BACTERIA RECOVERED FROM AQUACULTURE IN OMAN, WITH EMPHASIS ON *AEROMONAS* SPP.

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BY

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DECLARATION

I hereby declare that the work and results presented in this thesis were conducted by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not been previously submitted for any other degree or qualification. The literature consulted has been cited and where appropriate, collaborative assistance has been acknowledged.

Signature: _____

Signature of Principal Supervisor: _____

Signature of Supervisor: _____

Date: _____

In loving memory of my mother

To my husband Hamood Al-Ghabshi and My lovely sons Yousuf and Mohammed

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ABSTRACT

Aquaculture is being seriously considered as a promising sustainable industry in the Sultanate of Oman. Fish farming commenced in Oman in 1986, but it was only in 2011 that it became a more commercially driven sector. While worldwide aquaculture production is expected to rise to meet the shortage in capture fisheries, there is a parallel requirement to identify potential threats to the health and welfare of existing aquatic farmed stocks and to take appropriate steps to mitigate them.

As aquaculture in Oman is in an early stage of development, it is important to acquire baseline data on the existence and prevalence of aquatic diseases and pathogens to help the Government make policy decisions to develop health management regimes applicable for Omani aquaculture. Therefore, this study was conducted to evaluate current farming practices of tilapia in Oman, to investigate the bacterial species composition and distribution from different sites in some of the economically important fish species, and to study the characteristics and pathogenicity of *Aeromonas* species.

The current practices were studied for 9 Nile tilapia (*Oreochromis niloticus*) farms from four areas (Al Batinah, Ad Dhahirah, Ad Dakhiliyah and Ash Sharqiyah North) during the period of September to November 2012 by using questionnaires and interviews with the farm owners and staff.

In total 417 fish representing 5 target species were chosen on the basis of the commercial importance and their potential for aquaculture in Oman, including red spot emperor (*Le-*

thrinus lentjan), king soldier bream (*Argyrops spinifer*), white spotted rabbit fish (*Siganus canaliculatus*), abalone (*Haliotis mariae*) and tilapia (*Oreochromis niloticus*). The fish were collected from 5 main sampling areas in Oman (Muscat, Mudhaibi, Manah, Sohar and Salalah) based on the Atlas of suitable sites for aquaculture in Oman to investigate the bacterial species composition and distribution. The animals were examined for clinical signs of disease prior to routine bacteriology. Bacterial isolates were recovered using traditional methods and identified to species level using phenotypic and molecular approaches using 16S rDNA, 16S rDNA RFLP and 16S rDNA sequencing. Experimental fish challenge studies were also conducted using both live bacterial cells and ECP protein to investigate the pathogenicity of *Aeromonas* isolates. In addition, the presence of some virulence factors was investigated using both phenotypic and genotypic methods.

The results of this study showed that, the most farms in the Oman follow very similar farming practices. The major proportion of the tilapia is consumed within the local communities. A number of farmers have experienced mortalities, which were considered to be attributable to poor water quality, overcrowding or due to excessive feeding. Farmers facing fish mortalities tended not to record the problems due to a lack of understanding of the concept of fish farm management. There is a regulation about aquaculture and related quality control, but it has not yet been implemented in an appropriate manner in Oman.

From the diverse group of bacteria recovered from wild and farmed fish, 83% of the total isolates comprised Gram negative, rod-shaped bacteria. The most frequently isolated groups from marine and cultured fish were *Aeromonas* spp., *Vibrio* spp., *Sphingobacterium* spp., *Micrococcus* spp. and *Staphylococcus* spp., with *Aeromonas* spp. being the

predominant group representing 25% of the isolates recovered in this study. Identification of the *Aeromonas* spp. showed 57% agreement between the results of phenotypic and genotypic methodologies, and determined 6 species as the dominant organisms, i.e. *A. veronii, A. jandaei, A. caviae, A. trota, A. encheleia* and *A. salmonicida*. 65% of the isolates shared 99% 16S rDNA sequence similarity with the closest sequences in GenBank, and the dominant species was *A. veronii.*

In conclusion, the *Aeromonas* isolates recovered from fish with clinical signs of disease showed heterogeneity in their identification profiles and their pathogenicity.

LIST OF ATTENDANCE CONFERENCE AND PUBLISHED ARTICLES

Presentation

- Alya AL-Ghabshi, Brian Austin and Mags Crumlish (18-2-2015). *Aeromonas* species diversity in the aquatic environment of Oman. PhD Research Conference on sustainable aquaculture 2015 (Stirling University).

Article

- Alya AL-Ghabshi. (March 2015). Disease data in young industry. *fish farmer*, www.fishamer-magazine.com ,38,P.49

Middle East – Research

BY ALYA AL-GHABSHI

Disease data in young industry

Stirling PhD student gathers information to help improve the aquatic environment of Oman

FISH farming commenced in Oman in 1986, but it was only in 2011 that it became a more commercially driven sector, due to investment and the development of a regulatory body to govern aquaculture practices.

Currently, two species are farmed in the country, Indian white shrimp (Penaeus indicus) and Nile tilapia (Oreochromis niloticus).

The majority of aquaculture production was exported as whole fish or shrimp products to the United Arab Emirates, with a small quantity sold on the domestic market. The total production of farmed tilapia and shrimp combined was 353 tons, with the approximate value of $2 million, in 2013 (according to the Oman fisheries ministry).

An emerging industry can learn valuable lessons from other aquatic farming sectors, and it's important to have baseline data on the existence and prevalence of aquatic diseases and pathogens.

Therefore, a questionnaire based study was conducted to understand the strengths and weakness of the current tilapia production. This study was performed from September to November 2012 using a questionnaire designed to include both open and closed questions, supplemented by individual interviews with the farm owners and staff.

This was conducted in nine farms from four areas, all active in aquaculture (Al Batinah, Ad Dhahirah, Ad Dakhiliyah and Ash Sharqiyah North). The study showed that most farms in the area follow very similar husbandry practices.

The information generated from this survey identified some water quality issues faced by the farmers and lack of general knowledge on optimal fish health management.

The second study investigated the aquatic bacterial species composition and distribution in different aquatic environments in Oman during a seven-month period from December, 2011, to May, 2012. In total, 417 fish representing five target species were chosen on the basis of the commercial importance and their potential for aquaculture in Oman, including red spot emperor (Lethrinus lentjan), king soldier bream (Argyrops spinifer), white spotted rabbit fish (Siganus canaliculatus), abalone (Haliotis mariae) and tilapia (Oreochromis niloticus).

The fish were collected from five main sampling areas in Oman (Muscat, Madhaibi, Manah, Sohar and Salalah). Bacterial isolates were recovered using traditional methods and identified to species level using phenotypic and molecular methods.

The results gave a total of 420 different bacteria isolates, with 83 per cent of the total isolates identified as Gram negative, rod-shaped bacteria. The most frequently isolated groups from marine and cultured fish were Aeromonas spp., Vibrio spp., Sphingobacterium spp., Micrococcus spp. and Staphylococcus spp. with Aeromonas spp. being the predominant group, representing 25 per cent of the isolates recovered in this study.

A third study focused on characterising the Aeromonas spp using a range of molecular and traditional bacterial methods and determined seven species as the dominant organisms: A.veronii, A. jandaei, A. caviae, A. trota, A. encheleia and A. salmonicida. Of these, the sequence homologies were 99 per cent and the dominant species was A. veronii.

In conclusion, the aquaculture sector in Oman is emerging and the study will help support the development of appropriate field and laboratory based tools to identify bacterial infectious diseases impacting production levels.

As found in other aquatic farming systems, the Aeromonas isolates recovered from fish with clinical signs of disease showed heterogeneity in their identification profiles and their pathogenicity as determined by experimental challenges. It is anticipated that the data from this study will assist in the development of good health management practices for fish farmers.

"The survey identified lack of general knowledge on optimal fish health management"

Above: Collecting data from Omani fish farmers

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38

TABLE OF CONTENTS

Table of contents

Table of contents

Table of contents

LIST OF FIGURES

List of Figures

List of Figures

List of Figures

LIST OF TABLES

LIST OF ABBREVIATIONS

µg	Microgramme
µl	Microlitre
µm	Micrometre
16S rDNA	Ribosomal DNA of the 16 subunit
wa	water activity
API-20E	Analytab system of 20 biochemical test for identifying Enterobacteriaceae
BSA	bovine serum albumin
bp	Base pair
CFU	colony forming unit
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EDTA	Ethylene Diamine Tetraacetic Acid
ECP	Extracellular products
e.g.	Example
et. al.	*"et alia":* and others
FAO	Food and Agriculture Organization of the United Nations
g	gramme
× g	multiples of gravity

List of Abbreviations

h	Hour
H	Height
H&E	Haematoxylin and Eosin
Ha	Hectare
kg	Kilogramme
L	Length
MAF	Ministry of Agriculture and Fisheries
min	Minute
NCBI	National Centre for Biotechnology Information
No.	Number
OD	optical density
^{o}C	Degree Celsius
W	width

General introduction

1.1 Background

The Sultanate of Oman is located in the southeast corner of the Arabian Peninsula with a total surface area of approximately 309,500 km^2, and comprises continental land and the main islands of Masirah and Al-Halaniyat. Oman has more than 3,165 km of coastline stretching from Musandam in the north to Salalah in the south. Oman overlooks the Indian Ocean, Sea of Oman, Persian Gulf and Arabian Sea (Figure 1.1).

Oman has nine administrative regions of which six are coastally located. The whole coastal belt represents a rich and valuable source of renewable natural resources of which fisheries from Omani waters provide the major source of income and livelihood option for the Omani population after oil revenues.

The Omani ocean shelf along the coastline has excellent natural conditions featuring an unpolluted coastline. The result is an environment with a variety of fish populations, including a variety of staple and financially rewarding species as well as rare and valuable delicacies, such as kingfish, lobster and abalone.

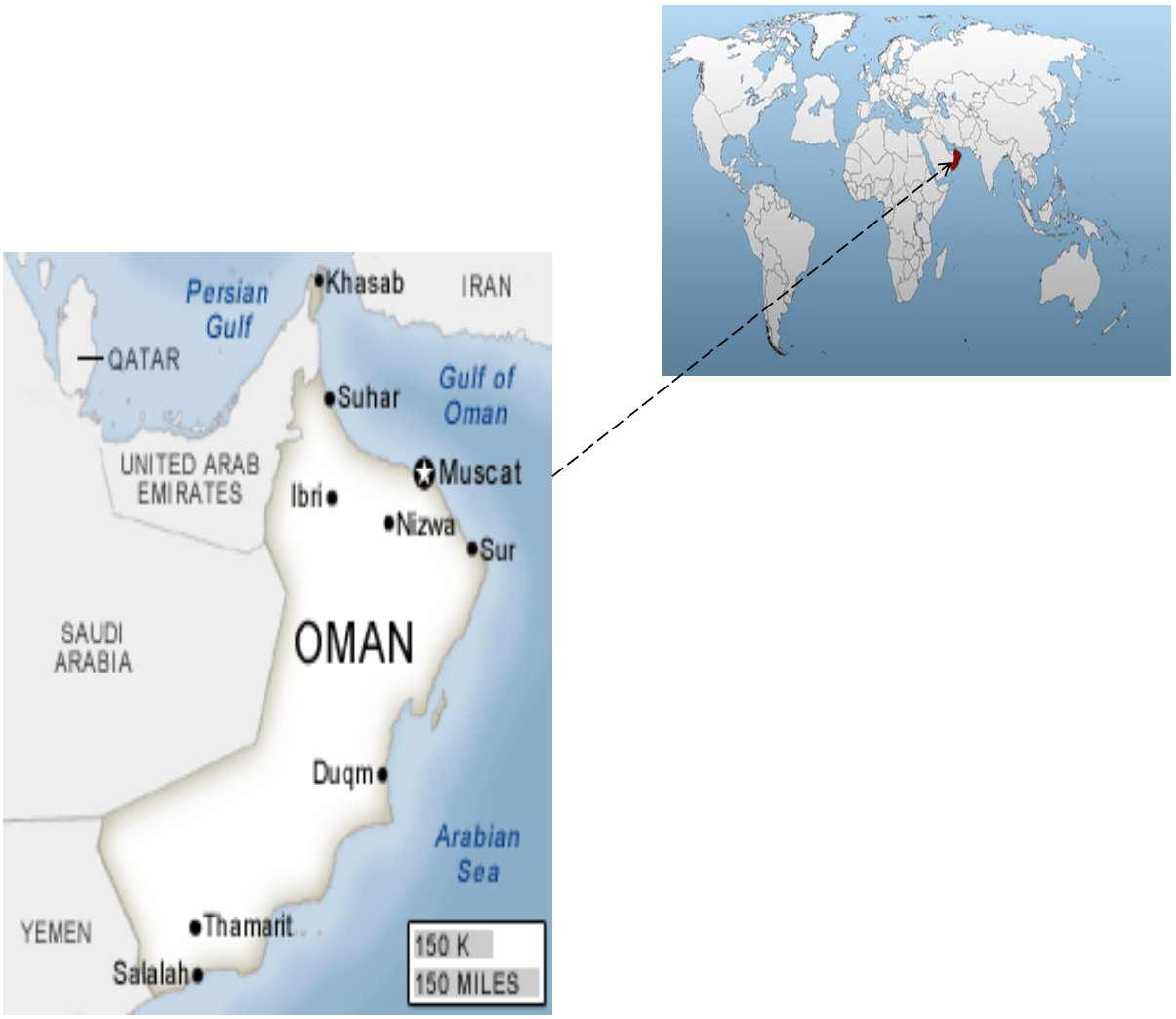

Figure 1. 1 Map of Oman and its geographical position in the world

The artisanal fishermen rely on the natural fisheries resources of Oman to supply fisheries products to the local consumers or for export mainly to neighbouring countries. The fish export business is a significant foreign exchange provider to the national economy, ranking first among non-oil export products.

1.2 Fish and fisheries in Oman

Fish consumption in Oman is ~28 kg /capita /year, which is ranked high compared with the world average of 18.9 kg /capita in 2010 (FAO, 2014). The current fish production in

Oman is 206,000 metric tonnes (MT) annually (MAF, 2014), which cannot continue to meet the heavy demand for seafood in the country and in international markets. Also, most fisheries are being harvested at or near the maximum sustainable yield, and some were even overexploited by the late 1990s. This included the fisheries for sharks (Al-Kharusi *et al*., 2002), demersal and pelagic species (Al-Oufi *et al*., 2002; De Rodellec *et al.*, 2002), shrimp and other invertebrate species, such as abalone and sea cucumber, which have similarly become depleted (Al-Rashdi, 2009). Hence, the only way to substantially and cost-effectively increase the fish supply in the country is by developing an aquaculture industry.

Aquaculture is the fastest growing food-producing sector worldwide with a sustained annual growth rate of 6.2 % per annum between 2000 and 2012 (FAO, 2014). The Fisheries and Aquaculture Department of the United Nations Food and Agriculture Organization (FAO) reported that aquaculture production worldwide was over 66.6 million tonnes in 2012 (FAO, 2014). However, aquaculture production in Oman accounts for only 353 tonnes (MAF, 2014), which is minimal compared with the estimated potential production of 200,000 metric tonnes by 2020 (MAF, 2010). Aquaculture has tremendous potential for growth and development throughout the Sultanate of Oman by the private and public sector. This critical economic and environmental activity could contribute towards a healthy diet by providing seafood, as well as supporting employment and recreation within the country. Aquaculture could effectively protect wild fishery stocks and restore populations of aquatic animals that face extinction. Fortunately, Oman is blessed with a number of native species suitable for aquaculture production. Of the 991 species known to exist within Omani waters, some of these species are under active consideration for use in

aquaculture. Figure 1.2 shows the recommended candidate species for aquaculture in Oman. Species, such as sea bream (*Sparus aurata*), shrimp (*Penaeus indicus*) and tilapia (*Oreochromis niloticus*), are already produced in an industrial scale. Conversely, the other named species on the list (Figure 1.2) are either produced on a smaller scale or are already supported by existing research and development programmes.

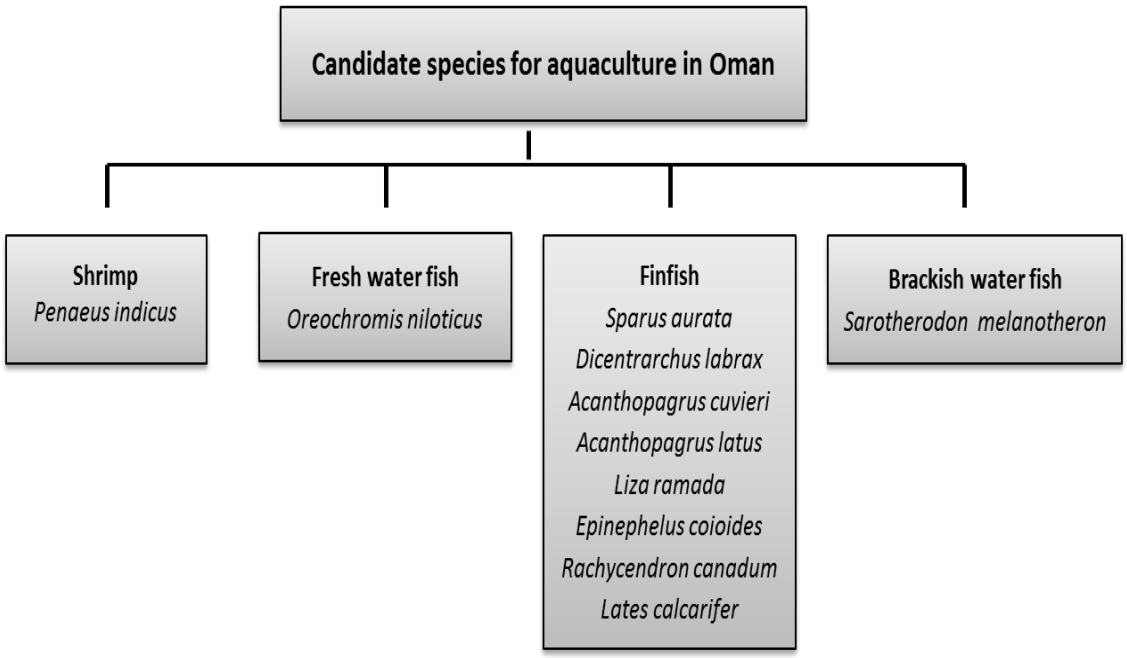

Figure1.2 List of candidate species considered for aquaculture in Oman (MAF, 2010).

Recently, aquaculture has been recognised as the best alternative to further support the fishery production in Oman. If properly implemented with a clear vision and planning process, the aquaculture industries can effectively double the seafood production in the country. A recent survey by the Ministry of Agriculture and Fisheries Wealth has mapped the coastline of the entire country, and identified many suitable sites for aquaculture and, indeed, placed ~15,000 ha of land for aquaculture development (Atlas of suitable sites for

aquaculture projects in the Sultanate of Oman, Ministry of Agriculture and Fisheries Wealth, 2010) **(**Figure 1.3).

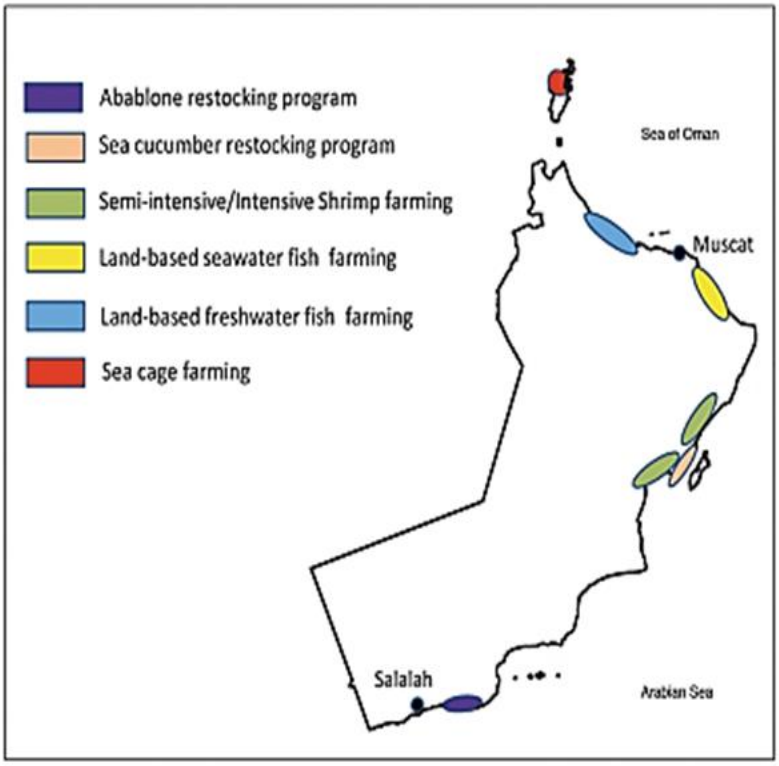

Figure 1.3 General layout of the suitable locations for aquaculture development in Oman (MAF, 2010).

1.3 Fish pathogens

Fish pathogens have a severe economic influence on the sustainability and resilience of aquatic animal production. Global estimates of disease losses to aquaculture by the World Bank (1997) were ~US$ 3 billion. Disease is the single largest cause of economic loss in aquaculture, and globally ⅓ to ½ of aquaculture production is lost because of poor health management of farmed fish and shrimps (Tan *et al.*, 2006). For example, during 2007 to 2009, Chilean salmon farms lost 350-400 thousand tonnes of production to disease. In

China, losses of 295,000 tonnes were reported in 2010. Moreover in Mozambique, disease wiped out production of marine shrimp in 2011 (FAO, 2014).

The presence of pathogens, *i.e.* viruses, bacteria, fungi and parasites, in the aquatic environment may lead to reductions in growth rate, in reproductive performance, or mass mortalities. This may be a potential risk to the emerging Omani aquaculture industry, leading to inferior products that either affect human (food) safety, result in the rejection of consignments of exported products, or lead to the catastrophic destruction of local wild populations of aquatic animals and plants. Unfortunately, such biosecurity threats are not uncommon in countries where aquaculture has developed (Roberts, 2003).

Large scale losses by pathogenic organisms have been reported. For example, infectious salmon anaemia virus (ISAV) and the caligid copepod, *Caligus rogercresseyi*, had a devastating impact resulting in the decimation of Chilean salmon stocks from 385,086 tonnes in 2006 to 230,678 tonnes in 2010 (FAO FishStatJ, 2013). Six percent of farmed Atlantic salmon production in Scotland from 1995 to 1998 was affected with pasteurellosis caused by *Pasteurella. skyensis* (Jones & Cox, 1999; Birkbeck *et al*., 2002). Additionally, there have been several reports of bacterial infections in eels in Korea that were caused by *Edwardsiella tarda*, *Aeromonas hydrophila*, and *Vibrio* spp. (Hah *et al*., 1984; Yoo *et al*., 1990 ; Kim *et al.*, 2011; Joh *et al*., 2011).

Arguably, bacteria are serious causes of infectious disease in aquatic animals. In virtually every type of aquaculture systems, bacterial diseases rank number one among all the possible aetiological agents. Septicaemias and cutaneous lesions are among the common

manifestations of bacterial infections (see Austin & Austin, 2012). Fish are susceptible to a wide variety of Gram-negative and Gram-positive bacterial pathogens, (Palmeiro & Roberts, 2009; Noga, 1996). An understanding of the potential pathogens and early disease recognition is important to prevent significant losses (McAllister *et al*., 1987). It is also important to realise that many disease outbreaks are caused by opportunistic pathogens, and thus may be prevented by proper husbandry and management techniques.

During previous routine investigations by the Microbiology Laboratory at the Fish Quality Control Center in Oman, a number of bacterial species were detected of which *Aeromonas* was one of the most prominent groups recovered.

1.4 *Aeromonas*

The genus *Aeromonas* consists of Gram-negative, straight, coccobacillary to bacillary shaped bacteria with rounded end, and measure 0.3-3.5 µm in length. They are asporogenous, facultatively anaerobic bacteria, which give positive reactions with the oxidase and catalase tests (Daskalov, 2006) and ferment maltose, and give negative reactions for urease, pectinase, ornithine decarboxylase, tryptophan and phenylalanine deaminases (Holt, 1994). Growth occurs at 22-35ºC (Ghenghesh *et al*., 2008), but some can grow at 0-45ºC (Mateos *et al*., 1993). However, *A. salmonicida* do not grow at >35ºC (Martin-Carnahan & Joseph, 2005). Most species grow at pH 4.5 to 9 (Isonhood & Drake, 2002) and in 0 to 4% (w/v) sodium chloride, and are resistant to 150 µg concentration of the vibriostatic agent O/129 (Joseph & Carnahan, 2000). They grow at refrigerator temperatures and may well produce exotoxins at these low temperatures (Kirov, 1997). The genus *Aeromonas* was first described by Zimmerman in 1890 (Zimmermann, 1890) and placed in the family *Vibrionaceae* (Demarta *et al*., 2000). However, genetic studies based on 16S rRNA

cataloguing, 5S rRNA sequencing and RNA-DNA hybridization data (MacDonell *et al*., 1985;1986), and phylogenetic studies (Martínez-Murcia *et al*., 1992; Ruimy *et al*., 1994) provided enough evidence to support the position of *Aeromonas* in a new family, i.e. the *Aeromonadaceae* (Colwell *et al*., 1986). The organisms share many biochemical characteristics with the *Enterobacteriaceae,* and are primarily differentiated by being oxidase-positive, production of acid or acid and gas (CO_2 and H_2) from carbohydrates, and reduce nitrates to nitrites. They can be differentiated from *Plesiomonas* due to the fermentation of inositol, and from *Vibrio* due to their capability to grow in the absence of NaCl (Martin-Carnahan & Joseph, 2005) .

Numerous studies based on DNA-DNA hybridization and 16S rDNA analysis show that members of the genus *Aeromonas* form a distinct line within the Gammaproteobacteria, and there is sufficient phylogenetic strength within the genus to warrant the elevation of the genus to the level of family (Ruimy *et al*., 1994; Jangid *et al*., 2007; Yáñez *et al*., 2003). The present edition of Bergey's Manual of Systematic Bacteriology lists three genera in the family *Aeromonadaceae,* including *Aeromonas, Oceanimonas,* and *Tolumonas* (Martin-Carnahan & Joseph, 2005) .

The taxonomy of *Aeromonas* has been confusing because of the lack of congruity between phenotypic and genotypic characteristics. Currently, *Aeromonas* consists of 17 DNA hybridization groups (HG), 25 species (Figueras *et al*., 2011), 12 subspecies and two biovars. The species are *A. bestiarum, A. salmonicida* (with 5 subspecies: *salmonicida, masoucida, smithia, achromogenes* and *pectinolytica*), *A. caviae, A. media, A. eucrenophila, A. sobria, A. veronii, A. encheleia, A. jandaei, A. schubertii, A. trota, A.*

allosaccharophila and *A. popoffii*. Also some species were synonimised with previously recognized species, such as *A. ichthiosmia* and *A. culicicola* with *A. veronii,* and *A. enteropelogenes* with *A. trota.* Since then, the genus has extended rapidly with another 11 new species (*A. simiae, A. molluscorum, A. bivalvium, A. tecta, A. aquariorum, A. piscicola, A. fluvialis, A. taiwanensis, A. sanarellii, A. diversa* and *A. rivuli*). Thus, the difficulty of the classification of the genus *Aeromonas* makes identification very complex. As a result, the assignment of exact species names to the genus *Aeromonas* will continue to be a challenge for microbial taxonomists.

Aeromonas species are divided into two main groups: the motile mesophilic group and are usually responsible for numerous human infections, and which is subdivided *into A. hydrophila, A. caviae and A. sobria,* and the non-motile psychrophilic group usually responsible for causing fish infections, i.e. *A. salmonicida* (Janda & Abbott, 1998). Aeromonads may also be grouped on the basis of the origin of the isolates, namely the environmental species that are recovered from water, fish, other animals, and industrial sources, such as *A. salmonicida* (HG 3), *A. sobria* (HG 7), *A. media* (HG 5), *A. eucrenophila* (HG 6), *A. trota*, *A. allosaccharophila*, *A. encheleia* (HG 11), *A. bestiarum* (HG 2) and *A. popoffii*; and the human species which are subdivided into major pathogens, *A. hydrophila* (HG 1), *A. caviae* (HG 4) and *A. veronii* biovar sobria (HG 8), and minor pathogens, *A. veronii* biovar veronii (HG 10), *A. jandaei* (HG 9) and *A. schubertii* (HG 12) (Janda & Abbott, 1998).

1.4.1 Disease distribution and clinical signs associated with *Aeromonas* infections

Aeromonas species are significant disease-causing organisms, worldwide. They are found in vertebrates, such as fish and other cold and warm-blooded organisms (Janda & Abbott,

2010; Montoya *et al*., 1992). They may be isolated from the aquatic environment, including brackish, fresh, marine, and chlorinated and non-chlorinated drinking water (Seidler *et al*., 1980). Furthermore, they may be isolated from a broad range of fresh fish and seafood (Abeyta & Wekell, 1988), meat and meat products, salad leaves, milk, ice cream and cheese (Freitas *et al*., 1993; Rhodes *et al*., 2000; Villari *et al.*, 2000). *Aeromonas* spp. are present in the faeces of healthy animals, including humans, most probably as the result of intake of food and water containing these organisms (Holmes *et al*., 1996; Demarta, *et al*., 2000).

1.4.2 Clinical signs of disease

Aeromonas spp. cause haemorrhagic and ulcerative diseases in fish (Austin & Adams, 1996). *A. caviae, A. sobria* and *A. schubertii* have all been implicated in diseases of aquatic finfish and crustaceans as well as infections in humans, whereas *A. salmonicida* is a fish pathogen, and has not been associated with infections in any terrestrial animal species (Janda & Abbott, 1996). Clinical sign of infections in fish are rarely specific, and include ulcerative lesions of the skin around the base of the fins and the anus, raised scales, abdominal distension, and exophthalmia; all of which are signs that are common with other bacteria. Depending on the severity of infection, anaemia, hepatomegaly, and ascites may develop in affected fish (Lowry & Smith, 2007). *Aeromonas* infections in fish are often secondary to other stresses, such as poor water quality, parasitism, and nutritional deficiencies.

Members of the genus *Aeromonas* have been known as important emerging human pathogens, which are responsible for numerous diseases including gastroenteritis, localized wound swelling, septicaemia, meningitis, and pneumonia (Galindo *et al*., 2006; Galindo

& Chopra, 2007; Lehane & Rawlin, 2000). Infections in people working in aquaculture or keeping fish as pets and following injuries from handling and direct contact with mucus and tissues from infected or carrier fish have also been reported (Lehane and Rawlin 2000). One detailed investigation in Spain spanning 1997 to 2006 found that *Aeromonas* spp. ranked fourth among the causes of total gastrointestinal diseases reported each year (Epidemiological Surveillance System, 2007).

Aeromonas salmonicida, which causes furunculosis in salmonids and ulcer disease in other fish species, affects marine and fresh water fish, such as salmonids, Atlantic cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*), turbot (*Scophthalmus maximus*), lamprey (*Petromyzon marinus*), carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and eel (*Anguilla anguilla*) (Austin & Austin, 2007; Bernoth, 1997; Godoy *et al*., 2010; Goldschmidt-Clermont *et al*., 2009; Noga, 2010; Wiklund & Dalsgaard, 1998). There are no reported cases of human infection with *A. salmonicida*.

Furunculosis is manifested in several forms, ranging from inappetence and melanesia to a sub-acute or a chronic disease in older fish, consisting of lethargy, slight exophthalmia, and severe septicaemia with additional haemorrhages at the bases of the fins and in muscle and internal organs (Austin & Adams, 1996). Sometimes infected fishes with furunculosis do not show any symptoms at all (Noga, 2010) .

A. hydrophila is a major cause of bacterial infections, causing diseases in fish, worldwide (Thune *et al*., 1993) and no fish species is known to be completely resistant to the organism (Plumb, 1994). The organism is responsible for septicaemia, and ulcerative diseases

affecting carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), striped bass (*Morone saxatilis*), largemouth bass (*Micropterus salmoides*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*), carp (*Cyprinus carpio*), gizzard shad (*Dorosoma cepedianum*), goldfish (*Carassius auratus*), golden shiner (*Natemigonus crysoleucas*), snakehead (*Ophicephalus striatus*) and tilapia (*Oreochromis niloticus*) (Bullock *et al.*, 1971; Egusa & Nakajima, 1978; Aoki, 1999).

A. veronii infects fish and crustaceans. In fish, such as carp, tilapia, perch, catfish, and salmon it produces a haemorrhagic septicaemia and ulcerative infections in catfish, cod, carp, and goby, whereas in bass and carp it produces red sore disease (Joseph & Carnahan, 1994). In other hosts, including humans, leeches and mosquitoes, *A. veronii* colonizes the digestive tract (Graf *et al*., 2006; Janda & Abbott, 1998) .

A. sobria has been implicated as a cause of epizootic ulcerative syndrome (EUS) characterized by the presence of severe, open dermal ulcers on the head, on the middle of the body, and on the dorsal regions of fish (Rahman *et al*., 2002). *A. sobria* was the causative agent of disease in farmed perch (*Perca fluviatilis*), in Switzerland. Here, focal skin lesions were observed on the lateral sides, and fin rot, and resulted in mass mortality (Juraj *et al*., 2012) . Also, *A. veronii* biovar sobria has produced severe disease problems in fish farms in Bangladesh and India (Rahman *et al*., 2002).

A. piscicola has been recovered frequently from diseased salmonids and turbot (Beaz-Hidalgo *et al*., 2010; Kozińska, 2007; Li & Cai, 2011; Martino *et al*., 2011; Nawaz *et al*.,

2006; Soriano–Vargas *et al*., 2010). Other mesophilic species, such as *A. encheleia, A. allosaccharophila, A. jandaei, A. media, A. eucrenophila, A. aquariorum* and *A. tecta,* have also been recovered infrequently from healthy or diseased fish (Beaz-Hidalgo *et al*., 2010; Kozińska, 2007).

1.4.3 Geographical distribution

Fish disease is a major risk factor in commercial aquaculture with millions of dollars lost annually (Boulanger *et al*., 1977; Fang *et al*., 2000; 2004). In the past decade, *A. hydrophila* has been associated with major fish kills worldwide due to climate and environmental change, and resulted in enormous economic losses. These die-offs included over 25,000 tonnes of carp lost in 2001 in the St. Lawrence River in North America; 820 tonnes of goldfish in 2002 in Indonesia resulting in losses of $37.5 million; and catfish mortality in 2007 in Minnesota, USA (http://www.promedmail.org). In another case, the protozoan *Ichthyophthirius multifiliis* (Ich) enhanced invasion of *A. hydrophila* in tissues of channel catfish in West Alabama. The loss due to this disease was estimated to be more than 3 million pounds of channel catfish in 2009 (Pridgeon & Klesius, 2011).

