Studies on the pathogenicity of *Yersinia ruckeri* biotype 2 to rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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Abstract

Recently outbreaks of enteric redmouth disease in previously vaccinated salmonids was attributed to biotype 2 (non-motile, lipase negative) isolates of *Yersinia ruckeri*. Currently, isolates of biotype 2 are the most widely encountered form in Europe and North America. Biochemical analysis of over 90 clinical isolates revealed that there are potentially eight novel phenotypes associated with disease. Cell surface characteristics of biotype 2 isolates displayed a unique O antigen structure. A multilocus sequence typing scheme was developed using 4 'housekeeping' gene loci in order to understand the phylogeny and population structure of *Y. ruckeri*. The scheme highlighted that although there is low species diversity, this is characteristic of a small population size which is not limited to fish species or geographical region. The study suggests that biotype 2 isolates have arisen due to changes in the population structure in the natural environment not as a direct mutation caused by vaccination. Analysis of extracellular products revealed that *Y. ruckeri* is a poor producer of excreted compounds. However, it was observed that these compounds are homogenous to all serotypes and biotypes. Antigenic characterisation highlighted that the O antigen is the serospecific antigen between biotypes although there was marked cross reaction with whole cell proteins. In this context, vaccination studies revealed that the O antigen is the dominant immunogenic molecule involved in protection against the disease. Virulence studies demonstrated that all serogroups and biotypes were virulent to rainbow trout. *Y. ruckeri* was able to stimulate the innate immune response in order to survive and proliferate in vaccinated rainbow trout.

To my family

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Finally, thanks and appreciations are due to my family, for their continued support and encouragement.

Declaration

I, John Tinsley, hereby declare that I am the author of this thesis. All the work described in this thesis is my own, except where stated in the text. The work presented here has not been accepted in any previous application for a higher degree. All the sources of information have been consulted by myself and are acknowledged by means of reference.

John Tinsley

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List of Abbreviations and symbols

~	Approximately
^{o}C	Degree centigrade
/	Divide
<	Less than
≤	Less than or equal to
µg	Microgram
µL	Microlitre
>	More than
≥	More than or equal to
x	Multiply/times
%	Percent
±	Plus or minus, error margin
Ab	Absorbance
bp	Base pair
bt	Biotype
cm	Centimeter
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
$_{d}H_{2}0$	Deionized water
dNTP	Deoxyribonucleic triphosphate
	Generic name for aATP, dCTP, cGTP and dTTP
e.g.	Example
ELISA	Enzyme linked immunosorbent assay

ECP	Extracellular Products
et. al.	*"et alia"*: and others
g	Gram(s)
× g	Multiples of gravity
h	Hour(s)
HRP	Horseradish peroxidase
ASP	Ammonium persulphate
HSF	Heat sensitive factor
HSWB	High salt wash buffer
i.e.	"*id est*": that is
Ig	Immunoglobulin
i.p	Intraperitoneal
IROMP	Iron regulated outer membrane protein
kbp	Kilobase pair
kDa	Kilo Dalton(s)
kg	Kilo gramme
L	Litre(s)
LD_{50}	Lethal dose 50%
LSWB	Low salt wash buffer
M	Molar
Mab	Monoclonal antibody
MDa	Mega Dalton
min	Minute(s)

mL	Millilitre
mL^{-1}	Per millilitre
mM	Millimolar
µM	Micromole
MW	Molecular weight
NCBI	National Centre for Biotechnology Information
NCIMB	National Collection of Industrial and Marine Bacteria
OD	Optical density
OMP	Outer membrane protein
Pab	Polyclonal antibody
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG_{8000}	Polyethylene glycol 8000
RNA	Ribonucleic acid
RPS	Relative percentage survival
sec	Second(s)
sp.	Species
TEM	Transmission electron microscopy
UV	Ultraviolet
V	Volt
v/v	Volume/volume
WCP	Whole cell proteins
w/v	Weight/volume

Publications and presentations

Publications

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EUROPEAN ASSOCIATION OF FISH PATHOLOGISTS

14th INTERNATIONAL CONFERENCE ON DISEASES OF FISH AND SHELLFISH

Prague, Czech Republic

POSTER AWARD
HIGHLY COMMENDED

J. W. Tinsley, M. Barker, F. G. Priest, B. Austin

Phylogeny and population structure of the fish pathogen *Yersinia ruckeri* as defined by multilocus sequence typing (MLST)

D. Alderman | M. L. Kent | I. Dyková

Prague, September 17, 2009

Chapter 1. Introduction

1.1. Aquaculture in the 21st century

Currently, aquaculture is one of the fastest growing food producing sectors, and accounts for over 47% of the worlds fish supply (Tacon *et al*., 2010). As demand for fish is increasing and pressure is being exerted upon natural fish stocks, aquaculture is playing an ever increasing part in meeting human demands for high quality protein, omega-3 (ω3) and omega 6 (ω6) long-chain polyunsaturated fatty acid (LC-PUFA) essential fatty acid (EFA) requirements (Tocher, 2009). Given the current human population growth estimates over the next two decades, it has been estimated that at least an additional 40 million tonnes of aquatic products will be required to maintain the current per capital consumption (FAO, 2006).

1.1.2. Rainbow trout aquaculture

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) as a source of protein is widely cultured throughout the world, even though it is only native to North West America. The ability of rainbow trout to grow well in culture is one of the main reasons why it has been successful as a cultured species. It is notable that the species grows well at a variety of temperatures, has less stringent water quality requirements than other salmonids, is fast growing. It has been subjected to genetic studies regarding reproduction, immunology and strain improvement. Globally in 2007, salmonid production was approximately 2 million tonnes, with an overall value of $1.1 billion USD, with rainbow trout contributing 600,000 tonnes of this figure (FAO, 2007). In 2008, 7600 tonnes of rainbow trout were produced in Scottish farms for both table and restocking purposes (Marine Scotland Science, 2008). Overall, most of the trout produced in the U.K. is farmed in fresh water, involving earthen ponds, raceways or tanks; a small quantity is farmed in sea cages.

1.2. Bacterial diseases in aquaculture

Infectious diseases are considered to be of paramount important to the development and sustainability of any aquaculture facility, in terms of direct losses of fish and indirectly as trade restrictions and poor product quality (Verschuere *et al.*, 2000). It is widely

regarded that diseases within aquaculture are multifactorial. The vast majority of diseases depend on particular interactions between the host, the disease agent or pathogen and environmental stressors; these interactions were represented in the 'epidemiological triad' of Snieszko (1974) (Figure 1.1). The influence of such 'stressors' as rearing densities, water quality, handling, genetic factors, stressors and nutrition are paramount in determining the impact of disease outbreaks. However, still relatively little is known about how bacterial pathogens cause disease within aquaculture. It has been shown that different strains, serotypes, genotype and biotypes of bacterial pathogens vary in their ability to cause disease (Austin *et al*., 2003, Esteve *et al*., 2004; 2007). Diseases may spread more easily within dense populations because of increased opportunities for association of infected and uninfected fish (Owens, 2003). Most economic losses from disease in aquaculture are due to morbidity and mortality. The World Organisation for Animal Health has defined notifiable diseases, depending upon the list status of the pathogen. Sites may have to be fallowed resulting in losses due to production, and any trade restrictions that may be implemented could lead to widespread socioeconomic issues. In 2009, £20 million of the Scottish salmon industry as it was affected by the viral disease Infectious Salmon Anaemia (ISA) in List 1. ISA is a highly virulent disease, which once confirmed, requires the eradication of stock, and cessation of fish movements until further inspection has confirmed the absence of the disease. The outbreak of ISA sparked the creation of an ISA recovery scheme worth over £1 million in order to aid recovery and protect against job losses. Bacterial kidney disease (BKD), which is caused by *Renibacterium salmoninarum*, is currently the only bacterial fish pathogen listed as notifiable by the Office international des epizooties (OIE), List 3. The organism was first isolated in the 1930's from wild fish in the River Spey, Scotland (Smith, 1964). To date, although it is a notifiable disease, it is not widespread and occurs sporadically.

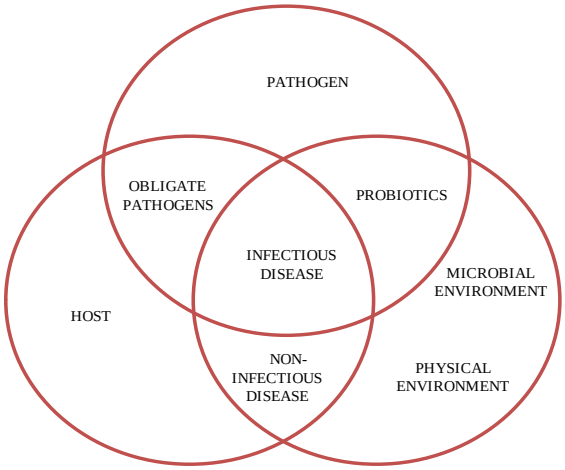


Figure 1.1 The epidemiological triad of Snieszko, indicating the factors of an aquaculture system that can lead to disease (after Owens, 2003).

Most of the problems associated with bacterial fish pathogens are caused by so called 'opportunistic pathogens', which are present naturally in the environment, but only invade a host once its immune system has been compromised by some stressful event or other disease process (Austin and Austin, 2007). Other pathogens, such as *Vibrio anguillarum* or *Aeromonas salmonicida*, do not require an immunocompromised host to establish an infection (Roberts, 2004).

The rapid worldwide increase in aquaculture in recent years has led to a corresponding increase in the incidence and severity of the long recognized bacterial conditions, as well as the emergence of a number of new infections, such a Red Mark Syndrome (Verner-Jeffreys *et al*., 2008). Table 1.1 includes details of the many different bacterial pathogens of cultured fish species. As aquaculture continues to grow all over the world, it is of paramount importance that research into bacterial diseases continues in order to prevent losses and to improve health management decisions.

Table 1.1 Principle bacterial pathogens of fish (After Roberts, 2004; Austin and Austin, 2007)

Pathogen	Disease
Gram-negative gliding bacteria	
Flavobacteriaceae:	
Flavobacterium branchiophilum	Bacterial gill disease: fin rot
Flavobacterium columnare	Columnaris disease
Flavobacterium psychrophilum	Cold water disease
Flexibacter maritimus	Saltwater columnaris
Gram-negative facultatively anaerobic rods	
Enterobacteriaceae	
Edwardsiella tarda	Edwardsiella septicaemia
Edwardsiella ictaluri	Edwardsiella septicaemia of catfish
Yersinia ruckeri	Enteric redmouth: ERM, Yersiniosis
Vibrionaceae and Aeromonadaceae	
Vibrio anguillarum	Vibriosis
Vibrio ordalii	Vibriosis
Vibrio (Aliivibrio) salmonicida	Cold water vibriosis: Hitra disease
Vibrio viscosus (= Moritella viscosa)	Cold water ulcer disease

Aeromonas hydrophila	Motile aeromonad septicaemia
Aeromonas salmonicida	Furunculosis
Photobacteriaceae	
Photobacterium damselae subsp. *piscicida*	Pasteurellosis
Gram-positive aerobic rods	
Renibacterium salmoninarum	Bacterial kidney disease: BKD
Carnobacterium piscicola	Lactobacillosis pseudo-kidney disease
Vagococcus salmoninarum	Lactobacillosis pseudo-kidney disease
Lactococcus piscium	Lactococcosis
Gram-positive anaerobic rods	
Endospore-forming bacteria	
Clostridium botulinum	Type E botulism
Acid Fast rods and filaments	
Mycobacterium marinum	Mycobacteriosis
Mycobacterium fortuitum	
Mycobacterium chelonae	

Nocardiaceae	
Nocardia asteroides	Nocardiosis
Nocardia kampachi	
Rickettsias: Chlamydias: Obligate intracellular parasites	
Piscirickettsia salmonis	Salmonid rickettsiosis
Epitheliocystis organism	Epitheliocystis
Microcystis organism	Microcystis
Red Mark Syndrome	Red Mark Syndrome

1.3. Enteric redmouth disease in aquaculture

Enteric redmouth disease (ERM) or yersiniosis is a significant condition of farmed fish, notably salmonids, and is caused by *Yersinia ruckeri*. The pathogen was first isolated from rainbow trout, in the Hagerman Valley of Idaho, USA, in the 1950s (Rucker, 1966). It is Gram-negative and a member of the family Enterobacteriaceae. *Y. ruckeri* differs from all other members of the genus *Yersinia* by the ability to produce lysine decarboxylase (Bottone, 2005). The clinical signs of the disease condition are generalised haemorrhagic septicaemia. The distinctive redmouth feature is caused by venous and capillary congestion of the brain and eye vessels (Roberts, 2004).

Salmonids, especially rainbow trout in coldwater aquaculture, are most susceptible to ERM (Furones *et al.,* 1993). At present ERM has been isolated in North-western USA, Canada, Europe, Australia, South Africa, and Asia. It is generally regarded that the organism has been transmitted throughout the world as a result of the spread of salmonid aquaculture. The pathogen produces a variety of toxins, such as haemolysins, endotoxins and cytotoxins (Romalde and Toranzo, 1993; Secades and Guijarro, 1999; Aussel *et al.,* 2000; Fernandez *et al.,* 2004).

In recent years, there has been an emergence of biotype (bt) 2 isolates of *Y. ruckeri* causing disease in previously vaccinated rainbow trout (Austin *et al.,* 2003). Currently, it is the most common form encountered throughout Europe. The bacterium responsible for these losses is different to the previous definition of bt 2 as it is non motile and positive for the Voges-Proskauer (VP) reaction. Isolates, which were VP positive, were deemed to be from a new biogroup termed 'EX5'. Little research has been undertaken regarding the importance of bt 2 or the 'EX5' isolates to aquaculture, the pathogenic mechanisms, and vaccination studies to reduce its impact.

1.3.1. Historical background

The origins of *Y. ruckeri* are uncertain. The bacterium was initially isolated from hatchery-reared rainbow trout in the Hagerman Valley of Idaho, USA in the Early 1950's (Busch, 1982). Subsequently, the organism was isolated from an increasing number of states of the USA and Canada (Ross *et al*., 1966; Wobeser, 1973; Busch, 1978; Busch, 1982). Busch (1982) described *Y. ruckeri* as being enzootic in many major trout and salmon raising areas of the USA and Canada by 1982.

Previous reports into the origin of *Y. ruckeri* strains in the UK were scarce until 1980. Indeed, Roberts (1983) provided the first report in *Y. ruckeri* infections in a rainbow trout hatchery in the U.K. An interesting suggestion was that the first isolations were achieved in the 1970's but the findings were never published (Roberts, 1983). Moreover, this was one of the first descriptions of the presence of non-motile isolates of *Y. ruckeri*. The nature of the epizootic was slightly different than other clinical outbreaks as water temperatures were around 8^{o}C and lower when outbreaks were first noticed. The pathogen was responsible for losses of 200-300 fish per day on one fish farm during a September-October outbreak.

Busch (1978) stated that ERM had been reported in Italy prior to 1978 although no citation was given. The first published report of ERM in Europe appears to be Lesel *et al.* (1983), who described isolation of the bacterium from rainbow trout in south-

western France in March 1981. Outbreaks of ERM throughout Europe and other parts of the world are sporadic. Subsequent isolations of *Y. ruckeri* were isolated in Denmark (1983) (Dalsgaard *et al*., 1984), Italy (1983) (Giorgetti *et al*., 1985), Norway (1985) (Sparboe *et al*., 1986), Spain (1985) (De la Cruz *et al*., 1986), and Ireland (McArdle and Dooley Martin, 1985). An outbreak of ERM was described by Meier (1986) among rainbow trout fingerlings in Switzerland. Serotype O1 strains were associated with causing 0.5%-5% mortalities. Changing environmental conditions were attributed to the outbreak of ERM as sources of eggs and fry from Germany were ERM negative, dismissing the carrier fish theory.

Llewellyn (1980) recorded an organism with similarities between *Y. ruckeri* and *Serratia liquefaciens* in salmonids in Australia. The author termed the disease 'salmonid blood spot'. Recovery was from hatchery reared brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*) during February – March when water temperatures were falling from 23^{o}C to 11^{o}C. Hatcheries were associated with poor water quality, which was polluted with urine and faeces. The organism was determined to be Gram negative, motile, utilised glucose and was catalase positive and oxidase negative, i.e. characteristics of the family Enterobactericaeae. Previously, it was thought that the organism was possibly a representative of *Enterobacter*, *Serratia* or *Pasteurella*. The organism differed from the characteristics of *Y. ruckeri* insofar as it did not utilise citrate and hydrolyse gelatin. At the time, there was not any previous knowledge of the biochemical differences between *Y. ruckeri* isolates, therefore, it is possible that this was the first case of *Y. ruckeri* in Australia.

Although South America is one of the largest producers of salmonids, reports regarding the recovery of *Y. ruckeri* are scarce. Bravo and Kojagura (2004) reported the first isolation of *Y. ruckeri* in Peru during 2004. Diagnosis was based on biochemical reactions and whole cell agglutination (WCA) with serotype O1 antiserum. No hypothesis was given as to the source of the outbreak. Two surveys were undertaken in order to investigate the fish health status of hatcheries producing rainbow trout in Peru. The outcome was that this study highlighted that the number of hatcheries with a positive diagnosis of *Y. ruckeri* increased from 9 in 1998 to 22 in 2000. Affected fish showed classical redmouth symptoms with highest mortalities occurring in fish of 10 g

average weight rather than larger specimens of 30 g. It was stated that transportation involving the movement of fry between 3 main hatcheries to a smaller one could be the reason for the spread of the disease. The first recorded case of ERM in Iran was reported by Soltani *et al.* (1999) from a chronic infection in rainbow trout. Mortalities ranged from 10 – 20 % in rainbow trout. *Edwardsiella* sp. and *Cytophaga* sp. were also isolated from diseased organs of fish, suggesting that there could have been another underlying disease problem at the farm.

Biotype 2 isolates were initially recovered in the 1980's (Davies and Frerichs, 1989). Although these isolates were noted for their ability to cause disease in rainbow trout, little attention was given to them until they started to cause disease in previously vaccinated fish. An emerging biogroup of bt 2 was proposed following a study of ERM outbreaks among vaccinated rainbow trout in Southern England (Austin *et al.,* 2003). Outbreaks of this new biogroup were associated with poor water quality and husbandry practises i.e. high stocking density and handling. The precise origins of this new biogroup are unknown and whether VP positivity confers an advantage over standard bt 2 isolates. Spanish isolates, described by Fouz *et al.* (2006), were classified as belonging to serotype 01, bt 2. The VP test was not carried out, therefore making it difficult to hypothesise whether it was similar to isolates recovered from the U.K. The first report on bt 2 isolates of *Y. ruckeri* in the USA was described by Arias *et al.* (2007). Isolates were collected over a three year period and typed using biochemical characteristics, fatty acid methyl ester analysis (FAME), 16S rDNA sequencing and Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). It was found that strains isolated between 2003-2005 were representative of bt 2 due to the lack of motility and their lack of ability to hydrolyse Tween 20/80 and negativity to the VP reaction. Interestingly, a disease outbreak caused by *Y. ruckeri* bt 2 was observed in fish that had been previously vaccinated. As VP negative results were obtained from this study, this is the first indication that there are biochemical differences between bt 2 isolates which could be useful in diagnosis. Recently, bt 2 isolates of the bacterium were isolated from Finland (Ström-Bestor *et al*., 2010). researchers nothed that using molecular techniques, researched demonstrated that isolates were similar to isolates from Demark which had not been previously reported from Finland.

Other serogroups have been associated with ERM in Europe. Although Spain has had a history of ERM, Romalde *et al.* (2003) described the first isolation of serotype O2b from unvaccinated rainbow trout. This strain was previously thought to be restricted to North America and the Baltic countries (Stevenson, 1997). The isolates were characterized as serotype O2b because cross reaction with serum O1a antiserum disappeared when heat stable O-antigens where used in the agglutination reactions. The origins of the strain could not be traced as the facility sourced eggs and fry from many farms. One hypothesis was that the facility where the fry had been raised had been vaccinated with serotype O1 vaccine; a booster vaccine was not administered. The authors suggested that selection pressure exerted by vaccination could have led to a change in serotype.

1.4. Characteristics of *Y. ruckeri*

1.4.1. Taxonomic position of *Yersinia ruckeri*

The taxonomic position of *Y. ruckeri* has been subject to debate since its first classification in the genus *Yersinia* by Ewing *et al.* (1978). This research demonstrated that *Y. ruckeri* isolates had a G + C ratio of 47.5 - 48.5%, and on this basis would be included in the genus *Yersinia*. However, investigations of the biochemical and serological reactions highlighted that the inclusion was really not so clear cut. *Y. ruckeri* is typically distinguishable from other members of the *Yersinia* by the ability to produce lysine decarboxylase. Other researchers found that the biochemical characteristics of *Y. ruckeri* bore similarities with *Serratia* and *Salmonella* (Llewellyn, 1980). Serological cross reactions with *Hafnia alvei* and *Salmonella* sp. have indicated that there is work still needed on the precise taxonomic position of the organism (Ross *et al.* 1966, Stevenson and Daley, 1982). Recently, Kotetishvili *et al.* (2005) used multilocus sequence typing (MLST), and considered that *Y. ruckeri* was the most genetically distant species within the genus *Yersinia*, and that the taxonomic status possibly needs to be reassessed. From the results of many studies and the similarities with many other members of the Enterobacteriaceae, *Y. ruckeri* could possibly belong in its own separate genus.

1.4.2. Growth characteristics

Y. ruckeri is a Gram-negative rod of 0.5-0.8 x 1.0-3.0 µm in size. Peritrichous flagella are observed in the motile bt 1 forms of the pathogen (Davies and Frerichs, 1989). *Y. ruckeri* does not form endospores, and a capsule is not present (Tobback *et al*., 2007). The organism may be isolated readily on a variety of different media from the internal organs of diseased fish (Table 1.2). Colonies are 1-1.5 mm in diameter, and glistening after 24-48 h incubation at 22^{o}C. However, the organism will grow in a wide range of temperatures and has an optimum temperature of 28^{o}C (Stevenson *et al.*, 1993). Early investigations into the pathogen by Austin *et al.* (1982) observed that altering culture media formulations had an effect on overall cell morphology of *Y. ruckeri*. Isolates varied in the number of flagella observed under transmission electron microscopy (TEM). For TSA media supplemented with 3% (w/v) NaCl, it was observed that there was a marked difference in cell size, from 0.5 – 4 µm.

Table 1.2. Methods of isolation for *Y. ruckeri*

Medium	Author
Tryptone soya agar	Stevenson and Daley, 1982
Nutrient agar	Secades and Guijarro, 2007
BHIA (brain heart infusion agar)	Arias *et al*., 2007
Columbia blood agar	Gibello *et al.*, 2004
McConkey agar	Gibello *et al.*, 1999

1.4.3. Biochemical characteristics

As with the other members of the Enterobacteriaceae, *Y. ruckeri* is glucose-fermentative, catalase-positive, oxidase-negative and nitrate-reductive (Ross *et al*., 1966). Differentiation of *Y. ruckeri* from other members of the *Yersinia* can be achieved by a positive result for the production of lysine decarboxylase. Strains of *Y. ruckeri* are fairly homogeneous in biochemical reactions although there is variability in the methyl red (MR) test, VP reaction, and the ability to ferment sorbitol and hydrolyse Tween. Table 1.3 highlights the biochemical characteristics of *Y. ruckeri*. To date, there is not any standard procedure for differentiation between bt 1 and bt 2 apart from tests for

motility and lipase activity. Table 1.4 indicates the various characteristics of both biotypes that have been reported in the literature. The main tests to distinguish between the biotypes centre on motility and the ability to hydrolyse Tween (Davies and Frerichs, 1989). *Y. ruckeri* may also be identified using the API 20E rapid identification system (Coquet *et al.*, 2002). Thus, *Y. ruckeri* may be identified from *Hafnia alvei* by the inability to ferment xylose on the API 20E system (Coquet *et al.*, 2002).

Table 1.3. Biochemical characteristics of *Yersinia ruckeri* [a]after Ross *et al.* (1966), Ewing *et al.* (1978) Austin and Austin (2007).

Biochemical Characteristic	Result	Biochemical Characteristic	Result
Fermentative metabolism	+	Tributyrin	v
Production of:		Tween 20	v
Arginine dihydrolase	-	Tween 40	v
Catalase	+	Tween 60	v
ß-galactosidase	+	Tween 80	v
H_2S	-	Urea	-
Indole	-	Utilisation of sodium citrate	+
Lysine decarboxylase	+	*Production of acid from:*	
Ornithine decarboxylase	+	Fructose	+
Oxidase-phenylalanine deaminase	-	Glucose	+
Phosphatase	-	Inositol	-
Methyl red test	v	Lactose	-
Reduction of nitrate	+	Maltose	+
Voges-Proskauer reaction	v	Mannitol	+
		Raffinose	-

Degradation of :		Salicin	-
Aesculin	-	Sorbitol	-
Chitin	-	Sucrose	-
DNA	-	Trehalose	+
Elastin	v	Xylose	-
Gelatin	v		
Pectin	-		

+, - and v - variable correspond to ≥80%, ≤20% and 21-79% of positive responses, respectively.

Furones *et al*. (1990) described an interesting feature of serotype O1 isolates. They possess a virulence-associated heat sensitive factor (HSF), which was distinguishable by using a selective medium containing Congo red and bromophenol blue. HSF was described as being lipid in nature although a hypothesis was not given for its location or function (Furones *et al*., 1989). This HSF is thought to be associated with resistance to serum killing. Secades and Guijarro (1998) reported that the composition of the culture medium had an effect over the production of the 47-kDa protease, which was optimum in peptone medium suggesting that certain peptides are necessary for its induction.

Table 1.4. Biochemical characteristics of biotype1 1 and biotype 2 isolates of *Y. ruckeri* (after Davies and Frerichs, 1989, and Austin *et al*., 2003).

	Result for:	
Biochemical tests	Biotype 1	Biotype 2
Motility	+	-
Voges Proskauer reaction	v	+
Acid from sorbitol	v	v
Methyl red test	v	v

Lipase activity	+	-
Gelatinase	v	v
Growth at 22^{o}C	+	+

+, - and v - variable correspond to ≥80%, ≤20% and 21-79% of positive responses, respectively.

1.4.4. Serological classification

Various schemes involving serological, biochemical and phenotypic methods have been used to characterise *Y. ruckeri* strains. They vary depending upon the country and preferred method (Busch, 1978; Green and Austin, 1982; De Grandis *et al.*, 1988; Davies, 1990; 1991a). Most of the typing schemes were based around whole cell serological reactions. De Grandis *et al.* (1988) grouped isolates into 5 serovars (I, II, III, V and VI). Some doubt was cast about the validity of serovar VI as fresh isolates could not be linked to any serovar. Biochemically serovar IV was capable of fermenting arabinose and rhamnose, and these isolates were probably *H. alvei*. Romalde *et al.* (1993) revised this serological scheme by proposing a new typing scheme by identifying four different O-serovars. Serovar O1 was subdivided into two subgroups O1a (previously serovar I) and O1b (previously serovar III). Serovar O2 (serovar II) was divided into three subgroups O2a, O2b and O2c. The remaining serovars are designated as serovar O3 (serovar V) and serovar O4 (serovar VI).

Five O-serotypes have been identified (O1, O2, O5, O6, O7) from 127 isolates of *Y. ruckeri* from Europe and North America by Davies (1990). From these findings, a clonal complex scheme was proposed, which grouped isolates based on their biotype, O-serotype and outer membranes protein (OMP) type (Davies 1991a). The OMP-type is determined by the mobility on 1-D sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane proteins (OMP). Isolates possess a 36.5 or 38 kDa heat modifiable protein (HMP), and a peptidoglycan-associated protein (PAP) with a molecular weight of 36.5, 37.5 38.0, 39.5 or 40.5 kDa (Davies, 1990). The combination of biotyping, OMP typing and serotyping allowed the construction of a clonal group theory for *Y. ruckeri* O1 isolates (Davies, 1991a). The clonal concept of bacterial population structures was first introduced in the 1970's for describing *Escherichia coli* strains (Ørskov *et al.*, 1976). It was suggested that certain O :H

serotypes represent clones, which carried plasmids that were necessary to provoke diarrhoea. This method of classification proved useful in epidemiological studies as ERM outbreaks in rainbow trout farms were shown to be predominantly affected by *Y. ruckeri* serotype O1 strains. Serotype O1 may be sub divided by bt and OMP-type into six clonal groups based around the clonal complex theory. Two clonal groups are associated with ERM in rainbow trout. Clonal group 5 (O1:5) strains cause ERM in mainland Europe and the USA, and include the original Hagerman strain; clonal group 2 (O1:2) strains is predominantly responsible for ERM in the U.K. (= bt 2). The remaining O1 clonal groups have not been reported to cause disease in rainbow trout.

It is unknown to what extent bt 1 and bt 2 isolates differ in terms of serology. As the O antigen used in the Davies (1990) scheme is the sero specific antigen, there must be some sort of similarity between the molecules. There is some evidence that the O antigen within serotype O1 varies as described by Romalde *et al.* (1993). These workers were able to find evidence of a varying O antigen structure, but little biochemical or growth characteristics were given making it difficult to hypothesize if these were bt 2 isolates. If the O antigens are different then there must be some other common antigens present within the O1 serogroups, perhaps OMPs, which causes agglutination with specific antiserum. A comparison with biotypes isolates in the 1980's and present isolates has not been performed. Arguably, serological analysis of the two biotypes will allow for greater understanding of the cross protection between the two, and may provide an insight into why the current commercial vaccine is failing to cross protect against bt 2 isolates.

1.4.5. Molecular classification of *Y. ruckeri*

Technological advances in molecular diagnostics have led to a greater understanding of the relationships between bacteria. Molecular typing has become a rapid and simple procedure whereas previously these techniques were complex and time consuming (Towner and Cockayne, 1993). Molecular typing has been used to widely characterize bacterial pathogens in humans and animals, but they have been rarely employed to study bacterial diseases in fish and shellfish (Goarant *et al.,* 2007, Nicolas *et al.,* 2008). Phenotypic markers make it difficult to relate outbreaks of ERM with a potential source because the traits are inconsistently expressed. However, with the use of molecular

typing and diagnostics such as Multi locus enzyme electrophoresis (MLEE), MLST and ERIC-PCR the epidemiology of this organism is being better understood (Lucangeli *et al.*, 2000).

To date, there have been few molecular studies about the epidemiology and classification of *Y. ruckeri*. The typing scheme of Davies (1990) is insufficient for investigating the genetic relatedness of isolates. ERIC-PCR has been used to study genetic diversity between environmental isolates of *Y. ruckeri* (Coquet *et al.*, 2002). The data revealed that three strains were distinguishable from the 22 isolates studied by the presence of three fragments (721, 912, 1,015 bp). Unfortunately, ERIC-PCR has only been used as a tool for epidemiological purposes on a few occasions and at a national level (Coquet *et al.*, 2002; Arias *et al.*, 2007). Its use has been limited due to reproducibility issues.

Garcia *et al.* (1998) described using plasmids and ribotyping as useful to distinguish between isolates of *Y. ruckeri*. These workers used the technique with success when comparing ribotypes and plasmid profiles from 183 *Y. ruckeri* strains from around the world. Eleven different ribotypes and 8 different plasmid profiles were described. Large plasmids of 75 MDa were only found in serovar O1 isolates, and were hypothesised to correlate to virulence. Smaller plasmids were observed in other serovars. Plasmid profiles have been studied widely in *Y. ruckeri* and provided a better basis to type strains as serotyping systems do not correlate well (Stave *et al.*, 1987; Romalde *et al.*, 1993).

MLEE was one of the first methods used to study *Y. ruckeri* over a long time scale using a variety of isolates. The study by Schill *et al.* (1984) highlighted a considerable genetic homogeneity between *Y. ruckeri* isolates, suggesting that the population was a clone. However, caution must be taken when interpreting MLEE. Like other phenotypic and amplification based tests, differing environmental conditions can alter phenotypic expression of enzymes and amplificons under examination therefore distorting data analysis (Kotetishvili *et al.*, 2005). MLST uses a nucleotide sequence based approach centred around housekeeping genes, which can alleviate all of the

mentioned drawbacks of previously described tests. This approach was developed by Maiden *et al*. (1998) to characterize meningococcal infections, and has been adopted to study many other bacterial pathogens (O'Shea *et al.,* 2004). MLST works by sequencing protein coding loci 'housekeeping genes'. Generally, these genes have low rates of recombination due to slow evolution. Although the number of nucleotide polymorphisms is low, concatenating the sequences of several genes has been shown to provide high discriminatory power whilst still provide information on long-term evolution. The benefits of using MLST as a sequence-based typing method is that the results are highly reproducible between laboratories and the housekeeping genes sequenced are present in all isolates. Previous studies have primarily focused upon defining MLST schemes for the genus *Yersinia*. Kotetishvili *et al*. (2005) included only a limited number of *Y. ruckeri* isolates in their study, therefore making meaningful interpretation difficult. Molecular typing has been used to characterize bacterial pathogens of humans and animals, but the approach has been rarely employed to study bacterial diseases in fish and shellfish.

A comprehensive study of *Y. ruckeri* using pulsed field gel electrophoresis (PFGE) was carried out by Wheeler *et al*. (2009). Using the restriction enzyme *Not*1, 160 isolates were grouped into 44 different pulsotypes. This technique was able to distinguish between all previous serotypes from the Davies (1990) scheme. The hypothesis that *Y. ruckeri* was introduced into the U.K. from the USA was questioned in the report as related strains from the U.K. and USA formed distinct and non-overlapping sub populations (Wheeler *et al*., 2009). Different pulsotypes were observed from genetically and phenotypically similar isolates suggesting that they could have arisen separately. As an epidemiological tool, PFGE has distinct advantages over many molecular typing schemes, but its applications are limited due to poor reproducibility.

1.5. Epizootiology

1.5.1. Transmission and susceptible species

Y. ruckeri is transmitted from fish by direct contact with infected carriers. Initially, it was thought that the pathogen spread through the movement of asymptomatic carriers and eggs, but after isolation from mammals, it was considered possible that wild

animals, such as birds, wild fish, invertebrates, and even humans could act as vectors (Willumsen, 1989). Sauter *et al.* (1985) recovered *Y. ruckeri* from chinook salmon *(Oncorhynchus tshawytscha)* eggs highlighting that vertical transmission could be a possibility; further evidence of this has not yet been provided.

The role of carrier fish is important during *Y. ruckeri* infections. There is some evidence to suggest that *Y. ruckeri* was imported from asymptomatic carrier fish. For example, Michel *et al.* (1986) isolated *Y. ruckeri* from minnows, *Pimephales promelas*, from Belgium and France, respectively, which had been imported from Missouri and Arkansas, USA. The first report of ERM in Canada was found to be the result of infection through asymptomatic carrier fish (Wobeser, 1973). Hunter *et al.* (1980) looked into the importance of stress inducted transmission of *Y. ruckeri* from asymptomatic carriers to naïve fish. It was found that the organism was shed in the faeces over 36-40 day cycles. This shedding cycle was found to correlate with seasonal variations of water temperature, along with crowding, handling and other stressors. There is no evidence to link bacterial shedding to ecotoxicological problems. However, the authors suggested that it may initiate the cycle. The organism has been recovered from the aquatic environment, including water, faeces and sewage sludge (Willumson, 1989) and attaches to- and forms biofilms readily (Coquet *et al.*, 2002). These biofilms may be a source of recurrent infection in rainbow trout farms. Survival may be for up to 2 months in pond mud (Roberts, 2004).

The status of carrier fish and the reasons for the spread of the pathogen from the USA remain unclear. Bullock *et al.* (1978) questioned the idea and hypothesised that *Y. ruckeri* could have already existed previously as isolates recovered from the National Fisheries Center, Leetown, USA were found dating before the first isolation by Rucker in the 1960's, and an Australian isolate was also recovered in the 1960's.

Although the disease has been predominantly a problem for rainbow trout in intensive culture, all salmonids may be affected (Table 1.5). McDaniel (1971) provided a detailed report on different salmonids that could be potentially affected by the disease. In particular, the organism has been isolated from non-salmonid feral fish, such as pike

(*Esox lucius*), eels (Fuhrmann *et al.,* 1984), gudgeon (*Gobio gobio*), and sturgeon (Vuillaume *et al.,* 1987), which may suggest a source of infection for trout and salmonid stocks. The organism has also been isolated from various salt water fish including, turbot, coalfish, sole and gilthead sea bream (Michel *et al.*, 1986). Currently, there is not any published data regarding *Y. ruckeri* infections of Atlantic salmon (*Salmo salar*) in sea cages, although there are reports of the pathogen affecting farmed specimens in freshwater (Wheeler *et al*., 2009). There are not any species specific strains of *Y. ruckeri*. Moreover, it is currently unknown whether other fish species are susceptible to bt 2 isolates.

Table 1.5. Fish and other species susceptible to *Y. ruckeri* infections

Common name	Scientific name	Reference
Rainbow trout	*Oncorhynchus mykiss*	Rucker (1955)
Atlantic salmon	*Salmo salar*	Rintamäki *et al*. (1986)
Cod	*Gadus morhua*	Personal observation
Coalfish	*Pollachius virens*	Michel *et al.* (1986)
Sole	*Soleidae sp.*	Michel *et al.* (1986)
Arctic charr	*Salvelinus alpinus*	Personal observation
Sturgeon	*Acipenser sturio*	Vuillaume *et al.* (1987)
Brown trout	*Salmo trutta*	Mc Daniel (1971)
Gudgeon	*Gobio gobio*	McDaniel (1971)
Turbot	*Scophthalmus maximus*	Michel *et al.* (1986)
Goldfish	*Carassius auratus*	McArdle and Dooley-Martyn (1985)
Common carp	*Cyprinus carpio*	Fuhrmann *et al.* (1987)
Eel	*Anguilla anguilla*	Fuhrmann *et al.* (1987)
Pike	*Esox lucius*	McDaniel (1971)
Other species:		
Muskrat	*Ondatra zibethica*	Stevenson and Daley (1982)
Kestrel	*Falco (partim)*	Bangert *et al*. (1988)

Sea gulls	*Larus argentatus*	Willumsen (1989)
Human	*Homo sapiens*	Farmer *et al.* (1985)

1.5.2. Clinical signs of infection

To date, there is limited knowledge about the virulence of this pathogen, and hence clinical signs might not always suggest possible mechanisms of pathogenesis. Fish become lethargic, the food conversion ratio (FCR) is reduced, and therefore growth slows. The gross external changes produced by infection with *Y. ruckeri* were first reported by Rucker (1966). Both bt 1 and bt 2 infection are characterised as generalised haemorrhagic septicaemia. It was stated that infected rainbow trout become lethargic, develop melanosis/darkening of the skin along with congestion of the vessels of the oral area, with ulceration and haemorrhaging producing a distinctive pathognomic lesion (Roberts, 2004). Clinical signs vary, and cases have been found where the principle 'red mouth' feature is absent (Tobback *et al.,* 2007).

Internally, the disease causes generalised haemorrhaging over the internal organs, with kidney and spleen swelling. The stomach and intestine may contain watery and yellow fluid, respectively (Stevenson *et al.,* 1993; Roberts, 2004, Toddbeck *et al.,* 2009).

In chronic disease, granulomas may be found in the kidney, but in acute cases the spleen, liver and kidney are heavily infiltrated with leucocytes, and are associated with necrotic foci and haemorrhaging (Roberts, 2004). It was observed by Wobeser (1973) that the anterior and posterior kidney of infected fish showed distinct changes in density and structure of the haematopoietic tissue. Haematoxylin and eosin (H & E) stained tissue sections revealed general congestion of the posterior kidney, degeneration of renal tubules and a marked increase in melano-macrophages; coagulative necrosis may be observed in spleen sections (Toddbeck *et al*., 2009). Wobeser (1973) highlighted that packed cell volume and total blood protein was reduced by 50%, suggesting degradation of haematopoietic tissue in the anterior kidney.

1.6. Pathogenicity of *Y. ruckeri*

Research into the pathogenicity mechanisms of *Y. ruckeri* is limited. Much more is known about other members of the genus *Yersinia*, i.e. *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*, which cause disease in terrestrial mammals. *Y. pestis* is the aetiological agent of the bubonic and pneumonic plague, which are lethal diseases affecting the lymph nodes and respiratory tract of humans. Some estimates suggest that >200 million people succumbed to the Black Death of the Middle Ages **(**Perry and Fetherston, 1997). *Y. enterocolitica* is a common food-borne pathogen causing yersiniosis in humans (Bottone, 1997), Infections with *Y. enterocolitica* are characterised by gastro-enteritis, although extraintestinal manifestations and postinfectious sequelae, such as reactive arthritis, occur as well (Bottone, 1997). *Y. pseudotuberculosis* is an enteric pathogen that induces a variety of clinical symptoms, fever, scarlatiniform rash, diarrhoea, vomiting, and arthritis (Abe *et al.*, 1993). In comparison, ERM is generally a chronic condition, which causes grumbling mortalities. However, the cumulative mortality may reach 50% overnight following stressful events, such as handling, grading and poor water quality.

The virulence of *Y. ruckeri* isolates differs with location and experimental procedure used. Avci and Birincioğlu (2005) found that mortality occurred 5 to 10 days following i.p. injection with $3x10^6$ cells of *Y. ruckeri*; these findings are similar to those of other authors who studied i.p. injection of bt 1 isolates. The LD_{50} reported by Raida and Buchmann (2008b) in their challenge experiments was 5×10^5 CFU $fish^{-1}$ for i.p. challenge, and 1×10^7 CFU ml^{-1} *Y. ruckeri* in tank water for bath challenge. Initial experiments using the new biogroup of *Y. ruckeri* discovered that the organisms killed fish at a cell density of 1×10^4 CFU ml^{-1} (D. Austin, *pers. comms, 2007*). Yet to date, there have not been any published comparative studies between bt 1 and bt 2 isolates. Virulence studies conducted by Fouz *et al.* (2006) reported that bt 2 isolates from the USA had a LD_{50} dose of 5×10^2 CFU $fish^{-1}$ following i.p. administration.

1.6.1. Extracellular products (ECP)

Many bacterial pathogens, including *Y. ruckeri*, produce ECP's that contribute to their pathogenicity. *Y. ruckeri* has been considered to be a poor producer of cytotoxins and haemolysins (Romalde and Toranzo, 1993). Despite this, researchers have demonstrated

that *Y. ruckeri* is able to produce a variety of ECP's, including haemolysins which have been shown to play a role in pathogenesis (Romalde *et al*., 1993). When ECP's were injected into rainbow trout, haemorrhaging around the mouth developed. However, little information regarding the nature of these toxins and their role in pathogenesis has been published.

Most of the extracellular virulence factors are enzymes that enhance pathogen colonisation and growth (Madigan and Martinko, 2006). One important molecule to bacterial survival is iron. This is an absolute growth requirement for bacteria, and must be removed from iron binding proteins in the host, such as by the action of siderophores (Neilands, 1995). The siderophore, ruckerbactin, is the iron uptake system involved with virulence in *Y. ruckeri* (Fernandez *et al.,* 2004). Iron is generally tightly bound to molecules, such as haemoglobin and transferrin. Following secretion, siderophores sequester and solubilise the iron, which is then transported back across the cell well via various receptors.

Secretion of effector molecules, such as proteases, allows pathogenic bacteria to breakdown the surrounding environment as a source of nutrients. Secades and Guijarro (1998) identified a 47-kDa metalloprotease, termed *Yrp1*, which was produced at the end of the exponential growth phase. Metalloprotease is a histolytic enzyme, and it was proposed that it is involved in colonisation and virulence. The metalloprotease is not a general feature of *Y. ruckeri,* and is not related to serotype. Isolates have been described as Aso^+ and Aso^- according to the presence and absence of the *Yrp*1 proteolytic activity. Aso^+ strains have been described at virulent.

A haemolysin/cytolysin, termed *YhlA*, has been demonstrated to play a role in the pathogenicity of *Y. ruckeri* (Fernández *et al*., 2007). *YhlA* is able to lyse erythrocytes and cultured fish cells, and may be related to invasive properties as has been demonstrated with some *Serratia*-type toxins. LD_{50} experiments using *yhlB* and *yhlA* mutant strains demonstrated the role of the toxin in the virulence of *Y. ruckeri* (Fernández *et al*., 2007b). Here, levels of expression increased at 18°C and under iron

limiting conditions, suggesting that these two factors could be environmental clues that the pathogen uses in order to produce certain extracellular toxins.

1.6.2. The outer membrane and other surface components

The outer membrane of Gram-negative bacteria is composed of flagella, lipopolysaccharides (LPS), porin proteins, pili, fimbriae and various secretion systems (Bos *et al.,* 2007) (Figure 1.2). Bacterial LPS are diverse, functionally important components of the outer membrane which belong to a class of molecules call pathogen-associated molecular patterns (PAMPs) (Chisholm *et al.,* 2006) LPS is an amphiphilic molecule, consisting of three domains: the membrane-embedded lipid A, a core oligosaccharide and an distal O-antigen (Evans *et al*., 2010). Depending on whether O antigens are expressed, LPS types may be rough or smooth. Rough LPS types lack polysaccharide side chains (= O antigen), whereas smooth LPS structures are characterised by long ‘repeating O antigen side chains (Perez-Perez *et al.,* 1986). Smooth LPS has been linked with serum resistance in fish pathogens (Boeson *et al.,* 1999). Similar LPS structures in *Y. ruckeri* could provide evidence for the lack of specific immune response seen in ERM. Yet, these molecules have antigenic properties. The lipid component, lipid A, has been recognised as the principle toxic component in mammals. However, fish appear to be immune to the toxic nature of this molecule (MacKenzie *et al*., 2010). The polysaccharide components of LPS are associated with immunogenicity. LPS is regarded to be the serospecific antigen responsible for O-serologic specificity (Pyle and Schill, 1985). Indeed, LPS was considered to be the dominant immunogenic molecule in formalin-killed vaccine preparations for ERM (Amend and Johnson, 1983). Due to the presence of different sugars and sugar linkages, it is one of the most variable cell constituents which play an important role in bacterial evasion of host defence systems (Reeves, 1995).

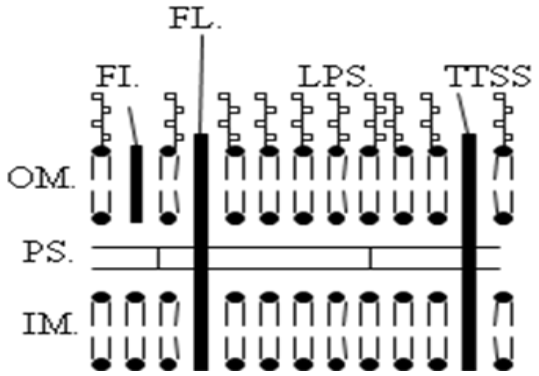


Figure 1.2. The cell surface of a Gram-negative bacterium with its major outer membrane components, including flagella, fimbriae, LPS, T3SS and OMPs. (Fi = fimbriae, FL = flagella, T3SS= type 3 secretion system, LPS = lipopolysaccharide, IM = inner membrane, OM = outer membrane, PS = periplasmic space.).

The outer membrane components of bacterial pathogens are key mediators in attachment to the host. Attachment may be mediated by cell surface features or adhesins, such as flagella, LPS and pili or by cell properties, i.e. hydrophobicity or cell surface charge. However, attachment is important in colonisation of the host but not necessarily a sign of pathogenicity as generally there are multiple factors involved. Of relevance, Mantle and Husar (1993) reported that virulence in isolates of *Y. enterocolitica* was correlated with attachment to human and rabbit mucus. Cell surface hydrophobicity is influenced by structures, polypeptides and proteins (An and Friedman, 2000). This association was hypothesised to be beneficial to the pathogen, as it allowed cells to come into contact with macrophages in which it was able to multiply. Mantle and Husar (1993) studied virulence, and concluded that there was a correlation with attachment; this may not be the case for *Y. ruckeri*. Moreover, Santos *et al.* (1991) suggested that hydrophobicity was absent in isolates of *Y. ruckeri,* suggesting that other cell components, such as LPS, could be important in the process.

Yersinia sp. possess a type 3 secretion system (T3SS) (Gunasena *et al.,* 2003). This comprises a series of proteins with a hollow tube like structure through which effector proteins are 'injected' into the host cell. This secretion system helps pathogens by protecting against phagocytosis and serum resistance. Toxins induce apoptosis, which in turn releases the pathogen from the phagocyte (Monack *et al.,* 1997).

1.7. Immune response to *Y. ruckeri* infections

There has been a dramatic increase of research into fish immunology over the last decades, driven by academia and industry (Bebak *et al*., 2009; Forlenza *et al.,* 2009). Knowledge about the immune defence mechanisms of fish against bacteria is important in terms of disease control and prevention (Toddbeck *et al.,* 2007). The immune system of fish, like mammals, may be separated in two distinct parts, i.e. the innate or specific immune systems. Each has further subdivisions between the humoral and cellular components (Ellis, 1999). Even though these two systems are often described separately, they may act together. In order to infect a host, a pathogen must overcome or avoid these systems. The immune responses of fish may be primed through vaccination in order to ready the fish against a challenge from a pathogen. However, since the first commercial vaccines became available in the 1970's, research is still needed particularly in view of the reduced efficacy against *Y. ruckeri* bt 2 isolates. Certainly, there is still a gap in knowledge regarding the nature of the optimal antigens and their immune functions necessary for protection against this isolate.

1.7.1. Non specific immune response

The non-specific or innate immune system in fish is really the first and ever present line of defence against invading pathogens. The non specific immune response has been stated to be of paramount importance when defending the host from *Y. ruckeri* infections (Raida and Buchmann, 2008b). The system is made up of humoral factors, such as inhibitors and lysins, and cellular factors, specifically neutrophils and macrophages. Young *et al.* (2006) described ladderlactin, a non specific humoral factor, which was able to bind to *Y. ruckeri*. It was hypothesised that lectins are able to bind to the pathogen and enhance complement activity and phagocytosis. The latter is of paramount importance when protecting a host from bacterial infection (Ellis, 1999). Neutrophils and macrophages are involved in the engulfing and internalisation of bacteria and killing them through the process of the respiratory burst (Neumann *et al*., 2001). It has been shown that injection with formalin killed whole cell vaccines of *Y. ruckeri* led to a large influx of macrophages and neutrophils (Alfonso *et al*., 1998). Phagocytic cells, such as neutrophils and macrophages, produce molecules of various reactive oxygen species, i.e. OH^-, H_2O_2 and superoxide anion (O_2^-). Macrophages may also produce nitric oxide, which has been shown to be an important antibacterial compound to *Y. ruckeri* (Campos-Pérez *et al.,* 2000). To date, there has not been any

work to determine the role of phagocytosis in *Y. ruckeri* infections. Certainly, the majority of research into the immune response against *Y. ruckeri* has involved motile bt 1 isolates; there is not any information about how bt 2 isolates interact with the fish immune system. It would appear that flagellin is a key molecule involved in stimulating the innate immune system, therefore, non-motile isolates could have a distinct advantage in evading detection. It is relevant to note that the lack of flagella in non-motile *Vibrio* sp. was hypothesised by Sallum and Chen (2008) to benefit the pathogen by evading detection by host molecular pattern receptors, such as Toll like receptors (TLR).

1.7.2. Specific immune response

The specific immune system is also known as the 'adaptive' immune system and forms the second line of defence against pathogens. This system employs both humoral components, such as antibodies, and cellular components and activated macrophages (Ellis, 1999). There is little evidence that circulating antibodies play a role in protection against *Y. ruckeri* infections (Crossarini-Dunier, 1986). This view was backed up by the study by Raida and Buchmann (1998a), who found that passive immunisation with antibodies raised against *Y. ruckeri* did not confer any protection. Similar findings were reported by Cipriano and Ruppenthal (1987) using brook trout. Indeed, it was suggested that protection was conferred by circulating cellular factors, other than IgM. Circulating antibodies could be important in opsonising bacteria and activating classical complement pathway in *Y. ruckeri* infections (Crossarini-Dunier, 1986). Moreover, Raida and Buchmann (2008a) highlighted that cellular specific immune responses were involved in secondary infections involving *Y. ruckeri*. Using RT-PCR, it was discussed how CD8α, a cytotoxic T cell, was upregulated during secondary infection, highlighting a potential role that the cellular adaptive protection mechanisms play against *Y. ruckeri*. Certainly, studies have researched the role of immunoregulatory gene expression during *Y. ruckeri* infections, and the role in antibacterial immunity (Rodriguez *et al.*, 2005; Wiens *et al.*, 2006). In this connection, cytokines comprise a group of secreted compounds, which regulate immunity. In particular, Rodriguez *et al.* (2005) and Wiens *et al*. (2006) found that various cytokines were upregulated during infection, i.e. Toll like receptor 2, *CXCd* and IL-1*β*2. Although upregulation was observed, the role of these molecules in protection has yet to be determined. Certainly, Bonn *et al.* (1998) noted that interleukin 12 (IL-12) exerted a role in protection against *Y. enterocolitica*

infections of mice. Thus, administration of IL-12 was observed to stimulate interferon gamma (IFN-γ), a pro inflammatory cytokine involved with activation of macrophages.

1.7.3. Factors affecting the immune response

The immune response in fish can be influenced by three main factors;

- extrinsic factors which relate to the environment and the nature of the antigen
- intrinsic factors which relate to the immunoregulatory mechanisms within the immune system
- ontogenic factors which relate to the maturation of the immune system in young fish (Roberts, 2004).

1.7.3.1. Extrinsic factors

Fish are poikilothermic vertebrates and have many vital functions including the immune response which is affected by changes in water temperature and chemistry. Many authors have detailed seasonal variations in the immune response (e.g. Le Morvan *et al.*, 1998; Bowden *et al*., 2007). Temperature, photoperiod and other environmental conditions, which are directly or indirectly controlled by the season, may affect fish in intensive culture (Bowden *et al.,* 2007). In particular, the effect of water temperature on the immune response is of paramount importance. Thus, within the thermal limits of a cultured fish species, it is regarded that the higher the temperature the faster the response and magnitude of the immune system (Roberts, 2004). Clearly, fluctuation in water temperature may affect the immune system in a number of ways, namely the strength of the actual response, the activation of specific immune cells, and the efficacy of vaccination.

