Detecting *Renibacterium salmoninarum* in cultured and wild salmonids

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Greining nýrnaveikibakteríu í öldum og villtum laxfiskum

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Ritgerð til meistaragráðu í Líf- og læknavísindum

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Ágrip

Renibacterium salmoninarum, bakterían sem veldur nýrnaveiki í laxfiskum (BKD), er landlæg um alla jörð, þar á meðal á Íslandi, bæði í villtum fiski og eldisfiski. Sjúkdómurinn veldur einkum vanda í eldi, þó að faraldrar og sjúkdómseinkenni séu þekkt í villtum fiski. Engin fullnægjandi lyfjameðferð er til gegn sýkingunni né bóluefni, sem gerir sjúkdóminn erfiðan viðureignar. Förgun hrogna undan sýktum hrygnum er sú aðferð sem gefst hvað best til að fyrirbyggja smit milli kynslóða og þ.a.l. eru hraðvirkar greiningaraðferðir afar mikilvægar í baráttunni gegn sjúkdómnum. Þær aðferðir sem helst eru notaðar eru greining með mótefnum (ELISA og FAT) og kjarnsýrumögnun (PCR). Ræktun er einkum notuð við sjúkdómsgreiningar, en þar sem hún tekur afar langan tíma hentar hún ekki við skimun. Algengast er að nota nýrnasýni við skimun og greiningu.

Megin markmið verkefnisins var að þróa ódýra, sérhæfða og næma PCR aðferð til að nota við skimun og greiningar ásamt ELISA prófi. Enn fremur að bera slíka aðferð saman við aðrar PCR aðferðir, sérstaklega þá aðferð sem er viðurkennd af Alþjóða dýraheilbrigðisstofnuninni (OIE). Einnig var ný aðferð við kjarnsýrueinangrun (DNA) prófuð. Aðferðirnar voru reyndar á tveimur sýnahópum. Í öðrum hópnum var eldislax með virka sýkingu en í hinum var villtur lax, bleikja og urriði úr Elliðaám og Elliðavatni. Einnig var uppsöfnun utanfrumuafurða (ECP) bakteríunnar í nýra laxfiska könnuð og notuð voru eldisseiði úr stöð þar sem nýrnaveiki var ekki til staðar.

Þróuð var aðferðin snPCR og reyndist hún jafnnæm og nPCR aðferðin sem OIE mælir með. Við greiningu á BKD reyndust snPCR og nPCR greina fleiri jákvæð sýni en aðrar PCR aðferðir sem prófaðar voru. Einnig reyndist vel ný aðferð við að einangra DNA, í samanburði við mikið notaða DNA einangrunaraðferð. Flest jákvæð sýni, í bæði eldisfiski og villtum fiskahópunum greindust hins vegar með pELISA prófi, þar sem notuð eru fjölstofna mótefni til að nema mótefnavaka bakteríunnar. Einnig var sýnt fram á að hvorki tálknasýni né hrognavökvi henta til skimunar.

Niðurstöður úr Elliðaám og Elliðavatni sýna að hlutfall sýktra laxfiska hefur aukist gríðarlega á undanförnum árum. Líklegt er að hækkun á meðalhita vatnsins sé aðal skýringin á þessum breytingum í Elliðavatni sem veldur auknu smiti í silungastofnunum jafnframt því sem aukið smitmagn berst í ána.

Könnuð var uppsöfnun mótefnavaka úr utanfrumuafurðum bakteríunnar í nýrum laxaseiða sem fengu þau í kviðarhol. Með pELISA, þar sem notuð eru fjölstofna mótefni og mELISA með einstofna mótefnum, var hægt að sýna fram á að mótefnavakarnir höfðu borist í nýra tveimur dögum síðar. Eftir 6 vikur sýndi mELISA að magn MSA próteinsins fór lækkandi, en pELISA sýndi ekki marktæka breytingu.

Abstract

Renibacterium salmoninarum, that causes bacterial kidney disease (BKD), is endemic in both wild and farmed stocks of salmonids worldwide, including Iceland. The disease is mainly a problem in culture, in fresh and marine water, although epidemics and clinical signs of BKD are observed in wild fish. Therapeutic measures, including the use of antibiotics or vaccines, have been tried with limited success. Brood stock culling, where fertilized ova from infected females are destroyed, is an important method in the battle against the disease and therefore rapid diagnostic tests are important. The diagnostic methods currently used for screening are Fluorescent antibody techniques (FAT), enzyme-linked immunosorbent assays (ELISA) and polymerase chain reactions (PCR) and for these analyses, kidney tissue is most commonly used. Culture is used in diagnostics, but the slow growth of the bacterium hampers the use of culture for screening purposes.

The main objectives of the study were to develop a cheap, specific and sensitive PCR method to use along with an ELISA test for screening as well as confirmative purposes. Also, to compare such a method with other PCR tests, esp. the nested PCR (nPCR) test recommended by OIE, the World Organization of Animal Health. The use of FTA minicard, an easy way to isolate and store DNA, was also tested and compared to conventional method.

The protocols were tested in cultured salmon with active infection and in wild Atlantic salmon, Arctic charr and brown trout from Lake Ellidavatn and River Ellidaár that flows from the lake. To investigate how long bacterial antigens can be detected in salmonid kidney after exposure to extracellular products (ECP) of the bacterium, Atlantic salmon fry from a BKD free farm were injected intra-peritoneally with two different doses of ECP and sampled over 6 weeks.

A semi-nested PCR (snPCR) method was developed in the study and proved to be as sensitive as nPCR. These two methods detected more positive samples than other PCR methods tested. Using FTA minicard for DNA isolation further increased number of positive samples in snPCR and nPCR. ELISA using polyclonal antibodies detected the highest number of positives in both the cultured and wild sample groups. Ovarian fluid or gill tissue can not replace kidney tissue for *R. salmoninarum* detection as fewer positive fish were detected and many samples gave inconclusive results. Results obtained from wild fish revealed a significant increase in the prevalence of *R. salmoninarum* positive fish than has been observed previously. Six weeks after ECP injection, a decline over time in the average amount of the MSA antigen was observed using ELISA with monoclonal antibodies, but ELISA with polyclonal antibodies did not show a significant change in OD values.

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Abbreviations

BKD	Bacterial kidney disease
CC-R7	CC chemokine receptor 7
CHSE	Chinook salmon fibronectic and embryo cells
Cox-2	Cyclo-oxygenase
Ct	Cycle threshold
CXC-R4	CXC chemokine receptor 4
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
gDNA	Genomic DNA
dpi	Days post injection
ECP	Extra cellular products
ELISA	Enzyme-linked immunosorbent assay
FAT	Fluorescence antibody test
IL-1β	Interleukin-1β
iNOS	Inductible nitric oxide synthase
i.p.	Intraperitoneal
kDa	Kilodalton
KDM	Kidney disease medium
KDM-C	Kidney disease medium supplemented with charcoal
MHC	Major histocompatibility complex
MSA	Major soluble antigen, protein
msa	Major soluble antigen, gene
MW	Molecular weight
nPCR	Nested PCR
OD	Optical density
p57	Protein, 57kDa in size, (MSA)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKD	Proliferative kidney disease
qPCR	Quantitative polymerase chain reaction
RNI	Reactive nitrogen intermediates
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
rpm	Rounds per minute
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SKDM	Selective kidney disease medium
snPCR	Semi-nested PCR
TGF-β	Transforming growth factor - β
TNF-α	Tumor necrosis factor - α

1 Introduction

1.1 Salmonids

Atlantic salmon (*Salmo salar* L.), Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) all belong to the family Salmonidae, order Salmoniformes, commonly referred to as salmonids. There are about 70 species in the order Salmoniformes, all classified in Salmonidae, the only family within the order. They are some of the most studied fish species of the world. Trout, salmon and charr comprise the subfamily Salmoninae. All salmonids spawn in freshwater but many are anadromous, spending part of their life at sea. Salmonids are caught for subsistence, commercial gain, and recreation both at sea and in freshwater. Some salmonid species are as well popular for aquaculture. Salmonids are native to cool waters of the Northern hemisphere. However, due to transplantation outside their native ranges, some salmonid species now occur worldwide. Atlantic salmon, Arctic charr and brown trout are found in Icelandic waters. The Atlantic salmon is anadromous but the Arctic charr and brown trout can be both anadromous or freshwater residents (Jonsson, 1983; Mecklenburg *et al.*, 2002).

1.2 Bacterial kidney disease

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, was first reported in Atlantic salmon in Scotland in 1933 in the rivers Spey and Dee (Smith, 1964). The bacterium is endemic worldwide in wild and farmed stocks of salmonids, including Iceland (Smith, 1964; Pippy, 1969; Evelyn *et al.*, 1973; Ellis *et al.*, 1978; Mitchum *et al.*, 1979; Banner *et al.*, 1986; Souter *et al.*, 1987; Sanders *et al.*, 1992; Jonsdottir *et al.*, 1998; Meyers *et al.*, 1999; Meyers *et al.*, 2003; Chambers *et al.*, 2008). The disease is mainly a problem in cultured salmonid fish, in fresh and marine water, although epidemics and clinical signs of BKD are observed in wild fish (Smith, 1964; Evelyn *et al.*, 1973; Mitchum *et al.*, 1979; Souter *et al.*, 1987).

1.3 BKD in Iceland

The bacterium was first detected in Iceland, in cultured Atlantic salmon fry in 1968 (Helgason, 1985) and in 1976, the bacterium was detected for the second time in cultured fish (Helgason *et al.*, 1992). During the 1980's the fish farming industry grew rapidly in Iceland and in 1985, BKD was diagnosed on four fish farms (Gudmundsdottir *et al.*, 2000). Systematic screening for the bacterium was started in 1986, a year later. In that epidemic, which lasted for around 6 years, the prevalence and the infection load increased in the fish farms and the overall percentage of positive brood fish in infected farms was around 35% when a brood stock culling program was initiated (section 1.7.5). After a few years of

brood stock culling, the prevalence figures in brood fish declined and remained below 2.0% (Gudmundsdottir *et al.*, 2000). The following years, there were sporadic incidents of BKD, mainly in wild brood fish. Between 2003 and 2007, a widespread BKD epidemic, affecting cultured Atlantic salmon, Arctic charr and rainbow trout (*Oncorhynchus mykiss* (W.)), was experienced in Iceland and the bacterium was detected in total of 18 fish farms, i.e. approximately half of all fish farms rearing salmonids in Iceland at the beginning of the epidemic. When looking at the epidemiology, the source of infections can be traced back to seemingly two separate initial incidents (records of the Fish Disease Laboratory, Keldur). Macroscopic symptoms were observed 1 to 28 months after the predicted initial infection, on the fish farms where the bacterium was detected, or not at all in few cases. With systematic screening for the bacterium and by practicing culling, the epidemic was brought under control. Gaining control of the epidemic was a costly procedure. Millions of fish were discarded in the process and the damage to the fish farming industry amounted to hundreds of millions of Icelandic krónur (ISK), or millions of Euros (€) (Kristmundsson *et al.*, 2008; Kristmundsson *et al.*, 2009).

A thorough study of the infection status of wild Arctic charr and brown trout, in 23 Icelandic lakes, was conducted at the end of the last century. A high proportion of the fish were infected but macroscopic symptoms of BKD were not observed. The prevalence of infection were 3-100% in Arctic charr and 6-81% in brown trout, but the antigen load in the fish kidney was low (Jonsdottir *et al.*, 1998). In Lake Ellidavatn, the prevalence was 16% in Arctic charr and 13% in brown trout.

Between 1991 and 2006, approximately 400 kidney samples from wild brood fish, caught in River Ellidaár located within the Reykjavík area, were screened for *R. Salmoninarum* antigens and only one fish tested low positive in ELISA. In the years 2006-2008, the prevalence in River Ellidaár had increased to 70-90% of all brood fish stripped for enhancement (Kristmundsson *et al.*, 2010a).

1.4 Disease symptoms

1.4.1 External symptoms.

Fish heavily infected with *R. salmoninarum* can suffer from various external symptoms such as exophthalmia, petechiae (Figure 1), pale gills, general haemorrhaging in areas around the fins and the lateral line, skin discolouration, distended abdomen, superficial blebs and blisters and loss of balance (Fryer & Sanders, 1981; Bruno, 1986; Hirvela-Koski, 2005).

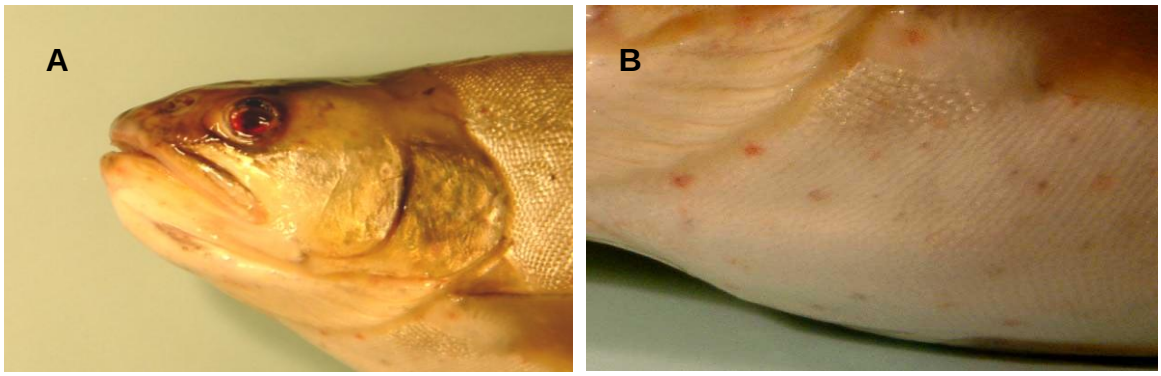


Figure 1. Exophthalmia (A) and petechia (B) observed in Arctic charr with BKD.
Photo: Keldur.

1.4.2 Internal symptoms

Macroscopic lesions are typically observed in some internal organs, in heavily infected fish. Greyish-white nodules or granulomas are commonly observed in the kidneys (Figure 2). Similar granulomas can also be seen in the spleen, liver and the heart. Other internal clinical signs include splenomegaly, light colored liver, pale kidney, increase in the peritoneal exudates, general anemia, and a formation of an opaque membrane around some of the internal organs. Accumulation of ascitic fluid is also often observed in the abdominal cavity. The kidney becomes greatly enlarged, grayish-white and necrotic in severe cases. Extensive tissue destruction in vital organs is presumably the direct cause of death (Fryer & Sanders, 1981; Bruno, 1986; Dale, 1994; Hirvela-Koski, 2005).