1.4.4 Diagnostic methods for the detection and characterization of *Aeromonas* spp.

Diagnosing disease and identifying the infectious agents are important for managing any disease situation. It has been recommended that detecting a bacterial fish pathogen requires analysis of samples from 4 to 10 infected fish and between 10 and 60 samples from apparently health populations (Noga, 2010). The target organs are generally the kidney, spleen, skin, and ovarian fluid. Sometimes, pathogens are detected in mucus, blood, fae-

ces and skin (Beaz-Hidalgo *et al*., 2008; Byers *et al*., 2002; Gustafson *et al.*, 1992; Kulkarni *et al*., 2009). The kidney and intestine are the recommended sites when screening for asymptomatic carriers (Roxana & María, 2012).

A number of methods have been reported for the detection/cultivation and identification of aeromonad pathogens, including traditional (phenotypic characteristics), immunological and molecular techniques. Non-selective culture media used for the isolation of *Aeromonas* strains include tryptone soya agar (TSA), brain heart infusion agar (BHIA) or Columbia blood agar with incubation at 15-25^{o}C for up to 7 days (Austin & Austin, 2007; 2012; Bernoth, 1997; Hiney & Olivier, 1999). Selective media used for the recovery of *A. salmonicida* include furunculosis agar (tryptone, yeast extract, L-tyrosine and NaCl), TSA or BHIA supplemented with L-tyrosine (0.1%) or TSA supplemented with 0.01% (w/v) Coomassie brilliant blue (Austin & Austin, 2012).

Numerous biochemical methods have been proposed for the idetnification of *Aeromonas* species, but they mainly recognize three phenotypic groupings i.e. *A. hydrophila* (including *A. hydrophila, A. bestiarum, A. salmonicida* and *A. popoffii), A. caviae (A. caviae, A. media* and *A. eucrenophila)* and *Aeromonas sobria (A. sobria, A. veronii, A. jandaei* and *A. trota*) (Abbott *et al*., 1992; 2003; Borrell, *et al*., 1998; Kozińska *et al*., 2002; Martin-Carnahan & Joseph, 2005; Martínez-Murcia *et al*., 2005). However, identifications based on phenotyping lack accuracy and tend to result in inconsistencies due to misinterpretation in some characters and because of issues with lack of reproducibility when they are carried out under different laboratory conditions, such as varying temperatures and incubation times (Figueras *et al*., 2011). In addition, the expression of biochemical character-

istics between different isolates of the same taxon, e.g. *A. hydrophila,* are not always the same (Figueras *et al*., 2011; Figueiredo & Plumb, 1977). Therefore with the issues surrounding the use of phenotyping, there has been a move towards molecular based identification.

Sequencing of the 16S rRNA gene is considered a strong taxonomic tool and is now widely used in bacterial taxonomy (Cascón *et al*., 1996; Khan & Cerniglia, 1997). However, research has indicated that phylogenetic analyses based on 16S rRNA gene sequencing may lead to difficulty with distinguishing between closely related taxa (Martínez-Murcia *et al*., 1999; 2005). Also, the data are compared with entries in the BLASTN data base, which are entered by authors and for which there is not any external verification (Austin, personal communication).

DNA probes and restriction fragment length polymorphism (RFLP) profiles designed from the 16S rRNA gene are useful to distinguish *Aeromonas* at the species level (Ash *et al*., 1993; Borrell *et al.*, 1997; Dorsch *et al*., 1994; Figueras *et al*., 2000; Khan & Cerniglia, 1997; Lee *et al*, 2002). However, some strains have produced unpredicted or different restriction patterns making their identification uncertain (Alperi *et al.*, 2008). Sequencing analyses based on the gene sequences of *gyrB* and *rpoD* are invaluable for the recognition of *Aeromonas* species and for the proper identification of novel closely related isolates (Soler *et al*., 2004; Yanez *et al*., 2003). Finally, the use of a combination of 16S rDNA-RFLP analyses (Borrell *et al.*, 1997; Figueras *et al*., 2000) and housekeeping genes (*gyrB, rpoD*), (Alperi *et al*., 2008; Figueras *et al*., 2009; Martínez -Murcia *et al*.,

2005; Tena *et al*., 2008) make suitable molecular markers for assessing the phylogeny of closely related *Aeromonas* species.

1.4.5 Pathogenicity and virulence factors

Aeromonas infections are complex and multifactorial. Many species are believed to be pathogenic since they satisfy most of the requirements of pathogenic bacteria. Severity of disease is influenced by a number of factors, including putative virulence factors, the type and degree of stress exerted on a population of fish, the physiological condition of the host, and the degree of genetic resistance inherent within specific populations of fish. Motile aeromonads vary in their relative pathogenicity (De Figueiredo & Plumb, 1977). Several virulence factors are required for the bacterium to colonize, gain entry into and produce damage in host tissues. Factors are also involved in evading the host defense systems, the ability to spread within tissues, and the ability to kill the hosts (Smith, 1995). The main pathogenic factors associated with *Aeromonas* are surface polysaccharides (capsule, lipopolysaccharide, and glucan), S-layers, iron-binding systems, exotoxins, extracellular enzymes, secretion systems, fimbriae and other nonfilamentous adhesins, and flagella.

The capsule is composed of polysaccharides covering the outer layer of the bacterial cell connecting it with the outer environment. It is described as a main virulence factor of many pathogens (Merino & Tomas, 2010). In some instances, it provides protection from host phagocytosis and its induction and expression increased the level of virulence for fish and provided resistance to serum killing (Merino *et al*., 2012; Magarinos *et al*., 1996).

Mesophilic *Aeromonas* spp., *A. hydrophila* AH-3 (serogroup O: 34), PPD134/91, JCM3980 stains of *A. hydrophila* (serogroup O: 18), *A. veronii* bv. sobria (serogroup O: 11) and *A. salmonicida* are all able to form a capsular polysaccharide when grown in a glucose-rich medium (Gardufio *et al.*, 1993; Garrote *et al.*, 1992; Martínez *et al.*, 1995). *A. salmonicida* and *A. hydrophila* serogroup 0:34 strains grown under conditions promoting capsule production showed significantly higher ability to attack fish cell lines compared with those grown under conditions, which did not promote capsule formation (Merino *et al*., 1996; 1997). *A. hydrophila* group II capsule (IIA and IIB) producing strains have been reported to possess resistance to serum and phagocyte killing (Zhang *et al*., 2003). It has also been shown that purified capsular polysaccharides and O-antigen of *A. hydrophila* PPD134/91 have the ability to confer resistance to serum-mediated killing (Zhang *et al*., 2002).

Lipopolysaccharide (LPS) is a surface glycoconjugate exclusively found in Gram-negative bacteria, and consists of lipid A, core oligosaccharide and O-antigen. The lipid A components anchor LPS in the outer membrane (Reeves *et al*., 1996). The core can be subdivided into two regions, the inner and the outer core, based on the sugar composition. The O antigen is a polysaccharide extending from the cell surface and consists of repeating oligosaccharide units made up of 1 to 6 sugars. It mediates pathogenicity by protecting infecting bacteria from serum complement killing and phagocytosis (Whitfield & Valvano, 1993; Joiner, 1988). The first constructive scheme for the serogrouping of *Aeromonas* strains based on O antigens separated 44 subgroups from a total of 307 *A. hydrophila* and *A. caviae* strains (Sakazaki & Shimada, 1984). Afterwards, it was extended to 97 O serogroups (Thomas *et al*., 1990). More than 60% of the septicaemia cases were

associated with four of these subgroups: (O: 11; O: 16; O: 18; O: 34) (Janda & Abbott, 1998). Serogroup O: 34, was the most common mesophilic *Aeromonas* associated with outbreaks of septicaemia in fishes and with wound infections in humans, whereas, serogroup O: 11 is related to severe infections in humans, as septicaemia, meningitis and peritonitis (Janda *et al*., 1996). *In vivo* experiments by Merino and co-workers (Merino *et al*., 1992; 1998) showed that the appearance of LPS in serogroups O:13, O:33 and O:44 of mesophilic *Aeromonas* strains can be affected by both temperature and osmolarity. Strains grown at 20°C (at high or low osmolarity) and at 37°C (at high osmolarity) carried a smooth LPS, whereas strains grown on low osmolarity carried a rough LPS (Merino *et al*., 1992). Also, these workers observed that the smooth strains were more resistant to the bactericidal activity of serum than rough strains, and showed better adhesion to Hep-2 cells and more virulence in fish and mice (Merino *et al*., 1998).

S-layers (originally termed the A-layer in *A. salmonicida*) were first described by Kay and coworkers (Kay *et al*., 1984). Belland and Trust (1987) provided the first molecular evidence of atypical strains of *A. salmonicida* losing ability to produce the A layer protein after growth at 30°C due to a genetic rearrangement and deletion. The S-layer was considered as a significant virulence factor in aeromonads. In some cases, the virulence was influenced by temperature. For example, *A. salmonicida* lost virulence at high temperature (Ishiguro *et al*., 1981; Noonan & Trust, 1995). In *A. hydrophila*, the S-layer may play a less significant role in its virulence. Thus, it was confirmed that spontaneous mutants of *A. hydrophila* missing a S-layer manifests lesser virulence in animal models (Kokka *et al*., 1991; 1992). *Aeromonas* S-layers have the ability to adhere and protect the bacteria against proteases. For example, the A-layer of *A. salmonicida* promoted significant ad-

herence to non-phagocytic fish cell lines (Garduño *et al*., 2000) by helping the bacteria to bind the host receptors, such as type IV collagen, laminin and fibonectin (Doig *et al*., 1992; Trust *et al*., 1993). The S-layer proteins of *A. hydrophila* have been shown to be antigenic and protective in common carp against a variety of virulent strains (Poobalane *et al*., 2010).

Iron is a vital nutrient for virtually all forms of life. It is also essential for bacteria, which can obtain their iron by direct contact between the bacterium and the exogenous iron/heme sources. Bacteria are also capable of scavenging iron or heme from several sources by using siderophores and haemophores (Wandersman & Delepelaire, 2004). There are two high affinity iron-sequestering mechanisms identified in *Aeromonas* strains: siderophore-dependent and siderophore-independent mechanisms (Beyers *et al*., 1991). Mesophilic *Aeromonas species* synthesize either enterobactin or amonabactin siderophores, but never together. The action of siderophores in *Aeromonas* spp. is also mediated by two types of siderophores: amonabactin and enterobactin (Barghouthi *et al*., 1989; Zywno *et al*., 1992). The enterobactin is found in different Gram-negative bacteria, but the amonabactin is only produced in *Aeromonas* spp. (Telford & Raymond, 1998). An understanding of the distribution of specific siderophores in *Aeromonas* spp. may help in the classification of the genetic species of *Aeromonas* and the evaluation of potential virulence properties (Zywno *et al*., 1992).

Aeromonas strains are able to produce a large number of toxins and extracellular enzymes associated with pathogenicity or the disease process. Enterotoxins are major virulence factors of *Aeromonas* spp. produced and secreted by several species targeting the intes-

tines. They consist of two categories: cytotoxic and cytotonic enterotoxins. Cytotoxic enterotoxins are described as heat-labile (*Act*). These toxins are pore-forming channel haemolysins able to modify cell permeability (Castilho *et al*., 2009). Haemolysins belong to two major classes; one class is the aerolysins (*aerA*), which are usually ß-haemolysins that produce clear zones of haemolysis on blood agar. The *aerA* genes specifying the aerolysins were cloned and sequenced from *A. hydrophila* (Howard *et al*., 1987) and *A.veronii* biovar sobria and *A. caviae* (Singh & Sanyal, 1992). α-haemolysin is the second class of haemolysins, and were first cloned and sequenced from *A. hydrophila* ATCC7966 (Hirono *et al*., 1997; Janda, 1991). These haemolysins produce opaque, incomplete zones of haemolysis that are often seen as the inside haemolytic zones of strains producing "double-zone" haemolysis. Elaboration is during the stationary phase, and expression does not occur when temperatures exceed 30°C. Both aerolysins and related β-hemolysins have the ability to lyse red blood cells in addition to enterotoxic activity, and cause tissue damage in a variety of eukaryotic cell lines, including Hep-2, HeLa, Chinese hamster ovary, Vero and erythrocytes (Asao *et al*., 1984; Chopra *et al*., 1993; Fujii *et al*., 1998; Wong *et al*., 1998). In contrast, cytotonic enterotoxins differ from aerolysins and aerolysin-related β-haemolysins both genetically and in biological action (Janda, 2001). They do not produce a cytopathic effect in eukaryotic cells. Instead, their action results in cell elongation or rounding (Janda, 2001).

Two types of cytotonic enterotoxins have been reported. The first type is the heat-labile enterotoxin (*Alt*) at 56°C, and is also known as lipase, extracellular lipase, or phospholipase (Chopra & Houston, 1999). The *Alt* protein consists of a single 44 kDa polypeptide (Chopra *et al*., 1994; 1996). The activity of this toxin may be neutralized by CT antise-

rum. Pre-incubation with anti-CT reduced the CHO cell titre of cell lysates 10-fold (Potomski *et al*., 1987). Heat-stable enterotoxin (*Ast*) is the second type of the cytotonic enterotoxin reported in *A. hydrophila* and *A. sobria,* and is stable at 56°C (Chopra & Houston, 1999). Both cytotonic enterotoxins (*Alt* & *Ast*) are unrelated to cholera toxin (Albert *etal*., 2000; McCarter, 1995) and cause higher intracellular cAMP and PGE2 levels in cultured CHO cells (Chopra *et al*., 1992;1996).

Aeromonas species secrete a wide range of extracellular proteins, including proteases, serine proteases, elastases [e.g. AhpB], lipases, DNAses, amylases, chitinases, xylanase, lecithinases and gelatinases. These secreted proteins are important molecules involved in a variety of pathogenicity mechanisms including tissue damage, evading host defences and deriving essential nutrients from the host for survival (Figueras *et al*., 2009; Figueras, 2005; Chopra & Houston, 1999; Scoaris *et al*., 2008).

Proteases are secreted enzymes commonly found in both pathogenic and non-pathogenic microorganisms. They are major virulence factors of certain pathogenic bacteria, and mediate the entry and invasion of host tissues by proteolysis and tissue damage (Miyoshi & Shinoda, 2000). *Aeromonas* spp. secrete a group of proteases, which degrade many diverse proteinaceous compounds, such as albumin, fibrin, gelatin and native elastin molecules (Janda, 1985). Two major proteases are produced by *Aeromonas* strains. One enzyme, a thermolabile serine protease that is inactivated by EDTA, appears to belong to the general class of thermostable metalloproteases; the other protease is the thermostable (56°C, 30 min) metalloprotease that is EDTA-sensitive or insensitive (Rivero *et al*., 1991; Ellis *et al*., 1997). The role of proteases in pathogenesis is not clearly understood. They

are implicated in colonization, invasion of host tissues during host-pathogen interaction and mediate tissue lysis during pathogen invasion by helping the bacteria to take essential nutrients from the host environment for further survival, multiplication and distribution inside the host (Bjornsdottir *et al.*, 2009)**.**

Researchers have confirmed that the toxic fraction of extracellular products (ECP) secreted by *A. hydrophila* is linked with the haemolytic activity and protease activity (Allan & Stevenson, 1981; Subashkumar *et al.*, 2006; Kanai & Wakabayashi, 1984; Sakai, 1985). *In vivo* experiments on rainbow trout, carp and rohu, have shown that the lethality of ECP was decreased and totally inactivated when boiled at 100°C for 10 min. In contrast, the presence of protease and haemolysin activities in the ECPs of *A. salmonicida* was correlated with the development of lesions, but not with the lethal toxicity of the ECPs in rainbow trout. Thus, an unidentified component of ECP was responsible for killing fish (Ellis *et al*., 1988).

Lipases contribute to bacterial nutrition and are involved in erythrocyte lysis (Chopra & Houston, 1999; Pemberton *et al*., 1997). *Aeromonas* spp. are capable of secreting several lipases, which hydrolyze esters of glycerol with long-chain fatty acids (Jaeger *et al*., 1994). An extracellular lipase purified from the culture supernatant of *A. hydrophila* H3 showed both esterase and lipase activities (Anguita *et al.*, 1993). Later, *A. hydrophila* strains PLA1, LipE, Lip and Apl-1 were found to secrete a group of lipases with high homology (Ingham & Pemberton, 1995; Merino *et al.*, 1999).

Glycerophospholipid-cholesterol acyltransferase (GCAT) is a well-known phospholipase isolated from both *A. hydrophila* and *A. salmonicida* (Thornton *et al*., 1988; Eggset *et al*., 1994). GCAT can digest plasma membranes of host cells, and leads to their lysis (Thornton *et al*., 1988). However, generation of an *A. salmonicida* GCAT isogenic mutant did not result in decreased virulence, demonstrating that they may be accessory rather than essential virulence factors (Vipond *et al*., 1998). Three groups of chitinases A, B and C are known to be secreted by *Aeromonas spp.* (Watanabe *et al*., 1993).

Nucleases have been long recognized as possible virulence factors, but their exact role in virulence is unclear compared with the role of other degradative enzymes (Pemberton *et al*., 1997). DNases are considered as possible nutritional enzymes in *Aeromonas* (Scoaris *et al*., 2008). They have been involved in bacterial infections, given that DNases are enzymes that directly attack DNA, a macromolecule essential for the function of any host cell. In addition, the degradation of DNA yields carbon and nitrogen molecules to the bacteria (Podbielski *et al.*, 1996).

Gram-negative bacteria have developed different secretion systems to transport proteins to the cell surface. Secretion systems in bacteria have been classified as type I, II, III, IV, V, and VI systems (Henderson *et al*., 2004). Type III (T3SS) and Type VI secretion systems (T6SS) have been recognized to play a significant role in the virulence of many Gram-negative bacteria, and are often activated upon contact with target cells and carry their toxin proteins directly into the host cell cytosol. In *Aeromonas* spp., the functioning of T3SS has only been described in *A. salmonicida* (Burr *et al*., 2002) and in *A. hydrophila* strains AH-1, AH-3 and SSU (Yu *et al*., 2004; Sha *et al*., 2005), while the func-

tioning of T6SS has been described in *A. hydrophila* strains SSU, AH-3 and ATCC7966 (Suarez *et al*. 2008).

Bacterial adherence to the host may involve either specific interactions between a receptor and a ligand or hydrophobic interactions. The receptors are usually specific carbohydrate or peptide residues on the eukaryotic cell surface, and the ligands termed adhesins are bacterial surface proteins or polysaccharides. Fimbriae/pili are micro-filamentous structures protruding from the bacterial surface. They are formed by individual subunits identified as pilin. The pili may be present singly or in bundles. They are generally described as adhesive organelles and are also involved in some other bacterial processes, as well as phage binding, DNA transfer, biofilm formation, cell aggregation, host cell invasion, and twitching motility (Tomás, 2012). *Aeromonas* spp. contain two different types of fimbriae identified based on morphology. They are short, rigid fimbriae (S/R) and long, wavy fimbriae (L/W). The S/R fimbriae are the predominant type in aeromonads with high pili numbers (Kirov *et al*., 1995). All Gram-negative bacteria possess type IV pili on their surface. These are highly antigenic and are involved in the host-pathogen cross talk. Also, they are recognized to play a significant role in adhesion to epithelial cells and virulence (Mattick, 2002). The *A. salmonicida* subsp. *salmonicida* genome contains a complete set of genes for two type IV pilus systems, referred to A Tap and Flp pili (Boyd *et al*. 2008). The arrangement of Tap pili is polar, whereas the Flp pili appear to be peritrichous in nature. The TAP pili may play an important role in the pathogenicity of *A. hydrophila* (Pepe *et al.*, 1996). *In vivo* experiments in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) showed that the Tap pilus contributes moderately to virulence, whereas the Flp pilus made little or no contribution to virulence in At-

lantic salmon (Boyd *et al*., 2008; Masada *et al*., 2002) In addition to type IV pili, *A. salmonicida* also has a type I or Fim pilus system. The type I pili are incapable of adhering to the salmon gastrointestinal tract, and hence the ability of the bacteria to invade the host was found to be not related to its presence in Atlantic salmon (Dacanay *et al*., 2010).

Depending on the environmental conditions, bacteria are able to move in a free way by swimming, swarming, gliding, twitching, and sliding among others or stay in the same place to form colony groups and colonize surfaces. *Aeromonas* spp. has both polar and lateral flagella. A polar flagellum is used for moving on solid surfaces and functions for adhesion, whereas multiple lateral flagella are mainly used for swarming over surfaces and serve as colonization factors. About 60% of mesophilic *Aeromonas* spp. contain lateral flagella (Kirov *et al*., 2002). *A. caviae* is able to produce polar and two lateral flagellins, whereas *A. hydrophila* produces two polar and only one lateral flagellin (Rabaan & Shaw, 2002 ; Canals *et al*., 2006), which are mostly responsible for the adhesion, invasion of the fish cell and also the formation of biofilm (Merino *et al*., 1997; Gavín *et al*., 2002).

1.5 Research objectives

Aquaculture is being seriously considered as a promising sustainable industry in the Sultanate of Oman. However, there is no baseline data or information on the existence and prevalence of aquatic animal disease or pathogens available in the country. Thus, the specific tasks of this thesis were:

- Evaluate current farming practices in Oman (chapter 2).
- Investigate the bacterial species composition and distribution from different sites in some of the economically important fish species (chapter 3).
- Characterize *Aeromonas* spp. isolated from wild and farmed fish in Oman by using phenotypic and molecular methods (chapter 4).
- Determine potential risk associated with the *Aeromonas* spp. recovered from wild and famed fish in Oman by investigating the pathogenicity following challenge experiments (chapter 5)

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Current Status of Aquaculture in Oman

2.1 Introduction

2.1.1 History of aquaculture in the region

The first aquaculture activities in Oman started in 1986, with the production of the giant tiger prawn *(Penaeus monodon)* in Sur (Ash Sharqiyah -South). It was relatively a short-lived activity which ceased in 1988 due to practical and management reasons. However, it was well recognized by the Omani Government that there was a need to engage with aquaculture to support the country's social and economic infrastructure. Therefore in 1992, the aquaculture laboratory of Marine Science and Fisheries Centre was established with the aim of supporting the sustainable development of aquaculture (Gindy *et al*., 1999). In 1994, research on the breeding and hatchery rearing of abalone, *Haliotis mariae*, commenced as this was considered one of the economically important species due to its high value, representing 6% of the whole fisheries sector income (Al-Hafidh, 2006). Abalone was followed by the initiation of rock oyster farming in 1995. Subsequently, preliminary experiments in the breeding and propagation of Indian white shrimp, *Penaeus indicus* and *P. semiculcatus*, were carried out during 1995-1998 (MAF, 1999). During 1997-1998, the Ministry of Fisheries in cooperation with a private company carried out a project on offshore cage farming of exotic gilthead sea bream *(Sparus aurata*) and the endemic sobaity *(Sparidentex hasta)* in the Bandar Khyran area in Muscat Governorate (Al-Qasmi *et al*., 1998**).** Also, studies were carried out on species distribution, reproductive biology, and culture of three oyster species, *Saccostrea cucullata*, *Crassostrea rhizoporae*, and *Crassostrea* spp. The European sea bass and thin lip gray mullet *(Liza ramada*) were in-

troduced by the Quriyat Aquaculture Company in 2001, and in the same year this company carried out successful trials on fattening local yellowfin tuna *(T. albacres)* (FAO, 2009). In 2008, the company abandoned this program because of a red tide phenomenon. This is a phenomenon in which an algal bloom develops, resulting from coastal upwelling or a result of increased nutrient loading from human activities (Trainer *et al*. 2000; Jump *et al*., 2000). As a consequence of this phenomenon, nearly 118 tonnes of fish died (MAF, 2009; 2012)

2.1.2 Current situation of aquaculture in Oman

2.1.2.1 Invertebrates

Currently, the main fish farming company in Oman is Bentoot Sea Food (Figure 2.1), which operates a pilot project on the culture of the white shrimp (*Penaeus indicus*) imported from Greece. The farm is located in Mahout (Al-Wusta). This farm was leased from the Government, and has 26 ponds with an area of 86 Ha**.** Currently, work is underway to expand the farm to 200 hectares, with completion set for 2015. Also, the farm has its own hatchery with an area of approximately 10,000 m^2. The company started production in early 2007, with the first harvest achieved in the last quarter of the same year. The current production is now between 100 and 300 tonnes of shrimp per year (Al-Qasmi *et al.*, 1998).

Figure 2. 1 White shrimp farm in Oman (Bentoot Sea Food Company).

2.1.2.2 Fish farming

Other than shrimp, integrated freshwater culture of Nile tilapia (*Oreochromis niloticus*) (Figure 2.2) is carried out in small farms where the presence of high levels of saline groundwater prevents or reduces agriculture activities. These sites are then available for other farming activities, including aquaculture. Tilapia is not an indigenous species, but has been introduced by the Ministry of Health in recent years as part of a mosquito control programme (McLean *et al*., 2011). Nowadays, wild populations of these fish are quite common, but are generally of unidentified genetic composition (McLean *et al*., 2002). However, due to the adaptability of this fish species to the local environment, it not surprising to find that their numbers have increased, and they are widely distributed in many wadis, falajs, natural springs and dams within various regions of Oman.

Figure 2. 2 Tilapia farms in Oman.

Small-scale Nile tilapia (*Oreochromis niloticus*) farming is becoming widespread in many villages. Currently, there are 12 tilapia farms operating, and most of these farms are located in the north and east of Oman, and a major proportion of the tilapia sold in the domestic market and consumed within the local communities. There is only one farm, which is located in the Manah (AD Dakhiliyah- north) that is considered as a major production farm. This site distributes seeds to other farms, and at the same time cooperates with the Ministry of Agriculture and Fisheries Wealth as a local field hatchery for breeding trials and development of techniques to increase the production of YY super male tilapia. In 2014, 10 farms obtained site license approval from the government to culture different species, including; grouper, sea bream, barramundi, cobia, white Indian shrimp, abalone and ornamental fish.

At present, the Ministry of Agriculture and Fisheries Wealth Create is a one-stop shop for fish farming in the Aquaculture Development Department at the Ministry of Agriculture and Fisheries, where the organisation provides information associated with investing in

the field of aquaculture. This includes the conditions to benefit from support programmes for aquaculture, the conditions for obtaining licenses for projects in aquaculture, and the regulations to invest in this area. Publications/ bulletins dealing with aquaculture fish and how to establish projects are provided (Figure 2.3 & 2.4). Also, summaries of scientific studies carried out by the Ministry for Aquaculture may be obtained. The activities lead to many projects and research programmes for the development and sustainability of aquaculture activity in Oman (Table 2.1).

Figure 2. 3 Economic indicators for the establishment of a fish farm for tilapia *(Oreochromis niloticus).*

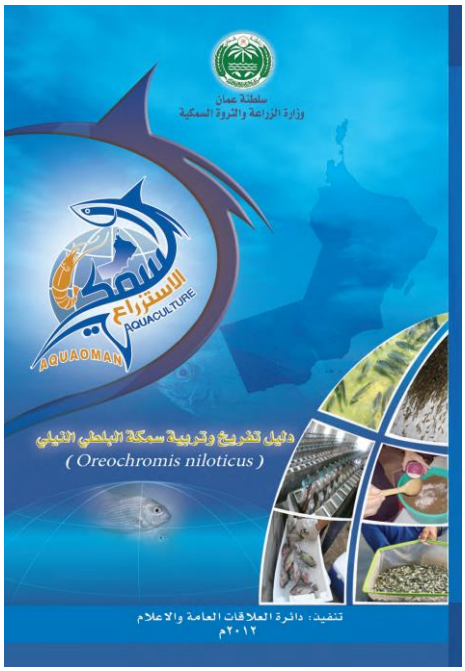

Figure 2. 4 Manual of hatchery production of tilapia (*Oreochromis niloticus).*

2.1.3 Commercial aquaculture production in Oman

Commercial aquaculture production in Oman started in 2003, and is dominated by sea cage farming of gilthead sea bream (*Sparus aurata*), and alone contributed 352 tonnes with a value of US$ 1.5 million (Figure 2.5). 2004 was considered as the most productive year to date, with a total production of 514 tonnes with a value of US$ 2.5 million. Unfortunately, production of tuna suffered lost production of 118 tonnes in the same year as a result of the occurrence of harmful algal blooms along the Omani coastline, which led to huge mortalities in sea cages (MAF, 2009; 2012). The production of farmed fish totalled 353 tonnes in 2013, which was an increase of 110% from 2012 (Figure 2.6). Approximately, 99% of this production was by two types of white shrimp and tilapia with an approximate total value of US$ two million (MAF, 2013). There are no data regarding the total production of tilapia from 2003-2009 due to lack of field data in these years (MAF, 2013).

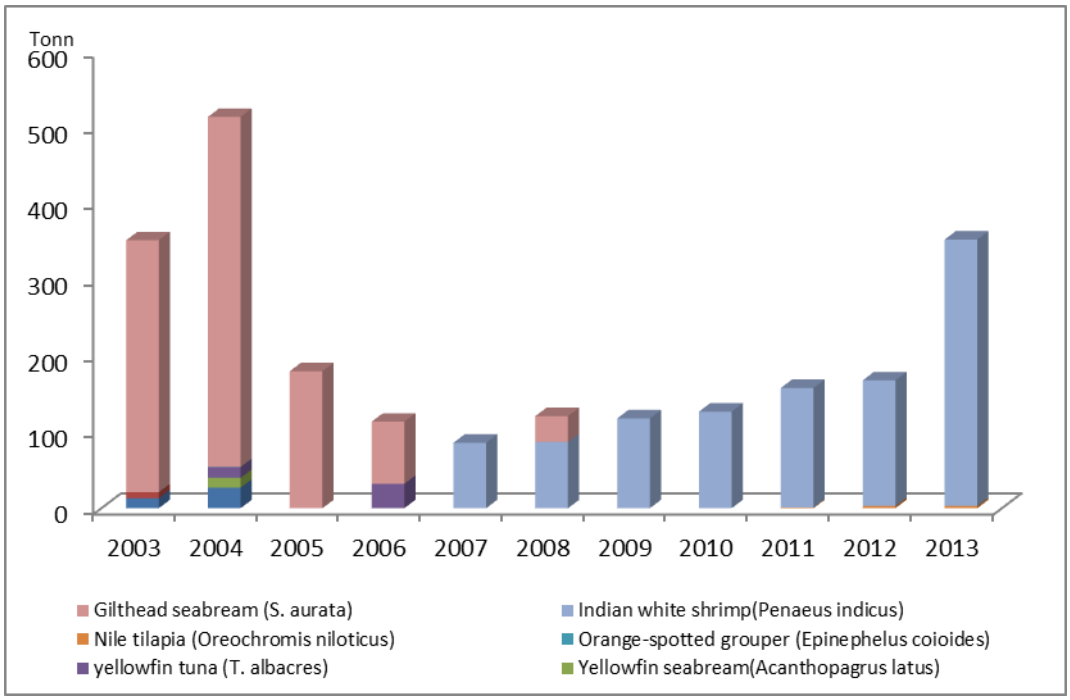

Figure 2. 5 Aquaculture productions in Oman (tonnes) for the period 2003-2013.

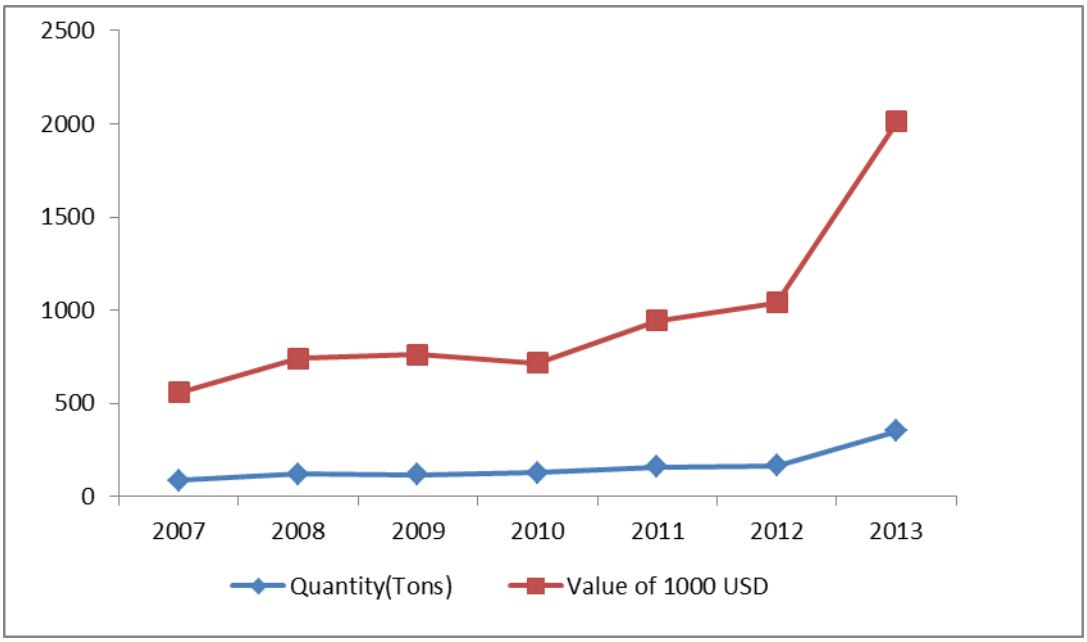

Figure 2. 6 Production and value from aquaculture for the period 2007-2013.

The majority of aquaculture production in Oman is exported to the United Arab Emirates; with a small quantity sold on the domestic market. This is due to the price and also to the

local customer preference for fresh fish. The export was in the form of whole fish (MAF, 2012).

The latest economic feasibility study conducted on fish farming in the Sultanate of Oman concluded that production from fish farming might yield approximately 10,000 tonnes per year during the first three years of development, and could be raised to 36,000 tonnes per year during the following ten years, and to more than 200,000 tonnes within 20 years (Atlas of suitable sites for aquaculture projects in the Sultanate of Oman, Ministry of Agriculture and Fisheries Wealth, 2010). By 2002, preliminary plans for national aquaculture development had been formulated (Mevel *et al*., 2002). This was followed by the preparation of a strategy document to assist in finalizing the process (FAO, 2007), and the preparation of environmental best management practice guidelines for various forms of aquaculture (MAF, 2010).