Many environmental factors are known to influence the immune response in teleost fish. In particular, studies have focused on the environmental effects on the production cycle of fish, but few have considered what effect this has upon the immune system. The effects of a changing environment can effect cultured fish in two ways, i.e. by inducing chronic stress or directly altering immune function. Environmental ammonia has been shown to increase the susceptibility of chinook salmon smolts to *V. anguillarum*

(Ackermann *et al.,* 2006). Here, ammonia was reported to reduce respiratory burst and lysozyme activities, both key nonspecific immune components. Formulated feeds produced for intensive aquaculture aim to provide the fish with a complete diet (Roberts, 2004), and it is generally regarded that adequate nutrition is crucial for the maintenance of a good immune system (Ashley, 2007). Comprehensive reviews have been carried out regarding the different aspects of nutritional diseases in fish (e.g. Lim and Webster, 2001; Roberts, 2004). Of importance here is the effect of nutrition on the immune response in fish. In particular, vitamins and minerals are key components that have been shown to affect the immune response and predispose fish to bacterial challenge. Of relevance, Hardie *et al*. (1990) found that feeding vitamin E depleted diets resulted in the impairing of Atlantic salmon complement, and therefore this led to the fish being more susceptible to *A. salmonicida* infection.

When multiple antigens are administered during vaccination, there is evidence that antigenic competition may reduce the immune response in fish. In particular, antigenic competition may affect the specificity, avidity and level of antibodies when administered with polyvalent vaccines (Nikoskelainen *et al.,* 2007). Common epitopes from LPS isolated from different bacterial species have been shown to cross-react with antibodies (Bøgwald *et al*., 1991). Thus, antibodies raised against LPS from *V. anguillarum* and *V. salmonicida* cross reacted with each other, suggesting a similar structure. Antigenic competition was certainly highlighted by Bøgwald *et al*. (1991) in that certain serotypes of *F. psychrophilum* were shown to be suppressive.

1.7.3.2. Intrinsic factors

Immunomodulation caused by the immune system has been reported in mammals (Goodburn *et al*., 2000). However, there is little information regarding this subject in fish. Diet, disease and environmental and ecotoxiological parameters may all cause immunomodulation in fish (Bols *et al*., 2001). Immune complexes (antibody-antigen complex) caused by aggregates of antigens and antibodies have been recognised to effect the immune system (Grayson *et al*., 2002). Phagocytic cells generally remove these complexes. However, if they are not phagocytosed, immune complexes may cause hypersensitivity reactions due to deposition within tissues, or acute inflammation occurs due to interactions with neutrophils (Lydard *et al.,* 2004). During the smoltification

process, it was observed that B-cells may be down regulated by cortisol, therefore compromising vaccination efficiency in Atlantic salmon (Espelid *et al*., 1996).

1.7.3.3. Ontogeny of the immune system

The development of the immune system in fish is not instantaneous, and differs between species. The thymus, kidney and spleen are the major lymphoid organs of teleosts (Zapat *et al.,* 2006). The development of these organs is related to the development of immunological components. Early vaccination is critical in protecting fish against pathogens at a very early age of development. Also, the effectiveness of vaccination reflects the age and size of fish; too early and the immune system may not be developed enough to build immunity; too late and fish will not be protected. Ellis (1998b) highlighted the importance of size in relation to vaccination. Of note, vaccination against *V. anguillarum* was ineffective when administered to 1 g salmonid fish, but full protection was achieved at the 4 g weight class regardless of age.

1.8. Diagnostics

Rapid diagnosis of any disease in aquatic animals is important for managing disease outbreaks. A number of methods have been reported for the detection and identification of *Y. ruckeri*, including traditional (phenotypic characteristics), immunological and molecular techniques. Traditional phenotypic diagnostic tests are still employed as the most cost effective ways of diagnosing *Y. ruckeri* infections (Austin *et al*., 2003). A rapid method based on biochemical analysis, using the API 20E rapid identification system has been routinely used for the identification purposes (De Grandis *et al.,* 1988). Yet, problems with interpreting the results of the API 20E systems have occurred when analyzing the bt 2 and new biogroups. In particular, the profile could be confused with *Hafnia alvei,* which is also a member of the Enterobacteriaceae (Austin *et al*., 2003). However, the tests have reproducibility issues as the biochemical characteristics expressed between different isolates of *Y. ruckeri* are not always identical (Ross *et al.,* 1966; De Grandis *et al.,* 1988; Austin and Austin, 2003). The lack of widely available antisera against the different serotypes and bt of *Y. ruckeri* makes serology difficult for many laboratories.

Immunological methods, including the enzyme linked immunosorbent assay (ELISA), have been developed for the detection of *Y. ruckeri* (Cossarini-Dunier, 1985). Thus, it was determined that rabbit antiserum was more specific in detecting antigens using the ELISA but there was no difference in agglutination reactions (Cossarini-Dunier, 1985). Although immunological methods have been useful in the identification of *Y. ruckeri*, it might only be possible to identify particular isolates/serotypes with the specific antibodies developed due to the heterogeneity of isolates (Romalde *et al*., 1993). The presence of antibodies in the serum suggests that they could play a role in protection (Tobback *et al*., 2007). Monoclonal antibodies (Mabs) have exerted an important role in diagnosis of many bacterial fish pathogens (Adams *et al.* 1995; Wagner *et al.* 1999). However, to date very few specific monoclonal antibodies have been developed for the detection or characterization of *Y. ruckeri* (Furones *et al*., 1993).

Molecular techniques, such as PCR and restriction fragment-length polymorphism (RFLP), have been developed for diagnostic purposes. Gilbello *et al*. (1999) developed a PCR based method for detecting *Y. ruckeri* in the tissues of artificially and naturally infected fish. A loop-mediated isothermal amplification (LAMP) assay was developed by Saleh *et al.* (2008) for the detection of *Y. ruckeri*. This technique is highly sensitive, being able to detect up to six copies of DNA per sample (Notomi *et al.,* 2000). LAMP has a clear advantage over standard PCR as it is simple to perform requiring only a set of primers and a water bath.

1.9. Treatments and control

Overall, a good health management strategy is paramount in preventing disease from entering into a farm (Bondad-Reantaso *et al*., 2005). Good farm management includes disinfection of tanks, control of water quality, the reduction of potential stressors and the use of vaccines play an important role in disease prevention (Conte, 2004). Ignoring any of these factors could lead to disease outbreaks (Ashley, 2007).

1.9.1. Antibiotics

The use of antibiotics for the control of ERM has been practiced ever since the bacterium was first recovered from diseased fish (Rucker, 1966). Compounds, such as

chloramphenicol or oxytetracycline, have been used to control infections (Rodgers, 1990). However, the misuse of antimicrobial agents has led to the emergence of drug resistant strains (Coquet *et al*., 2002). Moreover, by exposing bacteria to inadequate or variable dosage, failure to complete the recommended course or by repeated short term treatment, is likely to result in selection for drug resistance (Rodgers, 2001). This worker highlighted how resistance of bt 1 isolates to oxolinic acid, oxytetracycline and a potentiated sulphonamide was achieved through repeated exposure to the inhibitory compound. Clearly, the use of antibiotics in aquaculture should be minimised, and when needed administration should be in conjunction with good management practices.

1.9.2. Immunostimulants and probiotics

There has been a growing trend in the use of probiotics as an alternative to the use of antibiotics and vaccines (Verschuere *et al*., 2002). For example, Kim and Austin (2006) studied the effect of two carnobacterial cultures, which led to stimulation of the innate immune response in rainbow trout and protection against challenge with *Y. ruckeri*. It was demonstrated that the two cultures enhanced the cellular and humoral immune responses, notably phagocytic and respiratory burst activity of head kidney macrophages and lysozyme activity of serum and gut mucus. Moreover, Abbas *et al.* (2010) discussed that bt 2 infections of *Y. ruckeri* could be controlled by cellular components of certain probiotic bacteria.

The use of immunostimulants as a way of improving the effectiveness of vaccines has received growing attention since the shift away from the dominance of antimicrobial substances (Tobback *et al.,* 2007). Siwsicki *et al.* (1998) highlighted the effectiveness of dimerized lysozyme as an immunostimulant to improve the effectiveness of an ERM vaccine in rainbow trout. Thus, it was found that using lysozyme before, with or after the vaccine increased cell-mediated immunity and the specific immune response.

At present, there are numerous products designed to boost the immune system. For example, dietary application of ascorbic acid or vitamin E enhanced the innate immune response (Blazer and Wolke, 1984). Moreover, Wahli *et al.* (1998) determined that

rainbow trout fed diets high in vitamin E, alone or combined with high levels of vitamin C, enabled the best survival after challenge with *Y. ruckeri.*

1.9.3. Vaccination

Vaccines against ERM are one of the success stories of disease control in aquaculture, with the first commercial product released in the 1970's. The aims of vaccination are to induce memory in T and/or B lymphocytes through the administration of a non-virulent antigen preparation (Lydyard *et al.*, 2004). Therefore in the event of an infection, the infectious agent is met by a secondary response or specific immune system rather than a primary one, i.e. non –specific immune system. Overall, vaccination is one of the major methods for preventing infectious disease (Potter and Baiuk, 2001). Vaccination of fish is considered to be important in reducing economic losses caused by disease. Immunisation stimulates the immune system of the host against pathogens encountered during infections (Thompson and Adams, 2004). The protective mechanisms of vaccination may stimulate cell-mediated immunoprotection and humoral immunity. Certainly, there are many advantages over conventional drug treatment. However, there are not many commercial vaccines available to aquaculture.

The current vaccination strategy for ERM involves bathing or injection at the fry stage. Due to the nature of immersion vaccination, the protection falls short compared to injection vaccination. It is therefore standard practise to use an oral booster vaccine 6 months later. Booster vaccines against *Y. ruckeri* were observed to give increased protection in rainbow trout (Tatner and Horne, 1985). The route by which a vaccine is administered can exert a different effect over the immune response (Palm *et al.*, 1998). Administering vaccine via I.P, injection is regarded as providing the best levels of protection (Palm *et al*,. 1998). However, as fish are most susceptible to ERM at a size of ~4 g, bath vaccine is recommended (Hastein *et al.*, 2005). Certainly, the vaccination strategy should coincide with historical disease records for the specific farm; this could reflect yearly endemic infection or a seasonal outbreak (Rodgers, 1991). Seasonal outbreaks of disease are associated with low water temperatures at the start of the season and stressful events, such as handling and grading (Rodgers, 1991). Most situations require a bath vaccine which is administered to 5 g fish (SP Aquaculture, 2006). An oral in-feed booster should be administered 4-6 months after initial

vaccination to provide protection throughout the production cycle (SP Aquaculture, 2006). The end result is that vaccination will prevent clinical disease but unfortunately will not necessarily eliminate the carrier fish state (Bruno and Munro, 1989).

Currently, there is one bivalent vaccine (RELERA, SP Aquaculture) produced against bt 1 and bt 2 isolates of *Y. ruckeri*, which was released in 2008. The vaccine is a formalin-killed whole cell preparations of both biotypes. To date, there is no information regarding the efficacy of this vaccine in the field. Yet, formalin inactivated whole cells of the non-motile new biogroup have been shown to give protection following i.p challenge with a virulent culture (Austin *et al.*, 2003). Clearly, antigen dose and the nature of the antigen are closely related factors which affect the immune response in fish. In this connection, it is important to note that the immune response is dependent on dosage, route and water temperature (Roberts, 2004). Of relevance, it has been found that the antigen dose directly correlated with immunological memory in cultured fish species (Lamers *et al*. 1995).

Numerous studies have investigated the nature of the protective antigens in vaccines (Ross and Kontz, 1960; Anderson and Nelson, 1974; Vigneulle, 1990). Amend *et al.* (1983) highlighted that the culture conditions, including temperature, and methods of inactivation effected potency of *Y. ruckeri* whole cell vaccines. Also, lysing cells by heat led to increased potency (Amend *et al.*, 1983). These workers recommended that whole cell vaccines should be used as opposed to specific antigens, such as OMPs and LPS. Clearly, the nature of the antigen can determine the quantity and quality of the immune response, and chemical modifications of the preparation may result in differences in expression of the immune response (Amend *et al*., 1983).

Adjuvants are immunological stimulants, which do not have any specific antigenic effect. Adjuvants have been previously described as the '*dirty little secret'* of vaccines: 'dirty' because adjuvants contaminated the purified vaccine antigen recognized by T and B-lymphocytes, and 'secret' because their mode of action remains a mystery (Janeway, 1989). Freund's Complete Adjuvant (FCA) is widely used in fish immunology and stimulates higher and more prolonged antibody response (Jaio *et al*., 2010). Despite shortcomings including some granulomatous side-effects (Secombes *et al*., 1985), FCA

is still used in commercial vaccines especially in Norway. Overall, oil-adjuvanted vaccines are known to induce inflammation at the site of injection leading to migration of phagocytes, which could possible increases the amount of circulating leucocytes in general (Mutoloki *et al*., 2006). However, the use of adjuvants is not always beneficial, insofar as Horne *et al*. (1984) reported that using the aluminium salts and potassium alum adjuvant led to an increase in chronic peritonitis along with depressed growth rates.

As described previously, bt 2 infections were first identified from aquaculture sites which had previously vaccinated against bt 1 isolates of the disease (Austin *et al*., 2003). At the time, it was hypothesised that poor water quality and husbandry conditions played a part in the failure of the vaccine to induce protection. Similar findings were observed previously by Rogers (1990). Thus, a survey was carried out involving Atlantic salmon and rainbow trout farms in the U.K. with the intention of collecting data on the use of vaccination and antimicrobial agents to control ERM. This survey highlighted that the disease condition in previously vaccinated fish was associated with poor condition of the fish and low water temperatures, suggesting that the vaccination strategy employed and the immunocompromised hosts were still susceptible to the disease. Although new strains were not observed, the results highlighted the importance of good husbandry measures in ensuring that vaccines do not fail. Previous outbreaks of disease in mammalian and fish species have highlighted the possible of vaccine induced strain replacement (Martcheva *et al*., 2008). In particular, it has been demonstrated that selective pressure induced by intensive vaccination could cause changes in phenotype and immunogenic characteristics, which could cause disease in previously vaccinated fish (Bachrach *et al.,* 2001). However, it is unknown to what extent *Y. ruckeri* could be affected by vaccination, and whether this is a reason why bt 2 infections are increasing.

Subunit vaccines have potential to be useful in the future. Subunit vaccine are consist of a porin of a pathogen (typically peptides) (Winton, 1998). In this connection, Fernández *et al.* (2003) described the *Yrp1* protease as a key virulence factor in *Y. ruckeri* and which conferred significant protection when administered intramuscularly to fish.

Recently, interest in the use of live attenuated vaccines against bacterial fish pathogens has increased. In mammals, it is widely recognised that live vaccines have a number of advantages over dead preparations in that they are effective stimulators of cell mediated immunity (Marsden *et al.,* 1996). Of relevance, Temprano *et al.* (2005) discussed using a mutant of *Y. ruckeri* with a dysfunction of the *aroA* gene as a live vaccine. The *aroA* gene, which encodes the enzyme 5-enolpyruvylshikimate-3-phosphatesynthase, plays a role in the biosynthesis of aromatic amino acids. By causing a dysfunction in this gene, the organism can no longer proliferate within fish. Vaccination using this mutant conferred 90% relative percentage survival (RPS) against *Y. ruckeri* bt 1 (Temprano *et al.,* 2005).

1.10. Scientific aims

It is apparent that there is a need to examine *Y. ruckeri* bt 2 to further understanding of the epizootiology of this pathogen. Moreover, there is a need to learn more about the virulence determinants and pathogenesis.

The scientific aims of the study are:

i. To carry out in depth research into the biochemical characteristic of *Y. ruckeri* isolates.

ii. To understand the phylogeny of *Y. ruckeri* through the use of genetic techniques.

iii. To study the antigenic and pathology of bt 2 infections in rainbow trout.

iv. To investigate the immune response against *Y. ruckeri* bt 2 infection in order to elucidate why the conventional monovalent whole cell formalin-inactivated vaccine failed to provide protection in the fish farm environment.

Chapter 2. Biochemical and cell surface characteristics of *Yersinia ruckeri*

2.1 Introduction

Bacterial diseases cause extensive losses to the aquaculture industry each year (Muroga, 2001). The ability to accurately identify and characterise a pathogen is of paramount importance for both control and epidemiological investigations. Phenotyping is one of the main methods employed in bacterial taxonomy due to speed and cost advantages. This approach is valuable for differentiation of bacteria below the species level (Van Der Waaij *et al.,* 1977). Arguably, bacteria exist as populations, members of which exhibit different virulence characteristics which are displayed by the particular phenotype (Spratt and Maiden, 1999). Thus understanding the phenotypic characteristics of a bacterial isolate will ultimately lead to greater appreciation about its taxonomic position and the epidemiology of the disease that it may cause. Focusing upon the population and its evolution, epidemiological studies can provide important insights into the origins and spread of bacterial diseases (Spratt and Maiden, 1999).

Since the mid-1990's *Y. ruckeri* from Europe and USA have been apparently unaffected by commercial monovalent vaccines which are based around the Hagerman O1 type strain (Austin *et al*., 2003; Fouz *et al.,* 2006; Arias *et al.,* 2007). Evidence suggests that there are a number of difference variants of bt 2 causing disease within aquaculture (Austin *et al*., 2003, Arias *et al*., 2007). Thus, isolates recovered from the USA are VP negative whereas isolates from Europe are VP positive. To date there have been no investigation into the phenotypic characteristics of clinical non-motile isolates of *Y. ruckeri*.

Phenotyping of pathogenic bacteria has often been used to differentiate clinical from environmental isolates in epidemiological studies (Kozinsk *et al.*, 2002). Previously, bacterial pathogens had been misdiagnosed by relying on biochemical characteristics alone (Austin *et al*., 2003). Accurate phenotyping now relies upon knowledge of serology, cell surface properties and biochemistry of the bacterium. Initially before the availability of DNA sequencing, *Y. ruckeri* was confused with other bacterial taxa, namely *Serratia, Hafnia* or *Salmonella*, based only on phenotyping (Llewellyn, 1980;

Austin *et al.,* 2003). Certainly, the taxonomic position of *Y. ruckeri* has been subject to great debate since its first isolation, and it has been studied by many methods. By using phenotyping, serology and genotyping, confirmation of the taxonomic status of the pathogen may be verified (Ewing *et al.,* 1978). According to Cowan (1965), an organism cannot be characterised until it has been studied from a morphological, biochemical and genetic point of view. However, there are limitations to phenotyping, insofar as maintenance of strains in a laboratory. Repeated subculturing could result in a decrease in virulence and the loss or change of some characteristics (Thyssen *et al.,* 1998). Therefore, phenotyping is generally used in conjunction with serology and genotyping in order to identify and study bacterial pathogens (Coquet *et al*., 2002).

Serotyping is one of the classical methods used for intraspecific characterisation of bacteria, and is a useful tool used in conjunction with phenotyping for epidemiological and pathobiological studies. Surface antigens are the principal means of characterising bacterial pathogens (Spratt and Maiden, 1999). Using antibodies raised against these antigens, serotyping has been effective in the identification of many bacterial pathogens (López-Romalde *et al*., 2003). Specifically the O antigen or LPS molecule of bacterial pathogens has been used as this molecule varies considerably between strains. A variety of serotypes have been previous characterised using O antigens for a number of bacterial fish pathogens. Various schemes involving serological methods have been used to characterise *Y. ruckeri* strains (Busch, 1978; Green and Austin, 1982; De Grandis *et al.,* 1988; Davies, 1990; 1991a). Davies (1990) provided the most comprehensive characterisation of *Y. ruckeri* strains from the UK and Europe. Thus, 5 O-serotypes were identified, i.e. O1, O2, O5, O6 and O7, based around the O antigen reaction with specific antibodies (Davies, 1991a) This typing scheme is the most widely cited in the scientific literature. Romalde *et al.* (1993) produced another typing scheme for *Y. ruckeri*. Here, it was suggested that 4 O serotypes could be defined, with serotypes O1 and O2 subdivided into two and three subgroups, respectively. However, although this scheme divided serogroups based on their antigenicity no mention of biotyping was made, making it difficult to relate to current isolates

Davies (1991a) studied the relationship between O serotype, bt and OMP type and proposed a clonal complex theory for the O1 serogroup. This clonal concept of bacterial

population structures was first introduced in the 1970's for describing *Escherichia coli* strains (Ørskov *et al.* 1976). Thus, it was suggested that certain O:H serotypes represented clones which carried plasmids necessary to provoke diarrhoea. In the context of *Y. ruckeri*, two biotypes were recognised with differences in motility and the ability to hydrolyse Tween. The O-serotype is determined by agglutination of antiserum against heat stable O antigens. In comparison, the OMP-type was determined by SDS-PAGE of OMPs. Six clonal groups were identified based around character combinations of both bt and OMP type (Table 2.1). It was highlighted that only clonal groups 2 and 5 caused disease in salmonids. Clonal group 2 (O1: 2) was characteristic of 'Hagerman' like isolates from the USA, and clonal group 5 (O1: 5) was associated with bt 2 isolates from the U.K. At this point, bt 2 had only been identified in the U.K. and from Norway (single isolate).

Table 2.1. Clonal grouping of *Y. ruckeri* based around the Davies (1991b) scheme.

Clonal Group	Biotype	OMP type
1	1	1
2	2	1
3	1	2
4	2	2
5	1	3
6	1	4

In regards to ERM, the use of a bio-serotyping scheme must be used with caution as the vast majority of ERM disease outbreaks described in Europe and North America have been ascribed to strains belonging to bt 1 and serotype O1 (Lucangeli *et al.*, 2000). It is not known how these new biogroup isolates fit within the current clonal complex scheme defined by Davies (1991a). Wheeler *et al*. (2009) described differences in OMP profile between O1 bt 1 and O1 bt 2 strains. In particular, it was observed that variation in the 36.5 kDa porin protein was underexpressed in the bt 2 isolates. However, it was suggested that this could not be a distinguishing feature as laboratory conditions could change the expression of the protein.

From the information it is apparent that there is a need to examine the population structure of *Y. ruckeri* from a phenotypic point of view. Therefore the major objectives of this chapter were to:

i. To examine and compare all *Y. ruckeri* isolates from a wide range of geographical locations using standard morphological, biochemical and serological techniques. With the aim to improving standard identification methods for non-motile isolates.

ii. To analyse cell surface components, including WCP, LPS and OMP with the aim to identify cell surface features that would enable rapid identification of non-motile isolates.

iii. To relate the differences in phenotypic data, with the aim to update the current typing and clonal complex theories as defined by Davies (1991a).

2.2 Materials and methods

2.2.1. Bacterial isolates

The bacterial isolates used in study are listed in Table 2.2. Authenticity of *Y. ruckeri* was verified after Bottone *et al.* (2005). Cultures in tryptone soya broth (TSB, Oxoid) were supplemented with 15% (v/v) glycerol, and stored at -70^{o}C. Bench cultures were maintained in TSB without shaking or tryptone soya agar (TSA, Oxoid) with incubation at 22^{o}C for 48 h .

Table 2.2. Bacterial isolates used in study, indications country and date of isolation. All isolates were from rainbow trout unless stated.

Isolate		Country/Date	Comments
1	NCIMB 2194^{T}	USA	O1 'Hagerman' strain
2	NCTC 10476 (BA329)	USA	-
3	NCTC 10478 (BA19)	USA	-
4	NCTC 10976^{T}	UK	-
5	NCTC 12266	Iceland	-
6	NCTC 12266	University of Reading, UK	O2 Serogroup
7	NCTC 12268	University of Reading, UK	O5 Serogroup
8	NCTC 12269	University of Reading, UK	O6 Serogroup
9	NCTC 12270	University of Reading, UK	O7 Serogroup
10	Glennfinnes non-01	Scotland, 2003	-
11	48 non-01	Scotland, 2005	-

12	50 non-01	Scotland, 2005	-
13	MPM 04/178	France	Sturgeon
14	YR Tyre1 Atl.S	Chile, 1998	Atlantic salmon
15	16u01	France, 2006	-
16	1018 01	France, 2006	-
17	3535 01	France, 2005	-
18	5359 non 01	France, 1992	-
19	250181/2	UK, 1993	-
20	310 1/3	UK, 1993	-
21	Teppe 01 B. Trout	UK, 1993	-
22	787b. Atl. S	UK, 1993	-
23	P42/06	UK, 2003	-
24	EX5 (27)	Scotland, 2003	EX5 Isolate. Austin *et al*. (2003)
25	PR111	UK, 2003	-
26	PR1	UK, 2003	-
27	T1	UK, 2004	-
28	Brilford	UK, 2005	-
29	Trossachs	UK, 2006	-
30	FFF1	UK, 2007	-
31	IALT92	UK, 2008	-
32	IALT111	UK, 2009	-
33	RTF1B	UK, 2010	-

34	RTF 2AA	UK, 2011	-
35	GB01	Scotland, 2005	-
36	GBR11	Scotland, 2005	-
37	IALT11	UK, 2006	-
38	ITC1A	UK, 2006	-
39	GRE1A	England, 2006	-
40	GR1	England, 2006	-
41	GR2	England, 2006	-
42	RTF8A	England, 2006	-
43	RTF8B	England, 2006	-
44	RTF8C	France, 2006	-
45	TVT ITCHEN LT5	Scotland, 2004	-
46	TVT ITCHEN LT7	Scotland, 2004	-
47	TVT ITCHEN LT13	Scotland, 2004	-
48	Kinnaird 1	Scotland, 2004	-
49	Menther/rone	Ireland, 2005	-
50	YR449-4	Scotland, 2005	-
51	YR411-4	Scotland, 2005	-
52	DTF1	France, 2005	-
53	GK1	France, 2005	-
54	CFBR1/05	France, 2005	-
55	Loch Awe-LA2	UK, 2005	-
56	LA3	UK, 2005	-

57	250181/1	France, 2005	-
58	125 154	Iceland, 2006	-
59	125 928	France, 2006	-
60	M26 - France	France, 2006	-
61	FI75-05 Charr	France, 2006	Arctic charr
62	Teppe 08	France, 2006	Brown Trout
63	Teppe 10	Iceland	-
64	468	France, 2008	-
65	F175-05 Cod	UK, 2006	Cod
66	6038926 - France	UK, 2006	-
67	XAH 031.15.9	UK, 2006	-
68	XAH031.6.1	UK, 2006	-
69	Drum TFF1	France, 2006	-
70	Tres L31	Norway, 2006	-
71	Bassin 2A	Norway, 2007	-
72	VAL 50Y1 ATL. S	Scotland, 2007	Atlantic salmon
73	VAL 50Y2 Atl.S	Scotland, 2007	Atlantic salmon
74	F18/04	Scotland, 2007	-
75	K2	England, 2003	-
76	R3	England, 2003	-
77	R4	England, 2003	-
78	R5	England, 2003	-
79	R6	England, 2003	-

80	R8	Scotland, 2005	-
81	DTF2	Scotland, 2005	-
82	RTF7B	Scotland, 2005	-
83	RTF052	Scotland, 2005	-
84	TUTGB LT21	Scotland, 2008	-
85	H14 7/41	Scotland, 2008	-
86	H14 7/42	Scotland, 2008	-
87	H14 7/43	Scotland, 2008	-
88	H14 7/44	Finland	-
89	P42/07	Finland	Zander
90	XAH 02293 - uk07	Denmark, 2008	-
91	RD6	UK, 1983	Davies and Frerichs, 1989

2.2.2. Characterisation of the bacterial isolates

The isolates were examined by the Gram staining method, and viewed at x1000 magnification on a Kyowa phase contrast microscope. The Gram staining reaction and micro-morphology of the cells were recorded.

2.2.2.1. Motility

The presence of motility was determined from wet preparations using dense suspensions of bacteria (~10^8 cells ml^{-1}) in 0.9% (w/v) saline as determined using a haemocytometer slide (Improved Neubauer Type, Weber) at a magnification of x400 on a Kyowa phase contrast microscope.

2.2.2.2. API 20E

API 20E kits (BioMérieux) were inoculated with a light suspension(~ 10^6 CFU ml^{-1}) of bacteria in 0.9% (w/v) saline according to the manufacturer's instructions. After incubation for 48 h at 22^{o}C, the results were recorded.

2.2.2.3.API 50CH

API 20CH kits (BioMérieux) were inoculated with a light suspension of bacteria (~ 10^6 CFU ml^{-1}) in 0.9% (w/v) saline according to the manufacturer's instructions. After incubation for 48 h at 22^{o}C, the results were recorded.

2.2.3. Biochemical tests

2.2.3.1. Methyl red test

Isolates were grown on TSA plates. Then, single colonies were inoculated into methyl red Voges Proskauer medium (MRVP; Oxoid) and incubated at room temperature for 5 days. After incubation, 5 drops of methyl red reagent was added to the broth culture. A positive reaction was indicated by the development of a red colouration within 10 min.

2.1.3.2. Voges Proskauer reaction

Isolates were grown on TSA plates before single colonies were inoculated into MRVP medium (and incubated at room temperature for 5 days. Afterwards, 0.5 ml of 40% (v/v) KOH and 1.5 ml of α-naphthol was added to the culture. A positive reaction was indicated by the development of a red colouration within 1 h.

2.2.3.3. Sorbitol fermentation

Sorbitol fermentation was carried out in accordance with the method described in MacFaddin (1980). Briefly, a loopful of bacterial culture was inoculated into phenol red broth base (Oxoid). After 48 h incubation at room temperature, turbidity and a red coloration was recorded as evidence of positivity.

2.2.4. Degradation characteristics

2.1.4.1. Proteolytic activity

Casein hydrolysis was observed in plates made of double strength TSA mixed with an equal volume of 10% (w/v) of sterile skimmed milk. After incubation for up to 7 days, a positive response was recorded by the presence of clear zones around the bacterial growth.

2.2.4.2. Gelatin hydrolysis

Gelatin hydrolysis was examined using plates of TSA supplemented with 0.4% (w/v) gelatin (Oxoid). After incubation at 22^{o}C for 48 h, the plates were flooded with saturated ammonium sulphate (Sigma-Aldrich) solution and left for 1 h at room temperature. The presence of a clear zone surrounding the growth was indicative as a positive result.

2.2.4.3. Lipase activity

Lipase activity was examined using plates of TSA supplemented with 0.1% (v/v) Tween 20, Tween 40, Tween 60 and Tween 80 (Oxoid). After incubation at 22^{o}C for 48 h, the presence of an opaque zone surrounding the growth was indicative as a positive result.

2.2.4.4. Lecithinase activity

Lecithinase activity was demonstrated by the addition of 1% (v/v) egg yolk emulsion (Oxoid) to a peptone agar base (1.5% peptone [Oxoid], 1% agar [Oxoid], 1.5 % NaCl). A positive result was indicated by the presence of opacity surrounding colonies after 5 days incubation at room temperature.

2.2.4.5. Tributyrin agar

The medium comprised a solid medium base consisting of 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1% (w/v) agar and 0.1 ml (v/v) tributyrin. Lipase activity was

indicated by the presence of clear zones around the colonies after incubation at room temperature for 5 days.

2.2.4.6.Heat sensitive factor (HSF)

The presence of HSF was carried out according to Furones *et al.* (1993). HSF agar plates consisted of TSA supplemented with 1% (w/v) SDS (Sigma-Aldrich), 100 µg ml^{-1} of coomassie brilliant blue (Sigma-Aldrich) and 100µg ml^{-1} of congo red (Sigma-Aldrich). A blue halo surrounding the growth after 5 days at room temperature was indicative of a positive result.

2.2.5. Sensitivity to antibiotics

Antibiogrammes were performed on TSA plates spread with 200 µl volumes of the test organism (~ 10^7 CFU ml^{-1}). Thereafter, antibiotic sensitivity discs (MAST Diagnostics) were aseptically placed on the agar surface. The plates were incubated at 22^{o}C for 48 h, after which sensitivity (as indicated by the presence of a >3mm zone with no growth around the disc) profiles were recorded.

2.2.6. Growth in normal conditions

A single colony was inoculated into TSB at 22^{o}C. The optical density (OD) of the culture was determined using a UV spectrophotometer (Thermo Scientific) every 1 h at 600 nm. Growth was recorded for 14 h until stationary growth phase was observed.

2.2.7.O-antigen serotyping

Serotyping was carried out according to Davies (1990). O antigens were prepared by resuspending an overnight TSB culture in 10 ml of PBS and heating at 100^{o}C for 2 h. The cells were centrifuged at 500 x *g* for 10 min, and resuspended in fresh PBS. Then, 10 µ volumes of O antigen were mixed with equal amounts of undiluted monospecific rabbit polyclonal antiserum to the different serogroups of *Y. ruckeri* (Scottish Antibody

Production Unit) on glass microscope slides, and agglutination recorded after mixing for 30 sec.

2.1.8. Preparation of hyperimmune rabbit antisera

Formalin-killed cells were washed and resuspended in saline to an OD of 1.0 nm at 610 nm. Increasing volumes were injected intravenously into female New Zealand white rabbits as follows: day 1, 0.25 ml; day 4, 0.5 ml; day 8, 1.0 ml, and day 11, 2.0 ml. Washed live cells, adjusted to the same OD, were injected as follows: day 15, 0.5 ml; day 18, 1.0 ml; and day 22, 2.0 ml. The rabbits were bled from the marginal ear vein on day 29 and 36. The blood was allowed to clot at room temperature for 1 h and left at 4^{o}C overnight. After centrifugation at 100 x *g* for 5 min, the serum was separated, filter sterilized through 0.45 µm Millipore porosity filters, and stored at -20^{o}C.

2.2.9. Electron microscopy

Negative staining protocol

A drop of bacterial suspension was placed on to a formvar/carbon coated copper grid for 10 min before carefully blotting to remove excess liquid. The grid was then stained for 30 sec with 1% uranyl acetate (EMscope), blotted with filter paper to remove excess fluid, and air dried. The sample was viewed in a Phillips CM120 transmission electron microscope, and images captured on a Gatan Orius 1000 digital camera.

2.2.10. Whole cell preparations

Isolates were cultured in TSB at 30^{o}C for 18 h with shaking before centrifuging at 5000 x *g* for 20 min. Whole cell protein (WCP) samples were prepared by resuspending the bacteria in distilled water to an OD of 0.5 at 590 nm on a Thermo Scientific spectrophotometer. Then, 10 ml volumes were centrifuged at 5000 x *g* for 20 min. Double strength sample buffer (150 µl) (4 g SDS [Sigma-Aldrich], 20 ml glycerol [Sigma-Aldrich], 10 ml 2-mercaptoethanol [Sigma-Aldrich], 12.5 ml 1M TRIS-buffer (pH 6 8), and 20 mg bromophenol blue [Sigma-Aldrich]) per 100 ml dH_2O was added to the deposit. After boiling for 5 min, 150 µl of distilled water was added and boiling

continued for a further 5 min. The supernatant fluid collected after centrifugation at 10000 x *g* for 10 min represented WCP. Samples were stored at -20^{o}C until required.

2.2.11. Isolation of outer membrane proteins (OMP)

OMPs were isolated by following the procedure of Davies (1990) with slight modifications. Thus, OMPs were prepared by resuspending bacterial cells, cultured as above, in 5 ml of 50 mM TRIS-HCI buffer (pH 7.4) to an OD of 1.5 at 590 nm on a Thermo Scientific spectrophotometer. The suspended bacteria were lysed by sonication over ice for two one min periods at 12 µm peak-to-peak amplitude (MEC, Ultra Sonicator Unit). After centrifugation at 5000 x *g* for 10 min to remove cell debris, the OMPs were separated from the supernatant by adding 0.5 ml of 20% (w/v) sarkosyl (Sigma-Aldrich) and incubating at 22°C for 30 min. OMPs were collected by centrifugation at 50000 x *g* for 1 h at 4°C, and then resuspended in 50 µl of 50 mM TRIS-HCI buffer. Single strength sample buffer (150 µl) was added and then heated to 80^{o}C for 10 min. OMPs were separated on 4-12% SDS PAGE gels loading 15 µg protein per lane. Molecular size standards (New England Bio Laboratories) were run concurrently. Following electrophoresis, the gels were fixed and stained with coomassie brilliant blue G stain (Sigma-Aldrich).

2.2.12. Isolation of lipopolysaccharide (LPS)

LPS was isolated from cells after Romalde *et al.* (1993). Culturing was on TSA with incubation at 22^{o}C for 48 h. Cultures were suspended in PBS to an OD of 1.0 at 525 nm on a Thermo Scientific spectrophotometer. 1.5 ml of each suspension was pipetted into 1.5 ml volume Eppendorf tubes and centrifuged 3000 x *g* for 4 min. The pellets were suspended in 50 µl of Laemmli sample buffer (Sigma-Aldrich), and boiled for 10 min before the samples were centrifuged at 14000 x *g* for 10 min, and the supernatant retained. 10 µl of proteinase K (Sigma-Aldrich) was added to the supernatant with incubation at 60^{o}C for 60 min followed by re-centrifuging at 3000 x *g* for 4 min to remove any debris. The samples were then separated on 4-12% SDS-PAGE gels and silver stained according to Hitchcock and Brown (1983).

2.2.13. 1D SDS PAGE electrophoresis

One-dimensional denaturing SDS-PAGE separation of WCP, OMP's and LPS was carried out using 10–30 µl protein sample well^{-1} which were loaded onto Tris-HCl-SDS gels with 4% (v/v) polyacrylamide stacking, and 12% (v/v) polyacrylamide separating gels. Ten µl of prestained molecular-mass standards (Bio-Rad) were loaded in one lane on all gels. The resolving gel solutions (20 ml) contained 70 μl of 10% ammonium persulphate (APS; Sigma-Aldrich) and 15 μl of N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma-Aldrich), whereas stacking gels (10 ml) contained 50 µl 10% APS and 10 µl TEMED. Electrophoresis was carried out in a Mini Protean II electrophoresis chamber (Bio-Rad) for ~1.5 h at 150 V constant voltage in running buffer [12.0 g (w/v) Tris, 57.6 g (w/v) glycine (Sigma-Aldrich), 2.0 g (w/v) SDS; made up to 2.0 l with distilled water] at room temperature. After the electrophoretic separation, protein bands were visualized by staining the gel for 1 h with coomassie brilliant blue G solution followed by destaining in methanol-acetic acid-water solution (40:10:50) for 3 h. Densitometry of gels was performed with the aim of assigning relative molecular masses to the ECPs, CWPs and WCPs separated bands. Protein bands were digitally imaged using a Canon CanoScan 3000F scanner.

2.3 Results

2.3.1 Micromorphology and physiological characteristics of biotype 1 and biotype 2 isolates of *Y. ruckeri*

The characteristics of the isolates are detailed in Table 2.3. Motile and non-motile strains both produced circular, smooth, rounded, opaque colonies on TSA. Short, single rod shaped cells were visualised, which stained Gram negative. The optimum growth temperature was between 16^{o}C and 25^{o}C; although growth did occur between 4^{o}C- 37^{o}C and in 0% NaCl. Both biotypes were catalase positive and oxidase negative, and were facultative anaerobes. Biotype 2 isolates were non-motile in wet preparations and on semi solid agar (Figure 2.1).

Table 2.3. Physiological and morphological characteristics of *Y. ruckeri* biotype 1 and biotype 2.

Characteristics	Biotype 1	Biotype 2
Colony morphology		
Colour	cream	cream
Size	1-2 mm	1-2 mm
Shape	round, smooth	round, smooth
Motile	+	-
Rods	+	+
Cocci	-	-
Endospores	-	-
Capsule	-	-
Catalase	+	+
Oxidase	-	-
Fermentative	+	+

Oxidative	+	+
Gram-staining reaction	-	-
Growth at :		
4^oC		
10^oC	+	+
15^oC	+	+
25^oC	+	+
35^oC	+	+
45^oC	-	-
Growth in:		
0% (w/v) NaCl	+	+
1% (w/v) NaCl	+	+
2% (w/v) NaCl	+	+
3% (w/v) NaCl	+	+
4% (w/v) NaCl	-	-
5% (w/v) NaCl	-	-
6% (w/v) NaCl	-	-
7% (w/v) NaCl	-	-
8% (w/v) NaCl	-	-

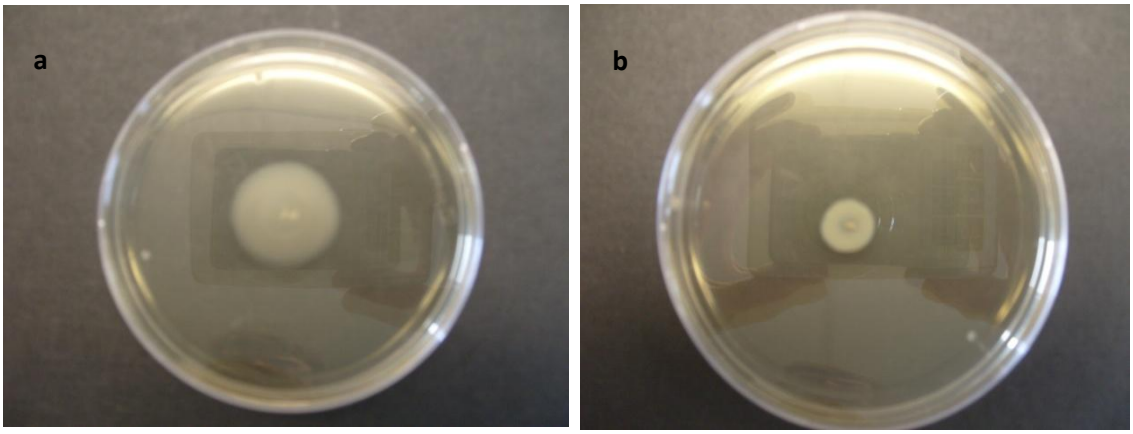


Figure 2.1. Motility biotype 1 and biotype 2 isolates of *Y. ruckeri* in semi solid agar (a, O1 motile bt 1 O1 'Hagerman' type strain NCTC 2197^{T}, (b), non-motile bt 2 EX5 isolate).

2.3.2 Transmission electron microscopy

Four *Y. ruckeri* isolates were studied using TEM of which 2 isolates were of the motile O1 Hagerman type strain and two were non-motile EX5-like cultures. Microscopy was carried out in order to detect the presence or absence of appendages, such as flagella, pili or capsules. TEM of motile isolates revealed the presence of peritrichous flagella, approximately four flagella per bacterium; broken flagella were also observed in the surrounding media (Figure 2.2). TEM of non motile isolates highlighted the lack of flagella or other appendages. The size of the non-motile organisms was slightly smaller than their motile counterparts, 0.8- 1 µm compared to 1-1.5 µm of the motile cells (Figure 2.3). Both cell shapes were similar, i.e. straight, single cells with rounded ends.

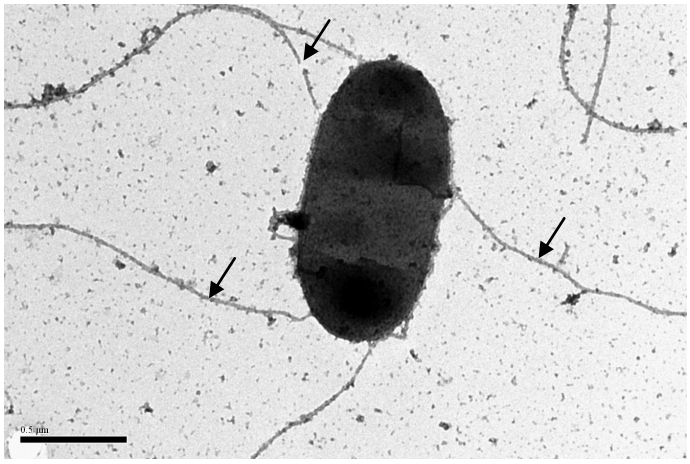


Figure 2.2. TEM of motile biotype 1 *Y. ruckeri* isolate. ← indicate flagella.

Bar = 0.5 µm.

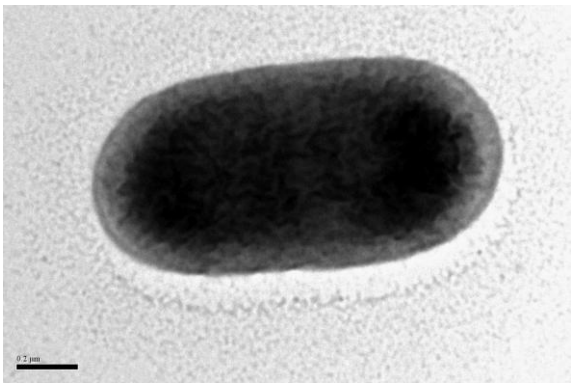


Figure 2.3. TEM of non-motile biotype 2 isolate of *Y. ruckeri*; flagella are not present.
Bar = 0.2 µm.

2.3.3. API 20E rapid identification system

The API 20E system was used for initial differentiation between serotypes and biotypes of *Y. ruckeri* (Table 2.4). Non-motile 'EX5' like bt 2 isolates differed from O1 bt 1 isolates in their ability to produce a positive reaction for gelatin hydrolysis and the VP reaction.

The relevant profiles were:

Serotype O1 bt 1	5104100
Serotype O1 bt 2	5106100
EX5 like bt 2	5107100
Serotype O2	5104100
Serotype O5	5107500
Serotype O6	5107500
Serotype O7	5104500

From the manufacturer's database, the profiles were identified as *Y. ruckeri*, with a 79.6% chance of *H. alvei*, a 14.8 % chance of *E. coli*, a 4.2 % chance of *S. marcescens* and a 1.2% chance of *V. mimicus*. The API 20E database indicated that the non-motile isolates were *Y. ruckeri*, although there was a 99.6% similarity with *H. alvei*, which is an acceptable identification according to the manufacturer.

Table 2.4. Characteristics of *Y. ruckeri* serotype O1 biotype 1 and biotype 2, EX5 biogroup, serotype O2, O5, O6, O7 using the API 20E rapid identification system.

Biochemical test	O1 Bt 1	O1 Bt 2	O1 EX5 bt 2	O2	O5	O6	O7
β-galactosidase	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+
Citrate utilisation	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-
Urease production	-	-	-	-	-	-	-

Tryptophan deaminase	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-
Voges-Proskauer reaction	-	-	+	-	+	+	-
Gelatinase production	-	+	+	-	+	+	+
Production of acid from							
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	+	+	+
Rhamnose	-	-	-	-	-	-	-
Saccharose	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-

(+ = positive positive for test, - = negative for test)

2.3.4. API 50 CH fermentation of carbohydrates

Carbohydrate fermentation (Table 2.5) was recorded after 48 h incubation. The manufacturers database gave an identification of *Y. ruckeri* (99.9% confidence) with a 0.1 % chance of *V. mimicus*. All *Y. ruckeri* isolates were able to ferment glycerol, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, N-acetyl glucosamine, maltose and trehalose. The only variable result was the fermentation of sorbitol. Motile isolates (O1, O2, O5, O6, O7) were able to ferment sorbitol, whereas biotype 2 did not.

Table 2.5. Carbohydrate fermentation patterns of *Y. ruckeri* serotype O1 biotype 1 and biotype 2, serotype O2, O5, O6, O7 using the API 50 CH identification system after 48 h.

Fermentation of:	O1 bt 1	O1 bt 2 RD6	O1 bt 2 EX5	O2	O5	O6	O7
Control	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	+
Erythitol	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-
L-Xylose	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
β Methyl-xyloside	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
L- Mannose	+	+	+	+	+	+	+
L-Sorbitol	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+
Sorbitol	+	-	-	+	+	+	+
α Methyl-D-mannoside	-	-	-	-	-	-	-
α Methyl-D-glucoside	-	-	-	-	-	-	-

N Acetyl glucosamine	+	+	+	+	+	+	+
Amygdalin	-	-	-	-	-	-	-
Arbutin	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-
Saccharose	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-	-
Amidom	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
β Gentibiose	-	-	-	-	-	-	-
D-Turanose	-	-	-	-	-	-	-
D- Lyxose	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-
D-Fucose	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-
2 ceto-gluconate	-	-	-	-	-	-	-

5 ceto-gluconate	-	-	-	-	-	-	-

(+ = positive positive for test, - = negative for test)

2.3.5. Antibiotic sensitivity

The antibiogramme data are included in Table 2.6. O1 bt 1 and O1 bt 2 isolates differed in their profile by their sensitivity or partial sensitivity to ampicillin, colistin sulphate and gentamycin. All isolates were resistant to sulphatriad and sensitive to tetracycline and cotrimoxazole.

Table 2.6. Antibiotic sensitivity of *Y. ruckeri* serotype O1 biotype 1 and biotype 2, and serotypes O2, O5, O6, O7.

Antibiotic sensitivity	Dose	O1 Bio 1	O1 Bio 2	O1 EX5 bio 2	O2	O5	O6	O7
Ampicillin	10 µg	S	S	P	S	P	P	P
Cephalothin	25 µg	R	R	R	R	R	R	R
Colistin sulphate	100 µg	S	S	S	P	S	P	S
Gentamycin	10 µg	S	P	P	S	P	P	P
Streptomycin	10 µg	P	P	P	R	R	P	R
Sulphatriad	200 µg	R	R	R	R	R	R	R
Tetracycline	25 µg	S	S	S	S	S	S	S
Cotrimoxazole	25 µg	S	S	S	S	S	S	S

(P= Partial sensitivity, R= Resistance, S = Sensitive).

2.3.6. Growth under normal conditions

The data on growth is included in Figure 2.4. The log phase was observed at 0-3 h, exponential phase from 3 h until 11 h, and stationary phase was recorded from 11 h until the end of the experiment at 24 h. All experiments were carried out using cultures from mid exponential phase (8 h).

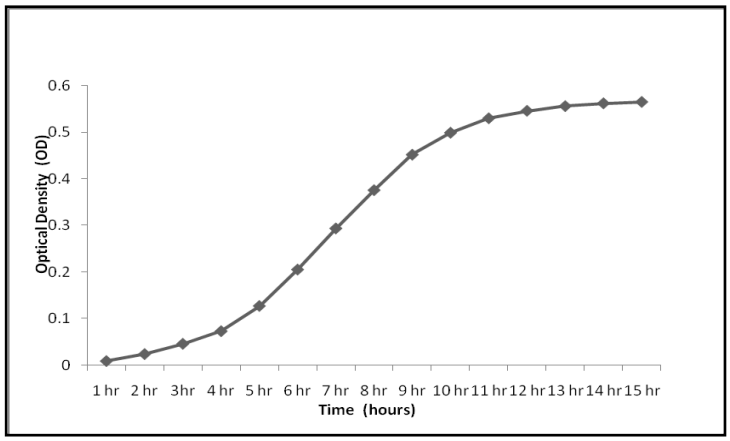


Figure 2.4. Growth curve for *Y. ruckeri* measured at 600 nm.

2.3.7. Biochemical characterisation

Biotyping was carried out on 90 isolates recovered between 1989 and 2009 using a variety of biochemical tests (MR, VP, Tween 20, Tween 40, Tween 60 and Tween 80, casein, lecithin, sorbitol, gelatin and trybutyrin) The results are included in Table 2.7. The *Y. ruckeri* isolates were recovered from 4 different salmonid species, i.e. rainbow trout from the U.K., Ireland, France, Germany, Switzerland, Italy, Finland, Denmark and Norway, Atlantic salmon from Scotland, Denmark, Chile, brown trout from France, and Arctic charr from Iceland, 1 gadoid species, cod, from Iceland, 1 Acipenseriformes, sturgeon (*Acipenser* sp.) although the species is unknown from France, and 1 *Percidae*, zander from Finland. It was noted that despite using isolates from a range of fish species and geographical locations that biochemical reactions were generally uniform. Twenty three isolates were motile, and 67 isolates were non-motile. Motile isolates were recovered from serogroups O1, O2, O5, O6, and O7. In comparison, non-motile isolates were recovered from serotype O1, O2, O5, O7. Motile isolates were recovered from rainbow trout, Atlantic salmon and sturgeon.

The biochemical reactions varied between the serogroup strains NCIMB 2194^{T} (O1), NCTC 12266 (O2), NCTC 12268 (O5), NCTC 12269 (O6), NCTC 12270 (O7) with respect to MR, VP, lecithin and the ability to ferment sorbitol. All serogroups were motile, hydrolysed Tweens, and were able to degrade casein except for the O7 isolates. All serogroup isolates were VP negative except for NCTC 10478 (O1). Serogroups O2, O5, O6 and O7 were able to ferment sorbitol. All isolates were negative on tributyrin agar. Serotypes O2, O5 and O6 had the same biochemical profiles in that they were all

motile, were MR and VP negative, hydrolysed Tween, and degraded casein and lecithin, fermented sorbitol, produced gelatinase and were negative on tributyrin agar. Serotype O7 differed from O2, O5, and O6 as this isolate was MR positive and did not degrade casein. Motile serogroups O2, O5, O6, and O7 were associated with the fermentation of sorbitol. In contrast, serotype O1 type strains were not associated with sorbitol fermentation. A number of non-motile isolates were also able to ferment sorbitol (n=7). The majority of VP positive isolate were also MR positive.

Biotype 1 and original bt 2 isolates (RD6) were differentiated on the basis of MR, VP, Tween hydrolysis and lethicinase production. Biotype 2 isolates were negative for MR, VP, Tween hydrolysis, and negative for sorbitol fermentation. Both biotypes were positive for caseinase, positive for gelatinase and negative for tributyrin degradation. Non-motile isolates collected between 1993-2009 shown differential biochemical characteristics although all were positive for casein degradation and gelatin hydrolysis. All isolates were negative on tributyrin agar apart from 1 isolate, i.e. 486, which was positive.