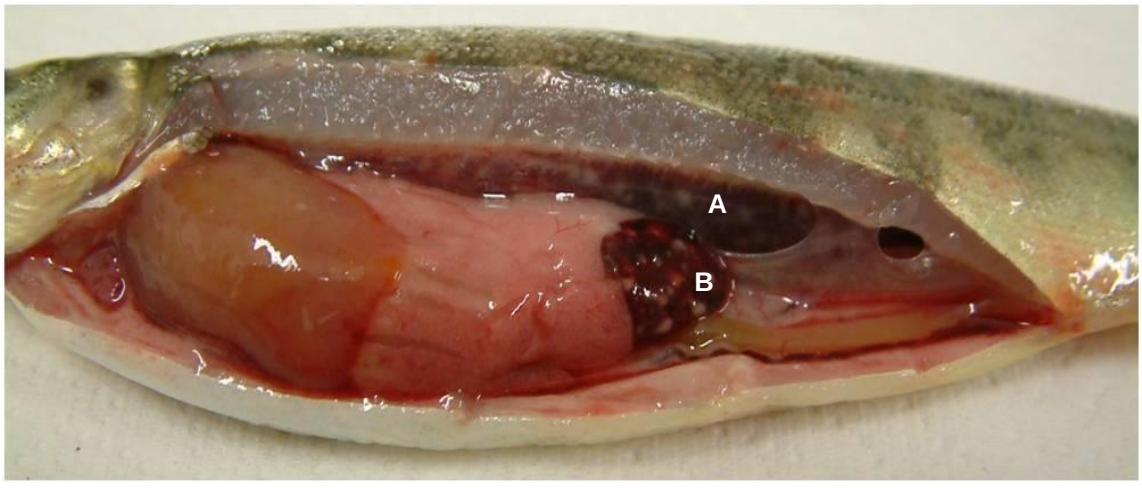


Figure 2. Granulomas in kidney (A) and spleen (B) observed in infected Arctic charr.
Photo: Keldur.

1.5 BKD susceptibility

The susceptibility to infection with *R. salmoninarum* can vary, depending for example on species, stage of life and variations in environmental factors. With higher susceptibility, the salmonid fish acquire more severe disease with higher mortality rate. Environmental factors, such as temperature (Smith, 1964; Sanders *et al.*, 1978), salinity (Fryer & Sanders, 1981), predation (Mesa *et al.*, 1998), and lack of nutrients (Jonsdottir *et al.*, 1998) can affect the host's stress responses which is somehow related to susceptibility to *R. salmoninarum*. Plasma cortisol, the indicator of a stress response, increases during BKD progression (Mesa *et al.*, 1999). What comes first is not fully known, the elevation of plasma cortisol followed by increased progression of the disease, or the other way around (Mesa *et al.*, 1998).

As mentioned before, clinical signs of BKD are mainly observed in cultured salmonids and rarely seen in wild fish (sections 1.4.1 & 1.4.2). Crowding in tanks and transfer of fish between locations are stress factors in cultured fish and may affect the susceptibility of the fish to diseases such as BKD. Hormonal changes and physiological stress during smoltification and spawning also affect the host's stress responses (Schreck, 2010). In a study on the antigen load in Arctic charr in 23 lakes in Iceland, it was demonstrated that the antigen load was higher in prospective spawners than in non-spawners (Jonsdottir *et al.*, 1998). Another study showed that an exposure of ova to MSA protein (major soluble antigen, see section 1.6.4.3) could result in long-term immunosuppression of the fish and increased vulnerability to subsequent challenges with *R. salmoninarum* (Brown *et al.*, 1996; Hamel, 2005). The differences in susceptibility to BKD between salmonid species indicate that Arctic charr, brown trout and Atlantic salmon are fairly resistant while rainbow trout is considered to be the most resistant species but chinook salmon (*Oncorhynchus tshawytscha* (W.)) considered to be one of the most susceptible species (Sanders *et al.*, 1978; Jansson *et al.*, 1996; Dale *et al.*, 1997; Starliper *et al.*, 1997; Meyers *et al.*, 2003).

1.6 *Renibacterium salmoninarum*

1.6.1 Bacterial characteristics

R. salmoninarum is a Gram positive, aerobic, non-sporulating, non-motile, not acid fast, 0.3 to 1.0 by 1.0 to 1.5µm short rod shaped bacterium, often occurring in pairs (Sanders & Fryer, 1980). The bacterium is a well known intracellular obligate pathogen in fish belonging to the subfamilies Salmoninae and Thymallinae of the Salmonidae family, order Salmoniformes (Bruno, 1988a). Recently the bacterium has also been detected in some Whitefish species members of the third subfamily of the Salmonidae, Coregoninae (Mecklenburg *et al.*, 2002; Rimaila-Pärnänen, 2002; Faisal *et al.*, 2010).

The cell wall of Gram positive bacteria consists of a single, thick, continuous layer. Peptidoglycan, the major polymer of the cell wall, mechanically strengthens the cell wall in most Gram

positive bacteria, in association with polysaccharides and teichoic acids. The amount of peptidoglycans in the cell wall of *R. salmoninarum* is relatively low and of the type A3α, containing lysine with N-acetylmuramyl residues linked by phosphodiester bridges to the polysaccharide. The polysaccharides are the major components of the cell wall, approximately 60-70% of the dry weight. The cell wall contains galactose as the major sugar component and the unique amino sugar N-acetyl-fucosamine, a rare component in the Gram positive cell walls (Kusser & Fiedler, 1983; Fiedler & Draxl, 1986). The resistance of the intact cell wall to lysozyme may be due to the covalently linked peptidoglycan to the polysaccharides. The polysaccharides (Fryer & Sanders, 1981) could also be the capsule-like material encapsulating the bacterium as seen by electron microscopy of immunostabilized bacterium (Dubreuil *et al.*, 1990a). The G+C content of the DNA was measured 55.5 mol% when comparing seven different isolates (Banner *et al.*, 1991) but when sequencing of the whole genome of the bacterium (section 1.6.3) which was concluded recently, the G+C content was determined to be 56.3 mol% (Wiens *et al.*, 2008).

1.6.2 Isolation and growth properties

KDM-2 medium (Evelyn, 1977), or the selective version of it, SKDM (Austin *et al.*, 1983) are commonly used for *R. salmoninarum* isolation and cultivation. The medium includes L-cysteine which is essential for the growth of the bacterium (Ordal & Earp, 1956). SKDM is supplemented with four antimicrobial compounds, D-cycloserine, polymyxin B, oxolinic acid and cycloheximide which reduces the problem with fast growing contaminating organisms. It has been reported either to reduce (Olsen *et al.*, 1992) or enhance (Gudmundsdottir *et al.*, 1991) the growth of *R. salmoninarum* itself. Serum (Fryer & Sanders, 1981) or activated charcoal (Daly & Stevenson, 1985) is added to the medium to enhance growth of the bacterium. Additionally spent broth from *R. salmoninarum* cultures enhances growth (Evelyn *et al.*, 1990; Teska, 1994). The bacterium can grow at temperatures between 5 and 22°C but the optimum temperature for growth is between 15 and 18°C (Sanders & Fryer, 1980). Being nutritionally fastidious, *R. salmoninarum* grows slowly and that is an important characteristic of the bacterium. In a study of infected herd of Atlantic salmon brood fish, it was shown that lengthening the incubation time from 6 to 12 weeks significantly increased the number of positive samples and that it could take up to 19 weeks for the bacterium to form colonies on the SKDM agar plate (Benediktsdottir *et al.*, 1991). The colonies of *R. salmoninarum* on solid medium are creamy-yellow, convex and smooth (Sanders & Fryer, 1980). The colony size is often around 2mm (OIE 2006) but can vary and all sizes contain bacteria with the same morphological characteristics (Ordal & Earp, 1956). Catalase test should give positive reaction and the oxidase test negative (OIE 2006). API-ZYM system (bio-Merieux), FAT (fluorescent antibody test) and Gram staining can also be used for the identification of the colonies (Benediktsdottir *et al.*, 1991; OIE, 2006). Atypical growth of *R. salmoninarum* has been observed. When cultured on KDM-2 agar plate, dry crystalline material is sometimes observed consisting of masses of hexagonal crystals on the surface of the agar surrounded by a thin layer of bacterial growth when seen in stereo microscope (Hirvelä-Koski *et al.*, 2006).

1.6.3 Taxonomy of *R. salmoninarum*

R. salmoninarum was originally placed in the genus *Corynebacterium* by its morphological appearance (Ordal & Earp, 1956). By analyzing the G+C mol% content of the DNA, cell wall sugar composition and the amino acid composition of the peptidoglycan cell wall layer, the bacterium was given a name and placed in a unique genus (Sanders & Fryer, 1980). A phylogenetic comparison of the 16S rRNA gene of *R. salmoninarum* and more than 165 Gram positive bacteria from 50 genera, using the cataloguing approach, revealed that *R. salmoninarum* is a member of the actinomycetes subdivision which includes for example the nonpathogenic Arthrobacter genus (Stackebrandt *et al.*, 1988). Another phylogenetic comparison study was conducted comparing the 16S rRNA gene of *R. salmoninarum* and 17 other Gram positive bacteria species, with either low (<50%) or high (>55%) G+C mol% content in the DNA. That study confirmed that *R. salmoninarum* is most similar to bacteria with high G+C mol% content (Gutenberger *et al.*, 1991). The genome of *R. salmoninarum* was recently sequenced (Wiens *et al.*, 2008). Phylogenetic analysis of the 16S rRNA gene and whole-genome alignment, using the sequenced genome, confirmed *Arthrobacter* species to be the closest relatives and *R. salmoninarum*, sharing 1562 protein ORF clusters or 7336 ORFs with both *Arthrobacter aurescens TC1* and *Arthrobacter sp. FB24 (Figure 3)*. As seen in figure 3, *R. salmoninarum* is also related to *Mycobacterium tuberculosis* and they share the characteristic of being slow growing, intracellular pathogens, surviving in the host's mononuclear phagocytes (Nuermberger *et al.*, 2004). According to Wiens *et al.* (2008) *R. salmoninarum* evolved into a pathogen from members of the genus *Arthrobacter*, via genome reduction and horizontal gene acquisition, some associated with pathogenesis. The genome of *R. salmoninarum* contains two or three identical copies of the msa gene (section 1.6.4.3) that was apparently acquired horizontally, as for example the nearby insertion elements indicate, and subsequently duplicated in two separate events forming msa 1, msa 2 and msa 3 (O'Farrell & Strom, 1999; Rhodes *et al.*, 2004a; Wiens *et al.*, 2008). The msa gene has no homolog in any other sequenced bacterial genome. Comparison of the msa gene between *R. salmoninarum* isolates from different geographical regions shows that the coding sequence of the gene is highly conserved among the isolates and that they are almost identical. Two strains are known to have a single nucleotide substitution in the gene resulting in a Ala^{139}-to-Glu codon mutation in the protein (Cook & Lynch, 1999; O'Farrell & Strom, 1999; Wiens *et al.*, 2002; Wiens & Dale, 2009). The strains with this mutation lack a known epitope of the protein and have increased agglutinating and binding activity to salmonid leukocytes and rabbit erythrocytes (section 1.6.4.3). Further genetic comparisons of *R. salmoninarum* isolates from different geographical regions by using the multilocus enzyme electrophoresis (MEE) or analyzing the sequence of the 16S-23S rDNA spacer region conclude that the bacterium is a highly conserved genospecies with low genetic diversity (Starliper, 1996; Grayson *et al.*, 1999).

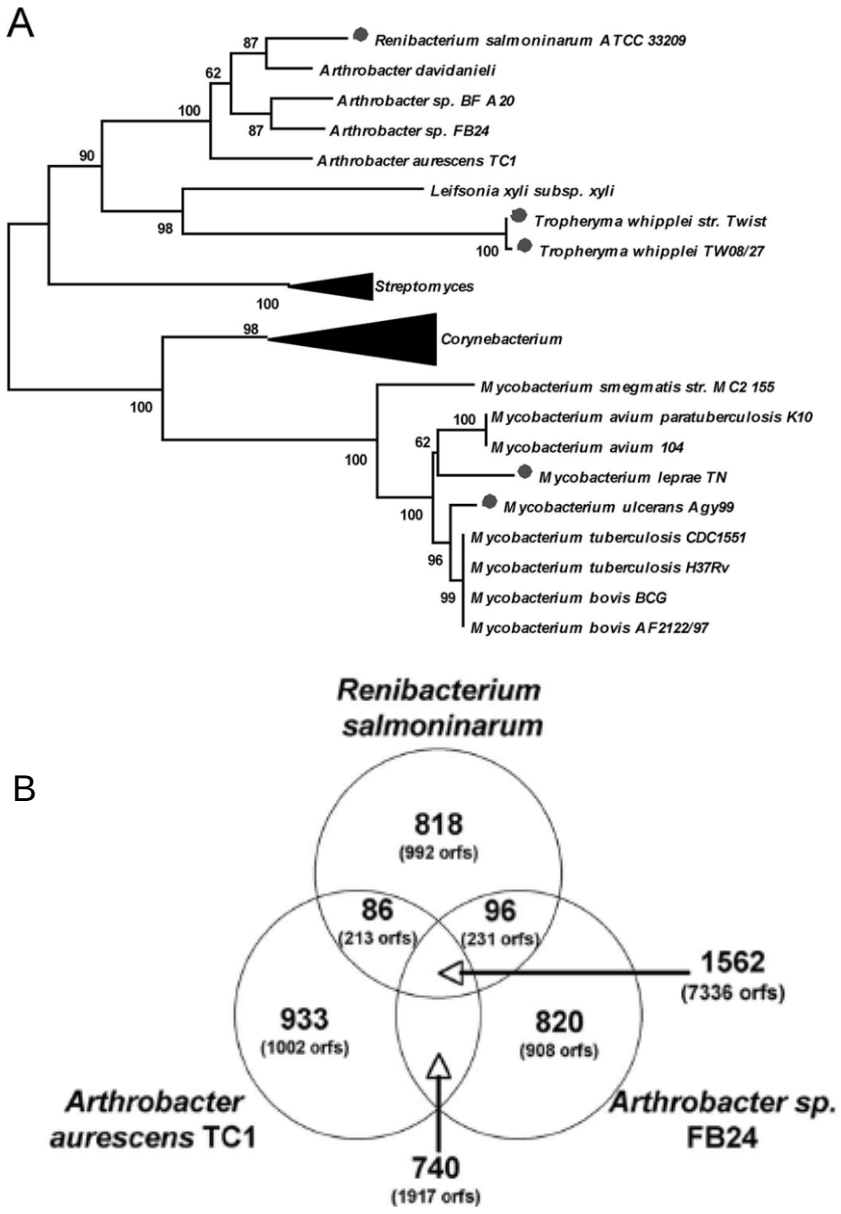


Figure 3. A phylogenetic tree and protein cluster analysis.