2.1.4 Laws and regulations for aquaculture

Although aquaculture began in the Sultanate in 1986, there was a delay in starting commercial investment because the country recognized the importance of initially focusing on legislation, regulations and developing a road map for aquaculture to promote fish farming on a sustainable basis. Also, the lack of specialized manpower and qualified specialists in this field has hampered its development in the country. The Ministry of Agriculture and Fisheries Wealth has issued aquaculture and related quality control regulations (Ministerial Decision no. 177/ 2012) and has issued regulations for aquaculture and the quality control of cultured organisms and licensing and quarantine procedures, which were prepared in cooperation with the Ministry and experts from the Food and Agricul-

ture Organization (FAO). In addition to the main law of Fishing and Protection of Living Aquatic Resources, the Ministry of Regional Municipalities Environment and Water Resources has prepared the Environment-Friendly and Better Management Practices (BMP) Manual for Aquaculture, which includes the guidelines for the production of eco-labeled and organically certified aquaculture products (MAF, 2010). The Ministry of Agriculture and Fisheries Wealth has also issued the Atlas for Establishing Aquaculture Sites that has evaluated all potential aquaculture sites in the whole Sultanate in relation to geographical, topographical, climatological and oceanographic features, as well as for the availability and utilization of water and land resources, relevant infrastructures and other characteristics (MAF, 2011). Investment Guidelines have been prepared by the Ministry of Agriculture and Fisheries Wealth (MAF) for the purpose of providing information to potential investors for development of aquaculture projects in Oman The information includes financial, geographic, commercial, and legal or technical matters (MAF, 2011), and there is a special chapter including a list of incentives for farming (Box 1) (investment guidelines for aquaculture development in the sultanate of Oman, 2011)

Box2.1 Incentives for farming

The government's incentives for farmers and investors include:

- ✓ Allocation of spaces of 3020 hectares for aquaculture projects
- ✓ Support and facilitate the procedures to be completed by the investor to obtain the necessary permits for projects aquaculture according to production capacity, the technical information, administrative and contribute to solving the problems faced by the investor during the construction and operation and provide the most appropriate solutions to overcome them.
- ✓ Provide the necessary support to the industry through the provision of production inputs and facilitate the role of the private sector.
- ✓ Supply of sites, soft loans, no tax to import equipment and farm inputs and no income tax during five years.
- ✓ Localization and transfer of fish farming techniques and results of research for investors.
- ✓ Giving a grace period of the lease
- ✓ Support the establishment of individual initiative projects in order to open the livelihoods for the young of Oman graduates who are capable of production in this sector.
- ✓ Support the fish farms integrated with rural agriculture for optimum result utilization of water and agricultural space.
- ✓ Helping to get funding for aquaculture projects from government and private banks.
- ✓ Periodic follow-up before and after the operation of these projects with specialists in this field to provide advisory services and technical guidance and the stages of production and marketing.
- ✓ Prepare and making seminars, conferences and scientific meetings and guidelines designed to support the industry.
- ✓ Issued many publications, articles and manuals in all areas of aquaculture facilitate the issuance of veterinary health certificates for farmed aquatic products exported to other countries.

2.2 study aims

The main objective of this study was to identify and evaluate current practices of tilapia farms in Oman.

2.3 Materials and Methods

2.3.1 Study area

Originally 12 tilapia farms were identified as potential study sites for this project. However, 3 farms were difficult to access, and hence not visited or included in the study. Therefore, the study sites included 9 farms located in four areas in Oman (Al Batinah – North, Ash Sharqiyah - East, Ad Dhahirah - North, Ad Dakhiliyah – North) (Figure 2.7). Most farms are located more than two to four hours' drive away from MAF. See Appendix 2.1 for a list of farms that have been studied.

Figure 2. 7 Map showing farms visited in the survey.

2.3.2 Data collection

The tilapia farms were selected from the fisheries profile records provided by the Ministry of Agriculture and Fisheries (Aquaculture Center), where a list of tilapia farms in the region and their locations was obtained. Each farmer was contacted through telephone calls prior to site visits to help with compliance. To facilitate data collection, questionnaire forms were completed with the farm owner or manager at the time of visit. The questionnaire interviews were conducted from September 2012 to November 2012, and were designed to obtain as much information as possible about the current status of tilapia farms in Oman. The questionnaires were divided into several sections. The first part focused on the farmers and farm information, the second section on husbandry, pond preparation, fish stocking and pond management, the third part on production, and the fourth section on disease and health management, and finally a small section about information and knowledge. See Appendix 2.2.

2.3.3 Water analyses

Through field survey between 9 -10 am in the morning, water parameters, including temperature, pH using a Model 3020, Jenwey, UK portable pH meter, dissolved oxygen, and total hardness were checked [the latter using a DR/890 Portable Colorimeter (HACH, USA)]. Water samples were collected in sterilized containers, and transported to the laboratory to check alkalinity and total ammonia according to the method proposed by APHA (1995).

2.4 Data analysis

All the data were categorized, summarized and analysed by MS-Excel 2003, and then presented as textual, tabular and graphical forms to evaluate the present status of tilapia farming in Oman.

2.5 Results

2.5.1 Farm information

Currently, there are 12 tilapia farms dispersed across different regions in Oman that were established between 2008 and 2012. Most farms started operation in 2011, and are located in Al- Mudhaibi (Ash Sharqiyah - East) as shown in Table 2.1. Nine farms were chosen for answering the questionnaire due to difficulties with accessing the other three farms. All farms use concrete ponds, and follow integrated farming systems. All the farmers employed workers, who were from Asian countries, particularly India and Bangladesh, with some background in fish farming and husbandry practices.

Table 2. 1 list of tilapia farms visited and surveyed by use of questionnaires.

Governorate	**wilayat**	**No. of farms**	**Operation year**
Al Batinah - North	Barka	1	2010
Al Batinah - North	Sohar	1	2008
Ash Sharqiyah - East	Al- Mudhaibi	5	2011-2012
Ad Dhahirah - North	Ibri	1	2011
Ad Dakhiliyah - North	Manah	1	2011

2.5.2 Background information about the farmers and their farms

The majority of the farmers interviewed has attained higher educational qualifications (Table 2.2), and have 2-5 years' experience of working on farms.

Table 2. 2 Educational level of the farmers.

Education level	No. of farmers
Primary school	1
Secondary School	4
Bachelor degree	3
Master's degree	1

All farmers showed a great deal of interest during interviews, which reflects the extent of their love and concern for tilapia farming. Most of them practice tilapia culture as a secondary activity in addition to their regular occupations. Mr. Haitham AL-Maskari, Al-Mudhaibi (Ash Sharqiyah - East) *began fish farming in his house with only one pond. Eventually he noticed a big market demand for the fish, especially from the expatriate communities, such as the Pakistanis, Bangladeshis and Indians. He then requested support for land from the MAF to build his farm in a wider and bigger scale of operation*.

Some of the farmers are hobbyists. Thus, Mr. ALHarthy (Al- Mudhaibi (Ash Sharqiyah - East) commented during the interview that *he practices tilapia culture as a hobby and now after his retirement he will find it as a source of livelihood.*

All farmers interviewed cultured fish in cement ponds. Three of the farmers used single ponds whereas others operate between 3 and 13 ponds. See Figure 2.8.

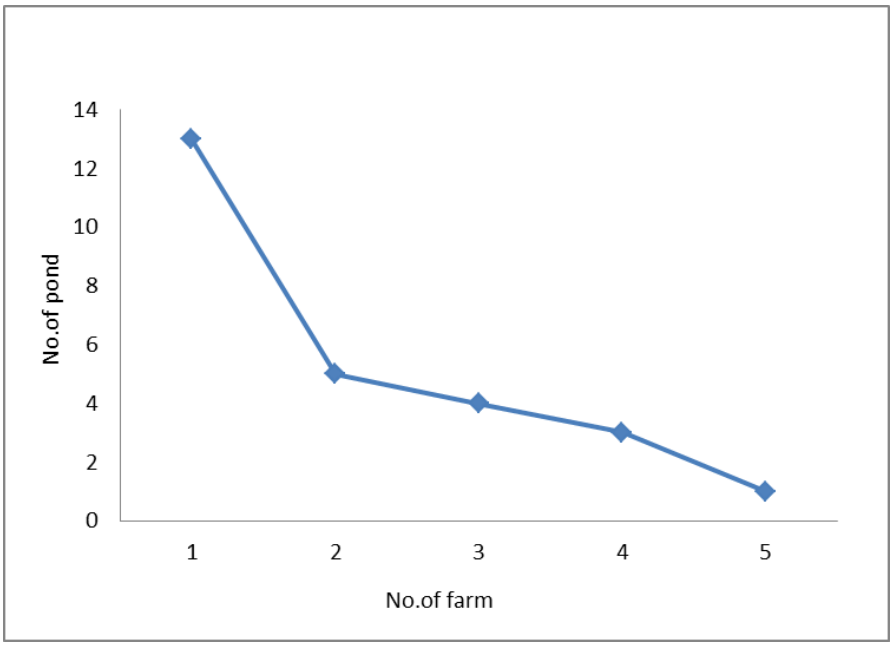

Figure 2. 8 Number of ponds per fish farm.

The size of tilapia ponds ranged from 4 m^3 to 254.8 m^3, with the average being 52.7 m^3. The average size of individual fish was approximately 600 g, with the largest fish being 1 kg, and the smallest of 450 g. The number of ponds, size and fish stocking density varied from farm to farm, and also varied within individual farms (Table 2.3). Most of the ponds are typically small. This is due to the fact that fish culture is often not the main occupation of the farmers, and also reflects the limitations of land area owned by the farmers. As a result of government encouragement and support for the aquaculture sector, all farmers considered that they would be likely to request larger land ownership, and some of them stated that they would request more government support to increase the number of ponds to 20-30 ponds for each farm.

Table 2. 3 Number of ponds per farm and range of pond dimensions and stocking density.

Farm code No.	No. of ponds	Dimensions (L x W x H)	Stocking Density (m3)
1	3	10m x 5m x 1.2m	2000
	1	2m x 1.3m x 1.2m	100 brood stock fish
2	2	4m x 1m x 1m	1500-2000
	1	4m x 2m x 1m	13 brood stock fish
3	1	13m x 5m x 1.8m	1200
4	4	12m x 5m x 1.8m	22000
5	2	3m x 3m x 1.8m	1140
	2	9m x 3m x 1m	1140
	1	12m x 5m x 1m	3738
6	1	4.60m x 4.60m x 1m	3000
7	1	4m x 6m x 1.20m.	3000
8	2	4m x 1m x 1m	1500-2000
	1	14m x 14m x 1.30m	4000
9	3	3m x 12m x 1.20m	100 male and female 300 fish
	1	3m x 12m x 1.20m	1000
	5	3m x 5m x 1.20m	15 male and 30 female fish
	4	3m x 12m x 1.20m	1200
	1	2m x 20m x 1.20m	35000 fingerling fish
	12	hatchery with 12 breeding basins	-

Explanation: L= length; W= width; H= height

2.5.3 Source of the tilapia

Currently tilapia farming is dominated by the culture of a single species, *Oreochromis niloticus*. The largest tilapia operation in Oman is Manah farm, which buys its brood stocks from Thailand. However, some farmers obtained tilapia seed stock from other sources, i.e. wadis, falajs and dams. None of the tilapia farmers possess their own hatcheries, and they mostly depend on one farm in Manah (Ad Dakhiliyah - North) belonging to Mr. Esa AL-Sultani for seed stocks. There is health certification provided along with the fingerlings imported from Thailand.

2.5.4 Water quality

Most farms followed a similar style of farming practices, from stocking to harvesting procedures with little if any variation between sites. Cleaning and leaving the pond to dry for periods of several hours to one day was the most commonly used form of preparation before stocking. Well water was the only source of water used on all farms, with the farmers exchanged water in their ponds weekly or on alternate days, or as desired depending on water quality requirements. Of all of the sites visited, just one farm checked water quality using a kit, which measured pH, temperature and dissolved oxygen whereas other farmers managed pond water mainly as a result of visual observations and personal experiences. Nevertheless, many farms experienced water quality issues in terms of turbidity and smell. Some farmers observed the frequent surfacing of fish which gulped for air indicating the lack of oxygen.

Mr Khalid AL-Meqbali and Mr. Khalfan AL-Sharji reported that they lost 50% and 7%, respectively, of their stock in one week due to deficiency in oxygen levels. So, they decid-

ed to exchange the water frequently, and additionally, decided to buy aerators to increase the dissolved oxygen levels in the water.

The values for physico-chemical parameters of the water samples taken from the culture ponds are shown in Table 2.4. All parameters are within Omani legislation for aquaculture and quality control of cultured organisms ((Ministerial Decision No.12/2009 2009) and (Ministerial Decision No.177/2012)).

Table 2. 4 The value for water parameters on tilapia farms.

parameter	**Farms codes No.**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Temperature c°	30.50	31.60	28.90	28.60	31.10	29.70	33.30	31.10	31.60
pH	7.80	7.69	9.19	7.58	7.81	7.59	7.75	7.61	7.690
Dissolved oxygen (mg/l)	5.7	4.82	3.5	3.871	6.06	8.30	6.30	7.50	4.820
Total ammonia (mg/l)	0.56	1.19	0.33	0.931	0.82	0.32	0.31	1.20	1.510
Total alkalinity (mg/l)	176.30	196.60	186.20	174.10	151.10	116.10	78.60	187.20	196.00
Total hardness(mg/l)	145.34	251.94	137.89	139.17	68.42	282.3	62.66	165.4	251.94

Approximately 60% of the farmers used electrically powered aerators to increase the dissolved oxygen in the water (Figure 2.9). Moreover, most of the farmers noticed increased water quality and better growth and welfare of the fish after they started using aerators. Water exchange was performed on most of the farms, except for two sites that used air circulation systems and exchanged 50% or 70% of the water as a husbandry practice.

Figure 2. 9 Aerators hanging from the roof of fish rearing tanks.

2.5.5 Feeding

The majority of farmers fed their fish with a combination of commercial fish feeds, which are imported from Saudi Arabia and distributed by MAF, and commercial chicken pellets and trash fish. Just one farmer (Manah farm) prepared his own recipe for the stock. All farmers observed that the growth rate and size of fish increased rapidly in a short period when they fed their fish with commercial fish feeds compared with chicken pellets and trash fish. Most farmers did not know exactly how much feed should be fed to the fish or the scientific basis of fish nutrition. They used empirical knowledge and experience to judge how much to feed. Just two farmers, Mr. Mohammed Al-Ryami from Barka (Al Batinah North Governorate) and Mr Esa AL-Sultani from Munah (Ad Dakhiliyah – North) used information on feeding 2.5 to 3% of the total fish biomass, daily. Farmers either fed their fish once every two days, once a day or twice a day. The fish were generally fed by scattering food widely to ensure that the food reached all the fish in the pond.

All farmers stored the fed on raised platforms or pallets in secure concrete or wooden rooms to ensure protection from rain, sunlight and humidity.

2.5.6 Production

Stocking, length of the culture cycle and harvesting times vary and depend mostly based on consumer demand. All farmers knew the individual size of the fish harvested, with an average size of 625 g at harvest, and with a selling price of US$ 3. The fish weight varied from one farm to another, ranging between 0.4 -1 kg being mainly dependent on market demand. If the fish were sold in the local market, the farmer was more likely to know the weight of individual fish compared to a farmer, whose fish harvest was for their own family's consumption. The results of the survey showed that farmers who sold their fish in local markets realized economic benefits from tilapia farming, expressing high levels of satisfaction and financial returns.

Mr Majid AL-Mangi from Al- Mudhaibi (Ash Sharqiyah east) sells his fish to restaurants at a price of 5USD for each fish and in his own words; there is great demand for his fish.

Simple harvesting methods are employed to harvest the stock from tanks. Usually one third of the water is emptied, and two workers drag a net along the length of the tank to concentrate and trap all the fish in the net before removal. See Figure 2.10.

Figure 2. 10 Harvesting of fish on a tilapia farm.

2.5.7 Knowledge of health management practices

The questionnaire was designed to determine the ability of farmers to recognize and identify fish diseases and other health problems. It was concluded that the farmers did not know much about fish diseases, however, they knew that their fish were not really healthy based on simple observations of mortalities, abnormal appearance, reduced growth, change in skin colour and abnormal behavior. Most farmers (56%) experience mortalities on their farms, and they related this mortality to water quality, overfeeding, and change in skin colour (Table 2.5).

Mr. Haitham AL-Maskari said that he lost 10% of his stock in one week because the cultured fish were in high stocking density; Mr. Sultan AL-Abri lost 5% of his fish because his workers overfed the fish.

The survey revealed that most farmed fish exhibited stunted growth and deformities, which may be attributed to poor fish nutrition.

Table 2. 5 Mortalities experienced by each farm.

Farm No.	Mortality (%)	Frequency	Farmer's description of the reasons for fish mortalities
1	No mortality		
2	50%	One time	turbid water/ lack of oxygen
3	No mortality		
4	2%	One time	lack of oxygen
5	10%	One time	change in skin color
6	10%	One time	high stocking density
7	No mortality		
8	5%	One time	overfeeding
9	No mortality		

2.5.8 Response of fish farmers to disease outbreaks.

Not any of the farmers, who experienced fish mortalities, contacted MAF. Usually, the farmers collected dead fish and used them as manure on the farm. It was apparent that the majority of farmers relied on their own experiences to apply what they felt were appropriate disease control measures. This was reflected in the fact that most of the farmers had not received any form of training on fish health and disease prevention.

2.5.9 Disease prevention measures used in aquaculture in Oman

All farmers took some form of proactive measures to prevent the outbreak of disease in their ponds. This included reducing stocking density, improving water quality, reducing water turbidity, and improved pond preparation methods, such as cleaning and drying. Some of the farmers covered the ponds with green shades to protect fish from high temperatures, especially during the summer months.

2.5.10 Fish disease treatment

Through interviews with the farmers, all of them claimed not to treat their fish before or when mortalities occur with any kind of treatment (chemical or antibiotic).

2.5.11 Information and knowledge

Two of the farmers were trained in tilapia aquaculture in Thailand as part of a training program arranged by MAF, whereas the other farmers received some tilapia farming information from MAF mainly on pond preparation, fish seed, stocking density, fish feed and water quality. Not any of the farmers received any information regarding fish health. The majority of farmers were not familiar with best practices of pond preparation. All farmers felt strongly that they needed to get training about culture techniques and farm management. The majority of farmers gained aquaculture information by interacting with other farmers, and by searching the Internet.

2.6 Discussion

There is no doubt that Oman is still in the very early stages of aquaculture development. However, the country is poised to make heavy investments and technical and manpower development to provide the infrastructure for sustainable development of this food production sector. No doubt, the importance of tilapia farming will increase in Oman, and will provide much needed protein for consumers. The offshoot will be economic progress in the rural coastal areas. Aquaculture will also help solve the problem of water shortage and salinization of the soil in some farms within the Sultanate. Farming tilapia will reduce the losses resulting from salinization of agricultural land as well as providing another income stream in rural areas. Although the government currently has finalized and publicized rules and legislation that guarantees sustainable aquaculture, there is a lack of application at the individual farm level.

The results of this study show that the first tilapia farm in Oman started in 2008, and the number of farms has continued to increase to 6 sites in 2011. A continuous increase in the number of tilapia farms due to governmental encouragement reflects financial support particularly in the form of loans, training, and the provision of fingerlings. Currently, integrated farming systems using concrete ponds are the only type of freshwater system used in Oman for culturing Nile tilapia (*Oreochromis niloticus*). This system is carried out in land where high levels of groundwater salinity preclude other agricultural activities. Water quality as well as water source is considered as a key factor in good aquaculture practices.

Effective water management in any fish holding facility is certainly important for successful aquaculture operations (Ahmed *et al*., 2009; Hossain *et al*., 2006). The survey re-

vealed that water quality problems were found in some farms as monitoring had not been conducted adequately. Farmers do not have any tools for checking water quality (oxygen, pH and temperature), which are essential aquaculture practices and although all farmers knew the importance of monitoring water quality, only one of them (Manah farm) checked it regularly. Even this farm used only the basic parameters of water temperature and pH.

A number of farmers have experienced mortalities, which were considered to be attributable to poor water quality, overcrowding or due to excessive feeding. Unfortunately, none of the farmers communicated data to the Ministry in order to verify and diagnose the problem. Many fish were suffering from congenital deformities and stunted growth, which may be due to hereditary or nutritional deficiencies. Some farmers raised fish obtained from falaj and wadi, which are unknown sources. Actually, there is a plan by the Aquaculture Centre to be the sole supplier for all fish fingerlings in the country to control the biosecurity, health and quality of tilapia grown in Oman. Certainly, disease is recognised to be one of the major constraints resulting from intensification of aquaculture, and may eventually become a limiting factor to the economics of a successful and sustainable aquaculture industry.

Intensive fish production often results in increased disease due to poor water quality and the high stock densities used. Tilapia is susceptible to a number of infectious agents including bacteria and parasites (Shoemaker *et al.*, 2006). Streptococcosis, *Flavobacterium columnare*, *Aeromonas* septicaemia (MAS), vibriosis, columnaris, edwardsiellosis, saprolegniasis, ciliates, iridovirus, protozoan parasites and monogenetic trematodes were the

major disease threats recognised as relevant to tilapia culture (Lim & Webster, 2001; Komar *et al*., 2008). In the present study, there were only a few reports of diseases in Omani fish farms, but this may reflect the lack of understanding of the concept of disease and water quality problems, and the importance of communicating information to the authorities. The farmers were unaware that the presence of unhealthy fish could have implications for the overall health of the stock, and could lead to higher mortalities. This was reflected in the absence of any form of training on fish health and disease prevention.

The results of this work showed that most farmers relied on their own experience and knowledge in growing fish; the farmers gained their knowledge from various sources including the Internet as well as from local advice on the management of tilapia culture. However, the survey was instrumental in creating awareness among the farmers by making them realize the importance of communicating their problems in farming operations. As a consequence of the survey, many farmers started communicating with the Ministry in order to obtain technical information on farming methods, health and disease information and for submitting samples for fish disease diagnostics. The Ministry has been trying to provide support to farmers, but there are issues with communication. Indeed, there is a regulation about aquaculture and related quality control, but it has not yet been implemented in an appropriate manner. In order to develop a sustainable tilapia culture in Oman, it is certainly necessary to implement rules and regulations at the farm level, and to ensure that the regulations are adhered to by all tilapia farmers.

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Bacterial species diversity and distribution in the aquatic environment in the Sultanate of Oman

3.1 Introduction

Aquaculture is an emerging industry in Oman, which presents a promising proposition for diversifying and augmenting Oman's oil-based economy. Although the Omani fishery sector is a renewable natural resource, constant monitoring, management actions and regulation policies are required to ensure the sustainability of Omani stocks.

While global aquaculture production is expected to rise to meet the shortfall in wild catches, there is a parallel requirement to identify potential threats to the health and welfare of existing commercial fisheries and of farmed stocks, and to take appropriate steps to mitigate/minimize them (FAO, 2012). Fish losses through infectious diseases may have serious impact on the sustainability of wild and farmed aquatic animals. Disease dynamics in farmed aquatic animal populations is not always a simple cause and effect relationship. There are many related factors including the interactions between the environment, host susceptibility and the presence of a virulent pathogen, prior to establishing a disease outbreak. There is a wide range of clinical agents responsible for a diverse array of diseases affecting all farmed fish species. Differentiation of the causes of infectious and non-infectious fish losses is an important aspect of developing an optimal farmed fish health management strategy, and should be fundamental when dealing with an emerging aquaculture sector.

In aquaculture, behavioral changes are often the first signs that the farmers use to identify an abnormality in their population (Martins *et al*., 2012). The cause of the behavioural changes may be multifactorial, and it is essential to recognize these changes as early as possible and check the stocks for the presence or absence of clinical signs, often presented grossly as abnormalities in the appearance of the fish. The clinical signs presented on the affected fish will depend on the type of host, age of the fish and stage of disease (acute, chronic, carrier) as well as infectivity of the pathogen. Furthermore, in some cases, there is no connection among the rate of mortality and significant physical damage (internal and external injuries). In fact, some infected fish with high mortality rates have a healthy appearance while others have low mortality with significant physical damage (Yiagnisis & Athanassopoulou, 2011).

Bacterial species are ubiquitous in the aquatic farming environment where the general consensus is that most aquatic bacterial pathogens are opportunistic pathogens. Infections due to opportunistic pathogens occur when environmental conditions are less favourable for the fish resulting in a stress response in the host and allowing the bacterial species to establish and cause an outbreak resulting in disease. A typical example of this type of aquatic bacterial pathogen is *A. hydrophila*, which causes motile *Aeromonas* septicaemia (MAS), and is one of several species comprising the MAS complex. Conversely, some bacteria are considered to be obligate pathogens, being rarely found in the absence of a host, and include *A. salmonicida, Renibacterium salmoninarum* (the aetiological agent of bacterial kidney disease) and *Mycobacterium* spp. (Austin & Austin, 2012 ; Palmeiro & Roberts, 2009).

Bacterial diseases are caused by Gram negative and Gram positive organisms. Gram-negative bacterial fish pathogens include representatives from *Aeromonas, Edwardsiella, Flavobacterium, Francisella, Photobacterium, Piscirickettsia, Pseudomonas, Tenacibaculum, Vibrio and Yersinia,* whereas Gram positive bacterial fish pathogens include *Enterococcus, Lactococcus, Mycobacterium, Nocardia, Renibacterium* and *Streptococcus* (Klesius & Pridgeon, 2011). Most bacterial infections of fish are caused by Gram negative organisms (Palmeiro & Roberts, 2009; Noga, 1996).

Clinical signs of bacterial infection in fish are rarely pathognomic. Infection may be internal and/or external. Internal infections result in septicaemias, involving many different organs/systems, with signs including the presence of fluid in the abdominal cavity, swollen kidneys and haemorrhaging. External infections may result in erosion of the finage, tail and mouth, and ulceration on the skin. Although more than 92 bacterial genera have been considered as pathogens for cultured and wild fish, just a small number of them have been reported as responsible for economic losses in cultured fish worldwide (Toranzo *et al*., 2005).

In 2009, > 10 million farmed channel catfish in the southeastern USA died as a result of a highly virulent form of MAS, caused by *A. hydrophila* (Hemstreet, 2010). In China between 1990 and 1992, > $120 million was lost due to three bacterial fish pathogens, i.e. *A. hydrophila, Yersinia ruckeri* and *Vibrio fluvialis*, that affected silver carp, bighead carp and crucian carp (Wei, 2002). Several *Aeromonas* spp. are pathogenic to fish causing epizootics, and may be isolated from naturally infected fish, including mullets and sea bream, and from brackish water (Manal, 2010). *Aeromonas* spp. (70.0%), *Flavobacte-*

rium. spp. (20%) and *Vibrio* spp. (10%) were recorded in the red spot emperor (*Lethrinus lentjan*) along the Kanyakumari coast of south India during August 2009 (Lipton *et al*. 2011).

Sudden environmental changes, such as the development of algal blooms, may lead to the development and spread of bacterial diseases (Hiney & Oliver, 1999). This phenomenon was recorded in the Sea of Oman and in the Arabian sea during 1976 to 2011 (Al-Azri *et al.* 2012). As a result of this sudden change to the environment, massive mortalities in fish and crustaceans (crab and shrimp) attributed to *V. anguillarum, Pseudomonas fluorescens, Streptococcus pyogenes, A. sobria* and *Yersinia ruckeri* were recorded in Qaroun Lake in Egypt (Abou El-Geit *et al.* 2013).

Sustainable aquaculture relies on the avoidance of infectious diseases (Salama & Murray, 2011), which, in turn, will reduce the costs of treatments, carcass removal and economic losses (Murray & Peeler, 2005). As aquaculture is being seriously considered as a promising sustainable industry in Oman, the government wants to make sure that proper control measures are in place for major risks. However, there are not any baseline data or information on the existence and prevalence of aquatic animal pathogens in the country. An understanding of the existence and distribution of aquatic animal pathogens will be extremely helpful in understanding the extent of involvement of farmed fish in the spreading of diseases, and to inform decision makers on the potential risk to wild populations. This will also provide policy makers with baseline information on the prevalence of fish pathogens in wild fish populations before any industrial level aquaculture activity is initiated in the country.

3.2 Study Aims

The main objective of this study was to investigate the bacterial flora in farmed and wild fish from different aquatic environments in Oman, and to establish a database of disease causing agents of Omani fish to serve the government in their plans to establish an aquaculture industry.

3.3 Material and Methods

3.3.1 Study Locations

The sampling locations are based on the zoning plan for aquaculture development as proposed in the Atlas of suitable sites for aquaculture projects in the Sultanate of Oman (MAF, 2010). Wild fish were sampled from three regions (Muscat, Sohar and Salalah), whereas farmed fish were examined from two aquaculture sites (Mudhaibi and Salalah). The sources of water in the aquaculture sites for tilapia were from underground systems, and sea water for abalone. The sampling of wild fish was from three main landing sites, each facing a different water source. Thus, Muscat and Sohar faces the Sea of Oman, whereas Salalah faces the Arabian Sea (Figure 3.1).

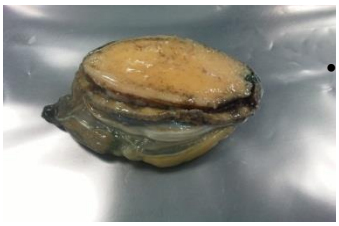
Figure 3. 1 Map showing the geographic locations of sampling sites in Oman.

(Wild fish ● , Farmed fish ●)

3.3.2 Animal samples

The fish and shellfish included in the study were chosen on the basis of commercial importance and their potential for use in Omani aquaculture (Table 3.1). Live fish samples were collected weekly from the study sites between 7.00 and 10.00 am over a period of 6 months, starting December, 2011 to May, 2012. All samples obtained from fishing boats at sea or from farms were immediately transported to the laboratory in transport containers with aeration and refrigeration to reduce stress. In some cases, moribund or freshly dead fish were transported chilled on ice in clean containers. In total, 417 fish (369 wild and 48 cultured animals) were examined in this study. All samples were transported within 1-4 h to the microbiology laboratory in the Fishery Quality Control Center in Oman.

Table 3. 1 Fish species, location and number of samples examined.

Type of sample	Fish species		Location	Weight (g) and Length of fish (cm)	Total per area	Total per species
Wild sample	Red spot emperor (*Lethrinus lentjan*),		• Muscat • Sohar • Salalah	537.22 ± 445.90g 32.11 ± 8.73cm	41	123
	King soldier bream (*Argyrops spinifer*),		• Muscat • Sohar • Salalah		41	123
	Spotted rabbit fish (*Siganus canaliculatus*)		• Muscat • Sohar • Salalah		41	123
Farmed sample	Abalone (*Haliotis mariae*)		• Salalah	116.06± 7.86 g 9.26±0.06cm	10	10
	Tilapia (*Oreochromis niloticus*)		• Mudhaibi	238.92± 145.32 g 23.40± 6.37 Cm	38	38
Total						417

3.3.3 Bacteriological Examination

On arrival at the laboratory, the fish were weighed and the length measured. Individual fish were necropsied according to Noga (1996), and five types of samples were obtained from each fish. Only muscle samples were taken from the abalone. From the fish, samples were taken by means inoculating loops from skin, gill and kidney, and were directly streaked onto the surface of tryptone soya agar plates (TSA; CM0131, Oxoid). Blood samples (0.1 ml) were taken from the caudal vein using a syringe and then streaked onto TSA plates. Additional samples were taken from external lesions. The plates were incubated at 30°C (similar to the field conditions) for 24–48 h. From each plate, colonies representing the most commonly occurring morphological types were picked and subcultured for purity. Smears were prepared for the Gram staining reaction and micro-morphology Catalase production and the determination of motility followed the methods of Frerichs and Millar (1993). Oxidase production was determined using Oxidase Strips (Ox-iStrips™, Oxoid). Then the biochemical profiles of the isolates were determined using a VITEK® 2 Compact bacterial identification system (bioMerieux, France) following the manufacturer's instructions.

3.4 Data Analysis

Microsoft Excel 2010 was used to store all the data, produce charts, tables, means with ± standard deviation (SD) and percentages (%).

3.5 Results

Most of the fish sampled in this study appeared normal as determined by gross examination of the external and internal organs. Tilapia was the only exception. Here, 10 tilapia displayed clinical signs of disease (Figure 3.2). The external signs of disease included the presence of body lesions, which were observed in 4 fish. External haemorrhages in the head, skin and at the bases of the fins were present in 4 fish; exophthalmia occurred in 1 fish; and anaemia was recorded from 1 fish.

Figure 3. 2 External symptoms of disease on tilapia.

A total of 417 fish were collected from the wild and aquaculture sites [n = 369 and 48 recovered from wild and farmed fish and shellfish, respectively], and from these aquatic animals, 420 bacterial isolates were recovered [n = 312 and 108 from wild and farmed fish and shellfish, respectively]. The total sample size of farmed fish (tilapia) and shellfish (abalone) was less compared with wild fish because of expense, low abundance, and sea-

sonal distribution in only selected sampling areas. The highest total of bacterial isolates recovered between fish and shellfish species was from wild king soldier bream (n = 132) and from cultured tilapia (n = 99) and shellfish (Figure 3.3).

