment, we were able to develop a highly specific visual LAMP assay for detection of the bacterial fish pathogen *A.hydrophila*. This assay did not amplify any other species of motile aeromonads or other related genera. However, it was interesting to observe the amplification of LAMP products in *A.aquariorum*, a novel *Aeromonas* species published

by Martinez-Murcia *et al* in 2008, which was isolated from aquarium fish. Later, *A. aquariorum* was considered a synonym of the species *A. hydrophila* subsp. *dhakensis* (Figueras *et al*, 2011). However, in the recent past, phenotypic, DNA-DNA hybridization and phylogenetic analysis of *Aeromonas aquariorum* and *Aeromonas hydrophila* subsp. *dhakensis* suggested that they belong to a unique taxon, different from the other *A. hydrophila* subspecies (Beaz-Hidalgo *et al*, 2012). Therefore, a formal synonymization of *A. aquariorum* and *A. hydrophila* subsp. *dhakensis* and a reclassification of both as *Aeromonas dhakensis* sp. nov. comb nov. was proposed by Beaz-Hidalgo et al in 2012. However, our findings suggest that these two are closely related in their *gcat* sequence as revealed by the high similarity observed in the alignment of *gcat* sequences of *A.hydrophila* and *A.aquariorum* which could have resulted in detecting both species/organisms using a single primer set.

As the next step, we aim to evaluate the specificity of this primer set as well as the other four primer sets developed based on *gcat* for the genus/species identification of *Aeromonas*. We will continue the same procedure to evaluate *OmpA*, *gyrB* and 16S-23S rDNA intergenic spacer region that have shown promising results in *Aeromonas* identification. Sequencing of the 16S–23S rDNA intergenic spacer region (ISR) is considered as a robust and sensitive taxonomic tool and has been used for strain typing of *Aeromonas* by several authors (Martínez-Murcia *et al*, 2005; Pridgeon and Klesius, 2011). Some recent studies have suggested ISR might be a better taxonomic tool to differentiate closely related *Aeromonas* species compared to very conserved house-keeping genes such as gyrB (Pridgeon and Klesius, 2011).

Our final objective is to design an assay that will correctly identify *A.hydrophila* and

A.veronii from fresh water ornamental fish. We believe that the selection of genome informed target signature sequences would be a more meaningful way to avoid misidentifications that can result from the genetic heterogeneity within this genus. A LAMP assay that can detect motile aeromonads would allow the rapid screening for the presence of *Aeromonas* in ornamental fish not only in routine disease monitoring but also in epidemiological investigations and in fish health certificates issuing as a quarantine and certification measure to prevent the international spread of pathogens during import/exports. On the other hand, an accurate diagnosis will be helpful to avoid the unnecessary use of antimicrobials on fish diseases due to other non bacterial etiologies, and also to clarify the diversity of aeromonads involved in causing septicaemia in ornamental fish.

CHAPTER 5

CONCLUSION AND OUTLOOK

5.0 CONCLUSION AND OUTLOOK

The growing interest in keeping aquarium/ornamental fish as a hobby has resulted in a significant rise in the number of countries culturing and exporting ornamental fish. At present, over 1 billion ornamental fish worth of US$200–300 million are traded globally each year involving more than 100 countries. Today, the international trade of ornamental fish has become a vast, lucrative global business with a great potential for development in most parts of the world. As the industry expands, the disease incidences and associated costs have become hindrances to the profitable culture of ornamental fish. A thorough understanding of the pathogens associated with the commercial production of ornamental fish is the prerequisite to develop more effective diagnostic techniques targeted at the field level pathogen identification and to improve the disease control strategies.