The majority of the isolates were MR+/VP+ (n =38). These isolates were non-motile and were isolated between 1966 and 2009 from over Europe and North America. Seventeen isolates (10, 11, 12,13,14, 18, 22, 55, 61, 65, 72, 73, 85, 86, 87, 88 and 89) were MR-/VP+; these were associated with serogroups O1, and recovered from Atlantic salmon (14), rainbow trout (10, 11, 12, 18, 22, 55, 61, 72, 73, 85, 86, 87, 88 and 89), Atlantic cod (65), and sturgeon (13) from the U.K., France, Finland Chile between 1992-2008. MR+/VP- isolates (n = 19) were associated with serotype O1 and O1 strains and also with non-motile isolates. MR-/VP- isolates (n = 5) were associated with serotypes O2, O5, O6 and the original bt 2 (RD6) isolates.

Some strains gave profiles that were different to the Hagerman O1 type strain, and bt 2 isolates. Strain 250 181/2 had a similar profile to EX5 isolates, although the isolate was motile.

Fifty nine *Y. ruckeri* isolates collected between 1993-2009 were characterised as bt 2 (non-motile and lipase negative) according to the current scheme, and were recovered from rainbow trout. Nine strains were non-motile, but positive for lipase activity (468, F175 Cod, F175 Charr, H147/41-44 Val Sal 1 and 2). Eight isolates fermented sorbitol; these isolates were recovered from Atlantic salmon in Chile and from rainbow trout in the U.K., Finland and Denmark.

Table 2.7. Biochemical characteristics of *Y. ruckeri* isolates (motility, methyl red (MR), Voges Proskauer (VP) reaction, Tween hydrolysis, caseinase, lecithinase, sorbitol, gelatinase, tributyrin and Heat Sensitive Factor (HSF)).

Isolate		Motility	MR	VP	T 20	T 40	T 60	T 80	Casein	Lecithin	Sorbitol	Gelatin	Trib	HSF
1	NCIMB 2194^{T}	+	+	-	+	+	+	+	+	-	-	+	-	-
2	NCTC 10476 (BA329)	+	+	-	+	+	+	+	+	+	-	+	-	-
3	NCTC 10478 (BA19)	+	+	+	+	+	+	+	+	+	-	+	-	+
4	NCTC 10976t	+	+	-	+	+	+	+	+	-	-	+	-	-
5	NCTC 12266	+	+	-	+	+	-	+	+	-	-	+	-	+
6	NCTC 12266	+	-	-	+	+	+	+	+	+	+	+	-	-
7	NCTC 12268	+	-	-	+	+	+	+	+	+	+	+	-	-
8	NCTC 12269	+	-	-	+	+	+	+	+	+	+	+	-	+
9	NCTC 12270	+	+	-	+	+	+	+	-	+	+	+	-	-
10	Glennfinnes non 01	+	-	+	+	+	+	+	+	+	+	+	-	-
11	48 non-01	+	-	+	+	+	+	+	+	+	+	+	-	-
12	50 non-01	+	-	+	+	+	+	+	+	+	+	+	-	-
13	MPM 04/178	+	-	+	+	+	+	+	+	+	+	+	-	-
14	YR Tyre1	+	-	+	+	+	+	+	+	+	-	+	-	-

15	16u01	+	+	-	-	-	+	+	+	-	+	+	-	+
16	1018 01	+	+	-	-	-	-	+	+	-	+	-	-	+
17	3535 01	+	+	-	-	-	-	+	+	-	+	+	-	+
18	5359 non 01	+	-	+	+	+	+	+	+	+	+	+	-	-
19	250181/2	+	+	+	-	-	-	-	+	-	-	+	-	+
20	310 1/3	+	+	-	+	+	+	+	+	+	+	+	-	+
21	Teppe 01	+	+	+	+	+	+	+	+	+	-	+	-	+
22	787b. Atl. S	+	-	+	+	+	+	+	+	+	-	+	-	-
23	P42/06	+	+	+	+	+	+	+	+	-	+	+	-	+
24	EX5 (27)	-	+	+	-	-	-	-	+	-	-	+	-	+
25	PR111	-	+	+	-	-	-	-	+	-	-	+	-	+
26	PR1	-	+	+	-	-	-	-	+	-	-	+	-	+
27	T1	-	+	+	-	-	-	-	+	-	-	+	-	+
28	Brilford	-	+	+	-	-	-	-	+	-	-	+	-	+
29	Trossachs	-	+	+	-	-	-	-	+	-	-	+	-	+
30	FFF1	-	+	+	-	-	-	-	+	-	-	+	-	+

31	IALT92	-	+	+	-	-	-	-	+	-	-	+	-	+
32	IALT111	-	+	+	-	-	-	-	+	-	-	+	-	+
33	RTF1B	-	+	+	-	-	-	-	+	-	-	+	-	+
34	RTF 2AA	-	+	+	-	-	-	-	+	-	-	+	-	+
35	GB01	-	+	+	-	-	-	-	+	-	-	+	-	+
36	GBR11	-	+	+	-	-	-	-	+	-	-	+	-	+
37	IALT11	-	+	+	-	-	-	-	+	-	-	+	-	+
38	ITC1A	-	+	-	-	-	-	-	+	-	-	+	-	+
39	GRE1A	-	+	-	-	-	-	-	+	-	-	+	-	+
40	GR1	-	+	-	-	-	-	-	+	-	-	+	-	+
41	GR2	-	+	-	-	-	-	-	+	-	-	+	-	+
42	RTF8A	-	+	-	-	-	-	-	+	-	-	+	-	+
43	RTF8B	-	+	-	-	-	-	-	+	-	-	+	-	+
44	RTF8C	-	+	-	-	-	-	-	+	-	-	+	-	+
45	TVT ITCHEN LT5	-	+	-	-	-	-	-	+	-	-	+	-	+
46	TVT ITCHEN LT7	-	+	-	-	-	-	-	+	-	-	+	-	+

47	TVT ITCHEN LT13	-	+	-	-	-	-	-	+	-	-	+	-	+
48	Kinnaird 1	-	+	+	-	-	-	-	+	-	-	+	-	+
49	Menther/rone	-	+	+	-	-	-	-	+	-	-	+	-	+
50	YR449-4	-	+	+	-	-	-	-	+	-	-	+	-	+
51	YR411-4	-	+	+	-	-	-	-	+	-	-	+	-	+
52	DTF1	-	+	+	-	-	-	-	+	-	-	+	-	+
53	GK1	-	+	+	-	-	-	-	+	-	-	+	-	+
54	CFBR1/05	-	+	+	-	-	-	-	+	-	-	+	-	+
55	Loch Awe-LA2	-	-	+	-	-	-	-	+	-	-	+	-	+
56	LA3	-	+	+	-	-	-	-	+	-	-	+	-	+
57	250181/1	-	+	+	-	-	-	-	+	-	-	+	-	+
58	125 154	-	+	-	-	-	-	-	+	-	-	+	-	+
59	125 928	-	+	-	-	-	-	-	+	-	-	+	-	+
60	M26 - France	-	+	-	-	-	-	-	+	-	-	+	-	+
61	FI75-05	-	-	+	+	+	+	+	+	+	-	+	-	+
62	Teppe 08	-	+	-	-	-	-	-	+	-	-	+	-	+

63	Teppe 10	-	+	-	-	-	-	-	+	-	-	+	-	+
64	468	-	+	+	+	+	+	+	+	+	-	+	+	+
65	F175-05	-	-	+	+	+	+	+	+	+	-	+	-	-
66	6038926	-	+	-	-	-	-	-	+	-	-	+	-	+
67	XAH 031.15.9	-	+	-	-	-	-	-	+	-	-	+	-	+
68	XAH031.6.1	-	+	+	-	-	-	-	+	-	-	+	-	+
69	Drum TFF1	-	+	+	-	-	-	-	+	-	-	+	-	+
70	Tres L31	-	+	-	-	-	-	-	+	-	-	+	-	-
71	Bassin 2A	-	+	+	-	-	-	-	+	-	-	+	-	+
72	VAL 50Y1	-	-	+	+	-	-	+	+	-	+	+	-	-
73	VAL 50Y2	-	-	+	+	-	-	+	+	-	+	+	-	-
74	F18/04	-	-	-	+	+	+	+	+	+	+	+	-	+
75	K2	-	+	-	-	-	-	-	+	-	-	+	-	+
76	R3	-	+	-	-	-	-	-	+	-	-	+	-	+
77	R4	-	+	-	-	-	-	-	+	-	-	+	-	+
78	R5	-	+	-	-	-	-	-	+	-	-	+	-	+

79	R6	-	+	-	-	-	-	-	+	-	-	+	-	+
80	R8	-	+	+	-	-	-	-	+	-	-	+	-	+
81	DTF2	-	+	+	-	-	-	-	+	-	-	+	-	+
82	RTF7B	-	+	+	-	-	-	-	+	-	-	+	-	+
83	RTF052	-	+	+	-	-	-	-	+	-	-	+	-	+
84	TUTGB LT21	-	+	+	-	-	-	-	+	-	-	+	-	+
85	H14 7/41	-	-	+	+	+	+	+	+	-	+	+	-	-
86	H14 7/42	-	-	+	+	+	+	+	+	-	+	+	-	-
87	H14 7/43	-	-	+	+	+	+	+	+	-	+	+	-	-
88	H14 7/44	-	-	+	+	+	+	+	+	-	+	+	-	-
89	P42/07	-	-	+	-	-	-	-	+	-	+	+	-	+
90	XAH 02293 - uk07	-	+	-	-	-	-	-	+	-	+	+	-	-
91	RD6	-	-	-	-	-	-	-	+	-	-	+	-	+

VP – Voges Proskauer reaction T = Tween hydrolysis, T= Tributyrin, Casein = Caseinase.

2.3.8. Grouping of non-motile isolates of *Y. ruckeri*

Eight different phenotypes were defined based around combinations of the phenotypic tests (Table 2.8). Phenotype 1 isolates (n = 42) were non-motile, VP positive, negative for Tween hydrolysis and negative for sorbitol fermentation; OMP was type 2. Phenotype 2 isolates (n = 9) were non-motile, VP negative, negative for Tween hydrolysis and negative for sorbitol fermentation, OMP was type 2. Phenotype 3 isolates (n = 3) were non-motile, VP positive, positive for Tween hydrolysis and negative for sorbitol fermentation; OMP was type 2. Phenotype 4 isolates (n = 1) were non-motile, VP positive, negative for Tween hydrolysis and negative for sorbitol fermentation; OMP was type 5. Phenotype 5 isolates (n = 2) were non-motile, VP positive, positive for Tween hydrolysis and positive for sorbitol fermentation; OMP was type 5. Phenotype 6 isolates (n = 4) were non-motile, VP positive, negative for Tween hydrolysis and negative for sorbitol fermentation. Phenotype 7 isolates (n = 1) were non-motile, VP positive, negative for Tween hydrolysis and positive for sorbitol fermentation. Phenotype 8 isolates (n = 1) were non-motile, VP positive, negative for Tween hydrolysis and positive for sorbitol fermentation. Most phenotypes were associated with salmonids (Table 2.9). However, phenotype 4 was recovered from Atlantic cod, and phenotype 5 was exclusively from Atlantic salmon. Although phenotype 1 accounted for the largest number of isolates, cultures comprising phenotype 2 were recovered 4 years earlier in 1989.

Table 2.8 Groupings of non-motile *Y. ruckeri* isolates determined by biotyping and serotyping.

Group	Motility	VP	T 20	T 80	Sor	API 20E	Sero-group	OMP	Origin	No. isolates
1	-	+	-	-	-	5107100	O1	2	UK, Italy, France, Germany, Switzerland	42
2	-	-	-	-	-	5106100	O1	2	UK, France	9
3	-	+	+	+	-	5107100	O1	2	France, Iceland	3
4	-	+	+	+	-	5107100	O1	5	Iceland	1

5	-	+	+	+	+	5107500	O5	4	Chile	2
6	-	+	+	+	+	5107500	UT	UT	UK	4
7	-	+	-	-	+	5107500	O1	O1	Finland	1
8	-	-	-	-	+	5106500	O7	2	Denmark	1

VP – Voges Proskauer reaction; UT – Untypeable, T – Tween hydrolysis, Sor – Acid from sorbitol.

Table 2.9. Year and isolation source of non-motile *Y. ruckeri* phenotypes.

Phenotype	Source of isolates	Year isolated
1	rainbow trout	1993-2009
2	rainbow trout, brown trout	1989-2009
3	rainbow trout, Arctic charr	2005-2008
4	cod	2005
5	Atlantic salmon	2007
6	rainbow trout	2008
7	rainbow trout	2005
8	rainbow trout	2008

2.3.9. HSF factor

Data for the production of HSF is included in Table 2.7. Colonies producing HSF on HSF agar is demonstrated in Figure 2.5. Serogroup O1, including bt 2, isolates were all HSF positive; only serogroup O6 (one isolate) was HSF positive. The remaining serogroups were HSF negative.

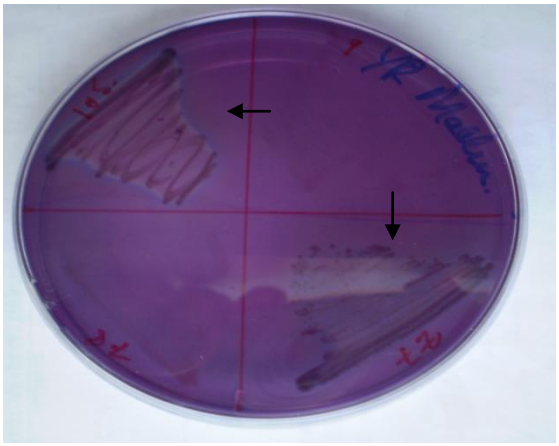


Figure 2.5. HSF agar plate demonstrating that biotype 1 and biotype 2 isolates possess HSF. The positive result is indicated by the presence of a blue halo around the colonies. (Arrows indicate positive colonies).

2.3.10. Whole cell protein (WCP) profiles

WCP profiles were analysed by 1D SDS PAGE electrophoresis, and the data presented in Figure 2.6. From this, it is clear that the WCP profiles for all *Y. ruckeri* serogroups including non-motile EX5 are homogeneous. Only O1 bt 1 and bt 2 differed slightly insofar as there was the presence of a single low molecular weight band at ~40 kDa. Serotype O2 over expressed two high molecular weight proteins of ~150 kDa and ~130 kDa.

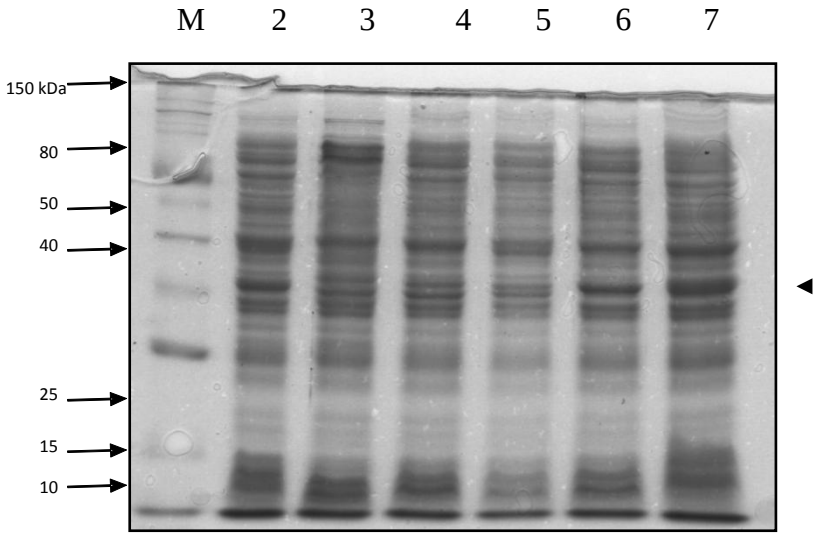


Figure 2.6. 12 % SDS-PAGE of whole cells proteins of *Y. ruckeri* isolates stained with Coomassie brilliant blue. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (EX5). Arrows indicate molecular weight (kDa). Arrow head indicate overexpressed porin protein in O1 bt 2 isolate (Lane 7).

2.3.11. Outer membrane protein (OMP) profiles

OMP profiles were analysed by 1D SDS PAGE electrophoresis (Figure 2.7). EX5 isolates (Lane 6) had a similar profile to O1 (Lane 1) isolates apart from a variation in the expression of the 36.5 kDa porin protein; bt 2 isolate (lane 7) appeared to have a similar OMP profile to that of serotype O7 (lane 6) although differing in the expression of a ~35 kDa protein.

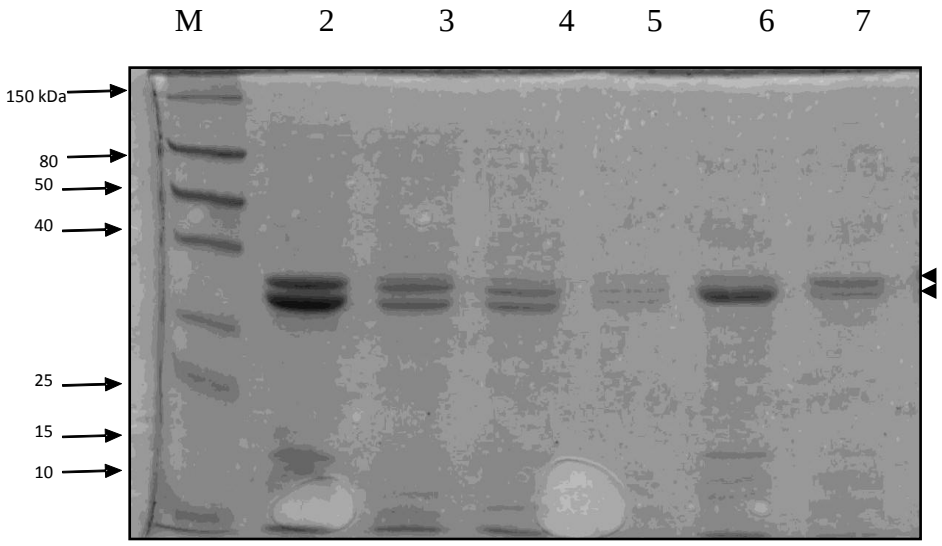


Figure 2.7. 12% SDS-PAGE of OMP profiles of *Y. ruckeri* isolates stained with Coomassie brilliant blue. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (EX5). Arrow heads indicate major OMPs used for identification different isolates of *Y. ruckeri* (Lane 7)

2.3.11.1. Outer membrane protein (OMP) profiles of non-motile *Y. ruckeri* isolates

The SDS-PAGE profiles of the OMP's revealed 10 major protein bands of 10 - 100 kDa (Figure 2.8). There was a distinct difference between the profiles of serogroup O1 Hagerman strains (OMP type 5) and bt 2 isolates (OMP type 2). The serogroup O1 bt 1 OMP profiles were used as a standard to compare all other OMP types. Thus, phenotypes 1, 2 and 3 had the same OMP type (= type 2). In contrast, phenotype 6 had a different OMP profile due to the presence of a 37 kDa protein.

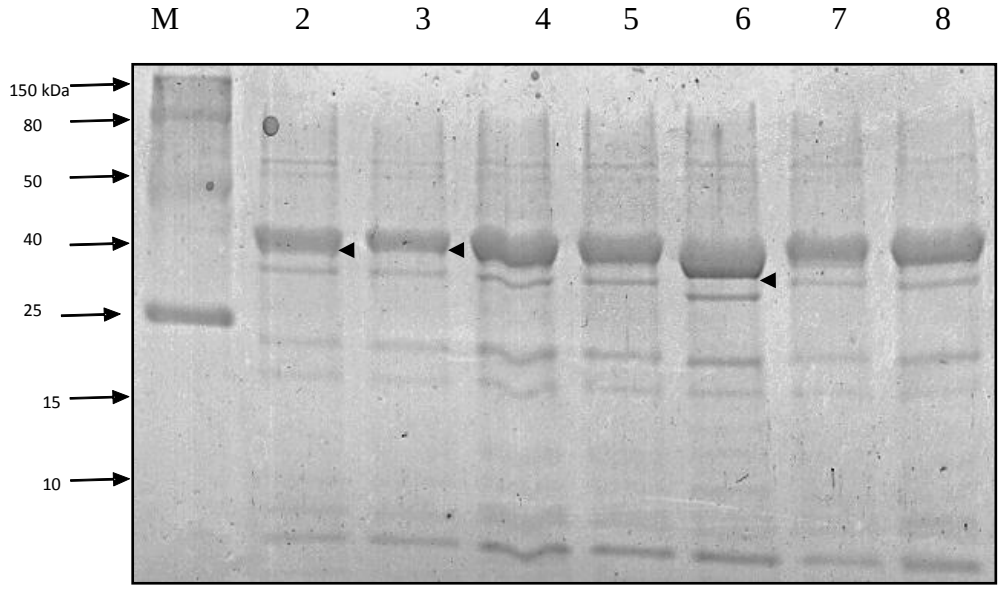


Figure 2.8. Coomassie brilliant blue stained SDS PAGE gel highlighting OMP profiles from different bt 2 phenotype of *Y. ruckeri*. Lane 1 (molecular marker), Lane 2 (O1 Hagerman); Lane 3 (phenotype 1), Lane 4 (phenotype 2), Lane 5 (R4), Lane 6 (phenotype 6), Lane 7 (phenotype 3), Lane 8 (phenotype 4). Arrow heads indicate different OMP types observed in bt 2 isolates.

2.3.12. LPS profiles

All serogroups and new non-motile biogroup gave different banding patterns (Figure 2.9). LPS profiles of *Y. ruckeri* serogroups were made up of long repeating polysaccharide units with more than 20 bands in each profile. All serotype and bt had similar LPS ladders at high molecular weight. Variation occurred within the spacing and number of bands for each serotype and bt in the low-molecular weight region (~ 15-40 kDa). Banding patterns from serotypes O5, O6, O7 showed fewer bands and spacing between bands was larger than O1 and O2 serotypes.

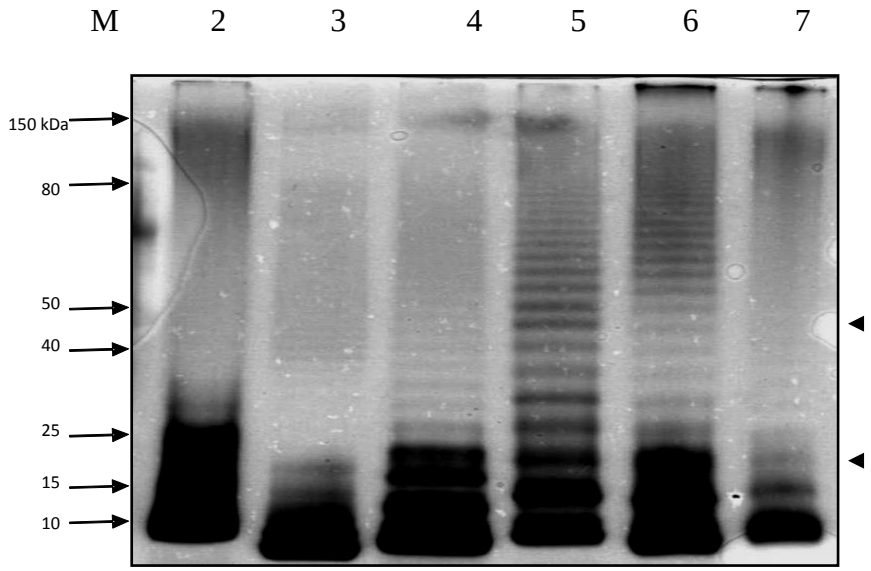


Figure 2.9. Lipopolysaccharide patterns of *Y. ruckeri* isolates in silver stained SDS-PAGE gels Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (EX5). Arrow heads indicate main regions of difference between LPS types of *Y. ruckeri.*

2.3.13. LPS profiles of biotype 2 isolates

From Figure 2.10, it is apparent that there are distinct differences between the LPS profiles of non-motile isolates. The number of bands and the space between them differed between isolates. The LPS profile for serogroup O1 bt 1 (Lane 2) and serogroup O1 bt 2 (Lanes 3, 4 and 5) differ. It was observed in bt 2 isolates, that the inter-band spacing was much smaller in and with more bands in the 20-40 kDa regions. Phenotype 6 possessed a banding pattern similar to serogroup O1 Hagerman isolates but with low molecular weight differences in banding spacing and number (Lane 2). Isolate H147/42 had a unique profile different to that of any other O1 serogroup isolates. Figure 2.11 highlights the different LPS profiles between the 8 proposed phenotypic groups. It can be observed that regardless of origin or isolation year all phenotypes of non-motile isolates had a similar LPS profile.

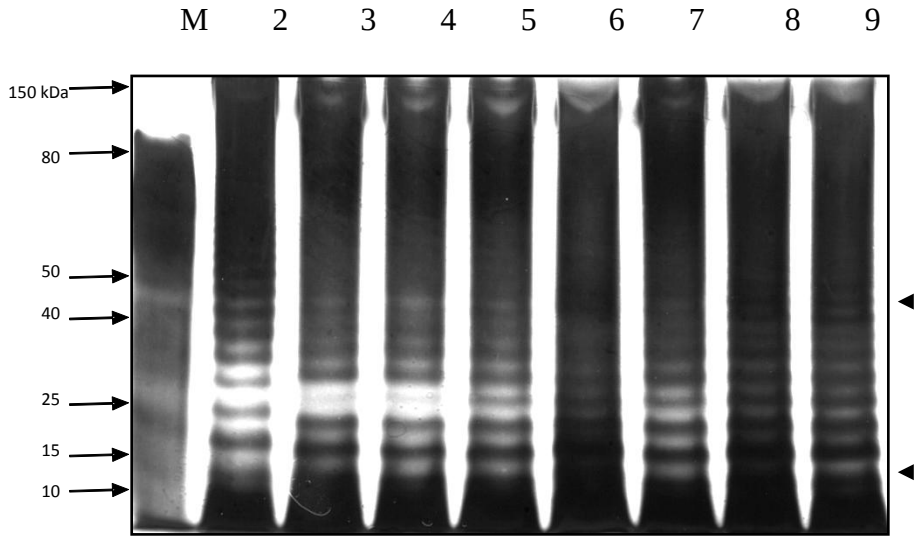


Figure 2.10. Silver stained SDS PAGE gel highlighting lipopolysaccharide patterns from different bt 2 isolates of *Y. ruckeri*. Lane 1 (molecular marker), Lane 2 (O1 Hagerman); Lane 3 (Ex5), Lane 4 (RD6), Lane 5 (R4), Lane 6 (H147/42), Lane 7 (486), Lane 8 (Arctic charr), Lane 9 (Val A. Sal). Arrow heads indicate main regions of difference between LPS types of *Y. ruckeri.*

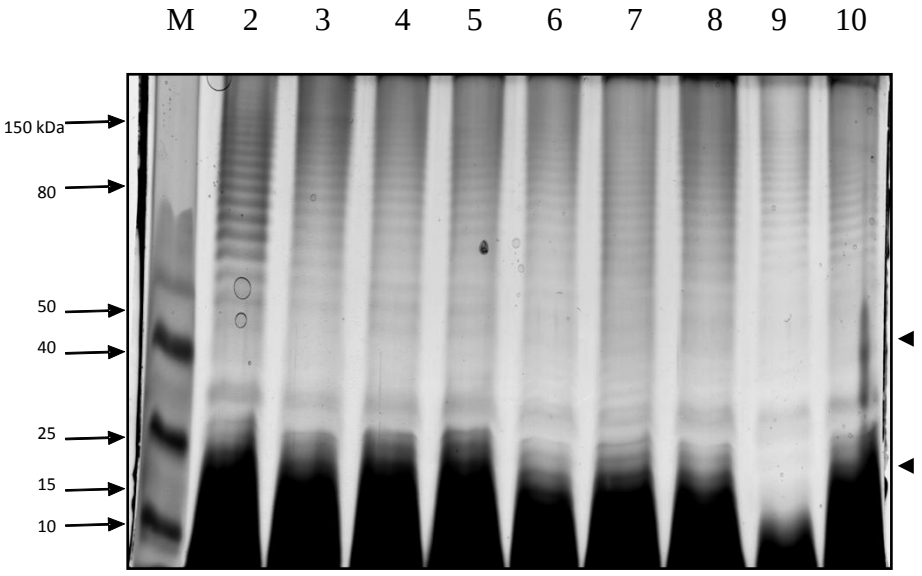


Figure 2.11. Silver stained SDS PAGE gel highlighting lipopolysaccharide patterns from different bt 2 phenotypes of *Y. ruckeri*. Lane 1 (molecular marker), Lane 2 (O1 Hagerman); Lane 3 (phenotype 1), Lane 4 (phenotype 2), Lane 5 (phenotype 3), Lane 6 (phenotype 4), Lane 7 (phenotype 5), Lane 8 (phenotype 6), Lane 9 (phenotype 7), Lane 10, (phenotype 8). Arrow heads indicate main regions of difference between LPS types of *Y. ruckeri.*

2.3.14. Serology

Data for slide agglutination for anti-O1 and anti EX5 serum is given in Table 2.10. Problems occurred with autoagglutination, and cross reactions both of which were eliminated when microplate agglutination was used. Data for microplate agglutination titre to O antigens is given in Table 2.11. Serotype O1 did not react with serotype O2 antiserum. Like with slide agglutination, cross reactions with O1 and O5 isolates were removed by the use of cross absorbed antiserum.

Table 2.10. Results of slide agglutination tests with O-antigens against rabbit antisera.

		Antiserum					
Serotypes	No. Isolates	a-O1	a-O1 bt 2	O2	O5	O6	O7
O1 bt1	22	++	++	-	-	-	-
O1 bt2	67	++	++	-	-	-	-
O2 bt1	2	-	+	+	-	-	-
O5bt 1	3	+	+	-	+	-	-
O6 bt 1	4	-	-	-	-	+	-
O7 bt 1	5	-	-	-	-	-	+

. (++ = strong reaction < 5 sec, + = reaction 30 sec, - = >5 min)

Table 2.11. Results of microplate agglutination assays with O-antigens against rabbit antisera.

		Agglutination titre [a]					
Serotypes	No. Isolates	a-O1	a-O1 bt 2	O2	O5	O6	O7
O1 M	22	640-2560	320-640	0	NT	NT	NT
O1 NM	67	320-640	640-2560	0	NT	NT	NT
O2	2	0-40	0	1024-2560	NT	NT	NT
O5	3	160-320	160-320	0-20	NT	NT	NT
O6	4	0-40	0	0	NT	NT	NT
O7	5	0-40	0	0	NT	NT	NT

([a] = ranges of agglutination reactions expressed as the reciprocal of the highest dilution showing a reaction. M= Motile, NM= Non-motile, NT = not tested)

2.4 Discussion

ERM is one of the most important diseases effecting salmonid aquaculture throughout Europe and North America (Tobback *et al*., 2007). Since the publications of the organism in the 1970's, there have been many attempts aimed at the taxonomy and intraspecific structure of the taxon (Ewing *et al*., 1978; Stevenson and Airdrie, 1984; Davies, 1990; Romalde *et al*., 1993). Since the first description of non-motile isolates in the 1980's, a few studies have focused on its taxonomic position within the species description (Davies and Frerichs, 1989). Therefore, it is of importance that any study includes non-motile isolates to facilitate better diagnosis and health management decisions. Because of the economic importance of the disease, the aquaculture industry is increasingly relying on vaccines for disease control.

TEM highlighted that bt 2 (non-motile) isolates did not possess flagella or other external structures, including pili and capsules. Motile bt O1 isolates revealed the presence of peritrichous flagella. These are often regarded as key virulence mechanisms in Gram-negative bacteria, (Ormonde *et al*., 2000). These authors highlighted that invasion of fish cell lines by *V. anguillarum* was correlated to the presence of flagella although attachment rates between motile and non-motile isolates did not differ significantly. Taking this into context, perhaps flagella are not connected with invasion or virulence of *Y. ruckeri*. Other structures, such a somatic antigens and cell surface proteins, could be necessary for attachment and invasion. Flagella are closely linked with the T3SS; however, it is unknown to what extent the type three secretion system is effected by the lack of flagella in *Y. ruckeri.* Motility by flagella is a conserved mechanism that may have evolved from the same ancestor as the T3SS (Aizawa, 2001). The presence of a flagellar apparatus in *Y. enterocolitica* has been demonstrated to deliver a virulence protein (the lipoprotein *YlpA*) into the surrounding medium *in vitro* (Young *et al.,* 1999). One suggestion is that although the presence of flagella would be energetically expensive, the proteins may play a role in another pathogenic mechanism (Aizawa, 2001). Although *C. pneumoniae* is thought to be non-motile, it has been shown to contain at least three orthologs of flagellar genes, namely *flhA, fliF, and fliI* (Peters *et al*., 2007). Flagella are key molecules for host recognition; the flagellum has been noted to induce pro-inflammatory cytokines, specifically IL-8 (Zhou *et al.,* 2003). The lack of motility due to the loss of flagella has been hypothesised to confer an advantage

to non-motile pathogens (Sallum and Chen, 2008). The lack of this component may be contributing to a reduced immune response and ultimately reduced protection against *Y. ruckeri*. A comparative study investigating the differences between the immune response towards flagellated bt 1 isolates and non- flagellated bt 2 isolates would be beneficial.

The size of bt 1 and bt 2 isolates of *Y. ruckeri* varied as confirmed by TEM. Biotype 2 isolates were observed to be slightly smaller in size (0.6-0.8 µm) compared to bt 1 isolates (1.0-1.5 µm). Gram-staining highlighted the presence of very short, coccobacilli rods regardless of serotype or source of isolation. However, it was not possible to differentiate the serotype or bt based around the shape of Gram stained cells. Austin *et al*. (1982) noted that some isolates of *Y. ruckeri* were long, thin rods as well as filamentous cells; these two cell types were not observed in this study. Variation in cell morphology as a result of age of culture was not studied.

The use of rapid identification kits, such as the API 20E rapid identification system, is often one the first steps taken in order to identify a Gram-negative bacterial pathogen. Isolates in this study were identified as *Y. ruckeri* albeit with the possibility of *H. alvei*, *E. coli* or *S. marcescens*. However, the API 20E system was initially developed for the identification of enteric pathogens of humans; therefore it is not necessarily appropriate for fish pathogens. Consequently, each test must be carried out in the most suitable environment for the bacterial pathogen in question. Incubation of the API 20E strips was carried out over a 48 h period due to the gelatinase tube being negative after 24 h in some cases. Similar findings were observed by Austin *et al.* (2003). Of relevance, previously API 20E strips have been associated with misdiagnosis of *Y. ruckeri*. Similarly, Santos *et al*. (1993) reported that the API 20E strip only positively identified *Y. ruckeri* isolates 17% of the time. However, it must be noted that the study was conducted in the 1990's and over time the database has become updated. Certainly, the API 20E strip was useful in differentiation between bt 1 O1 isolates and bt 2 O1 isolates. The results were reproducible suggesting the importance of this system for initial identification.

Using the API 50CH in conjunction with the API 20E systems gave a better identification than using the API 20E alone as isolates were consistently identified as *Y. ruckeri*. Consequently, it is argued that microbiologists should use both tests in conjunction to achieve more reliable identification of *Y. ruckeri*. Investigations using the API 50CH carbohydrate fermentation highlighted that there was a difference in profile between bt 1 and bt 2 isolates of *Y. ruckeri* with the former fermenting sorbitol. The API 50CH has been noted to be a useful tool in distinguishing between biotypes of *E. coli* pathogenic to rabbits (Okerman and Devriese, 1985). Yet, limitations of the API 50CH system have been highlighted by Boyd *et al*. (2005); only 4% of *Lactobacillus* isolates were positively identified by the system. The use of genetic tests was proposed to be superior for identifying bacteria within the same genus. Although it is difficult to distinguish between biochemically heterogeneous species of bacteria, the low diversity between *Y. ruckeri* isolates could be useful in rapid identification. It is important to note that, although useful, both the API 20E and the API 50CH test strips must be used with caution when diagnosing *Y. ruckeri* infections. This highlights the importance of using other conventional biochemical tests that are often more laborious and involve the preparation of large volumes of media. It is, therefore, recommended that conventional microbiological tests be used in conjunction with the API systems due to the discrepancy between results observed in this study. For example, it was noted that VP reactions on the API 20E tube often gave negative results. Sorbitol tests should be duplicated by conventional methods when the API 20E system is used. The variation in VP test suggests that an analysis of fermentation end-products is useful in taxonomic and epidemiological studies.

Biochemical testing is of great importance because often other techniques, such as serotyping and phage typing, only identify a specific group and do not yield information regarding variation between single groups. Biotypes within serogroups have been linked to a number of important disease outbreaks within cultured fish species (Tison *et al.*, 1982). Conventional biochemical methods were carried out on all *Y. ruckeri* isolates due to the variation in results from the API test strips.

Non-motile isolates of *Y. ruckeri* have been associated with disease in aquaculture since the 1980's. Initial reports by Davies and Frerichs (1989) suggested that they were only

associated with aquaculture in the U.K. The data collected in this report described isolates recovered from 10 different countries and the literature highlights that disease in Spanish trout farms has been attributed to bt 2 isolates of *Y. ruckeri* (Fouz *et al.,* 2006). The observed shift from motile to non-motile isolates being recovered from disease situations could be due to vaccine induced strain replacement; a concept proposed by Martcheva *et al*. (2008). As the prevalence of bt 1 isolates decreases, there is a commensurate rise in incidence of bt 2 possibly for competitive reasons, i.e. bt 2 does not have to compete with bt 1.

Conflicting reports about the biochemical nature of non-motile isolates were highlighted by Austin *et al.* (2003) from U.K. isolates, and by Arias *et al.* (2006) involving the USA. These two studies highlighted that isolates from the U.K. tended to be VP positive whereas isolates from the USA were VP negative. From the results presented in this study, it is clear that there is biochemical heterogenicity between non-motile isolates of *Y. ruckeri,* and that these factors should be considered to avoid misdiagnosis. The largest number of isolates was characteristic of the EX5 biogroup as defined by Austin *et al*. (2003). Non-motile VP negative isolates identical to that of the original Davies and Frerichs (1989) bt 2 isolates were recovered, although in much smaller numbers (n= 9) suggesting although they are still prevalent in the environment. These isolates are the same as those identified by Arias *et al.* (2006) from the USA suggesting that *Y. ruckeri* in the environment could be made up of a number of dominant phenotypes. Genetic studies looking into the phylogeny of the species would be beneficial in researching this point. In this context, it is possible that a shift in phenotypic traits between non-motile isolates has occurred. It is unknown whether VP positivity has any benefit to bacterial fish pathogens. In the case of *A. hydrophila,* it has been noted that VP positive strains produced different cytotoxic enterotoxins to their VP negative counterparts (Cumberbatch *et al.,* 1979). Here, the cytotoxins produced were linked to the cause of diarrhoea in humans. Similar mechanisms could be occurring between VP positive and negative isolates of *Y. ruckeri*. This shift in VP negativity to positivity and indeed motility to non-motility could be due to vaccine induced strain replacement.

Biotype 2 isolates were uniform in their inability to hydrolyse Tween. These observations are in accordance to other findings (Davies and Frerichs, 1989). However,

Busch (1982) noticed this previously when he mentioned that isolates were non-motile and did not hydrolyse Tween 80 although there was not any correlation between the two tests. Of relevance, Evenhuis *et al*. (2009) demonstrated that the production of lipase enzymes was associated with motility.

Although *Y. ruckeri* is most reported in rainbow trout aquaculture, the study has highlighted that other salmonids are susceptible to the condition. Thus, isolates were recovered from rainbow trout, brown trout, Atlantic salmon and Arctic charr. The occurrence of *Y. ruckeri* in a wide range of fish species could suggest an extension of the host range. The presence of the organism may not always be linked to disease, being instead indicative of an asymptomatic carrier state. In this connection, Busch and Lingg (1975) demonstrated that ~25% of rainbow trout still carried *Y. ruckeri* in the lower intestine 45 days after surviving initial infection. If survivors are transported to other locations then the possibility of spread of the pathogen is increased. It is interesting to note that *Y. ruckeri* has been demonstrated to form biofilms (Coquet *et al*., 2002). These biofilms could form a reservoir of infection.

The fermentation of sorbitol by *Y. ruckeri* isolates was associated with serogroups O2 - O7, although a small number of O1 isolates (n=4) from Finland, Norway, Canada and France (Davies and Frerichs, 1989). Sorbitol fermenting isolates were only found in a small number of isolates (n =3) from the U.K. and Finland. These results are in agreement with previous findings that O1 serogroups isolates do not ferment sorbitol (O' Leary *et al*., 1977). Michel *et al*. (1986) isolated a sorbitol-fermenting serotype O1 isolate in France from imported bait fish from the USA. Previously *Y. ruckeri* strains were biotyped on the basis of fermentation of sorbitol. This method of biotyping was abandoned when the Davies (1989) scheme was introduced. Moreover, sorbitol fermentation was also attributed to disease outbreaks caused by *Y. ruckeri* in chinook salmon and brook trout (Cipriano *et al.*, 1986). From an epidemiological perspective, extending the biotyping scheme to include both sorbitol fermentation and VP reactions would reduce confusion during identification and help build an accurate view of presence of *Y. ruckeri* in Europe.

Although the biochemical reactions were able to differentiate between isolates of *Y. ruckeri,* often these are time consuming and misdiagnosis can occur. Serology was considered to be a suitable approach for rapid and accurate diagnoses. Serological reactions were clearly invaluable in positively identifying isolates from the different serogroups. For this, O antigens were used because of the problems that have been recorded previously with cross reactions with whole cell antigens (Toranzo *et al.,* 1987). This team eliminated cross reactions by using somatic O antigens. Also, Pyle and Schill (1985) used O antigens and did not record any cross agglutination. The results described by these authors, together with the results of the current study, highlight that O antigens are effective molecules in the typing of *Y. ruckeri* isolates. Regardless of bt, both motile and non-motile isolates from serotype O1 reacted with both antisera. Analysis using microplate agglutination allowed for the comparison between isolates from different serotype. Whereas bt 2 was responsible for the majority of ERM cases throughout Europe, it was difficult to distinguish between the two biotypes using serology alone due to the similarity in agglutination titres.

Although there are biochemical differences between isolates and serogroups, analysis of WCP profiles revealed a homologous pattern. Other studies highlighted the use of this technique to distinguish *Enterococcus*, although it was not possible to distinguish between isolates of the same species (Merquior *et al*., 1994).

The surface of a bacterium is the site in which interactions occur with the host. From the results using SDS PAGE for the separation of OMPs, it is clear that there are 5 main OMP groups that *Y. ruckeri* can be grouped. Biotype 1 and 2 isolates gave the same profile although there was some difference in protein expression. Biotype 2 isolates had a underexpressed porin protein at ~36 kDa. It is well known that most bacterial pathogens are able to change their proteins and ECP expression under different environmental conditions (Crosa and Hodges, 1981). Key factors, such as temperature, pH and salinity, have been previously shown to influence the way in which bacteria grow and how this is linked to the expression of their proteins and related virulence factors (Cheng and Chen, 1999). Although it is unknown to what extent the expression of certain proteins is correlated to virulence in *Y. ruckeri*, it has been demonstrated that virulent strains of *R. salmoninarum* significantly overexpressed the p57 antigen

compared to avirulent cultures. Similar findings were also observed by Wheeler *et al*. (2009). Porin proteins are located in the outer membrane and form a channel to allow molecules to pass from the inner membrane to the outer membrane by passive diffusion (Nguyen *et al.,* 2006). Porins can be potent inducers of the immune system and are closely related to LPS structure (Wilson *et al*., 2001). A 28 kDa porin protein was purified from *A. salmonicida* by Lutwyche *et al*. (1995), who observed that this protein bore similarities with other *Aeromonas* sp. although did not cross react with other bacterial species by western blot. This suggests that the structure is unique to bacterial species, and could be a potential target vaccine antigen. Indeed in the same paper it was observed that vaccination with the protein provided protection against disease.

The analysis of LPS using SDS PAGE and silver staining showed that *Y. ruckeri* possesses a smooth LPS structure with long repeating polysaccharide O side chains, which is similar to other members of the Enterobacteriaceae (Perez-Perez *et al.,* 1986). However, the analysis of the LPS structure indicates that there are differences between serogroups and biotypes. The differences in frequency and spacing of bands in the bt 2 profile highlight that the O antigen is different from that of bt 1. It is possible to subdivide serogroup O1 into O1a and O1b as has previously been done with *V. anguillarum* (Grisez and Ollevier, 1995). These differences were observed by Romalde *et al.* (1993) although no mention was given to the bt/biochemical nature of the isolates. To accurately split the serogroup into parts O1a and O1b, the antigenic nature of the LPS using western blotting must be carried out. On this basis, it could by hypothesized that the differences in the LPS profile might have an effect over the virulence of the pathogen or the immune response of the host towards this molecule. In mammalian diseases, rough LPS phenotypes of *Brucella* are internalised by leukocytes and stimulate a higher amount of chemokines and pro inflammatory cytokines (Rittig *et al*., 2003). Not all the *Yersinia* possess smooth LPS, *Y. pestis*, which is the causal agent of bubonic and pneumonic plague, is unusual when compared with other pathogens of the Enterobacteriaceae in that it does not possess smooth LPS. Instead, its outer membrane is composed mainly of lipo-oligosaccharide (LOS) possibly reflecting its lifestyle as a pathogen transmitted by an arthropod vector (Oyston *et al*., 2003).

It was regarded by Reeves (1995) that the O antigen is extremely variable in Gram-negative bacteria. Although there is potential for using LPS profiling for a typing scheme, misdiagnosis and culture conditions could affect the LPS profile. Culture conditions have been known to change the structure of LPS and, therefore, could alter antigenic characteristics which could lead to misdiagnosis. Kawaoka *et al*. (1983) noted that cultures of *Y. enterocolitica* grown at 37^{o}C, as compared with 25^{o}C, expressed dramatically different LPS structures. It is certainly possible that the differences in composition of the LPS structure could explain the differences in virulence and protection amongst isolates of *Y. ruckeri*.

Analysis of the repeating O polysaccharide side chains indicated that there was a difference in frequency and spacing of bands from different serogroups and biotypes. Hitchcock and Brown (1986) demonstrated that differences in LPS profile are due to differences in biochemical composition of the molecule, superficially the polysaccharide component. Aussel *et al*. (2000) proved this by highlighting that the lipid component of the LPS from *Y. ruckeri* serotypes O1 and O2 was identical and that this lipid component has similarities with *Y. enterocolitica* O:11,23 and O:11,24. Differences in the antigenic structure or immunogenic structure of these LPS molecules could be due to differences in the polysaccharide side chains. Zamze and Morton (1987) demonstrated that different serotypes of *Haemophilus influenzae* had quantitative differences in the chemical composition of their LPS, which correlated with their antigenic specificity and their mobility in polyacrylamide gels.

There is considerable evidence from the biochemical and cell surface characteristics to suggest that the current clonal complex theory as put forward by Davies (1991a) is expanding. By extending the biotyping scheme to include both VP and sorbitol tests in conjunction with OMP profiles, eight phenotypic groups could be produced. The clonal concept of bacterial population structures was first introduced in the 1970's for describing *E. coli* strains (Ørskov *et al.,* 1976). It was suggested that certain O :H serotypes represent clones which carried plasmids which were necessary to provoke diarrhoea. The combination of biotyping, OMP typing and serotyping allowed the construction of a similar clonal group theory for *Y. ruckeri* (Davies, 1991a). Previously, bt 2 isolates from the UK and Europe were associated with OMP type 2 serogroup O1

which placed them into clonal group 2. Davies (1991a) highlighted that disease outbreaks were only associated with O1 clonal group 2 and 5; this study provides evidence that this is not the case. From the data presented here, it is clear that this clonal complex scheme is in need of revision, as bt 2 can be observed among other OMP and serological types, therefore highlighting the existence of new clonal groups. Previously only clonal group 2 and 5 were associated within disease in fish, the data presented that this is not the cases. From the serotyping and biochemical analysis it is clear that bt 2 isolates may be associated with serogroups other than O1. In context, it was noted by Davies (1991a) that non-motile, lipase negative isolates were mostly found in serogroup O1, although a single bt 2 isolate was recovered in serogroup O2. The data presented here suggests that although the majority of non-motile isolates belonged to serogroup O1, a number of strains were recovered in serogroup O5 and O7, with four isolates being untypeable. Indeed, the untypeable isolates (Phenotype 6) expressed a unique OMP profile. Certainly, it is reasoned that isolates, which possess a different OMP type, should be considered as a new clonal group. If these isolates are to be included within the Davies (1991a) scheme, then the clonal groups should be altered accordingly. Sorbitol fermentation was found to be a characteristic of serogroup O1 bt 1, OMP type 4, clonal group 6 isolates as based on the Davies (1991a) scheme. The sorbitol fermenters found in this study do not fit into this grouping as they are clearly bt 2 and have a different OMP profile, and would therefore be regarded as a new clonal group. There is a case based around the evidence in this article for excluding OMP from the typing scheme and diagnostic procedures. In particular, OMP profiling is time consuming, and expression of these proteins is subject to change to due culture conditions, and although the technique is easily replicated in laboratories, genetic studies such as conducted by Wheeler *et al.* (2009) using PFGE have provided better insights into the relatedness between *Y. ruckeri* isolates.

In summary, the results from this chapter suggest that there are novel biogroups arising within non-motile isolates of *Y. ruckeri*. Original isolates were VP negative and now VP positive isolates seem to be the most widely encountered form of the bacterium. By incorporating sorbitol and VP reactions of diagnostic tests, eight different phenotypes can be distinguished. These observations have demonstrated that the clonal complex theory needs to be expanded. For accurate identification a combination of serotyping,

API 20E and conventional microbiological methods must be used in order to differentiate between bt 1 and bt 2 isolates. The O antigen variation between bt 1 and bt 2 highlights possible antigenic differences. Further work is needed in order to understand the antigenic characteristics in terms of vaccination and immunological consequences of these non-motile *Y. ruckeri* isolates. This variation could allow the subdivision of the Davies (1990) typing scheme into groups O1a and O1b. Genotypic information regarding the phylogeny of the species would be useful understanding the relatedness of serotypes, clonal groups and the evolution of biotype 2 isolates.

Chapter 3. The phylogeny and population structure of selected *Yersinia ruckeri* isolates as defined by Multi-locus sequence typing (MLST)

3.1. Introduction

The nature of phenotypic and serological methods used in bacterial identification makes it difficult to understand phylogeny and population structure of bacterial species. Technological advances in molecular diagnostics have led to greater understanding of bacterial taxonomy. Molecular typing has become a rapid and simple procedure whereas previously these kinds of techniques were complex and time consuming (Towner and Cockayne, 1993). With regards to the clonal complex scheme developed in the 1990's, phenotypic and serological markers make it difficult to relate outbreaks of disease with a potential source because phenotypic traits are inconsistently expressed. The genes that control these antigens are subject to strong diversifying selection by the host immune system and therefore evolve rapidly. Moreover, recombinational events can alter antigenicity, making serological data difficult to interpret at a population level (Spratt and Maiden, 1999).

Unambiguous genotyping systems are key to describing epidemiological and ecological patterns and describing the evolutionary processes that shape microbial populations (Margos *et al.*, 2008). Although DNA:DNA hybridisation has been regarded as the 'gold standard' in defining bacterial species the technically challenging, labour-intensive method has allowed for the development of other techniques for studying bacterial population dynamics. (Mehlen *et al*., 2004; Martens *et al*., 2008). MLST was developed in 1998 (Maiden *et al.,* 1998). Previous studies employed multilocus enzyme electrophoresis (MLEE) to identify lineages within bacterial populations, and use was made of the technique for epidemiological studies. The study by Schill *et al*. (1984) using MLEE highlighted a considerable genetic homogeneity between *Y. ruckeri* isolates. However, as typing schemes have been subsequently developed or refined, it is timely for an update. The main problem with MLEE and other DNA amplification methods is that it is difficult to compare results between laboratories. Recently, Wheeler *et al.* (2009) investigated *Y. ruckeri* isolates throughout Europe using PFGE. The study highlighted that the non-motile isolates differed in pulsotype than the traditional Hagerman strains and other bt 2 strains suggesting separated ancestral origins.

The MLEE method of taking housekeeping enzymes from each strain was adopted and developed in MLST. PCR was used to amplify 450 bp to 500 bp regions from each gene, and the gene fragments were sequenced and compared (Maiden *et al.* 1998). Housekeeping genes are generally selected for MLST as they code for essential proteins and therefore are present in all strains without insertions or deletions (which could cause alignment difficulties). Maiden *et al*. (1998) compared data from MLEE with those from MLST in the case of *Neisseria meningitidis*. It was determined that the resolution using the sequence data from six alleles was equivalent to using twelve restriction enzymes in MLEE. Any nucleotide polymorphism in a MLST scheme produces a new sequence type. However in an MLEE scheme ~26 nucleotide changes are needed to produce a new electrophoretic type (Boyd *et al.,* 1994). The use of seven genes has been deemed optimum for a MLST scheme, although other studies have obtained good resolution using fewer genes (Kotetishvili *et al.,* 2005). The data from a MLST scheme is easily transferable between laboratories; the first MLST database was set up for meningococcal infections. The data from MLST studies have been found to be suitable for the study of population structures and the examination of the recombination by comparing phylogenetic tress for each allele and sequence type. Any population with enough nucleotide changes to generate different alleles and sequence types could be typed by MLST (Maiden *et al.* 1998). Recently, Kotetishvili *et al.* (2005) using multilocus sequence typing stated that *Y. ruckeri* was the most genetically distant species within the group, and that taxonomic status needs to be reassessed. *Y. ruckeri* is a highly clonal and biochemically homogeneous species (Schill *et al.,* 1984; Wheeler *et al.,* 2009).