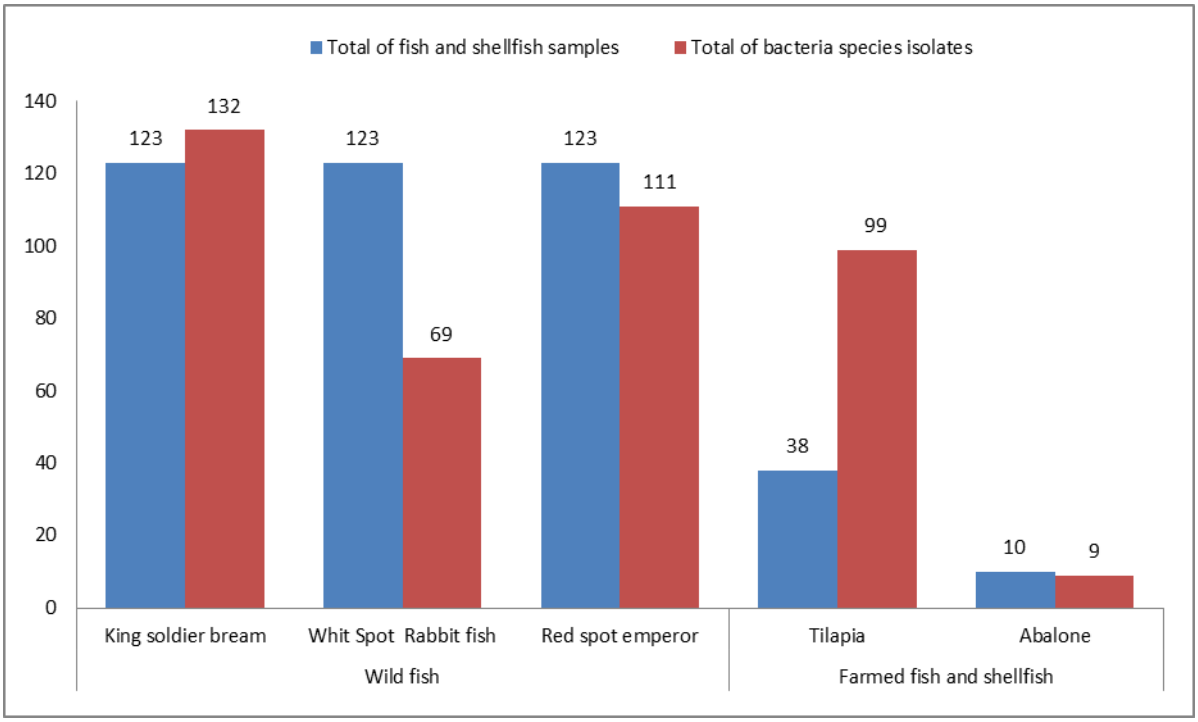
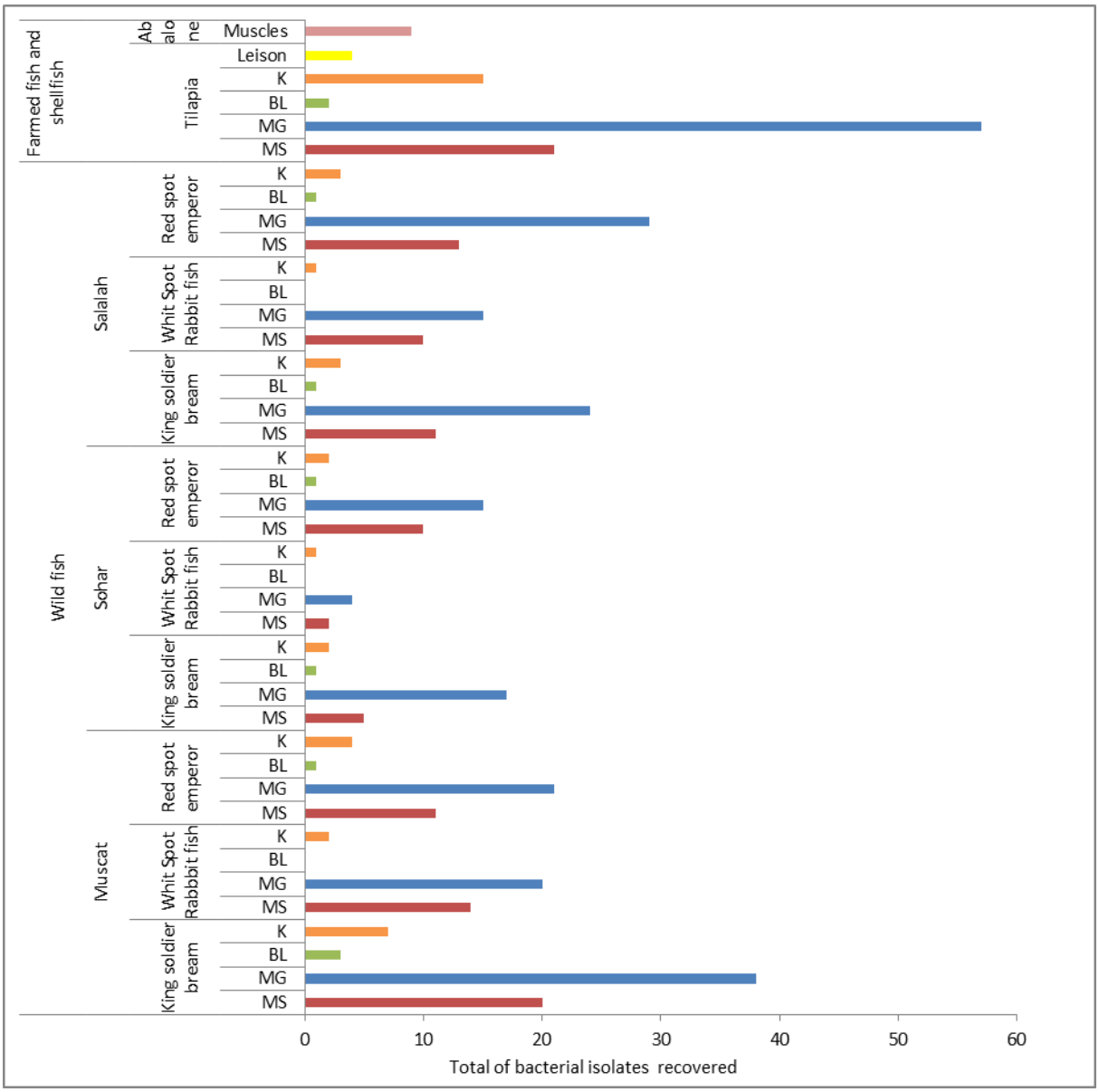


Figure 3. 3 Total number of bacterial isolates recovered from wild and farmed fish and shellfish.

From the tissue and biological samples examined, the highest number of bacterial isolates was recovered from the gill mucus (n = 240), and accounted for 57% of the total bacteria isolates. Tilapia gill mucus revealed the highest number of bacterial isolates (n = 57; 14% of the total), followed by king soldier bream gill mucus from Muscat area (n = 39; 9% of the total). Blood samples permitted the recovery of the lowest number of isolates (n = 10;

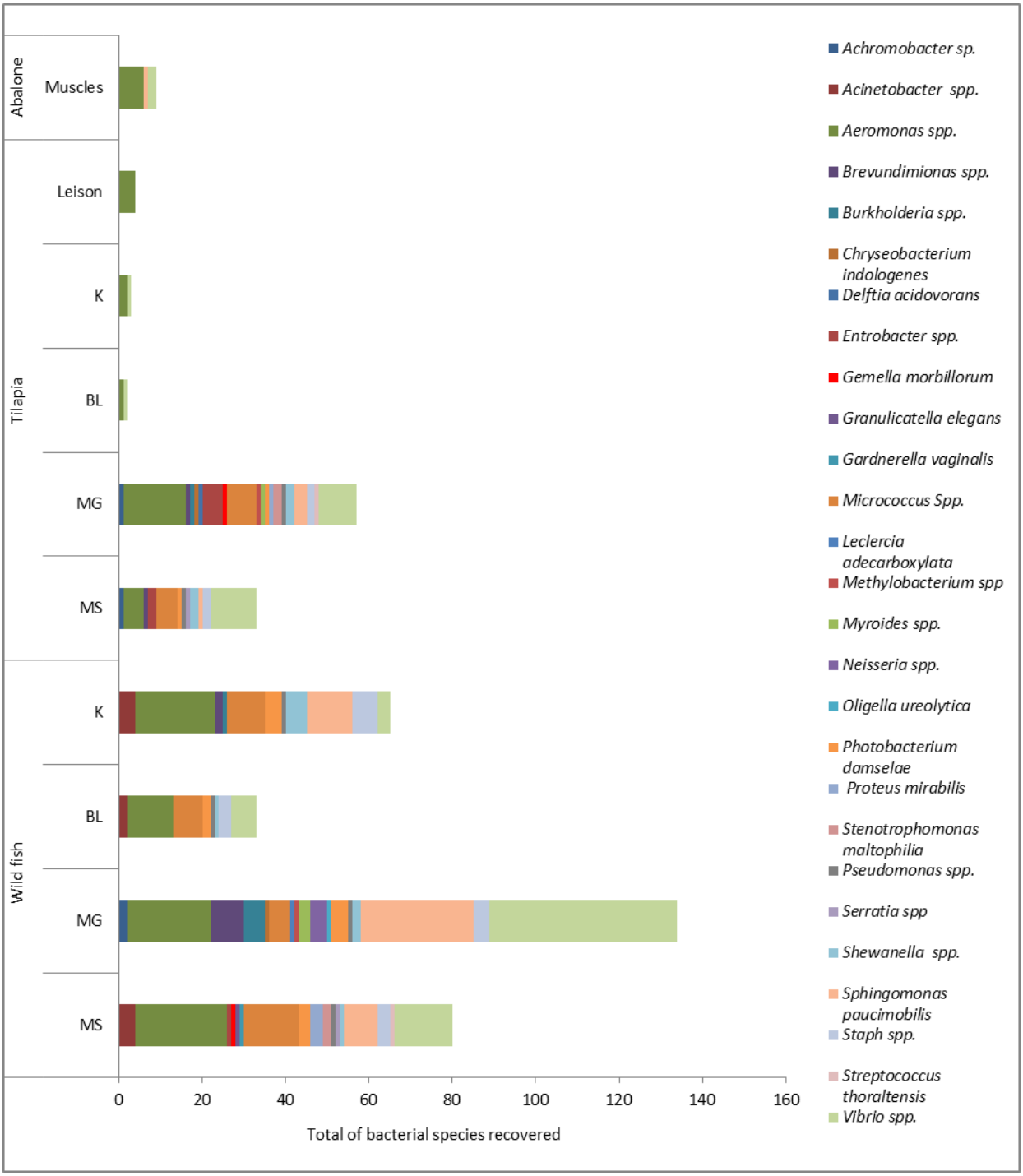
2% of the total). Moreover, in some white rabbit fish samples, bacterial isolates were not recovered from blood samples (Figure 3.4 & 3.5).



Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 4 Total numbers of bacterial isolates from fish and shellfish.

In Figure 3.5, it may be seen that wild and farmed fish gill mucus had the highest bacterial diversity, followed by skin mucus with 17 groups of bacteria. Also, abalone muscle, the lesions from tilapia, kidney and blood samples of both wild fish and farmed tilapia possessed between one to three species of bacteria and revealed the lowest number of bacterial species.



Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 5 Distribution of bacterial isolates among wild and farmed fish and shellfish.

The range of bacteria recovered from the different aquatic habitats over the study period has been summarized in Table 3.2. Thus, most of the isolates (n = 350) comprised Gram negative, rod-shaped bacteria; the remaining (n = 69) of the isolates were Gram-positive spherical organisms, and one comprised Gram variable rod shaped organisms. Of the Gram-negative bacteria, *Aeromonas* and *Vibrio* predominated. Conversely *Micrococcus* spp. and *Staphylococcus* spp. were the most common Gram-positive bacteria.

Table3. 2 Bacteria recovered from wild fish and farmed fish and shellfish from Oman

Total number of bacterial isolates species recovered		Phenotypic characteristics			Name of bacteria species recovered based on the Vitek system biochemical profile
Wild fish	Farmed fish and shellfish				
Gram-negative		**Motility**	**Oxidase**	**Catalase**	
2	2	+	+	+	*Achromobacter* spp.
10	0	-	-	+	*Acinetobacter* spp.
72	33	v	+	v	*Aeromonas* spp.
10	2	-	+	+	*Brevundimionas* spp.
6	1	+	+	+	*Burkholderia* spp.
1	1	-	+	+	*Chryseobacterium indologenes*
0	1	+	+	+	*Delftia acidovorans*
1	7	+	-	+	*Entrobacter* spp.
1	1	+	-	-	*Gemella* spp.
1	0	+	-	+	*Leclercia adecarboxylata*
1	1	+	+	+	*Methylobacterium* spp.
3	1	-	+	+	*Myroides* spp.
4	0	-	+	+	*Neisseria* spp.
1	0	+	+	+	*Oligella ureolytica*
13	2	+	+	+	*Photobacterium damselae*
3	1	+	-	+	*Proteus mirabilis*
2	2	+	-	+	*Stenotrophomonas maltophilia*
4	2	+	+	+	*Pseudomonas* spp.
1	1	+	-	+	*Serratia* spp.
9	4	+	+	+	*Shewanella* spp.
46	5	+	+	+	*Sphingobacterium* spp.
68	24	+	+	+	*Vibrio* spp.
Gram-positive					
16	4	-	+	-	*Staphylococcus* spp.
1	1	-	-	-	*Streptococcus thoraltensis*
1	0	-	-	-	*Granulicatella elegans*
34	12	-	+	+	*Micrococcus* spp.
Gram-variable					
1	0	-	-	-	*Gardnerella vaginalis*

Explanations: + = positive reaction, - = negative reaction, v= variable reaction

3.5.1 Results in terms of geographical location, fish and shellfish samples, and site of isolation

3.5.1.1 Wild fish

The distribution of bacterial species in wild fish collected from aquatic sites in Oman is shown in Figure 3.6. Thus, the results revealed that the species richness of bacteria associated with fish collected from Muscat (n = 141; 45% of the total number from wild fish), was higher than specimens from Salalah (n = 111) [36% of the total number from wild fish] and Sohar (n = 60) [19% of the total number from wild fish].

According to fish species within the different geographical area, the highest total of bacterial species recovered was from Muscat king soldier bream (n=68; 22% of the total]; conversely in Salalah and Sohar, red spot emperor exhibited the highest total of bacterial species at (n=46; 15% of the total) and (n=28; 9% of the total, respectively) (Figure 3.6).

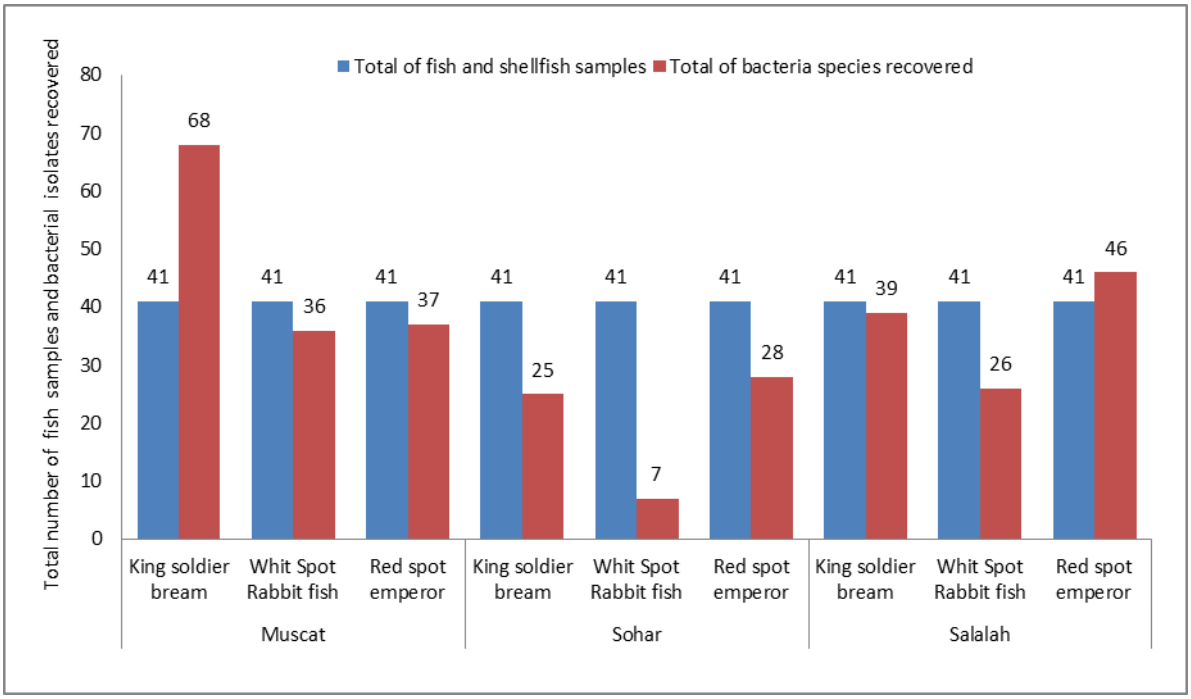


Figure 3. 6 Total number of bacterial isolates recovered from wild fish species with respect to the geographical sites.

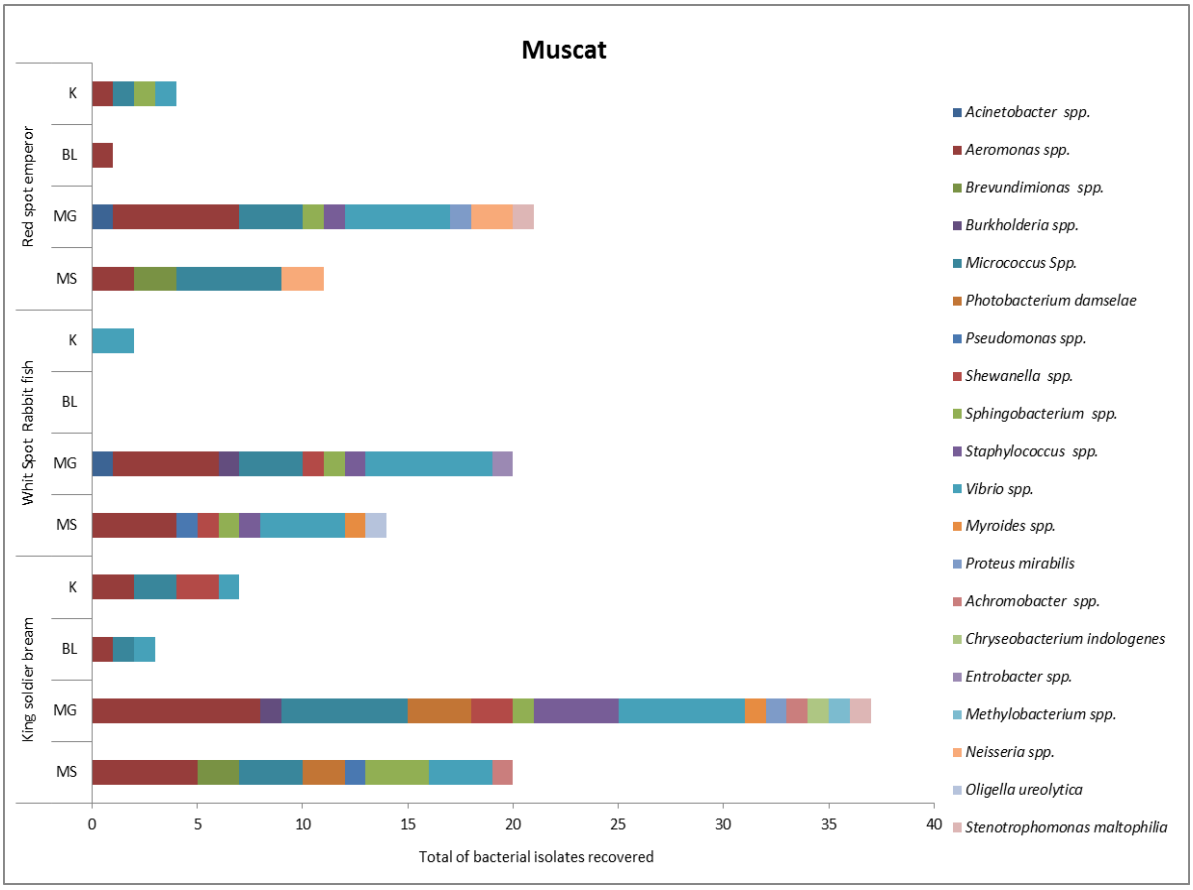
Figure 3.7, 3.8 and 3.9 shows that 21 bacterial groups were recovered from the total fish species examined from Muscat, and 16 and 13 bacterial groups were recovered from Salalah and Sohar respectively. It was also observed that the highest number of bacteria species was recovered from gill mucus samples, following by skin mucus, kidney and then blood samples. The last mentioned contained the lowest number of bacterial isolates recovered. In addition, gill mucus included a high number of bacteria species type ranging from between 9 and 15 species from Muscat, 3 to 10 species from Sohar, and 4 to 9 species from Salalah.

Generally, the blood samples revealed the lowest number of bacteria and the lowest diversity; sometimes bacteria were not recovered from blood as was the case with white

spot rabbit fish. Blood samples from Muscat contained *Vibrio* spp. from red spot emperor, and 3 species (*Aeromonas* spp., *Vibrio* spp. and *Micrococcus* spp.) from king soldier bream. In Salalah, *Aeromonas* spp. was recovered from king soldier bream, and *Sphingobacterium* spp. from red spot emperor whereas one species (*Aeromonas* spp.) was recovered from king soldier bream and red spot emperor (from Sohar).

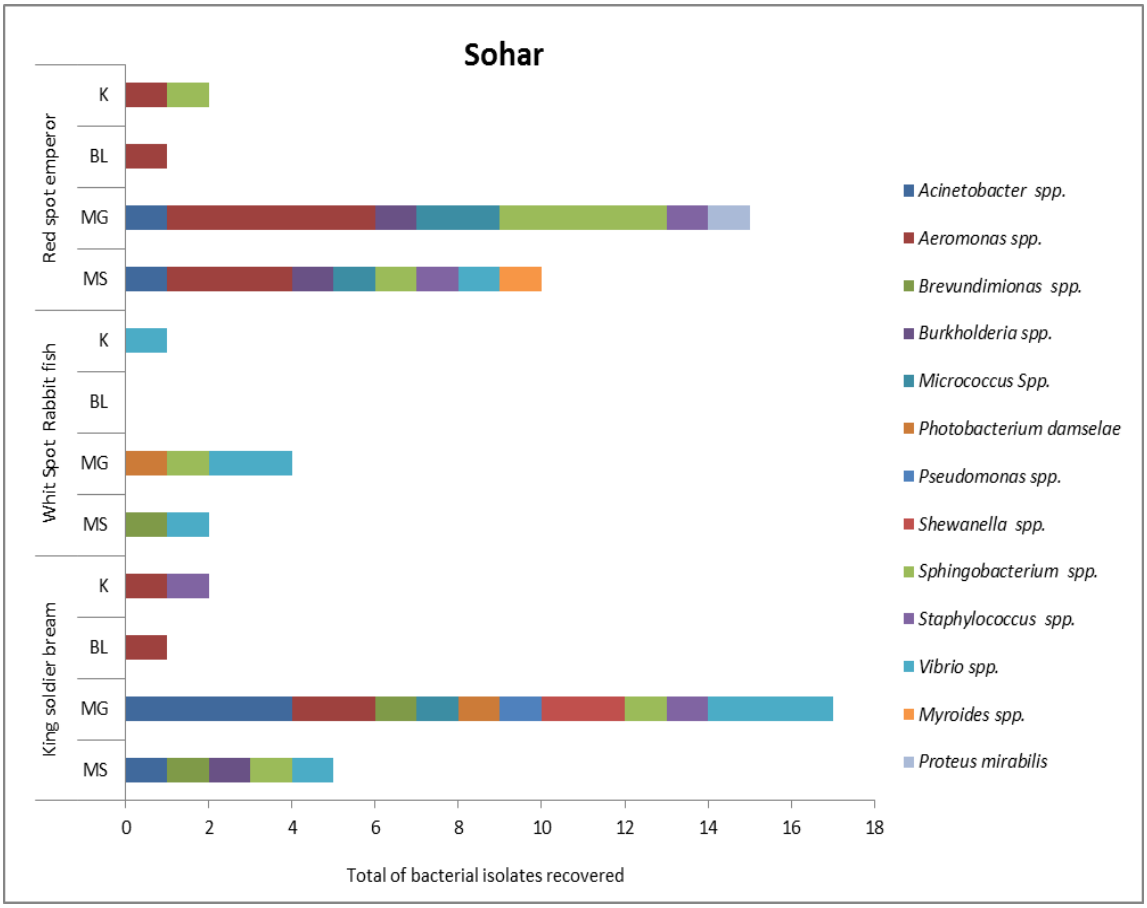
In some fish from Muscat, single groups of bacteria were recovered, e.g. *Vibrio* spp. were isolated from the kidney of white spot rabbit fish, and *Aeromonas* spp. were identified in red spot emperor blood samples. In Sohar, *Aeromonas* spp was recovered from the blood samples of king soldier bream and red spot emperor. *Vibrio* spp. were isolated from the kidney of white spot rabbit fish. In Salalah, *Aeromonas* spp. was recovered from blood sampled from king soldier bream, and the kidney of white spot rabbit fish. *Sphingobacterium* spp. were found in red spot emperor blood samples.

Sometimes, just one bacterial species was recovered from single whole fish samples and geographical locations, particularly in the case of fish from Muscat. Thus, *Chryseobacterium indologenes, Methylobacterium* spp. and *Streptococcus thoraltensis* were recovered only from king soldier bream; *Enterobacter* spp. was found only in white spot rabbit fish and *Neisseria* spp. was identified in red spot emperor blood. Also, *Gardnerella vaginalis* was recovered once from king soldier bream in Salalah.



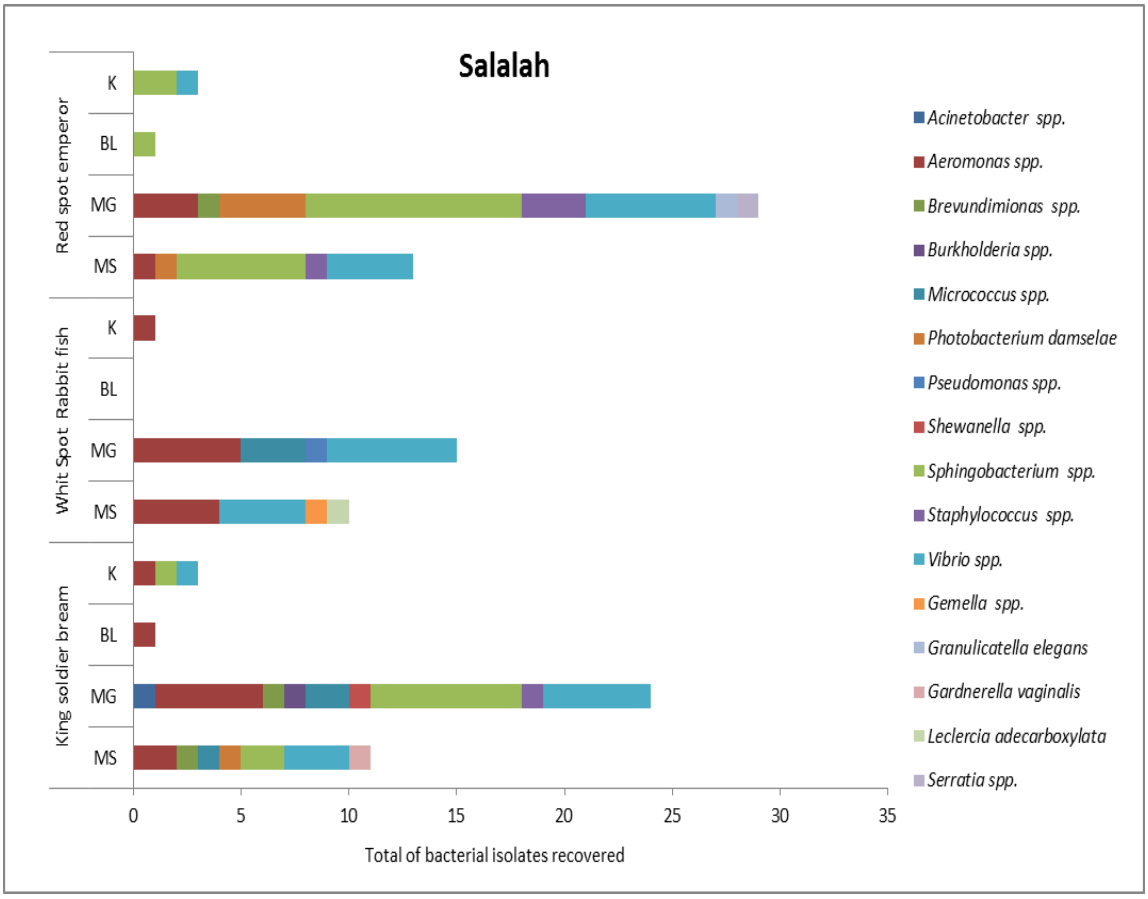
Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 7 Total of bacterial isolates recovered from Muscat, based on fish species and sample type.



Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 8 Total of bacterial isolates recovered from Sohar based on fish species and sample type.

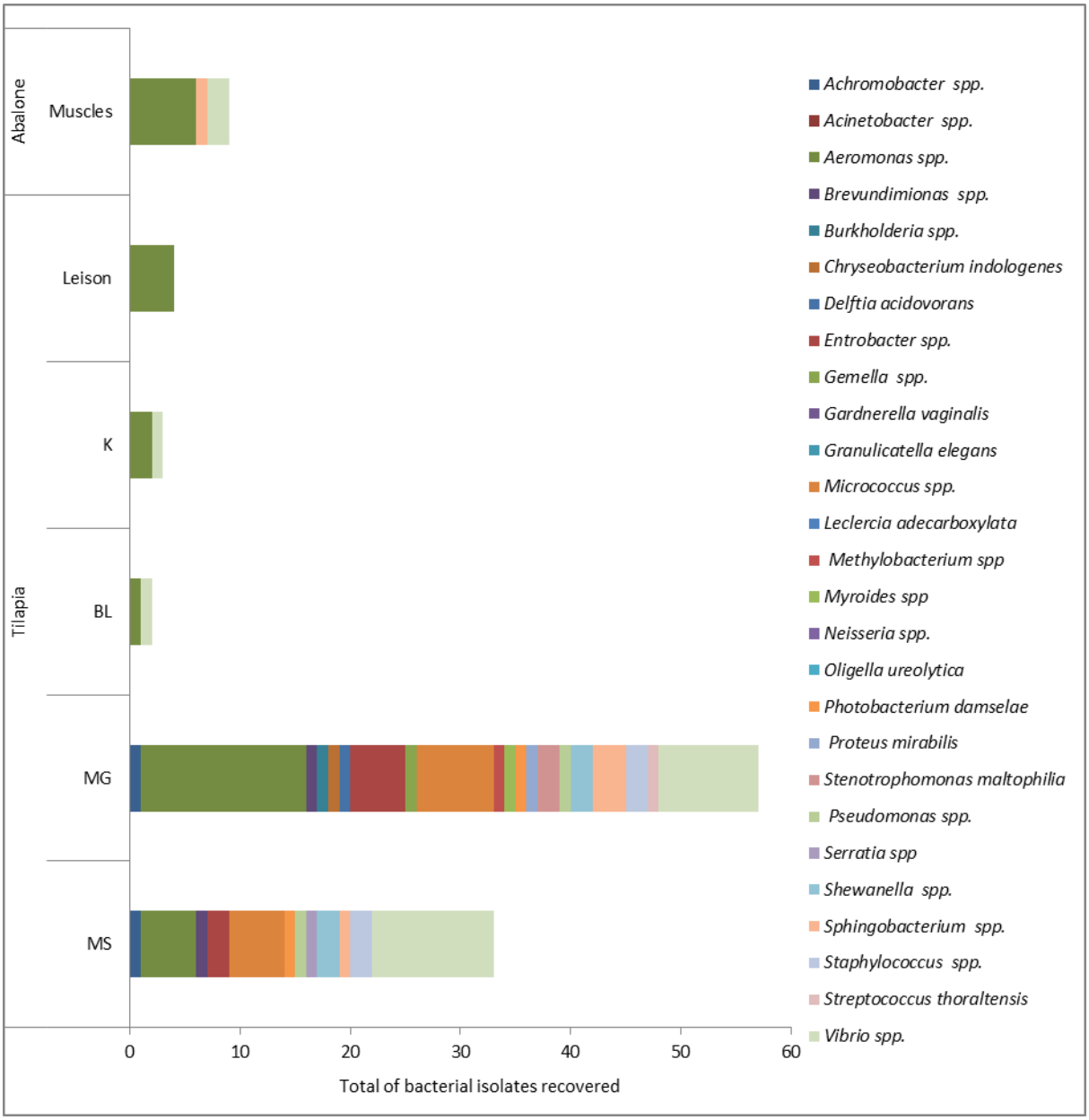


Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 9 Total of bacterial isolates recovered from Salalah based on fish species and sample type.

3.5.1.2 Farmed fish and shellfish

From Figure 3.10, it may be seen that 21 bacterial groups were recovered from tilapia, and three bacterial groups were isolated from abalone. It was observed that the highest number of bacterial species was recovered from tilapia gill mucus samples. Twelve bacterial species were present in tilapia skin mucus, whereas kidney and blood samples revealed two groups, i.e. *Aeromonas* spp. and *Vibrio* spp. Only *Aeromonas* was present in lesion samples from tilapia. In total, three groups were recovered from abalone muscle, i.e. *Aeromonas*, *Vibrio* and *Sphingobacterium*.

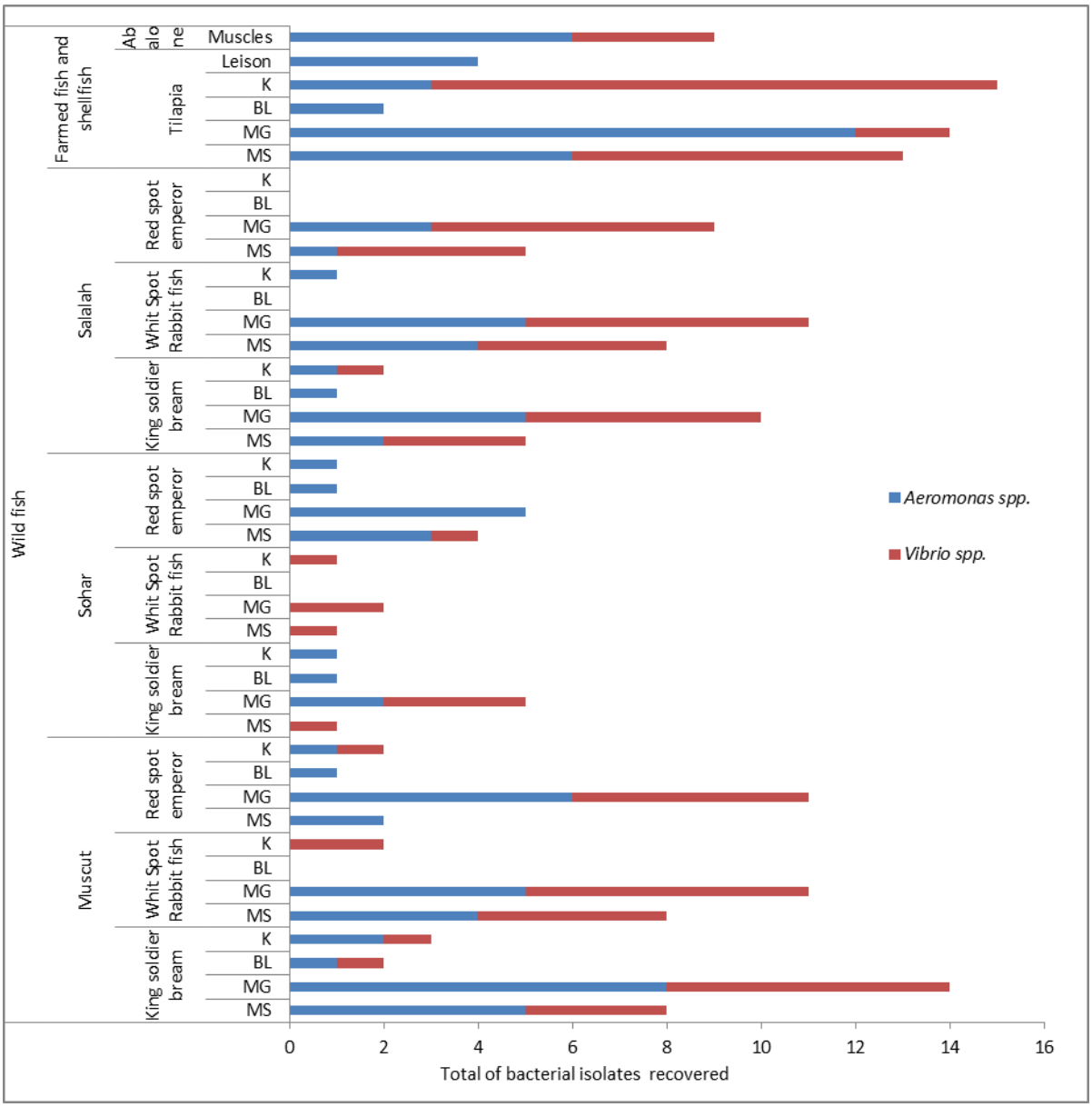


Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 10 Bacterial isolates recovered from tilapia and abalone.