A: R. salmoninarum is closely related to *Arthrobacter* spp. based on 16S rRNA sequence analysis. 16S rRNA sequences were aligned using ClustalW, and a tree was constructed using the neighbor-joining method with 1.000 bootstrap replications.

B: Venn diagram showing the numbers of common and unique protein clusters for *R. salmoninarum* and the sequenced *Arthrobacter* spp. The numbers of protein clusters and the total numbers of ORFs in the clusters are indicated.

Picture A and B: Wiens *et al.*, (2008).

1.6.4 Pathogenesis

1.6.4.1 Transmission of R. salmoninarum

R. salmoninarum can be transmitted between fish both horizontally, from individual fish to another, and vertically, from the female parent to her progeny via the eggs (Evelyn *et al.*, 1984; Armstrong *et al.*, 1989; Pascho *et al.*, 1998). There is evidence that the role of the male brood fish in the vertical transmission is none (Klontz, 1983; Evelyn *et al.*, 1986b; Elliott, 2002). As reviewed by Elliot *et al.* (1989) the disease can be transmitted horizontally from infected fish sharing the same water supply but also via the fecal-oral route (Balfry *et al.*, 1996). The use of binary coded wire tags for identification and management of the anadromous salmonid population, seemed to enhance the horizontal transmission of the pathogen (Elliott & Pascho, 2001). The horizontal transmission can occur in both fresh- and seawater (Murray *et al.*, 1992) and when outside the host, the bacterium can survive for weeks (Balfry *et al.*, 1996; Hirvela-Koski, 2004).

1.6.4.2 Intracellular existence

R. salmoninarum survives intracellularly in mononuclear phagocytes, such as macrophages (Bruno, 1986; Gutenberger *et al.*, 1997). The process of the invasion and intracellular survival of the bacterium is still poorly understood. It has been demonstrated that the adherence of *R. salmoninarum* to rainbow trout macrophages was enhanced by complement-mediated opsonisation (Rose & Levine, 1992) although the internalization of the bacterium can take place in the absence of serum factors (Bandin *et al.*, 1995). The MSA protein seems to function as a nonfimbrial adhesion for the bacterial attachment to cellular receptors allowing uptake by phagocytic cells (Wiens & Kaattari, 1991). In a recent study, a sortase homolog (srtD) was found in the genome of *R. salmoninarum*. The gene encodes a specific enzyme found in Gram-positive bacteria and enables them to anchore themselves to the host's cell surface (Sudheesh *et al.*, 2007). The intracellular survival of the bacterium depends on its ability to escape from the host's cell phagosome into the cytoplasm. Formalin-killed *R. salmoninarum* was able to escape into the cytoplasm which indicates that the surface molecules of the bacterium are involved in the internalization (Gutenberger *et al.*, 1997). Reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) are induced in the phagocytosis of *R. salmoninarum* (Campos-Perez *et al.*, 1997; Campos-Perez *et al.*, 2000). One study demonstrated that both virulent and avirulent strains were able to survive and possibly multiply within rainbow trout phagocytic cells for 3-4 days before decrease in number of viable bacteria was observed. During the first 5 days or while *R. salmoninarum* was able to survive in the phagocytes, the infected phagocytes were still able to kill *Aeromonas salmonicida*, indicating that mechanisms other than O_2^- mediated bactericidal systems are important in *R. salmoninarum* killing (Bandin *et al.*, 1993a). In contrast, one study demonstrated that O_2^- mediated bactericidal systems are indeed important in *R. salmoninarum* killing but nitric oxide radicals are not important (Hardie *et al.*, 1996). Another study demonstrated that the survival of *R. salmoninarum*, inside rainbow trout macrophage, can be prolonged if the bacterium is opsonised prior to

phagocytosis. The survival could be prolonged even more if the bacterium was exposed to immune sera, compared to exposure to non-immune sera (Bandin *et al.*, 1995). Infected circulating macrophages can serve as a transporter and disseminate the bacterium throughout the fish, and it can conceal the bacterium from the host's immune functions (Dale, 1994).

1.6.4.3 Virulence factors and host responses

There are several studies on potential virulence factors in the *R. salmoninarum*'s surface proteins and/or extra cellular products (ECP). The MSA protein plays a vital role in the bacterial virulence (Bruno, 1988b). The MSA protein is known to be a part of the ECP as well as being the predominant cell surface antigen, reportedly 70% of the total surface proteins of the bacterium (Dubreuil *et al.*, 1990b; Wood & Kaattari, 1996). The MSA protein is 57 kDa in size and is also called p57 or SM-40 and is encoded by two identical msa genes (O'Farrell & Strom, 1999). Both genes are needed for full virulence (Coady *et al.*, 2006). Some strains have the third copy of the gene and have increased virulence compared to strains with two copies of the gene (Rhodes *et al.*, 2004a). *R. salmoninarum* produces and secretes the MSA protein constantly and in large quantities during infection and growth (O'Farrell & Strom, 1999; Grayson *et al.*, 2002). Low-virulent strains of *R. salmoninarum* have been shown to have reduced levels of the MSA protein on the bacterial cell surface (O'Farrell *et al.*, 2000). *In vitro*, the MSA protein haemagglutinates rabbit red blood cells (Daly & Stevenson, 1990) and has agglutinating properties on salmonid leucocytes (Wiens & Kaattari, 1991). It has been demonstrated that the MSA protein suppresses the host's immune response (Turaga *et al.*, 1987; Grayson *et al.*, 2002). A surface protein, the size of 22 kDa produced by the bacterium, is also thought to have a suppressive effect on the host's immune system. The protein agglutinates salmon leucocytes as well (Fredriksen *et al.*, 1997). Two genes in *R. salmoninarum* are associated with haemolytic activity, rsh and the metalloprotease hly, though haemolytic activity has not been observed *in vivo* (Bandin *et al.*, 1991; Grayson *et al.*, 2002). During the first few hours of internalization of the bacterium into rainbow trout macrophages, the expression of both rsh and hly genes were suppressed before being upregulated again (Grayson *et al.*, 2002). In one study, the expression of genes in rainbow trout associated with the host's immune system were analyzed after injection with DNA vaccine containing the msa gene (Grayson *et al.*, 2002). The expression of the msa gene suppressed the expression of interleukin-1β (IL-1β), inducible cyclo-oxygenase (Cox-2), and major histocompatibility complex class II (MHC-II) but enhanced the expression of tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), CXC chemokine receptor type 4 (CXC-R4), and CC chemokine receptor type 7 (CC-R7). In the same study, the expression of genes *in vitro*, in rainbow trout macrophages, associated with the host's immune system were analyzed during the internalization and infection with live *R. salmoninarum*. The expression of IL-1β, Cox-2, MHC-II and inducible nitric oxide synthase (iNOS) were upregulated, while the expression of tumour necrosis factor-α (TNF-α) was downregulated rapidly during the first few hours of internalization. After the rapid down regulation, the expression of TNF-α was enhanced and stayed stimulated throughout the experiment (5 days). The suppression of TNF-α during the first few hours of the internalization, can affect both the TNF-α-dependent-iNOS-

dependent pathway (RNI) and the TNF-α-dependent-iNOS-independent pathway (ROI), both been shown to be important in the killing of intracellular bacteria although the importance in the killing of *R. salmoninarum* is controversial (section 1.6.5). The presence of TNF-α is required for the enzymatic function of iNOS (Bekker *et al.*, 2001), therefore the suppression of TNF-α during the first few hours of internalization may assist in the survival of *R. salmoninarum*. According to the DNA vaccine experiment mentioned above, the constituent expression of msa is probably not causing the suppression of TNF-α in the first few hours of *R. salmoninarum* infection in the macrophages. It is more likely causing the prolonged stimulation of TNF-α in the later stages of the infection. The chronic inflammatory pathology of BKD may be due to the prolonged stimulation of TNF-α. The long-term suppression of IL-1β may play a vital role in the suppression of other immune functions, such as phagocyte function, cytokine and eicosanoid production, lymphocyte proliferation and activation including T-cell-dependent antibody production. Prolonged suppression of MHC II expression may change the T-cell response into MHC I-dependent pathway and therefore induce the cytolytic T-cell response which may worsen the pathology of BKD (Grayson *et al.*, 2002).

The formation of lesions are believed to be due to the interaction between the bacterium itself and the hosts immune response (Young & Chapman, 1978). Extensive tissue destruction in vital organs can lead to death but mortalities due to BKD are not only directly attributed to the pathogen itself in the wild, but as well the vulnerability to predation in its environmental surroundings. Alteration in behavior in fish infected with *R. salmoninarum* leads to an increased vulnerability to predation (Mesa *et al.*, 1998).

1.7 BKD disease control

BKD is one of the most difficult of bacterial fish diseases to control. It's a slow evolving disease, asymptomatic in some cases in the host. The infectious agent is able to transmit vertically, and no effective vaccine or chemotherapy is available against the bacterium. As reviewed by Elliot *et al.* (1989) many therapeutic measures have been tested for its control with limited success. The intracellular survival of the pathogen is thought to be the biggest factor why the usefulness of these therapeutic measures has been so limiting.

1.7.1 Husbandry practices

Good husbandry is important in disease control. Crowding, bad feed quality and stressful handling may increase disease susceptibility. Stocking density over 22 kg m^{-3} resulted in lower welfare scores, which was measured by analyzing the condition of their bodies and fins and the plasma concentration of glucose and cortisol via multivariate analysis (Turnbull *et al.*, 2005). Good sanitary procedures, including year class separation and avoiding contact with other farms and wild fish further decrease the chances of introduction of the bacterium.

1.7.2 Diet modification

Several diet modification studies have been conducted in *R. salmoninarum* infected fish, as reviewed by Elliott *et al.* (1989). Substitution of dry diet containing corn gluten with cottonseed meal afforded some resistance to the Pacific salmon (*Oncorhynchus spp.*). The results suggested that fish fed corn gluten suffered more from nonspecific stress than fish fed cottonseed meal. In another study it was shown that an experimental semi-purified diet supplemented with iodine and fluorine reduced the occurrence of BKD (Elliott *et al.*, 1989).

1.7.3 Chemotherapy

The macrolide antibiotic erythromycin has proven to be the most effective compound tested against *R. salmoninarum*. Conversely, the drug has inadequate effect on infected fish since infection is not eliminated from all treated fish. Consequently there might still be a number of asymptomatic infected fish remaining, serving as reservoir of infection. The intracellular existence of *R. salmoninarum* may protect and limit exposure of the bacterium to therapeutic agents. Erythromycin has been used to try to prevent-vertical transmission of the bacterium (section 1.6.4.1) (Bullock & Leek, 1986; Evelyn *et al.*, 1986a; Lee & Evelyn, 1994; Gudmundsdottir *et al.*, 2000). Reduced susceptibility of *R. salmoninarum* to macrolide antibiotics was reported recently (Rhodes *et al.*, 2008).

In a recent study conducted by Sudheesh *et al.* (2007) it was discovered that *R. salmoninarum* cells treated with a sortase inhibitor (section 1.6.4.2), phenyl vinyl sulfone (PVS), prior to infection, reduced the adherence to Chinook salmon fibronectic and embryo cells (CHSE-214) significantly *in vitro* compared to untreated bacteria. PVS-treated bacteria were also unable to invade the CHSE-214 cells. No pathological changes were observed in the CHSE-214 cells challenged with PVS-treated *R. salmoninarum* cells, whereas CHSE-214 cells challenged with untreated *R. salmoninarum* showed pathological changes within few days. The sortase inhibitor could be a potential therapeutic agent in the battle against BKD (Sudheesh *et al.*, 2007).

1.7.4 Vaccines

As reviewed by Elliot *et al.* (1989) several vaccination studies have been conducted to try to develop a preventive strategy against BKD but unfortunately they have not been promising. Some vaccines give a measurable elevation in antibody response, although two closely related salmonid species can elicit very different antibody response to a particular protein antigen (Alcorn & Pascho, 2002). The protection antibodies against MSA protein offer to the host is none or quite limited. On the contrary, it seems to favor the survival of *R. salmoninarum* inside the host's macrophage (section 1.6.4.2) Deposits of immunocomplexes in the glomeruli of the kidney can develop into a chronic membranous glomerulonephritis (Sami *et al.*, 1992) although an attempt to experimentally produce glomerulopathy

in rainbow trout by repeated immunization of killed *R. salmoninarum* was unsuccessful (Lumsden *et al.*, 2008). Evidence has shown that the main virulence factor of the bacterium, the MSA protein, is able to suppress the immune response (section 1.6.4.3). Therefore, using the unattenuated bacterium or it´s ECP for immunization, can inhibit the host's immune response instead of improving it (Grayson *et al.*, 2002) (section 1.6.4.3). The MSA protein might actually mask other important surface proteins of the bacterium which would otherwise elicit stronger immune response in the host. By removing the bacterial cell surface-associated 57 kDa protein, the host's antibody response elevated 20-fold (Wood & Kaattari, 1996). The increased antibody activity was mainly directed at sites previously blocked by the presence of the MSA protein. Many vaccination studies have focused on using avirulent or attenuated whole cells of *R. salmoninarum* without the MSA surface proteins (Evelyn, 1971; Paterson *et al.*, 1981; Griffiths *et al.*, 1998; Piganelli *et al.*, 1999; Daly *et al.*, 2001; Rhodes *et al.*, 2004b; Alcorn *et al.*, 2005). A vaccine with combined antigens from different bacterial species has been tested with various affect on BKD protection (Kaattari *et al.*, 1987; Kaattari *et al.*, 1988; Rhodes *et al.*, 2004b). Renogen, a commercial vaccine against BKD is available (Griffiths *et al.*, 1998). Renogen contains live *Arthrobacter davidanieli* (proposed species nomenclature) which is nonpathogenic and closely related to *R. salmoninarum* (Figure 3). The protective effect it provides against BKD is controversial. Both positive effects (Rhodes *et al.*, 2004b; Salonius *et al.*, 2005) and no effects have been observed (Alcorn *et al.*, 2005).