The MLST databases are available at http://pubmlst.org. The largest collection of isolates is represented by *N. meningitidis,* which has ~7800 strains. Recent developments to help organise large sample sizes include ‘eBurst’ software, which uses the allelic profile obtained for each isolate, and divides the data into clonal complexes made up of isolates which share identity at six or seven alleles with at least one other member of the group. The founder of the complex is defined as the genotype that differs from the highest number of other genotypes in the clonal complex at only one locus out of seven.

It is clear that from the literature there is need for a thorough study into the genetic makeup of the *Yersinia ruckeri* species. The main objectives of this study were:

i. To develop a MLST scheme for *Y. ruckeri* in order to clarify the phylogenetic structure of the species.

ii. To develop a robust and reproducible typing scheme for the species.

iii. To provide some explanation into the increased incidence of biotype 2 isolates in aquaculture over the past 20 years.

3.2 Material and methods

3.2.1. Bacterial isolates

Culture of bacterial isolates is demonstrated in chapter 2 section 2.2.1. Thirty one random isolates were used for analysis by MLST.

3.2.2. Strain collection

A total of 31 isolates representing 5 host fish species, 10 countries and different biochemical traits were used in the study (Table 3.1).

Table 3.1. *Y. ruckeri* isolates used for MLST analysis.

Isolate No.	Id	Fish Species	Country/Date	Comments
2	NCTC 10476 O1	-	USA	O1 'Hagerman' strain
6	NCTC 12266 O2	-	University of Reading, UK	O2 serogroup
7	NCTC 12268 O5	-	University of Reading, UK	O5 Serogroup
8	NCTC 12269 O6	-	University of Reading, UK	O6 Serogroup
9	NCTC 12270 O7	-	University of Reading, UK	O7 Serogroup
10	Glennfinnes non01	RT	Scotland, 2003	-
11	48 non-01	RT	Scotland, 2005	-
18	MPM 04/184	S	France	SPA
21	YR Tyre1 Atl.S	AS	Chile, 1998	-
29	PR1 'EX5'	RT	UK, 1993	O1 bt 2 ‘EX5’
36	T1 (30)	RT	UK, 2005	O1 bt 2 ‘EX5’
38	RTF8A	RT	UK, 2007	O1 bt 2 ‘EX5’
43	RTF8B	RT	England, 2006	O1 bt 2 ‘EX5’
45	RTF8C	RT	England, 2006	O1 bt 2 ‘EX5’
46	TVT ITCHEN LT5	RT	England, 2006	O1 bt 2 VP - ve

55	YR411-4	RT	Scotland, 2003	O1 bt 2 'EX5'
56	DTF1	RT	France, 2008	O1 bt 2 'EX5'
63	LA3	RT	Scotland, 2007	O1 bt 2 'EX5'
65	250181/2	RT	Scotland, 2004	O1 bt 1 'Lipase -ve
72	Teppe 08	RT	Scotland, 2004	O1 bt 2 VP -ve
78	F175-05	C	Iceland	O1, Non-motile, Lipase +ve
80	468	RT	France, 2005	O1 bt 2 p
103	TUTGB LT21	RT	France, 2006	O1 bt 2 'EX5'
Ger2	Germany2	RT	Germany, 2009	O1 bt 2 'EX5'
It2	It2	RT	Italy, 2009	O1 bt 2 'EX5'
IT4	IT4	RT	Italy, 2009	O1 bt 2 'EX5'
Sw1	Sw1	RT	Switzerland, 2009	O1 bt 2 'EX5'
SW2	SW2	RT	Switzerland, 2009	O1 bt 2 'EX5'
P18	P18	AS	Norway	Motile, serotype O5
P42/06	F06	Zander	Finland	Motile, VP +ve
P42/07	F07	RT	Denmark	O1 bt 2 Sorbitol +ve
ATCC 29473^{T}	-	RT	USA	Chen *et al*., 2010

(RT = rainbow trout, S = sturgeon, AS = Atlantic salmon , C = cod, Z = zander).

3.2.3. Total-DNA extraction

Bacterial isolates were grown in TSB for 24 h at 25^{o}C. DNA was extracted using QIAGEN DNEASY (Qiagen) extraction kit according to manufacturer's instructions.

3.2.4. DNA sequencing and sequence analysis

PCR amplification and sequencing of the six housekeeping genes *AroA, gnlA, gyrB, HSP60, recA* and *thrA,* was performed using the following oligonucleotide primers using varying annealing temperatures (Table 3.2). Each 50 µl reaction contained 0.1

pmol forward primer, 0.1 pmol reverse primer, 0.2 5mM dNTP's, 2.0 mM $MgCl_2$, 0.3 µl Bioline *Taq* polymerase (Bioline). PCR was carried out with a Veriti 96 well thermocycler (Applied Biosystems) under the following conditions: 94^{o}C for 5 min, followed by 94^{o}C 45 sec, followed by 35 cycles of 45 sec at varying annealing temperatures then 1 min at 72^{o}C and final extension at 72^{o}C for 5 min (Table 3.2). Five µl PCR product was mixed with 2.5 µl gel loading buffer (Bioline). Six µl of 100 bp ladder (Gene rule, Fermentas) were loaded onto a 1% agarose gel (0.5M TAE, 1µl ethidium bromide). Gels were run at 60 V for 1.5 h. Bands were visualised under UV light.

Table 3.2. Primers used for MLST analysis.

Locus	Annealing temp	Forward primer (5'-3')	Reverse primer (5'-3')
AroA	58^{o}C	ACGTTTACGTGTGGTTTCC	GGCAAATAATGCCGTAGTCG
gyrB	51^{o}C	CGGCGGTTTGCAGGGTGG	CAGGTCGGTCATGCCG
glnA	69^{o}C	CGATTGGTGGCTGGAAAGGC	TTGGTCATGTTTGAAGCG
HSP60	58^{o}C	GACGTGTAGAAGGTATGAG	CGCCGCCAGCCAGTTTAG
recA	58^{o}C	GGGCCAAATTGAAAACATT	CGCCATTCATCGATG
thrA	51^{o}C	CAGATCAGTTTGGTCC	GCTTTTGTTGGCGTAC

3.2.5. Gel electrophoresis

A 1.5% gel was made using 1.05 g agarose (Bioline); 70 ml Tris-Boric-EDTA (TBA) buffer and 0.7 µl of ethidium bromide was added to the gel when in liquid form, whilst wearing protective clothing, using a sterile pipette tip. Molecular size marker 1 kp and 100 bp were used (Gene rule, Fermentas). Samples (15 µl) were mixed with 4 µl of loading buffer (Bioline) and pipetted into the wells of the gel. The same TBA buffer as used in the agarose gel was used as the buffer and was poured over the gel until covered. The gel was electrophoresed at 70 v for 90 min.

3.2.6. DNA purification

Gene fragments of around 400-600 bp were obtained by PCR for each gene. The PCR products were purified by PEG_{8000} precipitation to remove any unreacted primers and

dNTP's from the solution. Forty µl PCR product was mixed with 50 µl of PEG_{8000} (Sigma-Aldrich) in 1.5 ml Eppendorf tubes and kept overnight at 4°C. The tubes were centrifuged at 13000 (10,000 x *g*) rpm for 20 min, the supernatant was removed and 500 µl of ice cold 70% ethanol was added to the tube and centrifuged for 10 min at 13000 rpm (10,000 x *g*). The previous step was repeated, supernatant was removed and the tubes were placed into a heating block at 87.5°C for 1.5 min to dry. Once the tube was cool, 40 µl of Millipore d H_20 was added to resuspened the DNA pellet. The tubes were left overnight at 4°C to allow resuspension to occur. To check the quality of the purification, 5 µl of purified product was mixed with 2.5 µl gel loading buffer (Bioline), and loaded onto a 1% agarose gel (0.5 M TAE, 1 µl ethidium bromide). Gels were run at 60 V for 1.5 h. Bands were visualised under UV light. Quantity of DNA was compared to standard from 100 bp marker (Gene rule, Fermentas).

3.2.7. Processing of sequence data

Sequencing of the gene fragments obtained from PCR was carried by MWG-EUROFINS. The reading of trace files and contigs was performed using the 'STADEN' software package. Forward and reverse sequences were obtained for each primer set and assembled using STADEN software to provide unambiguous sequence over the required length of the allele. (The resulting DNA sequences were trimmed by removing low quality nucleotide sequences from the ends) The consensus sequence was saved and compared with existing alleles. If there were any base changes at all, the sequence was given a new allele number. Allele numbers were added to the MLST database, each completed strain had 6 allele numbers, forming its allelic profile, or sequence type (ST).

3.2.8. Analysis of sequence data

Concatenated sequences were aligned using Clustal W. Neighbour-joining tree construction was performed using concatenated sequences of loci sequenced in Clustal W. Treeviw was used in order to visualise concatenated sequence data. The START 2 (sequence type analysis and recombinational test) program was used to determine the number of polymorphic sties between isolates. eBurst was carried out in order to identify clonal complexes.

Several computer programs were used in the analysis of the sequence data:-

'Clustal W'

Available on-line at the European Bioinformatics Institute.

(http://www.ebi.ac.uk/Tools/clustalw2/index.html)

ClustalW2 is a general purpose multiple sequence alignment program for DNA or proteins. A multiple alignment is built up heuristically by a series of pairwise alignments. The most closely related sequences are aligned first and the most distant ones added. Clustal W uses the neighbour joining method (NJ) for tree construction. In addition to producing alignments, the Clustalx web site also provides a tree file, with a .dnd extension, which can be opened with Treeview.

'Treeview'

(http://taxonomy.zoology.gla.ac.uk/rod/treeview.html)

Treeview enables the results of the multiple sequence alignment to be viewed as a phylogenetic tree and can be represented unrooted or rooted. Tree visualized by Treeview can be saved as a .wmf or .emf file and be inserted into Powerpoint or Word.

'START' (Sequence Type analysis and Recombinational Test) (Jolley *et al.* 2001).

(http://pubmlst.org/software/analysis/start2/)

The 'START' program includes techniques for data summary, polymorphisms, G + C content, lineage assignment, and selection. The sequences of the alleles, in FASTA format and the allelic profile are entered directly from an excel file.

'**EMBOSS Transeq**'

(http://www.ebi.ac.uk/Tools/emboss/transeq/index.html)

Transeq translates nucleic acid sequences to the corresponding peptide sequence.

'**SAS'** Sequence Annotated by Structure

(http://www.ebi.ac.uk/thornton-srv/databases/sas/)

SAS is a tool for applying structural information to a given protein sequence. It uses FASTA to scan a given protein sequence against all the proteins of known 3D structure in the Protein Data Bank (PDB).

'eBURST' version 3

(http://eburst.mlst.net/)

The **BURST** algorithm first identifies mutually exclusive groups of related genotypes in the population (typically a MLST database), and attempts to identify the founding genotype of each group. The algorithm then predicts the descent from the predicted founding genotype to the other genotypes in the group, displaying the output as a radial diagram, centred on the predicted founding genotype**.** The eBURST algorithm is implemented as a Java applet at 'http://eburst.mlst.net', and detailed guidance in its use is available at this website.

3.3. Results

3.2.1. Choice of alleles

Six loci, *aroA, glnA, gyrB, HSP60, recA, thrA,* were derived from the sequences described by Kotetishvili *et al*. (2005). These 7 genes were distributed evenly around the chromosome of *Y. pseudotuberculosis* genome, as shown below (Figure. 3.1).

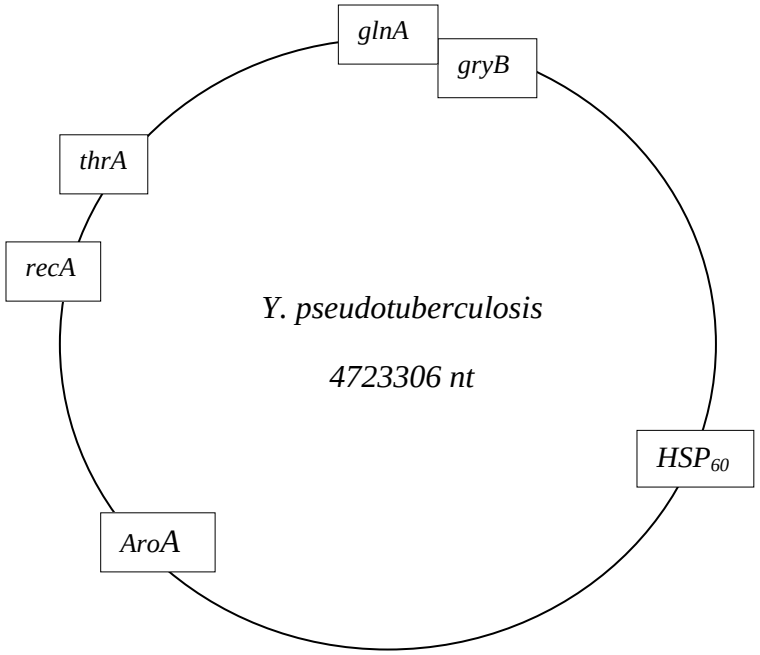


Figure 3.1. Position of alleles in *Y. pestis* chromosome.

3.2.2. Protein function

The function of the protein coded for by the MLST alleles was investigated using on-line databases available through Pubmed with links to Prosite. The sequences for the complete genes were derived from the whole genome of the type strain of *Y. ruckeri* ATCC 29473^{T} (Chen *et al*., 2010) and used in BLAST searches.

AroA (3-phosphoshikimate-1-carboxyvinyltransferase) catalyses the sixth step in the biosynthesis from chorismate of the aromatic amino acids (the shikimate pathway) in bacteria (gene *aroA*), plants and fungi (where it is part of a multifunctional enzyme which catalyses five consecutive steps in this pathway).

glnA (glutamine synthetase) plays a role in the nitrogen metabolism of bacteria, glutamine synthetase (GS) plays a central role, as it catalyzes one of the main reactions by which ammonia is assimilated L-glutamate + NH4' + ATP → L-glutamine + ADP + Pi. (Jansson *et al*., 1970).

gyrB (DNA gyrase B subunit) DNA gyrase regulates supercoiling of double-stranded DNA. It is necessary for DNA replication, and the enzyme is distributed universally among bacterial species (McMacken *et al.,* 1987).

HSP$_{60}$ (Heat shock protein) functions as a molecular chaperone role in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation and prevention of unwanted protein aggregation.

recA (DNA repair and recombination) is essential in the repair and maintenance of DNA. RecA protein catalyses an ATP-dependent DNA strand-exchange reaction that is the central step in the repair of dsDNA breaks by homologous recombination (Selbitschka *et al.*, 1991).

thrA (bifunctional aspartokinase) is involved biosynthesis of threonine, aspartokinase I and homoserine dehydrogenase.

3.3.3. Determination of primers/sequences

Primers for PCR amplification of gene fragments of ~500 bp were designed. The primers were all tested on a small set of isolates to determine amplification products. The most reliable and reproducible primers were used in the study. Some examples of PCR products obtained are shown in Figure. 3.2 and 3.3.

3.3.4. DNA sequencing

Gene fragments of ~400-600 bp were obtained by PCR for each gene (Figure. 3.3). A clean band obtained by PCR is important for sequencing. Primer dimers were observed if annealing temperature was too low. Primers dimers resulted in two bands being amplified (Figure. 3.2). The PCR products were purified by PEG 2000 precipitation to remove any unreacted primers and dNTP's from the solution. Forward and reverse sequences were obtained for each primer set and assembled using STADEN software (Staden, 1996) to provide unambiguous sequence over the required length of the allele.

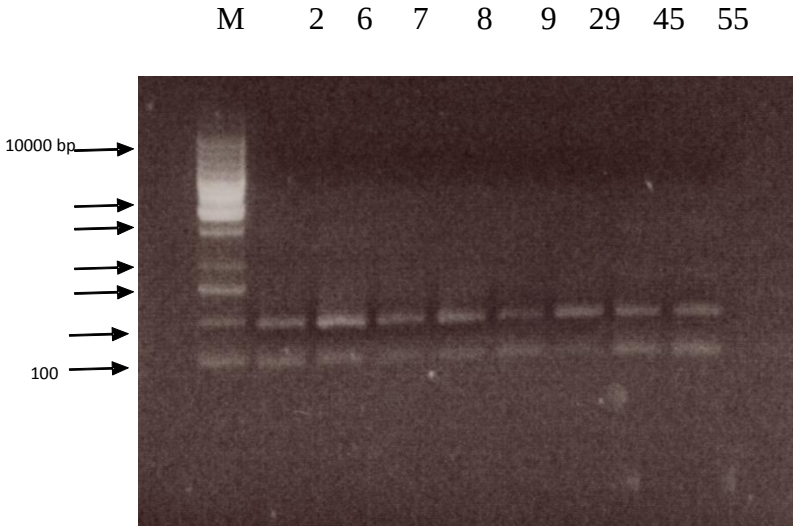


Figure 3.2. PCR primer dimers of amplified *recA* gene fragments of *Y. ruckeri* isolates. (M = molecular marker, 2,6,7,8,9,29,44,45 = *Y. ruckeri* isolates).

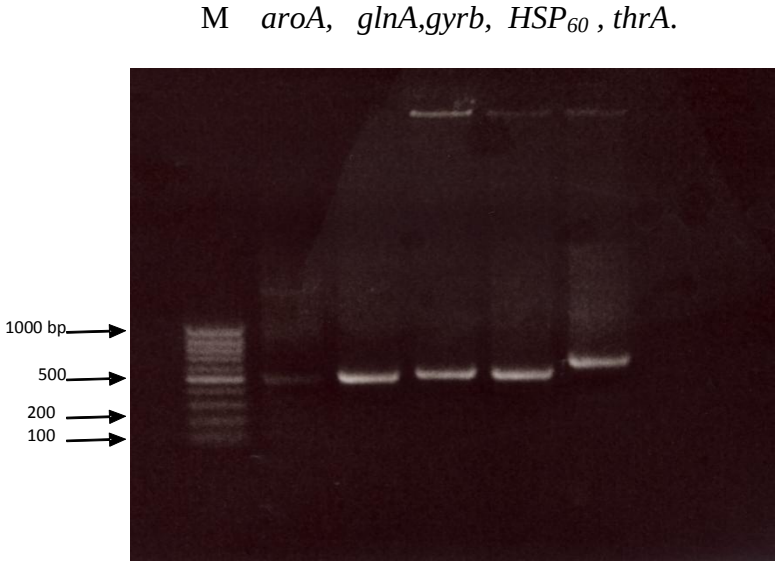


Figure 3.3. PCR amplification of *aroA*, *glnA*, *HSP*$_{60}$, *gyrB*, *thrA* ~ 500 bp gene fragments from bt 2 (EX5) isolate of *Y. ruckeri*.

3.3.5. Determination of sequence types

Six housekeeping genes (*aroA, glnA, gyrB, HSP60, recA, thrA*) were analysed using a total of 31 field isolates. Only *aroA, glnA, gyrB, thrA* were polymorphic. Sequences obtained from genes *HSP60* and *recA* only had one allelic profile each. For this reason, these genes have been omitted from the results as not to dilute findings. The results obtained from sequencing of the 4 genes gave 10 different sequence types (ST) (Table 3.3).

The DNA sequences for each allele were compared. Alleles that differed by one or more nucleotide were allocated arbitrary numbers, as shown in Table 3.3. The four allele numbers that formed the allelic profile were allocated a unique sequence type. By using this method, each strain was defined by a series of numbers, for examples strain NCTC 2197^T = (1,1,1,1,1) ST1. The sequenced type strain ATCC 29473^T gave a allelic profile of (1, 1, 1, 1) ST1. The number of strains studied were 31, and a total of 10 sequence types were recovered (Table 3.3). ST 1 was represented twenty times, and at a frequency of 64.52% of the total isolates examined. ST 2 occurred three times during the study at a frequency of 9.68%. The remaining eight ST (ST3-10) were occurred only once during the study (Table 3.4).

Table 3.3. Sequence types and allelic profiles of *Y. ruckeri* as derived by MLST.

Isolate No.	Id	Fish species	Country of origin	ST	*gnA*	*gyrB*	*thrA*	*aroA*
2	NCTC 10476 O1	Rainbow trout	USA	1	1	1	1	1
6	NCTC 12266 O2	Rainbow trout	UK	5	4	1	5	2
7	NCTC 12268 O5	Rainbow trout	UK	3	1	2	2	1

8	NCTC 12269 O6	Rainbow trout	UK	6	4	1	6	1
9	NCTC 12270 O7	Rainbow trout	UK	7	2	3	7	2
10	Glennfinnes non01	Rainbow trout	Scotland	2	1	1	3	1
11	48 non-01	Rainbow trout		2	1	1	3	1
18	MPM 04/184	Sturgeon	France	2	1	1	3	1
21	YR Tyre1 Atl.S	Atlantic salmon	Scotland	4	1	4	4	1
29	PR1 'EX5'	Rainbow trout	England	1	1	1	1	1
36	T1 (30)	Rainbow trout	England	1	1	1	1	1
38	RTF8A	Rainbow trout	England	1	1	1	1	1
43	RTF8B	Rainbow trout	England	1	1	1	1	1
45	RTF8C	Rainbow trout	England	1	1	1	1	1
46	TVT ITCHEN LT5	Rainbow trout	England	1	1	1	1	1
55	YR411-4	Rainbow trout	UK	1	1	1	1	1
56	DTF1	Rainbow trout	UK	1	1	1	1	1
63	LA3	Rainbow trout	UK	1	1	1	1	1
65	250181/2	Rainbow trout	UK	1	1	1	1	1
72	Teppe 08	Rainbow trout	France	1	1	1	1	1
78	F175-05 Cod	Cod	Iceland	10	1	5	4	1
80	468	Rainbow trout	France	1	1	1	1	1
103	TUTGB LT21	Rainbow trout	England	1	1	1	1	1

Ger2	Germany2	Rainbow trout	Germany	1	1	1	1	1
It2	It2	Rainbow trout	Italy	1	1	1	1	1
IT4	IT4	Rainbow trout	Italy	1	1	1	1	1
Sw1	Sw1	Rainbow trout	Switzerland	1	1	1	1	1
SW2	SW2	Rainbow trout	Switzerland	1	1	1	1	1
P18	P18	Atlantic Salmon	Denmark	8	1	2	3	1
P42/06	F06	Zander	Finland	9	3	2	1	1
P42/07	F07	Rainbow trout	Finland	1	1	1	1	1
ATCC 29473^{T}	-	Rainbow trout	USA	1	1	1	1	1

Table 3.4. Frequency and allelic profiles of *Y. ruckeri* sequence types.

Yersinia ruckeri ST	*AroA*	*glnA*	*gyrB*	*thrA*	Frequency
1	1	1	1	1	20 (64.52%)
2	1	1	1	3	3 (9.68 %)
3	1	1	2	2	1 (3.23 %)
4	1	1	4	4	1 (3.23 %)
5	2	4	1	5	1 (3.23 %)
6	1	4	1	6	1 (3.23 %)
7	2	2	3	7	1 (3.23 %)
8	1	1	2	3	1 (3.23 %)
9	1	3	2	1	1 (3.23 %)
10	1	1	5	4	1 (3.23 %)

3.3.6. Analysis of alleles

The frequency of each allele, length and number of polymorphic sites are included in Table 3.5 and 3.6. *AroA* had 2 alleles at 1 polymorphic site a length of 517 bases. *glnA* had 4 alleles at 2 polymorphic site a length of 469 bases was used. *Gyrb* has 5 different alleles and 11 polymorphic sites a length of 447 bases, and *thrA* has 7 alleles at 6 polymorphic sites.

Table 3.5. Frequency of alleles from *Y. ruckeri* strains used in MLST study.

Allele	*aroA*	*glnA*	*thrA*	*gyrB*
1	29	27	21	25
2	2	1	1	3
3	-	1	4	1
4	-	2	2	1
5	-	-	1	1
6	-	-	1	-
7	-	-	1	-
Unique	2	4	7	5

Table 3.6. Length of alleles, frequency and number of polymorphic sites obtained from MLST analysis.

Allele	Length (number of bases)	frequency (number of alleles)	Number of polymorphic sites
aroA	517	2	1
glnA	469	4	2
gyrB	447	5	11
thrA	601	7	6

3.3.6. Amino acid alignments and protein coding function

All alleles for the four loci were compared to detect non-synonymous nucleotide changes which may result in the formation of a different protein structure due to alterations in the amino acid structure (Table 3.7). No amino acid changes were detected in the *glnA* allele. In the *AroA* allele one conserved amino acid, change was observed (Table 3.7). *AroA* had an amino acid change from L (Leucine) to S (Serine) in *AroA*, ST2 affected the structure by a helix developing into a short strand. (Figure 3.4). Four non-conserved amino acid sequences were identified in the gyrB allele (Table 3.7). Allele *gyrB* had an amino acid change from G (Glycine) to W (Tryptophan) in *gyrB* ST5 (Table 3.7) I (Isoleucine) to T (Threonine) in *gyrb* ST3, H (Hisitdine) to L (Leucine) in *gyrb* ST3 and D (Aspartic acid) to V (Valine) in *gyrb* ST 4. There were four semi conserved amino acid changes in the *gyrB* allele, none of which produced a change in the predicted secondary structure of the protein (Figure 3.5). These changes were: M (Methionine) to V (Valine) in *gyrB* ST 1 and ST4, N (Asparagine) to K (Lysine) in *gyrB* ST5, H (Histidine) to D (Aspartic acid) in *gyrB* ST 3 and S (Serine) to N (Asparagine) in *gyrB* ST5. In the *thrA* allele, one conserved amino acid change was observed. In *thrA* St 5 H (Histidine) changed to (Glutamine), this change had no effect over the protein structure (Figure 3.6).

Table 3.7. Amino acid changes in frequently occurring alleles.

Allele	Number of alleles compared	Alleles	Number of amino acid changes	Amino-acid change	Type of change
aroA	1	1, 2	1	L (1) to S (2).	Conserved
glnA	4	1, 2, 3, 4,	none	none	none
gyrB	5	1, 2, 3, 4, 5	8	M (5, 2, 3) to V (1,4)	Conserved
				N (1, 2, 3, 4,) to K (5)	Semi conserved.
				H (1, 2 4, 5) to D (3)	Semi
				S (1, 2,3, 4,) to N (5)	

				G (1, 2, 3, 4) to W (5)	conserved.
				I (1, 2, 3, 4,) to T (3)	Not conserved
				H (1, 2 4, 5) to L (3)	Not conserved
				D (1, 2, 3, 5) to V (4).	Not conserved
					Not conserved
thrA	7	1, 2, 3, 4, 5, 6, 7	1	H (1, 2, 3, 4, 6, 7) to Q (5).	Conserved

AroA

```
ST4_aroa1_1       LFQLMKINISSSLRLPTIRMPIKFVIIQRSSPLFYYYAYRLFGSSPKYPIHSSDFVKTPM
120
ST5aroA2_1        LFQSMKINISSSLRLPTIRMPIKFVIIQRSSPLFYYYAYRLFGSSPKYPIHSSDFVKTPM
120
                  *** **********************************************************
```

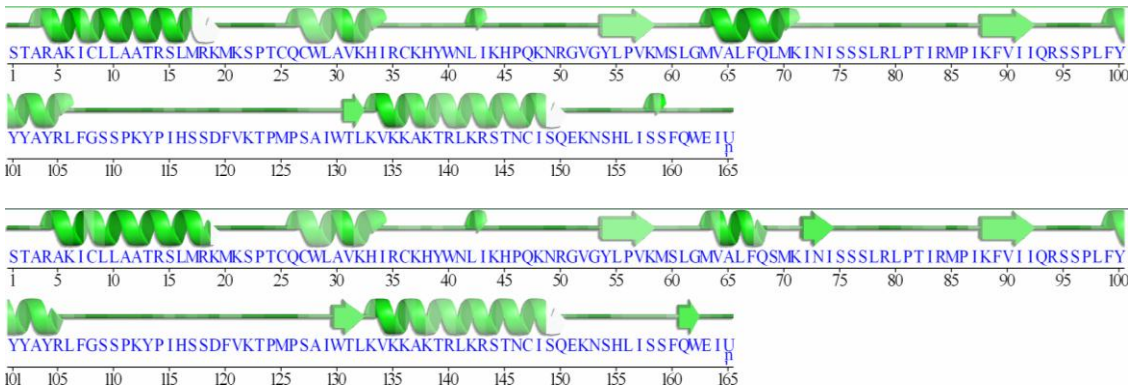


Figure 3.4. Clustal W alignment of amino acid sequence of part of the *aroA* gene and SAS predicted protein structure. * = nucleotides in the column are identical in all sequences, " ." semi conserved substitution, " " not conserved substitution.

In the *AroA* allele, there was one amino acid change. *Semi conserved* L (1) to S (2), this change affected the structure by a helix developing into a short strand.

gyrB

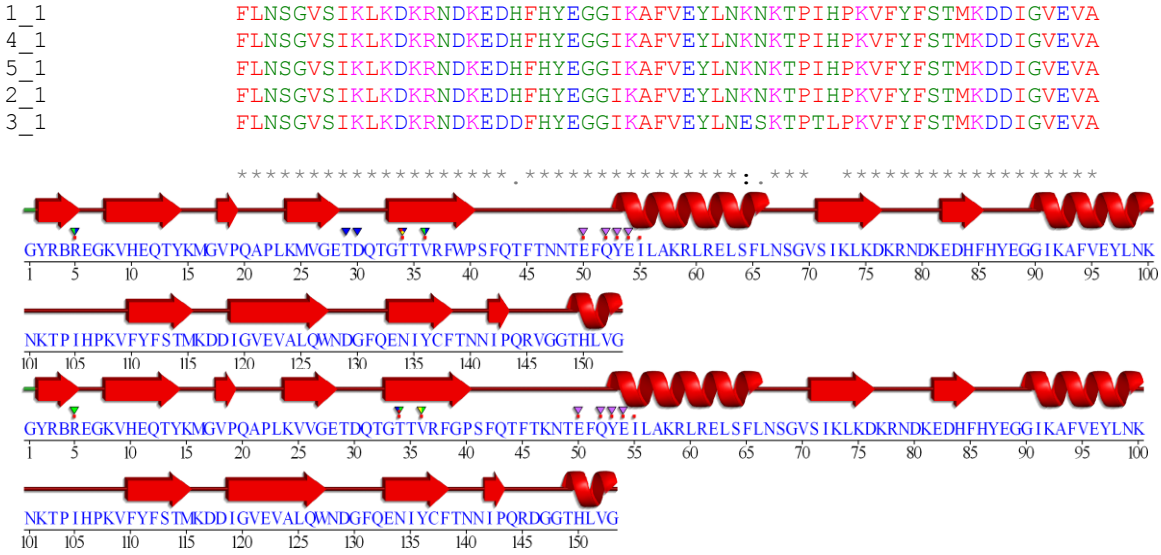


Figure 3.5. Clustal W alignment of amino acid sequence of part of the *gyrB* gene and SAS predicted protein structure. * = nucleotides in the column are identical in all sequences, " ." semi conserved substitution, " " not conserved substitution.

In the *gyrB* allele, there was one amino acid change :-

Conserved M (1, 4) to V (5, 2, 3) N (1, 2, 3, 4,) to K (5)

Semi conserved H (1, 2 4, 5) to D (3), S (3) to N (1, 2,3, 4,)

Not conserved. G (5) to W (1, 2, 3, 4), I (1, 2, 3, 4,) to T (3), H (1, 2 4, 5) to L (3), D (1, 2, 3, 5) to V (4). These changes did not affect the structure of the protein.

thrA

```
1_1 QGSSERSISVVVNNDSVTTGVRVCHQMLFNTDQVIEVFVIGAGGVGGALIEQIHRQQPWL
2_1 QGSSERSISVVVNNDSVTTGVRVCHQMLFNTDQVIEVFVIGAGGVGGALIEQIHRQQPWL
3_1 QGSSERSISVVVNNDSVTTGVRVCHQMLFNTDQVIEVFVIGAGGVGGALIEQIHRQQPWL
4_1 QGSSERSISVVVNNDSVTTGVRVCHQMLFNTDQVIEVFVIGAGGVGGALIEQIHRQQPWL
6_1 QGSSERSISVVVNNDSVTTGVRVCHQMLFNTDQVIEVFVIGAGGVGGALIEQIHRQQPWL
7_1 QGSSERSISVVVNNDSVTTGVRVCHQMLFNTDQVIEVFVIGAGGVGGALIEQIHRQQPWL
5_1 QGSSERSISVVVNNDSVTTGVRVCHQMLFNTDQVIEVFVIGAGGVGGALIEQIHRQHPWL
```

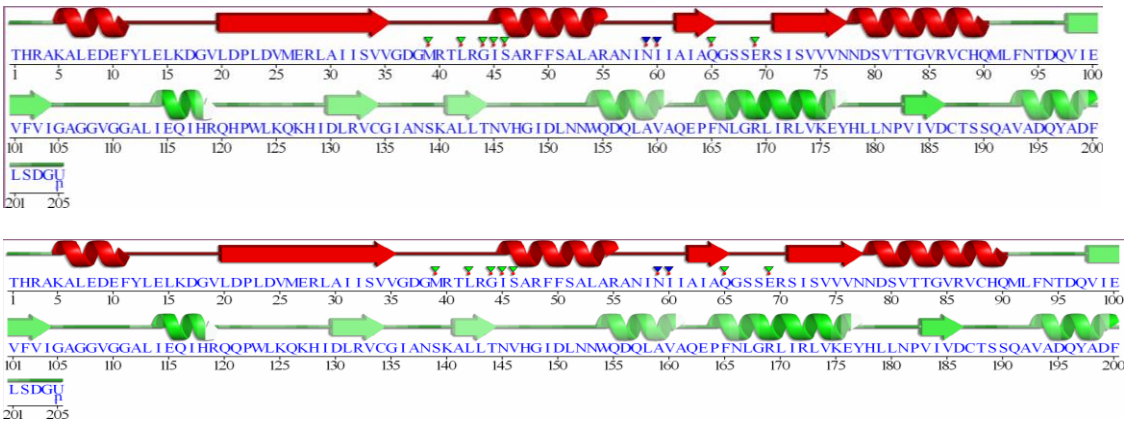


Figure 3.6. Clustal W alignment of amino acid sequence of part of the t*hrA* gene and SAS predicted protein structure. * = nucleotides in the column are identical in all sequences, : conserved substitution.

In the *thrA* allele, there was one amino acid change. *Conserved* H (5) to Q (1, 2, 3, 4, 6, 7). This change did not affect the overall structure of the protein.

3.3.7 Phylogenetic structure of *Y. ruckeri*

Interspecies diversity amongst *Y. ruckeri* isolates was examined used a neighbour-joining tree generated from concatenated sequences using START 2. The concatenated tree was constructed using 31 isolates for the 4 genes that showed nucleotide polymorphisms. The diagram highlights a single clone structure with potentially 3 groups occurring within the population. (Figure 3.7.) ST 7 is the most variable isolate of *Y. ruckeri* which seems to be forming or part of another clonal group. The UPGMA tree was produced based on allelic profiles for *Y. ruckeri* isolates (Figure 3.8). UPGMA tree highlights that ST 7 is the most distant or therefore genetically different *Y. ruckeri* isolate. Bootstrap values are low highlighting low genetic diversity between isolates. Caution must be advised when interpreting UPGMA tree as they do not take into account different rates of evolution.

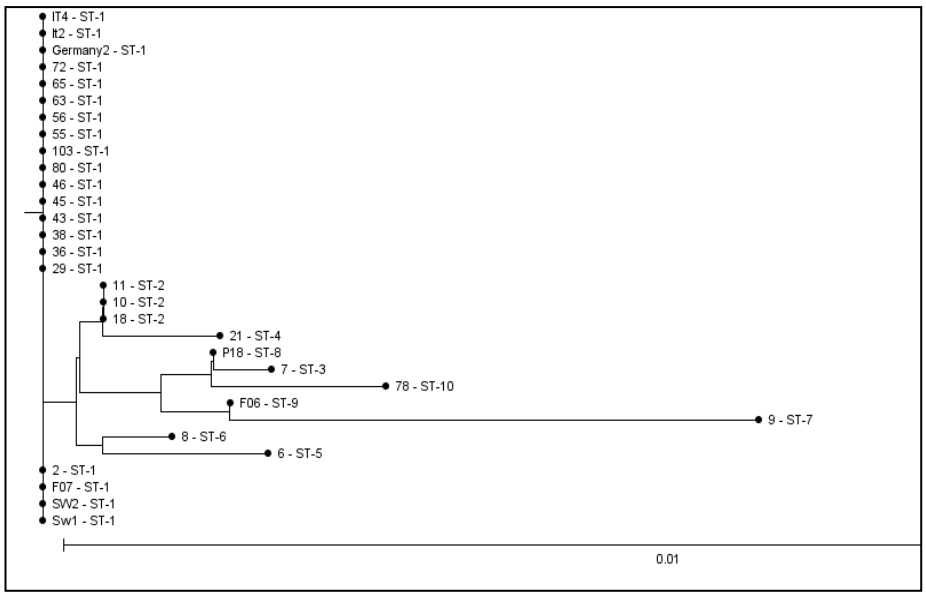


Figure 3.7. Unrooted neighbour joining tree constructed from concatenated allele sequences of 10 ST (31 strains) of *Y. ruckeri*. Bootstrap values are given as a percentage.

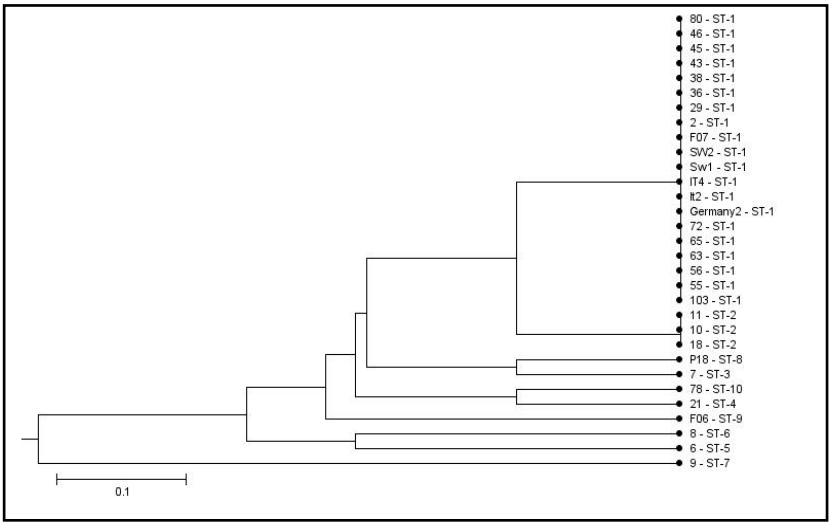


Figure 3.8. UPGMA tree constructed from concatenated sequences of 10 ST (31 strains) of the *Y. ruckeri* group. Bootstrap values are given as a percentage.

3.3.8. eBurst

A Burst diagram was produced from allelic profile data. The ancestral type was defined as ST 2 (Isolated from rainbow trout, Scotland 2003, UT serotype) although ST 1 was the most commonly identified ST. ST 7 was designated a singleton as no single-locus or

double locus variants were found (SLV, DLV) (Figure 3.9) ST 5, ST6, ST7 and ST 9 were designated as satellite populations.

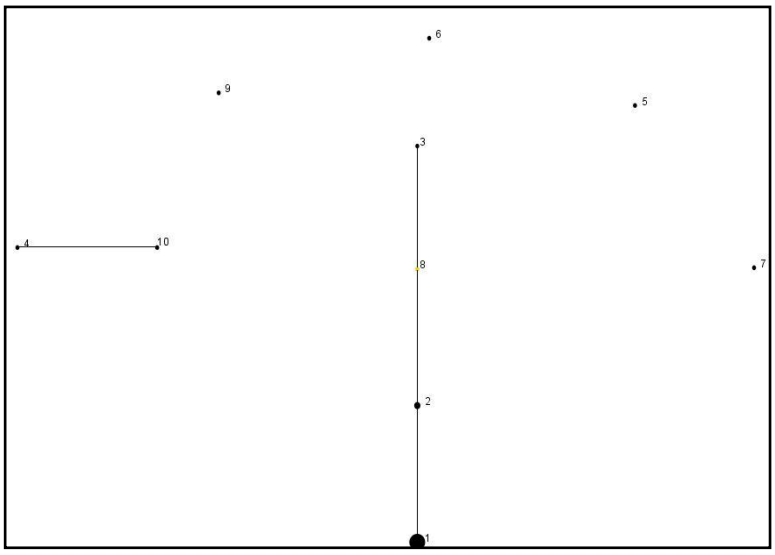


Figure 3.9. Analysis of *Y. ruckeri* isolates by eBURST. The area of each circle in the eBURST diagram corresponds to the abundance of the isolates of the ST in the input data; ST2 is the predicted founder of the group.

3.3.9. Further analysis of four gene loci

The phylogenetic analysis presented earlier was carried out on concatenated sequence data. In order to gain some insight into gene transfer among the ST's, trees were prepared for each gene. To simplify analysis, the 10 defined ST's as presented in Figure 3.10, 3.11, 3.12, and 3.13. Concatenated sequences of the 10 ST's are provided in a neighbour joining tree (Figure 3.14). The *gyrB* trees were found to be different from the concatenated tree indicating that this gene is possibly the most influenced by lateral gene transfer as they do not reflect the concatenated gene tree. However, most of the trees are similar to that of the concatenated tree indicating that recombinational events have not occurred.

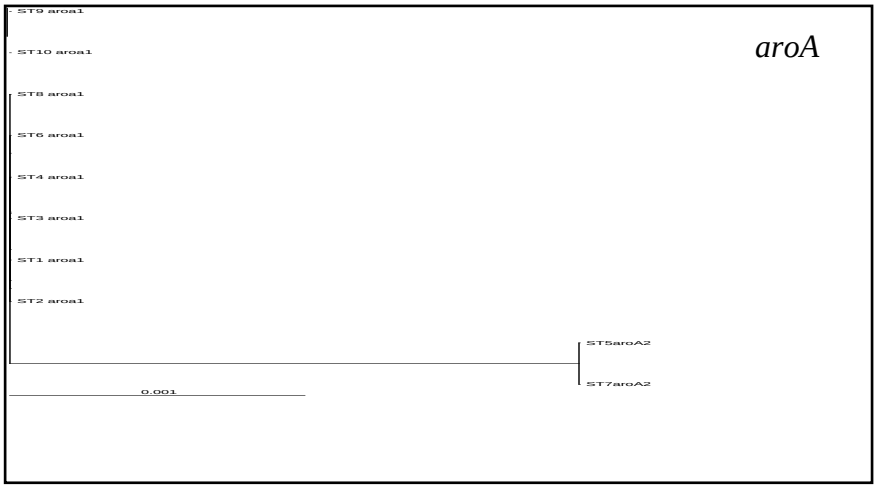


Figure 3.10. Neighbour joining tree for *aroA* alleles from 10 representative ST's. Bootstrap values are indicated.

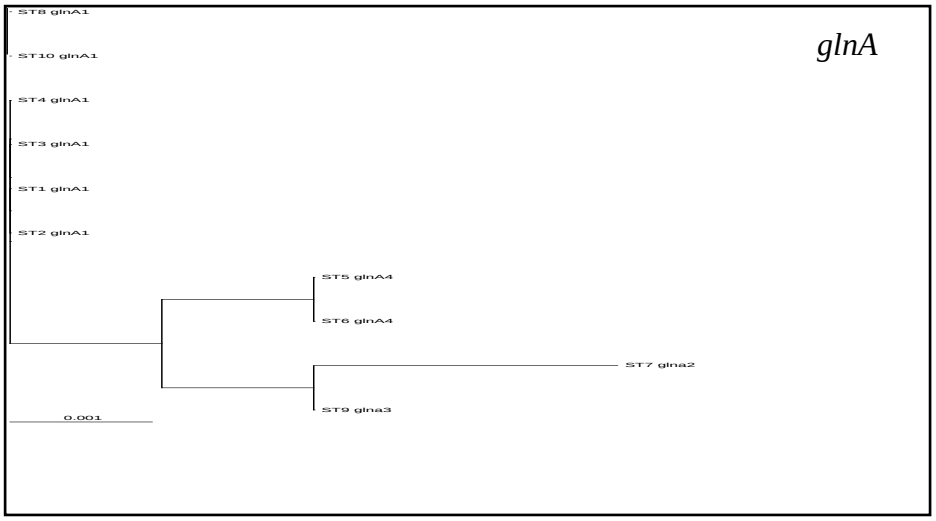


Figure 3.11. Neighbour joining tree for *glnA* alleles from 10 representative ST's.

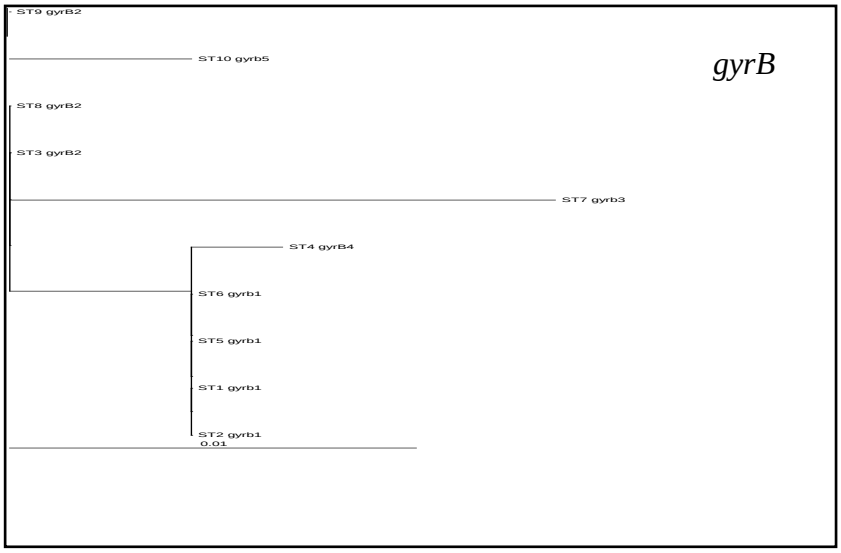


Figure 3.12. Neighbour joining tree for *gyrB* alleles from 10 representative ST's.

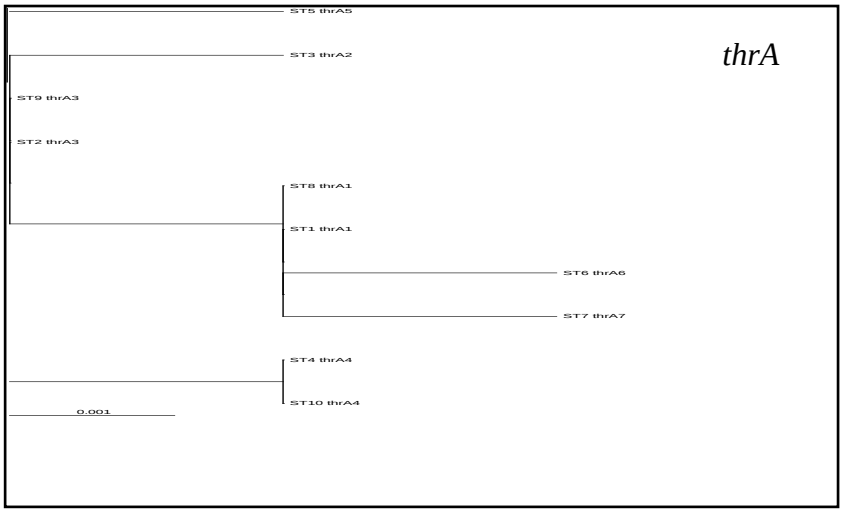


Figure 3.13 Neighbour joining tree for *thrA* alleles from 10 representative ST's.

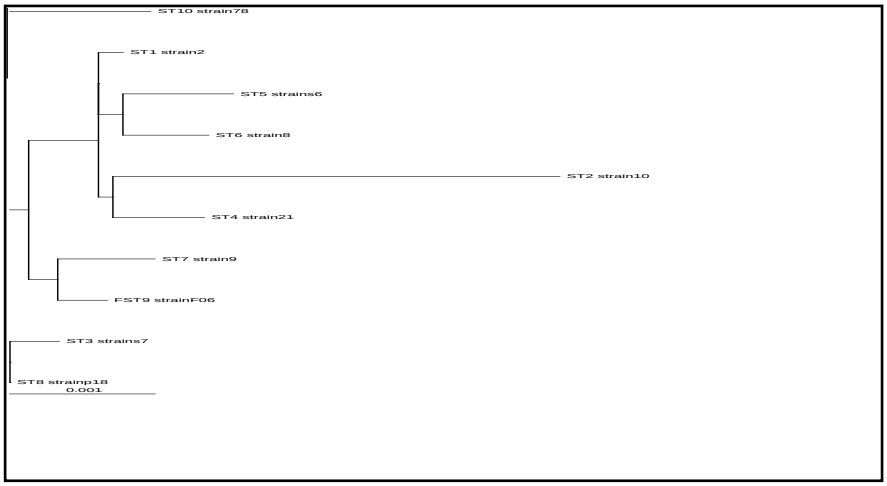


Figure 3.14 Neighbour joining tree for concatenated sequences alleles from 10 representative ST's.

3.4. Discussion

Although useful in the diagnosis of bacterial pathogens, phenotypic and serological methods are limited to understanding the genetics behind bacterial populations. Ever since the first classification and the clonal grouping theory was put forward, researchers have been trying to understand the phylogeny and population structure of *Y. ruckeri*. Biotype 2 isolates have been associated with disease in the UK since the 1980's (Davies and Frerichs, 1989). Recent evidence suggests a change in bacterial population dynamics (Austin *et al*., 2003, Arias *et al*., 2007). MLST was developed to investigate the long term evolution of the *Y. ruckeri* species and to provide a hypothesis to why bt 2 cases are increasing.

Sequence comparisons revealed 18 single nucleotide polymorphisms (SNP's) across 2034 bp surveyed. However, the housekeeping genes selected, particularly HSP_{60}, *recA* had no genetic variability which therefore could not be included within the scheme. Future investigations could focus upon using genes which are under greater selective pressure, such as virulence genes. Tankouo Sandjong *et al*. (2007) developed a MLST scheme using virulence genes as well as housekeeping genes termed MLST-v. Workers used a combination of housekeeping genes as well as flagellin genes in order to identify serovar-specific differences in *Salmonella* isolates. The use of virulence gene enabled researches to identify that lateral gene transfer *fliC* gene transforming one serovar into a new one. It is described that serotyping did not always recognise this change in serotype suggesting this method was a suitable method for resolving these problems. Similar genes could be used with the MLST scheme proposed here. However, the gene studies need to be present in all isolates in order to use them within a scheme. Therefore it is necessary to check find a suitable gene in *Y. ruckeri* that is present in all isolates. Studies are required into the antigenic characteristics of the organisms to identify key antigens which may suitable for incorporation into a MLST-v study. It has been demonstrated that the O antigen in *Y. ruckeri* isolates is variable and a possible virulence factor (Romalde *et al*., 1993). Although these would appear to be suitable virulence factors, it has been suggested that variations of the O antigen are due to different genes of these clusters being present and absent. It is thus not possible to find a single gene which would on its own reflect the O antigenic properties of the carrying strain (Tankouo-Sandjong *et al*., 2007).

The low diversity displayed by *Y. ruckeri*, suggests that there is a small population size that is a single clone and that the lineage has persisted in the environment over many years, only being isolated from places where aquaculture was practiced. In clonal population chromosomal variation occurs by *de novo* mutations, which can spread only by being passed on to the descendants of the cells in which they arose, and new lineages emerge by the accumulation of such mutations over successive generations (Spratt and Maiden, 1999). Clonal structures are largely thought to be stable, *S. enterica* which has a highly clonal structure, has been around for many thousands of years, isolates for different dates have a largely identical MLEE and antigenic profile (Spratt and Maiden, 1999). This stability suggests that intensive vaccination may not be the cause of bt 2 isolates arising. Other members of the genus *Yersinia* are often described as clones (Achtman, 1999). *Y. pestis* is often referred to as a 'young' species arising for *Y. pseudotuberculosis* due to the presence of similar alleles over the past 10,000 years or so, maybe due to changes in its lifestyle (Achtman, 1999; Kotetishvili *et al.*, 2005).

In comparison with the biochemical analysis of *Y. ruckeri* isolates in chapter 2, the MLST scheme does not correlate with the number of phenotypes found during the biochemical analysis. A larger set of isolates would be needed in order to observe any relationships between biochemical phenotype and ST. The work carried out within the project has to some extent found some correlation between serotypes of *Y. ruckeri* and their designated ST. From the dataset it was observed that ST1 correlates with all O1 serogroup strains, ST 5 corresponded to O2 serogroup, ST3 corresponded to O5 serogroup, ST6 corresponded to O6 serogroup and ST 7 corresponded to O7 serogroup. Biotype 2 isolates was unable to be distinguished from O1 bt 1 isolates based on MLST. Previous work has found some correlation between serotype and genomic typing. Priest *et al.* (2004) found that different ribotypes of *Bacillus* sp. corresponded to different serotypes. In comparison with other organisms, serotypes of *Campylobacter jejuni* were found in the study to be associated with more than one clonal lineage. *C. jejuni* isolates from poultry were found to be spread out throughout the study whereas isolates recovered from humans were found to be closely associated. Due to the availability for specific antiserum, not all ST could be correlated with serotype. Future studies would

require a large amount of sero-specific antiserum to fully understand the relationship between serotype and ST.

The small population size is not limited to geographic origin like other bacterial pathogens (Nicolas *et al.,* 2008). Some correlation between ST and fish species indicates that there are probably preferred routes of transmission for *Y. ruckeri.* ST 1 only seen in rainbow trout and ST4 and 8 Atlantic salmon. This difference in the ability of different ST to infect certain fish species could be linked with their lifestyle, ecological niche or some difference in immune response between species. Some 'atypical' strains coming from non-native fish species (cod, zander) highlights the potential of *Y. ruckeri* isolated to infect other fish species and pose future problems for other cultured species.