Through this dissertation we investigated into the aspects of aetiology (causative agents) and diagnosis of motile *Aeromonas* septicaemia (MAS) in fresh water ornamental fish, a disease that received a least priority and attention despite its common occurrence and potential adverse impacts. In an attempt to identify the different *Aeromonas* species that could possibly be involved in MAS in ornamental fish, we sampled tropical fresh water ornamental fish showing signs of septicaemia from aquaria in geographically diverse locations in Sri Lanka. Aseptically isolated *Aeromonas* sp. were then characterized using a polyphasic approach that included conventional phenotypic identification, 16SrDNA-Restriction Fragment Length Polymorphism (RFLP) analysis, molecular fingerprinting and the sequence analysis of *gyrB* and *rpoD* (two housekeeping genes that have already been used successfully as accurate, unequivocal molecular chronometers for phylogenetic identification of the members of the genus *Aeromonas*). Through this approach we aimed to prevent the possible misidentification

of aeromonads at the species level, attributed to the extremely complicated taxonomy of this genus. Our results indicated the diverse range of aeromonads that could potentially be associated with motile aeromonad septicaemia in ornamental fish. The isolates characterized in this study (n=53) which were originated from 10 different species of ornamental fish were found to belong to 6 different species of motile *Aeromonas* species adding more evidences to the wide host range and geographical distribution of this bacterium. Our findings emphasize the emerging role of *Aeromonas veronii* as an ornamental fish pathogen, in contrast to the long standing view that *A.hydrophila* is the classical pathogenic aeromonad in fish. We also isolated *A. dhakensis* from a septicaemic ornamental fish, the first isolation of this recently described species in a clinical case since its original description from the same host. However, the ability of rare species to act as fish pathogens need to be explored by further studies in order to clarify their role. Isolation of diverse species of *Aeromonas* that are genetically heterogeneous in terms of their virulence profiles (genetic determinants of virulence) with no clonal relationships with each other proves the opportunistic pathogenic role of mesophilic aeromonads in ornamental fish. This highlights the importance of fish health management in first place, since the opportunistic pathogenicity could have been the end result of the loss of fine balance between the fish, pathogen and the environment, but not caused by the mere presence of virulent bacteria in the culture environment.

Motile Aeromonads, being a taxonomically and antigenically diverse group often present multiple problems in the area of identification. Standard laboratory diagnostic methods including culture isolation and, identification through phenotypic, serological or genetic methods are being used to identify motile aeromonads with different levels of success. However, all these methods have their own advantages and disadvantages and therefore are being subjected to continuous debate by many researchers over decades. Our study also

added evidences to the poor correlation between phenotypic and genotypic characterization of *Aeromonas* spp emphasizing the need for incorporating polyphasic molecular approaches in precise species identification since phenotype alone will often lead to erroneous results. While the exact taxonomic position of fish-pathogenic *Aeromonas* species might not be of interest to all fish pathologists, its use is invaluable in epidemiological studies and in recognizing new pathogenic species and subspecies. Accurate identification of aeromonads involved in causing septicaemia in ornamental fish will help to clarify the true diversity of each pathogen and variations in their seasonal/geographical prevalences.

Resistance of *Aeromonas* species to commonly used antibiotics is an emerging problem in the ornamental fish industry. Antimicrobial susceptibility testing of the *Aeromonas* spp. isolated in the present study provided interesting insights into the emerging nature of development of antibiotic resistant phenotypes in bacteria dwelling ornamental aquaculture settings. Presence of antibiotic resistant *Aeromonas* species harbouring multiple virulence genes and transferable genetic elements such as integrons, in aquarium waters and ornamental fish raises concerns about the possible treatment failures in fish disease outbreaks and the public health threats they may pose, given the importance of aeromonads as emerging human pathogens. The relatively high prevalence of antimicrobial-multi resistance (49%) observed among our isolates, could likely be a result of their use (or misuse) in the aquarium fish industry, however, resistance can also arise from gene mutations or by acquisition of transferable genetic elements such as integrons. Overall, the results obtained highlight the need to promote responsible ornamental fish ownership, good husbandry practices and prudent use of antimicrobials in ornamental fish industry.