1.7.5 Brood stock selection, segregation and culling

R. salmoninarum can be transmitted vertically so infected female brood fish may produce infected eggs. Vertical transmission can be avoided by using brood stock free of the bacterium but for infected stocks, segregation (Pascho *et al.*, 1991; Elliott *et al.*, 1995; Maule *et al.*, 1996; Gudmundsdottir *et al.*, 2000; Meyers *et al.*, 2003) or culling (Gudmundsdottir *et al.*, 2000) of ova from infected females can be considered. These practices require extensive screening and facilities where gametes of different parentage can be kept apart while brood fish samples are being screened. Long-term storage of the eggs, that await segregation or culling, will affect the quality of the eggs. For that reason, rapid diagnostic methods are required for the screening of the bacterium (Gudmundsdottir *et al.*, 2000).

1.8 Diagnostic methods

In fish infected with *R. salmoninarum*, the bacterium may be present in different parts of the body and various tissue samples such as kidney, ovarian fluid, spleen, heart and blood are used for the detection (Austin & Rayment, 1985; Pascho *et al.*, 1987; Magnusson *et al.*, 1994; Rhodes *et al.*, 1998). The kidney, especially the hematopoietic posterior part (Ferguson, 1989), is the tissue most commonly used. The diagnostic methods currently used for detection of the bacterium are cultivation, fluorescent antibody techniques (FAT), enzyme-linked immunosorbent assay (ELISA) and polymerase

chain reaction (PCR). According to OIE (2006), screening should be carried out using ELISA and FAT. OIE requires that confirmation of positive results should be made using PCR or cultivation on KDM-2 medium in cases where the bacterium is detected on a previously uninfected fish farm (OIE, 2006).

1.8.1 Cultivation

The first reliable cultivation medium reported contained extra cystein (Ordal & Earp, 1956). Cultivation for detection of *R. salmoninarum* is widely used for diagnostic and screening purposes. Cultivation takes weeks, 12 weeks or even 19 have been reported (Benediktsdottir *et al.*, 1991; Rimaila-Pärnänen, 2002) and is therefore not preferred for screening (section 1.7.5).

1.8.2 Fluorescent antibody techniques

Two types of FAT are used for the staining of *R. salmoninarum*, direct and indirect FAT staining. Staining the antigens on the surface of the bacterial cell reveals the morphology of the bacterium when seen in a fluorescent microscope. Several studies have reported problems arising with the use of FAT staining, such as false positive and false negative results and low sensitivity (Cipriano *et al.*, 1985; Yoshimizu *et al.*, 1987; Armstrong *et al.*, 1989; Bandin *et al.*, 1993b). When examining healthy population of fish where the bacterial load is low, the examination can be laborious because it is recommended to examine at least 50 microscopic fields per sample and 100 microscopic fields or more to increase the sensitivity of the method (Elliott & McKibben, 1997).

1.8.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a widely used method to detect antigens from *R. salmoninarum* and most of the methods used are based on the double antibody sandwich method (Pascho & Mulcahy, 1987; Gudmundsdottir *et al.*, 1993). ELISA is a fast, cheap and convenient method to screen large populations of fish. Polyclonal or monoclonal antibodies are used in different ELISA protocols. Monoclonal antibodies detect one epitope and are usually made against the MSA protein while polyclonal antibodies detect multiple epitopes of the MSA protein as well as other antigens produced and secreted by the bacterium. Polyclonal ELISA (pELISA) is more commonly used than monoclonal ELISA (mELISA) for *R. salmoninarum* detection and a double-sandwich pELISA as described by Pascho (1991) is recommended by OIE (2006) to use for screening. Several modifications of polyclonal ELISA have been developed (Dixon, 1987; Pascho & Mulcahy, 1987; Pascho *et al.*, 1991; Gudmundsdottir *et al.*, 1993; Meyers *et al.*, 1993b; Olea *et al.*, 1993; Jansson *et al.*, 1996) and one such method is used for screening in Iceland (Gudmundsdottir *et al.*, 1993).

1.8.4 Polymerase chain reaction (PCR)

Many PCR methods have been developed for *R. salmoninarum* detection. Most of them target the msa gene for amplification (Brown *et al.*, 1994; Miriam *et al.*, 1997; Chase & Pascho, 1998; Cook & Lynch, 1999; Chase *et al.*, 2006; Bruno *et al.*, 2007; Suzuki & Sakai, 2007; Altinok *et al.*, 2008; Chambers *et al.*, 2009; Gahlawat *et al.*, 2009; Halaihel *et al.*, 2009). The 16S ribosomal RNA gene in *R. salmoninarum* has as well been targeted for amplification (Magnusson *et al.*, 1994; Rhodes *et al.*, 1998; Nilsson & Strom, 2002; Konigsson *et al.*, 2005; Jansson *et al.*, 2008). Several RT-qPCR and qPCR methods for *R. salmoninarum* detection have been developed (Magnusson *et al.*, 1994; Rhodes *et al.*, 1998; Cook & Lynch, 1999; Powell *et al.*, 2005; Chase *et al.*, 2006; Rhodes *et al.*, 2006; Bruno *et al.*, 2007; Suzuki & Sakai, 2007; Jansson *et al.*, 2008). OIE (2006) recommends the use of nested PCR (nPCR) developed by Chase & Pascho (1998) for *R. salmoninarum* detection. nPCR is a modified version of the conventional PCR, with increased sensitivity and specificity. The sensitivity of the nPCR was determined to be 10 *R. salmoninarum* cells per reaction (Chase & Pascho, 1998). In nPCR there is increased risk of contamination because the amplified product of the first reaction has to be transmitted to a second tube. (section 3.17.2). Modifications of nPCR methods have been developed focusing on resolving the high risk of contamination (Chan *et al.*, 1996; Berg *et al.*, 2001; Gookin *et al.*, 2002; Tao *et al.*, 2004; Saini *et al.*, 2009).

2 Aims of the study.

The aims of the study were to:

- Develop a convenient, cheap, specific and sensitive PCR method to detect *R. salmoninarum*.
- Test a new method to isolate nucleic acids from kidney, gills and ovarian fluid.
- Compare a new DNA isolation method and a new PCR method with methods recommended by OIE (2006) and other detection methods.
- Test the methods on cultured fish infected with *R. salmoninarum*.
- Test the methods on wild fish infected with *R. salmoninarum*.
- Study the accumulation of *R. salmoninarum* antigens in the fish kidney and evaluate the ability of the immune system to dispose of them.

3 Materials and methods

3.1 Fish

Three groups of fish were sampled for the study

3.1.1 Sample group 1

In group 1, there were farmed Atlantic salmon female brood fish (n=40). The fish were hatched in 2003 on farm A and moved to farm B in 2004, where they presumably got infected. Before that was suspected, the fish had been moved to sea cages where it was cultured during the years 2004-2006. Sampling in June 2005 resulted in low positive ELISA values in sporadic fish, but no macroscopic symptoms were observed. In the summer of 2006 the fish were moved to land based tanks on farm C. The following autumn they were stripped and samples collected from kidney, ovarian fluid and gills. Granulomas were observed in kidneys of several fish and one of them was included in sample group 1.

3.1.2 Sample group 2

In sample group 2 there were three salmonid species caught at different times and places in the Ellidaár river system (Figure 4). Subgroup 2a was wild Atlantic salmon from River Ellidaár consisting of samples from emigrating smolts taken in June 2007 (n=20). In 2008, samples from returning adults were sampled in July (n=22), August (n=22), September (n=21) and December (n=26). The smolts were kept in a releasing pond until sampled (section 3.2). The returning adults were caught upon return. Some of the fish caught in September were sampled immediately but others were kept in tanks fed with river water, and kept there until sampling took place in December. In subgroup 2b there were wild Arctic charr (n=18) and brown trout (n=60) from Lake Ellidavatn, sampled in October 2008. Subgroup 2c consisted of wild brown trout (n= 28) from Lake Ellidavatn, sampled in August 2009. All fish were free of disease symptoms.

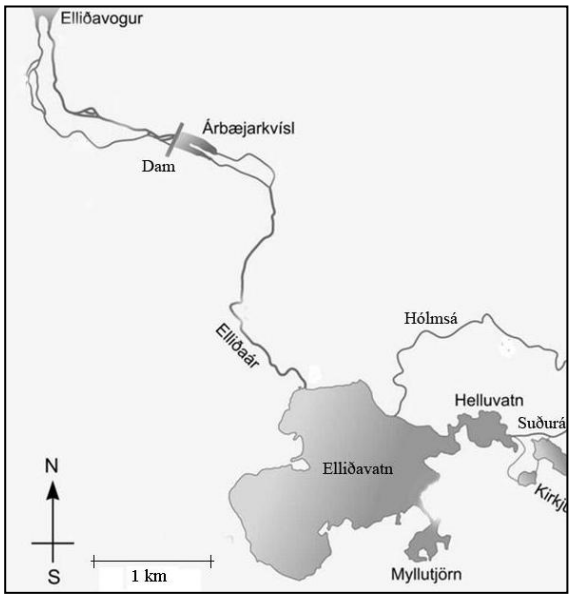


Figure 4. Lake Ellidavatn and its river system in the vicinity of Reykjavík.

3.1.3 Sample group 3

Sample group 3 contained cultured Atlantic salmon fry (n=102) originating from a BKD free farm. The fish was transported to the Institute for Experimental Pathology at Keldur and kept in 170 L tanks in running aerated fresh water at 9°C. After a week of acclimatization, the fish were challenged with extra cellular products (ECP) of *R. salmoninarum* broth culture, either by bathing or injected intra peritoneally (i.p.). Fry in each group were evenly divided into two 170 L tanks and fed 2mm dry feed pellets (Laxá hf, Akureyri, Iceland).

3.2 Catching and sampling of fish

The emigrating Atlantic salmon smolts in River Ellidaár, in sample group 2a, were caught in a smolt trap as they migrated to sea. The returning adults in sample group 2a were caught by trapping, netting or angling.

The Arctic charr and brown trout in sample groups 2b and 2c were caught in Lake Ellidavatn by using a series of gillnets of monofilament nylon with mesh sizes 12 to 50 mm (knot-to-knot).

Prior to sampling, the fish in sample groups 1 and 2 were killed by a blow on the head but in sample group 3 the fish were placed in a bucket of water containing excessive amount of the anaesthetic TMS (Tricaine methane sulphonate (Pharmaq Ltd)) resulting in killing by overdose of the anaesthetic.

Kidney samples for ELISA and DNA isolation were placed in sterile stomacher bags and stored in -20°C freezer until used. Ovarian fluid samples for ELISA and DNA isolation were placed in sterile 10 ml test tubes and stored in a -20°C freezer until used. Kidney samples for RNA isolation and gill samples for DNA isolation were stored in RNAlater (Ambion, Texas, USA) in -80°C until used. Samples were transferred chilled to the laboratory.

3.3 Bacterial strain

R. salmoninarum, strain S-182-90, used in this study, was originally isolated in 1990 from farmed Atlantic salmon fry in Iceland (Grayson *et al.*, 1999). The isolate was used in the sensitivity test for a new PCR method developed in the current study, to produce positive control sample for PCR tests and for production of ECP. *Arthrobacter globiformes* and *Williamsia* ssp. were also tested in the sn-PCR for specificity evaluation. (section 4.1.4). *Arthrobacter globiformes* is a close relative of *R. salmoninarum* (section 1.6.3) and ELISA samples made from rich growth of *Williamsia* spp. had previously shown low positive OD values in pELISA (records from the Department of Fish diseases).

3.4 *R. salmoninarum* isolation.

3.4.1 Culture on agar

R. salmoninarum was isolated and cultured on selective kidney disease medium, SKDM-2, or kidney disease medium, KDM-2 (OIE, 2006). Kidney tissue was diluted 1/10 with 0.1% peptone in saline and homogenized. After homogenization the samples were centrifuged at 2200 x g for 20 min at 4°C. Supernatant was discarded and pellets resuspended in peptone saline in ratio 1:1. Loopful of each homogenate was spread on SKDM agar plates, incubated at 14°C for at least 16 weeks and read once every week.

3.4.2 Broth culture for ECP isolation

A KDM-2 broth culture was seeded with a single colony of *R. salmoninarum* strain S-182-90 and cultured for 12 weeks at 14°C. The cultures were then centrifuged at 8000 rpm at 4°C for 20 min, the supernatant collected and frozen at -20°C. The supernatant was thawed, placed in Amicon Ultra-15 filter, 10K (Millipore) and centrifuged at 4000 x g for 30 min. The flow-through was discarded and the retained components of the ECP collected and analyzed for quality and quantity of proteins using nanodrop (section 3.12). The ECP was also analyzed in Western blot and by silver staining (sections 3.7 & 3.8). For the control group, fresh and sterile KDM-2 broth was filtered with Amicon Ultra-15 filter, 10K and analyzed for quality and quantity in nanodrop before it was used in the i.p. injection experiment (section 3.18.1).

3.4.3 Drop plate counting

A solution containing an unknown number of *R. salmoninarum* cells was serially diluted and 10 drops (10µl each) for each dilution inoculated onto SKDM agar plates. After 12 weeks incubation at 15°C, plate count was performed to determine the number of colony forming units in the original solution.