Using this sequenced based approach, this investigation has shown that all of the isolates of *Y. ruckeri* are from a single lineage. From the nearest neighbour tress draw from concatenated sequences highlights that *Y. ruckeri* is composed from a single clone. This makes the hypothesis for the global spread of the pathogen from North America where it was initially isolated difficult. It has been shown that the scheme is able to distinguish between all serogroups of *Y. ruckeri*, identify specific ST's that are linked to certain fish species and highlights the evolutionary relationships between ST. By examining each individual allele tree, the extent of gene transfer can be determined. If no horizontal gene transfer has occurred then each individual tree will be congruent, but if the genes have transferred then the trees will vary (Feil and Spratt, 2001). In this project, individual allele trees were constructed from the set of 10 STs from *Y. ruckeri*. Examination of four trees revealed that the structure of the trees differed only slightly suggesting that there is probably no evidence of recombination.

Dendrograms are often described as providing a poor representation of the relationships among isolates with similar genotypes and do not contain easily interpreted information about ancestry or pathways of recent evolutionary descent (Spratt *et al.,* 2006). Using eBurst (v3) the approach identifies clonal complexes within bacterial populations, and predicts their founding genotypes, and the evolutionary pathways by which the other

genotypes within the clonal complex may have arisen. The eBurst diagram produced from *Y. ruckeri* indicated that there were two lineages within the *Y. ruckeri* isolates examined highlight by the 2 horizontal lines produced in the diagram. Interestingly ST 7 (serogroup 7) was designated a singleton from the eBurst results, this could be a different clone of *Y. ruckeri* providing support for the hypothesis of a clonal population structure (Schill *et al*., 1984). As more MLST data is added to over time, more new ST should be seen, and it is also likely that conclusions concerning the clonal complex founder will have to be modified.

In relation to other members of the *Yersinia* sp., recently the genome of serotype O1 biotype 1 ATCC 29473^{T} (ST 1) was sequenced (Chen *et al*., 2010). In this context it was demonstrated that *Y. ruckeri*, is the earliest branching member of the genus and has the smallest genome (3.7 Mb), in comparison to *Y. pestis* (4.8 Mb). *Y. ruckeri* has similar characteristics to other members of the *Yesinia* sp. Like *Y. pestis*, *Y. ruckeri* lacks functional urease, methionine salvage genes, and B12-related metabolism. These losses may reflect adaptation to a lifestyle that does not include colonization of the mammalian gut (Chen *et al*., 2010). Shotgun sequencing of bt 2 isolate would be beneficial for future studies into the differences in pathogenesis between the two biotypes.

The discriminatory power of MLST for studying *Y. ruckeri* seems to be lower than that of other techniques used. Wheeler *et al*. (2009) demonstrated that PFGE was able to differentiate between bt 1 and bt 2 of *Y. ruckeri*. The study suggested that biotype 2 cases from Europe could have emerged from two different lineages. Although PFGE looks to be a more suitable technique to studying the phylogeny of *Y. ruckeri,* there are issues of portability surrounding the technique making it difficult for laboratories to share information. Other authors have noted a difference in discrimination levels between the two techniques (Vimont *et al.,* 2008; Melles *et al.,* 2007). One of the main advantages of sequence based approaches is that the data can be uploaded onto the internet and isolates added to the scheme constantly (Maiden *et al*., 1998).

The changes accumulated in the four genes resulted in amino acid changes but most of these were synonymous. Amino acid changes are rare events in the 'housekeeping' genes selected for MLST. This is a reflection of the fact that the conservation of these genes is necessary for cellular metabolism and non-synonymous mutations could results in non-viable progeny. Non-synonymous SNPs that lead to an amino acid change in the protein product are of major interest, because amino acid substitutions currently account for approximately half of the known gene lesions responsible for human inherited disease (Ng and Henikoff, 2003). Changes resulting in an altered protein conformation are extremely rare events, although one was detected in this MLST scheme. The only predicted change in protein structure was detected in the *AroA* gene, here *AroA* ST2 changed from a strand to a short helix.

As bt 1 and bt 2 isolates all cluster together in the same ST (ST 1), it is difficult to hypothesise about the increase in bt 2 cases throughout the world. There are two possible explanations, firstly the increase in bt 2 cases could be due to vaccine mediated strain replacement, and secondly this change could be a natural consequence of intensive aquaculture. Martcheva *et al*. (2008) introduced the concept of 'vaccine mediated strain replacement'. Workers demonstrated that intensive vaccination could drive the emergence of new dominant pathogen strains. This theory could be applied to the emergence of bt 2 isolates within aquaculture. As the prevalence of biotype 1 isolates decreases the prevalence of bt 2 increases possibly due to bt 2 not having to compete with biotype 1 as the standard monovalent vaccine is keeping biotype 1 cases down. Potentially treatment against the disease condition may positively select for the most virulent forms depending upon timing of treatment in relation to bacterial transmission.

A shift towards more virulent phenotypes as a result of intensive aquaculture was a theory put forward by Pulkkinen *et al*., (2010). In this context it was suggested that intensive aquaculture could be a reason for the evolution of pathogen virulence (Pulkkinen *et al*., 2010). In the paper by Pulkkinen *et al.* (2010), it was hypothesised that the increased occurrence in *F. columnare* was due to a due to an evolutionary change in virulence caused by intensive aquaculture in Finland. Where previously it has been suggested that vaccination could cause a change in virulence of bacterial

pathogens, this change could also be as a result of the changing ecosystems caused by intensive aquaculture or possibly a combination of the two. The evolution of pathogen virulence was associated with five observations of the pathogen. Firstly, the emergence was associated with increased severity of symptoms. Secondly, highly virulent *F. columnare* strains were associated with more severe symptoms prior to death. Thirdly, more virulent strains have greater infectivity, higher tissue-degrading capacity and higher growth rates. Fourthly, pathogen strains co-occur, so that strains compete. Fifth, *F. columnare* can transmit efficiently from dead fish, and maintain infectivity in sterilized water for months, strongly reducing the fitness cost of host death likely experienced by the pathogen in nature. Pulkkinen *et al*., (2010) also stated that treatment against the disease condition may positively select for the most virulent forms depending upon timing of treatment in relation to bacterial transmission. It also was suggested that the presence of several genetically distinct bacterial populations in one area may favour virulence, if the virulent strains have a competitive advantage. The explanation for the increase in bt 2 cases all over Europe could be associated with this change. It is however difficult to accurately account for this as data for the farming practises since the 1960's is unavailable. It is clear that from the biochemical analysis previously that there has been a change in phenotypic characteristics. It is unclear to what extent the VP reaction would allow biotype 2 isolates to proliferate within the external environment. Indeed it is yet to be determined how *Y. ruckeri* persists within the external environment in general. It was observed that *Y. ruckeri* was able to form biofilms and that this was correlated with flagellum production (Coquet *et al*., 2002). As biotype 2 isolates do not possess flagellum it is unclear to what extent biofilms are formed in the external environment and their role in pathogencity. Many bacterial fish pathogen are ubiquitous in the natural environment, *Y. ruckeri* has been previously described as an aquatic saprophyte incapable of living in water for long periods of time. It could be possible that over time and from selection from aquaculture and vaccination, virulent strains of *Y. ruckeri* are could become more saprophytic. Investigation into the excreted compounds of *Y. ruckeri* biotypes and serotypes would be useful in determining whether these new biotypes are producing any different substances that may give them a selective advantage over bt 1 isolates. Cross streaking could determine if bt 2 isolates are antagonistic towards bt 1 isolate, further providing evidence for the emergence of a more virulent phenotype due to aquaculture practises.

From the phenotypic and genotypic studies, it is difficult to hypothesize whether vaccination is having a direct affect upon *Y. ruckeri* phenotypes. Although theories have been put forward previously for vaccination or intensive farming practises altering bacterial population dynamics, there is also direct evidence that vaccination can induce changes in bacterial pathogens. Bachrach *et al.* (2001) identified a new serogroups of *S. iniae* arising after new outbreaks of the disease was observed within previously vaccinated fish. The new isolates unlike previous isolates were shown to be ADH negative. RAPD PCR patterns confirmed that difference between strains collected pre vaccination (1989-1995) and post-vaccination (1997-200) as band length estimated at 750 bp was seen in the previously vaccinated fish. Eyngor *et al.* (2004) described similar processes occurring in *L. garvieae.* It was hypothesized that selective pressure induced by specific vaccination against *L. garvieae* caused serological variation in the bacterium. The main virulence factors in many streptococci are capsular polysaccharides. It was hypothesised that selective pressure induced by vaccination caused changes in capsular composition. Similar findings have been seen in mammalian Gram-negative bacterial infections. Hallander *et al.* (2005) described a change in serotype with Swedish *Bordetella pertussis* strains over a 17 period without vaccination (1979 - 1996) and a period after the introduction of general vaccination among newborn children with acellular pertusis vaccines (1997 - 2003). Although there was a shift in serotype vaccination was still successful. It has been argued that bacterial polymorphism over time may be due to natural variation rather than immunity-driven selection. Halling-Brown *et al*. (2008) put forward the theory that bacteria might have evolved to evade the adaptive immune system by mutating their peptide sequences so that they either do not bind to MHC molecules or alternatively bind to them and produce a T-cell response protective to the bacterium.

In summary, the data presented here using the MLST scheme suggest that more work and isolates are needed in order to study the evolution, epidemiology, and phylogeny of *Y. ruckeri*. As allelic variation and recombination events are low MLST is probably not as useful as PFGE to understanding long term population dynamics. The data highlights that there is although the scheme shows evolutionary change between serotypes it cannot distinguish between bt 1 and bt 2 isolates. In this context it would be useful to incorporate virulence factors into future studies. This may create a scheme which can distinguish between the biotype, making identification faster. The data also highlights

the importance of ‘traditional’ microbiological techniques for the identification of bt1 and bt2 isolates of *Y. ruckeri*.

Chapter 4. Pathological and antigenic characterisation of *Y. ruckeri* isolates from Europe

4.1. Introduction

In the past decade, non-motile isolates of *Y. ruckeri* have emerged as a causative agent of severe ERM in previously vaccinated fish (Austin *et al*., 2003). Currently there is little information regarding the pathogenesis and antigenic characteristics of *Y. ruckeri*. The pathogenesis of *Y. ruckeri* is not well understood, and knowledge is limited to publications about exotoxins and plasmids (Degrandis *et al*., 1982, Fernandez *et al.*, 2004; 2007). It is unknown to what extent the antigenic differences of bt 1 and bt 2 differ. Antigenic characterization of the two biotypes will make clearer how 'vaccine failure' occurred in the past. Research into the antigenic makeup of the pathogen would be of great importance to the industry for a number of different reasons. Firstly, by identifying common antigens that cross protect against other isolates, the number of cases of vaccine failure could be reduced by the production of better vaccines. Secondly, the identification of key antigens or pathogen associated molecular patterns (PAMPS) will be a great use in terms of understanding molecular interactions with the immune system. Finally, the identification of common antigens may enable subunit vaccines in the future.

In the natural environment, bacteria interact with each other in an attempt to establish themselves and dominate their environment (Brook, 1999). Of paramount important are antagonistic interactions which cause bacterial phenotypes to grow and compete for ecological space. Antagonistic substances produced by bacteria can be in the form of, bacteriocins, organic acids, hydrogen peroxide and siderophores (Riley and Wertz, 2002; Ringø, 2008; Eberl and Collinson, 2009). Several microorganisms have been able to reduce bacterial diseases in challenge trials with fish or fish larvae (Verschuere *et al.*, 2000). Intraspecies antagonism in the natural environment is unknown, and it is unclear whether intensive aquaculture has caused a change in *Y. ruckeri* phenotype, allowing bt 2 isolates to competitively exclude other serotypes and bt in order to establish themselves as the dominant phenotype in the aquatic environment.

Many bacterial pathogens produce extracellular products that contribute to their pathogenicity. Most of these virulence factors are enzymes that enhance pathogen colonisation and growth (Madigan and Martinko, 2006). Iron is an absolute growth requirement for many bacterial pathogens, and must be removed from the iron binding proteins of the host (Wooldridge and Williams, 1993). Siderophores are important in the acquisition of iron from a host, which are secreted iron chelating compounds that can be regarded as virulence factors (Neilands, 1995) Iron is generally tightly bound to various molecules such as haemoglobin and transferrin. Following secretion, siderophores sequester and solubilise the iron. The sequestered iron is then transported back across the cell well via various receptors (Neilands, 1995). Until recently it was though the *Y. ruckeri* was not able to produce siderophores (Davies, 1991b). However, Fernandez *et al.,* (2004) demonstrated that the organism was able to produce siderophores. The siderophore, ruckerbactin is the iron uptake system that has been stated to be involved with virulence (Fernandez *et al.,* 2004).

The number of published articles about the excreted products and their ability to cause disease has been limited to a few studies namely involving bt 1 isolates (Romalde and Toranzo, 1993). *Y. ruckri* has been described as being a poor producer of extracellular products (Romalde and Troranzo, 1993). It would be of great importance to understand whether these excreted products differ between the biotypes and whether differences give a select advantage. Secretion of molecules, such as proteases, allows pathogenic bacteria to breakdown the surrounding environment as a source of nutrients. Secades and Guijarro (1998) identified a 47-kDa metalloprotease, described as *Yrp1,* which was produced at the end of the exponential growth phase. Metalloprotease is a histolytic enzyme, and it was suggested that it is involved in colonisation and virulence. Isolates have been described as Aso^+ and Aso^- according to the presence and absence of the *Yrp1* proteolytic activity. This metalloprotease is not a general feature of *Y. ruckeri* and is not related to serotype. Variation was found within all serogroups; the protease activity was not a characteristic of a single serogroup. The study did not use bt 2 isolates therefore it is difficult to relate this protease to pathogenicity in bt 2 isolates. Aso^+ strains have been described as virulent. Metalloproteases have been described as a pathogenicity mechanism and involved in the gross pathology of a number of fish pathogens (Zuo and Woo, 1998a). The metalloprotease identified by Secades and

Guijarro (1999) could be similarly associated with the characteristics clinical signs of ERM.

Outer membrane proteins (OMP) of Gram negative bacterial pathogens have been studied intensely in terms of their role in the virulence of the organism (Aoki and Holland, 1985; Actis *et al*., 1985). Consequently, OMPs are good vaccine candidates, since they are usually abundant proteins and are in direct contact with the host immune system (Qian *et al*., 2008). Such proteins are known to have pivotal functions related to adhesion and invasion of host tissues, weakening of the host immune response and acquisition of host metabolites necessary for bacterial survival (Evenberg and Lungtenberg, 1982; Simon *et al.,* 1996). In previous studies, these proteins have been found to be highly immunogenic and antigenic (Dumetz *et al.,* 2008). OMP's of *Y. ruckeri* could be potential antigenic and immunogenic molecules but the knowledge is lacking. Currently little is known of the role that OMP's play in pathogenicity or their antigenic nature, most studies using have focused upon characterization of isolates using these proteins (Davies, 1990; Ström-Bestor *et al*., (2010). In other species of *Yersinia*, protective outer membrane proteins have been studied widely as they play a vital role in pathogenicity (Lamaitre *et al*., 2006).

Y.ruckeri possesses endotoxins in the form of LPS, which is a major component of the outer membrane, like other Gram-negative bacteria (Toranzo and Barja, 1993). The lipid component has been associated with toxicity and the polysaccharide components of LPS are associated with immunogenicity in mammals (Aussel *et al.,* 2000). The breakdown of the outer membrane releases LPS which is associated with septic shock syndrome in mammals, although the toxic effects of LPS in fish are different (Gérard *et al*., 1993). Fish seem to be immune to the septic shock effects of LPS (MacKenzie *et al.,* 2010). It was stated by Reeves (1995) that the O antigen is extremely variable in Gram-negative bacteria. Culture conditions have been known to change the structure of LPS, and therefore could alter antigenic characteristic. It has been noted that the O antigen in other members of *Yersinia* sp., is highly variable at different temperatures (Kawaoka *et al.,* 1983). The LPS of *Y. ruckeri* has also been stated to be the protective antigen in vaccines (Amend *et al.,* 1983). In this context, LPS could be a suitable

vaccine candidate for *Y. ruckeri* infections. LPS vaccines have been used to some success against bacterial pathogens in cultured fish species (Kawakami *et al.* 1997).

Regulation of bacterial virulence is dependent upon an array of control mechanisms, some of which may not be evident *in vitro*, therefore, bacterial proteins expressed during growth in culture media may not be the same as those expressed within the host and should be treated with slight caution (Ishiguro *et al.*, 1981). Regulation of multiple virulence factors may arise from a response of the pathogen to a variety of host signals such as temperature, osmolarity, inorganic ion concentration, specific nutrient limitation, pH or proximity to mucosal surfaces (Griffiths, 1990). Any of the processes may have associated with it the expression of new membrane proteins or enhancement of existing proteins (Griffiths, 1990). It is therefore important that the antigenic properties of *Y. ruckeri* are investigated using various iron sources and conditions to deduce if there are any change in antigenic characteristics.

Due to the lifestyle of *Y. ruckeri,* it has previously been described there is a distinct lack of an antibody response during *Y.ruckeri* infections (Cossarini Dunier, 1986). This fact may make it difficult to understand the molecules which are immunogenic within the fish host during infections. Previous research into the antigenic nature of fish pathogens has been based around the ability of fish antiserum to bind with antigens from bacterial pathogens (Crump *et al.*, 2001; Jung *et al.*, 2008). In this context, the antigenic nature if *Y. ruckeri* has been somewhat hindered. Therefore, currently research into the antigenic characteristics has focused upon the reaction using rabbit antiserum (Romale and Toranzo, 1993; Crump *et al.*, 2001; Jung *et al.*, 2008). The humoral response stimulated in fish was found to be considerably weaker than that in the rabbit, with only four antigens eliciting a strong humoral response. It has been suggested that the immune system of fish may recognise different antigens associated with phenotypic changes in the bacterium due to culture conditions (Jung *et al.*, 2008).

The use of vaccination has been hypothesized to have profound effects on bacterial populations. Selective pressure from vaccination and intensive aquaculture has been suggested to cause accelerated pathogen evolution, with more virulent phenotypes

evolving due to a select advantage over competitive organisms (Martcheva *et al*., 2008). Yet to date, there have not been few comparative studies between bt 1 and bt 2 isolates. Virulence studies conducted by Fouz *et al.* (2006) reported biotype 2 isolates from the USA to have a LD_{50} dose of 5 x 10^2 colony forming units (CFU) $fish^{-1}$ following intraperitoneal administration. Recently, Ström-Bestor *et al*. (2010) noted that Finnish isolates of bt 1 and bt 2 isolates demonstrated no clear difference in their virulence to rainbow trout. Comparative virulence studies between representatives from each biotype and serotype could support this pathogen evolution hypothesis.

It is clear that there is a need to update the current knowledge of the pathological and antigenic characteristics of *Y. ruckeri to* include representatives of bt 2. The result of this study will be to improve vaccines and health management strategies in the future. Therefore, the objectives of the study were:

i. Investigation into the general enzymatic patterns of both WCPs and ECPs from representatives from all serotypes and biotypes of *Y. ruckeri*, in an attempt to differentiate between biotypes and serotypes.

ii. To identify whether *Y. ruckeri* isolates produce siderophores and iron-regulated OMP's, and to correlate these with virulence and antigenicity.

iii. To identify important antigenic components of the WCP, OMP and LPS structures of *Y. ruckeri*, using western-blotting. Different surface antigens between biotypes may be important for future vaccine development.

iv. To examine the virulence properties of biotype 1 and biotype 2 O1 isolates of *Y.ruckeri*. Differences in virulence could be a direct link between aquaculture practices altering phenotypes.

v. To examine the cross protection of a commercial monovalent and bivalent vaccine towards different biotypes of *Y. ruckeri*.

4.2. Material and Methods

4.2.1. Bacterial isolates

Bacterial isolates used within the study are given in Table 4.1. Culture of bacterial isolates is demonstrated in Chapter 2 section 2.2.1.

Table. 4.1 *Y. ruckeri* isolates used for study, indicating serotype, origin and biotype.

Y. ruckeri isolate	Serotype	Origin	Biotype
NCIMB 2194^T	O1 'Hagerman'	USA	1
NCTC 12266	O2	University of Reading, UK	1
NCTC 12268	O5	University of Reading, UK	1
NCTC 12269	O6	University of Reading, UK	1
NCTC 12270	O7	University of Reading, UK	1
TVT ITCHEN LT5	O1 bt 2	England, 2007	2
RD6	O1 bt 2	Davies & Frerichs, 1989	2

4.2.2. Cross-streaking method

The antagonism of bt 1 and bt 2 isolates of *Y. ruckeri* was assessed using the previously described cross streaking method of Robertson *et al*. (2000). Suspensions in saline (~10^7 cells ml $^{-1}$) were inoculated across inocula of the bacterial isolates on TSA plates, as appropriate, with incubation at 15-22^{o}C for up to 7 days. Antagonism by the isolates were indicated by overgrowth or an interruption in the growth of the pathogen.

4.2.3. Preparation of extracellular products

The ECP from *Y. ruckeri* were obtained by a cellophane overlay method (Sudheesh and Xu, 2001). Bacteria were grown overnight on TSA plates, and the actively growing cells were harvested into TSB to form a suspension. Sterile cellophane sheets

overlayered on TSA plates were prepared onto which was swabbed with the culture and incubated at 25^{o}C for 72 h. Bacteria grown on the cellophane sheet overlay were washed into 2 ml of PBS and centrifuged for 20 min at 15 000 × *g*. Following centrifugation, the supernatant containing ECP was filter sterilized with a 0.2 µm membrane filter (Millipore, Millex) and stored at –20°C until needed. The protein concentration of whole bacterial cells and ECP were determined using a Bradford protein assay kit.

4.2.4. Degradation characteristics of extracellular products

4.2.4.1. Haemolytic activity

Blood agar base (Oxoid) supplemented with 5% (v/v) sheep blood (Oxoid) was used. The plates were incubated at 22^{o}C and examined daily for 7 days. α-haemolysis was recorded by the appearance of an opaque greenish/brown zone around the area of growth. ß-haemolysis was recorded by the appearance of a clear zone around the areas of growth. α and ß haemolysis was recorded by the appearance of a clear zone around the growth surrounded by a zone of partial destruction of erythrocytes.

4.2.4.2. Proteolytic activity

Casein hydrolysis was observed in plates made of double strength TSA mixed with an equal volume of 10% (w/v) of sterile (115^{o}C 20 min^{-1}) skimmed milk (Oxoid). After incubation for up to 7 days, a positive response was recorded as the presence of clear zones around the bacterial growth.

4.2.4.3. Lipase activity

Lipase activity was examined using plates of TSA supplemented with (0.1% v/v) Tween 20, or Tween 80 (Oxoid). After incubation at 22^{o}C for 48 h the presence of a opaque zone surrounding the growth was recorded as evidence of positivity.

4.2.5. Enzymatic profile of WCPs and ECPs of *Y. ruckeri* using API ZYM

Sixty five µl of supernatants were inoculated to each well of API enzyme substrate (Bio-Mérieux) and incubated for 4 h at 37°C. The reaction was determined from the colour development following incubation for 5–10 min after addition of 1 drop each of reagents ZYM A and ZYM B at room temperature. The resulting colours were estimated under natural light and scored as 0–5, according to a colour scale supplied by the manufacturer.

4.2.6. Siderophore production

Bacterial strains were grown onto TSA plates before harvesting into TSB broth supplemented with (0.5 mmol) dipiridin (Sigma-Aldrich). Bacterial strains were starved of iron in this medium for 48 h. After incubation isolates were centrifuged at 4000 x *g* for 10 min. Then, 100 µL of the culture supernatant was added directly to well cut into the CAS agar plate. CAS siderophore detection agar medium was prepared as follows for 500 ml quantities - 2.65 g of NaOH and 15.12 g of pierazine-N,N'-bis (2-ethanesulphonic acid) were dissolved in 375 ml dH_2O. To this, 15 g agar and 50 ml of stock salt solution containing the following (per 500 ml): KH_2PO_4, 1.5 g; NaCl, 2.5 g; NH_2Cl, 5.0 g. The agar was dissolved by heating; the solution then was sterilized by autoclaving at 121^{o}C for 15 min and cooled in a water bath to 50^{o}C. Filter sterilized (0.22 µm Millipore, Millex porosity filters) solutions of the following components were added: Casamino acids (10%) 15 ml; glucose (20%), 5 ml; thiamine (200 mg ml^{-1}), 5 ml; nicotinic acid (200 mg ml^{-1}); 5 ml, $MgCl_2$ (1 M), 0.5 ml; $CaCl_2$ (0.1 M), 0.5 ml. Then, 50 ml of a sterile solution containing a complex of chrome azurol S, iron, and hexadecyltrimethylammonium bromide was added; its preparation is described below. The medium then was mixed gently and plates were poured. CAS agar medium used to grow *Y. ruckeri* strains AB3 and AB3-25 also contained 10 µM pantothenic acid to satisfy the growth requirement. The CAS-iron-hexadecyltrimethylammonium bromide solution was prepared by dissolving 0.030 g of CAS (Fluka) in 25 ml of distilled water and adding 5 ml of 1 mM $FeCl_3$ $6H_2O$ (in 10 mM HCl). This solution then was added slowly to a solution containing 0.036 gmg of HTDA (Fluka) in 40 ml distilled water. The resulting solution was sterilized by autoclaving (121^{o}C 15 min^{-1}). Siderophore production was apparent as a yellow-orange halo around the colonies; absence of this halo indicated the inability to produce siderophores.

4.2.7. Virulence studies

The virulence of different preparations (WCP, ECP, LPS) of *Y. ruckeri* isolates was examined in rainbow trout. These trout were anaesthetized with 1g MS222 / l^{-1} (Sigma-Aldrich). Groups of 10 fish were given intraperitoneally with 0.1 ml of *Y. ruckeri* preparations. Control fish were injected with sterile 0.9% saline. Fish were transferred to tanks and the mortality was recorded over 7 days. *Y. ruckeri* was confirmed phenotypically, as before, from kidney swabs.

4.2.8. Cross protection studies

4.2.8.1. Vaccination and challenge experiments.

Vaccines used were the standards monovalent 'Hgerman' Vaccine (Aquavac ERM ™) and a bivalent (Aquavac Relera™) vaccine produced against biotype 1 and biotype 2 isolates of *Y. ruckeri*. The vaccines were unadjuvanted. Vaccination was carried out following manufacturer's instructions. Thus, fish were vaccinated by 30 sec immersion in 1:10 dilution of vaccine. After vaccination, the fish were equally distributed between tanks. Control fish were subjected to 30 sec immersion in aquarium water. Challenge studies commenced 28 days after first vaccination. Groups of 30 fish were challenged i.p with 0.1 ml fish $^{-1}$ of *Y. ruckeri* isolates at ~10^6 CFU ml^{-1} (Table 4.2). Fish were held in separated tanks for the course of the experiment. Mortality was recorded after 21 days and expressed as accumulated mortality. The relative percentage survival (RPS; Amend, 1981) was also determined, as follows:

$$RPS = (1 - \text{vaccinate mortality/control mortality}) \times 100.$$

Table 4.2. Isolates used for cross protection study indicating origin, biotype and challenge dose.

Isolate No.	Isolate Code	Origin	Biotype	challenge dose
1	NCIMB 2194^{T}	UK,	1	1.8 x 10^6
29	TVT	UK, 1993	2	5.2 x 10^6
19	250-181/2	UK	1	4.0 x 10^6
71	Bassin 2A	France, 2005	1	2.1 x 10^6
	66542/2		2	4.5 x 10^6
-	Den A	Denmark	2	5.1x 10^6

4.2.9. Sampling for histology

Fish were initially exposed then resampled once every day for seven days. Fish injected with PBS were used as a negative control. Fish were killed, and kidney (posterior), spleen, gill, intestine, liver, and skeletal muscle along the lateral line were excised and fixed in 10% neutral buffered formalin. These samples were routinely processed into paraffin wax for histology, sectioned at 5 µm and stained using haematoxylin and eosin.

4.2.10. Lipopolysaccharide isolation

The isolation of lipopolysaccharide from *Y. ruckeri* isolates is described in Chapter 2 section 2.2.12.

4.2.11. Whole cell lysates

The isolation of whole cell lysates from *Y. ruckeri* isolates is described in Chapter 2 section 2.2.10.

4.2.12. Preparations of outer membrane proteins (OMP)

The preparation of OMPs from *Y. ruckeri* isolates are described in Chapter 2 section 2.2. 11.

4.2.13. Preparation of extracellular products (ECP)

Bacteria were grown overnight on TSA plates before actively growing cells were harvested into TSB to make a suspension. Sterile cellophane sheets overlayered onto TSA plates were prepared onto which was swabbed the bacterial culture with incubation at 25^{o}C for 72 h. Bacterial growth was washed into 1 ml volumes of PBS and centrifuged for 20 min at 15 000 × *g*. Then, the supernatant containing ECP was filter sterilized with a 0.2 µm Millipore Millex porosity filter and stored at –20°C until needed. Next, 20 µl volumes of ECP for use in 1D SDS PAGE were mixed with 20 µl 2× Laemmli sample buffer [2.5 ml (w/v) 0.5 M Tris-HCl (pH 6.8), 2 ml (v/v) glycerol, 4 ml (w/v) 10% SDS, 0.31 g (w/v) dithiothreitol (DTT, Sigma-Aldrich), 0.04% (w/v) bromophenol blue); and stored at –20°C until needed. The protein concentration of whole bacterial cells and ECP were determined using a Bradford protein assay kit.

4.2.14. 1D SDS PAGE electrophoresis

The procedure for 1-D SDS PAGE electrophoresis of various protein/LPS preparations from *Y. ruckeri* isolates is described in Chapter 2 section 2.2.13.

4.2.15. Western blot analysis of fish antiserum

Bacteria grown *in vivo* and *in vitro*, and the ECP of the bacteria grown *in vivo* were subjected to SDS-PAGE as outlined above, and the separated bacterial components were transferred to a nitrocellulose membrane using 60 V for 70 min. Prestained molecular weight markers were used as standards (New England Bio Labs) Proteins were visualised by soaking gel in Ponceau red (Sigma-Aldrich) for 10 mins to visualise

bands. Membranes were then washed in dH_2O and allowed to air dry. Non specific binding sites were covered by soaking the nitrocellulose paper in a solution of 1% w/v BSA (Sigma-Aldrich) in Tris-buffered saline (TBS) for 60 min at 22^oC.The membrane was washed 3 times with TTBS (Tris buffered saline + 0.1 % Tween – 20 (v/v); 5 min per wash. Membranes were then incubated with fish serum (diluted 1/10 in TBS) for 60 min at room temperature. Each membrane was washed as described above and then incubated with an anti-trout IgM monoclonal antibody (Aquatic Diagnostics) for 3 h at 20^oC The membrane was washed as previously described and incubated with an anti – mouse IgG – HRP (R & D systems) at 1/1000 for 60 min at room temperature. The membrane was washed again including a fourth rinse of 1 min using TBS without Tween 20. The blot was developed by adding chromogen and substrate (2 mL of 4 – chloro-naphthol solution with 10 mL of PBS and 10 µl of H_2O_2 (Sigma-Aldrich) at 37°C until bands materialised. The reaction was stopped by soaking the blot in distilled water for 10 min. Blots were allowed to air dry before scanning.

4.2.16. Western blot for rabbit polyclonal antiserum

Bacteria grown *in vivo* and *in vitro*, and the ECP of the bacteria grown *in vivo* were subjected to SDS-PAGE as outlined above, and the separated bacterial components were transferred to a nitrocellulose membrane using 60 V for 70 min. Prestained molecular weight markers were used as standards (New England Bio Labs) Proteins were visualised by soaking gel in Penso red (Sigma-Aldrich) for 10 min to visualise bands. Membranes were then washed in dH_2O and allowed to air dry. Non specific binding sites were covered by soaking the nitrocellulose paper in a solution of 1% w/v BSA (Sigma-Aldrich) in Tris-buffered saline (TBS) for 60 min at 22^oC.The membrane was washed 3 times with TTBS (Tris buffered saline + 0.1 % Tween – 20 (v/v); 5 min per wash. Membranes were then incubated with rabbit anti-*Y. ruckeri* serum (diluted 1/1000 in TBS) for 60 min at room temperature. The membrane was washed as previously described and incubated with an goat anti – rabbit IgG – alkaline phosphatase (Sigma-Aldrich) at 1/1000 for 60 min at room temperature. The membrane was washed again including a fourth rinse of 1 min using TBS without Tween – 20. The blot was soaked for 10 min in 0.05 M Tris-HCl, pH 9.8. The blot was developed by adding 10 mL of 10 µl DCIP (Sigma-Aldrich) and NBT (Sigma-Aldrich) at 37°C until

bands materialised. The reaction was stopped by soaking the blot in distilled water for 10 min. Blots were allowed to air dry before scanning.

4.2.17. Detection of total carbohydrate

Bacterial cells and the ECP and OMP of *Y. ruckeri* isolates were subjected to SDS-PAGE and blotting as described above. Bacterial components from the gel were transferred to a nitrocellulose membrane, and the total carbohydrate present was determined using a glycoprotein determination kit (Sigma-Aldrich) as previously described by Jung *et al*. (1998). Briefly, nitrocellulose membranes were oxidized for 60 min, washed twice with ultrapure water, the water was replaced with Schiff's reagent for 2 h until bands appeared magenta in colour, and then agitated briefly. Incubated with a reduction component (sodium metasulphite) wash for 60 min. Then, membranes were washed twice in ultrapure water for 60 min. HRP was used as a positive control.

4.3. Results

4.3.1. Cross-streak method

Antagonistic activity of *Y. ruckeri* isolates are given in Table 4.3. Selected isolates of *Y. ruckeri* showed no antagonistic activity against other isolates.

Table 4.3. Antagonistic activity of *Y. ruckeri* isolates as determined by the cross-streak method.

			Inhibitory activity to*					
Isolate No.	Serotype	Biotype	O1	O2	O5	O6	O7	O1 bt2
1	O1 'Hagerman'	1	-	-	-	-	-	-
6	O2	1	-	-	-	-	-	-
7	O5	1	-	-	-	-	-	-
8	O6	1	-	-	-	-	-	-
9	O7	1	-	-	-	-	-	-
29	O1 bt 2	2	-	-	-	-	-	-

*Cross-streak method; + overgrowth, - no overgrowth, +/- slight overgrowth.

4.3.2. Enzymatic activities of whole cell preparations of *Y. ruckeri* isolates

Enzymatic activities determined by API ZYM for whole cells of *Y. ruckeri* (Table 4.4). All strains were positive for naphthol-AS-BI-phosphohydrolase, acid phosphatase, alkaline phosphatase, leucine arylamidase, *N*-acetyl-β-glucosaminidase and α-glucosidase. It was observed that isolates from serogroups O1, O2 and O5 were positive β-galactosidase. All strains were negative for α-galactosidase, β-glucuronidase, α-

mannosidase, α-fucosidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, esterase (C4), esterase Lipase (C8) and lipase (C14).

Table 4.4. Enzymatic activities of whole cell preparations of *Y. ruckeri* isolates.

		[2]Enzyme activity of ECP:						
Enzyme:	[1]Substrate	O1bt1	O2	O5	O6	O7	O1 bt2 RD6	O1 bt 2 TVT
Control		–	-	-	-	-	-	-
Glycosidases:								
α-galactosidase	A = 13	-	-	-	-	-	-	-
β-galactosidase	B = 14	+	+	+	-	-	+	+
β-glucuronidase	C = 15	-	-	-	-	-	-	-
α-glucosidase	D = 16	+	+	+	+	+	+	+
β-glucosidase	E = 17	-	-	-	-	-	-	-
N-acetyl-β-glucosaminidase	F = 18	+	+	+	+	+	+	+
α-mannosidase	G = 19	-	-	-	-	-	-	-
α-fucosidase	H = 20	-	-	-	-	-	-	-
Peptide hydrolases:								
Leucine arylamidase	I = 6	+	+	+	+	+	+	+
Valine arylamidase	J =7	-	-	-	-	-	-	-
Cystine arylamidase	K = 8	-	-	-	-	-	-	-
Trypsin	L = 9	-	-	-	-	-	-	-
Chymotrypsin	M= 10	-	-	-	-	-	-	-
Ester hydrolases:								

Esterase (C4)	N = 3	-	-	-	-	-	-	-
Esterase Lipase (C8)	O = 4	-	-	-	-	-	-	-
Lipase (C14)	P= 5	-	-	-	-	-	-	-
Phosphohydrolases:								
Alkaline phosphatase	Q= 2	+	+	+	+	+	+	+
Acid phosphatase	R = 11	+	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	S= 12	+	+	+	+	+	+	+

(+ = positive, - = negative)

4.3.3. Enzymatic activities of ECP preparations of *Y. ruckeri*

Enzymatic activities of extracellular products as determined by API ZYM and classical biochemical methods (Table 4.5). All strains tested were positive for naphthol-AS-BI-phosphohydrolase. It was observed that isolates from serogroup O1 were positive for *N*-acetyl-β-glucosaminidase; the remaining serogroups were negative. All strains were negative for α-galactosidase, β-galactosidase, β-glucuronidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, esterase (C4), lipase (C14), alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, Tween 20, Tween 80, and gelatinase. Bt 2 isolates were positive for the production of caseinase. Isolates were not able to haemolyse blood from fish or sheep. A variable result for the production of lethicinase was not recorded.

Table 4.5. Enzymatic activities of ECP preparations of *Y. ruckeri* isolates**.**

Enzyme:	[1]Substrate	O1	O2	O5	O6	O7	O1 bt2 TVT	O1 bt 2 RD6
Control	–	-	-	-	-	-	-	-
Glycosidases:		-	-	-	-	-	-	-
α-galactosidase	a	-	-	-	-	-	-	-
β-galactosidase	b	-	-	-	-	-	-	-
β-glucuronidase	c	-	-	-	-	-	-	-
α-glucosidase	d	-	-	-	-	-	-	-
β-glucosidase	e	-	-	-	-	-	-	-
N-acetyl-β-glucosaminidase	f	+	-	-	-	-	+	+
α-mannosidase	g	-	-	-	-	-	-	-
α-fucosidase	h	-	-	-	-	-	-	-
Peptide hydrolases:		-	-	-	-	-	-	-
Leucine arylamidase	i	-	-	-	-	-	-	-
Valine arylamidase	j	-	-	-	-	-	-	-
Cystine arylamidase	k	-	-	-	-	-	-	-

Trypsin	l	-	-	-	-	-	-	-
Chymotrypsin	m	-	-	-	-	-	-	-
Ester hydrolases:		-	-	-	-	-	-	-
Esterase (C4)	n	-	-	-	-	-	-	-
Esterase Lipase (C8)	o	-	-	-	-	-	-	-
Lipase (C14)	p	-	-	-	-	-	-	-
Phosphohydrolases:		-	-	-	-	-	-	-
Alkaline phosphatase	q	-	-	-	-	-	-	-
Acid phosphatase	r	-	-	-	-	-	-	-
Naphthol-AS-BI-phosphohydrolase	s	+	+	+	+	+	+	+
Tween 20	N/A	-	-	-	-	-	-	-
Tween 80	N/A	-	-	-	-	-	-	-
Gelatinase	N/A	-	-	-	-	-	-	-
Blood (sheep)	N/A	-	-	-	-	-	-	-
(fish)		-	-	-	-	-	-	-
Lethicinase	N/A	-	+	+	+	+	+	+
Caseinase	N/A	-	-	-	-	-	+	+

(+ = positive, - = negative)

4.3.4. Siderophore production

All isolates produced siderophores which was indicated by an orange halo around the well cut into the agar plate (Figure 4.1). All isolates were able to produce siderophores in similar quantities.

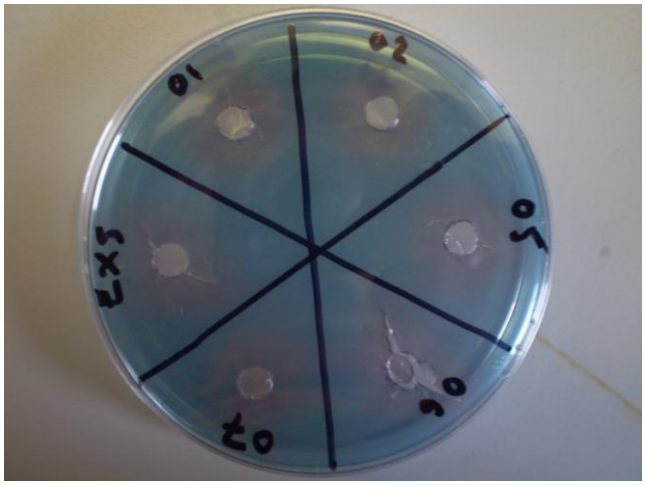


Figure 4.1. Siderophore production by *Y. ruckeri* isolates as indicated on CAS medium. Presence of siderophores is indicated by orange halo around the wells.

4.3.5. Toxicity of *Y. ruckeri* extracellular products

Toxicity of ECP's and HT ECP's from selected serotypes and biotype at varying concentrations are highlighted in Figures 4.2; 4.3; 4.4. Most of the ECP's gathered from *Y. ruckeri* were avirulent to fish. The lethal effects were lost after heating the ECP samples at 100^{o}C for 10 min. ECP's from bt 2 were virulent to fish at concentrations of 46.6 mg ml^{-1} after 48 h. Heat inactivated ECP's from bt 1 serogroup O1 caused mortality after 24 h, which could be associated with injection of the ECP's into the fish.

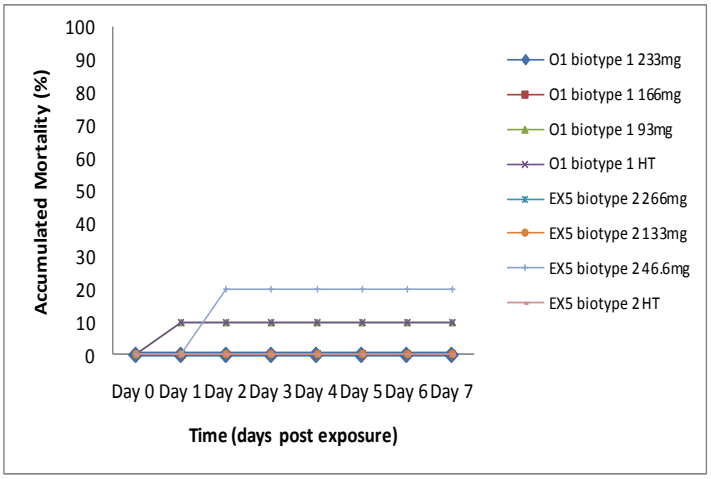


Figure 4.2. Accumulated mortality of rainbow trout following i.p. injection of ECP and HT ECP of *Y. ruckeri* serogroups O1 bt 1 and EX5 bt 2. HT – Heat treated.

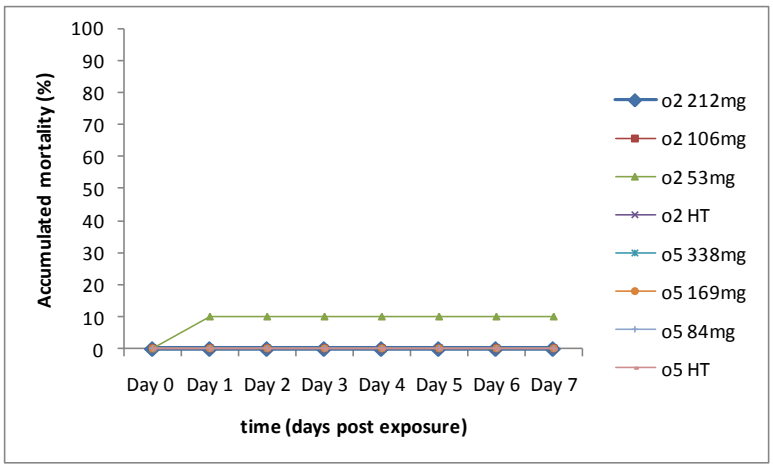


Figure 4.3. Accumulated mortality of rainbow trout following i.p. injection of ECP and HT ECP of *Y. ruckeri* serogroups O2 bt 1 and O5 bt 1. Heat treated.

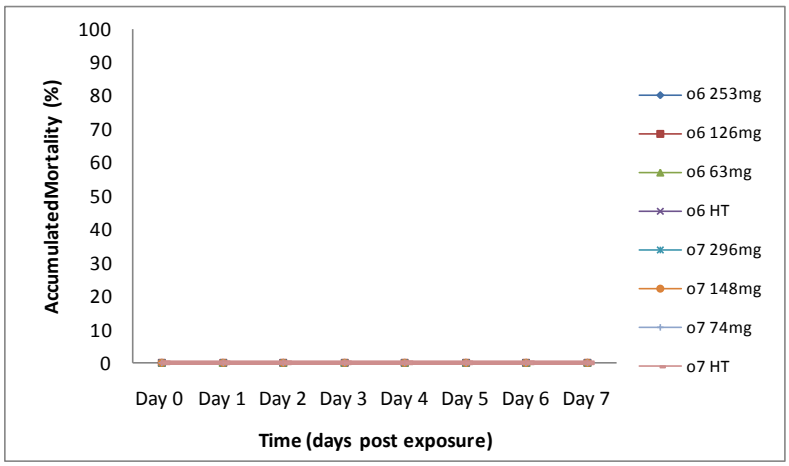


Figure 4.4. Accumulated mortality of rainbow trout following i.p. injection of ECP and HT ECP of *Y. ruckeri* serogroups O6 bt 1 and O7 bt 1.

4.3.6. Toxicity of *Y. ruckeri* LPS to rainbow trout

LPS from both biotypes was a virulent to fish when administered by i.p. injection at a concentration between 50 mg and 500 µg (Figure 4.5). LPS isolated from *E. coli* was not toxic to fish at a concentration of 1 mg.

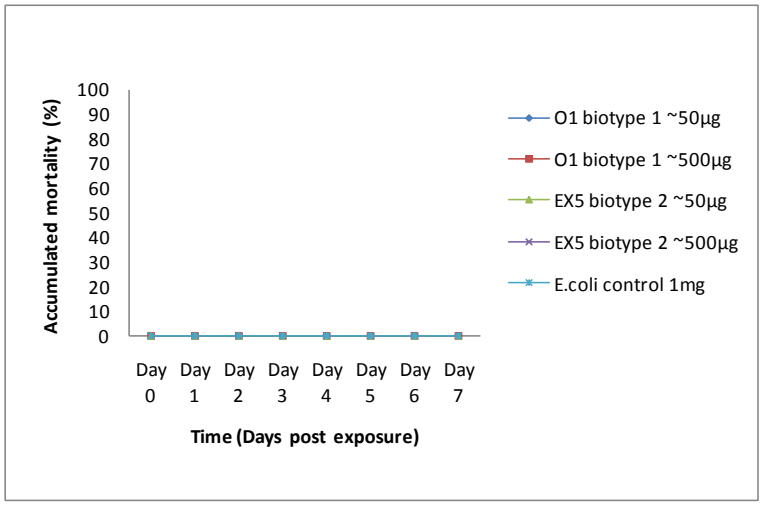


Figure 4.5. Accumulated mortality following I.P injection of LPS's of *Y. ruckeri* serotype O1 biotypes 1 and 2 to rainbow trout.

4.3.7. Subcelluar components

SDS PAGE analysis of WCP, OMPs, ECP proteins and LPS revealed some variation between the main serogroups of *Y. ruckeri* and the non-motile bt 2 isolates.

4.3.7.1. Whole cell protein profiles (WCP)

Analysis of whole cell proteins of *Y. ruckeri* isolates by SDS PAGE has been previously described in Chapter 2.

4.3.7.2. Outer membrane protein profiles under iron limiting conditions

OMP profiles in cultures grown in iron limiting conditions were analysed by 1D SDS PAGE electrophoresis (Figure. 4.6). It was observed that the expression of OMPs increased when cultured in iron limiting conditions. A number of high molecular weight proteins, i.e. 97-100 kDa in size, were overexpressed in all isolates. O1 bt 1 and O1 bt 2 had similar profiles, whereas IROMPS for serotype O5, O6 and O& varied in molecular weight. The differences between the proteins used for typing remained that same.

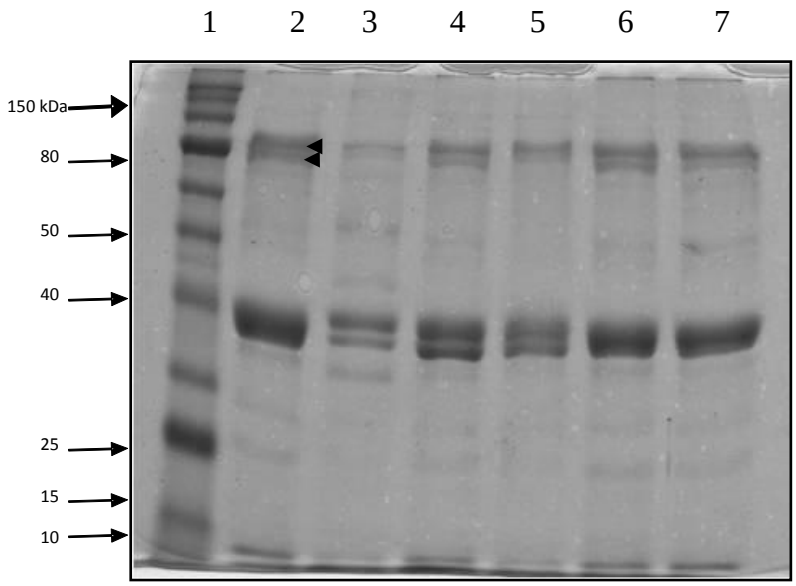


Figure 4.6. 12 % SDS-PAGE of OMP profiles *of Y. ruckeri* grown under iron limiting conditions. Staining was with Coomassie brilliant blue. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (EX5). Arrows indicate molecular weight (kDa). Arrows head indicate IROMPS.

4.3.7.3. SDS PAGE of ECPs

SDS PAGE of ECP's from bt 1 serogroups O1, O2, O5, O6, O7 and O1 bt 2 isolates of *Y. ruckeri* revealed that excreted protein profiles were fairly homogeneous for all isolates as highlighted in Figure 4.7. A dominant protein band was observed at ~50 kDa. Two other bands of between 15 and 25 kDa were expressed in all the serogroups and biotypes.

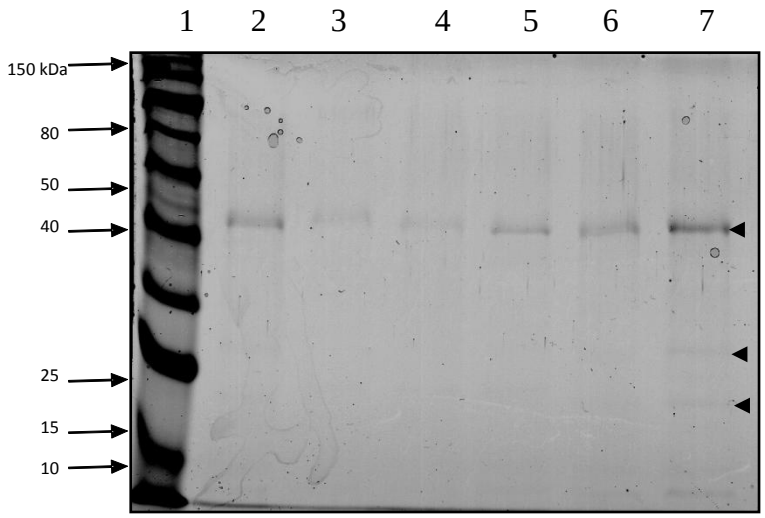


Figure 4.7. 12 % SDS PAGE of ECP profiles of *Y. ruckeri* isolates. Protein banding patterns detected using commassie brilliant blue. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (TVT) Arrows indicate molecular weight (kDa). Arrows indicate dominant bands.

4.3.7.4. LPS profiles

Analysis of lipopolysaccharide profiles of *Y. ruckeri* isolates by SDS PAGE has been previously described in Chapter 2.

4.3.8. Western blotting of cellular components

4.3.7.1. Whole cell proteins

Western blot analysis of six *Y. ruckeri* isolates (O1, O2, O5, O6, O7, EX5) strains was carried out using polyclonal rabbit serum raised against *Y. ruckeri* O1 NCTC 2091 (Figure 4.8). The five other geographically diverse *Y. ruckeri* strains had almost identical Western blot profiles. In all, there were 11 dominant antigenic bands observed. Serotype o1 bt 1 and bt 2 had two dominant bands at ~ 150 kDa which were not observed in the other serogroups.

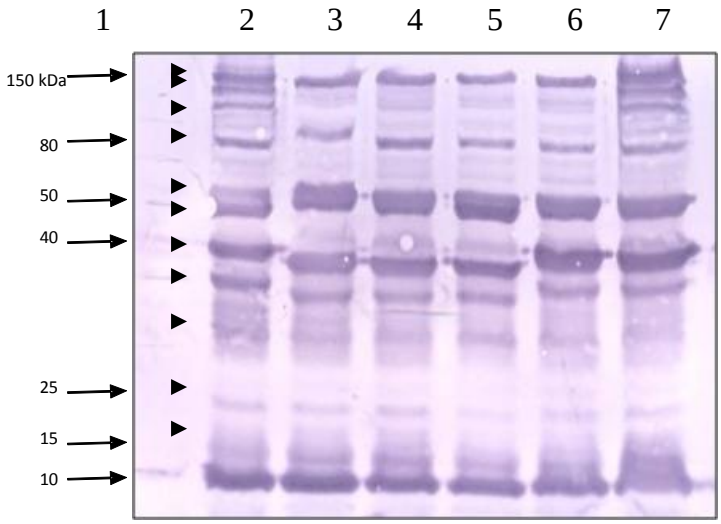


Figure 4.8. Western blot analysis of *Y. ruckeri* isolates polyclonal rabbit serum raised against *Y. ruckeri* O1 NCIMB 2194^{T}. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194$^{T);}$ Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (TVT). Arrows indicate molecular weight (kDa). Arrow heads indicate main recognition bands.