3.5.2 Dominant bacteria recovered from wild and farmed fish and shellfish

The most frequently bacteria recovered from wild and farmed fish and shellfish were *Aeromonas* spp. (n=105; 25% of the total) and *Vibrio* spp. (n=92; 22% of the total) (Figure 3.11). Most isolates were recovered from tilapia kidney following by tilapia and king soldier bream gill mucus from Muscat.

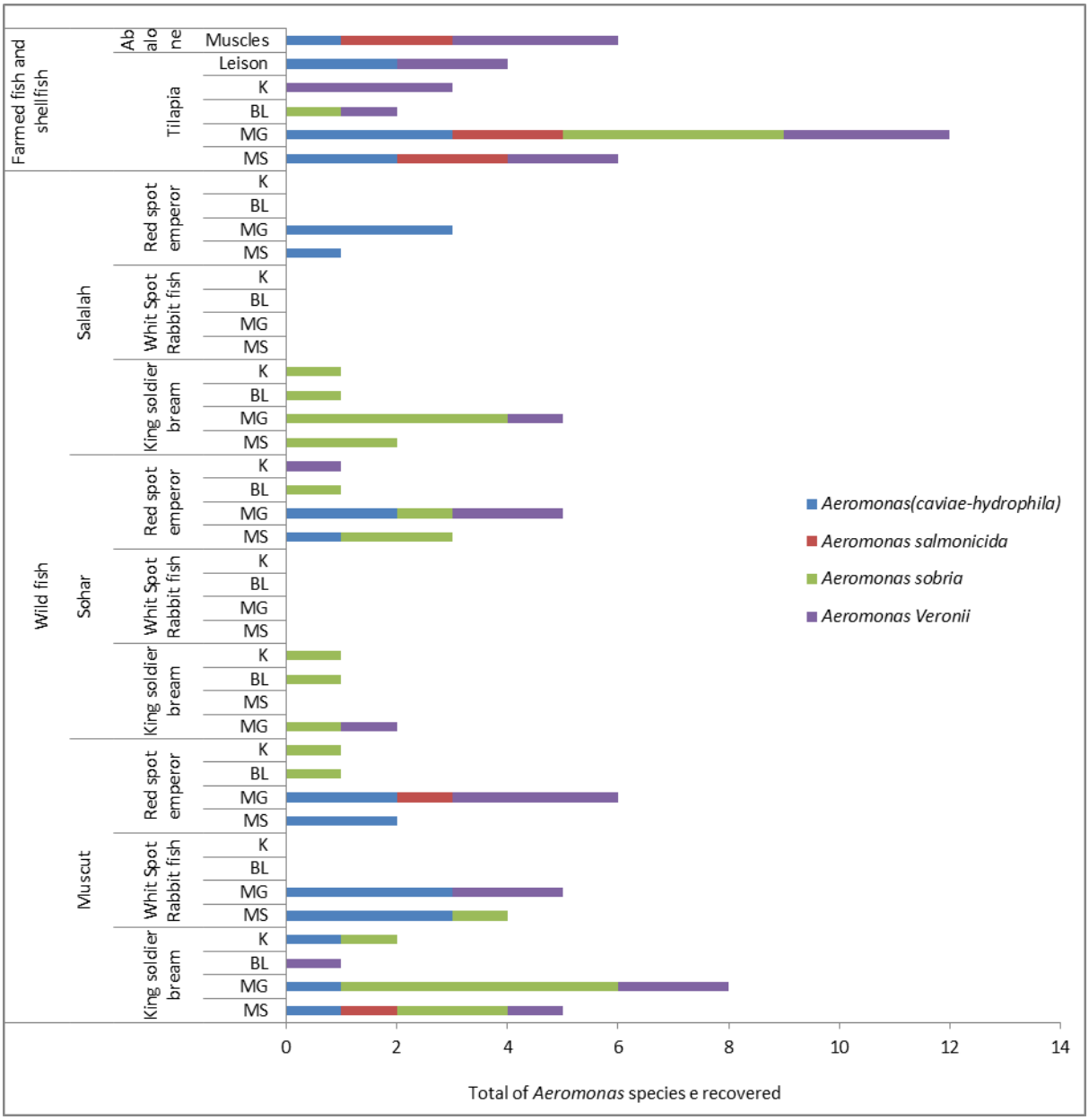


Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 11 Dominant bacteria in wild and farmed fish and shellfish.

3.5.2.1 *Aeromonas*

Aeromonas predominated in the fish and shellfish, representing 25% of the total isolates. Cultures produced light creamy to yellowish coloured colonies on TSA, and contained Gram-negative straight motile and nonmotile rods, which produced oxidase and demonstrated variable results in the catalase test (Table 3.2). The biochemical profile obtained with VITEK® 2 Compact bacterial identification systems identified the isolates to 4 species, i.e. *A. caviae/hydrophila*, *A. sobria*, *A. salmonicida* and *A. veronii*. *A.caviae/hydrophila* could not be distinguished further using the biochemical profile of VITEK® 2 Compact. The cultures were recovered from many tissue samples of fish and shellfish (Figure 3.12) However, *Aeromonas* was absent from kidney and blood samples of white spot rabbit fish and red spot emperor from Muscat and Salalah, respectively. Also, the organism did not occur in white spot rabbit fish from Sohar and Salalah. *A. sobria* (n=38; 36% of the total isolates) dominated. Interestingly, organisms identified phenotypically as *A. salmonicida* were recovered only from gill and skin mucus samples and from abalone muscle.

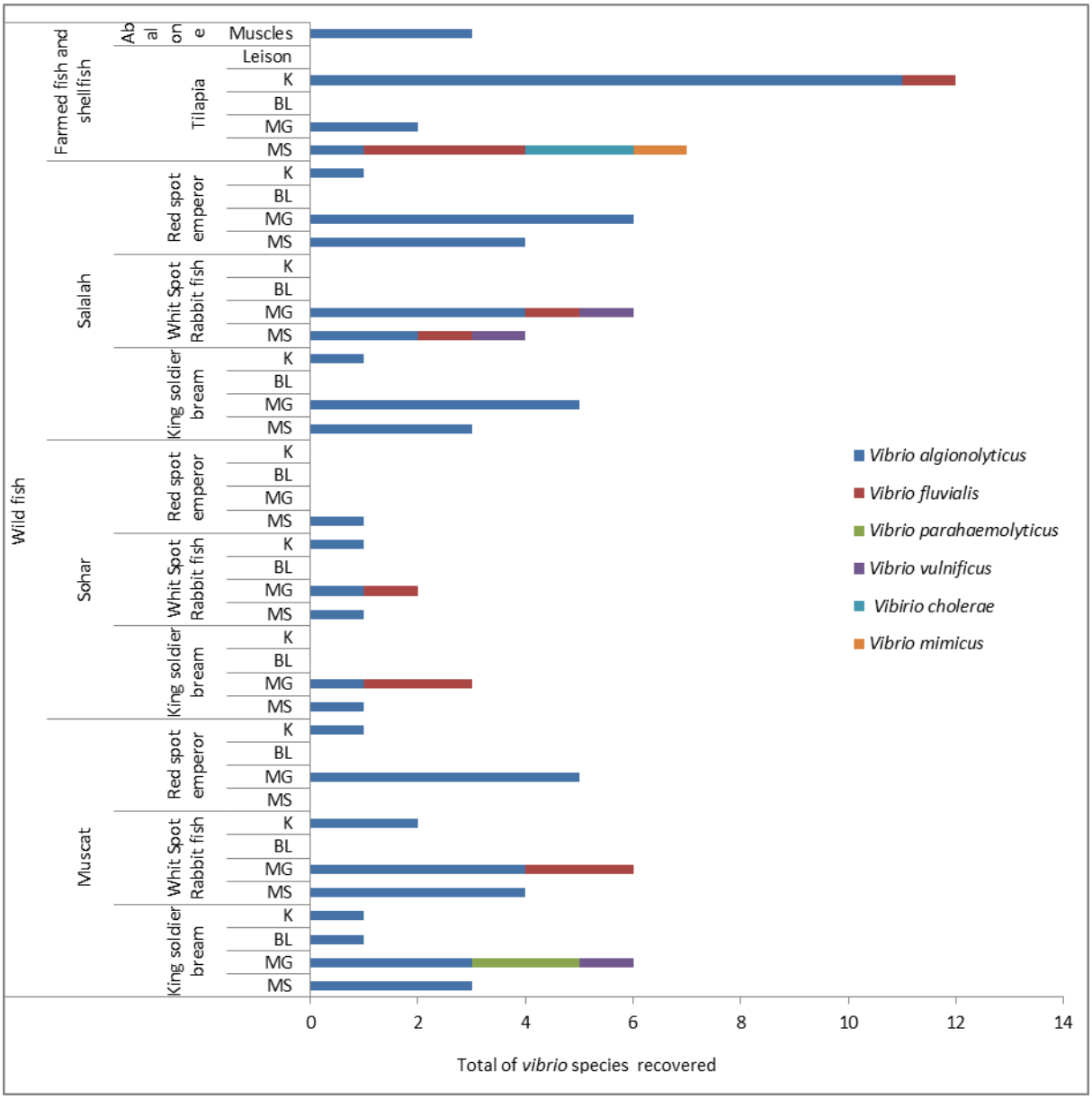


Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 12 The prevalence of *Aeromonas* spp. from fish and shellfish species and their geographical locations.

3.5.2.2 *Vibrio*

Vibrio was the second most common group to be recovered from fish and shellfish, and represented 22% of the total number of isolates. Cultures comprised motile curved or straight rods, which produced light creamy coloured colonies on TSA. Oxidase and catalase were produced (Table 3.2). The biochemical profile obtained with the VITEK® 2 Compact bacterial identification system differentiated 6 species, i.e. *V. alginolyticus, V. fluvialis, V. parahaemolyticus, V. vulnificus, V. cholerae* and *V. mimicus*. Generally, the fish and shellfish samples contained 1-4 groups of vibrios (Figure 3.13). *V. alginolyticus* (n=61; 90% of the total *Vibrio* isolates) was the most common *Vibrio* being present in all tissue samples of fish and shellfish. *V. parahaemolyticus* was recovered only from gill mucus of king soldier bream from Muscat, whereas, *V. cholerae* and *V. mimicus* were recovered only from tilapia skin mucus. *V. vulnificus* was isolated only from gill mucus of Muscat king soldier bream, and from gill and skin mucus of Salalah white spot rabbit fish.



Explanations: MG = gills mucus, SM = skin, BL = blood, K = kidney

Figure 3. 13 The prevalence of *Vibrio* spp. from fish and shellfish species and their geographical location.

3.5.3 Diseased tilapia

Some tilapia demonstrated clinical signs of disease, including external haemorrhages on the head, skin and at the bases of the fins. From these fish, *Aeromonas*, *Enterobacter*, *Sphingobacterium* and *Micrococcus* were isolated. Of these, the dominant isolates were equated with *A. veronii*, *A. caviae/hydrophila* and *A. salmonicida* (Table 3.3).

Table3. 3 List of bacterial species recovered from tilapia showing clinical signs of disease.

No.	Isolates Code	Clinical signs of disease	Site of isolation	Type of bacteria species recovered based on biochemical profile of VITEK® 2 Compact system
1	385L	Lesion	L	*A. veronii*
2	392L	Lesion	L	*A. veronii*
3	390L	Lesion	L	*A. caviae*
4	391L	Lesion	L	*A. caviae*
5	50BL1	External haemorrhages in the head, skin and at the bases of the fins	MG	*A. veronii*
6	189MG1	External haemorrhages in the head, skin and at the bases of the fins	BL	*A. veronii*
7	44MS1	External haemorrhages in the head, skin and at the bases of the fins	MS	*A. veronii/ Sphingobacterium* spp.
8	14MG3	External haemorrhages in the head, skin and at the bases of the fins	MG	*A. caviae/ Enterobacter* spp./ *Micrococcus* spp.
9	326MG	External haemorrhages in the head, skin and at the bases of the fins	MG	*A. salmonicida*
10	42MS1	Anemia	MS	*A. caviae*

Explanations: MG = gills mucus, MS = skin mucus, BL = blood, M = muscle, L = Lesion

3.6 Discussion

The aim of this study was to gather data on the bacterial species composition, and distribution of viable isolates recovered from a wide range of fish species located from different sites in Oman. Certainly it was recognised that obtaining a sufficient sample size was a common problem with the field studies especially when some species were in low abundance, e.g. abalone. This problem is not unique to the present investigation insofar as others have reported issues with obtaining sufficient specimens to study (e.g. Hudson & Peters, 2005). Also, the variation in microbial loading between farmed and wild fish and shellfish has been reported previously (e.g. Shewan & Hobbs, 1967).

In comparison with previous work, tilapia contained the highest number of bacterial isolates (Eissa *et al.,* 2010; Torimiro *et al.,* 2014). Also, cultured fish revealed more signs of disease than wild fish (Eissa *et al.,* 2010; Torimiro *et al.,* 2014). Certainly, it may be argued that microbial populations reflect the nature of the host and the interaction with the aquatic environment (Shewan & Hobbs, 1967; Maugeri *et al.,* 2000). It is possible that the higher number of bacterial isolates obtained from Muscat compared with other locations may be attributed to stress caused by water pollution resulting from municipal sewage (Al-Bahry *et al.,* 2009) or crude oil pollution in the sea attributable to shipping activities (Oman Daily Observer, 2013). It is conceivable that these factors could compromise the fish immune system resulting in the development of disease (Jobling & Tyler, 2003; Murakami *et al.,* 2008).

The larger diversity of bacterial species recovered from wild compared with farmed fish and shellfish has been recognized previously from the Andaman and Nicobar Islands, In-

dia (Jai Sunder *et al*., 2006). Moreover, the high proportion of Gram-negative rod-shaped bacteria identified from farmed fish and shellfish found in the study was similar to the work of Jai Sunder *et al.* (2006). Certainly, both wild and farmed fish have been shown to support large populations of bacteria particularly on the gills compared with internal tissues, namely kidney and blood (Austin, 2002). In this study, the total number of bacterial isolates recovered in fish gill mucus was higher than skin mucus, with the lowest number recovered from kidney and blood of the farmed and wild fish. Also, the greatest microbial diversity occurred in the gill and on the skin, which may reflect the environment where the fish occurred as well as to possible contamination during handling. *Aeromonas*, *Vibrio* and *Sphingomonas paucimobilis* were recorded more so from wild fish gills, whereas *Aeromonas*, *Vibrio* and *Micrococcus* occurred on tilapia gills. It is relevant to note that some of these bacteria, i.e. *Vibrio* and *Micrococcus*, were recorded from fish samples collected in Norwegian and India water (Shewan & Hobbs, 1967).

Internal tissues from healthy fish have been considered to be sterile (Apun *et al*., 1999). Yet, other investigators have reported the presence of some bacteria within internal fish organs of both healthy and unhealthy fish (e.g. Evelyn & McDermott, 1961). This is in agreement with the present study when low numbers of bacteria were isolated from kidney and blood samples of healthy fish. In addition, *A. veronii* was isolated from tilapia blood samples with external haemorrhages on the head, skin and at the base of the fins. Possibly, bacteria within the internal tissues may be involved in a reduction of immunological defense mechanisms, by so called stress factors (Cahill, 1990).

Aeromonas and *Vibrio* were recovered from apparently healthy abalone. This is not surprised in view of their previous recovery from abalone obtained from the Salalah region of Oman (personal observation). Moreover, *Vibrio* spp. have been isolated from blacklip abalone (*Haliotis rubra*) from southern waters of Australia during 2003 (Hayashi *et al.*, 2003), and from an outbreak of disease in abalone post larvae in China during 2006 (Cai *et al.*, 2006). In comparison, *Aeromonas* spp. were collected during an outbreak of post-larval abalone disease in July 2010 from Guangdong Province, China (Jing & Junpeng, 2013). The dominance of *Aeromonas*, and in particular *A. sobria*, may be due to the capability of adapting to the challenging conditions in different environments, including the presence of pollutants (Araujo, 1991; Bernoth *et al.*, 1997; Mateos *et al.*, 1993; Rahman *et al.*, 2002). Overall, *Aeromonas* and *Vibrio* are common inhabitants of healthy fish, but may be potential fish pathogens particularly when external stressors are present (Shotts & Gratzek, 1984; Austin & Austin, 2012; Mac Farlane *et al.*, 1986; Cahill, 1990; Sakata, 1990; Blanch *et al.*, 1997; Martin-Antonio *et al.*, 2007; Ward *et al.*, 2009). The presence of organisms, identified as *A. salmonicida* was surprising in view of its association as an obligate fish pathogen (Austin & Austin, 2012). However, the identification was based on phenotype rather than genotype, which will be described separately. Overall, it is argued that aeromonads pose major threats to wild and farmed fish (Bernoth *et al.*, 1997; Mateos *et al.*, 1993), and exhibit a range of pathogenic traits. Infections may cause mass mortalities with consequent high economic losses for aquaculture (Beaz-Hidalgo & Figueras, 2013; Shotts & Gratzek, 1984; Austin & Austin, 2012). Similarly, vibrios may cause serious outbreaks of disease mostly in marine fish and invertebrates especially when the animals are confined in heavily stocked, commercial systems where morbidity may reach 100% (Austin & Austin, 2012; University of Florida, 2009). It is

noteworthy that *V. alginolyticus*, which was the dominant vibrio recovered in this study, has been associated with disease, particularly septicaemia resulting in high mortalities among farmed and wild fish (Egidius, 1987; Larsen & Olsen, 1991). The widespread occurrence of this taxon is in line with previous studies that reported its presence in wild and farmed fish from a wide range of geographic locations (Baffone *et al.,* 2000; Arijo *et al.,* 1998; Barbieri *et al.,* 1999) .

Sphingobacterium spp. were commonly recovered in this study, being isolated from 15% of wild fish. Previously, the organism has been recovered from the outer membrane of Chinese salmon eggs (Barnes *et al*., 2005). Also, the presence of *Micrococcus* and *Staphylococcus* in this study matches previous work involving freshwater and wild fish (Austin & Austin, 1999).

In conclusion, a diverse group of bacteria was found in wild and farmed fish from the waters in and around Oman. Moreover, there is a similarity in the taxonomic composition of the bacterial populations with those reported by other workers.

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Phenotypic and molecular characteristics of *Aeromonas* spp. isolated from Oman

4.1 Introduction

Aeromonas is classified in the family Aeromonadaceae, and contains representatives that have been recognised as animal pathogens (Gosling, 1996).The genus *Aeromonas* may be divided into two groups based on their phenotypic characteristics, and may be referred to as motile mesophilic and non-motile psychrophilic aeromonads (Janda & Duffey,1988; Janda & Abbott, 2010). Mesophilic *Aeromonas* is a heterogeneous group of species collectively regarded as the *A. hydrophila* complex, and grow optimally between 35°C and 37°C and sometimes from 4°C and 42 °C. These organisms are frequently isolated as members of the normal flora from healthy fish, and as primary or secondary pathogens from diseased specimens (Trust & Sparrow, 1974; MacMillan & Santucci, 1990). In contrast, psychrophilic strains may be equated with the fish pathogen *A. salmonicida* that causes furunculosis and ulcer disease, and grows well at temperatures between 22°C and 28°C (Austin *et al.,* 1989).

Aeromonas spp. grow in many selective, non-selective and differential types of artificial media. On non-selective agar containing media, *Aeromonas* colonies are typically buff-coloured, smooth, convex and 3–5 mm in diameter after overnight incubation at 35–37^{o}C. Cultures are described as containing Gram-negative, rod-shaped bacteria that are asporogenous, catalase and oxidase positive, facultatively anaerobic, nitrate reducing, and unable to grow in 6.5% (w/v) NaCl (Austin *et al.*,1989). Most strains ferment sucrose and/or

lactose, exhibits β-haemolysis when grown on sheep blood agar (Pavlov *et al.*, 2004), and are resistant to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine). The taxonomy of *Aeromonas* is changing continuously, and the number of validly described species has increased to 31 according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) website (www.bacterio.net).

Identification of *Aeromonas* is considered to be complex (Popoff, 1984) due to changes in taxonomy, phenotypic heterogeneity and lack of a universally accepted identification scheme. There is a lack of congruity between phenotypic and genotypic characteristics of species, with many methods used for identification purposes (Abbott *et al.*, 1998; Ghatak *et al.*, 2007). In particular, there are often issues with phenotypic-based identification methods due to reproducibility with some tests, and variation among the characteristics of isolates of the same taxon (Baron, 1996). Molecular methods have gained widespread use (Abbott *et al.*, 1998; Janda *et al.*, 1996), and include the polymerase chain reaction (PCR) targeting 16S rDNA; these molecular techniques offer many advantages over traditional methods including ease, rapidity and reliability (Borrell *et al*., 1997) .

Restriction fragment length polymorphism 16S rDNA - RFLP is a more powerful method that has enabled the identification of *Aeromonas* spp. using endonucleases, i.e. *AluI, MboI, NarI, HaeIII, NlwNI* and *PstI* (Borrell, *et al*., 1997; Figueras *et al.*, 2000; Lee *et al*., 2002). 16S rDNA sequencing has been confirmed to be valuable in the identification of *Aeromonas* spp. (Martínez-Murcia *et al*., 1992; Demarta *et al*., 1999). However, sequence databases are not always accurate because the sequences deposited in the databases may result from strain misidentification, and there may be quality issues with the data, hence a

verification test is necessary to support the genetic classification of species (Blocka & Anthony, 2010).

4.2 study Aims

The present study was undertaken to characterize *Aeromonas* spp. isolated from wild and farmed fish in Oman by using phenotypic and molecular methods (16S rDNA-RFLP and 16S rDNA sequencing).

4.3 Materials and Methods

4.3.1 Bacterial isolates

Aeromonas strains (n=46), which were isolated from wild, farmed and shellfish from Oman with and without any apparent clinical signs of disease, were used in this study (Appendix 4.1).

4.3.2 Primary and biochemical identification tests

The isolates were presumptively identified as *Aeromonas* spp. Following growth on plates of TSA with incubation at 28ºC for 24-48 h. Then, primary identification tests were carried out as described by Frerichs and Millar (1993), which included examination of micro-morphology and the Gram staining reaction, motility (by the hanging drop method), and production of catalase (3% H_2O_2) and oxidase. For each test, the inoculum was always adjusted to an optical density of 0.5 McFarland standard units. The density (turbidity) of the suspension was checked by using a calibrated turbidity meter (DensiChek; BioMérieux, France).

The susceptibility to the vibriostatic agent O/129 (150 µg and 10 µg; Oxoid discs) were achieved by the disc method of Kirby and Bauer on TSA agar plates (Buller, 2004). After 24 h incubation at 28 °C, zones of clearing of < 7 mm and >7 mm were recorded as indicative of resistance and sensitivity, respectively (Whitman, 2004). Then, the biochemical profiles of the isolates were determined using a VITEK® 2 Compact bacterial identification system (BioMérieux, France) following the manufacturer's instructions. However, in some cases the VITEK® 2 Compact systems could not separate species, so additional biochemical tests were carried out to differentiate between these species. According to the manufacturer's instructions the VITEK® 2 Compact system was not able to differentiate between *A. hydrophila* and *A. caviae,* which was accomplished by examination of acetoin production in the Voges-Proskauer test (Buller, 2004). Thus, *A. hydrophila* was positive whereas *A. caviae* was negative. Additional biochemical tests were used to differentiate between biovars of *A. veronii* (*A. veronii* bv. sobria and *A.veronii* bv. veronii) involving use of arginine dihydrolase, and lysine and ornithine decarboxylase production (Moeller's method; Moeller, 1955). Growth in the presence of 0% and 6%, NaCl in TSB with incubation at 28ºC for 24-48 h was used to discriminate *Aeromonas* from *Vibrio* spp. (Lee & Donovan, 1985) when the VITEK® 2 Compact system was unable to do so. For each test performed a positive control *A. encheleia* NCIMB 13442 (National Collection of Industrial and Marine Bacteria, Aberdeen UK) was included. Additionally *A. hydrophila* ATCC 19570 (American Type Culture Collection, Maryland, USA) was used as an additional positive control.

4.3.2.1 Distinguishing typical and atypical strains of *A. salmonicida*.

Nine isolates of *A. salmonicida* strains identified by phenotyping using the VITEK® 2 Compact Bacterial Identification System were examined further to differentiate typical from atypical strains Thus, the bacteria were cultured on plates of TSA with incubation at 28ºC for 24-48 h **,** then the biochemical profiles were examined using the API 20E rapid identification kit (BioMérieux, France) following the manufacturer's instructions except that inoculated strips were incubated at 28ºC and the results read after 24 and 48 h (Crumlish, 2011). The serology was determined by indirect agglutination reactions using the MONO-As kit (BIONOR AS, Skien, Norway), following the manufacturer's instructions. This kit consists of antibody-coated latex beads and is designed for specific identification of *A. salmonicida*. Growth at 5, 20, 22, 28, 30 and 37 ºC and the ability to produce brown diffusible pigment were evaluated on TSA (Austin *et al.*, 1998). Here, inoculated media were examined every 24 h for up to 5 days to determine the presence of growth, and up to 14 days to assess for pigment production. All tests were carried out in duplicate, and *A. salmonicida* subsp. *salmonicida* NCIMB 1102 was included as a positive control.

4.3.3 Molecular identification

4.3.3.1 DNA extraction

All 48 *Aeromonas* isolates were examined for purity by growth on nutrient agar (Oxoid) plates. Then, single colonies were inoculated into 5 ml volumes of TSB and incubated aerobically overnight at 28°C before the cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C. The supernatant was discarded carefully, and the DNA extracted using a DNeasy™ Tissue Kit (Qiagen™, Crawley, UK) following the manufacturer's instructions. Briefly, the bacterial culture was pelleted, re-suspended using 200 µl volumes of PBS, and the cells lysed with 20 µl proteinase K (Qiagen). The released bacterial DNA was passed through a DNeasy Mini spin column and washed to remove impurities. The purified bacterial DNA was eluted with 100 ml of AE buffer. Purity of the extracted DNA was checked by agarose gel electrophoresis, and the total DNA concentration of each sample was determined by Nano drop spectrophotometer (ND-1000, Lab Tech International, East Sussex, UK) at 260 and 280 nm wavelengths, and adjusted to 50-80 ng/µl then stored at -20ºC until required.

4.3.3.2 Polymerase chain reaction (PCR) amplification

All *Aeromonas* cultures were examined by using the 16S rDNA assay. The specific primers selected for PCR analysis of 16S rRNA gene are shown in Table 4.1. The primers were suspended with nuclease-free water following the manufacturer's instructions (Eurofins MWG Operon, USA).

Table 4. 1 The sequence of forward and reverse primers of 16S rRNA gene (Borrell *et al.*, 1997)

Primer type	Primer sequence	Product size
Forward primer AeroRFLP-F.	5'-AGAGTTTGATCATGGCTCAG-3'	1509 to 1491
Reverse primer AeroRFLP-R.	5'-GGTTACCTTGTTACGACTT-3'	

The PCRs were carried out by using Ready-To-Go™ PCR beads (GE Healthcare) which contained freeze-dried DNTPs, and *Taq* polymerase enzyme was used for the PCR following the manufacturer's instructions. Accordingly, 5 µl of extracted DNA (50ng/µl) and 1 µl of each forward and reverse primer (10 pm/µl) and made up to 25 µl with nuclease-free water was added to each tube. The PCR reaction was performed with a thermal cycler (Biometra T gradient^{TM}, Goettingen, Germany), using the PCR conditions of 1 cycle of denaturation at 93°C for 3 min, followed by 35 cycles of denaturation at 91°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min, and then rested at 4^{o}C (Borrell *et al.*,1997). Five µl of each PCR product were mixed with 1 µl of 6x gel loading dye (Thermo Scientific) and electrophoresed on 1% agarose gel (Invitrogen, UK), stained with 0.5 µg/ml of ethidium bromide and visualized under ultraviolet light (UV). The PCR products were identified in comparison with the 1kb DNA ladder (Thermo Scientific GeneRuler) to determine 1509 to 1491 bp. A positive control *A. encheleia* (NCIMB 13442), *A. hydrophila* (ATCC 19570) and an internal control (no DNA template) were included.

4.3.3.3 DNA purification

The PCR-amplified 16S rRNA gene (1502 bp) was cleaned up before restriction digestion using the PCR purification kit (QIAGEN) according to the manufacturer instructions. Briefly, the binding buffer (Buffer PB) was added as a five times volume of the DNA sample to be cleaned up. The PB buffer containing the DNA was placed in the centre of a universal spin filter column assembled in a 2 ml collection tube. The column was then centrifuged at room temperature for 1 minute at 10,000 x g. The flow- through was then discarded, and 750 µl of the wash buffer (Buffer PE) containing ethanol was added to the column, and another centrifugation step was carried out as described above. The flow-through was discarded followed by addition of 50 µl of the H_2O to the column. For a more concentrated DNA sample, 30 µl of H_2O was added. The column was allowed to stand for 1 minute followed by its placement in a clean microfuge tube. A final centrifugation step was carried out, as described above, and the eluted DNA sample was stored at -20^{o}C until needed.

4.3.3.4 Discrimination of *Aeromonas* spp. by 16S rDNA RFLP analysis

The purified PCR-amplified 16S rRNA gene (1502 bp) were examined for restriction fragment length polymorphism patterns (RFLP) following the method described by Borrell *et al.* (1997) and Figueras *et al.* (2000). Thus, restriction digestions were performed first by incubating 10 µl of the amplified PCR product with 5 U of each enzyme (*AluI* and *MboI* or *NarI* , *HaeIII, Alw*NI, *PstI*) (Promega, Madison, WI, USA) and 2 µl of the corresponding 10X buffer (New England Biolabs, Beverly, MA) in a total volume of 20 µl. The reaction mixture was incubated at 37ºC for one hour. Ten µl aliquots of each

digestion was mixed with 1 µ of 6x gel loading dye (Thermo Scientific) and electrophoresed on 3% MetaPhor® agarose, and stained with 0.5 µg/ml ethidium bromides to confirm the presence of specific single bands. With some isolates, there was a need to repeat the process in order to obtain clear single bands. Positive controls of *A. encheleia* (NCIMB 13442) and *A. hydrophila* (ATCC 19570) and an internal control (no DNA template) were included.

4.3.3.5 Sequencing and computer analysis 16S rDNA genes

For the validation of *Aeromonas* identification, samples were sent to GATC Biotech (London BioScience Innovation Centre, UK) for sequencing. The samples were prepared by the following company protocol: 5 µl of purified PCR product was added with 5 Mm (5 pm/µl) of each primer (forward and reverse) to 1.5 ml Eppendorf tubes and sent to the company for sequencing. Species identification was confirmed by comparing the DNA sequence to the GenBank database using BLAST (basic local alignment search tool, standard nucleotide comparison (http://www.ncbi.nlm.nih.gov/ BLAST/) *(*Altschul *et al*., 1990).

Validation of *Aeromonas* spp. also done with a phylogenetic analysis whereas the Sequences were aligned using ClustalW for Multiple Sequence Alignment with the DNASTAR computer program and phylogenetic analyses (Maximum likelihood method and neighbour-joining methods) were conducted by the MEGA software version 6.0 (Tamura *et al*., 2013). Standard errors were obtained with 1,000 bootstrap replicates.

4.4 Data Analysis

MS Excel was used to store all the data, and to produce tables.

4.5 Results

4.5.1 Phenotypic identification

Results (presented in Appendix 4.1) revealed that 38 isolates examined in this study produced deep to light creamy colour smooth convex colonies on TSA agar plates. An additional 8 isolates produced yellowish coloured colonies**,** and were identified as *A. salmonicida*. Cultures comprised motile, Gram negative, straight rods, which were positive for catalase and oxidase production and resistant to the vibriostatic agent O/129. There was some variation in motility among the isolates, with 2 being non-motile, and 9 isolates producing only weak catalase reactions (Figure 4.1).

Figure 4. 1 Strong and weak reactions of *Aeromonas* spp. to catalase activity.

Five strains were sensitive to the vibriostatic agent O/129, with three showing inhibition zones around both the 150 µg and 10 µg discs, whereas two isolates demonstrated growth inhibition only around the 150 µg discs (Table 4.2).

Table 4. 2 *Aeromonas* spp. with susceptibility to the vibriostatic agent O/129

Reference number	*Aeromonas* species	Result of O/129 150 µg/ 10 µg	Inhibition (diameter [mm] size)
26/MS2	*A. salmonicida*	S/S	26/20
340M	*A. salmonicida*	S/R	0/8
373MG	*A. salmonicida*	S/R	0/20
388MS	*A. salmonicida*	S/S	25/23
389MS	*A. veronii*	S/S	30/26

Explanations: R, Resistant; S, sensitive

All strains that were sensitive to 10 µg and 150 µg of O /129 and did not grow in the presence of 6 % (w/v) NaCl were considered as *Aeromonas* spp. Sixteen isolates were identified by VITEK® 2 Compact as *A. hydrophila/A. caviae*; the Voges-Proskauer reaction was used to differentiate these isolates further. All 16 isolates that were positive to the Voges-Proskauer reaction were equated with *A. caviae* and so these isolates have been labelled as *A. caviae* in Appendix 4.1.

A. veronii bv *sobria* and bv *veronii* was labelled as *A. sobria* and *A. veronii* using VITEK® 2 compact, being unable to differentiate between them. Based on the positive reaction of ornithine decarboxylase, 22 isolates were identified as *A. veronii* bv *veronii*. For convenience, these isolates have been labelled as *A. veronii* in Appendix 4.1.