3.5 FAT staining.

A colony of *R. salmoninarum* from agar culture was smeared onto a drop of sterile PBS on a microscopic slide (Starfrost, Knittel) and dried at 60°C. Then, 50 µl of the fluorescein-labelled affinity purified antibody (Kirkegaard & Perry laboratories Inc.) diluted 1/10 was added and incubated for 10 minutes in a dark and moist chamber. The slides were rinsed with PBS and placed in a slide carrier filled with PBS and shaken gently for 5 minutes, removed and thoroughly drained. One drop of antifade buffer (2ml of glycerol, 80 mg of n-propyl gallate, 800 µl of 1M Tris-HCl, pH 8.0 and 1.2 ml of

ddH_2O) was placed on the slide and coverslip put on top. The preparation was examined using 1250 fold magnification in a fluorescent microscope.

3.6 SDS-PAGE

Reduced SDS-PAGE analysis was conducted by using Mini-PROTEAN® II 2-D cell system (Bio-Rad). Samples were diluted 1:25 in water. The diluted samples were mixed with equal volume of reducing sample buffer (0.125M Tris, pH 6.8, 0.5% 2-mercaptoethanol and 2% SDS) and heated for 3 min at 100°C. The final dilutions of samples were 1:50. The samples were loaded on stacking gel (4.5% acrylamide) and separated on separation gel (12% acrylamide) by electrophoresis. PageRuler prestained protein ladder (Fermentas) was used to evaluate the size of the proteins. After separation, gels were either silver stained (section 3.7) or proteins were transferred from the gel to a nitrocellulose (NC) membrane for Western blotting (section 3.8).

3.7 Silver staining

Silver staining kit from BioRad was used in this study. After electrophoresis, gels were placed in solution containing 5ml of Fixative Enhancer Concentrate and 95 ml of 50% methanol-10% acetic acid for 20 min. The gels were washed with distilled water for 2 x 10 min. Thereafter gels were placed in a solution containing 2.5 ml of Silver Complex Solution, 2.5 ml of Reduction Moderator Solution, 2.5 ml of Image Development Reagent, 17.5 ml of distilled water and 25 ml of Development accelerator reagent for development. After development, the reaction was stopped with 5% acetic acid for 15 min and finally the gels were washed in water.

3.8 Western blotting

Transfer of proteins from the separation gel to a NC membrane (Immobilon-P transfer membrane, Millipore) was performed in Trans-blot SD, semi-dry transfer cell (Bio-Rad) for 30 min at room temperature following the manufacturer‘s instructions. After transfer, residual sites of the NC membrane were blocked with 0.1% skimmed milk powder in washing buffer (0.1M Tris buffered saline, pH 7.8, containing 0.1% Tween®20 (Sigma-Aldrich)) incubated overnight at 4°C. The NC membrane was immersed in a solution of primary antibodies diluted 1/500 in washing buffer for 1 hour at room temperature. The primary antibodies used were 1A1, a monoclonal antibody raised against the MSA protein, a generous gift of Dr. Greg Wiens, Leetown, USA (Wiens & Kaattari, 1991) and B-6-5, a polyclonal antibody against *R. salmoninarum* produced in rabbits at Keldur (Gudmundsdottir *et al.*, 1993). Secondary antibodies conjugated with alkaline phosphatase, polyclonal goat anti-rabbit IgG/AP for B-6-5 and polyclonal goat anti-mouse IgG/AP for 1A1 (Dako), were diluted 1/3000 in washing

buffer and added to the NC membrane for 1 hour at room temperature. The blots were finally developed in a substrate buffer containing 100 µg ml^{-1} NBT (nitro blue tetrazolium), 60 µg ml^{-1} BCIP (5-bromo-4-chloro-3-indonylphosphate), and 4mM of $MgCl_2$ in and 0.1M ethanolamine-HCl buffer, pH 9.6. Extensive washing steps were carried out between all steps.

3.9 ELISA

3.9.1 Sample preparation

Kidney tissue was weighed, diluted in 1 to 3 wt/vol in Dulbecco's PBS and homogenized using Stomacher 80 micro-Biomaster (Seward). For each ml of homogenate, 25µl of Tween®20 was added. The samples were heated at 100°C for 15 minutes, centrifuged at 2200g for 20 minutes at 4°C and the supernatant collected for testing.

3.9.2 ELISA tests using polyclonal antibodies, pELISA

The pELISA test is a double sandwich ELISA using polyclonal antibodies as in Gudmundsdottir *et al.* (1993). Wells of the test plate (Maxisorp immunoplate, Nunc) used were coated overnight at 4°C with 100µl catching antibodies diluted 10µg ml^{-1} in 0.05M carbonate-bicarbonate buffer, pH 9.6. The catching antibodies were both normal and specific rabbit IgG (B-6-5). Washing was carried out between all steps with 3 changes of the washing solution (Dulbecco's PBS, pH 7.2 with 0.7M NaCl and 0.1% Tween®20) every 5 minutes. Following incubation and washing, 100µl of samples, including negative and positive samples, were put in each pair of wells (i.e. the test well and the control well) and incubated for two hours at room temperature. Following incubation the wells were washed, adding an extra washing step, before the conjugate was added. The detecting antibodies made in goat, were polyclonal, affinity purified, horse radish-peroxidase conjugated IgG against heat-treated whole cells of *R. salmoninarum* (Kirkegaard & Perry Laboratories Inc.). The antibodies were diluted in a buffer containing PBS, 0.05M NaCl and 0.05% Tween®20 to a concentration of 2ug ml^{-1}. Samples were incubated with the conjugate for 1 hour at room temperature. One hundred µl of the substrate buffer for conjugate staining (8 mg of the substrate o-phenylenediamine dihydrochloride (OPD, Daco), 12 ml of water with 5 µl of 30% hydrogen peroxide (H_2O_2)) was added to all test wells and incubated for 15 min at room temperature. The enzymatic reaction was stopped by adding 50 µl of 3N hydrochloric acid to each well. The OD values were read at 490 nm in $Victor^3$ 1420 multilabel counter from PerkinElmer. The cut off value for determination of positive samples was 2.3 times the average of three negative samples.

3.9.3 ELISA test using monoclonal antibodies, mELISA

The mELISA, a double sandwich ELISA was purchased in a kit (GrupoBios, Chile). Provided in the kit are antibody-coated wells and all antibodies and reagents needed. Washing was carried out between all steps. Samples were put in the wells and incubated for 60 minutes at 37°C. The conjugate was added and incubated for 30min at 37°C. At last the two substrate components provided (substrate A and B) were mixed together and added to the wells followed by 30min incubation time at room temperature. The reaction was stopped by adding 3N H_2SO_4 to each well. The OD values were read at 450 nm in Victor^3 1420 multilabel counter from PerkinElmer. The cut off value for determination of positive samples is defined as the OD value of the negative control samples + 0.260.

3.10 Isolation of DNA

3.10.1 FTA Minicards

FTA minicard (Whatman), a product for nucleic acid isolation was used following the manufacturer's protocol. In short (Figure 5), 50 µl of homogenized kidney samples diluted 1/4 in sterile PBS, or ovarian fluid, was put on the FTA minicard and dried for at least 1 hour at room temperature. A small disc (2mm) was punched out from the FTA minicard, using a special gadget (included in the kit), and placed in a PCR amplification tube. The disc was then washed three times in the tube with 200µl of FTA purification reagent and two times with 200µl of TE^{-1} buffer with 5 minutes interval. The disc was dried for 1 hour at room temperature and thereafter used as a template in the PCR reactions.

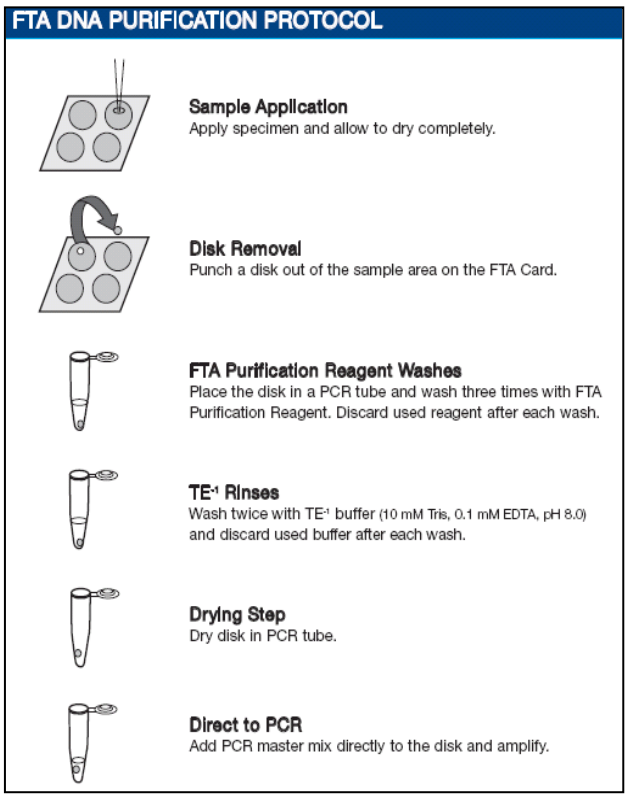


Figure 5. FTA DNA purification protocol.

A step by step demonstration of DNA isolation using FTA minicard. The figure was copied from a FTA minicard advertising booklet from Whatman.

3.10.2 Genomic DNA purification kit

Genomic DNA purification kit (Puregene) was used for DNA isolation from kidney and gill tissue following the "solid tissue protocol" with few exceptions. The proteinase K in the protocol was replaced by achromopeptidase (Sigma-Aldrich) which is known to function well with the Gram positive *R. salmoninarum* (Magnusson *et al.*, 1994). Approximately 10mg of kidney tissue or 20mg of gill tissue

were homogenized in cell lysis solution. Homogenized samples were treated with 100 U (units) of achromopeptidase for 1 hour at 37°C, followed by incubation in RNase A solution for 30 minutes in 37°C. Protein precipitation solution was added to the solution and centrifuged after mixing. Supernatant was collected and placed in a new tube and isopropanol added to the solution and centrifuged again. The supernatant was discarded and 70% ethanol added to the tube and centrifuged. The supernatant was discarded and finally 50 µl of DNA hydration solution was added to the tube. The dissolved DNA was then analyzed for quality and quantity in nanodrop (section 3.12) and stored at -20°C before use.

3.10.3 DNeasy Blood & Tissue Kit (Qiagen)

DNA from kidney tissue in one group, sample group 2c, was isolated using DNeasy Blood & Tissue Kit. Approximately 20 mg of samples were added to 180 µl of ATL buffer and 20 µl of Proteinase K solution. Samples were incubated at 37°C overnight. After incubation, 50 µl of 4 x lysozyme lysis buffer was added to the solution and incubated at 37°C for 1 hour with occasional vortexing during the incubation. Four µl of RNase A was added to the solution, vortexed and incubated at room temperature for 2 min. Then, 250 µl of Buffer AL was added to the solution and incubated at 70°C for 10 min. The samples were centrifuged for 1 min at full speed (all centrifugation steps were carried out at room temperature) and the supernatant transferred to a new microfuge tube containing 250 µl of 95% ethanol and thoroughly mixed. The solution was transferred to a QIAamp spin column which was placed in a collection tube and centrifuged at 8000 rpm for 1 min. The spin column was placed in a new collection tube and 500 µl of Buffer AW1 added to the column followed by centrifugation at 8000 rpm for 1 min. The spin column was placed in a new collection tube and 500 µl of Buffer AW2 added and spun for 3 min at full speed. The spin column was placed in a clean microfuge tube, 400 µl of preheated (70°C) Buffer AE added, incubated for 1 min at room temperature and centrifuged at 8000 rpm for 1 min. The dissolved DNA was then analyzed for quality and quantity in nanodrop and stored at -20°C before use.

3.11 RNA isolation.

3.11.1 TRIzol

TRIzol (Invitrogen) for RNA isolation was used to isolate RNA from cultured *R. salmoninarum* cells from SKDM agar or from homogenized kidney tissue, following the manufacturer's protocol with slight modifications. The tissue samples (approximately 30 mg) were placed in sterile eppendorf tube containing 100µl of buffer (10mM Tris-HCl, pH 8.65) and 80 U of RNase inhibitor (New England Biolab). The samples were homogenized in the eppendorf tube using sterile rods. After homogenization, 100 U of achromopeptidase were added to each sample tube before incubation at 37°C for 60 minutes. After incubation, 900 µl of TRIzol was added to the solution and stored for 5 min

at room temperature. For phase separation, 0.2 ml of chloroform was added to the solution and the tube shaken vigorously for 15 seconds before incubation at room temperature for 3 minutes. The samples were centrifuged at 12.000 x g for 15 min at 4°C. In the centrifugation the solution separates into 3 phases. The top phase (aqueous) was transferred to a new fresh tube. The RNA in the aqueous phase was precipitated by adding 0.5 ml of isopropyl alcohol. The samples were then incubated at room temperature for 10 minutes and centrifuged at 12.000 x g for 10 min at 4°C. The supernatant was discarded and 1 ml of 75% alcohol added to the tube. The sample was vortexed and centrifuged at 7.500 x g for 5 min at 4°C. The supernatant was discarded and the RNA pellet was air dried for 5 min before 50 µl of sterile water was added to the tube. The dissolved RNA was analyzed for quality and quantity in nanodrop and placed in -80°C for storage.

For RNA isolation of bacterial culture used as a positive control for the RT-qPCR method, a loop full of *R. salmoninarum* (strain S-182-90) was taken from SKDM agar and added to 100 µl of buffer (10mM Tris-HCl, pH 8.65) along with 100 U of achromopeptidase. The samples were incubated at 37°C for 60 min. Phase separation, RNA precipitation, RNA wash and RNA was dissolved again as in the protocol described above for kidney tissue RNA isolation using TRIzol.

3.12 Nanodrop

Nucleic acids and proteins were analyzed in NanoDrop®ND-1000 spectrophotometer (NanoDrop technologies, Inc.) for quantity and quality following the manufacturer‘s user manual.

3.13 DNase treatment and cDNA synthesis.

Before cDNA synthesis, the template RNA was treated with DNase (Fermentas) following the manufacturer‘s protocol. In an eppendorf tube, 7 µl of sterile water, 1 µl of template RNA (between 1000-1500ng/µl of RNA) 1 U of DNase and 1 µl of 10x reaction buffer were mixed together and incubated at 37°C for 30 minutes. After incubation, 1 µl of 25mM EDTA solution was added to the solution and incubated at 65°C for 10 min.