4.3.7.2. Iron regulated outer membrane proteins

Western blot analysis IROMPs by Y. ruckeri isolates is displayed in Figure 4.9. Reactive IROMP's were detected using a-bt 2 rabbit antiserum. Two bands were observed in bt 2 isolate at ~ 150 kDa. No antigenic bands were observed from the remaining serotypes.

Figure 4.9. Western blot analysis of IROMPs of *Y. ruckeri* isolates. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194$^{T);}$ Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (TVT). Arrows indicate molecular weight (kDa). Arrow head indicate main recognition bands.

4.3.7.3. Lipopolysaccharide

Western blot analysis of the LPS from six *Y. ruckeri* isolates (O1, O2, O5, O6, O7, TVT) strains (Figure 4.10) was carried out using polyclonal rabbit serum raised against *Y. ruckeri* TVT bt 2. Here, it was observed that the LPS profile for bt 2 was unique and did not cross react with any epitopes from other serogroups. Banding opattern was similar to that of the silver stained profiles, as long repeating units were observed.

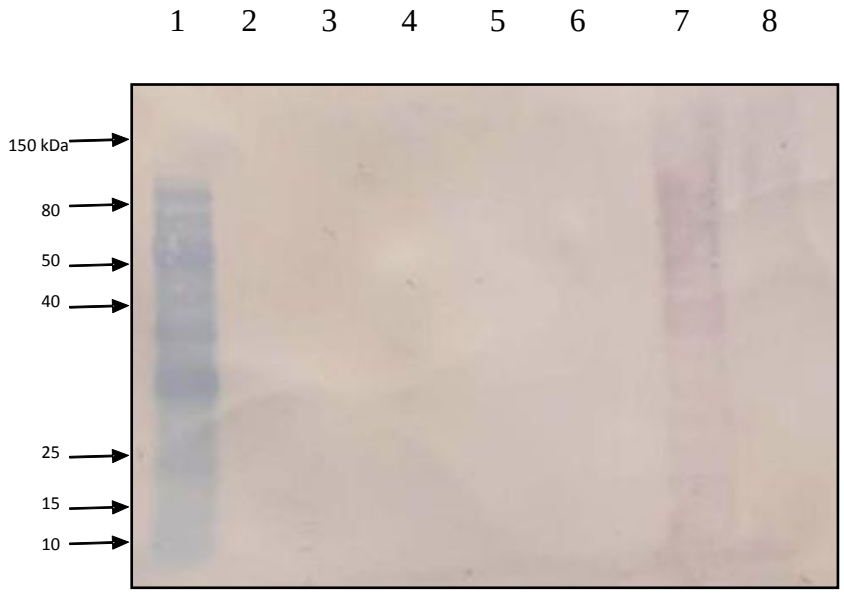


Figure 4.10. Western blot analysis of LPS from *Y. ruckeri*. LPS was separated by SDS-PAGE, blotted onto nitrocellulose, and reacted with anti-*Y. ruckeri* rabbit serum followed by immunochemical staining. Ladder banding patterns detected using silver stain. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (EX5) Lane 8 (RD6).

4.3.7.4. Lipopolysaccharide from biotype 2 isolates

Western blotting various worldwide isolates of bt 2 are displayed in Figure 4.11.

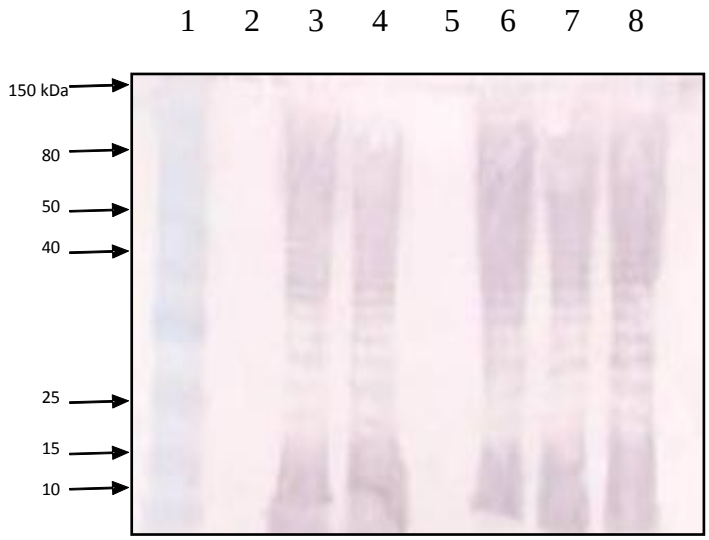


Figure 4.11. Western blot analysis of LPS *Y. ruckeri* bt 2 isolates. LPS was separated by SDS-PAGE, blotted onto nitrocellulose, and reacted with anti-*Y. ruckeri* rabbit serum followed by immunochemical staining. Ladder banding patterns detected using silver stain. Lane 1 (molecular marker), Lane 2 (O1 Hagerman); Lane 3 (Ex5), Lane 4 (RD6), Lane 5 (R4), Lane 6 (H142/2), Lane 7 (486), Lane 8 (Arctic charr). Arrows indicate molecular weight (kDa).

4.3.7.5. Effect of temperature on Lipopolysaccharide structure

Y. ruckeri O1 and EX5 bt 2, LPS was extracted from cells grown at 16^{o}C, 22^{o}C and 27^{o}C (Figure 4.12). With these cells, it was found that temperature did not alter the LPS profile or in the reaction with the polyclonal antiserum. A slightly stronger reaction was observed in the isolate grown at 27^{o}C.

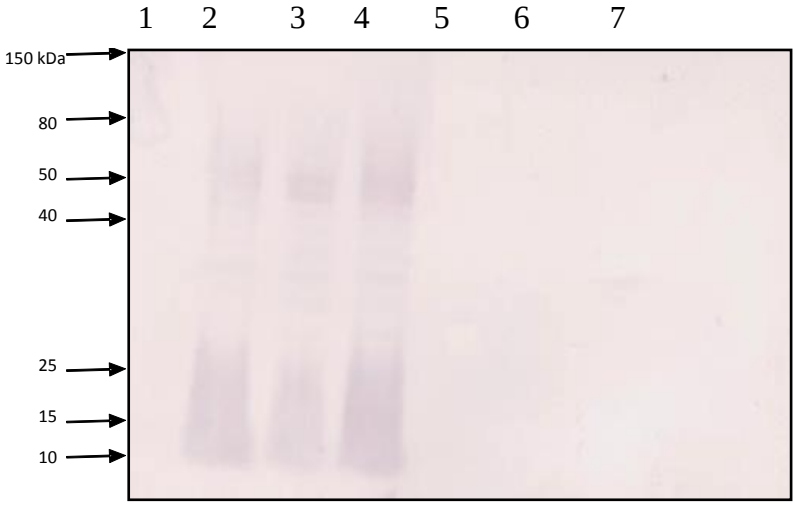


Figure 4.12. Western blot analysis of O1 and EX5 *Y. ruckeri* isolates strains grown at 16^{o}C, 22^{o}C and 27^{o}C. Ladder banding patterns detected using silver stain. Lane 1 (Molecular marker); Lane 2 (O1 NCIMB 2194^{T} 16^{o}C); Lane 3 (O1 NCIMB 2194^{T} 22^{o}C); Lane 4 (O1 NCIMB 2194^{T} 27^{o}C); Lane 5 (EX5 16^{o}C); Lane 6 (EX5 22^{o}C); Lane 7 (EX5 27^{o}C).

4.3.8. Carbohydrate content of extracellular products

Figure 4.13 demonstrates that regardless of serotype or bt, no carbohydrates were detected in the ECP's of selected *Y. ruckeri* isolates.

Figure 4.13. Nitrocellulose paper stained with Schiff reagent for carbohydrate visualisation of ECP's. Lane1 (Molecular Marker) Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (EX5) Lane 8 (RD6). Lane 9 (HRP positive control). Arrows indicate molecular weight (kDa). Arrow head indicate positive control.

4.3.9. Carbohydrate content of OMPs

Figure 4.14 demonstrates that regardless of serotype or bt, no carbohydrates were detected in the OMP's of selected *Y. ruckeri* isolates.

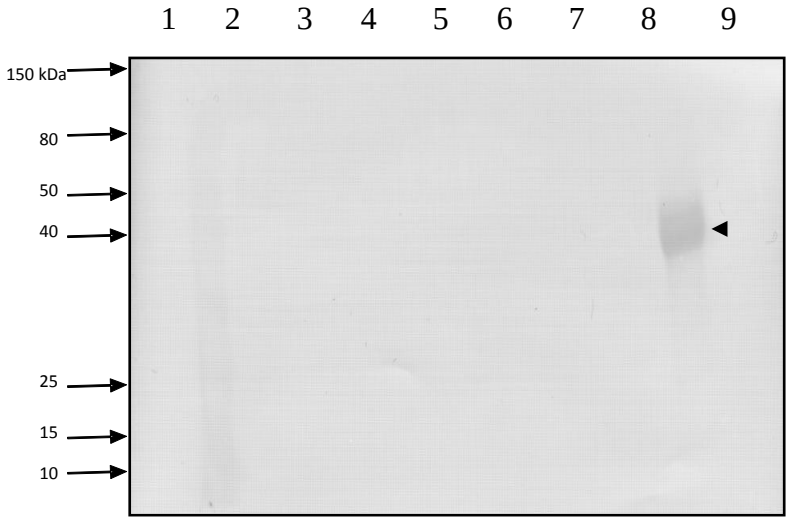


Figure 4.14. Nitrocellulose paper stained with Schiff reagent for carbohydrate visualisation of OMP's. Lane 1 (Molecular Marker) Lane 2 (O1 NCIMB 2194^{T}); Lane 3 (O2 NCTC 12266); Lane 4 (O5 NCTC 12268); Lane 5 (O6 NCTC 12269); Lane 6 (O7 NCTC 12270); Lane 7 (EX5) Lane 8 (RD6). Lane 9 (HRP positive control). Arrows indicate molecular weight (kDa). Arrow head indicate positive control.

4.3.10. Virulence studies

Virulence studies between bt 1 and bt 2 isolates of *Y. ruckeri* in naïve rainbow trout suggest that both biotypes of serotype O1 are extremely pathogenic to fish when challenged i.p. with 1 x 10^4 CFU ml^{-1}. Total, i.e. 100%, mortality was achieved after 2 days (Figure 4.15). Serogroups O2 and O5 caused 80% mortality after a similar period using 1 x 10^4 CFU ml^{-1} (Figure 4.16).

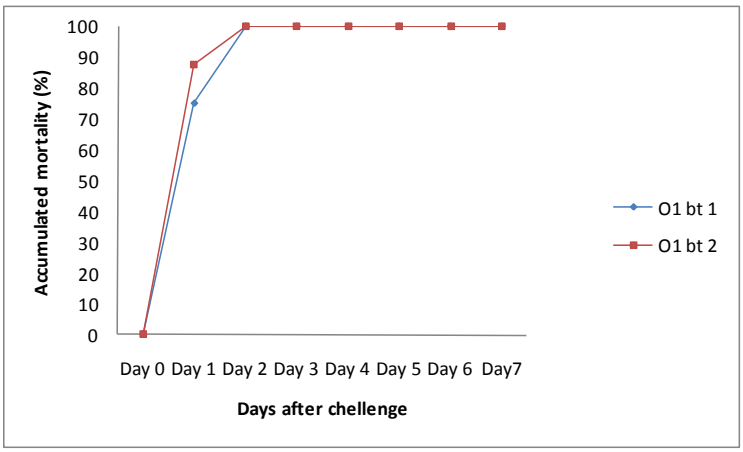


Figure 4.15. Accumulated mortality following I.P injection of of *Y. ruckeri* serogroups O1 bt 1 and O1 bt 2. Fish had previously not been vaccinated against ERM.

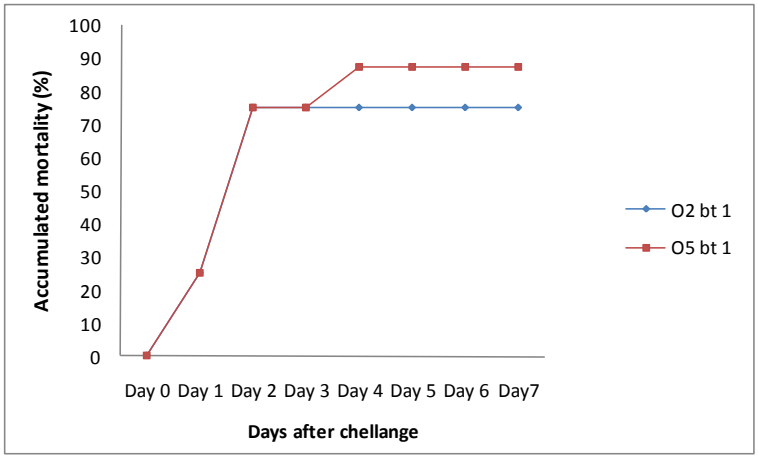


Figure 4.16. Accumulated mortality following I.P injection of *Y. ruckeri* serogroups O2 bt 1 and O5 bt 1. Fish had previously not been vaccinated against ERM.

4.3.11. Cross protection studies

Cross protection studies were carried out in order to determine the virulence of bt 2 isolates in previously vaccinated fish. Initially isolate was YR1 highly virulent to control rainbow trout (Figure 4.17). Control fish began to die on day 1 and the mortality continued exponentially until day 5. Cumulative mortality of reached 86.6% after 12 days, and then remained constant until the end of the experiment, fish began to die at

day 2 and 3 in the monovalent and bivalent vaccinated groups respectively. Comparison of the RPS against the homologous isolates revealed that the bivalent vaccine induced much higher protection (RPS of 92%) than the standard monovalent vaccine (RPS of 80%) when challenged against YR1 isolate.

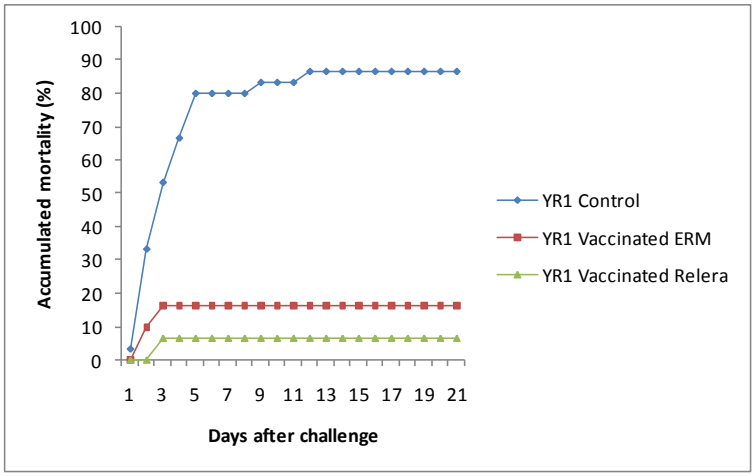


Figure 4.17. Accumulated mortality following I.P injection of *Y. ruckeri* O1 bt 1 (YR1) isolate. Fish had been vaccinated using standard Aquavac ERM ™ and Aquavac Relera™ bivalent vaccine.

R1 bt 2 isolate was highly virulent to control rainbow trout (Figure 4.18). Control fish began to die on day 1 and the mortality continued exponentially until day 3. Cumulative mortality of reached 83.3 % after 18 days, and then remained constant until the end of the experiment, fish began to die at day 2 in the monovalent and bivalent vaccinated groups respectively. Comparison of the RPS against the homologous isolates revealed that the bivalent vaccine induced much higher protection (RPS of 96%) than the standard monovalent vaccine (RPS of 76%) when challenged against R1 bt 2 isolate.

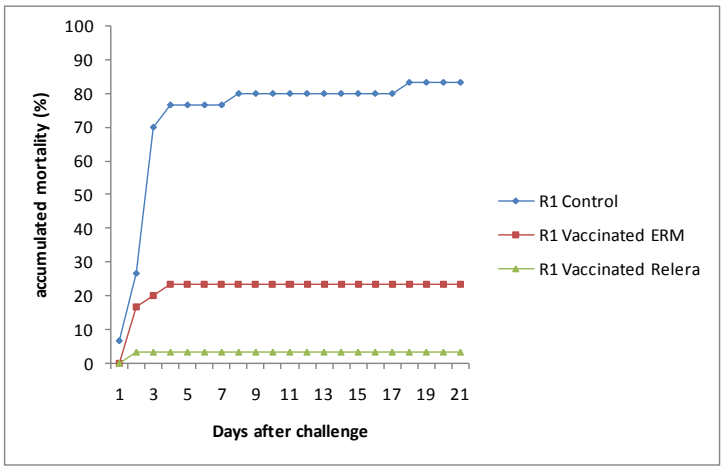


Figure 4.18. Accumulated mortality following I.P injection of *Y. ruckeri* O1 (TVT) bt 2 isolate. Fish had been vaccinated using standard Aquavac ERM ™ and Relera bivalent vaccine.

UK 250-181/2 bt 1 isolate was highly virulent to control rainbow trout (Figure 4.19). Control fish began to die on day 1 and the mortality continued exponentially until day 6. Cumulative mortality of reached 79.7 % after 11 days, and then remained constant until the end of the experiment, fish began to die at day 2 in both vaccinated groups respectively. Comparison of the RPS against the homologous isolates revealed that the bivalent vaccine induced much higher protection (RPS of 86%) than the standard monovalent vaccine (RPS of 79%) when challenged against UK 250-181/2- isolate.

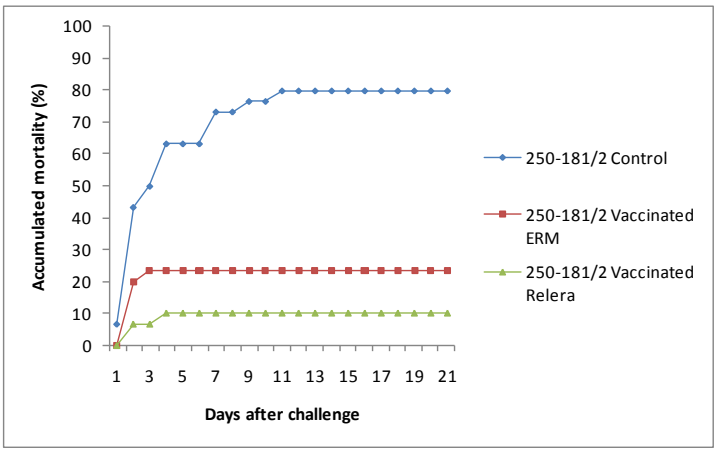


Figure 4.19. Accumulated mortality following I.P injection of *Y. ruckeri* O1 (250-181/2) bt 2 isolate. Fish had been vaccinated using standard Aquavac ERM ™ and Relera bivalent vaccine.

Bas-2a bt 2 isolate was highly virulent to control rainbow trout (Figure 4.20). Control fish began to die on day 1 and the mortality continued exponentially until day 5. Cumulative mortality of reached 69 % after 7 days, and then remained constant until the end of the experiment, fish began to die at day 1 in the monovalent vaccinated group respectively. No mortality was observed in the bivalent vaccine group. Comparison of the RPS against the homologous isolates revealed that the bivalent vaccine induced much higher protection (RPS of 100%) than the standard monovalent vaccine (RPS of 86%) when challenged against Bas 2a isolate.

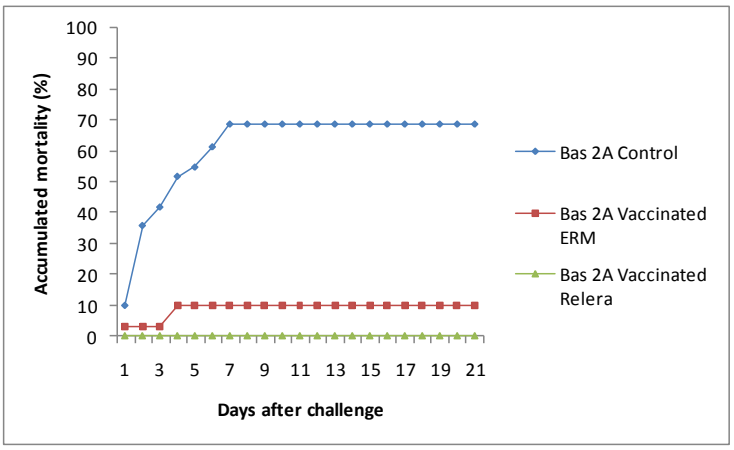


Figure 4.20. Accumulated mortality following I.P injection of *Y. ruckeri* O1 (BAS 2A) bt 2 isolate. Fish had been vaccinated using standard monovalent Aquavac ERM ™ and Relera bivalent vaccine.

Isolate 66542 bt 2 isolate was highly virulent to control rainbow trout (Figure 4.21). Control fish began to die on day 1 and the mortality continued exponentially until day 3. Cumulative mortality of reached 73.2 % after 8 days, and then remained constant until the end of the experiment, fish began to die at day 2 in both vaccinated groups respectively. Comparison of the RPS against the homologous isolates revealed that the bivalent vaccine induced much higher protection (RPS of 91%) than the standard monovalent vaccine (RPS of 70%) when challenged against 66542 bt 2 isolate.

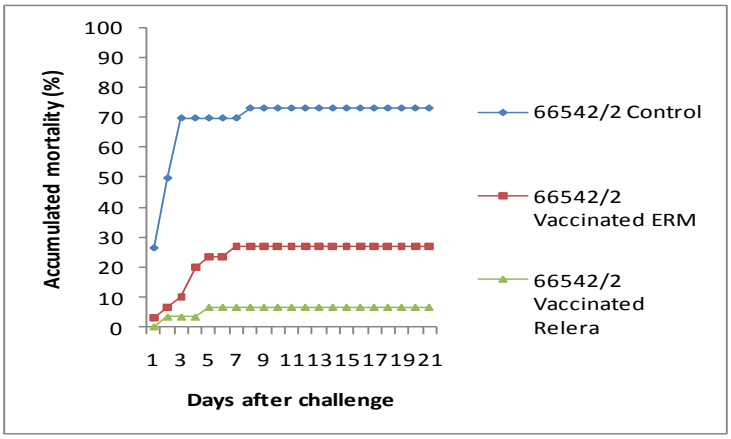


Figure 4.21. Accumulated mortality following I.P injection of *Y. ruckeri* O1 (6542/2) bt 2 isolate. Fish had been vaccinated using standard monovalent and Relera bivalent vaccine.

Danish DenA bt 2 isolate was highly virulent to control rainbow trout (Figure 4.22). Control fish began to die on day 1 and the mortality continued exponentially until day 11. Cumulative mortality of reached 66.6 % after 13 days, and then remained constant until the end of the experiment, fish began to die at day 2 in both vaccinated groups respectively. Comparison of the RPS against the homologous isolates revealed that the bivalent vaccine induced much higher protection (RPS of 100%) than the standard monovalent vaccine (RPS of 96%) when challenged against Danish DenA- isolate

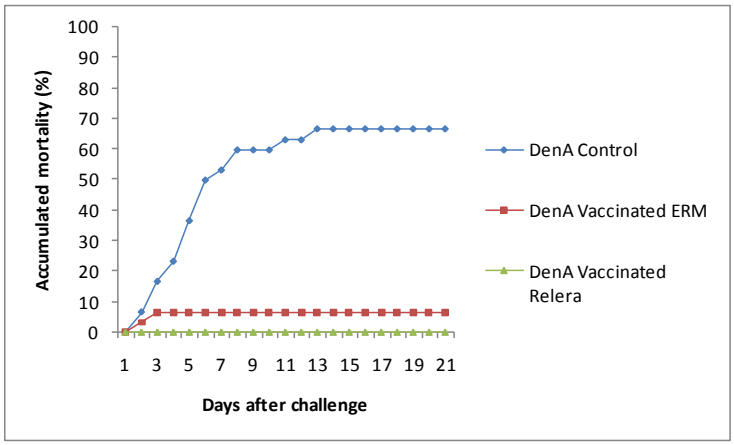


Figure 4.22. Accumulated mortality following I.P injection of Danish *Y. ruckeri* O1 (DenA) bt 2 isolate. Fish had been vaccinated using standard monovalent and Aquavac Relera™ bivalent vaccine.

4.3.12. Internal & External examination of infected fish

4.3.12.1. External examination

Externally infected fish exhibited abdominal distension, exophthalmia and darkening of the skin in most fish. A small number of fish displayed marked reddening around the base of the operculum, and fins or bleeding at the anal vent 2 days after infection. 'Classical' redmouth symptoms (haemorrhages around eyes, and mouth) were not observed. Pale and anaemic gills were observed.

4.3.12.2. Internal examination

Internal examination of rainbow trout revealed classic haemorrhagic septicaemia conditions (Figure 4.23). Infected fish displayed slight swelling of the kidney along its length. The liver was pale, mottled with hemorrhagic areas (a). Haemorrhagic areas were observed on the heart and visera (b). Spleens varied in size and colour from pale to dark red, with various degrees of enlargement (c). Accumulation of clear ascitic fluid was found in the peritoneal cavity.

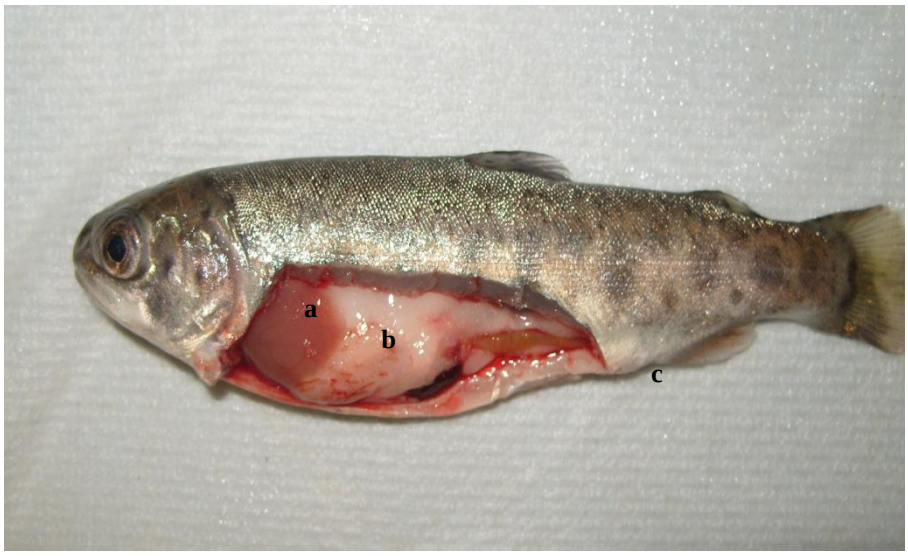


Figure 4.23. Internal examination of rainbow trout 5 days post infection with bt 2 of *Y. ruckeri*. Note: reddening of the operculum (a), fins (b) and the protruded anal vent (c).

4.3.12.3. Histology

Many histological sections exhibited non-specific pathological changes. Pathological changes observed from skin section of fish exposed to *Y. ruckeri* are shown in Figure 4.24. Observed on the skin of infected rainbow trout namely an increase in mucus cells and this was associated with marked epidermal hyperplasia. Thinning of the stratum spongiosum and muscle degradation was also observed 5 days post exposure.

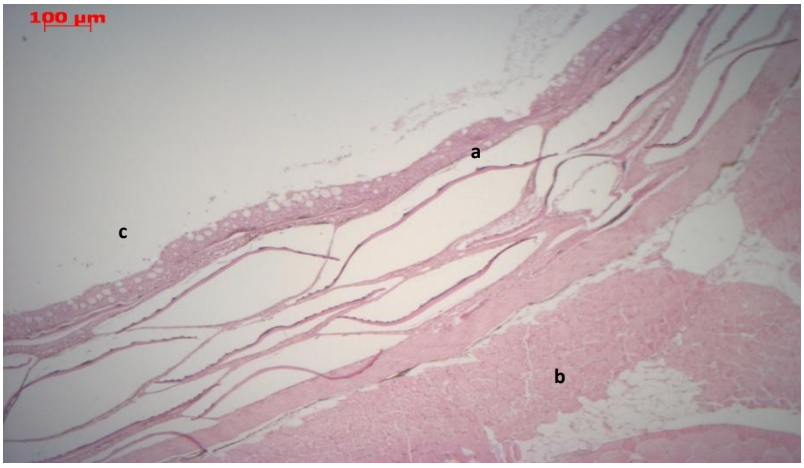


Figure 4.24. Light microscopy of rainbow trout skin after 5 days infection *Y. ruckeri* bt 2 post showing Separation of the stratum spongiosum (a), degradation of red muscle (b), and increased hyperplasia and mucus production on the epidermis (c). H & E stain x 400 (Bar = 100 µm).

Pathological observations of rainbow trout gill sections after exposure to *Y. ruckeri* are displayed in Figure 4.25. Hypotrophy and hyperplasia of the epithelial cells on the filaments and lamellae were observed along with lamellae fusion and clubbing. Furthermore, capillary dilatation of the lamellae and diffuse haemorrhages in both the lamellae and filaments were observed.

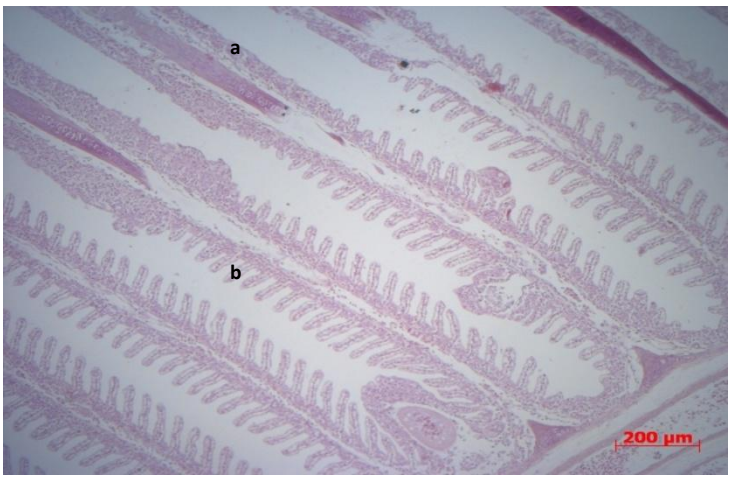


Figure 4.25. Light microscopy of rainbow trout gill 5 days post infection with *Y. ruckeri* bt 2 showing lamellae destruction and fusion (a), haemorrhages in the filaments (b) H & E stain x 100 (Bar = 200 µm).

Noticeable changes were observed in the posterior kidney. Sections revealed large amounts of congestion with blood suggesting loss of haemopoeitic tissue (Figure 4.26). A change in kidney tubule structure in sample later into the infection was due to slight interstitial haemopoeitic hyperplasia. Pyknotic cells in the lumen and glomerulus were observed, suggesting that the cells were going through apoptosis. Basophilic immature kidney tubules were often seen, highlighting signs of regeneration. Melano-macrophages in the kidney increased throughout the course of the infection.

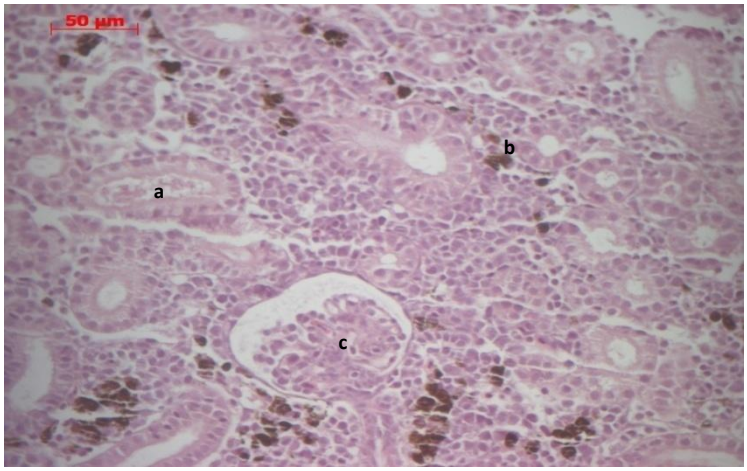


Figure 4.26. Light microscopy of rainbow trout kidney 5 days post infection *with Y. ruckeri* bt 2 showing glomerular nephritis, melanin deposits and destruction of the renal tubules. H & E stain x 400 (Bar = 50 µm). (Destruction of the renal tubules (a) melanin deposits (b) and glomerular nephritis(c)).

Pathological changes of bt 2 infections of the liver of rainbow trout as shown in Figure 4.27. Liver sections displayed marked areas of necrosis with diffused areas lipidosis of hepatocytes.

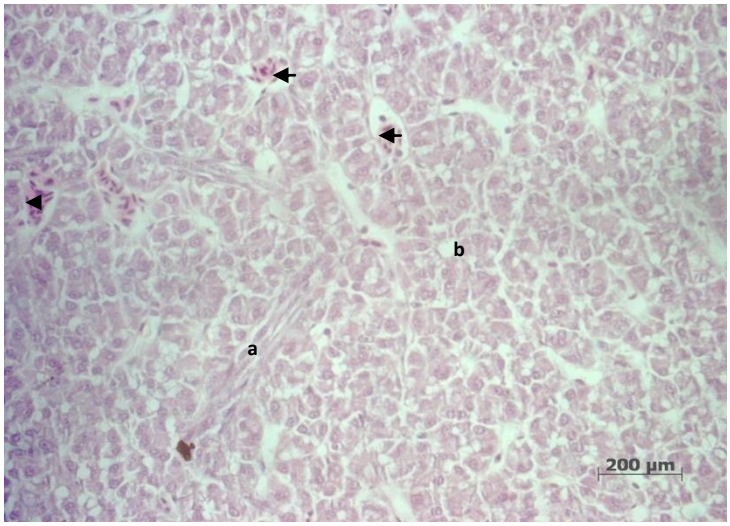


Figure 4.27. Light microscopy of rainbow trout Liver 5 days post infection with *Y. ruckeri* bt 2. Hyperplasia of necrotic foci (a), lipidosis of hepatocytes (b) H & E stain x 100 (Bar = 200 µm). Arrow indicate blood congestion.

Pathological observations in rainbow trout heart are displayed in Figure 4.28. Infection resulted in hyperplasia of the epicardium. Myocardial necrosis was observed in acute cases.

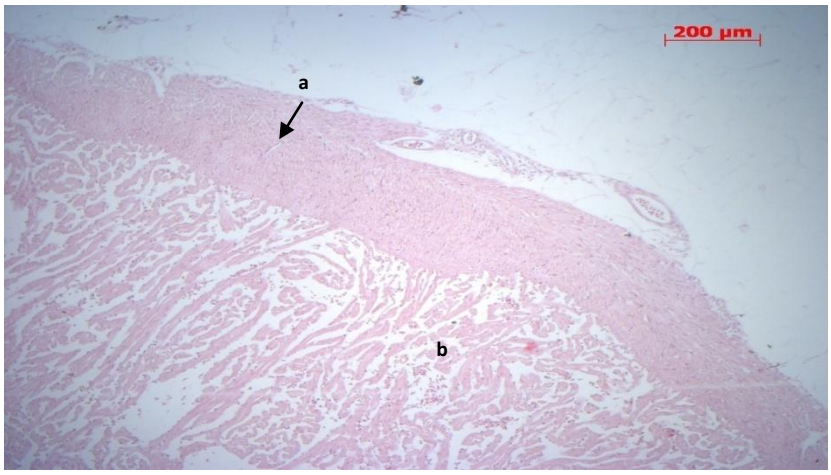


Figure 4.28. Light microscopy of rainbow trout heart 5 days post infection with *Y. ruckeri* bt 2. Hyperplasia of epidardium (a), Myocardial necrosis (b) H & E stain x 100 (Bar = 200 µm).

4.4. Discussion

Incidences of bt 2 isolates of *Y. ruckeri* causing diseases in cultured fish species have been increasing ever since their initial isolation (Davies and Frerichs, 1989; Austin *et al*., 2003). It has been stated that intensive aquaculture could potentially cause the increase in more virulent/pathogenic phenotypes which aid their spread in the natural environment (Pulkkinen *et al.,* 2010). With respect to bacterial antagonism, it has been shown that isolates of *Y. ruckeri* are not antagonistic to each other. Thus, disproving any suggestion that bt 2 isolates have arisen due to their ability to competitively exclude bt 1 isolates through antagonism caused by aquaculture practices.

Overall, the whole cells of *Y. ruckeri* displayed a homogeneous enzymic profile; with the whole cells of all serogroups and biotypes producing strong phosphohydrolases reactions. Indeed, alkaline phosphatase, a key phosphohydrolase, has been identified as a key virulence factor in a number of pathogens. Thus, Saha *et al.* (1985) demonstrated that cell-associated phosphatase may play a role in the virulence of *Legionella micdadei* by blocking the production of ROS by human neutrophils. Alkaline phosphatases have also been linked to a number of parasitic infections. For example, Volety and Chu (1995) demonstrated that the protozoan parasite *Perkinsus marinus* avoided the host immune defences by blocking the reactive oxygen intermediates of the eastern oyster, *Crassostrea virginica.* As *Yersinia* sp. have been shown to block the respiratory burst within macrophages, it is necessary to determine if alkaline phosphatases play a role in virulence with *Y. ruckeri*. It was recorded that all isolates and biotypes were positive for leucine arylamidase activity, which has been identified in a number of Gram negative fish and crustacean pathogens (Ridgway *et al.* 2008). Of relevance, leucine arylamidase has been described as a key virulence factor in the ECP's of *Vibrio pelagius* (Farto *et al.,* 1998).

Comparative studies between the excreted products of bt 1 and bt 2 isolates of *Y. ruckeri* are lacking. ECP's from selected isolates displayed fewer enzymic activities than the live cells, which suggests that many of these activities were associated with the cell envelope. In the phenotypic characterization of *Y. ruckeri* ECP's by the API ZYM and standard techniques, variability was low and homogenous reaction profiles were

observed from the serotypes and biotypes used. Overall the strains of *Y. ruckeri* used produced low amounts of ECP's the lack of which was mirrored in the low number of bands observed in the gel electrophoresis. Isolates from serotype O1 including bt 1 and bt 2 both showed strong N-acetyl-β-glucosaminidase reactions, whereas the other remaining serotypes did not. N-acetyl-β-glucosaminidase is a glycosidase enzyme that is associated with bacterial metabolism and the degradation of biomass in order to acquire nutrients, chiefly sugars. Glycosidase have been found in a variety of Gram negative organisms and have been implicated as key virulence factors (Olmsted and Mayen, 2003).

The ability of *Y. ruckeri* to produce phospholipase was detected by using lecithin agar. The role of secreted phospholipases is unclear in *Y. ruckeri.* In some bacterial pathogens, phospholipases appear to be the major cause of pathophysiological effects. Yet in other cases, phospholipases are key virulence factors, contributing to bacterial survival or dissemination without causing tissue destruction (Schmiel and Miller, 1999). The role of phospholipases as the main extracellular toxin responsible for virulence has been clearly demonstrated in fish pathogens such as *A. salmonicida* (Lee and Ellis, 1991), which produces a glycerophospholipid cholesterol acyltransferase (GCAT). Phospholipase A in *Y. enterocolitica* has been identified as a key virulence factor and is involved in flagellum regulation (Schmiel *et al.* 2000). The correlation between bt 2 and phospholipase activity is curious. It is possible that phospholipase activity in *Y. ruckeri* isolates is not associated with flagellum regulation like with other members of the species.

It has been highlighted in this study that regardless of serogroup or bt *Y. ruckeri* is able to produce siderophores under iron limiting conditions. Previously it was thought that *Y. ruckeri* did not produce siderophores (Davies, 1991b*).* It was not until later that it was discovered that *Y. ruckeri* was able to produce siderophores, although only in times of iron limitation (Romalde, 1991). All isolates were screened for the production of siderophores. These findings agree with previous authors that *Y. ruckeri* is able to produce siderophores under iron limiting conditions (Romalde *et al.,* 1991). Siderophores have been isolated from other members of the *Yersinia* genus (Perry *et al.,* 1999, Chambers *et al.,* 1996). Siderophores are not all structurally similar which

probably relates the bacterium's environment and lifecycle. Siderophores are small iron-chelating molecules and can be divided into three major classes, catecholates, hydroxamates, and heterocyclic compounds (Fernandez *et al.,* 2004). Other members of the *Yersinia* sp., only produce and utilize is the heterocyclic compound yersiniabactin, which has been related to pathogenicity (Perry *et al.*, 1999). West and Buckling, (2003) put forward the theory that there could be some correlation between pathogenesis and siderophore production. It was hypothesized that greater production of siderophores could lead to greater scavenging of iron molecules therefore leading the better bacterial growth which ultimately leads to increased virulence. It is unclear whether there is a difference between bt 1 and bt 2 in terms of their siderophore production. It was observed that in bt 1 and 2 of *Vibrio vulnificus* that siderophore mediated iron acquisition mechanisms were similar and probably reflected the nature of the pathogen and not an indicator of virulence (Biosca *et al.,* 1996).

There seems to be little difference in terms of virulence and ECP's produced by bt 1 and bt 2 isolates, suggesting that other factors are involved with the pathogenicity of *Y. ruckeri* in fish. There appears to be some correlation between the lack of excreted compounds *in vitro* and their ability to cause mortality *in vivo*. The toxicity of ECP's from other bacterial fish pathogens has been demonstrated by a number of studies (Magarinos *et al.,* 1992). The toxicity of ECP's to fish in the study by Magarinos *et al*. (1992) highlighted that toxicity was due to the protein components of the excreted products and not the LPS. From the lack of production of extracellular products and haemolysins coupled with the lack of toxicity to fish, it is possible that *Y. ruckeri* require cell to cell contact in order to overcome the immune system in fish. It is unknown whether ECP's secreted from *Y. ruckeri* are having some kind of immunomodulatory effect over the host. It is clear that they are not causing mortality however they could be having some immunomodulatory effect. As *Yersinia* sp. have been known to replicate within the phagolysosome of phagocytes, extracellular products could be involved in protecting the pathogen against certain components of the respiratory burst. It was observed by researchers that extracellular products produced by *R. salmoninarum* significantly decrease the respiratory burst in brook trout (*S. fontinalis*) (Densmore *et al*., 1998). Studies into the immune system after exposure to *Y. ruckeri* would help prove this hypothesis.

LPS from bt 1 and bt 2 isolates was unable to cause mortality in fish. LPS is known to be highly immunogenic and has been described to have a number of different modes of action despite being non-toxic to fish (MacKenzie *et al.,* 2003). Therefore, the data suggest that free LPS from lysed *Y. ruckeri* cells is not toxic to fish and this has been observed in many other studies. It is unclear to what extent this 'free' LPS is causing immunomodulation. In mammals, LPS is recognized by Toll-like receptor 4 (TLR4) and results in stimulating a variety of pro-inflammatory cytokines as a result (Rebl *et al.* 2010). To date, TLR 4 mediated endotoxin has not been identified in salmonids although a number of proinflammatory cytokines and acute-phase proteins (APP) have been known to be stimulated (MacKenzie *et al.,* 2003; Swain *et al.,* 2008). Pro-inflammatory cytokine studies would be useful in explaining the differences these antigens have in term of interaction with the immune system.

Western blotting was carried out using polyclonal rabbit anti serum raised against O1 bt 1 and O1 bt 2. It has been shown from the results that there is scope in the use of western blotting as an approach to typing strains of *Y. ruckeri*. The study provides evidence that there are numerous cross reactive antigens with identical structures with whole cell *Y. ruckeri* isolates regardless of serotype. The immunoblotting using anti O1 rabbit antiserum highlighted 7 identical bands common to all serotypes at 150, 75, 70, 60, 50, 35, 20 and 15 kDa. There was some cross reactivity at high molecular weights between serotype O1 bt 1 and bt 1. A band appeared to be present in only O1 bt 1 and bt 2 isolates at approximately 100-120 kDa.

The data suggest that *Y. ruckeri* produces IROMP's during culture in iron limiting medium. It was demonstrated that they are immunogenic and are specifically recognized by anti-bt 1 and anti-bt 2 antiserum. These reactions underline the fact that these antigens are likely expressed *in vivo* and that *Y. ruckeri* grows in the host under iron-restricted conditions. It is difficult to hypothesise the functions of these molecules although it could be a receptor for siderophores are previously stated by Crosa (1989) in enteric bacteria. From the western blotting it has been observed that these are expressed *in vivo* by the presence of a double band at ~100 kDa, supporting this hypothesis.

This approach was able to distinguish between major serotypes of *Y. ruckeri* by analyzing the LPS (O antigen structure). Western blotting using rabbit anti-EX5 antiserum highlights that bt 2 isolates are distinguishable from bt 1. Regardless of isolation source or isolation year bt 2 has a uniform LPS profile that western blotting detects. Although the O antigen is the basis for the serogroups, some cross reaction is observed when agglutination with O antigen preparations suggesting that it shares some common antigens. It could be possible to based on this data divide O1 into serogroups O1a and O1b as done previously by (Grisez and Ollevier, 1995) The sero-specificity of the LPS antigen also provided the basis of the O-agglutination reactions (Davies, 1990). The findings in this report suggest that there are differences within the serogroup. The use of specific antibodies to LPS has been used previously to characterize isolates of *F. psychrophilum* due to their slow growing nature and cross reactive antigens (Crump *et al*., 2003). The differences in the composition of the LPS structure could be a reason lack of cross protection due to recognition by the host immune system.

It has been shown that the production of LPS of *Y. ruckeri* is stable at various temperatures unlike other members of the genus. Kawaoka *et al.* (1983) noted that cultures of *Y. enterolitica* grown at 37^{o}C as opposed to 25^{o}C, expressed dramatically different LPS structures. From the western blot profiles it is clear that temperature has little effect over the O antigen structure, although expression of the molecule is. The differences observed in the profile are most likely due to the influence on temperatures effecting production of LPS. The bands in 16^{o}C are less predominant than bands grown at 22^{o}C or even 27^{o}C. However, it has been stated previously by Lloret *et al.* (1995) that the LPS structure of *Rhizobium meliloti* was influenced by ionic stress and osmotic pressure. The researchers believed that an adaptive mechanism of this strain for the special environment from which it was isolated. The variation in the O antigen between bt1 and bt 2 isolates could be further explained by the development of specific monoclonal antibodies toward these structures. The use of rabbit polyclonal antiserum as a diagnostic tool must also be treats with caution because of the potential differences in reactivity with the immune system.

Knowledge about the protective roles of LPS suggests that this could be a reason for the lack of cross protection between the biotypes and serotypes. It has been previously

suggested that the molecule is the protective antigen, and comparisons with other bacterial pathogens in the literature suggests that this is the case. It has been observed that in *Francisella tularensis* infections in mammals that the LPS profile of the two main sub species, *F. tularensis* subsp. *tularensis* (type A) and *holarctica* (type B) is identical and that a vaccine provide good cross protection against these types (Thirumalapura *et al.* 2005).

Carbohydrate was not found to be excreted by *Y. ruckeri*. Chen *et al*., (2010) demonstrated that in comparison with other members of the *Yersinia* sp., *Y. ruckeri* O-antigen operon contained a *neuB* sialic acid synthase gene. Researchers hypothesized that the bacterium was able to produce a sialated outer surface structure. The results from this study suggest that this is not the case. Carbohydrate has been observed from other bacterial fish pathogens. Thus, Jung *et al.* (2008) identified carbohydrate bands at low and high molecular weights from the ECPs of *in vivo* and *in vitro* grown cultures of *Ph. damselae* subsp. *piscicida,* these were hypothesised to be salic acids and carbohydrate capsular material.

Virulence studies between bt 1 and bt 2 isolates of *Y. ruckeri* in naïve rainbow trout suggest that both biotypes of serotype O1 are pathogenic to fish when I.P challenged, these results are in accordance to previous authors (Arias *et al*., 2007). On the basis of the histopathological evidence, it appears that there is little difference in the pathological characteristics of bt1 and bt 2 infections.

Cross protection studies indicated that regardless of isolate used, that good protection levels can be achieved through the use of a commercial bivalent vaccine against bt 1 and bt 2 isolates of *Y. ruckeri*. However, there is no evidence to date that a vaccine can protect against several different serotypes and biotypes of *Y. ruckeri*. The antigenic results appear to indicate that the LPS structure is indeed to dominant protective antigen in *Y. ruckeri* vaccines. This has been indicated by the similarities between bt 1 and bt 2 isolates in terms of their ECP's, WCP and OMP profile. These results agree with other publications (Wheeler *et al*., 2009; Ström-Besto *et al*., 2010). It is currently unknown to what extent the role of the flagellum is having upon the immune system in terms of

protection. The lack of cross protection has been noted against vaccines of other fish pathogens. It was recently demonstrated that vaccinated eels with serovar E of *Vibrio vulnificus* can be infected by other less frequent serovars of the pathogen possessing low degree of virulence which act as a secondary pathogen (Fouz and Amaro, 2003). It is currently not known whether bt 2 isolates are acting as a secondary pathogen. The data suggests that there has been a shift in bacterial population dynamics and due to the lifestyle of *Y. ruckeri*, LPS of the specific bt needs to be included the a vaccine to provide protection. Although the study has highlighted antigens that are protective to a serotype of biotype, the identification of antigens critical to the induction of protective immunity against multiple serotypes is necessary for development of vaccines. However, future vaccines may have to contain a wide range of isolates (multivalent) in order to protect from the disease due to the protective nature of the O and IROMP antigens.

Bath vaccination appears to be a suitable method of vaccination against ERM. The route of administration probably has some effect over the way that the antigens are presented to the relevant recognition and effector molecules within the host (Palm *et al.,* 1998). I.P injection has been demonstrated to provide better protection againt the disease (*Data not shown*) although, the use of i.p. injection however is unsuitable to use on a commercial scale to the number of fish and the time consuming nature of the procedure.

In summary, it appears that there is marked homogeneity between serogroups and biotypes of *Y. ruckeri* in terms of their production of ECP's. The results from the ECP's and LPS analysis suggests that the excreted compounds by *Y. ruckeri* may not be as important as cell surface characteristics in term of virulence *in vivo* due to their lack of toxicity. Studies into the immune response will help understand their role in pathogenesis. The O antigen appears to be highly antigenic and is the dominant sero-specific antigen which does not change with geographical region Western blotting could be a method used in the future to accurately type isolates as rabbit antiserum was able to accurately type each isolate. The LPS molecules was unaffected by the temperature and iron limiting conditions. Outer membrane proteins were altered culture in iron limiting medium and were found to be antigenic. Virulence studies suggest that there is little difference between all isolates, supporting the hypothesis that there has been a change in

bacterial population dynamics in the natural environment. The production of a commercial bivalent vaccine has shown that using isolates from both biotypes provided excellent protection against the disease.

Chapter 5. Innate immune response towards *Y. ruckeri* infections in vaccinated rainbow trout

5.1 Introduction

Commercial vaccines produced against bt 1 isolates of *Y. ruckeri*, have highlighted good levels of protection by using bath and oral vaccination strategies (Johnson and Amend, 1983; Rodgers, 1990). As fish are most susceptible to ERM at ~4 g, bath vaccine is often recommended (Hastein *et al.*, 2005). Most experimental studies are concerned with challenging naive fish with bacterial pathogens in order to elucidate pathogenicity mechanisms or virulence factors (Toranzo *et al*., 1995; Eldar *et al*., 1997). The interaction of bacterial pathogens with vaccinated fish is something that has been greatly overlooked. Understanding how bt 2 isolates of *Y. ruckeri* interact with previously vaccinated fish will be of paramount importance in understanding some of the reasons why mortalities occur in the field.

The nonspecific immune response has been stated to be of paramount importance in defending the host from *Y. ruckeri* infections (Raida and Buchmann, 2008b). Instead of relying on antibodies for pathogen recognition, innate immunity relies upon pattern-based recognition of non-self cells (Ewart *et al*., 2001). The innate immune system relies on both the cell mediated immunity and humoral factors (Magnadóttir, 2006). Cell mediated immunity (CMI) relies upon the activation of phagocytic cells, such as macrophages and neutrophils (Nakanishi *et al*., 1999). Evasion of the microbial action of phagocytes has been studied in other members of the *Yersinia* sp. but to date no research has focused or hypothesised on how *Y. ruckeri* evades the phagocytic processes (Ruckdeschel *et al*., 1997). The humoral immune response is associated with serum of the host, and there are arrays of soluble substances, such as inhibitors and lysins, which protect the host by inhibiting the growth of micro-organisms (Bayne and Gerwick, 2001). Young *et al*. (2006) described ladderlactin, a non specific humoral factor, which was able to bind to *Y. ruckeri*, which in turn enhanced complement activity and phagocytosis.

The process of phagocytosis is of importance when protecting a host from bacterial infection (Aderem and Underhill, 1999). Neutrophils and macrophages are involved in

the internalisation of bacteria, killing them through the process of respiratory burst (Aderem and Underhill, 1999). It has been shows that following injection with whole cell inactivated vaccines of *Y. ruckeri* there was a large influx of macrophages and neutrophils (Alfonso *et al*., 1998). Phagocytic cells, such as neutrophils and macrophages, produce molecules of various reactive oxygen species (ROS), such as OH^-, H_2O_2 and superoxide anion ($O2^-$) during the respiratory burst which have been shown to be inhibitory to range of microbial pathogen (Babior, 1984). Currently work has not been carried out on the role phagocytosis and the involvement of ROS during *Y. ruckeri* infections.

The reactive oxygen intermediate (ROI) pathway is another oxygen-dependent pathways used by phagocytes to fight against microbial infection (Bogdan *et al*., 2000). Reactive oxygen intermediates such nitric oxide (NO) are produced by a family of enzymes known as nitric oxide synthases (NOS) (Campos-Perez *et al*., 2000). NO has been demonstrated to be inhibitory to a wide range of viral, bacterial and parasitic infections (Clark and Rockett, 1996). It has also been shown that NO can combine with species ROS, particularly superoxide anion ($O2^-$) produced during the respiratory burst, to produce peroxynitrite, a potent free radical (Carreras *et al*., 1994). The formation of peroxynitrite has been shown to cause cell wall disruptions, cell lysis and morphological changes in bacteria (Tecder-Ünal *et al*., 2008). Cell surface antigens such as LPS of Gram negative bacterial pathogens have been shown to be potent induces of nitric oxide (Neumann *et al*., 1995). Macrophages can also produce nitric oxide (NO), which has been shown to be important antibacterial compound against bacterial pathogens (Campos-Pérez *et al.*, 2000).