4.5.1.1 *A. salmonicida* identification

The putative *A. salmonicida* isolates were examined further using additional phenotypic tests including those in the API 20E rapid identification kit. All these results were in agreement with those reported in Table 4.3. However, 5 isolates, i.e. 16MG, 26MS2, 291MS, 340M, 373MG and 388MS, differed from other isolates. Some of the cultures were non-motile, fermented rhamnose and melibiose, and were sensitive to the vibriostatic agent O/129. Isolate 340M produced light creamy colonies on TSA whereas the other isolates (16MG, 26MS2, 291MS, 293 MS, 295MS, 373MG, 388MS, and 395M) produced yellowish colonies (Figure 4.2). Only isolate 291MS autoagglutinated in saline.

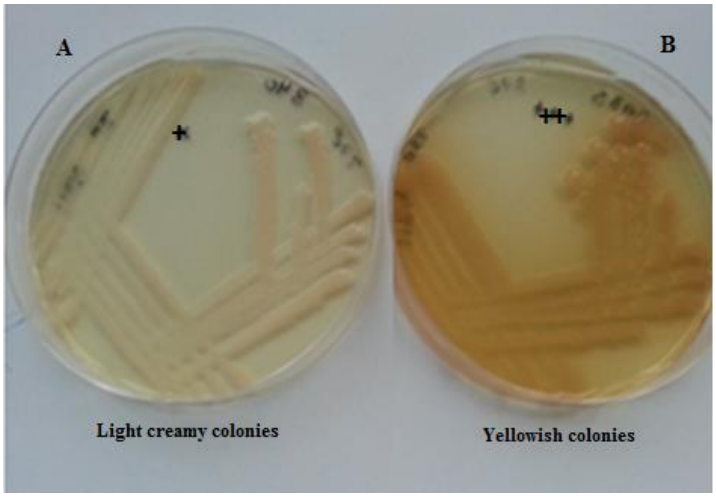

Figure 4. 2 Colonies of *A. salmonicida* A (340M) and B (291MS) on TSA agar,

Table 4. 3 Phenotypic characteristics of *A. salmonicida* isolates.

Explanations: A = light creamy colonies; B = yellowish colonies; + = Positive; - = Negative; V= Variable result; *

Isolate No.	*A.salmonicida***	16MG	26MS2	291MS	293MS	295MS	340M	373MG	388MS	395M
Colonial colour on TSA	*	B	B	B	B	B	A	B	B	B
Gram stain	-	-	-	-	-	-	-	-	-	-
Motility	v	-	+	+	+	+	-	+	+	+
pigment production	v	-	-	-	-	-	-	-	-	-
Autoagglutination	v	-	-	+	-	-	-	-	-	-
production of: Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Growth at: 5 °C	v	-	-	+	+	+	-	-	+	-
15/22/28/30°C	+	+	+	+	+	+	+	+	+	+
37 °C	v	+	+	+	+	+	+	+	+	+
API20E : ONPG	+	+	+	+	+	+	+	+	+	+
ADH: arginine dihydrolase	v	+	+	+	+	+	+	+	+	+
LDC: lysine decarboxylase	v	+	+	+	-	-	+	+	+	+
ODC: ornithine decarboxylase	v	-	+	-	-	-	-	+	-	-
CIT: utilization of citrate as sole carbon source	–	-	-	-	-	-	-	-	-	-
H_2S:production of hydro-gen sulphide	v	-	-	-	-	-	-	-	-	-
UREA: urease production	–	-	-	-	-	-	-	-	-	-
TDA: tryptophan deaminase	–	-	-	-	-	-	-	-	-	-
IND: production of indole	v	+	+	+	+	+	+	+	+	+
VP: the Voges-Proskauer reaction	v	+	+	+	+	+	+	+	+	+
GEL: gelatinase	+	+	+	+	+	+	+	+	+	+
GLU: fermentation of glucose	+	+	+	+	+	+	+	+	+	+
MAN: fermentation of mannose	+	+	+	+	+	+	+	+	+	+
INO: fermentation of inositol	–	-	-	-	-	-	-	-	-	-
SOR: fermentation of sorbitol	v	+	-	+	-	-	+	+	-	-
RHA: fermentation of rhamnose	–	+	-	-	-	-	+	-	-	-
SAC: fermentation of sucrose	v	+	+	+	+	+	+	+	+	+
MEL: fermentation of melibiose	–	+	-	-	-	-	+	-	-	-
AMY: fermentation of amygdalin	v	+	-	+	+	-	+	+	+	+
ARA: fermentation of arabinose	v	+	-	+	+	+	+	+	-	-
Susceptibility to the vibriostatic agent O/129:150 µg/ 10 µg	R/R	R/R	S/S	R/R	R/R	R/R	S/R	S/R	S/S	R/R

=No data; R= Resistant; S=sensitive

**= Data are from the following references: Abott *et al*., 1992; Austin *et al*., 1989; Carnahan & Altwegg, 1996; Huys *et al.*, 1996; Janda *et al.*, 1996; Popoff, 1984; Griffith *et al*, 1953; Schubert, 1967,1974; McCarthy, 1977,1980; Pavan *et al.*, 2000; Yamada *et al.*, 2000.

4.5.2 Molecular identification

4.5.2.1 Polymerase chain reaction (PCR)

All 46 isolates plus the positive controls *A. hydrophila* (ATCC 19570) and *A. encheleia* (NCIMB 13442) produced by PCR an amplicon at (1502 bp); all isolates were therefore confirmed as *Aeromonas* species (Figure 4.3).

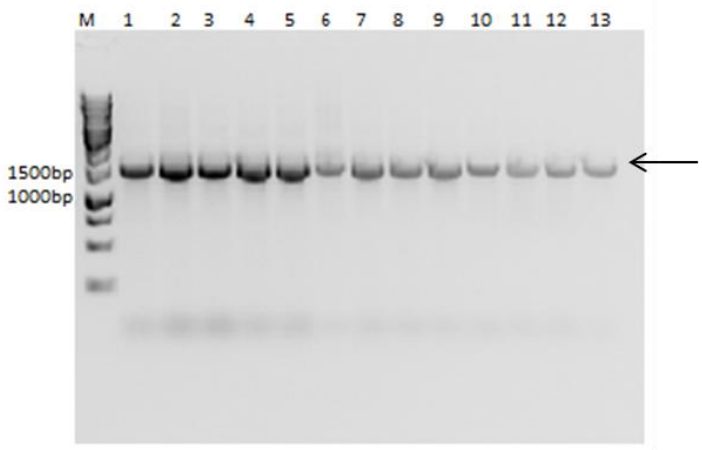

Figure 4. 3 Electrophoresis image of the 16S PCR results to detect *Aeromonas* spp. Lanes M = molecular weight marker (1kb), lanes1= *A. hydrophila* ATCC 19570 **(**positive control), lanes 2-13 are *Aeromonas* species.

4.5.2.2 16S rDNA RFLP analysis

The PCR-RFLP pattern analysis using the 6 restriction enzymes, *AluI, MboI, NarI, PstI, HaeIII* and *AlwNI*, and the simultaneous digestion of the amplified fragment by *AluI* and *MboI* (Figure 4.4 & 4.5) allowed the recognition of *A. veronii* (24/46), *A. jandaei* (4/46), *A. caviae* (2/46) and *A. trota* (2/46)**.** Four out of 46 of the isolates were identified as *A. encheleia* after digesting by *HaeIII* and *AlwNI*, respectively (Figure 4.6 & 4.7), and 10/46 cultures were equated with *A. salmonicida* after digestion with *NarI* followed with *PstI* (Figure 4.8).

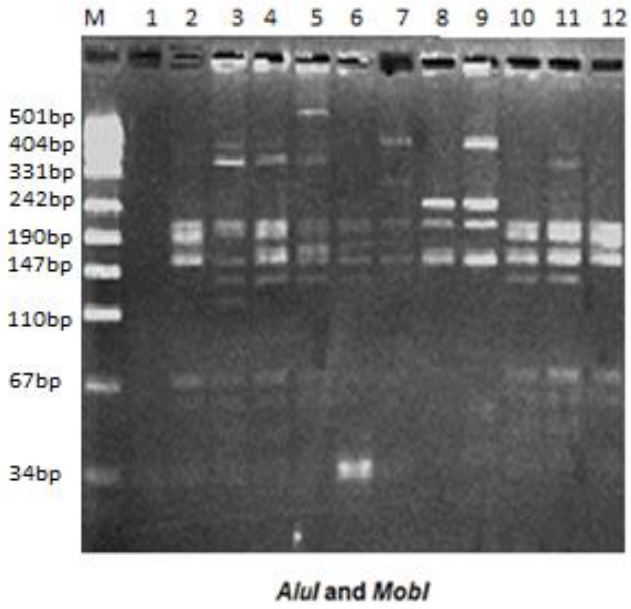

Figure 4. 4 RFLP patterns obtained using endonucleases *AluI and MboI*, of 16S rRNA genes amplified by PCR. Lanes: M = pUC19 DNA ladder; Lanes: 1= No template; Lanes 2,6,10,12 = *A. veronii*; Lanes 3,4,5,11= *A. salmonicida* ; Lanes 8= *A. trota*; Lanes 7,9 =*Aeromonas* spp.

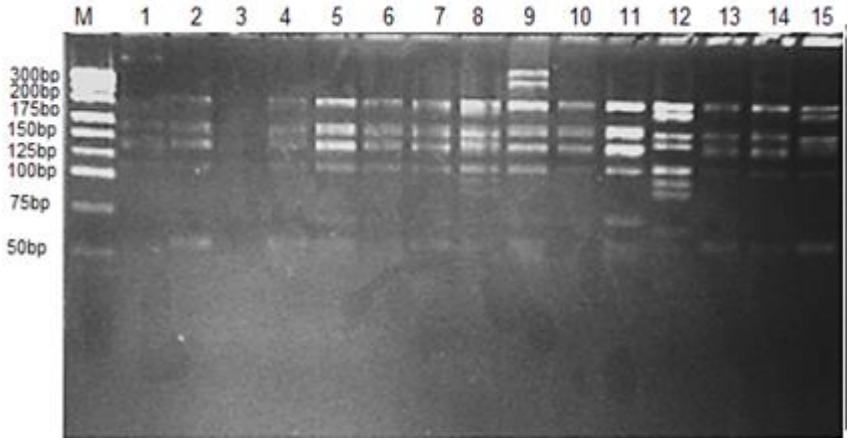

Figure 4. 5 RFLP patterns, obtained by using endonucleases *AluI and MboI*, of 16S rRNA genes amplified by PCR. Lanes M = 25bp step ladder; Lane 1, *A. salmonicida*; Lanes (2, 4, 5, 6, 7, 8, 10, 11) = *A. veronii;* Lanes 9= *A. trota;* Lanes 3= No template.

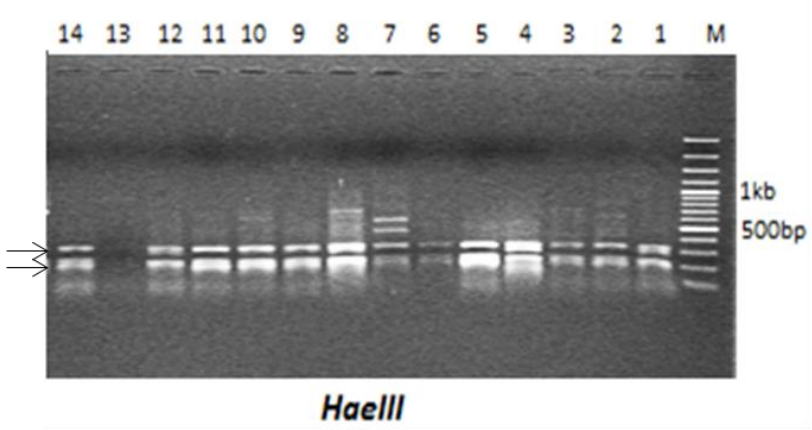

Figure 4. 6 RFLP patterns, obtained by using endonucleases *HaeIII*. Lane M=1kb DNA ladder; Lane (1, 2, 3, 4, 5, 6, 9, 10, 11, 12) = Positive isolates of *A. encheleia* and *A. popoffii*, Lane 13 = No template; Lane 14= positive control of *A. encheleia* NCIMB 13442.

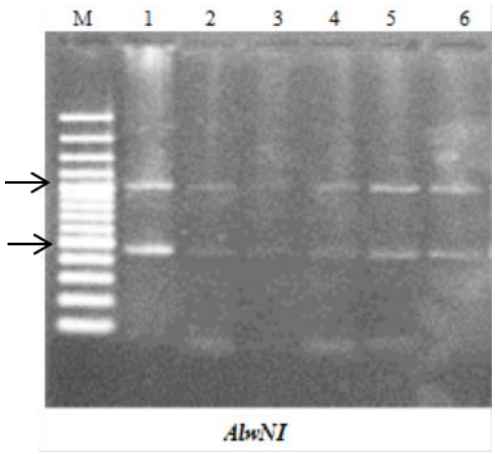

Figure 4. 7 RFLP patterns, obtained by using endonucleases *AlwNI*, Lane M= 1kb DNA ladder; Lane 1 = positive control of *A. encheleia* strain NCIMB 13442**;** Lane (2, 3, 4, 5, 6) = Positive isolates of *A. encheleia*.

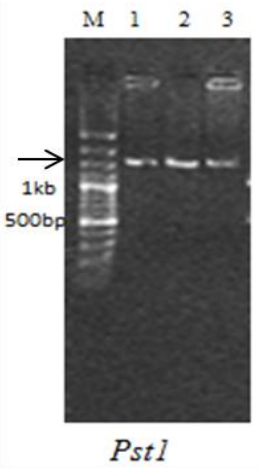

Figure 4. 8 RFLP patterns, obtained by using endonucleases *Pst1,* Lane M=1kb DNA ladder; Lane (1, 2, and 3) = *A. salmonicida*.

4.5.2.3 Comparison of PCR-RFLP results with 16S rDNA sequence data

According to the 16S rDNA sequencing analyses, the highest similarity between the *Aeromonas* isolates was 99%. The sequence similarity between species diverged from 92% to 99% of gene level, and 88% to 99% of species level. 65% of the isolates shared 99% 16S rDNA sequence similarity with the closest sequences in GenBank, whereas 35% of the isolates had between 88% - 98% sequence similarity (Table 4.4) and (Figure 4.9 & 4.10). The highest homologies of the *Aeromonas* sequences in the GenBank were isolates from fish, including trout, goldfish and sea lamprey from Korea, China and Iran.

Table 4. 4 Sequence similarity percentages between *Aeromonas* species.

Aeromonas spp.	Sequence similarity (%)										
	99%	98%	96%	95%	94%	93%	92%	91%	90%	89%	88%
A. caviae N = 2	1	0	1	0	0	0	0	0	0	0	0
A. salmonicida N = 10	3	2	0	1	0	0	3	0	0	0	1
A. veronii N = 24	18	0	0	0	1	1	0	3	0	1	0
A. encheleia N = 4	3	0	1	0	0	0	0	0	0	0	0
A. trota N = 2	1	0	0	0	0	0	0	0	1	0	0
A. jandaei N = 4	4	0	0	0	0	0	0	0	0	0	0
Total: N=46	30	2	2	1	1	1	3	3	1	1	1

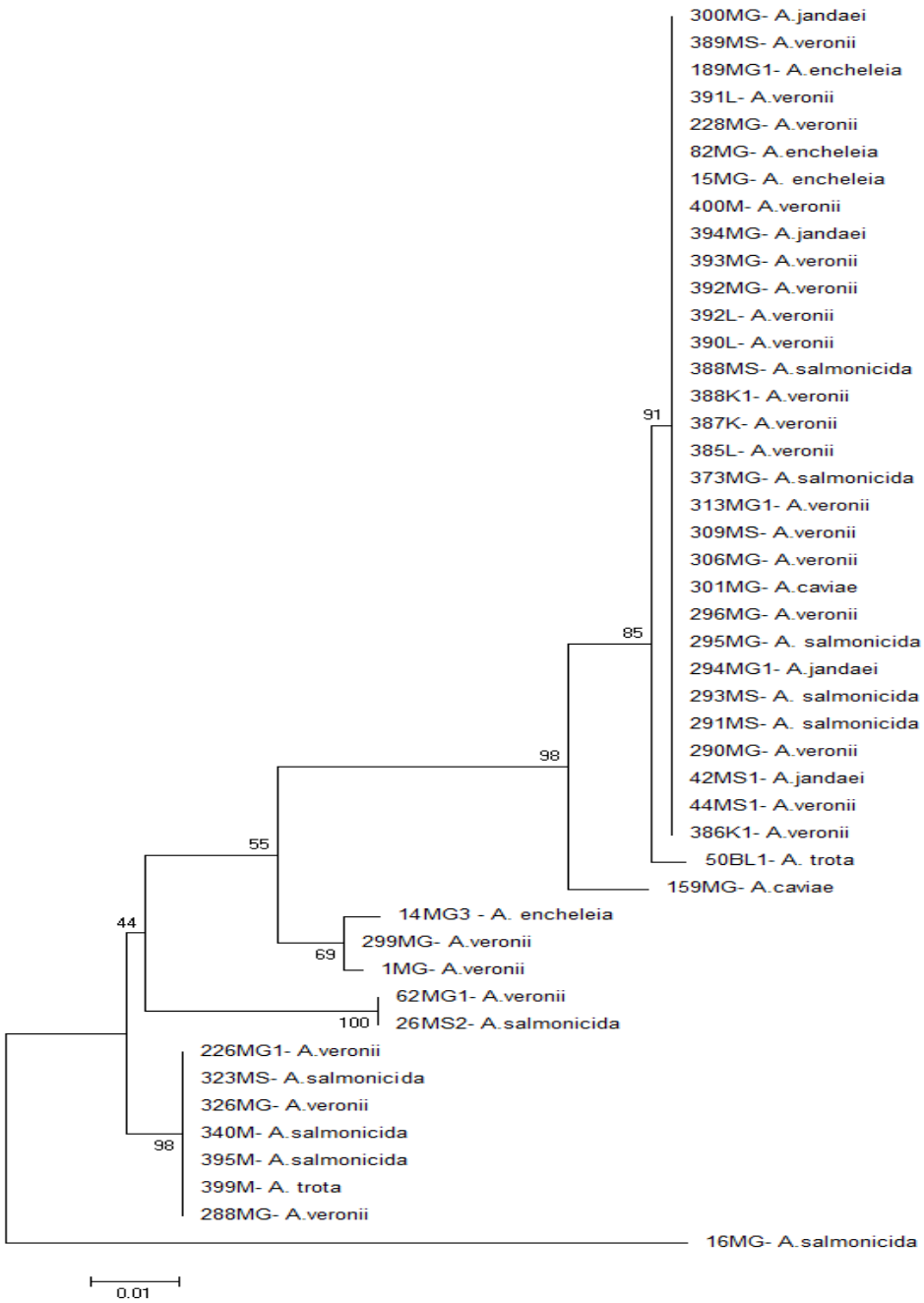

Figure 4. 9 The phylogenetic tree based on 16S rDNA fragment sequences, showing relationship of the genus *Aeromonas* (constructed by Maximum likelihood method using MEGA6 software); scale bar 0.01 represents sequence divergence.

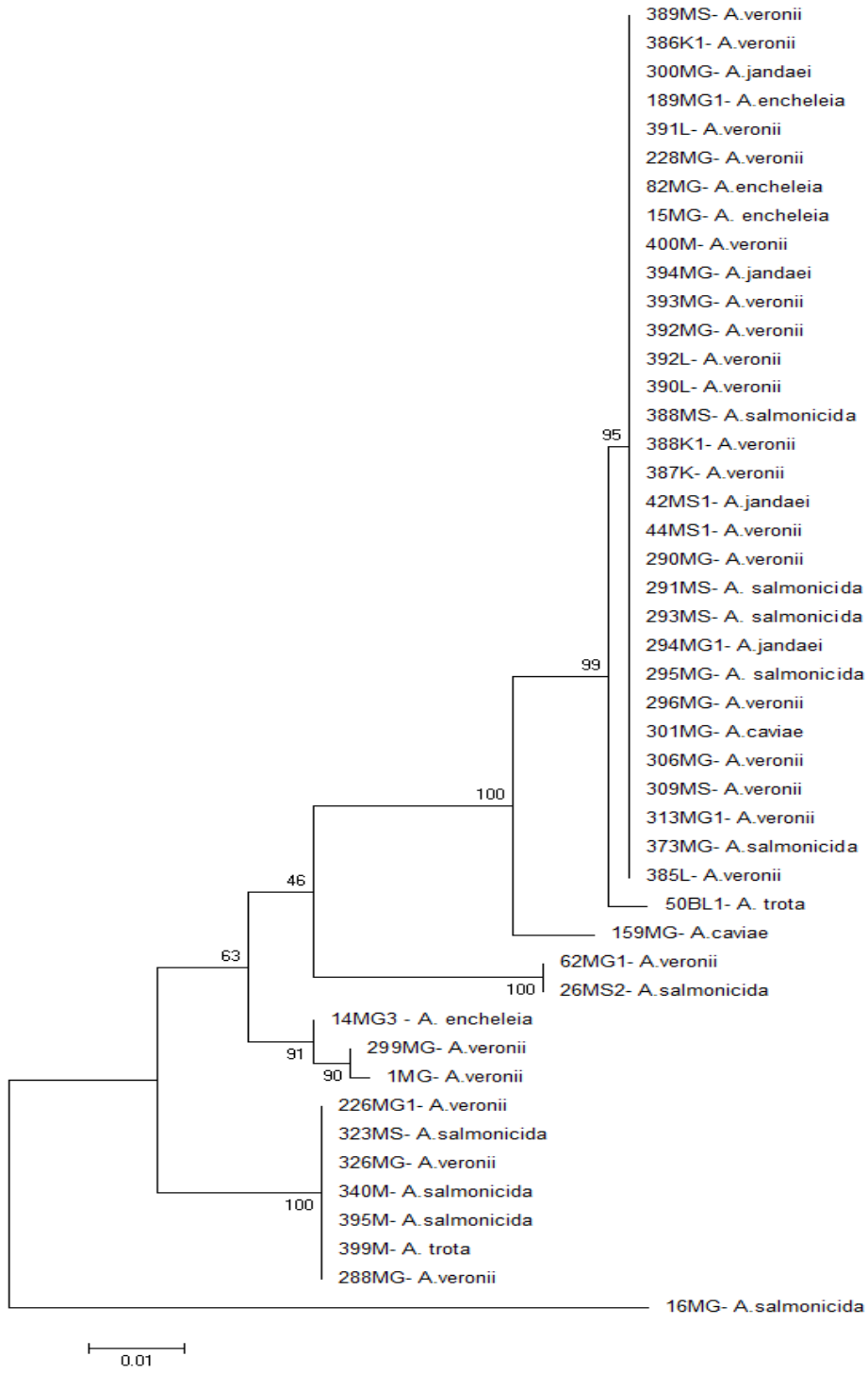

Figure 4. 10 The phylogenetic tree based on 16S rDNA fragment sequences, showing relationship of the genus Aeromonas (constructed by the neighbour-joining method using MEGA6 software) ; scale bar 0.01 represents sequence divergence.

4.5.3 Comparison of phenotypic and molecular data.

The phenotypic traits of 57% of the isolates agreed with data from the molecular genetic methods. Thus, the phenotypic approach resulted in the 46 isolates being equated with *A. veronii, A. caviae* and *A. salmonicida*. By molecular approaches, these isolates were identified as *A. caviae, A. veronii, A. salmonicida, A. encheleia, A. trota and A. jandaei* (Tables 4.5 and 4.6).

Table 4. 5 Identification of the 46 presumptive *Aeromonas* isolates.

	Total	**percentage**
BIO+RFLP+SEQ	26	57%
RFLP+SEQ-16SrDNA	20	44%

Explanations: BIO, Biochemical; RFLP, Restriction fragment length polymorphism; SEQ-16S rDNA, 16S rDNA gene sequencing

Table 4. 6 Total number of *Aeromonas* spp. identified by using phenotypic and molecular genetic (16S rRNA-RFLP and 16S rDNA sequencing) methods.

	16S rDNA RFLP/ 16S rDNA sequencing					
Phenotypic:	***A. caviae***	***A. salmonicida***	***A. veronii***	***A. encheleia***	***A. trota***	***A. jandaei***
	N = 2	**N = 10**	**N = 24**	**N = 4**	**N = 2**	**N = 4**
A. caviae (16)	2/16	3/16	7/16	2/16	0/16	2/16
A. salmonicida (8)	0/8	7/8	1/8	0/8	0/8	0/8
A. veronii (22)	0/22	0/22	16/22	2/22	2/22	2/22

4.5.4 Distribution of *Aeromonas* isolates between wild and farmed fish and shellfish in Oman

Most of the *Aeromonas* spp. identified in this study was recovered from both farmed and wild fish, except for two isolates of *A. caviae* that was recovered from wild fish (Table 4.7).

Table 4. 7 Distribution of *Aeromonas* isolates in aquatic samples in Oman

	Aeromonas spp.						Total
	A. caviae	***A. salmonicida***	***A. veronii***	***A. encheleia***	***A. trota***	***A. jandaei***	
Sample type	**N = 2**	**N = 10**	**N = 24**	**N = 4**	**N = 2**	**N = 4**	
Farmed fish and shellfish N=(26)	0	5	13	2	2	2	24
Wild Fish N= (20)	2	5	11	2	0	2	22

4.6 Discussion

The taxonomy of *Aeromonas* is complex due somewhat to their phenotypic and genotypic heterogeneity (Janda & Abbott, 2010; Beaz-Hidalgo *et al.*, 2010; Figueras *et al.*, 2000); a view that is supported by this study. *Aeromonas* cultures are generally sensitive to the vibriostatic agent O/129. However, some authors including Cahill & MacRae (1992); Ezaki *et al.* (1989) and Haruo Sugita *et al.* (1994) suggested that susceptibility to 150 µg of O/129 is not always sufficient to exclude isolates from *Aeromonas*. In agreement with these authors, 5 cultures in the present study were indeed sensitive to O/129. Certainly, many studies have reported discrepancies in phenotypic and molecular data (e.g. Borrell *et al*., 1997; Castro-Escarpulli *et al.,* 2003). Most studies concerning the identification of fish bacteria rely on biochemical tests, particularly using commercial identification systems, such as VITEK® 2 Compact, which has been suggested to be not particularly useful for the identification of *Aeromonas* (Lamy *et al*., 2010; Soler *et al*., 2003). This may be explained because many taxa are missing from the databases, which are biased towards medical rather than environmental isolates, i.e. those that grow at 35-37^{o}C rather than 15-25^{o}C. Certainly, molecular methods are in vogue, and are routinely used in many coun-

tries. The restriction enzymes used in this study showed the same characteristic patterns reported previously by Borrell *et al.* (1997). Overall in the present study, 57% of the *Aeromonas* isolates identified by phenotypic means were in agreement with the genetic methods. Conversely, there was disagreement with the remaining 43% of the isolates. Thus isolates identified phenotypically as *A. caviae* were delineated by molecular methods as *A. caviae*, *A. veronii, A. salmonicida, A. encheleia* and *A. jandaei*. In parallel, the 8 cultures of *A. salmonicida* identified by phenotypic means were equated with *A. salmonicida, A. veronii* and *A. encheleia* by molecular approaches. Similarly, 22 isolates identified phenotypically as *A. veronii*, were placed in *A. veronii, A. jandaei, A. encheleia* and *A. trota* by molecular genetic methods. Moreover, some authors have been reporting *A. veronii* bv. sobria as *A. sobria* (Martin-Carnahan & Joseph, 2005; Janda & Abbott, 2010) because of the result for ornithine decarboxylase, positivity infers *A. veronii* bv. *veronii;* negativity *A.veronii* bv. sobria (Martin-Carnahan & Joseph, 2005; Huys & Swings, 2005). Additionally, the restriction profile for the *A. veronii* bv. sobria and bv. veronii was similar because of their identical nucleotide sequences (Borrell *et al.,* 1997; Martínez-Murcia & Collins 1992,1993; Popoff *et al.,* 1981).

Sequencing of the 16S rRNA gene is not always useful for identifying *Aeromonas* (Figueira *et al*., 2011; Han *et al.,* 2011). Thus, a low percentage of sequence homology may lead to misidentification when comparison is made with the data in the BLAST program databases (Blocka & Anthony, 2010). Furthermore, it is worth remembering that the data contained within the BLAST databases are entered by individual authors/scientists, with no quality control. Nevertheless, sequence homology is regarded as useful for confirmation of membership in *Aeromonas* (Figueira *et al*., 2011; Han *et al.,* 2011).

The large differences between phenotypic and genotypic identification was most profound in the case of *A. veronii,* which predominated in this study. This lack of congruence between different methods has been recorded previously (Sugita *et al*., 1995). It is noted that *A. veronii* is present widely in the aquatic environment, and is known to pose a health risk to aquatic vertebrates, specifically being pathogenic to rainbow trout (Orozova *et al*., 2009). Moreover, the organism has been recovered from wild spawning gizzard shad (*Dorosoma cepedianum*) in Maryland, USA during 1987 (Toranzo *et al*., 1986; 1989) and from Siberian sturgeon (*Acipenser baerii*) (Ma *et al*., 2009).

A. salmonicida is a fish pathogen able to cause furunculosis in salmonids, and ulcer disease in cyprinids and marine flatfish (Austin & Austin 1993; Austin & Adams, 1996, Janda *et al.*,1996; Sørum *et al.,* 2000; Garduño *et al.*, 2000). The potential recovery of isolates in this study was surprising in view of the lack of disease signs in fish normally associated with the organism. Moreover, the initial identification by phenetic means invoked a more detailed study of the organisms (Table 2). Thus, it was observed that they did not produce brown pigmentation on TSA. In addition, there was not any evidence of variation in colonial morphology expected of many isolates of *A. salmonicida.* Therefore in terms of the current classification if confirmed as *A. salmonicida* the isolates would correspond with the description of atypical representatives of the taxon (Austin *et al.*, 1998; Figueras, 2005; Martin-Carnahan & Joseph, 2005; Austin & Austin, 2012). It should be emphasised that the traditional expectation of *A. salmonicida* is that the organism is found only in fish and never in the aquatic environment (Austin & Austin, 2012). There is a tendency that the organism is only recoverable as colony-forming-units from clinically diseased fish and not from the asymptomatic carrier state (Austin & Austin, 2012). However by using phenotyping and molecular methods, some isolates were equated with *A. salmonicida*

although there was not always congruence between the data. Clearly, there is more to be learnt about the biology of *A. salmonicida.*

4.7 References

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Pathogenicity and virulence of *Aeromonas* spp. isolated from Oman

5.1 Introduction

Aeromonas isolates are widespread in the aquatic environment, with many cultures being associated with both diseased and healthy fish (Torres *et al*., 1993; Esteve *et al*., 1995). Isolates have been associated with ulcerative disease, furunculosis, fin/tail rot, and internal and external haemorrhagic legions of fish (Beaz-Hidalgo and Figueras 2013). The pathogenesis of *Aeromonas* infections may be correlated with many factors, including stress and the production of cell associated and extracellular virulence determinants, e.g. proteases and haemolysins, which often lead to outbreaks of disease with concomitant mortalities and huge economic losses to aquaculture (Janda & Abbott, 2010). Among the cell-associated structures are pili, polar and lateral flagella, outer membrane proteins and plasmids (Janda & Abbott, 2010). *Aeromonas* extracellular products (ECP) contribute to pathological effects in fish, including rainbow trout (Ellis *et al*., 1981; Allan & Stevenson, 1981), and centre on cytotoxic, cytolytic, haemolytic and enterotoxin properties (Allan & Stevenson, 1981; Santos *et al*., 1988; Thune *et al*., 1986). It has been considered by some (e.g. Burke *et al*. 1983; Subashkumar *et al*., 2006) that haemolysin represents the main ECP virulence factor implicated in toxicity, whereas others, e.g. Kanai & Wakabayashi, 1984 and Sakai (1985), considered protease to be the responsible factor. Many virulence factors are involved consecutively for the bacterium to colonize, gain entry to the host, replicate and cause damage in host tissues, and thus obtain nutrition from the host, evade the host defense systems, spread, and finally damage/kill the host (Smith, 1995). The virulence factors are difficult to classify, and some of the toxins have an unclear role in pathogenicity (Scoaris *et al*., 2008).

In addition, no single virulence factor has been clearly correlated to virulence in fish. Variations in the distribution of potential virulence genes between *Aeromonas* isolates may well contribute to their degree of virulence (Albert, *et al*., 2000). Certainly, *in vivo* and *in vitro* biological assays have been used to identify potential pathogenesis. Although numerous virulence factors contribute to pathogenesis of fish and human diseases caused by *Aeromonas* spp., none of the factors alone are responsible for all the signs of disease (Ali *et al*., 1996). Therefore, there is a need to continuously assess the presence of several accepted virulence factors in *Aeromonas* isolates for better understanding of the overall pathogenesis of *Aeromonas* infections (Sen & Rodgers, 2004)

5.2 Study Aims

Determine pathogenicity and characterise the major putative virulence factors of *Aeromonas* spp. recovered from wild and farmed fish in Oman.

5.3 Materials and methods

5.3.1 Fish

Two challenge experiments were carried out, as follows:

A. A challenge experiment involving use of tilapia and rainbow trout. The challenge experiment with tilapia was carried out in the Fishery Quality Control Center (Oman) whereas the challenge with rainbow trout was conducted in the University of Stirling (Scotland).

B. Challenge experiment with ECPs from *Aeromonas* was conducted in the University of Stirling, and involved use of tilapia and rainbow trout.

The fish used for challenge experiment in Oman had been quarantined in the wet laboratory of the Aquaculture Center for 14 days prior to use. The experiments in Stirling involved use of a Home Office licensed challenge aquarium, and the fish were obtained from a stock held in quarantine facilities. These fish had been certified as of disease-free status prior to use. The weight of the tilapia and rainbow trout was 30 to 50 g, respectively. The fish were maintained in 100 l aquaria with aerated fresh water at 26◦C ± 1◦C and 15◦C ± 1◦C for tilapia and rainbow trout, respectively, and fed with commercial diet throughout the experiments. The fish were examined twice daily.