RevertAid H minus first strand cDNA synthesis kit (Fermentas) was used for cDNA synthesis in RT-qPCR following the manufacturer’s protocol. The solution contained 4 µl of DNase treated RNA (around 400-600ng of RNA), 1 µl or 100pmol of random primers and 7 µl of sterile water. The solution was then incubated in 65°C for 5 minutes. After incubation, 4 µl of 5x reaction buffer, 0.5 µl (20 U) of RNase inhibitor, 2 µl (1mM final concentration) of dNTP mix and 1 µl (200 U) of RevertAid^{TM} reverse transcriptase was added to the solution in the total volume of 20 µl and mixed gently. The solution was then incubated at room temperature (25°C) for 10 min followed by 42°C incubation for 60min. Finally the reaction was terminated by heating the mix at 70°C for 10 minutes.

3.14 Uracil-DNA Glycosylase (UNG enzyme)

To test the UNG enzyme in semi-nested PCR (snPCR, section 3.17.1), the positive control sample was treated with 1 U of UNG enzyme and incubated at 37°C for 20 min before the usual thermal cycling program for snPCR. In the mastermix, dTTP was replaced by dUTP. Samples on FTA minicard were used as templates for the PCR reactions. To test the efficiency of the UNG enzyme, 0.5 µl of DNA template from a positive control sample from a previous dUTP PCR reaction was added to one reaction tube.

For comparison, a regular snPCR was conducted using the same material and mastermix with dTTP instead of dUTP and UNG enzyme was not included.

3.15 TOPO cloning

TOPO cloning was performed using pBAD TOPO® TA Expression kit (Invitrogen). Prior to the TOPO cloning, PCR products, a part of both the msa genes and 16S rRNA genes from *R. salmoninarum*, were amplified with Platinum taq DNA polymerase (Invitrogen). Platinum taq polymerase produces A-overhang on the amplified PCR products, which is needed for the cloning. The reaction solution for the TOPO cloning consisted of 3 µl of the amplified PCR products, 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 1.5 µl of sterile water and 0.5 µl of the TOPO vector. The solution was gently mixed together and incubated at room temperature for 10 minutes before it was placed on ice. To facilitate transformation of pBAD TOPO construct into component *E. coli*, One Shot® TOP10 Chemically component *E. coli*, included in the kit and stored at -80°C, was used. The component *E. coli* cells were placed directly on ice when removed from -80°C freezer. The reaction solution was added to the tube containing the component *E. coli* cells and stored on ice for 30 minutes, followed by heat shock at 42°C for 30 seconds and cooled again on ice for 2 minutes. 250 µl of SOC medium was then added to the tube and horizontally shaken at 37°C for 1 hour. The SOC medium containing the cells was divided in 50 µl, 100 µl and the rest and spread on LB agar plates (containing 100 µg ml^{-1} ampicillin) and cultured at 37°C overnight. The following day 10 colonies were picked for each target gene and added to 2ml LB-amp medium (containing 100 µg ml^{-1} ampicillin) and cultured overnight, shaking at 37°C. The picked colonies were also analyzed with PCR to make sure that the TOPO cloning worked.

3.16 Isolation of plasmids.

Plasmids DNA were isolated using Qiaprep miniprep kit (Qiagen). The LB culture containing the desired E. coli was centrifuged for 15 min at 3000 rpm to pellet the cells. Bacterial cells were then resuspended in 250 µl P1 buffer with RNase A included and transferred to a microcentrifuge tube. Then 250 µl of P2 buffer was added to the tube and mixed gently but thoroughly or until solution

became viscous and slightly clear. Thereafter 350 µl of N3 buffer was added and the solution was mixed immediately and thoroughly to avoid localized precipitation. The solution was then centrifuged for 10 min at 5000 rpm to form a white pellet. The supernatant was transferred to a Qiaprep spin column and centrifuged for 1min at 5000 rpm. The flow-through was discarded. Spin column was washed again by adding 0.75ml of PE buffer and centrifuged again at 5000 rpm for 1 min. Flow-through discarded and centrifuged again to remove residual wash buffer. The Qiaprep column was placed in a new tube. DNA was finally eluted by adding 50 µl of EB buffer to the column, let stand for 1min, and centrifuged for 1 min. The DNA was analyzed for quality a quantity in nanodrop before it was placed in -20°C for storage.

3.17 PCR

All the samples in sample groups 1 and 2c were tested in duplicate in the PCR's but only one reaction per sample was performed for the samples in sample groups 2a and 2b. If both reactions were positive, the sample was considered positive and when both reactions were negative, the sample was considered negative. If one of the reactions was positive and the other negative the sample was registered as negative but such samples are presented separately in the data as semi-positive ((+)). The samples in sample group 1 tested with PCR from DNA isolated with FTA minicard or DNA kit, were retested in the case of semi-positive reaction. The samples were then tested in quadruplicate and if one or more of the four reactions was positive, the sample was considered positive. Each set of samples in nPCR, snPCR, qPCR and RT-qPCR included two negative and two positive control samples. The negative control samples in all PCR methods included sterile water instead of DNA or cDNA template. The positive control sample in nPCR, snPCR and qPCR was prepared from a kidney tissue heavily infected by *R. salmoninarum*. For positive control samples in RT-qPCR, the RNA was isolated from *R. salmoninarum* cells and transferred into cDNA via reverse transcriptase reaction (section 3.13). For the standard curve in qPCR and RT-qPCR, the target genes, or msa and 16S rRNA from *R. salmoninarum,* were cloned into *E. coli* vectors via TOPO cloning (section 3.15) and subsequently isolated (section 3.16). A serial dilution, from 5 to 50.000 template copies of the target gene, was conducted on the isolated vectors.

Table 1. A list of all the primers and probes used in this study.

Information of target genes, names, location numbers corresponding to the nucleotide of the ORF, sequence and reference of the method are given for each primer or probe.

PCR methods	Target gene	Name	Location	Sequence	Reference
Semi-nested PCR	msa				This study
Forward primer		For_msa	996-1015	5'-AGATGGAGCAACTCCGGTTA-3'	
Reverse primer		Rev_msa	1247-1266	5'-GGGATTACCAAAAGCAACGA-3'	
Nested reverse primer		nRev_msa	1175-1191	5'-TCTCTCAACGCCAATAC-3'	
Nested PCR	msa				Chase et al 1998
Forward primer		P3	75-93	5'-AGCTTCGCAAGGTGAAGGG-3'	
Reverse primer		M21	438-458	5'-GCAACAGGTTTATTTGCCGGG-3'	
Nested forward primer		P4	95-119	5'-ATTCTTCCACTTCAACAGTACAAGG-3'	
Nested reverse primer		M38	394-415	5'-CATTATCGTTACACCCGAAACC-3'	
qPCR and RT-qPCR	16S rRNA				This study
Forward primer		Left16S	976-997	5'-ACCAAGGCTTGACATGGATTAG-3'	
Reverse primer		Right16S	1028-1046	5'-GCACCACCTGTGAACCAAC-3'	
Hydrolysis probe		Hyb16S	1003-1027	VIC-5'-TGCAGAAATGTACTCCCCCTTTTGG-3'-TAMRA	
qPCR and RT-qPCR	msa				Chase et al 2006
Forward primer		RS1238	968-989	5'-GTGACCAACACCCAGATATCCA-3'	
Reverse primer		RS1307	1018-1037	5'-TCGCCAGACCACCATTTACC-3'	
Hydrolysis probe		RS1262	992-1007	FAM-5'-CACCAGATGGAGCAAC-3'-NFQ-MGB	
qPCR	msa				Powell et al 2005
Forward primer		250F	142-165	5'-CAACAGGGTGGTTATTCTGCTTTC-3'	
Reverse primer		344R	215-236	5'-CTATAAGAGCCACCAGCTGCAA-3'	
Hydrolysis probe		300T	192-211	FAM-5'-CTCCAGCGCCGCAGGAGGAC-3'-TAMRA	
qPCR	ABC transporter ATPase gene				Rhodes et al 2006
Forward primer		ABCtransfor2		5'-CTAAACGATTTCCCGGTCAA-3'	
Reverse primer		ABCtransrev2		5'-GATTTTGCCTGCTGGTATTTCC-3'	
Hydrolysis probe		ABCtrans		FAM-5'-AAGCGCCAGCAGTCGACGGC-3'-TAMRA	

3.17.1 Semi-nested PCR

For semi-nested PCR (snPCR), three primers were designed (Table 1) for two reactions carried out in the same PCR tube to amplify a part of the msa gene of *R. salmoninarum.* The primers (Table 1) were designed from the published sequence of the msa 1 gene using Primer3 program (GenBank accession number: AY986794.1). Two primers, For_msa and Rev_msa, amplify the first fragment of 270 base pairs. The third primer, nRev_msa, amplifies the second fragment of 199 base pairs within the first fragment along with the For_msa primer. The optimal melting temperature for these three primers are 57.3°C for the For_msa primer, 55.3°C for the Rev_msa primer and 50.4°C for the nRev_msa primer.

Table 2. Thermal cycling program for snPCR.

PCR program		
1. Denaturing	94°C	10 min
	+	
2. Denaturing	94°C	30 sec
3. Annealing	61°C	2 min
(↓0.5°C for each cycle)		
4. Elongation	72°C	30 sec
	+	
5. Denaturing	94°C	30 sec
6. Annealing	55°C	2 min
7. Elongation	72°C	30 sec
	+	
8. Denaturing	94°C	15 sec
9. Annealing	45°C	15 sec
10. Elongation	72°C	15 sec
	+	
11. Elongation	72°C	10 min
Cycle (step 2-4) was repeated 11 times.		
Cycle (step 5-7) was repeated 13 times.		
Cycle (step 8-10) was repeated 16 times.		

The platinum taq DNA polymerase (Invitrogen), used in nPCR and snPCR, is an antibody-mediated, hot-start enzyme and therefore an initial denaturation step for 10 minutes was required before beginning the amplification cycles. The samples tested were either one µl of extracted nucleic acid, diluted 10^{-1} or a punctured disc from FTA minicard containing the sample. The reaction mixture, in total volume of 25 µl, contained 0.24mM of each nucleotide, 2mM of MgCl, 24mM of Tris-HCl (pH8.4), 60mM of KCl, 1.6 µM of For_msa 1 and nRev_msa3 primers, 0.8 µM of Rev_msa2 primer, and 0.625 U of platinum taq DNA polymerase. The thermal cycling was done with Peltier thermal cycler (PTC-200) from MJ research (Table 2).

Gel electrophoresis for snPCR and nPCR amplicons separation was performed on 2% agarose gels (Saekem) that included ethidium bromide (Sigma) and then visualized under UV light. Sizes of amplicons were estimated with comparison to 1kb DNA standard (Invitrogen) or GeneRuler 100bp DNA ladder (Fermentas).

3.17.2 Nested PCR

Table 3. Thermal cycling program for nPCR.

PCR program		
1. Denaturing	94°C	10 min
	+	
2. Denaturing	94°C	30 sec
3. Annealing	60°C	30 sec
4. Elongation	72°C	1 min
	+	
5. Elongation	72°C	10 min
Cycle (step 2-4) was repeated 29 times.		

In the protocol for nested PCR (nPCR) as in Chase et al. 1998, there are two separate reactions producing two products (Chase & Pascho, 1998). There are two sets of primers (Table 1) that amplify a part of the msa gene of *R. salmoninarum*. The first set of primers, amplify a fragment as seen in conventional PCR. The amplified fragment is 383 base pairs long. When amplification is complete the product is used as a template in a separate reaction. The second

pair of primers amplifies a fragment within the first PCR product. The second fragment is 320 base pairs long.

The amplification mastermix for both the first and the second reaction was in total volume of 25µl. In the first reaction, 1µl of extracted nucleic acid using DNA kit isolation (Puregene) with tenfold dilution, or a punctured disc from FTA minicard containing the sample that is being tested, was included to serve as template DNA. In the second reaction, 1µl of the amplified product from the first reaction was used as template DNA. Platinum taq DNA polymerase (Invitrogen) was used in the PCR reaction mixture.

The reaction mixture contained 0.2mM of each nucleotide, 2mM of MgCl, 20mM of Tris-HCl (pH 8.4), and 50mM of KCl, 1 µM of each primers, and 0.625 U of platinum taq DNA polymerase. Thermal cycling was carried out with Peltier thermal cycler (PTC-200) from MJ research for both the first and the second reaction (Table 3).

3.17.3 qPCR

Quantitative PCR was performed on the kidney's gDNA isolated with DNA kit. The DNA for the qPCR runs was isolated from the samples in sample group 1 by using the Puregene DNA kit (section 3.10.2) and the DNA template was diluted 10^{-2} before added to the reaction mixture. Both msa and 16S rRNA genes were targeted for magnification. For msa magnification, published primers and probe as in Chase *et al.* (2006) were used (Table 1). For 16S rRNA magnification, primers and probe were designed in the study (Table 1) from the published sequence of the 16S rRNA gene (GenBank accession number: AF180950.1) using Primer3 program. In sample group 2c, two additional published qPCR methods were tested on the samples, Powell *et al.* (2005) targeting the msa gene, and Rhodes *et al.* (2006) targeting the ABC transporter ATPase gene (Table 1). The DNA for the qPCR runs from samples in sample group 2c isolated with the DNeasy Blood & Tissue Kit (section 3.10.3) was not diluted before it was added to the reaction mixture. For magnification of target genes, Maxima probe/rox qPCR mastermix from Fermentas (2x) was used in the reaction mixture which was in total of 12µl. Five µl of the diluted gDNA template was used in the reaction mixture along with 0.9 µM of forward and reverse primers and 0.25 µM of the probe. The thermal cycling was done with StepOnePlus Real-time PCR system, thermal cycling block from Applied Biosystem as described in Chase *et al.* (2006). The cut off in qPCR and RT-qPCR was at Ct 38 (Cycle threshold), or samples with Ct value at 38 or lower were considered positive but otherwise negative.

3.17.4 RT-qPCR

RT-qPCR was performed on the kidney's cDNA. Everything was the same as in qPCR (section 3.17.3), except for the difference in template used. In the reaction mixture, five µl of undiluted cDNA

template from cDNA synthesis solution was used in the reaction mixture instead of the diluted gDNA as in qPCR.