Apoptosis or programmed cell death (PCD) is a vital part of life in multicellular organisms (Wilson *et al*., 2002). Apoptosis is involved in a wide variety of different biological systems, for example, cell turnover and modulating the pathogenesis of many infections. (Cohen, 1997). In this process, cells within the body of an organism die without inducing any untoward response. Cells can be induced into apoptosis by specific signals. Two pathways, the intrinsic and extrinsic death pathways, both involving caspases, have been identified in most cases of caspase-dependent apoptosis. The caspases can be divided three groups, the inflammatory caspases (-1, -4, -5, and -

11), the initiator caspases (2, -8, -9, and -10), and the executioner caspases (-3, -6, and -7) (Jin *et al*., 2008). The intrinsic death pathway involves mitochondrial release of cytochrome *c*, which interacts with Apaf-1 (apoptosis proteinase-activating factor-1) and dATP to promote procaspase-9 autoactivation, which in turn activates downstream effectors such as caspase-3, -6 and -7 (Stoka *et al*., 2006). The extrinsic death pathway is initiated by the engagement of cell surface death receptors (CD95/Fas/APO-1 and TNFR). The complex of death receptors and ligands leads to the recruitment of the adapter molecule FADD, and the activation of caspases 8 which in turn mediates with caspase 3 (Fulda and Debatin, 2006). In both pathways, after activation, caspases cleave various cellular substrates (such as PARP and Lamin A/C) resulting in membrane blebbing, chromatin condensation, and the formation of apoptotic bodies (Lauber *et al*., 2003). Once a signal is received cell shrinkage and rounding are shown because of the breakdown of the proteinaceous cytoskeleton by caspases, pyknotic nuclei can be observed as the chromatin condenses (Lauber *et al*., 2003). Karyorrhexis follows as the pyknotic nucleus breaks apart. The cell membrane 'blebs' characteristically. The cell then breaks apart into apoptotic bodies which are actively phagocytosed.

Certain pathogens of both mammals and fish have evolved ways of targeting the mechanisms of apoptosis to either stimulate it or inhibit it. Bacterial pathogens induce apoptosis in host's cells for three reasons: firstly, activation of apoptosis to destroy cells, secondly utilization of apoptosis to initiate inflammation and finally, inhibition of apoptosis (Zychlinsky and Sansonetti, 1997). Pathogens can induce apoptosis by a variety of mechanisms such as, interaction with certain cell receptors, activation of cellular signal transduction systems, disruption of membrane integrity by hemolysin and regulation of caspase functions by a bacterial protein translocated via T3SS directly into the cytoplasm of infected cells (Ojcius *et al*., 1998, Galan and Collmer, 1999; Minamino *et al*., 2003, Bai *et al*., 2010). With regards to other fish pathogens, it has been demonstrated that the intracellular pathogen *P. salmonis* was able to induce apoptosis in rainbow trout macrophages (Rojas *et al*., 2010). Similarly, the virulence plasmid of *A. salmonicida* was associated with apoptosis in goldfish (*Carassius auratus*) macrophages (Shao *et al.*, 2004). Apoptosis has been studied in great detail in *Yersina* sp. infections of mammals (Monack *et al*., 1997). OMPs associated with the T3SS of *Yersinia pestis* has been shown to cause apoptosis in macrophages which in turn disrupts to induction of proinflammatory cytokines hampering the innate immune

system (Lemaître *et al*., 2006). It is unclear whether similar processes are occurring within *Y. ruckeri*. It has been hypothesized that since the bacteria possess a T3SS and have similar OMPs that these processes may occur (Tobback *et al*., 2007). It is not only the OMPs that are responsible for inducing apoptosis in immune cells. LPS has been demonstrated to induce apoptosis by 2 independent mechanisms: firstly, through the secretion of TNF-α and secondly, through the production of NO (Xaus *et al*., 2000).

Apoptotic cell death is accompanied by a change in plasma membrane structure and characterized by surface exposure of phosphatidylserine (PS) (van Engeland *et al*., 1998). Using the Annexin-V affinity assay is one method of detecting PS changes. Annexin-V is not able to bind to normal, 'live' cells since it cannot penetrate the phospholipid bilayer. In dead cells, however, the integrity of the plasma membrane is lost, and PS in the inner leaflet of the membrane is available to bind applied Annexin-V. To detect 'dead' cells, a membrane impermeable DNA stain, such as propidium-iodide (PI), is used. As apoptotic cells expose PS at the outer leaflet of the plasma membrane the PI cannot permeate the cells membranes as they are still intact (van Engeland *et al*., 1998). Using flow cytometry, bivariate analysis shows that vital cells are negative for Annexin-V and PI, apoptotic cells are Annexin-V positive, but PI negative, while dead cells are positive for both Annexin-V and PI. Using flow cytometry to discriminate between cellular states using these stains has been used widely to understand apoptotic processes (Eray *et al*., 2001).

To reveal the immune and pathological pathways involved in *Y. ruckeri* infections, it is important to quantify the cytokines involved. Cytokines are glycoproteins that act as signalling molecules with vertebrate body, they control many aspects of cellular behaviour such as proliferation, differentiation and function (Secombes *et al*., 1996; Wood, 2001). Cytokines play a central role in inflammation, apoptosis, lymphocyte activation, proliferation, differentiation and modulating the immune response (Giulietti *et al*., 2001).

Cytokines are grouped based upon their family such as interferons (IFN), interleukins (IL), chemokines, colony stimulating factors (CSF), and tumour necrosis factor (TNF).

And within these families there are many members (Secombes *et al*., 1996). Cytokines do not act on an endocrine level like hormones, they act locally and are produced by local tissues or act of cells in that tissue (Wood, 2001). The introduction of bacteria into a normal sterile tissue results in the formation of a wide range of cytokines. IL-6 and TNF-α are normally the first to be produced as they deal with inflammation and activate other cells within the area of infection. Cytokines Il-1 and TNF-α also help the liver produce a wide range of proteins that acts as opsonins (Wilson *et al*., 2002). The activation of phagocytes by cytokines, such as Il-6, helps the process of bacterial killing (Tanaka *et al*., 1995).

Cytokines and the innate immune system are signalled though pathogen recognition receptors (PRR) and pathogen-associated molecular patterns (PAMP's). PAMP's are molecules such as flagellins, LPS, OMP, capsules and pili which are recognized by the innate immune system. They activate innate immune responses through PRR such as toll-like receptors (TLR). TLR-5 is a key receptor in recognizing bacterial flagellin (Tsujita *et al*., 2006). In fish, TLR 5 has been demonstrated to be highly upregulated in the liver following exposure to flagellin (Tsujita *et al*., 2004). One of the potential reasons why non-motile *Y. ruckeri* isolates are not being removed by the host's immune system is that the lack of flagellum is not stimulating TLR's. It is known that repression of flagellin expression once inside the mammalian host provides a selective advantage for enteropathogenic *Yersinia* as flagellin is a potent inducer of innate immunity (Minnich and Rohde, 2009). Furthermore, *Y. pestis* was motile at some point in its history but became non-motile after it acquired a mutation in the *flhD* gene. This mutation is thought to confer a selective advantage to *Y. pestis* and contributed to its virulence. (Minnich and Rohde, 2009).

The objective of this chapter is to examine the innate immune response towards bt 2 infection in previously vaccinated fish with the following objectives in mind:

i. To compare the innate immune response towards bt 1 and bt 2 isolates in vaccinated fish.

ii. To demonstrate modulation of the respiratory burst during infection;

iii. To demonstrate whether *Y. ruckeri* isolates induce apoptosis in phagocytes using the Annexin-V Vs PI staining assay;

iv. To ascertain whether flagella trigger the immune response in vaccinated fish by monitoring the proinflammatory cytokine response towards bt 2 infections.

5.2 Material and methods

5.2.1. Bacterial isolates

Bacterial isolates used for this study are described in Table 5.1. Culture of bacterial isolate is demonstrated in Chapter 2 section 2.2.1.

Table 5.1 *Y. ruckeri* isolates used for immune response study, indicating serotype, origin and bt.

Y. ruckeri isolate	Serotype	Origin	Bt
NCIMB 2194^T	O1 'Hagerman'	USA	1
NCTC 12266	O2	University of Reading, UK	1
NCTC 12268	O5	University of Reading, UK	1
NCTC 12269	O6	University of Reading, UK	1
NCTC 12270	O7	University of Reading, UK	1
TVT ITCHEN LT5	O1	England, 2007	2

5.2.2. Preparation of formalin killed bacteria

O1 bt 1 (NCIMB 21974^T) and bt 2 (TVT isolates were grown overnight in TSB at RT. Broth cultures were centrifuged at 4000 x *g* for 4 min, resuspended and washed three times with saline. Then, 0.5% formalin (v/v) was added with incubation at room temperature for 72 h. The resulting bacterial preparation was then cultured on TSA to ensure that all bacteria had been killed. Bacterial suspensions were washed with 0.9% saline three times and stored at 4^{o}C until required.

5.2.3. Fish

Unvaccinated rainbow trout with an average weight of 7.86 g were used for this study. Fish were kept in free flowing freshwater at $14^{o}C \pm 2^{o}C$. The fish were acclimatised for two weeks prior to vaccination.

5.2.4. Vaccination

Vaccines used were the standards monovalent 'Hagerman' vaccine (Aqua Vac ERM™) and a bivalent (RELERA™) vaccine produced against bt 1 and bt 2 isolates of *Y. ruckeri*. The vaccines contained no adjuvants. Vaccination was carried out following manufacturer's instructions. Thus, fish were vaccinated by 30 sec immersion in 1:10 dilution of vaccine. After vaccination, the fish were equally distributed between tanks. Control fish were subjected to 30 sec immersion in aquarium water. Fish were left for 28 days post vaccination before starting challenge studies.

5.2.5. Challenge protocol and sampling

Groups of 50 fish were challenged i.p with 0.1 ml fish $^{-1}$ of *Y. ruckeri* bt 1 (NCIMB 2194^{T}) and bt 2 (TVT) suspensions containing 2×10^{4} cells ml^{-1} respectively. Fish were held in separate tanks for the course of the experiment. Sampling of livers was taken over a 3 week period with time points taken at 1 h, 9 h, 24 h, 72 h, 1 wk, 2 wk and 3 wk. Negative control fish were injected with 0.1ml fish $^{-1}$ PBS.

5.2.6. Serum collection

Blood was collected by venepuncture using syringes coated with heparin (Sigma-Aldrich) and transferred immediately into 9 ml capacity lithium heparin vacuettes (Greiner) on ice. For serum, the blood was transferred into vacuettes containing Z Serum Clot Activator (Greiner) and allowed to clot at 4°C for 4 h. The sera were separated by centrifugation (2,000 × *g* for 25 min at 4°C) and stored at −70°C until required.

5.2.7. Head kidney macrophage isolation

Head kidney (HK) macrophages were isolated largely according to the method of Secombes (1990). Thus using aseptic techniques, the anterior HK was removed, placed into a glass homogenizer (30 mL capacity; VWR-Jencons) containing 5 mL of L-15 medium (Sigma-Aldrich), crushed and passed through a 100-µm nylon mesh into a Petri dish. The mesh was rinsed with additional 1 ml quantities of L-15 medium. The resulting cell suspension was slowly layered onto a 51% Percoll cushion [51 ml Percoll (Sigma-Aldrich), 10 ml 10% Hank's balanced salt solution (HBSS; Sigma-Aldrich), 39 mL sterile distilled water] and centrifuged at 400 × *g* for 30 min at 4°C. The band of cells lying at the medium/Percoll cushion interface was collected with a Pasteur pipette and washed twice with L-15 medium by centrifugation at 800 × *g* for 10 min. The number of cells was adjusted to ~10^6 cells ml^{-1} (by microscopy and use of a haemocytometer slide) in fresh L-15 medium supplemented with 0.1% (v/v) foetal bovine serum (FBS; Sigma-Aldrich) and 10 µl ml^{-1} of heparin (Sigma-Aldrich). Cell viability was determined at ×400 magnification of preparations containing equal mixtures of the cell suspension and 0.4% (w/v) aqueous trypan blue solution (Sigma-Aldrich). Dead cells developed an intracellular blue coloration within 5 min.

5.2.8. Head kidney bactericidal assay

Briefly, 100 µl of macrophage cell suspension (1 x 10^6 cells ml^{-1}) was added to 96-well 'U' bottom microtitre plate. Microplates were centrifuged at 700 x g for 20 min to adhere cells and non-adherent cells were washed with sterile PBS 2 times at 700 x g for 5 min. The resulting monolayers of macrophages were resuspended with 100 µl L-15 containing 5 % FBS. 20 µl bacterial suspension 105 cells ml^{-1} were added to triplicate wells containing macrophages or medium only which served as a control. The plates was incubated at 27^{o}C for 0 h, 3 h and 5 h for killing. After incubation, the supernatants were removed from the wells and the killing process was stopped by lysing the macrophages by the addition of 50 µl 0.2% Tween-20 in dH_2O per well. 100 µL of TSB was added to each well to support overnight growth (16 h) of the surviving bacteria at 27^{o}C. The amount of the surviving bacteria present in each well was determined by the addition of 10 µl of 5mg ml^{-1} MTT (dimethlthiazoyldiphenyltetrazolium bromide). The plate was shaken and reaction was allowed to occur for 15 min. OD was read at 570/600 nm exactly 15 min after addition of MTT. The data was converted to a killing index (KI) by taking the ratio: KI = OD_{th}/OD_{0h}. The lower this index the more bacteria have

been killed. Control wells without leucocytes were analysed to give 100% survival of bacteria.

5.2.9.Superoxide anion assay

The ability of macrophages to produce ROIs when stimulated by live bacterial cells was carried out in accordance to Mathew *et al*. (2001). Head kidney macrophages were isolated as previously described above. Approximately 0.5 x 10^6 head kidney cells were seeded into well of a 96-well tissue culture plate. Plates were incubated for 2 h at 25^{o}C in a 5% CO_2 atmosphere. Bacteria grown overnight at 22^{o}C in TSB were washed twice by centrifugation and adjusted to 1 x 10^6 cell ml^{-1}. The phagocytes were inoculated with bacteria at a ratio of 1:1 and incubated for 30 min at 25^{o}C. All tests were carried out in triplicate. 100 µl of 1mg ml^{-1} nitroblue tetrazolium (NBT, Sigma-Aldrich) was added to each well and incubated for a further 30 min at 25^{o}C. The reaction was arrested with 100 % methanol followed by a single wash with 70% methanol. The plate was dried for 1 min, 120 µl 2M potassium hydroxide (Sigma-Aldrich) and 140 µl dimethyl sulphoxide (Sigma-Aldrich) were added to each well. The A_{630} was measured using a microplate reader (Tecan). The results were expressed as means ±SEM from triplicate wells.

5.2.10. Nitric oxide production by fish phagocytes

Nitric oxide production by macrophages was measured by the Griess reaction with a commercial kit (Promega) according to the protocol provided. Macrophages were obtained using the method above. A sub sample of these macrophages were washed and resuspended in cold L-15 medium (Sigma-Aldrich). Aliquots of 100 µl of macrophage suspension were adjusted to 1 x 10^6 cells were transferred into 3 microcentrifuge tubes and 100 µl of *Y. ruckeri* were added to each tube to provide a macrophage: bacteria ratio of 1:20. At intervals of 0, 0.5, 1, 1.5 and 2 h following bacterial challenge, the reaction was stopped by placing the tubes onto ice. Fifty µl of the macrophage/bacterial suspension was transferred to a well of a 96 well flat-bottom plate. Each test was performed in triplicate. Sulphanilamide in a volume of 50 µl was added to each test well and to wells containing the nitrite references standard provided in the test kit and run in parallel. PMA stimulated macrophages, macrophages alone in L-15 medium and

bacteria in L-15 medium were used as controls. The test plates were incubated at room temperature for 10 min in the dark. Fifty µl of the Griess reagent [N-1-napthylethylenediamine dihydrochloride (NED)] equilibrated to room temperature was added to each well of the plate and incubated for 10 min in the dark. The colorimetric reactions were measured with a microplate reader (Tecan) at A_{550}. The average absorbance per test sample for each time interval was determined and compared with the nitrite standard reference curve generated for each assay.

5.2.11. Cytotoxicity assay

Head kidney leucocytes were used for the cytotoxicity effects of bt 1 and bt 2 isolates of *Y. ruckeri* using CytoTox96® Assay (Promega) according to the manufacturer's instructions. The optimum number of HK leucocytes (target cells) isolated from rainbow trout was determined by a preliminary experiment. The cytotoxicity of bt 1 ($NCIMB2194^T$) and bt 2 (TVT) to target cells (5 x 10 ml^{-1}) was performed at a multiplicity of infection (MOI) of 2000 and 200 (ratio of bacteria to cells), respectively. The bacteria (50 µl) and leucocytes (50 µl) were incubated together with L-15 medium (Sigma-Aldrich) supplemented with 15 mM HEPES (Sigma-Aldrich) and 5% foetal calf serum (FCS) (Sigma-Aldrich) on a 96 well round-bottom plate (Nalge Nunc) at 18^oC for 5 h and 24 h. After incubation the culture medium was collected, centrifuged at 10,000 x *g* for 5 min to remove cells, and transferred to a 96 well flat-bottom plate (Nalge Nunc). Then, the concentrations of lactate dehydrogenase (LDH) in the resulting supernatants were measured using the CytoTox96® Assay. A_{490} was determined using a microplate reader (Tecan). Cytotoxicity caused by bacteria was expressed as % cell death. Percentage of cytotoxicity was calculated using the formula:

Percentage cytotoxicity= *100×[(experimental release–target spontaneous release-effector spontaneous release)/(100% target maximum release–target spontaneous release)]*.

The spontaneous release reflects the amount of LDH released from the cytoplasm of uninfected macrophages, whereas the maximum release is the amount of LDH released from detergent-lysed uninfected macrophages. For comparison, 100% release corresponds to the amount of LDH released by kidney cells lysed with 10x Lysis buffer (9% (v/v) Triton® x-100).

5.2.12. H_2O_2 inhibition zone test

Y. ruckeri strains were grown overnight at 25°C in TSB, and harvested by centrifugation. After washing, the cells were resuspended in fresh TSB at A_{540} 0.5. Four mL of this suspension was added into 16 mL top-agar medium containing TSB and 1% agar at 50°C. After solidification, sterile Whatman 3MM disks (0.6 cm diameter) containing 10 µl 2 mM, 20 mM and 200 mM H_2O_2 were placed on the surface. Zones of inhibition were visualised after incubation overnight at 25°C.

5.2.13. Flow cytometry

To measure apoptosis, head kidney macrophages were obtained as described above. The experiment was also carried out using whole blood of rainbow trout. Bt 1 and bt 2 isolates of *Y. ruckeri* grown overnight at 22°C in TSB were washed twice by centrifugation and adjusted to 1 x 10^6 cell ml^{-1} in PBS. Whole blood and head kidney macrophages were combined with the bacteria at a ratio of 1:10. Two hundred µl cell suspension was added to 800 µl L-15 medium (Sigma-Aldrich), 9.6 µl Annex V-FLUROS (Roche) Ca^{2+}, 2µl propidium iodide (Sigma-Aldrich). Samples were analyzed on a Partec *CYFlow* flow cytometer, and data acquisition was performed using Flomax® software. Gating was carried out on propidium iodide negatively stained cells. The experiments were carried out at 0 h, 3 h, 6 h and 24 h time points. Gating was based on forward scatter (FS) versus side scatter (SSc) signals. The experiments were carried out in triplicate. Macrophages with no bacterial incubation were used as a negative control. Formalin killed O1 bt 1 and O1 bt 2 isolates were used as bacterial controls.

5.2.14. Light microscopy

Cytospin preparations were carried out for each of the time points. Apoptosis was visually confirmed by using cytospin preparations of rainbow trout leukocytes stained using Giemsa stain (Sigma-Aldrich). Briefly cytospin preparations were carried out using Cytospin 3 apparatus (Shandon). One hundred µl of leukocyte suspensions were added into cytospin chamber and centrifuged at 250 rpm for 3 min. Cells were fixed onto glass slides with methanol for 2 min. Methanol was tapped off glass slides and then cell were stained using Giemsa stain for 10 min. After incubation, slides were rinsed with H_2O, allowed to dry then a cover slip was placed over the sample using histomount (National Diagnostics) therefore allowing for long term storage.

5.2.15. RNA extraction

Fish were sacrificed using a lethal dose of MS222 (Sigma-Aldrich) and livers were dissected aseptically. Initially, tissue samples were preserved in RNAlater (Qiagen) and stored at -20^{o}C until required. Total RNA was isolated from homogenized liver samples using the Qiagen RNEasy mini kit according to manufacturer's instructions (Qiagen).

5.2.16. Reverse transcription PCR

For the reverse transcription, 0.5 µg of total RNA from each sample quantified by UV spectrophotometry was mixed with 0.55 µg of Oligo $(dT)_{15}$ primer (Fermentas) and annealed at 70^{o}C for 5 min. Resulting cDNA was aliquoted and stored at -70^{o}C until required.

5.2.17. Primer design

Details of primers used in the study are given in Table 5.2. The default parameters of the program were modified in order to eliminate primer dimer formation and get the best for PCR and SYBR green fluorescence. Conditions for choosing primers were the following: amplification product size 100-250 bp, primer size 20 ± 2 bp, GC content 50 ± 5%, primer T_m 60 ± 2^{o}C.

Table 5.2. Sequences, accession numbers and conditions of oligonucleotides primers used for real-time PCR.

Gene target	Primer name	Product size	Sequence (5'-3')	Anneal temp.	Accession No.
β-actin	β-actin_F	181	GGACTTGAGCAGGAGATGG	60^{o}C	AJ438158
	β-actin_R		ATGATGGAGTTGTAAGGTGATCT		
IL- 6	IL6_F	91	ACTCCCCTCTGTCACACACC	61^{o}C	DQ866150
	IL6_R		GGCAGACAGGTCCTCCACTA		
TNF-ά	TNF-ά_ F	75	CAAGAGTTTGAACCTCATTCAG	60^{o}C	AJ277604
	TNF-ά-_R		GCTGCTGCCGCACATAAAG		

5.2.18. Real-time PCR

The real-time (RT) PCR analysis was carried out by the 7900 HT Fast Real-Time PCR System (Applied Biosystems). For the RT PCR, IQ SYBR Green mix (BioRad) was used following manufacturer's instructions. The PCR mixture was composed of 10 µl of SYBR Green mix, 1µl cDNA (30 ng of total RNA) and each primer (final concentration: 70 nm), and then Millipore water was added to the final volume of 12 µl. The mixtures were run with the following thermal cycling program: enzyme activation 95^{o}C for 10 min, initial denaturation at 95^{o}C for 5 min, followed by 50 cycles of denaturation at 95^{o}C for 20 sec, annealing at 60^{o}C (for primer) for 30 sec. Then the PCR program was followed by a melt curve program with a heating rate of 0.5^{o}C per sec (for 10 sec) and continuous fluorescent measurement starting at 60^{o}C until 95^{o}C. Randomly selected samples were analysed by gel electrophoresis, which always showed the PCR product of the predicted molecular weight, indicating specific amplification. The resulting amplification curves were processed immediately with the 7900 HT Sequence Detection Systems software v2.3 (Applied Biosystems).

5.2.19. Data analysis

Relative quantification was carried using Relative Quantification Manager (Applied Biosystems) using β-actin as the endogenous controls and 0 h samples as calibrators. RT quantitative (q) RT-PCR was performed, and the comparative cycle number threshold (C_T) method ($\Delta\Delta C_T$) was used [where $\Delta C_T = C_T$ (gene) – C_T (*β-actin*)] for quantification of the data. The $\Delta\Delta C_T$ calculation involved finding the difference between each sample's ΔC_T and the mean ΔC_T for the time point.

5.2.20. Statistics

Each experiment was replicated three times and within the experiment three replicates were performed. The results are presented as means ± SD (standard deviation of the mean). Initial 0 h values were statistically analysed by one-way analysis of variance (ANOVA) and a Bonferroni post Hoc test. Only when values were not statistically significant, results were statistically analysed by two-way analysis of variance with bonferroni and tukey post Hoc tests. Percentage data and ratio values were transformed to square-root arcsine values to homogenise variance. All statistical tests were conducted using the software package SPSS (Statistical Package for Social Sciences).

Differences were considered significant when $P < 0.05$. The data was plotted using Microsoft Excel.

5.3 Results

5.3.1. Head kidney bacteroicidal assay

The capacity of macrophages to phagocytose and remove *Y. ruckeri* serotypes O1, O2, O5, O6 & O7 and biotypes 1 and 2 was carried out by measuring a reduction of MTT. Results from the experiment are displayed in Table 5.3. Apparent differences were observed in the ability of macrophages to kill selected *Y. ruckeri* serotypes and biotypes. no change in bacteriocidal activity was noted for bt 1 isolates. One way analysis of variance indicated that bt 2 isolates significantly increased in numbers at 5 and 24 h post infection. One way analysis of variance indicated that serotypes O2 and O5 were significantly killed after a 24 h period compared to the 0h time point.

Table 5.3 Bacteriocidal activity of rainbow trout macrophages exposed to *Y. ruckeri* isolates. Values represent OD, with highest values relating to highest number of surviving bacteria. Results are expressed as mean ±SD of triplicate experiments. Mean with '*' are significantly different ($p<0.05$) from the control (0 h) post exposure point.

Isolate	Bt	0 h	3 h	5 h
NCIMB 2194^T (O1)	1	0.71±0.17	0.79±0.06	0.77±0.05
NCTC 12266 (O2)	1	1.17±0.07	0.92±0.05	0.59±0.38*
NCTC 12268 (O5)	1	1.11±0.01	0.92±0.05	0.49±0.36*
NCTC 12269 (O6)	1	0.8±0.06	0.63±0.08*	0.65±0.01*
NCTC 12270 (O7)	1	0.65±0.15	0.55±0.04	0.67±0.15
TVT ITCHEN LT5	2	1.02±0.10	1.45±0.09*	1.66±0.04*

The OD from incubations was converted into a killing index by $KI = OD_{th}/OD_{0h}$ (Figure 5.1). Incubation with bt 1 and bt isolates of *Y. ruckeri* with rainbow trout macrophages

highlights that there is a difference between the killing between motile and non motile organisms. Compared with the bt 1 controls there is a marked increase in the population of bt 2 isolates as detected by MTT. Serogroups O2, O5, O6 all appeared to be readily killing ability rainbow trout macrophages after a period of 5 h. Bacterial isolates from serogroups O1 and O7 appeared to remain stable after 5h. Bt 2 isolate demonstrated a marked increase in survival and reproduction after 5 h.

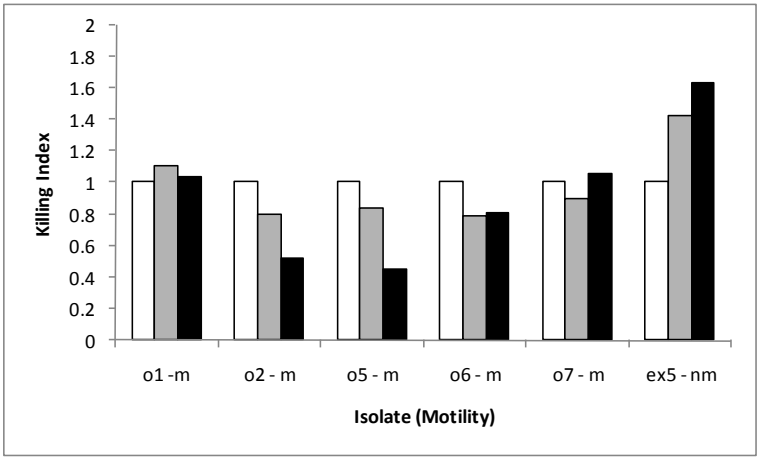


Figure 5.1. Macrophage bacteriocidal activity against *Y. ruckeri* isolates. Results are expressed as a killing index. Macrophage bacteriocidal acitivty of rainbow trout macrophages in response to bt 1 and bt 2 challenge of *Y. ruckeri* at 0 h (□), 3 h(■) and 5 h(■). Values given as index (ODt/OD). Results are the means three separate experiments.

5.3.2. Nitric oxide production by fish phagocytes

The production of nitric oxide by rainbow trout macropahges was was carried out by measuring the Griess reaction (Table 5.4). The reaction was measured over a 24 h period. Macrophages were artificially stimulated using PMA as a positive control. Macrophages alone were used as a negative control. The PMA stimulate macrophages produced a high concentration of NO which began to tail off after decrease after 4 h. PMA stimulated macrophages were significantly higher than all other tests. Mean NO production peaked after 24 h in bt 1 samples Although O1 bt 1 and bt 2 were able to induce as higher release of NO statistical analysis by two-way analysis of variance revealed that results were not significant.

Table 5.4. Nitric oxide production by rainbow trout phagocytes following infection with bt 1 and bt 2 isolates of *Y. ruckeri*.Values represent amount of NO produced by phagocytes (mM). Results are the means ±standard deviations of triplicate experiments.

	Nitric Oxide production					
	0 h	1 h	2 h	4 h	6 h	24 h
Ctr	11.8 ± 0.3	11.5 ± 3.4	13.7 ± 6.9	8.5 ± 1.2	11.1 ± 5.5	9.4 ± 3.5
PMA ctr	29.7 ± 0.8*	30.1 ± 0.4*	29.5 ± 2.12*	30.8 ± 1.2*	27.1 ± 1.2*	23.6 ± 1.9*
O1 bt1	10.8 ±1.3	9.7 ± 1.6	13.3 ± 3.3	12.8 ± 1.1	16.7 ± 1.7	19.1 ± 7.5
O1 bt 2	11.9 ± 0.6	9.7± 0.2	11.1 ± 3.5	14.6 ± 0.03	17.1 ± 3.9	13.9 ± 0.4
dead bt1	10.2 ± 0.7	7.4 ± 0.7	7.8 ± 2.72	6.7 ± 0.1	10.3 ± 4.3	6.7 ± 0.4
dead bt2	10.6 ± 2.9	10.8 ± 0.1	9.4 ± 3.51	8.5 ± 0.7	10.0 ± 4.2	7.6 ± 0.1

'*' Values are significantly different ($p<0.05$) from the control at that time point.

5.3.3. Superoxide anion production by fish phagocytes

The production of superoxide anion ($\dot{}O_2$) by rainbow trout macrophages as a response towards incubation with bt 1 and bt 2 isolates of *Y. ruckeri* was measured by the reduction of NBT over 30 min (Figure 5.2). The results demonstrated that isolates of Y. ruckeri were able to stimulate the production of $\dot{}O_2$. One way analysis of variance highlighted that serotypes O6 and O7 stimulated the production of $\dot{}O_2$ significantly higher than the control. Bt 2 stimulated the least amount of $\dot{}O_2$ although these results were not significant from the control group.

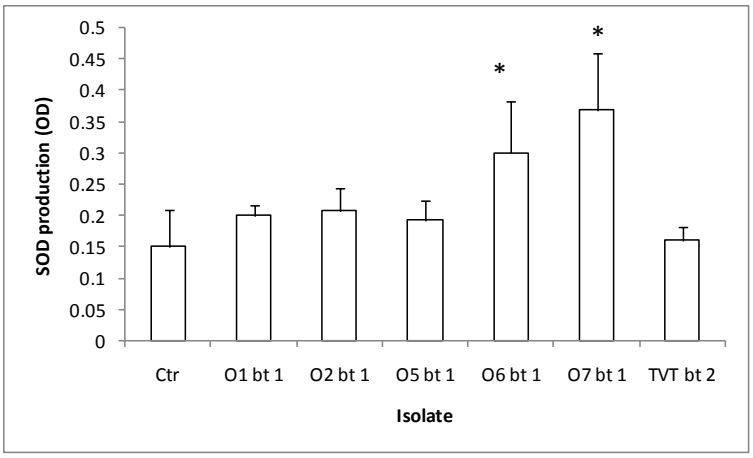


Figure 5.2. Superoxide anion oxide production of rainbow trout macrophages in response to bt 1 and bt 2 challenge *of Y. ruckeri* (± SD). Results are the means three seperate experiments. Mean with '*' are significantly different ($p<0.05$) from the control.

5.3.4. H_2O_2 inhibition zone test

The sensitivity of all serotypes and biotypes of *Y. ruckeri* to various concentrations of H_2O_2 was determined (Table 5.5). Serotype 1 bt 1 serotype O2 and O5 has the same resistance profile in that isolates were resistant up until 200 mM of H_2O_2 . Serotype O6 and O7 were both sensitive to 20-200mM of H_2O_2 . Serotype O1 bt 2 was resistant to all concentrations of H_2O_2.

Table 5.5. Inhibition of selected *Y. ruckeri* isolates in response towards various concentrations of H_2O_2.

Isolate	Bt	2mM	20mM	200mM
NCIMB 2194^T	1	R	R	S
NCTC 12266	1	R	R	S
NCTC 12268	1	R	R	S
NCTC 12269	1	R	S	S
NCTC 12270	1	R	S	S
TVT ITCHEN LT5	2	R	R	R

(R = resistant, S = sensitive).

5.3.5. Cytotoxicity of bacterial cell cultures to rainbow trout phagocytes

As various target cell types contain different amounts of lactate dehydrogenase (LDH), a preliminary experiment was carried in order to optimise the target number of cells. The concentration of target cells (2×10^5) yielded more than two times the background O.D value of the medium control (Table 5.6). Then optimum leucocyte cell number was determined as to ensure an adequate signal-to-noise ratio. Cytotoxicity of live and formalin killed preparations of bt 1 and bt 2 isolates of *Y. ruckeri* after 6 hrs and 24 h are given in Table 5.7, 5.8 . Co-cultures of the mixture between leucocytes and biotypes 1 and 2 of *Y. ruckeri* were examined for LDH production in the media after incubation for 6 h and 24 h. After 6 h, live bt 1 and bt 2 isolated has caused 5.4% and 2.5% LDH release from leucocytes. No detectable LDH was released from leucocytes incubated with formalin killed bt 1 and bt 2 isolates of *Y. ruckeri*. After 24 h, cytotoxicity increased to 95% for bt 1 and 40.5% for bt 2 isolates.

Table 5.6. Optimisation of leucocytes cell number for cytotoxicity assay. Values represent background % LDH release. Data represents mean and standard deviation of quadruplicate wells.

	Leucocytes number (cells ml^{-1})				
	Medium control[a]	2×10^7	2×10^6	2×10^5	2×10^4	Positive control[b]
% LDH release	0.95 ± 0.003	0.47 ± 0.2	0.26 ± 0.06	0.14 ± 0.01	0.13 ± 0.02	1.19 ± 0.16

[a] L-15 Medium; [b] LDH positive control.

Table 5.7. Cytotoxicity of live and formalin killed preparations of bt 1 and bt 2 isolates of *Y. ruckeri* after 6 h. Values represent % cytotoxicity after 6 h incubation. Results are expressed as the means of quadruplicate well in triplicate tests. [a] formalin inactivated .

	Bt 1	Bt 2	Killed Bt 1[a]	Killed Bt 2[a]
% Cytotoxicity	5.45 % ± 6.4	2.5 % ± 3.3	0 % [b]	0% [b]

[a] formalin inactivated . [b] = No result

Table 5.8. Cytotoxicity of live and formalin killed preparations of bt 1 and bt 2 isolates of *Y. ruckeri* after 24 h. Values represent % cytotoxicity after 24 h incubation. Results are expressed as the means of quadruplicate well in triplicate tests. Means with '*' are significantly different ($p<0.05$) from the 6 h post exposure time point.

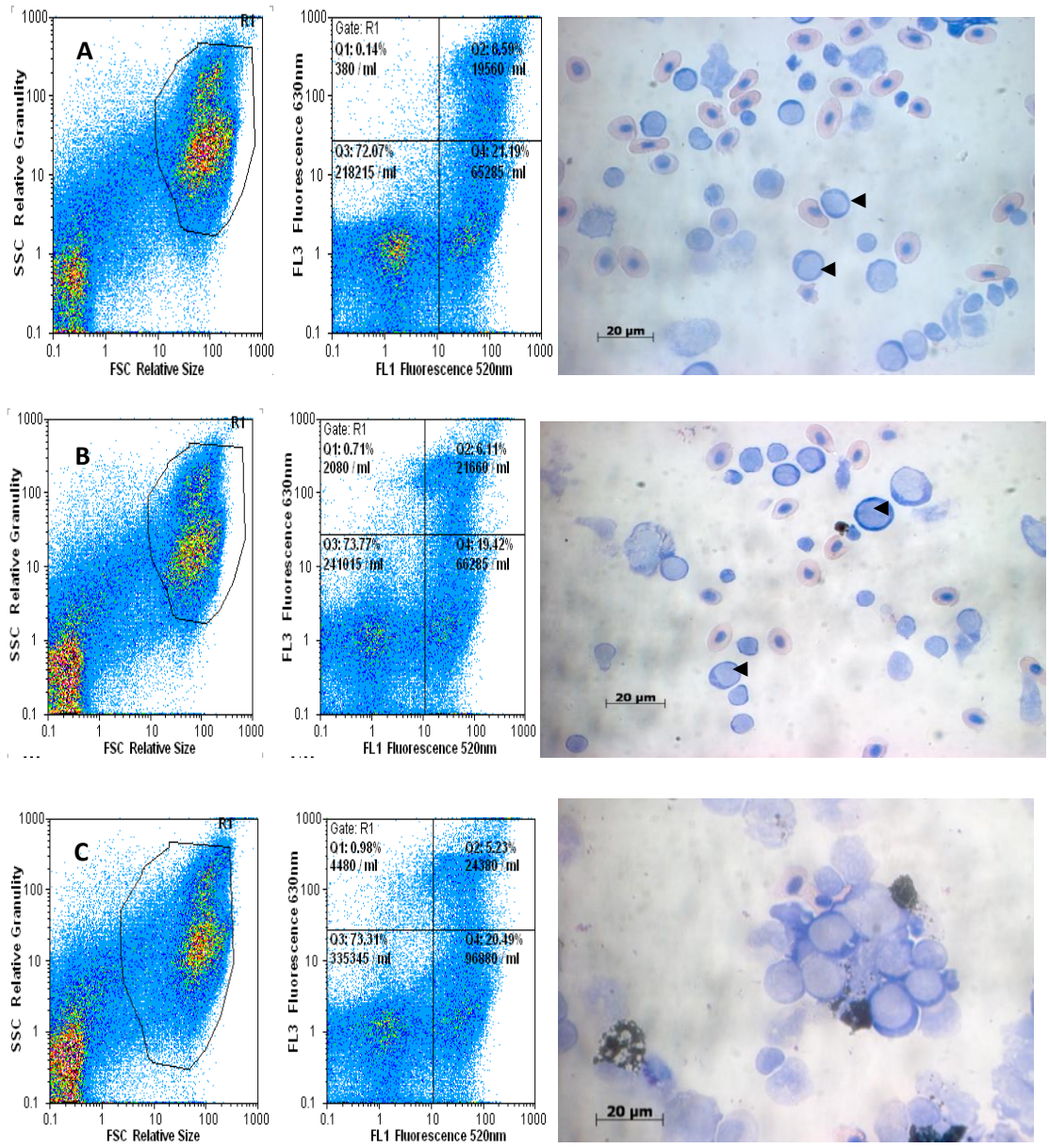
	Bt 1	Bt 2	Killed Bt 1[a]	killed Bt 2[a]
% Cytotoxicity	95 % ± 19*	40.5%± 16.2*	0% [b]	2%± 0.02

[a] formalin inactivated . [b] = No result.

5.3.6. Flow cytometry analysis of apoptosis induced by *Y. ruckeri* bt 1 & 2 in rainbow trout phagocytes

5.3.6.1. Control phagocytes

The ability of *Y. ruckeri* to induce apoptosis in rainbow trout macrophages was assessed by flow cytometry using the Annexin-V vs PI staining method. Figure 5.3 demonstrates the change in cellular characteristics of control phagocytes. 'Live' cells were identified as being negative for both Annexin and PI were gated in quadrant Q3, where as apoptotic cells were positive for Annexin-V but negative for PI, these were gated in quadrant Q4. Late apoptotic/necrotic cells were gated in Q2, these cells were positive for both Annexin and PI. Quadrant Q1 only contained PI stained genetic material.



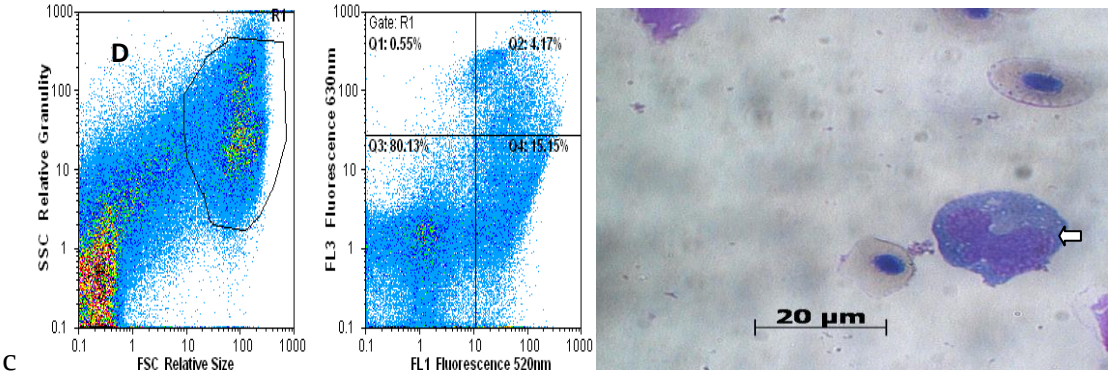


Figure 5.3. Flow plots and cytospin of control phagocytes (Giemsa stain at x 10000). (A = 0 h, B= 2 h, C= 6 h, D= 24 h. *Y. ruckeri* infected macrophages expose PS as a result of rapid membrane damage.The lower left area of the cytograms represents viable cells, which are negative for Annexin-V binding and exclude PI. The lower right area represents apoptotic cells that demonstrate Annexin-V binding, but are negative for PI indicating an intact cytoplasmic membrane. The upper area represents non-viable, dead cells that are positive for both Annexin-V and PI. (◄ = normal phagocytes, ⇦ indicates = loss of membrane structure. Scale bar = 20 µm.

5.3.6.2. Exposure of macrophages to killed bt 1 & bt 2 isolates of *Y. ruckeri*

Similar changes were also observed in phagocytes exposed to formalin-killed *Y. ruckeri* bt 1 and bt 2 isolates (Figure 5.4). The overall number of phagocytes decreased as displayed by lighter shade in flow plots. There were no visible differences between the two biotypes. Alterations in the membrane of phagocytes was observed with blebbing and degranulation observed in small number of granulocytes, which increased until 24 h (Figure 5.4 C). Large majority of the population of phagocytes remain intact.

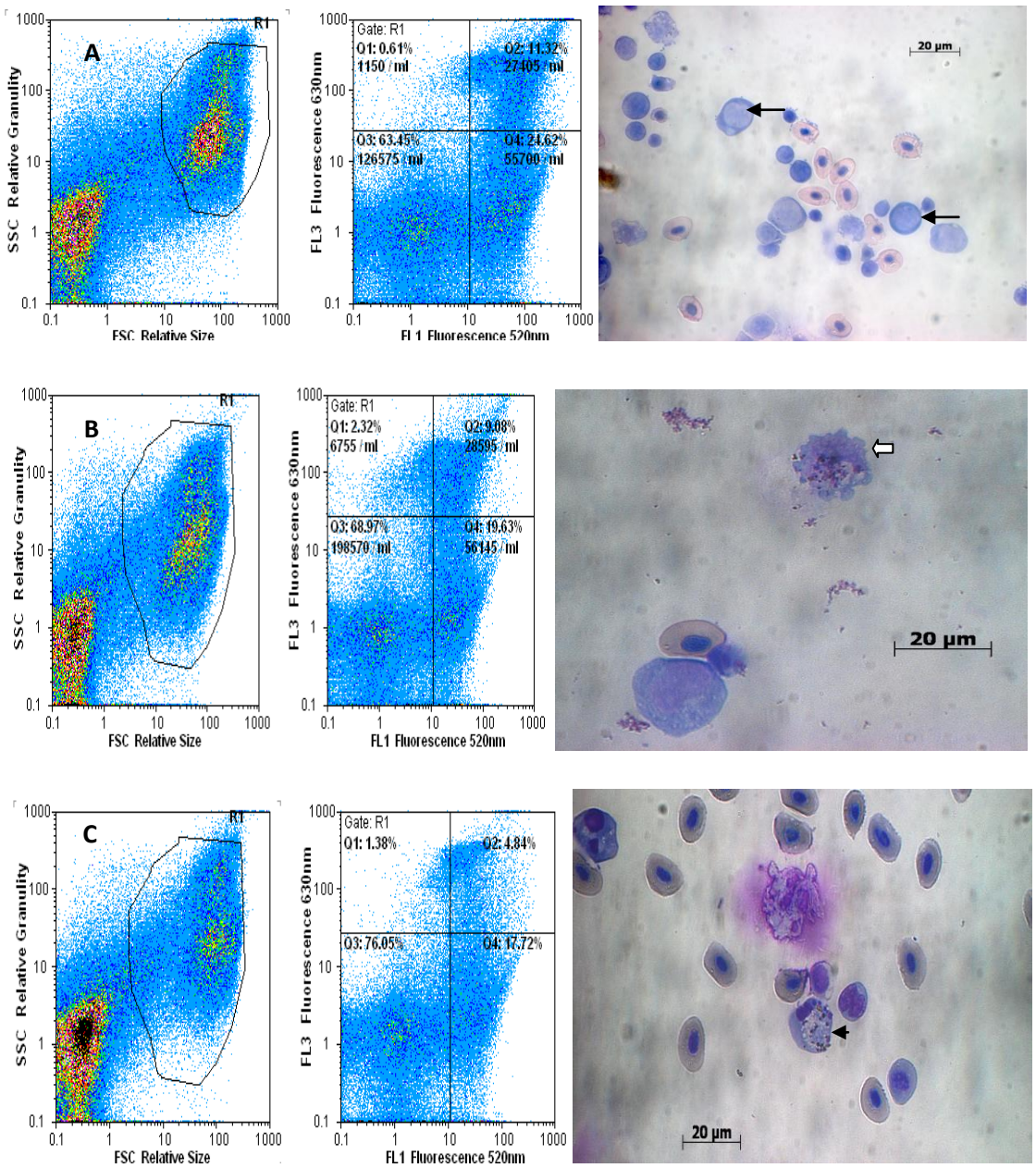
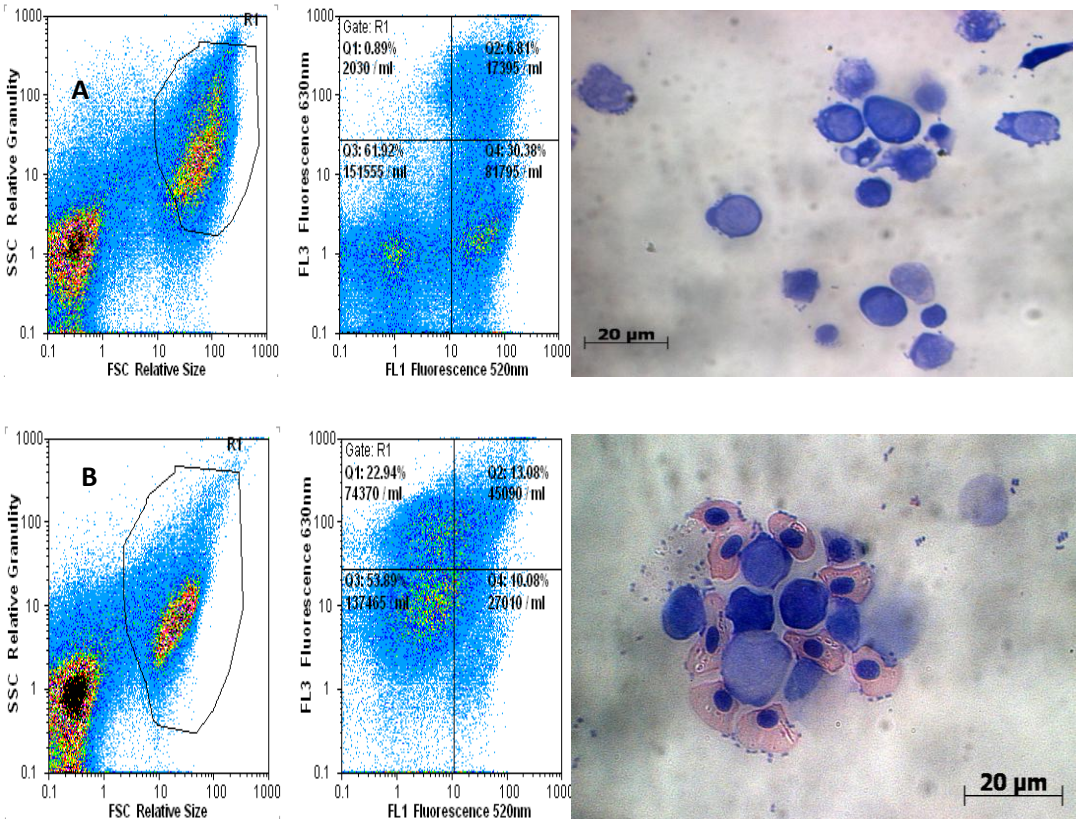


Figure 5.4. Flow plots and cytospin of phagocytes exposed to formalin killed O1 bt 1 and bt 2 isolates of *Y. ruckeri* (Gimsa stain at x 1000). (A = 2 h, B= 6 h, C= 24 h). *Y. ruckeri* infected macrophages cells expose PS as a result of rapid membrane damage. The lower left area of the cytograms represents viable cells, which are negative for Annexin-V binding and exclude PI. The lower right area represents apoptotic cells that demonstrate Annexin-V binding, but are negative for PI indicating an intact cytoplasmic membrane. The upper area represents non-viable, dead cells that are positive for both Annexin-V and PI.(← = normal phagocytes, ⇦ = loss of membrane integrity, ← = fragmented chromatin, apoptotic bodies). Scale bar = 20 µm.

5.3.6.3. Exposure of macrophages to bt 1 isolates of *Y. ruckeri*

Microscopic analysis of rainbow trout phagocytes demonstrated classical characteristics of apoptosis when exposed to bt 1 of *Y. ruckeri*. No change was observed between 0 -2 h. After 2 h post exposure to bt 1 isolates of *Y. ruckeri*, phagocytes could be observed to be undergoing characteristic apoptotic processes; bacteria could be observed attached to leukocytes and erythrocytes (Figure 5.5 A.). After 2 h post exposure, phagocytes appeared to lose membrane structure and lyse (Figure 5.6) phagocytes after 6 h post exposure, appeared to aggregate together (Figure 5.8 B) After 24 h post exposure to bt 1 isolates, macrophages were completely lysed, only fragmented cytoplasm remained (Figure 5.5 C).



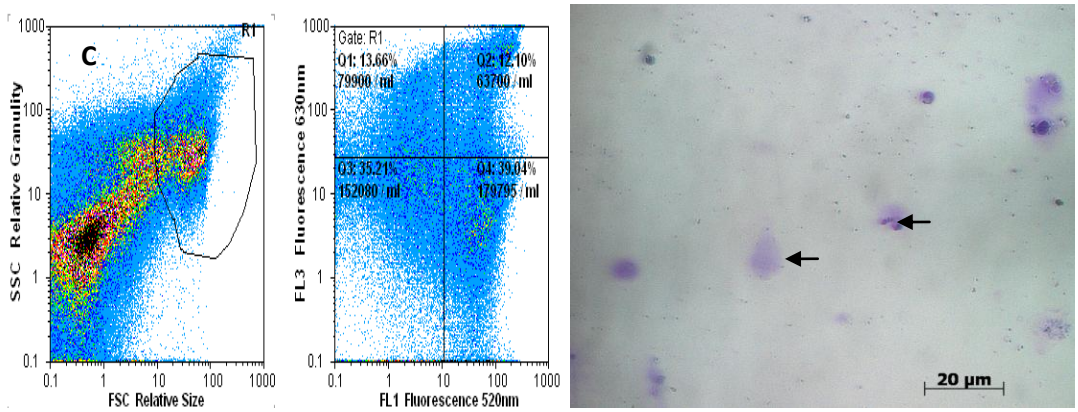


Figure 5.5. Flow plots and cytospin of phagocytes at various time points after exposure to O1 bt 1 isolates of *Y. ruckeri* (Giemsa stain at x 1000). (A = 2 h, B = 6 h, C = 24 h). The lower left area of the cytograms represents viable cells, which are negative for Annexin-V binding and exclude PI. *Y. ruckeri* infected macrophages cells expose PS as a result of rapid membrane damage. The lower right area represents apoptotic cells that demonstrate Annexin-V binding, but are negative for PI indicating an intact cytoplasmic membrane. The upper area represents non-viable, dead cells that are positive for both Annexin-V and PI. (← = cellular debris and nuclear material) Scale bar = 20 µm.

5.3.6.4. Exposure of macrophages to bt 2 isolates of *Y. ruckeri*

Microscopic analysis of rainbow trout phagocytes demonstrated classical characteristics of apoptosis when exposed to bt 2 isolates of *Y. ruckeri* (Figure 5.6). Between 2 h and 6 h post exposure to bt 2 isolates, characteristic apoptotic changes were observed, loss of membrane structure, blebbing of cell wall, condensed chromatic, pyknotic nuclei and de granulation (Figure 5.6 B). After 24 h post exposure to bt 2 isolates some phagocytes and erythrocytes were still present, many were necrotic (Figure 5.7 C). Cells had condensed nuclei, cytoplasmic material was observed.