In the recent past, high throughput whole genome sequencing has emerged as a rapid means of understanding the biology, evolution, virulence and diversity of bacterial pathogens. Availability of multiple genomes from closely related organisms serves as the basis for comparative genomics through which important inferences on genome diversity and evolution can be made. Genome sequencing could be considered as a promising avenue for solving many mysteries surrounding the genus *Aeromonas* and its genetic heterogeneity (Janda and Abott, 2010). The first complete genome of an *Aeromonas* strain, *A. hydrophila* ATCC 7966 isolated from a tin of milk with fishy odour (Seshadri *et al*, 2006) has been sequenced in 2006. Whole genome sequences of *Aeromonas* species published since then have been able to reveal important inferences regarding the evolution of aeromonads, in particular, the importance of lateral gene transfer (Hossain *et al*, 2013) as a governing mechanism that determine the emergence of virulent strains of *Aeromonas*. Genomic differences among different strains with respect to their toxin production, biofilm formation, antibiotic resistance, and other virulence properties could be a result of bacteriophage or hypothetical genes. Prophage induced incorporation of foreign genetic elements in to *Aeromonas* genomes might be a determining factor of their genetic heterogeneity that interfere with diagnostics. Despite the growing interest and economic importance of global aquarium fish trade, there is a dearth of information on the genome sequence of *A. hydrophila* isolated from tropical ornamental fish. Therefore, as a part of this dissertation, we sequenced *A. hydrophila* strain Ae34, an isolate recovered from the kidney of a septicaemic koi carp (*Cyprinus carpio koi*) with the view of identifying what specific genetic features would differentiate a 'fish pathogenic strain' from their 'environmental' counterparts (By comparing this genome to that of *A. hydrophila* ATCC 7966). Understanding of the complete array of virulence genes required for the *Aeromonas* pathogenicity in fish could only be accomplished by virulence genes comparisons among the genomes of many fish pathogenic strains. This is

not very far from now considering the numbers of fish pathogenic aeromonads sequenced each day. In time to come, the fundamental information on virulence gathered through genomic data would eventually help to formulate a mechanism to characterize the strains according to their pathogenic potential. In this way, it would be possible to estimate the virulence potential of a given isolate by screening for the presence of the required set of virulence genes and to detect novel virulence factors through sequence analysis alone (Grim *et al*, 2013).

Making a prompt and accurate diagnosis is vital for successful treatment and long term prevention of fish diseases. As the clinical signs of MAS are rarely pathognomonic, the diagnosis is accomplished by the isolation of the pathogen from the internal organs and identification using biochemical characterization which is laborious, time consuming and needs microbiology expertise. In a typical outbreak of septicaemia in fish, especially in motile *Aeromonas* septicaemia, symptoms occur gradually with an increasing number of fish becoming affected. To reduce the mortalities and to prevent the spread, it is important to identify the problem as early as possible to take corrective measures (both therapeutic interventions and environmental manipulations) within the shortest possible time. Molecular methods are rewarding in this respect, but the high cost limits their practical use in routine fish disease diagnosis. Loop mediated isothermal amplification (LAMP) is widely accepted as a rapid, easy to perform economical and specific method of identifying pathogens and have been used successfully for the fish and shellfish disease diagnosis at the field level. Using the draft genome sequence of *A.hydrophila* Ae34 and the publicly available genomes of *Aeromonas*, we attempted to evaluate the specificity of *gcat* (glycerophospholipid cholesterol acyl- transferase) gene which is reported to be present in >98% of aeromonads, as a target gene sequence specific for the genus *Aeromonas*, in identifying *Aeromonas* spp. in

our culture collection. We suceesfully identified a LAMP primer set specific for *A.hydrophila* and *A.hydrophila* subsp. *dhakensis*. In due course, we plan to evaluate several other genes for their ability to detect aeromonads, in particular, *A.hydrophila* and *A.veronii*, Our aim is to incorporate the most appropriate target sequences in a LAMP assay to detect the presence of *Aeromonas* in the kidney tissues of infected ornamental fish and to differentiate *A. hydrophila* and *A.veronii*, which are the two commonest mesophilic, motile aeromonads in fresh water ornamental fish.

We believe this LAMP assay would allow the rapid screening for the presence of *Aeromonas* in ornamental fish not only in routine disease monitoring but also in epidemiological investigations and in fish health certificates issuing as a quarantine and certification measure to prevent the international spread of pathogens during import/exports. This will not only help to take corrective measures for MAS early in the course of disease, but also to avoid the unnecessary use of antimicrobials on fish diseases due to other non bacterial etiologies.

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