3.18 Treatment with MSA protein

3.18.1 I.p. injection

The fry were divided into 5 groups. Three groups were control groups. The first group was sampled before injection (n=5). The second group (n=6) was injected with 0.1 ml of sterile PBS. The third group (n=4) was injected i.p. with 0.1 ml of fresh KDM-2 broth sterile-filtered before use and diluted 1/100. The fourth group (n=40) was inoculated i.p. with 0.1 ml of a solution containing 0.1 mg of ECP and the fifth group (n=32) with 1.0 mg of ECP. Fry in all groups were marked individually using Visible implant fluorescent elastomer (Northwest Marine Technology, Inc. USA) for labelling. Weighing and sampling was further done on days 2, 7, 14, 28 and 42 after challenge.

3.18.2 Bathing

The fry (n=15) were immersed for 150 min in 8 L of aerated freshwater containing 50 mg l^{-1} of ECP and transferred to a tank containing freshwater. Sampling and weighing was done on the same days as for the i.p. injection groups.

3.19 Statistical analysis.

Chi-square, testing for homogeneity or multinominal distribution, was used for statistical analysis of the differences recorded for different diagnostic methods in sample groups 1 and 2. Microsoft Office Excel 2007 was used to perform ANOVA for the statistical analysis in sample group 3 to compare the mean values in each sampling group. $P<0.05$ was the critical value of significance in both Chi-square and ANOVA.

4 Results

4.1 Semi-nested PCR

A novel snPCR test was constructed and various parameters considered and tested before the final protocol was ready. The products of a positive snPCR reaction, as seen in a gel, are two separate bands of sizes 270 and 199 base pairs as shown in Figure 6. Sequencing of the lower band confirmed that the amplified product belongs to the msa gene. More than 1800 snPCR reactions were performed in the study.

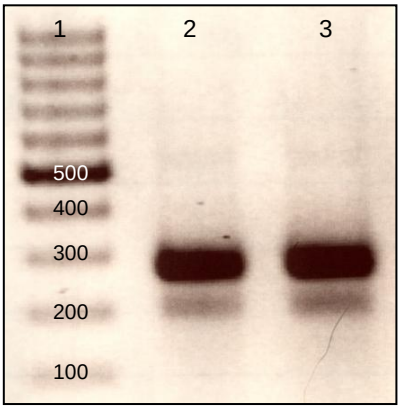


Figure 6. Gel electrophoresis of snPCR.

The two products of snPCR , 270 bp and 199 bp in size.

Lane 1: Ladder.

Lanes 2 & 3: Heat treated *R. salmoninarum cells.*

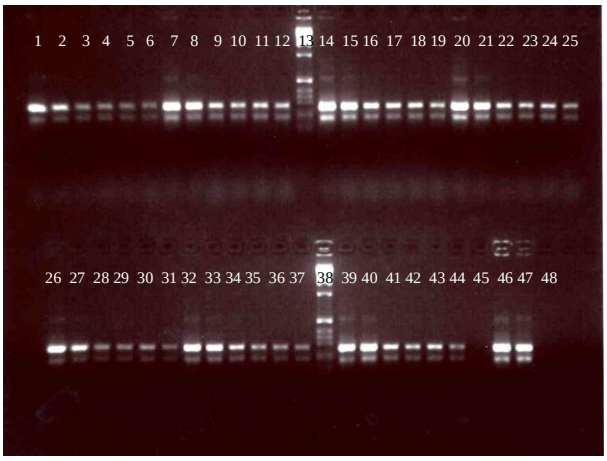


Figure 7. Gel electrophoresis, products of snPCR treated with increasing amount of achromopeptidase.

Serial dilutions of *R. salmoninarum* cells (undiluted: lanes 1, 7, 14, 20, 26, 32 and 39), 10^{-2} (lanes 2, 8, 15, 21, 27, 33 and 40), 10^{-4} (lanes 3, 9, 16, 22, 28, 34 and 41), 5.0×10^{-5} (lanes 4, 10, 17, 23, 29, 35 and 42), 2.0×10^{-5} (lanes 5, 11, 18, 24, 30, 36 and 43) and 10^{-5} (lanes 6, 12, 19, 25, 31, 37 and 44) treated with increasing amount of achromopeptidase and incubated for 1 hour at 37°C before heat-treated for 25 min at 70°C.

Lanes 1 - 6: 0 U of achromopeptidase.
Lanes 7 - 12: 25 U of achromopeptidase.
Lanes 14 - 19: 100 U of achromopeptidase.
Lanes 20 - 25: 250 U of achromopeptidase.
Lanes 26 - 31: 500 U of achromopeptidase.
Lanes 32 - 37: 750 U of achromopeptidase.
Lanes 39 - 44: 1000 U of achromopeptidase.
Lanes 45 & 48: Negative control samples.
Lanes 46 & 47: Positive control samples.
Lanes 13 & 38: Ladder.

4.1.1 Achromopeptidase in sample preperation

Semi-nested PCR was performed on serial dilutions of *R. salmoninarum* cells treated with various amounts of achromopeptidase. The results show that 100 U of achromopeptidase added to the solution gave the clearest results although the results for 25 U and 250 U differed only slightly (Figure 7). Consequently, 100 U of achromopeptidase was added to each sample when using the kit for DNA isolation or the TRIzol procedure for RNA isolation, in the current study. Treating samples with achromopeptidase before placing them on FTA minicard, did not enhance DNA extraction, but rather seemed to diminish the efficiency (data not shown) so this step was not added to the FTA protocol.

4.1.2 UNG enzyme treatment in PCR

The PCR efficiency, or the amount of products amplified in the PCR reaction, was poor when the samples were treated with UNG enzyme prior to the snPCR, compared to samples not treated with the enzyme (lanes 4 and 5 in figure 8). When previously amplified template was treated with UNG enzyme prior to the snPCR reaction, the enzyme degraded most of the template in the reaction mixture compared to the samples not treated with UNG enzyme where degradation was not observed (lanes 6 and 7 in figure 8). The previously amplified template is made of dUTP-containing DNA which is degradable by the UNG enzyme. Consequently, this method was not included in the snPCR procedure.

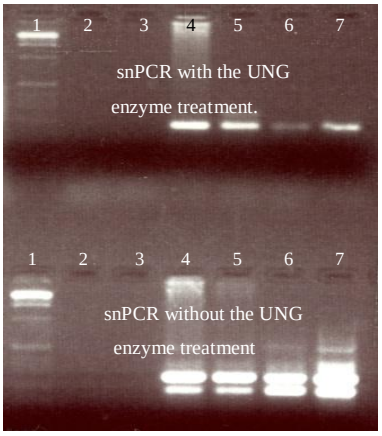


Figure 8. snPCR and UNG enzyme treatment.

Lanes are marked on the picture (1-7). The upper lanes have samples treated with UNG enzyme but the lower lanes have samples without UNG enzyme treatment.

Lane 1: Ladder.
Lanes 2 & 3: Negative control.
Lanes 4 & 5: Positive control (*R. salmoninarum*).
Lanes 6 & 7: (dUTP) Positive control (*R. salmoninarum*) made of dUTP-containing DNA which is degradable by the UNG enzyme.

4.1.3 Sensitivity of snPCR compared to nPCR.

To determine the detection limit of snPCR, DNA from uninfected kidney tissue, approximately 200ng per reaction, was mixed with serially diluted DNA from a known number of *R. salmoninarum* cells and tested in snPCR. The detection limit of snPCR was determined to be 5 *R. salmoninarum* cells per reaction. Previous study had estimated that nPCR can detect as few as 10 *R. salmoninarum* cells per reaction (Chase & Pascho, 1998).

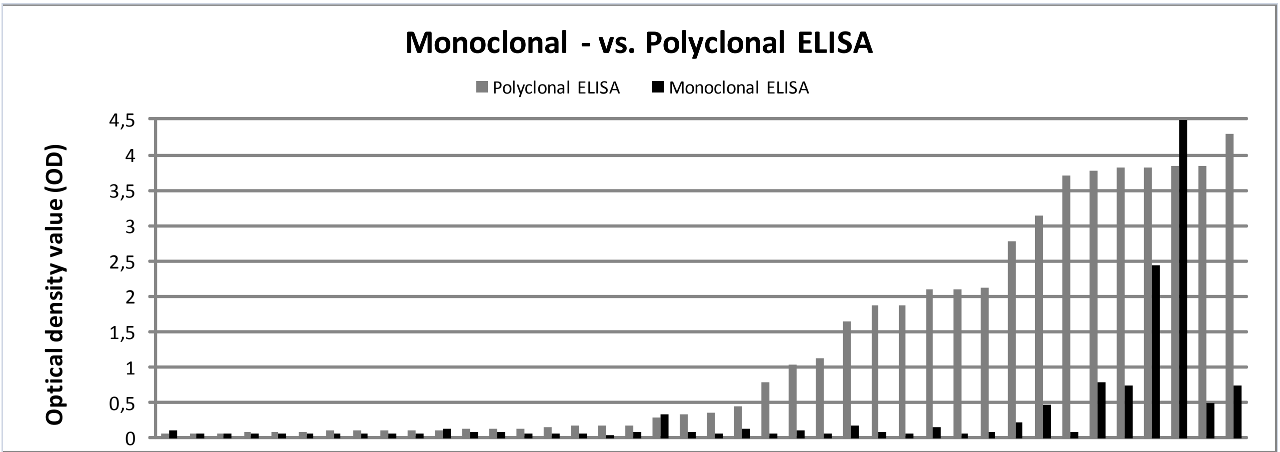
Serial dilutions of DNA from infected kidney tissue were run in both PCR methods and the endpoint observed was the same in two separate trials. Serial dilutions of DNA from infected kidney tissue were run in both PCR methods and the endpoint observed was the same in two separate trials.

4.1.4 Testing for cross reactivity

Arthrobacter globiformis and *Williamsia* spp. (with 99% identity to *Williamsia maris*) were tested for cross reactivity in snPCR and *R. salmoninarum,* strain S-182-90, was used as a positive control. The snPCR primers did not amplify any products, using DNA isolated from the bacteria on FTA minicards, except for the *R. salmoninarum* sample. FAT staining was conducted as well, on smears from all three bacterial cultures, with *R. salmoninarum* giving the only positive reaction.

4.2 Sample group 1: Cultivated Atlantic salmon brood fish

All results for sample group 1 are summarized in figure 9. The results show that kidney samples were most informative regarding *R. salmoninarum* infection in the 40 female brood fish studied. In snPCR, 17 kidney samples were positive compared to 4 ovarian fluid samples in the same test. In pELISA 26 positives were detected in kidney samples but merely one ovarian fluid sample. One gill sample of 30 samples tested was positive in snPCR.



Kidney																																								
pELISA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
mELISA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+
FTA snPCR	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
FTA nPCR	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
DNA kit snPCR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	+	-	+	-	+	+	+	+	+	+
DNA kit nPCR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	+	+	+	-	+	+	+	+	+	+
qPCR msa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	-	-	+	+	(+)	(+)	-	+	+	+	+	+	+	+	+	+	+
RT-qPCR msa	-	-	-	-	(+)	-	-	-	-	-	-	-	(+)	-	(+)	(+)	-	-	-	-	-	-	(+)	+	(+)	+	-	+	-	-	+	+	+	-	+	(+)	+	+	(+)	+
qPCR 16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	-	-	+	+	-	-	-	-	(+)	+	-	+	+	+	+	+	+
RT-qPCR 16S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+
Ovarian fluid																																								
pELISA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
FTA snPCR	-	(+)	-	-	-	(+)	-	-	-	-	-	-	(+)	-	(+)	-	+	-	(+)	(+)	(+)	-	(+)	-	-	-	-	-	-	-	(+)	(+)	(+)	+	-	+	-	+	-	-
FTA nPCR	-	-	-	-	-	(+)	(+)	-	(+)	+	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	-	+	-	+	(+)	+	(+)	(+)
Gills																																								
DNA kit snPCR	ND	-	-	-	-	-	-	-	-	-	-	-	(+)	-	ND	-	-	-	-	-	ND	(+)	(+)	ND	(+)	ND	(+)	ND	ND	-	(+)	ND	-	-	-	ND	ND	+	(+)	(+)
DNA kit nPCR	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	ND	-	-	ND	-	ND	-	ND	ND	-	-	ND	-	+	-	ND	ND	+	(+)	(+)
Fish no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40

Figure 9. Results of 10 detection methods tested on samples in sample group 1, farmed Atlantic salmon brood fish.

The OD value results in kidney tissue for both pELISA (grey columns) and mELISA (black columns) are shown on the chart. Every set of columns represents an individual fish. Samples are aligned on the chart from the left to the right with increasing OD values in pELISA. The results for all the other methods tested on the kidney tissue, ovarian fluid and gills tissue for every individual fish in this sample group are shown below the chart. Positive samples are marked with +, negative with – and semi-positive with (+). Samples marked with ND were not tested. Sample no. 36 was the only sample in this sample group with granulomas observed in the kidney.

4.2.1 Kidney tissue

4.2.1.1 ELISA tests

The highest number of positives, or 65% of the samples tested was detected in pELISA while mELISA detected 17.5%, the lowest percentage positive in all tests. The difference is statistically significant ($p<0.001$). The average OD value in pELISA was 1.140 but 0.327 in mELISA. All samples positive in mELISA were also positive in pELISA, five of 7 showing OD readings below 1.0 while their OD readings in pELISA all showed measurements above 3.0. One sample reached an OD value greater than 4.5 in mELISA and this was the only sample (fish no. 38) positive by all methods in all tissues tested. All but one sample below OD value of 3.7 in pELISA, or 60 times more than the average of the negative samples, were negative in mELISA except for sample no. 33 that had OD value of approximately 3.1 in pELISA.

4.2.1.2 Semi-positive samples in snPCR and nPCR.

Samples tested in nPCR and snPCR when using both FTA minicard and DNA kit for DNA isolation, were tested again in the case of semi-positive reaction to get either (clear) positive or (clear) negative results. The samples 2, 9, 13 and 28 were semi-positive in snPCR and samples 6 and 23 were semi-positive in nPCR when using FTA minicard for DNA isolation. Of these six samples retested, only sample no. 9 turned positive in snPCR. Samples no. 24, 29 and 32 were semi-positive in nPCR before retesting but no sample was semi-positive in snPCR when using DNA kit for DNA isolation. None of the three semi-positive samples in nPCR turned positive when tested again.