5.3.2 Pathogenicity of *Aeromonas* spp.

5.3.2.1 Bacterial challenge studies

In total, 18 *Aeromonas* cultures, which were isolated from wild and farm fish (see Appendix 5.1) was used for challenge studies. Generally, the isolates were derived from fish with clinical signs of disease; although cultures equated with *A. salmonicida* were not associated with any unhealthy fish (see Appendix 5.1). All 18 isolates were cultured in TSB and incubated overnight at 28ºC. Following centrifugation at 3500 rpm for 10 min, the cells were washed in 0.85 % (w/v) NaCl, and adjusted to an OD of 1.0 at 610 nm. The viable colony counts were performed using the Miles and Misra method (Miles & Misra, 1938) and then 10 -fold serial dilutions prepared to give ~1×10^8 /ml CFU for the challenge studies.

For each *Aeromonas* culture, one rainbow trout and one tilapia was injected intraperitoneally (i.p) with 0.1 ml of $l0^8$ CFU of bacteria/ml. Controls received 0.1 ml volumes of sterile 0.85% (w/v) saline. Fish were examined for 5 days whereupon the presence or absence of mortalities and disease signs were recorded. Bacterial recovery were carried out aseptically from the kidney

and spleen by inserting a sterile loop directly in to the organs and the material streaked onto TSA plates, which was incubated at 28ºC for 24 h. These were examined for bacterial growth, with identification as before (Chapter 4).

5.3.2.2 Preparation of extracellular products (ECP) from *Aeromonas*

ECP was prepared using the cellophane overlay method of Austin & Rodgers (1981); Austin *et al*., (1998) and Gudmundsdóttir (1996). Through the use of this method, bacteria are able to draw nutrients easily from below the cellophane sheet, and at the same time prevent mixing of the ECP proteins released by the bacteria with the proteins present in the underlying culture medium. Briefly, sterilized cellophane sheets (121ºC/15 min) were placed on the surface of TSA plates and inoculated by spreading 0.5 ml of overnight culture in TSB over the surface. After incubation at 28 ºC for 24 h, the cellophane overlay was transferred to an empty petri dish. Bacterial cells were washed off the cellophane film using 10 ml volumes of saline and removed by centrifugation at 10,000 x g at 4°C for 30 min. The supernatant containing the ECPs was sterilized by filtration through 0.22 μm (Millipore, Millex, Edinburgh, UK) porosity filters, and stored at -20 ºC until required. To confirm sterility, 0.2 ml volumes of the filtered supernatants were streaked over TSA plates for 48 h at 25^{o}C whereupon the absence of growth was indicative is sterility.

5.3.2.2.1 Protein determination

The protein concentration of the ECP was determined by the method of Bradford (1976) using a protein determination kit (Bio-Rad) according to the manufacturer's instructions with bovine serum albumin (BSA; Sigma-Aldrich) as a standard, and subsequently stored at -20 ºC until required.

5.3.2.2.2 Determination of ECP toxicity

The toxicity of ECPs was tested by using rainbow trout and tilapia. For each bacterial culture, one rainbow trout and one tilapia were injected i.p. with 0.1 ml volumes of ECP (0.1mg/ml). A similar number of fish were injected intramuscularly (i.m) with 0.1 ml volumes of ECP. Then, the fish were monitored, and mortalities recorded daily for a maximum of 5 days, and samples taken for histopathology. Controls were injected i.p. and i.m. with 0.1 ml volumes of sterile saline.

5.3.2.2.3 Extracellular proteolytic and haemolytic activity- *in vitro* assays

Filtered supernatants obtained from the ECP preparations were used. Protease production was detected as zones of clearing around colonies on TSA supplemented with 1% (v/v) skimmed milk. A clear zone around the inoculum of ECP was taken as a positive result (Pavlov *et al*., 2004). Haemolytic activity was determined by seeding ECP into well cut into 5% (v/v) horse blood agar plates (Oxoid) with incubation at 28°C for 24 h. The presence of clear areas around the wells indicated haemolytic activity (Brender & Janda, 1987).

5.3.3 Histopathological examination

Samples of spleen and kidney were dissected from the fish, and fixed in 10% (v/v) neutral buffered formalin for 24 h before embedding in paraffin following routine tissue processing. Five-micron thick sections were cut and stained with haematoxylin and eosin (H&E) (Oliveira Ribeiro *et al.*, 1981) and Gram stained for histological examination. All slides were examined by light microscopy.

5.3.4 Characterisation of virulence factors– *in vitro* assays

Single colonies of *Aeromonas* were inoculated onto TSA plates, and incubated at 28°C for 24 h. All isolates were tested in duplicate, and when results were different, a third experiment was carried out to resolve any discrepancies.

5.3.4.1 Haemolytic activity

Haemolytic activity were tested using 5% (v/v) horse blood agar plates (Oxoid) on which loopful's of overnight cultures were streaked with incubation at 28°C for 24 h. The presence of clear zones surrounding the colonies indicated haemolytic activity (Brender & Janda, 1987).

5.3.4.2 Proteolytic activity

Protease activity was determined on skimmed milk agar, in which 50% warmed skimmed milk (Marvel) was added to TSA with double the amount of agar No. 1 (Oxoid) just before pouring the medium into Petri dishes. The inoculated plates were incubated at 28 °C for 4 days when the presence of a transparent zone around the colonies indicated caseinase activity (Gudmundsdottir, 1996).

5.3.4.3 DNase

DNase agar plates (Oxoid) were inoculated and incubated at 28ºC for 24 h. The plates were then flooded with 1M of HCl and the formation of clear zones surrounding the areas of bacterial growth indicated a positive result.

5.3.4.4 Congo red dye uptake

To evaluate Congo red binding, bacteria were grown at 28º C for 24h on TSA supplemented with 0.01% (w/v) Congo red (Sigma-Aldrich) and 0.15% (w/v) bile salts (Sigma-Aldrich). Positive colonies appeared red, whereas negative colonies were pale. Based on the intensity of red colour, the binding was scored as + and ++ (Crump & Kay, 2008).

5.3.4.5 Coomassie Brilliant Blue

TSA supplemented with 0.1% (w/v) Coomassie Brilliant Blue (CBBA; Bio-Rad Laboratories, Richmond, CA, USA) was streaked with the cultures, and incubated for 24 h at 37°C (Cipriano & Bertolini, 1988). Dark blue colonies on CBBA plates were considered to express the surface A-layer protein. Based on the intensity of blue colour, the binding was scored as + and ++.

5.3.4.6 Detection of virulence genes

The presence of genes encoding the virulence factors aerolysin (*aer*), aerolysin-like proteins (*act*), cytotoxic enterotoxins (*ast*, *alt*), glycerophospholipid cholesterol acyltransferase (*gcat*), structural gene flagellin (*lafA* , *lafB*) and serine protease was determined by the polymerase chain reaction (PCR) using primers and conditions already published, as shown in Table 5.1. PCR amplifications for the genes were carried out by using Ready-To-Go™ PCR beads (GE Healthcare). According to the manufacturer's instructions, 5 µl of extracted DNA (50 ng/µl) and 1 µl of each Forward and Reverse primer (10 pm/µl) and made up to 25 µl with nuclease-free water was added to each tube. The PCR reaction was performed with a thermal cycler (Biometra T gradient^{TM}, Goettingen, Germany). Five microliters of each PCR reaction were mixed with 1

µl of 6x gel loading dye (ThermoScientific) and electrophoresed on 1% agarose gel (Invitrogen UK), stained with 0.5 µg/ml of ethidium bromide and visualized under ultraviolet light (UV).

Table 5. 1 Sequence of oligonucleotide (F: forward, R: reverse) used in the study, respective product size and reference.

Gene amplified	Primer	Primer set- Sequence of oligonucleotides (from 5' to 3' end)	Amplified fragment size in bp (gene)	Reference
Aerolysin gene	*areo*- F	CCAAGGGGTCTGTGGCGACA	209	(Pollard *et al*., 1990)
	areo- R	TTTCACCGGTAACAGGATTG		
GCAT	*gcat*-F	CATGTCTCCGCCTATCACAACAAGC	339	(In-Young & Kiseong, 2007)
	gcat-R	CCAGAACATCTTGCCCTCACAGTTG		
Serine protease	*Ser*-F	ACGGAGTGCGTTCTTCCTACTCCAG	211	
	Ser-P	CCGTTCATCACACCGTTGTAGTCG		
Nuclease	*nuc*-F	CAGGATCTGAACCGCCTCTATCAGG	504	
	nuc-R	GTCCCAAGCTTCGAACAGTTTACGC		
Lateral flagella B	*lafB*-F	GACCAGCAAGGATAGTGGGTTGGAG	624	
	lafB-R	AAGCACCATCGCGTTGGTATAAGG		
Lateral flagella A	*lafA*-F	CCAACTT(T/C)GC(C/T)TC(T/C)(C/A)TGACC	700	(Aguilera-Arreola *et al*., 2005)
	lafA-R	TCTTGGTCAT(G/A)TTGGTGCT(C/T)		
Cytotonic enterotoxins	*ast*-F	ATCGTCAGCGACAGCTTCTT	504	
	ast-R	CTCATCCCTTGGCTTGTTGT		
	alt-F	AAAGCGTCTGACAGCGAAGT	320	
	alt-R	AGCGCATAGGCGTTCTCTT		
	act- F	AGAAGGTGACCACCAAGAACA	232	(Sen & Rodgers, 2004)
	act-R	AACTGACATCGGCCTTGAACTC		
16S rDNA	16S-rDNA-F	AGAGTTTGATCATGGCTCAG	1,502	(Borrell *et al*., 1997)
	16S-rDNA-R	GGTTACCTTGTTACGACTT		

5.4 Data Analysis

MS Excel was used to store all the data, and to produce charts and tables. Categorical variables were presented as frequencies (N, %).

5.5 Results

5.5.1 Pathogenicity for fish

There was no evidence of mortalities in any of the fish injected with *Aeromonas* cultures. Moreover, there were not any disease signs or mortalities in fish injected with sterile saline (Figure 5.1). However, four of the cultures were recoverable from the kidney and spleen of fish at the end of the experiment at 7 days, suggesting that organisms had the ability of remaining within fish tissues in a viable state. In total, 4 isolates caused clinical signs of disease, albeit in the absence of mortalities, in tilapia and rainbow trout (Table 5.2).

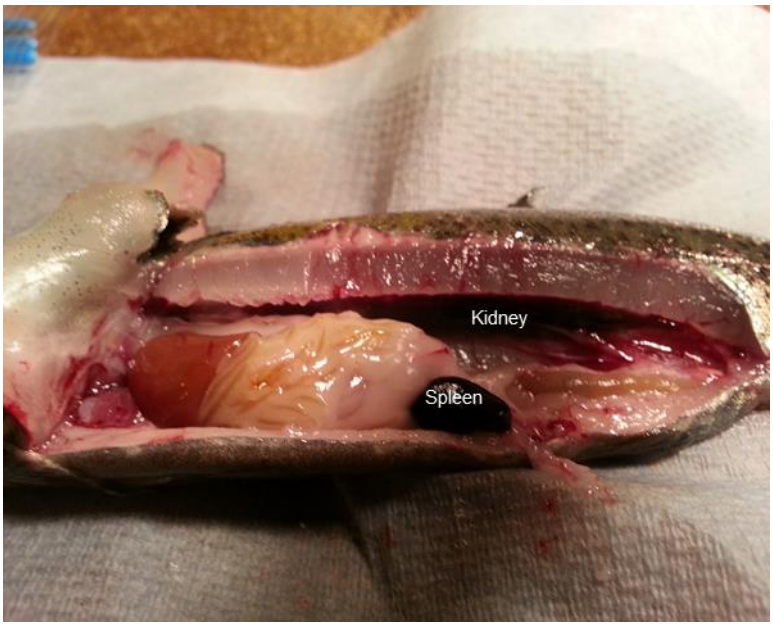


Figure 5. 1 Rainbow trout showing normal internal organs, skin and fins after challenge with *Aeromonas.*

Table 5. 2 Result of bacterial challenge studies – *in vivo*

Isolate ref no.	***Aeromonas* spp.**	**clinical sign of disease**		***Aeromonas* strain recovered**	
		Tilapia	**Trout**	**Tilapia**	**Trout**
14MG3	*A. encheleia*	NC	Haemorrhage internal	NR	NR
16MG	*A. salmonicida*	NC	NC	NR	√ from Kidney & Spleen
26/MS2	*A. salmonicida*	NC	NC	NR	NR
42/MS1	*A. jandaei*	NC	NC	NR	NR
44MS1	*A. veronii*	NC	NC	NR	NR
50BL1	*A. trota*	NC	NC	NR	NR
189MG1	*A. encheleia*	Internal haemorrhage	Exophthalmia	√ from kidney	√ from kidney
291MS	*A. salmonicida*	NC	NC	NR	NR
293MS	*A. salmonicida*	NC	NC	NR	NR
295MG	*A. salmonicida*	NC	NC	NR	NR
340M	*A. salmonicida*	NC	Pale Liver & dark Kidney	√ from kidney & spleen	√ from kidney
373MG	*A. salmonicida*	NC	NC	NR	NR
385L	*A. veronii*	NC	NC	NR	NR
388MS	*A. salmonicida*	NC	NC	NR	NR
390L	*A. veronii*	NC	NC	NR	NR
391L	*A. veronii*	NC	NC	NR	NR
392L	*A. veronii*	Internal haemorrhage	NC	√ from kidney	√ from spleen
395M	*A. salmonicida*	NC	NC	NR	NR

Explanations: NC= no clinical signs of disease; NR= not recovered

5.5.2 Toxicity of the ECP in fish

As a result of challenge experiments with live bacterial cells, 4 isolates were used to check the toxicity of the ECP. The ECP filtrate was adjusted to 0.1 mg/ml; the ECP standard concentration curve is shown in Figure 5.2. Following i.p. injection, small haemorrhages were noted in the internal organs of fish injected with one isolate (189G1; Table 5.3). In contrast, clinical signs did

not occur when injection was by the intramuscular route. However, ECP injected i.m. and i.p. did not result in any harmful effects in the fish. Moreover, mortalities did not occur in the control fish.

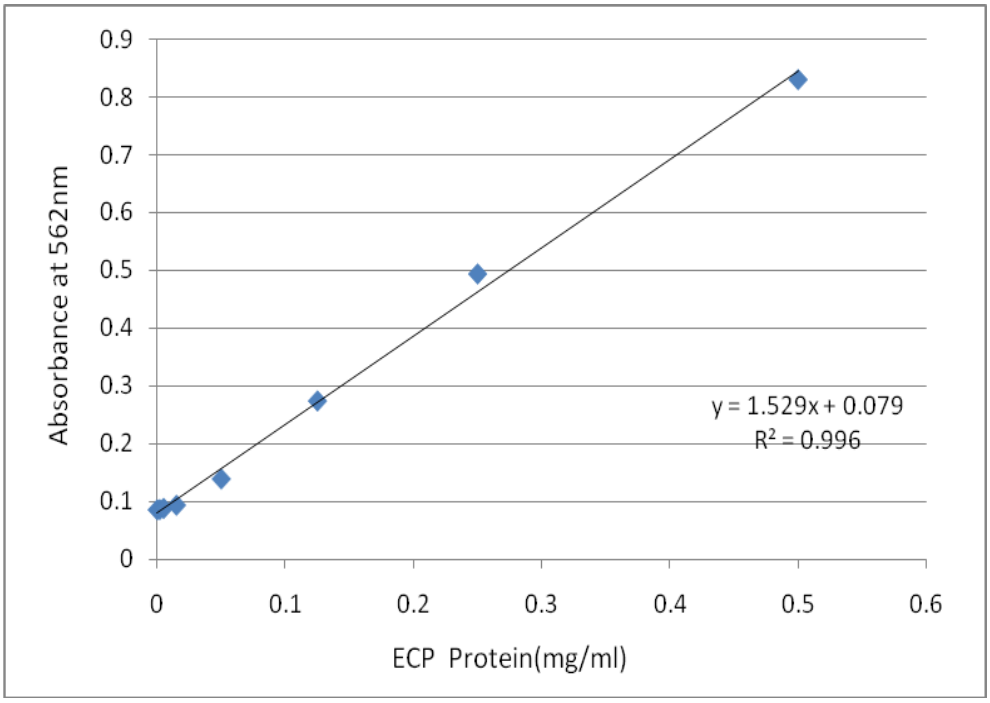


Figure 5. 2 ECP protein concentration standard curve**.**

Table 5. 3 ECP challenge experiment- in vivo

Isolate ref no.	Symptom of disease after ECP challenge			
	Intramuscular injection		Intraperitoneal injection	
	Rainbow trout	Tilapia	Rainbow trout	Tilapia
16MG	NC	NC	NC	NC
189MG1	NC	ND	Haemorrhage internal system	Haemorrhage internal system
340M	NC	NC	NC	NC
392L	ND	NC	NC	NC

Explanations: NC= No clinical signs of disease.

5.5.3 Histopathology

There were not any pathological signs observed from the histological material prepared from the kidney and spleen of fish injected with any of the *Aeromonas* isolates (Figure 5.3 A&B).

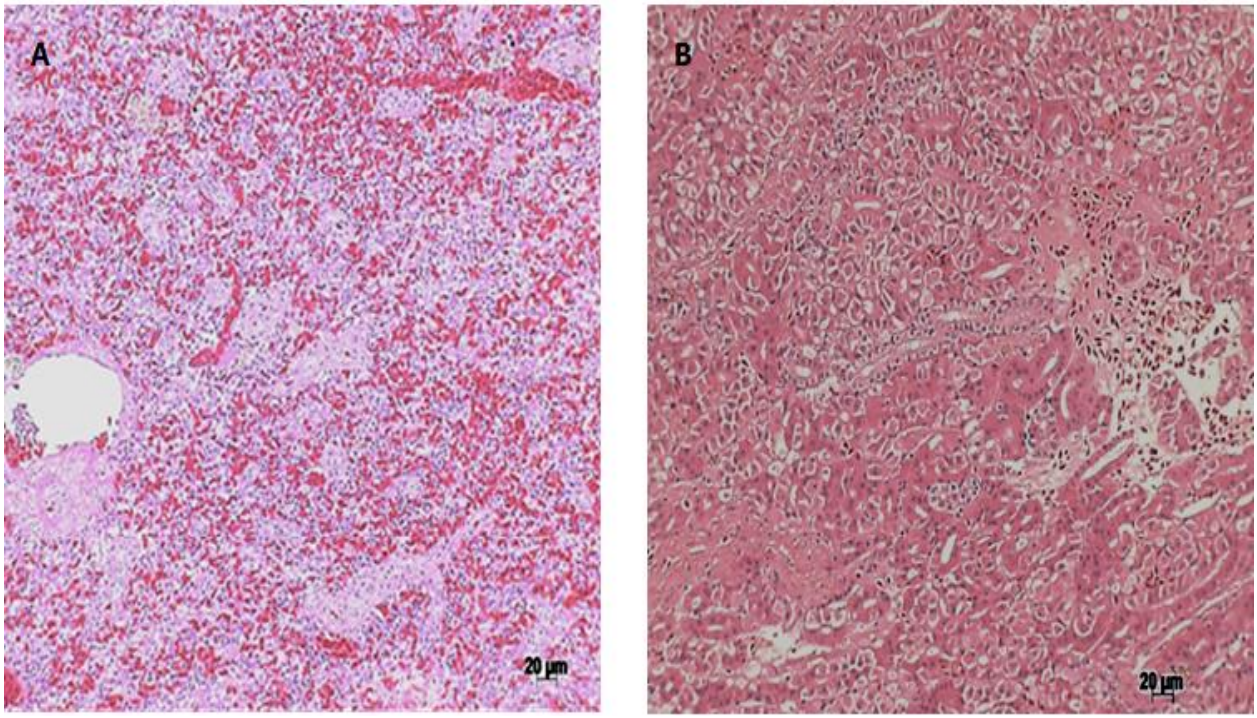


Figure 5. 3 Light microscopic appearance of the (A) spleen and (B) kidney of tilapia injected with extracellular products (ECP) of *Aeromonas* isolates. (H&E, scale bar = 20µm).

5.5.4 Putative virulence factors

Results of the examination of selected genes encoding putative virulence factors and their related phenotypic activity are shown in Appendix 5.1.

5.5.4.1 Detection of putative virulence factors

Eighteen *Aeromonas* cultures were screened for aerolysin, aerolysin-like proteins (*act*), cytotoxic enterotoxins (*ast,alt*), glycerophospholipid cholesterol acyltransferase (*gcat*), structural gene flagellin (*laf*A ,*laf*B) and serine protease (Figure 5.4 A,B,C,D). In general, there was not any similar distribution of putative virulence factors among all isolates, and also there was not any isolate

that had all six genes; however, all isolates possessed at least one gene (Table 2). The *ast* gene was found widely distributed among the isolates, with 78% of the total number of isolates containing the gene (Figure 5.5). Many (67%) contained aerolysin-like proteins (*act*). Also, the genes for *lafA, alt* were present in 50% of isolates, whereas *ser, lafB* and *aer* were present in 17% of the strains, and *nuc* and *gcat* were present in 11% of the strains. The *gcat* gene was recorded only in *A. salmonicida* cultures.

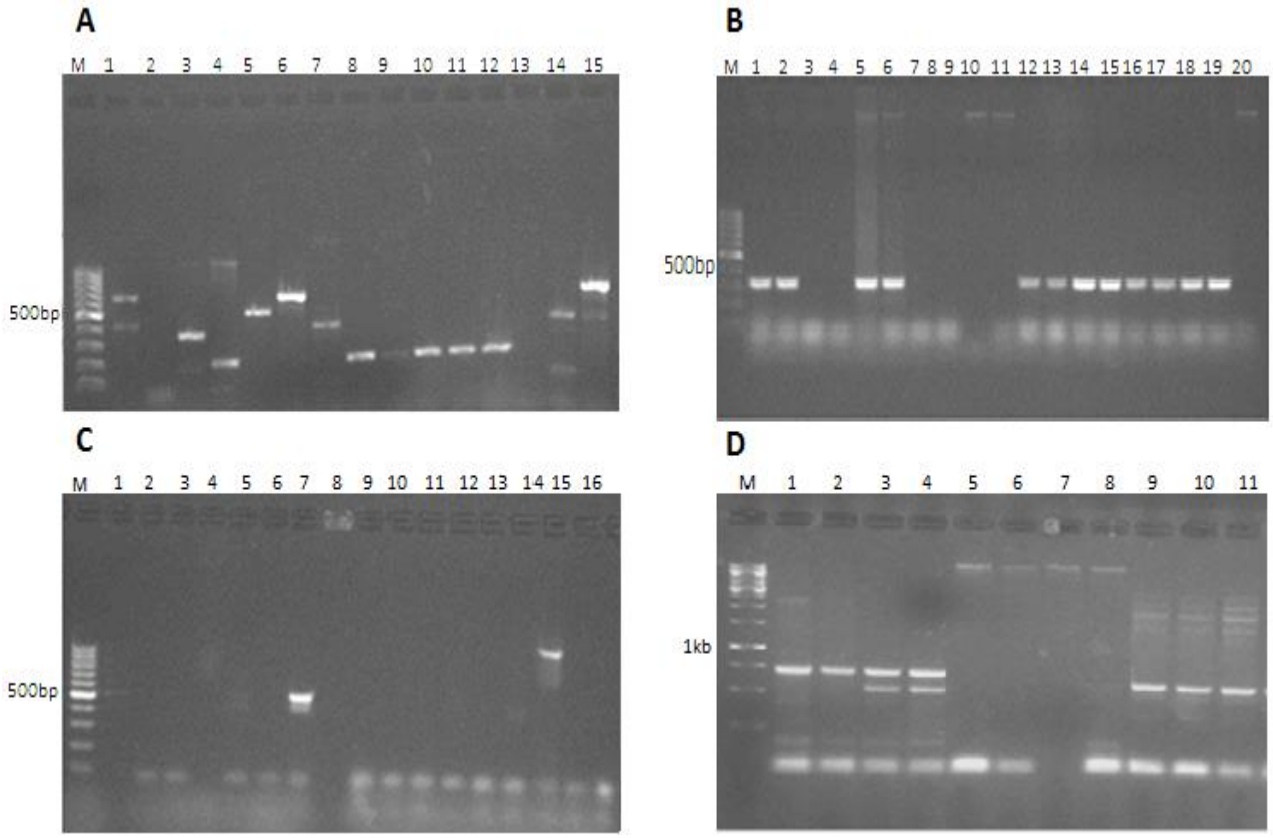


Figure 5. 4 (A) Amplification of the 209 bp region of the *aer* gene, Lane M= 500 bp DNA markers, Lanes 4, 8-12-10= *aer* gene amplified from *Aeromonas* strains. (B) Amplification of the 209 bp region of the *alt* gene, Lane M= 500 bp DNA markers, Lanes 1, 2, 5, 6, 12-19= *alt* gene amplified from *Aeromonas* strains. (C) Amplification of the 504 bp region of the *nuc* gene, Lane M=500 bp DNA markers, Lanes 7= *nuc* gene amplified from *Aeromonas* strains. (D) Amplification of the 700 bp region of the *lafA* gene, Lane M= 1kb DNA marker, Lanes 1, 2, 9-11= *lafA* gene amplified from *Aeromonas* strains.

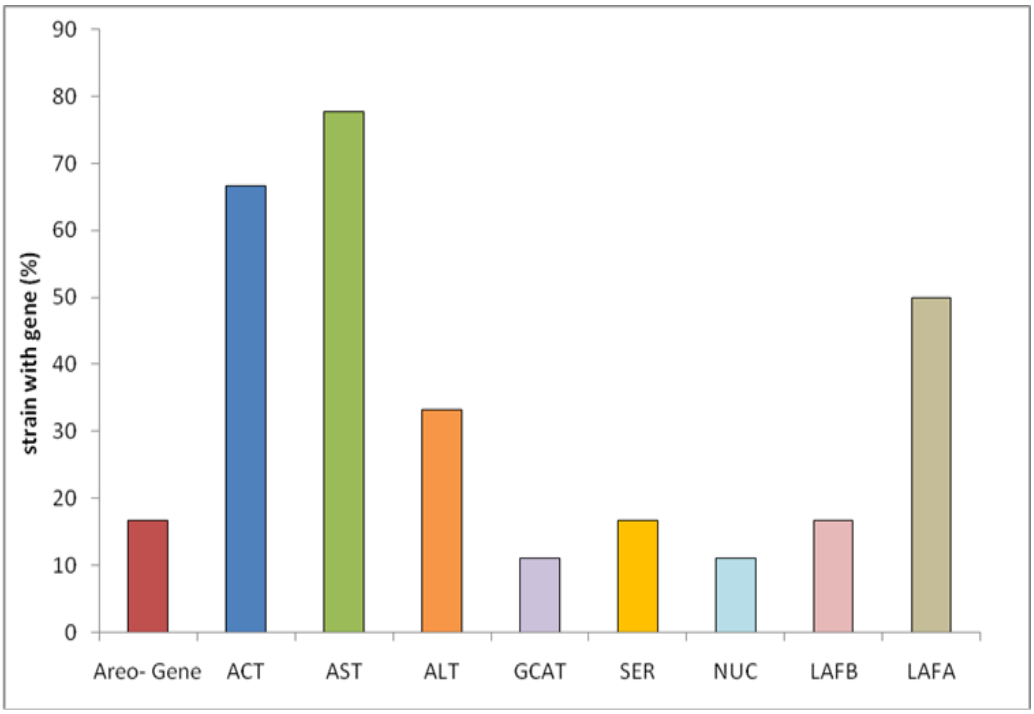


Figure 5. 5 Frequency of putative virulence genes in *Aeromonas* spp. isolates (results are given as percentage of total *Aeromonas* strains isolated).

5.5.4.2 Phenotypic determination of virulence factors

Extracellular nuclease activity was present in most isolates (67% of the total); extracellular protease and haemolysin activity was present in 56% of the total isolates. Of the latter, 44% of the isolates showed γ haemolysis, 39% β haemolysis and 17% α haemolysis (Figure 5.6). Uptake of Congo red was present in 39% of total isolates, whereas uptake of Coomassie brilliant blue was observed in 44% of the total number of isolates. Here, positive colonies appeared dark red for Congo red and dark blue for Coomassie brilliant blue, whereas negative colonies were pale coloured. Based on the intensity of red colour for Congo red uptake, the binding was scored as +, and ++ and negative for non-activity, as shown in Figure 5.7.

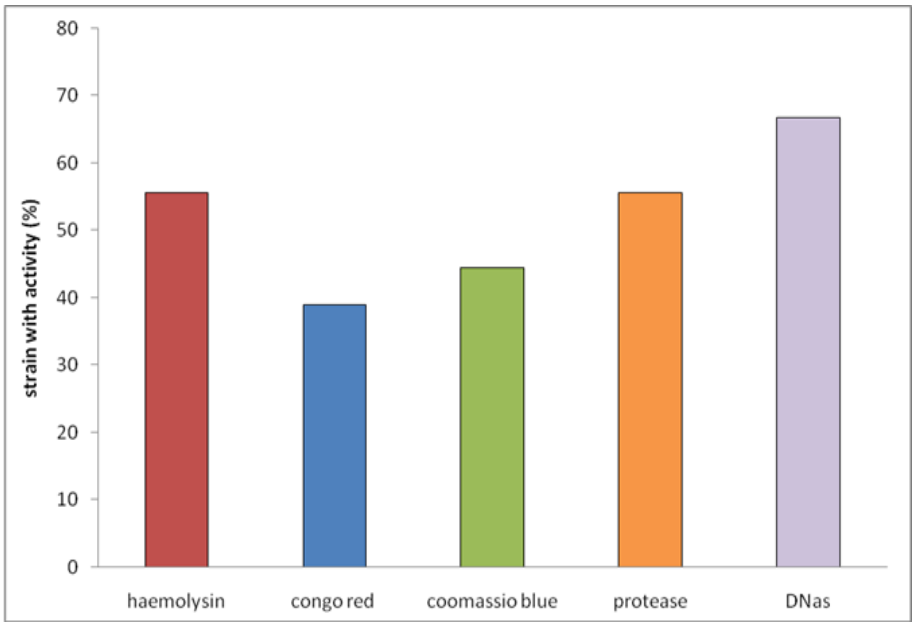
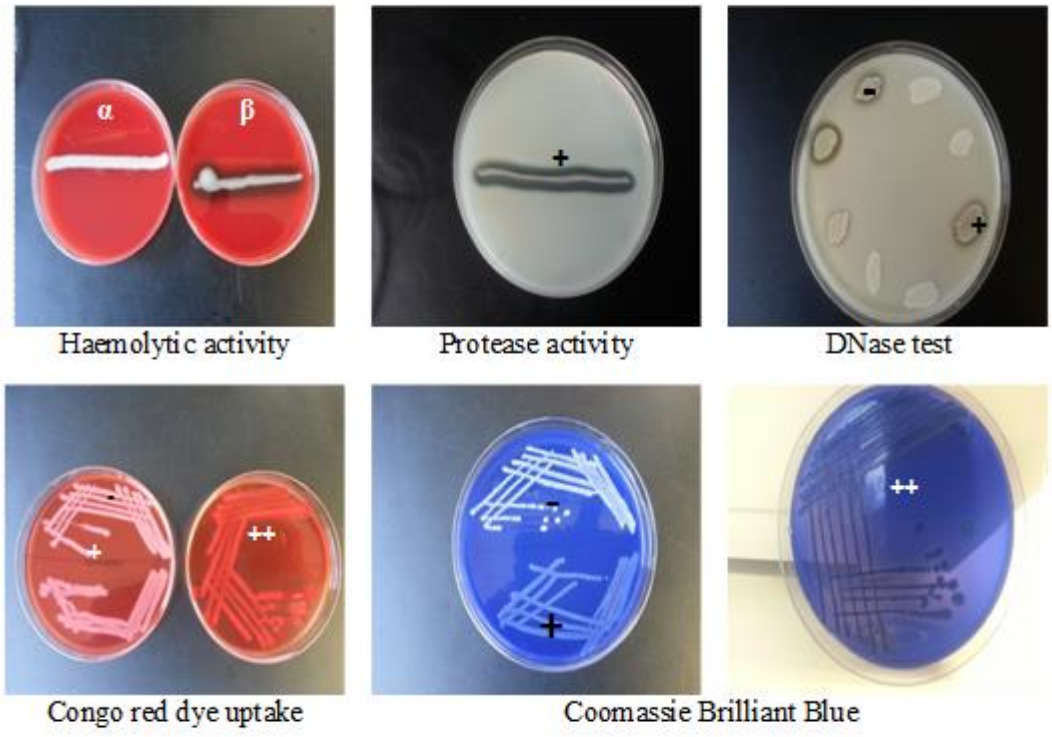


Figure 5. 6 Frequency of virulence factors in *Aeromonas* spp. isolated from wild and cultured fish.



Explanations: α = α haemolysis; β= β haemolysis; ++= positive activity and strong intensity of colour; += positive activity and less intensity of colour; - = negative or no activity

Figure 5. 7 Virulence factors expressed in *Aeromonas* spp.

5.5.4.3 ECP - proteolytic and haemolytic activity

The ECPs did not display haemolytic activity, although two isolates demonstrated proteolytic activity in the ECPs (Figure 5.8). The positive strains of proteolytic activity were identified as *A. veronii* and *A. encheleia.*

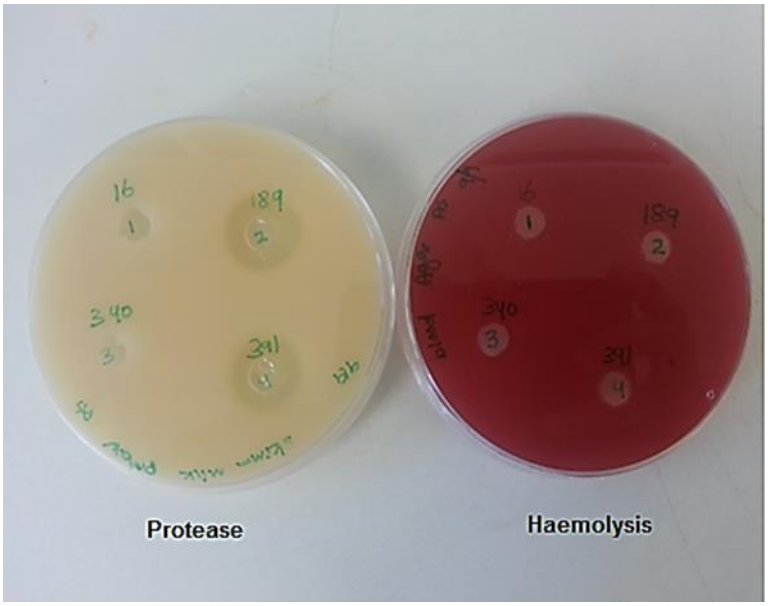


Figure 5. 8 Proteolytic and haemolytic activity of ECP product.

In order to determine the host susceptibility range of Aeromonas, infectivity trials were conducted using tilapia and rainbow trout. *Aeromonas* isolates used in this study originally recovered from tilapia while using rainbow trout as a second model as is cold water fishes and using intensively in the past as a model fish for *Aeromonas* challenge experiment.