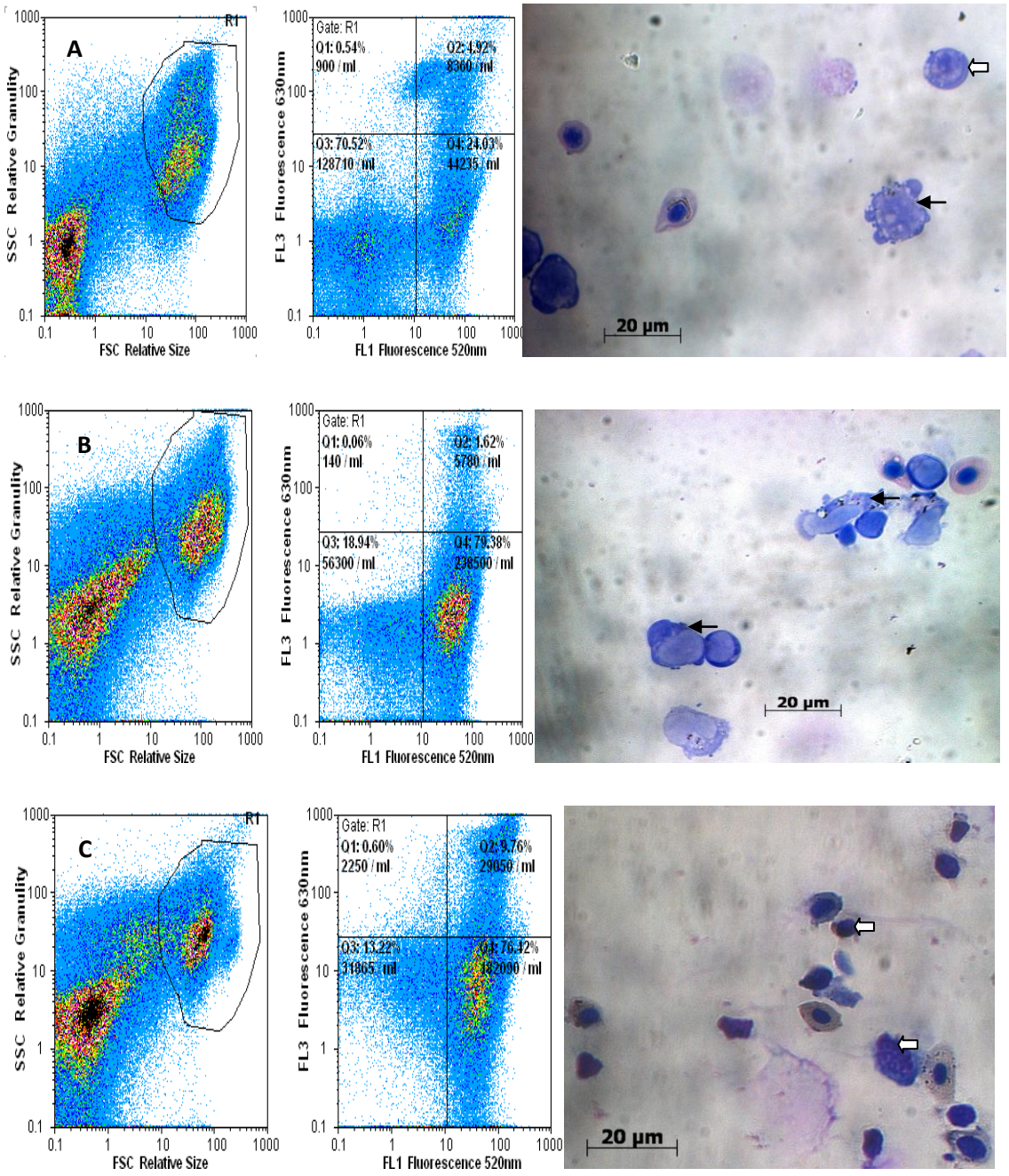


Figure 5.6. Flow plots and cytospin of phagocytes at various time points after exposure to O1 bt 2 isolates of *Y. ruckeri* (Giemsa stain at x 10000). (A = 2 h, B= 6 h, C= 24 h). *Y. ruckeri* infected macrophage-like cells expose PS as a result of rapid membrane damage.The lower left area of the cytograms represents viable cells, which are negative for Annexin-V binding and exclude PI. The lower right area represents apoptotic cells that demonstrate Annexin-V binding, but are negative for PI indicating an intact cytoplasmic membrane. The upper area represents non-viable, dead cells that are positive for both Annexin-V and PI. (⇦ = pyknotic nuclei and ← indicates membrane blebbing Scale bar = 20 µm.

5.3.7. Cellular changes in rainbow trout phagocytes following exposure of *Y. ruckeri* isolates

5.3.7.1. Induction of apoptosis by *Y. ruckeri* in rainbow trout phagocytes

In order to characterize the cell death induced by *Y. ruckeri* biotypes, we used a flow cytometric assay that discriminates between apoptosis and necrosis. The analysis of different groups of Annexin-V/PI double stained head kidney phagocytes after 0, 2, 6 and 24 hours of incubation is shown in Figures 5.7, 5.8, 5.9 and Tables 5.9, 5.10, 5.11.

For control macrophages, no change in over all cell populations was observed(Figure 5.7.). There was no significant difference between the percentage of live cells at 0 h, 2 h, 6 h and 24 h, likewise with apoptotic and necrotic cells. There was a slight increase in live cells over 24 h although not significant it is believed that the cells are generally more stable. For control phagocytes the 'live' percentage of phagocytes increased from 62% to 80% over a period of 24 h Apoptosis increased in control macrophages decreased from 27% to 15% over the 24 h period. Late apoptotic/necrotic cells decreased from 8% to 4.26% over a period of 24 h.

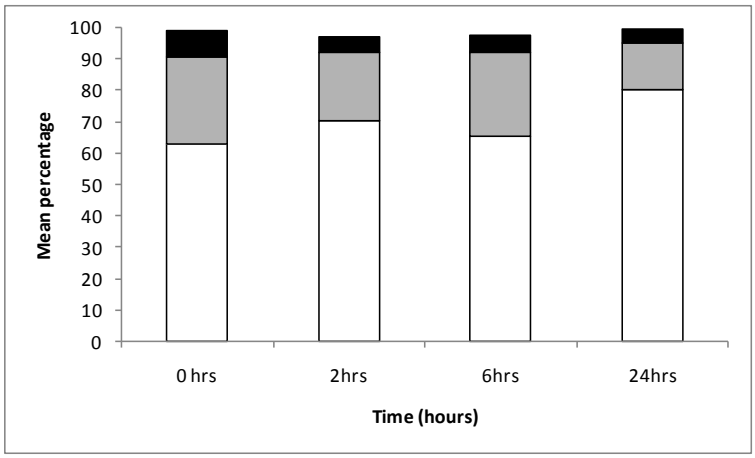


Figure 5.7 Mean percentage of normal (□), early apoptotic (■), late apoptotic (■) separated macrophages from rainbow trout. Macrophages were cultured in L-15 at 15^{o}C and analysed by flow cytometry at 0, 2, 6 and 24 h via Annexin-V Vs. PI staining. Each experimental run was performed in triplicate with pooled macrophages from rainbow trout.

5.3.7.2. Cellular changes in rainbow trout phagocytes induced by bt 1 isolates

Cellular changes induced by exposure to bt 1 isolates of *Y. ruckeri* are displayed in Figure 5.8. Exposure to live bt 1 isolates of *Y. ruckeri* caused the mean percentage of 'normal' phagocytes to decreased from 59% to 46% over a period of 24 h. Apoptosis increased in macrophages increased from 26% to 36 % over the 24 h period. Late apoptotic/necrotic cells increased from 6.38% to 6.65% over a period of 24 h.

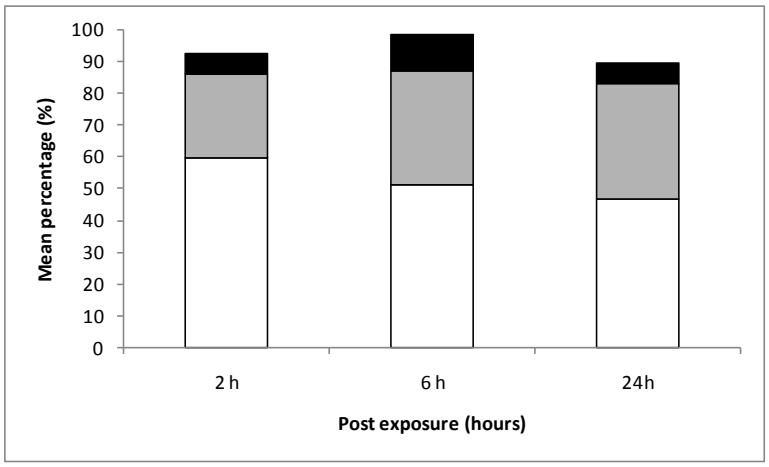


Figure 5.8. Mean percentage of normal (□), early apoptotic (■), late apoptotic (■) separated macrophages from rainbow trout cultured exposed to bt 1 *Y. ruckeri* isolate. Macrophages were cultured in L-15 at 15^{o}C and analysed by flow cytometry at 2, 6 and 24 h via Annexin-V Vs. PI staining.

5.3.7.3. Cellular changes in rainbow trout phagocytes induced by bt 2 isolates

Cellular changes induced by exposure to bt 2 isolates of *Y. ruckeri* are displayed in Figure 5.9. Exposure to bt 2 isolates of *Y. ruckeri* resulted in the percentage of 'normal' phagocytes to decrease from 62% to 9% over a period of 24 h. Apoptosis increased in macrophages increased from 33% to 69 % over the 24 h period. Late apoptotic/necrotic cells increased from 6% to 19% over a period of 24 h.

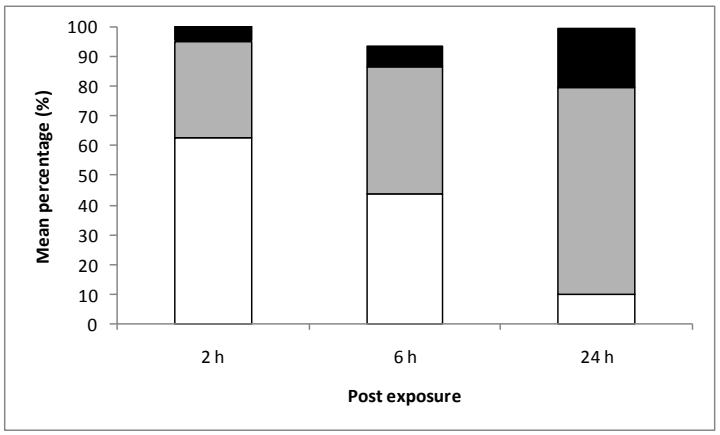


Figure 5.9. Mean percentage of normal (□), early apoptotic (■), late apoptotic (■) separated macrophages from rainbow trout cultured exposed to non-motile bt 2 *Y. ruckeri* isolate. Macrophages were cultured in L-15 at 15^{o}C and analysed by flow cytometry at 2, 6 and 24 h via Annexin-V Vs. PI staining.

5.3.7.4. Cellular changes in rainbow trout phagocytes

Table 5.9, demonstrates percentage of live phagocytes after exposure of to *Y. ruckeri* isolates. One way analysis of variance demonstrated that live cells bt 2 caused a significant reduction in the number of normal phagocytes after 24 h post exposure compared to the control. Two way analysis of variance indicated that there was a significant difference between bt 1 and bt 2 24 h post exposure.

Table 5.9. Percentage normal rainbow trout macrophages as a result exposure to various *Y. ruckeri* isolates as identified by Annexin-V vs PI staining. Values represent the mean percentage of cells ± standard deviation in the Live (Q3) (Annexin-V negative) quadrant) whether exposed to live or formalin killed *Y. ruckeri* isolates.

	Normal			
	0 h	2 h	6 h	24 h
Control	62.966 ±7.5	70.38 ± 6.3	65.5 ± 16.87	80.15± 0.04
Y. ruckeri bt 1	-	59.70 ± 5.23	51.18 ± 15.57	46.68 ± 11.99 [a]

Y. ruckeri bt 2	-	62.77 ± 6.85	44 ± 16.70	10.07 ± 11.01*[b]
dead *Y. ruckeri* bt 1	-	62.82 ± 8.02	52.19 ± 16.14	60.26 ± 11.01
dead *Y. ruckeri* bt 2	-	60.62 ± 7.93	50.67 ± 14.71	60.32 ± 8.88

'*' Values are significantly different ($p<0.05$) from the control at that time point. Means with common letter differ significantly between biotypes at a given time period.

Table 5.10, demonstrates percentage of live phagocytes after exposure of to *Y. ruckeri* isolates. One way analysis of variance demonstrated that live cells of bt 2 caused a significant increase in apoptotic cells compared to the control after 24 h. Two way analysis of variance indicated apoptotic cells significantly increased when exposed to bt 2 isolates compared to bt 1 isolates at 24 h post exposure.

Table 5.10. Percentage change rainbow trout macrophages as a result exposure to various *Y. ruckeri* isolates as identified by Annexin-V vs PI staining. Values represent the mean percentage of cells ±standard deviation in the Apoptotic (Q4) (Annexin-V positive) quadrant whether exposed to live or formalin killed *Y. ruckeri* isolates.

	Apoptotic			
	0 h	2 h	6 h	24 h
Control	27.48 ± 6.49	21.916 ± 6.8	26.4 ± 13.89	15.045 ± 0.16
Y. ruckeri bt 1	-	26.24 ± 13.18	35.71 ± 13.89	36.33 ± 13.31 [a]
Y. ruckeri bt 2	-	32.49 ±12.34	42.58 ± 9.37	69.56 ± 10.29*[b]
dead *Y. ruckeri* bt 1	-	30.13 ± 7.15	35.25 ± 13.67	36.33 ± 13.31
dead *Y. ruckeri* bt 2	-	32.49 ± 9.19	37.76 ± 12.76	39.34 ± 7

'*' Values are significantly different ($p<0.05$) from the control at that time point. Means with common letter differ significantly between biotypes at a given time period.

Table 5.11, demonstrates percentage of necrotic phagocytes after exposure of to *Y. ruckeri* isolates. One way analysis of variance demonstrated that live cells of bt 2 caused a significant increase in necrotic cells compared to the control after 24 h. Two way analysis of variance indicated that at there was no significant difference between bt 1 and bt 2 at 2 h , 6h and 24 h post infection.

Table 5.11. Percentage necrotic rainbow trout macrophages as a result exposure to various *Y. ruckeri* isolates as identified by Annexin-V vs PI staining Values represent the mean of percentage cells ± standard deviation in the necrotic (Q2) (Annexin-V positive, PI positive) quadrant whether exposed to live or formalin killed *Y. ruckeri* isolates.

	Necrotic			
	0 hr	2 hr	6 hr	24 hr
Control	8.68 ± 2.92	4.97 ± 1.35	5.56 ± 2.86	4.26 ± 0.08
Y. ruckeri bt 1	-	6.38 ± 3.80	11.71 ± 5.22	6.65 ± 3.97
Y. ruckeri bt 2	-	6.03 ± 0.86	6.72 ± 5.70	19.49 ± 10.89
dead *Y. ruckeri* bt 1	-	6.07 ± 4.22	9.42 ± 7.11	6.65 ± 3.97
dead *Y. ruckeri* bt 2	-	5.65 ± 3.49	9.94 ± 7.08	3.68 ± 1.80

'*' Values are significantly different ($p<0.05$) from the control at that time point. Means with common letter differ significantly between biotypes at a given time period.

5.3.7.4. Cellular changes in rainbow trout phagocytes induced by bt 1 isolates

Cellular debris changes after 24 h are given in figure 5. 10. Cellular debris was located in flow quadrant Q1, staining PI positive and Annexin V negative. After 24 h incubation with formalin-killed isolates of bt 1 and bt 2, cellular debris was 1.3% and 1.4% respectively. Following 24 h exposure to bt 1 isolate, one way analysis of variance highlighted that cellular debris significantly increased compared to the control. no significant difference was observed between groups.

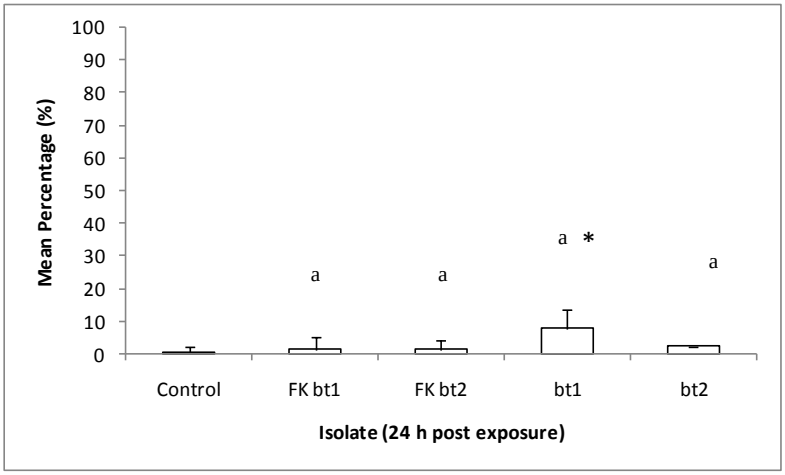


Figure 5.10. Cellular debris changes following 24 h exposure to *Y. ruckeri* isolates. Macrophages were cultured in L-15 at 15^{o}C and analysed by flow cytometry at 24 h via Annexin-V Vs. PI staining. '*' Values are significantly different ($p<0.05$) from the control at that time point. Common letter differ significantly between bacterial isolates.

5.3.8. Real Time PCR analysis of proinflammatory cytokines

5.3.8.1 Dissociation curve analysis (Melt curve)

Fluorescent measurement was obtained by a dissociation curve program with a heating rate of 0.5^{o}C (for 15 sec). Dissociation curves for each primer set are given in Figure 5.11. Melting temperatures for each product was 82.2^{o}C, for β-actin, IL-6, TNF-α, IFN-γ and TLR 5 respectively.

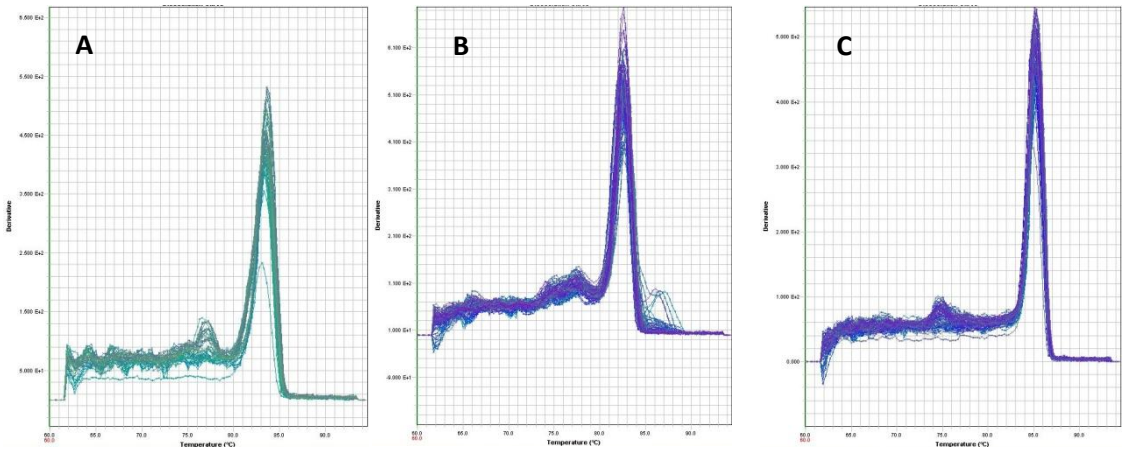


Figure 5.11. Dissociation curve for each primer set indicating amounts of fluorescence, vertical axis, at each temperature point. (a = β-actin, 82^{o}C, b= IL-6, 83^{o}C c = TNF-α, 85^{o}C).

5.3.8.2. Cytokine mRNA expression in liver in response to bt 1 and bt 2 infections

To evaluate whether infection with bt 2 isolates of *Y. ruckeri* were having an affect over the immune response in vaccinated fish, IL-6TNF-α, were studied using RT-PCR.

The mRNA expression levels of IL-6 in the liver of fish are displayed in Figure 5.16. The expression ration for IL-6 in the liver of vaccinated rainbow trout exposed to bt 1 and bt 2 isolates of *Y. ruckeri* are displayed in Figure 5.12 (A & B). Expression levels in fish exposed to bt 1 isolates increased 4 fold after 72 h and decreased to 1 fold after 3 weeks. IL-6 in the liver of fish exposed to bt 2 isolates were initially up regulated after 1 hr, a slight increase in gene expression was noticed after 1 wk post exposure.

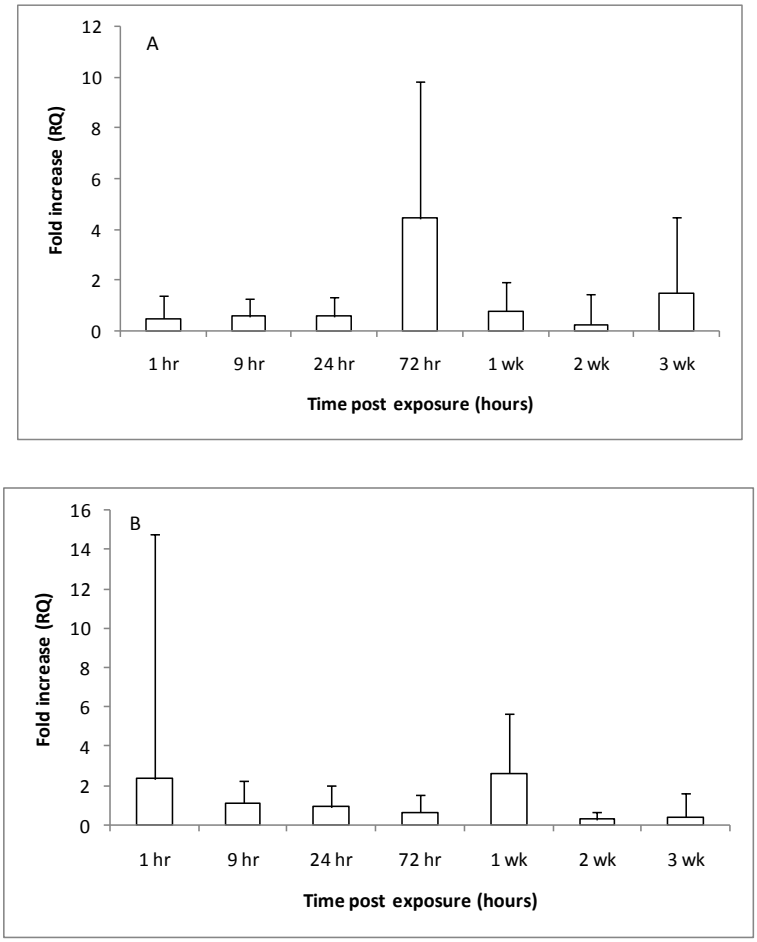


Figure 5.12. Expression ratio (mRNA) for IL6, in liver of rainbow trout following exposure to bt 1 (A) and bt 2 (B) isolates of *Y. ruckeri* over a 3 wk time course. Data shown as expression ration ± *SE* and were compared to the control group using * significant difference <0.05.

The expression ratio for TNF-α in the liver of vaccinated rainbow trout exposed to bt 1 and bt 2 isolates of *Y. ruckeri* are displayed in Figure 5.13 (A and B). Expression levers of fish exposed to bt 1 isolates was down regulated until 2 wks post exposure. In fish challenged with bt 2 mRNA expression levels increased 5 fold after 1 h.

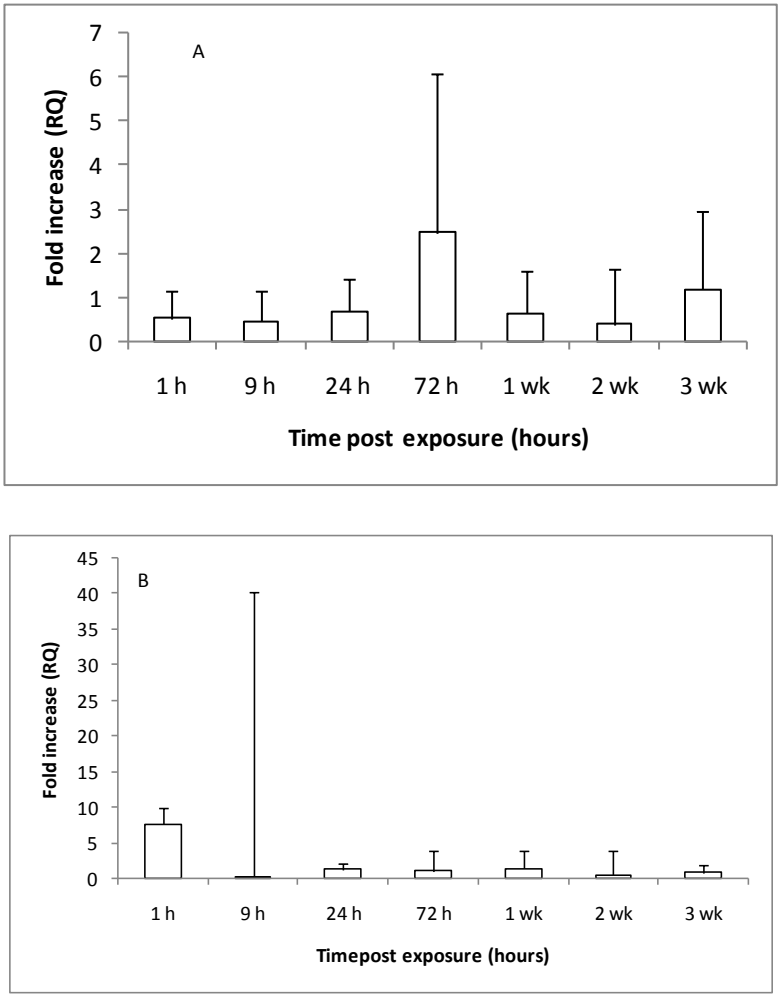


Figure 5.13. Expression ratio (mRNA) for TNF-α, in liver of rainbow trout following exposure to bt 1 (A) and bt 2 (B) isolates of *Y. ruckeri* over a 3 wk time course. Data shown as expression ratio. Error bar indicate RQ value. * indicated significant difference between control group at 0 h

5.4 Discussion

Although a successful commercial vaccine has been produced to protect against O1 bt 1 isolates of *Y. ruckeri*, cross protection against bt 2 isolates appears to be limited (Austin *et al*., 2003; Fouz *et al*., 2006;). It has been demonstrated previously that bt 2 isolates possess different antigenic profiles which may affect the way it interacts with the immune system. These antigens have also been demonstrated to vary in terms of their cross protection efficiency. Therefore it is possible that one of the reasons why vaccine efficacy was reduce against bt 2 infections is due to the interactions of these antigens with the immune system. Currently there is little material regarding the interaction with bt 2 isolates of *Y. ruckeri* with the immune system.

Although non-specific cell mediated immune responses are paramount in the defence against *Y. ruckeri*, little is currently known about the interaction of *Y. ruckeri* with phagocytes during infection (Ellis, 1999). The data presented represents one of the first studies into the bactericidal activity of phagocytes in vaccinated fish against *Y. ruckeri*. The data suggests that there is variability in macrophage killing of bt 1 and bt 2 isolates of *Y. ruckeri*. The difference in macrophage killing could be due to the resistance of non-motile isolates of *Y. ruckeri* avoiding stimulation of the respiratory burst, oxygen radicals and nitric oxide. It has been observed that the killing index of *Y. ruckeri* isolates is stable with bt 1 isolates suggesting that although the bacterium is surviving with the macrophages that they are not replicating intracellularly. This is the opposite observed for bt 2 isolates where the killing index and the number of bacteria are increasing over the 5h period, suggesting that bt 2 isolates are persisting within the macrophage. Ryckaert *et al*. (2010) reported on similar findings with bt 1 isolates. Macrophage killing of isolates coincided with an increase in superoxide anion production. The differences in killing of bacterial strains could be attributed to many factors, such as differential stimulation of the respiratory burst, phagocytosis and resistance to oxygen free radicals. The inability of macrophages to kill serotype O1 isolates of *Y. ruckeri* highlights the importance of other immune parameters such as cytotoxic T cells. Raida and Buchmann (2009) highlighted the importance of CD 8 cytotoxic T cells during *Y. ruckeri* infections.

Macrophages can also produce NO which has been shown to be important antibacterial compound to *Y. ruckeri* (Campos-Pérez *et al.,* 2000). Production of NO by macrophages suggests that there is little differences between bt 1 and bt 2 isolates in terms of triggering NO to be produced by macrophages. LPS has been demonstrated to be a key molecule in inducing NO in fish macrophages (Neumann *et al*., 2001). Artificially induced nitric oxide production through the addition of PMA highlighted that there is some sort of inhibition of production caused by the bacterium. Once such possibility is that, NO is generated in phagocytes as a response to bacterial ingestion and stimulation through cytokines and LPS. The lack of flagella in the non-motile isolates could be a hypothesis as to why a reduced NO response is observed. The lack of flagellum in non-motile bacterial pathogens was hypothesised by Sallum and Chen (2008) to provide benefit by evading detection by host molecular pattern receptors such as Toll like receptors (TLR).

It was demonstrated by Campos-Perez *et al*. (2000) that bt 1 isolates of *Y. ruckeri* were relatively resistant to NO compared with other bacterial fish pathogens. These results correlate with the findings of the bactericidal activity of macrophages against bt 1 isolates of *Y. ruckeri. Y. ruckeri* is a facultative anaerobic organism, this method of metabolism has been hypothesised to possibly protect it from NO during phagocytosis (James, 1995). Although the mechanisms involved in this are not clear, James (1995) suggested that some bacterial pathogens may switch from aerobic to anaerobic metabolism to avoid dependence on enzymes which may be deactivated by NO. Although some NO is produced by phagocytes when exposed to *Y. ruckeri* isolates, a strategy to avoid killing by this means could be to inhibit the accumulation of NO when *Y. ruckeri* is ingested. It has been stated that NO inside phagocytic vacuoles may have accumulated until it reaches the concentration required so that bactericidal killing can occur (Chan *et al*., 1992).The ability of O1 bt 1 and 2 isolates of *Y. ruckeri* to survive phagocytosis and NO production could be related to the production of a number of extracellular enzymes. It has been highlighted that the bacterial enzyme arginase, produced by *H. pylori* helps that pathogen evade the immune response by down-regulating eukaryotic NO production (Gobert *et al*., 2001). It is currently unknown to what extent the secreted products of *Y. ruckeri* play a role in protecting it from phagocytic processes.

The results, indicate that from the *in vitro* experiments, *Y. ruckeri* isolates are able to withstand and alter the production of ROS .The production of superoxide anion ($-O_2$) increased in serotypes O5, O6 and O7 where the levels remained low with serogroups O1 and O2 suggesting that some factors could be responsible for this. There appears to be some correlation with the levels of SOD produced, NO production and the bactericidal activity of phagocytes. To counteract the effects of phagocytic killing bacteria produce a wide range of enzymes that are able to neutralize reactive oxygen species, produced during aerobic metabolism or during respiratory burst in fish macrophages (Barnes *et al*., 1999). The production of differing amounts of superoxide dismutases and catalases in the non-motile isolate of *Y. ruckeri* could be causing this. These enzymes are key virulence factors and differential expression of these enzymes could be a reason for enhanced pathogenicity of bt 2 isolates. Other bacterial pathogens of fish such as *R. salmoninarum*, *V. anguillarum* and *P. salmonis* have been shown to survive within macrophages (Campos-Pérez *et al*., 1997; Boesen *et al*., 2001; Rojas *et al*., 2007). It was suggested that *R. salmoninarum* interferes with the production of ROS in macrophages therefore avoiding bactericidal killing. Sepulcre *et al*. (2007) demonstrated that *V. anguillarum* inhibited the production of ROS and apoptotic processes in order to provide them with a safe environment in which to grow. These pathogenic mechanisms enable the bacterium to survive and proliferate within a host by evading the innate immune response. The data suggest that some modulation of the phagocytic processes is occurring during *Y. ruckeri* infections. Another possibility for the variability of the production of ROS is that certain YOP's and the T3SS are interfering with the phagocyte function. It was demonstrated by Hartland *et al*. (1994) that *Y. enterocolitica* serogroup O:9 inhibits the respiratory burst through altering phagocytic capacity, macrophage morphology and respiratory burst capacity by using a series of YOP's and the T3SS.

The production of ROS such as superoxide anion and H_2O_2 is crucial for the optimal microbicidal activities of phagocytes in host defence. It has been demonstrated that there are differences between the serogroups of *Y. ruckeri* in terms of their resistance to H_2O_2. These differences could be explained by their cell surface characteristics such as OMP's and LPS. Secreted enzymes such as catalases have been suggested to protect bacterial

pathogens from ROI and H_2O_2 exposure during the respiratory burst. Mathew *et al*., (2001) observed that catalase deficient mutant of *E. tarda* were more sensitive to H_2O_2 than catalase producing organisms. The difference observed between the serogroups of *Y. ruckeri* could be explained by their differential production of catalase.

Cytotoxicity in bacterial pathogens is regarded as being a key pathogenic mechanism in enabling *Yersinia* sp. to proliferate within a host (Roquivst *et al*., 1990). The results obtain in this study are the first to highlight that *Y. ruckeri* is cytotoxic to fish phagocytes. By surviving phagocytic processes *Y. ruckeri* isolates are able to produce the necessary enzymes in order to induce a cytotoxic response in macrophages. Both bt 1 and bt 2 isolates were found to be cytotoxic for head kidney phagocytes from rainbow trout compared with formalin killed control isolates after 24 h. Due to the lack of excreted products produced by *Y. ruckeri*, virulence is associated with ingestion of the bacterium. Wang *et al.* (1998) stated that virulent strains of *Vibrio* sp. pathogenic to fish were to cytotoxic to fish cell lines through ingestion. Researchers demonstrated that non-cytopathic strains did not enter cells or induce any changes in cells through cytotoxicity. Other members of the *Yersinia* genus have demonstrated cytoxicity (Rosqvist *et al.,* 1990). As previously discussed, secreted YopE protein of *Y. pseudotuberculosis* induces a cytotoxic response in mouse macrophages where it influences the ability of the pathogen to resist phagocytosis (Rosqvist *et al.,* 1990). Not all members of the *Yersinia* are cytotoxic however, virulent strains of *Y. pestis* has been demonstrated by researchers to reduce the capacity of macrophages to undergo apoptosis (Zumerman *et al.,* 2009). The reduced cytotoxic potency may allow the bacterium to propagate within a shielded environment, suppressing cytokine release, consequently leading to increased virulence (Zumerman *et al.,* 2009). Nematollahi *et al.* (2005) noted that increased virulence in *F. psychrophilum* was correlated with increased cytotoxicity, resistance to ROS and bacteriocidal killing to rainbow trout macrophages. However, Wiklund and Dalsgaard (2003) noted that *F. psychrophiulm* was weakly cytotoxic after 2 h incubation. The reasons for the differences are unclear but it is possible that experimental protocols or bacterial strains could be possible reasons. It appears from other studies that killed bacteria did not cause a significant change in cell condition after 24 h suggesting that the proteins responsible for cytotoxicity need to be actively synthesized and exported by bacteria.

Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover and modulating the pathogenesis of many infections to name a few (Cohen, 1997). These findings appear to be the first demonstrating apoptosis and associated necrosis over time following macrophage exposure to *Y. ruckeri.* The results demonstrated that, like cytotoxicity, live bacteria are required during infection to induce apoptosis because the proteins responsible for apoptosis are actively synthesized and exported by bacteria. However, these results differ from those presented by Ryckaert *et al.* (2010). Workers demonstrated that although *Y. ruckeri* could survive within macrophages for a period of 24 h, after which no apoptosis was detected. Apoptosis is clearly observed by the binding of Annexin-V with PS on the cell membrane. Previous studies have demonstrated that surface expression of PS on macrophages is required for phagocytosis of apoptotic lymphocytes (Callahan *et al.*, 2000). Recent studies demonstrated that phagocytosis of *Escherichia coli* is a stimulus for the phagocyte to undergo apoptosis (Hacker *et al*., 2002). Similar findings were observed in the study by Suzuki *et al.* (2008) when macrophages were exposed to various *Lactococcus* isolates it was shown that ingestion of bacterial cells is required for the exposure of PS in cell surface.

Both O1 bt 1 and bt 2 were able to induce apoptosis which led to necrosis and cell lysis after a 24 h period post exposure to bacterial isolates. Induction of phagocyte necrosis and lysis is an important pathogenic mechanism, as it combines the pathogen evasion from phagocyte antimicrobial activities and the release of highly cytotoxic molecules (Silva, 2010). This pathogenicity mechanism therefore promotes the multiplication of the pathogen and contributes directly to the pathology of *Y. ruckeri* infections. After 24 h, macrophages underwent apoptosis and pronounced cytopathogenesis and necrosis. Potentially *Y. ruckeri* suppresses apoptosis and necrosis for a certain period, allowing sufficient time for replication and dispersion. Toddbeck *et al*. (2009) highlighted that virulent isolates of *Y. ruckeri* once entry was gained spread rapidly reaching the spleen and kidney after 6 h. These results appear to suggest that this could be a possible mechanism of dispersion of *Y. ruckeri* throughout the host as maximum apoptosis and necrosis was observed after 6 h. Other fish pathogens such as *V. anguillarum* have also been re-isolated from different organs within 1 h after contact exposure (Spanggaard *et al.,* 2000). In *Salmonella* sp. induction of macrophage cell death after infection promotes intestinal inflammation and attraction of new macrophages to the intestinal

mucosa (Nematollahi *et al.,* 2005). Monack *et al*. (2000) hypothesised that the induction of inflammation could possibly aid in the dispersal of *Salmonella* from the gastrointestinal tract. Once *Salmonella* has established a systemic infection, such rapid killing of its host cell would be detrimental to the pathogen. During this systemic phase, when bacteria are reliant upon macrophages as a site of intracellular replication, bacteria delay the onset of apoptosis to allow sufficient time to replicate and be distributed systemically, escape and invade new macrophages.

This information obtained goes someway to explaining what is occurring within a host following *Y. ruckeri* infection. Although apoptosis and necrosis have been observed from the results, it is difficult to accurately pinpoint the cause of excreted product profiles. It is likely that since the bacterium is phagocytosed then some kind of intrinsic pathway is involved. Only speculation can be made to whether *Y. ruckeri* is causing apoptosis through caspase involvement via DNA damage, TNF-α suppression or excreted products being injected into the cell. Other members of the *Yersinia* sp have been demonstrated to cause macrophage apoptosis through caspase activation and the intrinsic pathways (Denecker et al., 2001). YopJ in *Y. pseudotuberculosis* and *Y. pestis* injected into the cell via the T3SS, causes a variety of effects, such as suppression of TNF-α and IL-8 production, as the result of the suppression of mitogen-activated protein kinase (MAPK) kinases (MKKs), MAPK, and nuclear factor kB (NF-kB) (2–6) which finally leads to the induction of apoptosis in macrophages (Denecker et al., 2001). To correlate these findings with previous results, cytotoxicity and apoptosis appear to be correlated. Bt 1 isolates were cytotoxic to macrophages after 24 h and this correlates with the degree of apoptosis and necrosis observed. There seems to be a difference in the rates of apoptosis and cytotoxicity between bt 1 isolates and bt 2 isolates of *Y. ruckeri* serotype O1 in vaccinated fish. It could be possible that the motile bt 1 isolates are potentially more pathogenic for fish than bt 2 due to the rate of change in cytotoxicity and apoptosis.

The innate immune system has the ability to produce cytokines that induce inflammation and recruit other immune cells, including neutrophils (Secombes and Fletcher, 1992). The data collected in this study is the first to demonstrate the proinflammoatory cytokine profiles of both biotypes of *Y. ruckeri* in previously

vaccinated fish. Previously it has been demonstrated that following challenge of *Y. ruckeri* bt 1 after 24 h following vaccination at 15^{o}C resulted in increased IL-6 and TNF-α after 24 hrs (Raida and Buchmann, 1998). The result from this study suggested that this was the case with the highest expression ration after 72 h in IL-6 and TNF-α when challenged against bt 1. Fish challenged with bt 2 isolates of *Y. ruckeri* had peak expression levels of IL-6 after 1 h post exposure suggesting that inflammation is occurring or T cells have been stimulated. TNF-α expression in fish exposed to bt 2 isolates was significantly expressed at other sampling points suggesting that local inflammation and lack of cross protection against bt 2 is causing an acute immune response. The results from this study suggest that there is some correlation between apoptosis and TNF- α expression of fish exposed to bt 2 isolates of *Y. ruckeri*. It has been demonstrated in *Y.enterocolitica* infections that increased TNF-α expression has been linked apoptosis (Ruckdeschel *et al.*, 1998).

The results make it quite difficult to correlate these findings with other publications. Raida and Buchmann (2009) demonstrated gene expression within rainbow trout exposed to a motile BT1 *Y. ruckeri* strain 392/2003 via bath challenge. Weins and Vallejo (2010) demonstrated that during bt 2 infections TNF-α and *IL1-β1* transcripts in the spleen were significantly increased after 1 days post challenge with a maximum transcription level at 3 days post challenge. From these publications, it is clear that both bt 1 and bt 2 isolates of *Y. ruckeri* are capable of stimulating a strong pro-inflammatory and acute-phase gene transcription. Therefore it was stated that flagellin, motility and secreted lipase are not required for a rainbow trout pro-inflammatory innate immune response (Wiens and Vallejo, 2010). However, comparing studies must be treated with caution as in genetic makeup of fish, challenge model tissues examined, and also unknown differences between *Y. ruckeri* strains. The differences observed within this study compared to others could be due to the nature of the challenge protocol. It is widely considered that bath challenge is a more natural route of pathogen exposure due to possible stimulation of mucosal immunity at the surface of the host (Raida and Buchmann, 1998). Using I.P injection, although can be administered uniformly and to individual fish, it bypasses both innate and acquired immunity at the mucosal surfaces of the host (Nordmo, 1997).

To summarise, it has been demonstrated that O1 bt 1 and 2 isolates of *Y. ruckeri* have a pronounced effect over macrophage function in vaccinated rainbow trout. Although, the factors influencing whether fish succumb to bt 2 infections are often complex and interrelated with environmental stressors, it have been shown in this study that bt 2 isolates are less readily killed, and have the capacity to modulate the respiratory burst allowing them to survive and replicate within phagocytes. However, bt 1 isolates appear to be more cytotoxic to phagocytes. Both biotypes are able to induce apoptosis in macrophages although necrosis is also observed after 24 h. This apoptosis could be a key pathogenic mechanism of *Y. ruckeri* in order for it to survive and proliferate and survive within a host. Cytokine production in vaccinated fish highlights differences in the immune response, with bt 2 isolates inducing inflammation faster. This highlights the issue of cross protection, suggesting that antigenic profiles are key molecules in protection and stimulation of the immune system. These findings further support the hypothesis that there has been a shift in bacterial population dynamics within the natural environment.

Chapter 6: Conclusion

It is clear that *Y. ruckeri* serotype O1 bt 2 infections are serious to salmonid culture. Initial concerns were raised when non-motile isolates were found to cause disease in previously vaccinated salmonids (Austin *et al*., 2003). To-date, evidence has suggested that the incidence of infections is increasing throughout Europe and North America (Fouz *et al*., 2006; Arais *et al.,* 2007). Evidence presented in this study demonstrates that the reasons for vaccine failure in the field are numerous and complex. However, the data has provided information that will aid microbiologists and fish health experts to develop better disease management strategies in the future. Further work is required in order to reduce *Y. ruckeri's* impacts to the aquaculture industry in the future.

In this study, isolates were screened with phenotypic tests to improve identification methods for bt 2 isolates. Clinical cases have highlighted that serogroup O1 bt 2 is currently the most widely encountered form of the pathogen to date. The majority of isolates were recovered from salmonids, confirming other reports regarding host range (Tobback *et al*., 2007). However, some isolates from non salmonids were recovered suggesting that there is potential for the disease to cause problems in other fish species. Most commonly, the disease within salmonids is associated with serogroup O1, however, it was observed that serogroups O2, O5, and O7 may all cause disease, and it is speculative whether or not these forms of the organism will be problematical in the future. Certainly, the majority of isolates demonstrated a homogenous biochemical profile, therefore, confirming the results of previous authors (Stevenson and Airdrie, 1988; Furones *et al.,* 1993). Phenotypic studies highlighted that there is a need for new diagnostic information to accurately identify bt 2 isolates. The data suggests that by including both sorbitol and the Voges Proskauer reaction in typing schemes, 8 different non-motile phenotypes can be recognised. When analysed together using biotype, OMP type and serotype, the clonal complex theory expanded, suggesting that other virulence factors are responsible for the disease condition. The SDS PAGE of LPS (O antigen) profiles confirmed earlier work (Pyle and Schill, 1985; Romalde *et al.,* 2003), and highlighted variation between biotypes and serotypes of *Y. ruckeri*. This variation along with biochemical differences observed with O1 bt 2 isolates may be used for identification and epidemiological studies. Variation in the LPS molecule may explain some of the immunological and protective variation observed.

Caution must be advised when analysing phenotypic characteristics as their expression may be inconsistent and pose problems of reproducibility of results (Spratt & Madien, 1999). Genes that control these antigens and characteristics are subject to strong diversifying selection and, therefore, evolve rapidly making it difficult to interpret at a population level. Genotyping of *Y. ruckeri* carried out as DNA:DNA hybridisation techniques have been stated as being 'gold standard' in defining bacterial species (Mehlen *et al*., 2004). Due to the associated problems with reproducibility associated with electrophoresis based analysis a MLST scheme was developed in order to provide a platform for other researchers to share data regarding *Y. ruckeri*. Genotyping has identified that there is low genetic diversity between isolates of the organism between bt and serotype. The study failed to find a suitable combination of housekeeping genes that could discriminate between bt 1 and bt 2 isolates. However, the study revealed that the low diversity of bt 2 isolates suggests that they have been present in the environment for some time, and that it is likely that the lack of motility in bt 2 isolates is not due to a mutation caused by vaccination. It is, therefore, difficult to hypothesise how bt 2 became such a problem in aquaculture. Possibly, a combination of vaccine mediated strain replacement and the expansion of aquaculture and carrier fish are the most likely reasons for the distribution of bt 2 throughout Europe. As the prevalence of bt 1 is decreased due to vaccination, bt 2 isolates increase within the environment due to lack of competition. It is difficult to hypothesis whether intensive aquaculture practises have caused evolutionary changes in bt 2 isolate virulence, like other bacterial pathogens (Pullkinen *et al*., 2009).

The approaches of vaccine development for bacterial pathogens in aquaculture still follow some of the traditional principles set out by Pasteur in 1880. Understanding the protective antigens will allow better vaccines to be designed in the future. Until now, little has been published about the antigenic characterisation of *Y. ruckeri*. Western blot profiles of whole cell preparations of O1 bt 1 and bt 2 isolates against *anti-Y. ruckeri* O1 antiserum indicated that there is immunological similarity due to the strong cross reaction. However, surface antigen characterisation revealed that the O antigen varies antigenically between bt 1 and bt 2. Western blotting with anti-*Y. ruckeri* O1 antiserum was able to visualise this difference. This variation alongside the difference in phenotypic traits allows for the differentiation of the serogroup into O1a and O1b. Both biotypes are poor producers of extracellular toxins. This suggests that cell to cell

association is vital for *Y. ruckeri* pathogenicity and similar to other *Yersinia* sp. (Cornelis, 2002). Under iron limiting conditions, *Y. ruckeri* isolates produced four iron-regulated OMP's which confirms previous observations (Davies, 1991b). These were produced in all isolates tested, suggesting that they are not involved in virulence. Western blotting revealed that these molecules were highly immunogenic suggesting the vaccines could be enriched using preparations of these whole cell products.

Vaccination studies revealed that the O antigen is the dominant molecule involved in protection in *Y. ruckeri* products. This variation was backed up by cross protection studies. The newly licensed bivalent vaccine provided good levels of protects whereas monovalent vaccines provided little cross protection against bt 2 isolates. There is little difference in the virulence between bt 1 and bt 2 isolates. This observation supports the hypothesis for vaccine mediated strain replacement theory. It is unlikely that intensive vaccination strategies have produced mutations in the O1 phenotype creating a more virulent pathogen. Therefore, it is possible that vaccination has had an effect over the bacterial population dynamics in the aquatic environment. The lack of cross protection afforded by the monovalent vaccine in combination with a stressor, possibly led to the host succumbing to bt 2 infections. The identification and characterization of important antigens, both cellular and extracellular, should lead to a greater understanding of the pathogenesis of this bacterium as well as being a prelude to the development of recombinant vaccines against *Y. ruckeri* in the future. It is likely from the virulence data and what has been understood from the protective antigens that there is a possibility that bt 2 infections are increasing in prevalence due to the removal of bt 1 isolates from the environment through vaccination.

It has been suggested that the lack of flagellin in the non-motile phenotypes somehow bypasses the host's anti-flagellin immune response (Weins and Vallejo, 2010). It has been hypothesised that non-motile pathogens have a select advantages over motile pathogens in that they lack the appendages (flagella) in order to trigger the immune response (Sallum and Chen, 2008). The study did not provide evidence to support this theory, suggesting that there is little difference in the immune response between bt 1 and bt 2 isolates of the disease, and that vaccine strategy was a possible reason why little cross protection is observed. *In vitro* investigations into the innate immune

response towards *Y. ruckeri* infections highlighted that the bacterium is phagocytosed by macrophages and can subvert some of the respiratory burst processes. Of future interest is the association with the T3SS and YOP's and the ability to subvert macrophage function. Bt 1 isolates appear to be more significantly cytotoxic and cause necrosis of phagocytes after 24 h post exposure. Likewise, bt 2 isolates, although cytotoxic, induced apoptosis and significantly less necrosis. This furthered the argument that although bt 1 isolates are more pathogenic, due to their prevalence in the environment, bt 2 isolates are able to cause disease due to lack of cross reactive/protective antigens in the current monovalent vaccine. Pro-inflammatory cytokines appear to suggest that there is an initial difference between the response times of bt 1 and bt 2 infections in vaccinated fish due to recognition by PAMP's. The results from the study suggest that flagellum is not needed in order for the host to mount an immune response towards the pathogen, similar processes have been observed by other authors (Weins and Vallejo, 2010). These findings further back up the findings that the protective antigens (LPS) are key molecules that the fish require to recognise in order to mount a successful immune response.

Future studies into *Y. ruckeri* should re-evaluate the serology and typing scheme of the species. The data has highlighted numerous O antigen structures that do not fit into the current Davies (1990) typing scheme. Evidence suggests that there are an increasing numbers of biotypes and potential serotypes that could cause problems in the future. Therefore there is scope to develop a range of Pabs and Mabs to help restructure the typing scheme which would greatly aid diagnosis and vaccine development studies. The data in this study is concerned with the host pathogen interactions, there is still scope for investigation into the fate of bt 2 isolates within the environment and their reservoir of infection. Although this research has focused upon salmonids, it is clear from the isolates received and reports in the literature that *Y. ruckeri* is not just and infection of salmonids. As aquaculture and the cultured species list expand, there is scope for investigation into other cultured fish spices which may be subject to the disease.

As emerging technologies develop over time, these could be incorporated into future studies to better understand the nature of bt 2 infection and the *Y. ruckeri* species. The cost of whole genome shotgun sequencing is coming down, there it will be easier to

compare bt 1 and bt 2 isolates. It has been demonstrated that *Y. ruckeri* isolates share the same core genes (~2500) as other *Yersinia* sp., therefore as more information is gathered regarding mammalian infections new information about pathogenic mechanisms in salmonid fish may be eluded (Chen *et al*., 2010). The availability of whole bacterial genomic sequences for *Y. ruckeri* could help helped to identify potential new vaccine subunits (Movahedi and Hampson**,** 2008).

This research presented here has numerous practical implications for vaccine, epidemiological and immunological studies. The data has identified the O antigen as the main protective antigen which should be incorporated into vaccines. The data has also highlighted a number of iron regulated molecules that are immunogenic suggesting that vaccines could be enriched by using these preparations in whole cell vaccines. MLST studies have indicated that the phylogeny if this pathogen is far from understood. One of the overwhelming advantages of MLST is that sequence data is fully portable and stored in a single expanding central multilocus sequence database. This can be accessed and analysed electronically via the Internet to produce a powerful resource for global epidemiology. By making the data available over the internet, this will allow for information exchange and worldwide collaboration.

In conclusion:

1. Outbreaks of disease caused by biotype 2 infections are now observed all over Europe.
2. Biotype 2 isolates have a different O-antigen structure to biotype 1 isolates, which the dominant immunogenic antigen.
3. The likely reason for ‘vaccine failure was the lack of protective O antigen in monovalent vaccine.
4. Eight potential new non-motile phenotypes of *Y. ruckeri*
5. *AroA*, *glnA*, *gyrb* and *thrA* are all suitable loci for investigation into the phylogeny and population structure of *Y. ruckeri*.
6. *Y. ruckeri* species displays low levels of genetic diversity.
7. *Y. ruckeri* are poor producers of extracellular enzymes and toxins and there is little variation between biotypes and serotypes.
8. Protection achieved through the use of a bivalent vaccine containing both biotypes.
9. *Y. ruckeri* appear to be able to subvert macrophage function in-order to survive and proliferate. Results suggest that the respiratory burst including SOD and NO are down regulated during infection.
10. O1 biotype 1 isolates are more cytotoxic to phagocytes then biotype 2 isolates.
11. *Y. ruckeri* isolates are able to induce apoptosis and necrosis in rainbow trout phagocytes.
12. Flagellin and motility are not required for rainbow trout proinflammatory innate immune response.

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