5.6 Discussion

Pathogenesis of *Aeromonas* species is complex and multi-factorial, with the contribution of a comparatively large number of virulence factors (Aguilera-Arreola *et al*., 2007).

Experimental fish challenge studies were performed using both tilapia and rainbow trout as in Vivo models. The tilapias were a suitable species as the bacterial species under examination

were originally recovered from clinically affected tilapia in Oman. Furthermore these fish are susceptible to motile *Aeromonas* infection (Cipriano , 2001). The rainbow trout were used as an additional model to explore pathogenicity of the isolates as these fish are not commonly affected by motile *Aeromonas* as septicaemia but are used as a standard aquaria challenge model for freshwater bacterial infectivity studies (Iwama *et al*., 2011).

Thus to better understand the exact role of virulence factors in pathogenicity and the potential risk associated with the presence of *Aeromonas* isolates recovered from wild and farmed fish in Oman, isolates were examined for the presence of key virulence determinants. In agreement with previous studies, i.e. Albert *et al*. (2000), Krzymińska *et al.* (2001) and Sechi *et al*. (2002), heterogeneity was observed in the distribution of virulence factors among the isolates. Certainly, all *Aeromonas* isolates in this study were positive for at least one toxin gene and this observation is in agreement with the data for an *Aeromonas* strain recovered from aquatic fish Ottaviani *et al*. (2011). In contrast *ast* and *alt* genes were present only in some isolates. The *ast* gene was presented only in *A. veronii* and *A. salmonicida,* whereas the *alt* gene was present only in *A. enchelia*.

Haemolytic and proteolytic activities are considered to be virulence-associated factors in many *Aeromonas* species (Paniagua *et al*.,1990; Esteve *et al*. 1995; González-Rodríguez *et al*., 2002; Rahman *et al*., 2002). In this study, 56% out of total number of *Aeromonas* isolates were found to be β haemolytic in horse blood agar. The same proportion of isolates was accredited with proteolytic activities. However, the same isolates, when tested for the presence of aerolysin (*aerA*) and aerolysin-like proteins (*act*) genes by PCR, revealed only 17% and 67% positive results, respectively. Similarly, the nuclease gene was found in only 11% of the isolates tested, being present in A. *janda* and *A. veronii*, in comparison, DNase activity was present in 66% of the isolates. This suggested that the isolates may be negative phenotypically but nevertheless possess

but not express the genes (Wang *et al*. 2003). Perhaps, this reflects the methods used, and it is therefore important when describing taxa to highlight the method used.

Only three isolates, 17 % out of the total number of *Aeromonas* isolates and belonging to *A. encheleia* and *A. veronii,* expressed all three enterotoxin genes (*alt*, *ast* and *act* genes); data that are consistent with previous publications (Albert *et al*., 2000; Chang *et al*., 2008; Sha *et al*., 2002). These workers reported the presence and expression of these three genes in *Aeromonas* spp., and if presented together could lead to a 100% reduction in fluid secretion in a mouse model (Sha *et al*., 2002). Conversely, Sen and Rodgers (2004) reported that the mere presence of these toxins may not be sufficient for virulence, and this was agreement with the current study as the three isolates possessing all three genes together did not show any sign of disease or mortality through the use of live cells and ECPs in challenge experiments.

Glycerophospholipid cholesterol acyltransferase (*gcat*) is known to be secreted by *A. salmonicida* and is considered to be an important enzyme in view of its association with virulence (Moriarty, 2010). In this study, the *gcat* gene was detected only in 22% of *A. salmonicida* isolates, whereas it was absent in all other isolates. This result was in agreement with the work of Onuk *et al*. (2013), who reported the presence of this gene in *A. salmonicida* isolates, and not in *A. sobria* and *A. veronii*.

Congo red and Coomassie brilliant blue agar have been used as phenotypic markers for the presence of A-layer proteins, which distinguishes virulent and nonvirulent isolates of *A. salmonicida* (Ishiguro *et al*., 1985; Maurelli *et al*., 1984). The A^+ bacteria absorb the protein dye and develop as blue or red colonies, whereas the A^- bacteria produced white or pale colonies. In this study, most isolates displayed a white/pale colour or exhibited a low intensity of each virulence marker

except one isolate of *A. veronii* (44MS3). This isolates was originally recovered from tilapia with external haemorrhages in the head, skin and at the base of the fins, but in pathogenicity experiments, the isolate did not cause any sign of disease or any mortality. This was indicative of the presence of non-virulent isolates as discussed previously by Cipriano and Bertolini (1988). Conceivably, a loss in pathogenicity may be attributed to conditions such as routine passage on culture media (Phipps *et al*., 1983). Possibly, the virulence may have needed to be enhanced by passaging the bacteria repeatedly through a susceptible host (Cipriano *et al*., 1988).

Some studies reported a correlation between the higher number of virulence genes harboured in *Aeromonas* spp. and their potential for causing disease (Albert *et al*., 2000; Chang *et al*., 2008; Sha *et al*., 2002).

In this study, there was not any correlation between the number of virulence genes and the potential pathogenicity. A reasonable explanation could be that the experimental conditions used in this study affected the expression of the genes involved in pathogenicity and also the level of virulence has inevitably been correlated with the amount of enzymes and toxins produced (Hsu *et al*., 1981; 1983; Kozin´ska 1996). Another possibility is that the presence in aeromonads does not infer that disease is inevitable reflecting the susceptibility of the host, immune state, and actual number of bacterial cells in and around the host (Ottaviani *et al*. 2011). Notwithstanding, some isolates did lead to the development of small haemorrhages in/on the internal organs, as reported previously (Austin & Adams 1996). In this respect, it is worthwhile to consider the comments of Austin & Austin (1993) and Austin (2011), who considered that loss of virulence might well reflect the effects of storage, i.e. the transition to what are effectively laboratory cultures, and the inability to replicate conditions of the initial disease which led to the recovery of the cultures.

Certainly, it may be concluded that pathogenicity may be correlated with many factors, including the size of the inoculum, the method of administration of the bacteria to the host, host susceptibility and the virulence characteristics of the pathogen (Falkow, 1990).

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General conclusions, recommendation and future directions

6.1. General conclusions

This is the first study aimed at acquiring knowledge about the current status of aquaculture in Oman. One outcome is that the information will help government make policy decisions related to developing health management strategies for Omani aquaculture.

Additionally, some specific conclusions can be outlined:

- The initial focus of using a questionnaire to be completed by fish farmers provided an interesting insight into the knowledge base of the aquaculturists. In short, there was a lack of knowledge on fish health management and the need to communicate relevant information [on disease] to the Ministry of Agriculture and Fisheries. However, the role of water quality of the aquatic environmental conditions and the occurrence of disease are unclear. Yet, all water parameters appeared to be within the legal standards allowed for tilapia culture in Oman. For the future and in order to develop sustainable aquaculture in Oman, awareness and training in Good aquatic practise and management with emphasis on fish health management is important.
- From the primary biological data of the project (chapter 4), 420 different bacterial isolates were recovered from fish samples in Oman, with the region of Muscat and king soldier bream fish displaying the maximum number of bacterial taxa recovered. Overall, gill mucus yielded the highest number of isolates, with 84% comprising

Gram negative, rod-shaped bacteria. The most frequently isolated groups from wild and cultured fish were *Aeromonas* spp. (25% of the total), *Vibrio* spp. (22%).

- Differences between the results of phenotyping and molecular-based methods were highlighted, with the former recognising *A. caviae, A. salmonicida* and *A. veronii,* whereas *A. caviae, A. salmonicida, A. veronii, A. encheleia, A. trota* and *A. jandaei* were delineated by 16S rDNA-RPLP. The RFLP patterns of the *Aeromonas* isolates showed the characteristic patterns reported previously by Borrell *et al.* (1997).
- Many studies (Schloter *et al*., 2000; Figueras *et al*., 2006 ; Singholey & Zervos, 2006) agree with this study, and confirmed that molecular methods are powerful tools for discriminating bacterial groups, more so than phenotypic methods.
- The lack of congruence between the phenotypic and genotypic methods matched previous work (Austin *et al.*, 1989).
- The recovery of cultures identified by phenotyping and separately by molecular methods as *A. salmonicida* is interesting, and contradicts the commonly held view that the organism is an obligate fish pathogen (see Austin & Austin, 2012). However, this may reflect that scientists have focused recovery only on diseased fish rather than environmental samples. All the isolates recovered in this study had similar morphologies and lacked diffusible brown pigment production. Clearly, further research is needed to extend the knowledge of this group of organisms.
- Challenge experiments revealed that some aeromonads caused comparatively mild disease, without ensuing mortalities. The question to be answered concerns whether or not the cultures were responsible for disease in Omani fish or was virulence lost during storage in the laboratory.

- There was not any direct relationship between pathogenicity and the presence of putative virulence factors. Again, does this reflect the loss of activity during storage?

6.2 Recommendations at the farm level and Government Policy

From this thesis the following recommendations can be promoted at both the farm level to reduce fish/shellfish losses and support the development of an emerging aquaculture sector. These recommendations provide some measureable impact from the thesis.

1. **Implement long-term, routine field sampling to determine the presence of diseases in Omani aquaculture**. Future consideration should be given for a diagnostic capacity in Omani aquaculture to detect and mitigate infectious disease outbreaks affecting the farming sector. This could be achieved through on-going Train farmers in good management practices, including the need to maintain written records related to their fish farming activities. Outputs from this thesis would contribute towards a Prepare a manual about health management practice, written in language that the farmers would understand.

2. **Implement legislation for aquaculture at the farm level, and ensure that the regulations/rules are strictly adhered to by all farmers**. This could be achieved by

Create awareness among farmers about fish health management strategies and give training on disease diagnostic procedures and methods of controlling the diseases.

6.3 Further research

- Continue the microbiological examination of fish stocks in Oman to determine the presence of pathogens.
- Continue with the taxonomy of fish associated aeromonads, particularly cultures equated with *A. salmonicida.*
- Determine effective control methods for diseases of relevance to Omani aquaculture.

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APPENDIX

Appendix 2.1 List of farms

Fam code	Farm Location	Name of the owner
1	Barka (Al Batinah – North)	Mr. Mohammed Al-Ryami
2	Sohar (Al Batinah – North)	Mr. Khalid AL-Meqbali
3	Al- Mudhaibi (Ash Sharqiyah - East)	Mr. Majid AL-Mangi
4	Al- Mudhaibi (Ash Sharqiyah - East)	Mr. Khalfan AL-Sharji
5	Al- Mudhaibi (Ash Sharqiyah - East)	Mr. Said AL-Harthy
6	Al- Mudhaibi (Ash Sharqiyah - East)	Mr. Haitham AL-Maskari
7	Al- Mudhaibi (Ash Sharqiyah - East)	Mr. khalid AL-Rawahi
8	Ibri (Ad Dhahirah – North)	Mr. Sultan AL Abri
9	Munah (Ad Dakhiliyah – North)	Mr. Esa AL-Sultani

APPENDIX

Appendix 2.2 Farmer's Questionnaire

Sultanate of Oman Case Study: Tilapia Farmers Questionnaire

Enter Farm Code: ----------

1. Farmer

Name: ----------

Region: ----------

Number: ---------- Date of Interview: ----------

1.1 Is this an owned or rented farm?

1.2 Is this the (please tick all that apply)

Owner	Family member	Worker/Helper

1.3 Educational level (please tick all that apply)

Illiterate	
Primary School	
Secondary School	
High School	
Other	Please specify:

1.4 What year did you establish a fish farm?

1.5 Do you have previous farming experience?

Yes	No

1.6 How many years' experience do you have in fish farming?

APPENDIX

1.7 Is fish farming your main source of income?

Yes	No

1.8 Do you also do any of the following in the farm? (please tick all that apply)

Growth other Animal	
Grow plants	
Sell fry or fingerlings to other farmers	
Other	Give details:

1.9 Who works on the fish farm? (please tick all that apply)

Farm owner	
Worker	
Family members	
Other	

1.10 How often do they work on the fish farm? (Please tick one box only)

Daily	
Some time	Please specify:

2. Farm

2.1 Type of current farming system (please tick all that apply).

Monoculture	
Poly culture	
Integrated	
other	

2.1.1 If other, what is it?

2.2 Have you prepared the farm before every crop?(Please tick one box only)

Yes	No	Don't know	Cannot remember	Sometimes

2.3 If yes, do you do any of the following to prepare before each crop? (Please tick all that apply).

Remove sediment	
Lime	
Leave to dry	
other	Describe ?

2.4 What month do you normally harvest?

2.5 What is the weight of fish at harvest? Total Weight

2.6 What is the individual weight of fish at harvest?

2.7 What is the price per kg at harvest?

2.8 From stocking to harvest, what is the total input cost of the farm?

2.9 Does the farmer know the expected income from fish farming (per crop)? (Please tick one box only)

Yes	No

2.10 If yes, what is the expected income from fish farming (per crop)?

3. Production

3.1 What is the total number of cages/ponds on your farm?

3.2 What is the pond type? (please tick all that apply)

Earthen	
Concrete	
Other	Describe ?

3.3 What is the size of each cage/pond in use for fish farming?

3.4 What are the fish species in cages/ponds in use for fish farming at present?

3.5 Stocking density: _____ /m2

3.6 Source of fish seed (please tick all that apply).

Wild stocks	Which place?
Farm inside the country	Where?
Import	Where?
Other	Please specify:

3.7 What do you do with the fish you produce? (please tick all that apply)

Sell fish	
Eat Fish	
Market	Where?

3.8 If you sell the fish, to whom you sell it? (Please tick all that apply).

Family	
Local people	
Fish processing factory	Where?
Market inside the country	Where?
Export	Where?

3.9 What is the Total Weight of Fish you sold it (kg)?

3.10 What is the Weight Per Fish you sold it (kg)?

4. Husbandry

4.1 Do you feed the fish? (Please tick one box only)

Yes	No

APPENDIX

4.2 What do you use as feed (please tick all that apply).

Pellets	Source of pellete?
Chicken waste	
Animal manure	
Trash fish	
other	Give detailes:

4.3 How often do you feed fish

Once daily	
Twice daily	
Once weekly	
More than once weekly	
Other	Give detailes:
Don’t know	

4.4 How do you know how much feed to give?

4.5 Do you ever stop feeding the fish? (Please tick one box only)

Yes	No

4.5.1 If yes, why?

4.6 What is the Source of water?

4.7 Do you exchange water on the farm? (Please tick one box only)

Yes	No

4.8 If Yes, what are the exchange rates?

4.9 How many times?

4.10 Do you use fertilizer / manure for your ponds? (Please tick one box only)

Yes	No

4.10.1 If yes, what kind of it?

4.11 Do you always farm the same fish species? (Please tick one box only)

Yes	No

4.12 Do you ever change fish species? (Please tick one box only)

Yes	No

4.13 If yes, What factors would cause you to change fish species? (please tick all that apply)

Increased income	
Advice from another farmer	
Disease outbreak	
Media advice	
other	Give detailes:

4.14 Do you keep written records from your farm? (Please tick one box only)

Yes	No

4.14.1 If yes, what do you record?

4.14.2 Why you record?

4.15 Do you experience any water quality problems? (Please tick one box only)

Yes	No

4.16 If yes, give you answer or each question in each row?

4.16.1 What are they?	
4.16.2 What month do they occur?	
4.16.3 How long did this problem take time?	
4.16.4 What do you do? e.g. exchanges water/stop feeding…..	
4.16.5 Does it affect the fish?	
4.16.6 How does it affect the fish ?(E.g changes in behavior/appearance…..)	

5. Disease and Health Management

5.1 Do your fish ever get sick? (Please tick one box only)

Yes	No

5.1.1 If yes, how frequently?

5.2 How do you know your fish are sick or How do you recognize disease? (please tick all that apply)

Fish stop feeding	
Fish change color	
Fish change behavior	
Fish change shape or size	
Marks(lesions/ulcers/spots) On the fish surface	
Damage to the external surface including fins	
Fish die	
Don’t know	

5.3 During a mortality period, what percentages of fish were lost?

5.4 If disease occurs, what do you do? (please tick all that apply)

Remove died fish	
Change water	
Apply different type of treat-ment	
Nothing	

5.5 If you used treatment, What are the main types of treatments you use?

Traditional	What is it?	Where these are obtained from?
Chemical	What is it?	Where these are obtained from?
Antibiotics	What is it?	Where these are obtained from?

5.6 Does it work? (Please tick one box only)

Yes	
No	
Don’t know	
Sometimes	

5.7 Do you know when the fish are recovered and became better?

5.8 Do you ever treat before a disease occurs?

5.9 What happens to diseased or dead fish? (please tick all that apply)

Discarded	
Used as fish feed	
Other	Give detailes:

5.10 Where do you obtain information on disease from? (please tick all that apply)

Self	
Neighboring farmer	
Family	
MAF Research staff	
Media	
Other	

5.11 Who give you advice on disease and treatments? (please tick all that apply)

Self	
Other farmer	
Relatives	
Ministry	
Other	

6. Information and Knowledge

6.1 Have you received information about fish farming? (Please tick one box only)

Yes	No	Some times

6.2 If Yes, in which subject it was? (please tick all that apply)

Cover all subjects of Farm Management	
Husbandry practice	
Fish health management	
Marketing and Production	

6.3 Have you ever attended a training course on fish farming? (Please tick one box only)

Yes	No	Sometimes

6.4 If Yes, please describe (who ran it, when and in which subject it was?)

APPENDIX

Appendix 4.1 Identification of *Aeromonas* spp. recovered from wild fish, farmed fish and shellfish from Oman.

No	Isolates NO.	fish species	Source	Clinical Sign	Morphology	Gram stain	Motility	Cat	Ox	vibriostatic agent O/129		Biochemical Identification By VITEK® System	Accuracy %	list of bacteria growth in same plate with Aeromonas	16S rDNA RFLP	16S rDNA sequencing	sequencing length	species match %	genus match %
										150 µg	10 µg								
1	1 MG	Red spot sea bream	Muscat	NC	Convex/ light creamy colour	-	+	+	strong, +	R	R	*A.caviae*	97%	*Citrobacterfreundi*	*A.veronii*	*A.veronii*	1096	94%	99%
2	14MG3	*Oreochromis Niloticus*	Mudhaibi	weakness, swimming in one side, External haemorrhages in the head, skin and at the bases of the fins		-	+	+	WEAK,+	R	R	*A.caviae*	97%	*1. Mcrococcus spp.* *2.Enterobacter aerogenes*	*A. encheleia*	*A. encheleia*	1091	96%	99%
3	15MG	*Oreochromis Niloticus*	Mudhaibi	No clinical sign	Convex/ light creamy colour	-	+	weak,+	strong, +	R	R	*A. caviae*	99%		*A. encheleia*	*A. encheleia*	1104	99%	99%
4	16MG	*Oreochromis Niloticus*	Mudhaibi	Weakness, swimming in one side	Convex/ yellowish colour colonies	-	-	+	strong, +	R	R	*A.salmonicida*	90%		*A.salmonicida*	*A.salmonicida*	1092	88%	92%
5	26/MS2	*Oreochromis Niloticus*	Mudhaibi	NC	Convex/ yellowish colour colonies	-	+	+	strong, +	S	S	*A.salmonicida*	97%	*Vibirio fluvialis*	*A.salmonicida*	*A.salmonicida*	1094	92%	94%
6	42/MS1	*Oreochromis Niloticus*	Mudhaibi	Anaemia	Convex/ light creamy colour	-	+	+	strong, +	R	R	*A.caviae*	99%		*A.jandaei*	*A.jandaei*	1100	99%	99%
7	44MS1	*Oreochromis Niloticus*	Mudhaibi	External haemor-rhages in the head, skin and at the bases of the fins	Convex/ light creamy colour	-	+	+	strong, +	R	R	*A.veronii*	94%	*Sphingobacterium* spp.	*A.veronii*	*A.veronii*	1097	99%	99%

APPENDIX

No	Isolates NO.	fish species	Source	Clinical Sign	Morphology	Gram stain	Motility	Cat	Ox	vibriostatic agent O/129		Biochemical Identification By VITEK® System	Accuracy %	list of bacteria growth in same plate with Aeromonas	16S rDNA RFLP	16S rDNA sequencing	sequencing length	species match %	genus match %
										150 µg	10 µg								
8	50BL1	*Oreochromis Niloticus*	Mudhaibi	External haemor-rhages in the head, skin and at the bases of the fins	Convex/ light creamy colour	-	+	+	strong, +	R	R	*A.veronii*	95%		*A. trota*	*A. trota*	1107	99%	99%
9	62MG1	Redspot emperor	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	97%		*A.veronii*	*A.veronii*	1102	91%	94%
10	82/MG	King soldier bream	Sohar	NC	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.veronii*	94%		*A. encheleia*	*A.encheleia*	1100	99%	99%
11	159MG	Redspot emperor	Sohar	NC	Convex/ light creamy colour	-	+	+	weak,+	R	R	*A.caviae*	95%	*Photobacterium damselae*	*A. caviae*	*A.caviae*	1221	96%	96%
12	189MG1	*Oreochromis niloticus*	Mudhaibi	External haemor-rhages in the head, skin and at the bases of the fins	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.veronii*	97%		*A.encheleia*	*A.encheleia*	1100	99%	99%
13	226MG1	Redspot emperor	Sohar	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	99%	*Oligella ureolytrica*	*A.veronii*	*A.veronii*	1092	91%	95%
14	228MG	Redspot emperor	Sohar	NC	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.veronii*	99%		*A.veronii*	*A.veronii*	1094	99%	99%
15	288MG	White spotted rabbit fish	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	99%		*A.veronii*	*A.veronii*	1226	89%	93%
16	290MG	Redspot emperor	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	98%		*A.veronii*	*A.veronii*	1220	99%	99%

APPENDIX

No	Isolates NO.	fish species	Source	Clinical Sign	Morphology	Gram stain	Motility	Cat	Ox	vibriostatic agent O/129		Biochemical Identification By VITEK® System	Accuracy %	list of bacteria growth in same plate with Aeromonas	16S rDNA RFLP	16S rDNA sequencing	sequencing length	species match %	genus match %
										150 µg	10 µg								
17	291MS	White spotted rabbit fish	Muscat	NC	Convex/ yellowish colour colonies	-	+	+	strong,+	R	R	*A.caviae*	97%		*A. salmonicida*	*A. salmonicida*	1216	99%	99%
18	293MS	White spotted rabbit fish	Muscat	NC	Convex/ yellowish colour colonies	-	+	+	strong,+	R	R	*A.caviae*	94%	*Micrococcus* spp	*A. salmonicida*	*A. salmonicida*	1217	99%	99%
19	294MG1	King soldier bream	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	99%		*A.jandaei*	*A.jandaei*	1219	99%	99%
20	295MG	King soldier bream	Muscat	NC	Convex/ yellowish colour colonies	-	+	+	strong,+	R	R	*A.caviae*	93%		*A. salmonicida*	*A. salmonicida*	1219	99%	99%
21	296MG	King soldier bream	Muscat	NC	Convex/ light creamy colour	-	+	+	weak,+	R	R	*A.veronii*	89%		*A.veronii*	*A.veronii*	1220	99%	99%
22	299MG	Redspot emperor	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	99%		*A.veronii*	*A.veronii*	1220	93%	99%
23	300MG	Redspot emperor	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.caviae*	98%		*A.jandaei*	*A.jandaei*	1220	99%	99%
24	301MG	White spotted rabbit fish	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.caviae*	99%		*A.Caviae*	*A.caviae*	1211	99%	99%

APPENDIX

No	Isolates NO.	fish species	Source	Clinical Sign	Morphology	Gram stain	Motility	Cat	Ox	vibriostatic agent O/129		Biochemical Identification By VITEK® System	Accuracy %	list of bacteria growth in same plate with Aeromonas	16S rDNA RFLP	16S rDNA sequencing	sequencing length	species match %	genus match %
										150 µg	10 µg								
25	306MG	White spotted rabbit fish	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.caviae*	99%		*A.veronii*	*A.veronii*	1220	99%	99%
26	309MS	White spotted rabbit fish	Muscat	NC	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.caviae*	99%	*Pantoea* spp	*A.veronii*	*A.veronii*	1218	99%	99%
27	313MG1	White spotted rabbit fish	Muscat	NC	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.veronii*	94%		*A.veronii*	*A.veronii*	1221	99%	99%
28	323MS	King soldier bream	Muscat	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.salmonicida*	85%	*Sphingomonas Paucimobiles*	*A.salmonicida*	*A.salmonicida*	1217	95%	95%
29	326MG	*Oreochromis niloticus*	Mudhaibi	haemorrhages in skin surface/ history of high mortality	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.salmonicida*	98%		*A.veronii*	*A.veronii*	1217	91%	95%
30	340M	Abalone	Salalah	femal/history of high mortality with no clear clinical sign	Convex/ light creamy colour	-	-	+	strong,+	S	R	*A.salmonicida*	96%		*A.salmonicida*	*A.salmonicida*	1222	92%	95%
31	373MG	Redspot emperor	Muscat	NC	Convex/ yellowish colour colonies	-	+	+	strong,+	S	R	*A.salmonicida*	97%		*A.salmonicida*	*A.salmonicida*	1217	98%	98%
32	385L	*Oreochromis niloticus*	Mudhaibi	Lesion	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	93%		*A.veronii*	*A.veronii*	1224	99%	99%
33	386K1	*Oreochromis niloticus*	Mudhaibi	NC	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.veronii*	99%		*A.veronii*	*A.veronii*	1223	99%	99%

APPENDIX

No	Isolates NO.	fish species	Source	Clinical Sign	Morphology	Gram stain	Motility	Cat	Ox	vibriostatic agent O/129		Biochemical Identification By VITEK® System	Accuracy %	list of bacteria growth in same plate with Aeromonas	16S rDNA RFLP	16S rDNA sequencing	sequencing length	species match %	genus match %
										150 µg	10 µg								
34	387K	*Oreochromis niloticus*	Mudhaibi	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	93%		*A.veronii*	*A.veronii*	1221	99%	99%
35	388K1	*Oreochromis niloticus*	Mudhaibi	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	99%		*A.veronii*	*A.veronii*	1222	99%	99%
36	388MS	*Oreochromis niloticus*	Mudhaibi	NC	Convex/ yellowish colour colonies	-	+	+	Strong+	S	S	*A.salmonicida*	89%		*A.salmonicida*	*A.salmonicida*	1217	98%	98%
37	389MS	*Oreochromis niloticus*	Mudhaibi	NC	Convex/ light creamy colour	-	+	+	strong,+	S	S	*A.veronii*	95%		*A.veronii*	*A.veronii*	1217	99%	99%
38	390L	*Oreochromis niloticus*	Mudhaibi	Lesion	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.caviae*	97%		*A.veronii*	*A.veronii*	1219	99%	99%
39	391L	*Oreochromis Niloticus*	Mudhaibi	Lesion	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.caviae*	99%		*A.veronii*	*A.veronii*	1223	99%	99%
40	392L	*Oreochromis Niloticus*	Mudhaibi	Lesion	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	96%		*A.veronii*	*A.veronii*	1224	99%	99%
41	392MG	*Oreochromis Niloticus*	Mudhaibi	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.caviae*	99%		*A.veronii*	*A.veronii*	1219	99%	99%
42	393MG	*Oreochromis Niloticus*	Mudhaibi	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	*89%*		*A.veronii*	*A.veronii*	1219	99%	99%

APPENDIX

No	Isolates NO.	fish species	Source	Clinical Sign	Morphology	Gram stain	Motility	Cat	Ox	vibriostatic agent O/129		Biochemical Identification By VITEK® System	Accuracy %	list of bacteria growth in same plate with Aeromonas	16S rDNA RFLP	16S rDNA sequencing	sequencing length	species match %	genus match %
										150 µg	10 µg								
43	394MG	*Oreochromis Niloticus*	Mudhaibi	NC	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.veronii*	*94%*		*A.jandaei*	*A.jandaei*	1220	99%	99%
44	395M	Abalone	Salalah	NC	Convex/ yellowish colour colonies	-	+	+	strong,+	R	R	*A.salmonicida*	*96%*		*A.salmonicida*	*A.salmonicida*	1222	92%	95%
45	399M	Abalone	Salalah	NC	Convex/ light creamy colour	-	+	+	strong,+	R	R	*A.veronii*	*86%*	*Sphingomonas paucimobils*	*A. trota*	*A. trota*	1223	90%	95%
46	400M	Abalone	Salalah	NC	Convex/ light creamy colour	-	+	weak,+	strong,+	R	R	*A.caviae*	99%	*1.Sphingomonas paucimobils 2.Elizabethkingia meningoseptica*	*A.veronii*	*A.veronii*	1227	99%	99%

Explanations: M= Muscle; MG=Mucus of gill; MS= Mucus of Skin; BL=Blood; K=Kidney; L= Lesion; NC= no clinical signs of disease; += Positive; - = Negative; R= Resistant; S= sensitive; Cat=catalyse; Ox-oxidase

APPENDIX

Appendix 5.1 Result of bacteria challenge studies and present of different virulence factors expression among *Aeromonas* spp.

NO.	Isolates code	fish species	Source	Clinical Sign	*Aeromonas* spp.	pathogenicity results				genetic method									Phenotypic method				
						clinical sign of disease		*Aeromonas* strain recovered															
						Tilapia	Trout	Tilapia	Trout	Areo-Gene	ACT	AST	ALT	GCAT	SER	NUC	LAFB	LAFA	Haemo-lysin	Congo red	Coomassio blue	Protease	DNas
1	14MG3	***Oreo-chromis niloticus***	Mudhaibi	weakness, swim-ming in one side, External haemor-rhages in the head, skin and at the bases of the fins	*A. encheleia*	NC	Haemor-rhage internal	NR	NR	-	-	-	+	-	-	-	-	-	γ	-	-	-	-
2	16MG	*Oreochromis niloticus*	Mudhaibi	Weakness, swim-ming in one side	*A.salmonicida*	NC	NC	NR	√ from Kidney & Spleen	-	-	+	-	-	-	-	-	-	γ	-	-	+	-
3	26MS2	*Oreochromis niloticus*	Mudhaibi	No clinical sign	*A.salmonicida*	NC	NC	NR	NR	-	+	+	-	-	-	-	-	-	+β	+	+	+	+
4	42MS1	*Oreochromis niloticus*	Mudhaibi	Anaemia	*A.jandaei*	NC	NC	NR	NR	-	+	+	-	-	+	+	+	+	γ	-	-	-	-
5	44MS1	*Oreochromis niloticus*	Mudhaibi	External haemor-rhages in the head, skin and at the bases of the fins	*A.Veronii*	NC	NC	NR	NR	-	+	+	-	-	-	+	-	+	α	++	++	+	+
6	50BL1	*Oreochromis niloticus*	Mudhaibi	External haemor-rhages in the head, skin and at the bases of the fins	*A. trota*	NC	NC	NR	NR	-	+	+	-	-	-	-	-	+	+β	+	+	+	+

APPENDIX

NO.	Isolates code	fish species	Source	Clinical Sign	*Aeromonas* spp.	pathogenicity results				genetic method										Phenotypic method				
						clinical sign of disease		*Aeromonas* strain recovered																
						Tilapia	Trout	Tilapia	Trout	Areo-Gene	ACT	AST	ALT	GCAT	SER	NUC	LAFB	LAFA	Haemo-lysin	Congo red	Coomas-sio blue	Protease	DNas
7	189MG1	*Oreochromis niloticus*	Mudhaibi	External haemor-rhages in the head, skin and at the bases of the fins	*A. encheleia*	Internal haemor-rhage	Exophthal-mia (pop eye)	√ from kidney	√ from kidney	+	+	+	+	-	-	-	+	+	γ	+	+	+	+
8	291MS	White spotted rabbit fish	Muscat	No clinical sign	*A. salmonicida*	NC	NC	NR	NR	-	+	-	-	+	+	-	+	+	+β	-	-	-	+
9	293MS	White spotted rabbit fish	Muscat	No clinical sign	*A. salmonicida*	NC	NC	NR	NR	-	-	+	-	-	-	-	-	-	γ	-	-	-	+
10	295MG	King soldier bream	Muscat	No clinical sign	*A. salmonicida*	NC	NC	NR	NR	+	+	+	-	+	+	-	-	+	α	-	-	-	+
11	340M	Abalone	Salalah	femal/history of high mortality with no clear clinical sign	*A.salmonicida*	NC	Pale Liver & dark Kidney	√ from kidney & spleen	√ from kidney	-	-	+	-	-	-	-	-	+	γ	-	-	-	-
12	373MG	Redspot emperor	Muscat	No clinical sign	*A. salmonicida*	NC	NC	NR	NR	+	-	+	-	-	-	-	-	-	+β	-	-	-	-
13	385L	*Oreochromis niloticus*	Mudhaibi	Lesion	*A.veronii*	NC	NC	NR	NR	-	+	+	-	-	-	-	-	+	γ	-	-	-	-

APPENDIX

NO.	Isolates code	fish species	Source	Clinical Sign	*Aeromonas* spp.	pathogenicity results				genetic method									Phenotypic method				
						clinical sign of disease		*Aeromonas* strain recovered															
						Tilapia	Trout	Tilapia	Trout	Areo-Gene	ACT	AST	ALT	GCAT	SER	NUC	LAFB	LAFA	Haemo-lysin	Congo red	Coomassio blue	Protease	DNas
14	388MS	***Oreo-chromis niloticus***	Mudhaibi	NC	*A. salmonicida*	NC	NC	NR	NR	-	+	-	+	-	-	-	-	-	+β	+	+	+	+
15	390L	*Oreochromis niloticus*	Mudhaibi	Lesion	*A.veronii*	NC	NC	NR	NR	-	-	+	-	-	-	-	-	-	α	-	-	+	+
16	391L	*Oreochromis niloticus*	Mudhaibi	Lesion	*A.veronii*	NC	NC	NR	NR	-	+	+	+	-	-	-	-	+	+β	+	+	+	-
17	392L	*Oreochromis niloticus*	Mudhaibi	Lesion	*A.veronii*	Internal haemor-rhage	NC	√ from kidney	√ from spleen	-	+	+	+	-	-	-	-	-	γ	+	+	+	+
18	395M	Abalone	Salalah	No clinical sign	*A. salmonicida*	NC	NC	NR	NR	-	+	-	+	-	-	-	-	-	+β	-	+	+	+

Explanations: M= Muscle; MG=Mucus of gill; MS= Mucus of Skin; BL=Blood; K=Kidney; L= Lesion; NC= no clinical signs of disease; NR= not recovered; α = α haemolysis; β= β haemolysis; γ = γ haemolysis; + = presence; - = absence; ++ = positive activity and strong intensity of colour