4.2.1.3 DNA isolation methods tested in snPCR and nPCR

A higher number of samples were positive in snPCR and nPCR, using FTA paper for isolation, than using a DNA purification kit (Table 4). Using FTA, snPCR detected 42.5% positive samples and nPCR 45%. Using DNA kit for isolation, 30% of samples were positive by both PCR methods. Significantly more samples were positive in nPCR using FTA minicard for DNA isolation than using DNA kit for DNA isolation ($p<0.05$). When individual fish are compared (Figure 9), using FTA minicard for DNA isolation, 16 samples (84.2%) were positive in both PCRs, one in snPCR only and two in nPCR. Applying DNA kit isolation, the PCR methods agreed on 11 samples being positive (84.6%) but each one detected the bacterium in one sample that the other PCR method determined as negative.

Table 4. DNA isolated on FTA minicard or with DNA kit tested in snPCR and nPCR.

DNA isolation n=40	FTA		DNA kit	
	snPCR	nPCR	snPCR	nPCR
+	17	18	12	12
-	23	22	28	28
% pos.	42.5%	45%	30%	30%

Five samples were positive in both PCR methods using FTA minicard for DNA isolation but negative in both PCR methods when using DNA kit for DNA isolation, i.e. samples 10, 20, 24, 29 and 34. Their OD values in pELISA ranged between 0.11 and 3.7, i.e. from negative value to highly positive. Two samples, no. 9 and 28, were positive in either of the PCR methods when using FTA minicard for DNA isolation, but negative in both PCR methods when using DNA kit for DNA isolation. One sample was positive in both PCR methods using DNA kit for DNA isolation but negative in both PCR methods using FTA minicard for DNA isolation, i.e. sample 23.

4.2.1.4 Quantitative PCRs using gDNA (qPCR) and cDNA (RT-qPCR)

When targeting the msa gene, qPCR identified 30% of all samples as positive and RT-qPCR 25% of the samples (Table 5). Eight of 14 (57.1%) were positive using both methods while qPCR alone detected four and RT-qPCR two positive samples. When targeting the 16S rRNA gene, 22.5% were positive applying qPCR and 32.5% using RT-qPCR. A total of 9 samples out of 13, were positive by both methods (69.2%) and additional four by RT-qPCR only.

Table 5. Comparison between qPCR and RT-qPCR targeting both msa and 16S rRNA genes.

	msa gene		16S rRNA gene	
n=40	qPCR	RT-qPCR	qPCR	RT-qPCR
+	12	10	9	13
+/-	3	8	2	0
-	25	22	29	28
% pos.	30%	25%	22.5%	32.5%

When using qPCR, both target genes agreed on 9 samples out of 12, or 75%, being positive and additional three samples targeting the msa gene. When using RT-qPCR, both target genes agreed on 10 samples out of 13, or 77%, being positive and additional three samples when targeting the 16S rRNA gene. The difference between qPCR and RT-qPCR targeting the 16S rRNA gene was significantly different ($p<0.05$)

4.2.1.5 Comparison between detection methods in kidney tissue.

pELISA detected significantly highest number of positive samples when using kidney tissue compared to all other methods tested ($p<0.05$). When comparing pELISA with the PCR methods using FTA minicard for DNA isolation, 9 samples that were positive in pELISA were negative in both PCR methods. Two of those, no.18 and 30 with OD value of 0.17 and 2.1, were negative in all other methods and in all tissue types tested. On the other hand, two samples were positive in either or both of the PCR methods using FTA for DNA isolation, but negative in pELISA. One of them, no. 10 was positive in both PCR methods using FTA minicard for DNA isolation but negative in all other methods tested as well as in all sample types tested, except for nPCR in ovarian fluid. The OD value for that sample was 0.11 in pELISA and 0.052 in mELISA. The other sample, no. 9, was positive in snPCR but negative in all other methods tested and in all sample types. The OD value for that sample was 0.099

in pELISA and 0.059 in mELISA. All samples positive in mELISA were positive in all other methods tested on kidney tissue except for two samples that were semi-positive in RT-qPCR targeting the msa gene. Both snPCR and nPCR using FTA minicard for DNA isolation detected the bacterium in significantly more samples than either qPCR or RT-qPCR targeting the msa gene ($p<0.05$). Five samples were positive in both or either snPCR and nPCR but negative in qPCR targeting the msa gene. Five samples were positive in nPCR and six were positive in snPCR but negative in RT-qPCR targeting the msa gene.

4.2.2 Ovarian fluid

pELISA detected the *R. salmoninarum* antigens in one out of 40 ovarian fluid samples. The sample gave very high OD value, i.e. 106 times higher than the average OD value of three negative samples. Three samples were positive in both snPCR and nPCR. One was positive in snPCR but negative in nPCR and another one was positive in nPCR but negative in snPCR.

4.2.3 Gill tissue

Thirty gill tissue samples were available and tested in snPCR and nPCR. One sample was positive by both methods and one sample in nPCR only.

4.3 Sample group 2: Wild Atlantic salmon, Arctic charr and brown trout

4.3.1 Sample group 2a: Atlantic salmon in River Ellidaár

All smolts sampled in June 2007, in River Ellidaár, were positive for *R. salmoninarum* antigens in pELISA (Figure 10). The average OD value was 0.260. In the first sampling in returning adults in July 2008, 18.2% samples were positive in pELISA and the average OD value was 0.072. In August, numbers of positives had increased to 31.8% and the average OD value was 0.087. In September, 57.1% of samples were positive and the average OD value 0.117. And finally in December 69.2% samples were positive and the average OD value 0.162. All samples were further tested in snPCR and nPCR except for the migrating smolts, which were only tested in snPCR. All samples, both from migrating smolts and returning adults were negative. The pELISA diagnosed significantly more positive samples than either PCR method in all groups using Chi-square test for statistical analysis ($p<0.05$). Significantly more samples were positive in ELISA in the migrating smolts than in any group of returning adults in 2008 ($0.001<p< 0.01$). Significantly more samples were positive in ELISA in July and August compared to the samples in December ($p<0.01$).

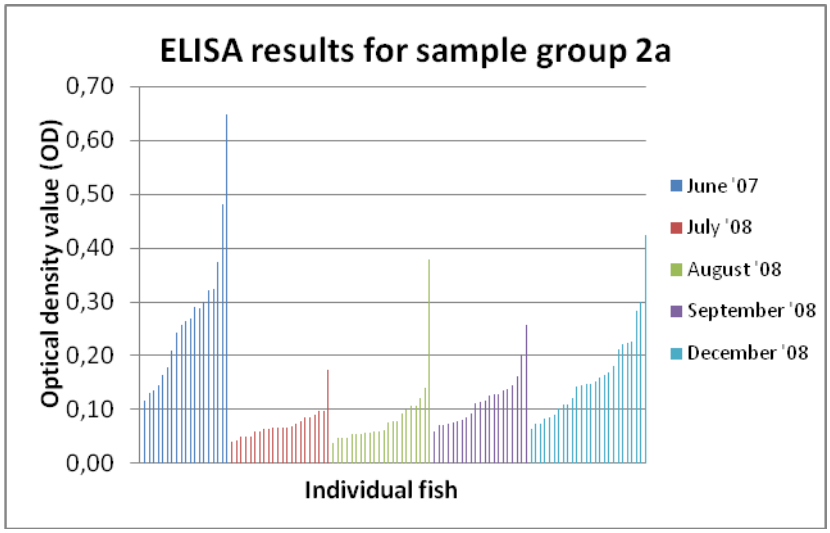


Figure 10. Results for polyclonal ELISA for the samples in sample group 2a.

The OD values for polyclonal ELISA in kidney tissue of wild Atlantic salmon, every column represents an individual fish. The samples are divided into 5 groups depending on sampling period, or June 2007, July, August, September and December 2008. The samples from June 2007 were taken from migrating smolts, but the other sampling periods were returning adults. Samples for each group are aligned on the chart from the left to the right with increasing OD values in polyclonal ELISA. All samples were negative in snPCR and nPCR.

4.3.2 Sample group 2b: Arctic charr and brown trout in Lake Ellidavatn in 2008

The Arctic charr samples had the average OD value of 0.485 and 89% of the samples were positive in pELISA. The brown trout samples had the average OD value of 0.514 and 98% of the samples were positive for the antigens (Figure 11). Two of the positive Arctic charr samples and three of the brown trout had OD value over 2.0 in pELISA. Both snPCR and nPCR were carried out on all samples. snPCR gave negative results in all samples but nPCR detected the bacterium in 11% of the Arctic charr samples and in 5% of the brown trout. The two Arctic charr samples that were positive in nPCR had OD readings of 0.229 and 0.510. The three brown trout samples that were positive in nPCR had OD values of 0.209, 0.281 and 1.528 (Figure 12). The difference in detection rates between pELISA and snPCR or nPCR was significant for both fish species ($p<0.001$) but the difference between the PCR methods was not statistically different. The differences between fish species for either pELISA results or nPCR results were not statistically significant.

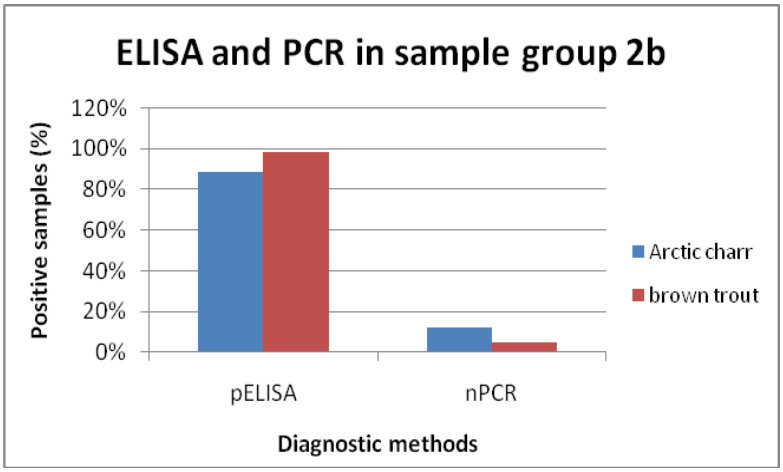


Figure 11. pELISA and nPCR results for Arctic charr and brown trout in sample group 2b.

Percentage of positive samples in sample group 2b for Arctic charr (blue columns) and brown trout (red columns) in pELISA and nPCR.

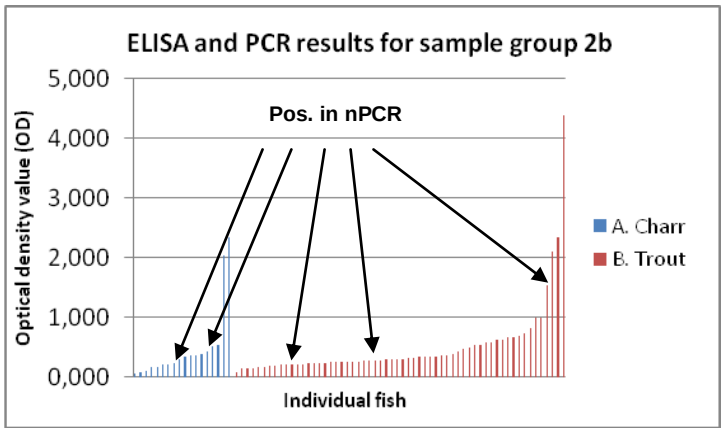
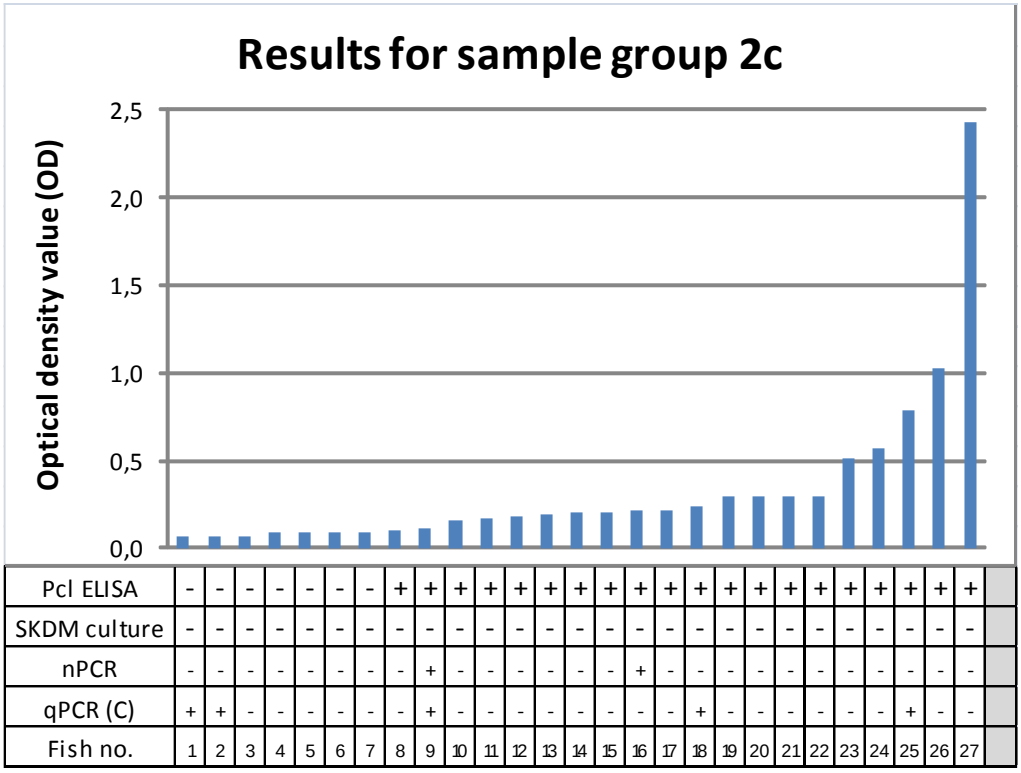


Figure 12. Results for pELISA and nPCR for samples in sample group 2b.

OD values in kidney tissue of Arctic charr (blue columns) and Brown trout (red columns) for pELISA are shown in the figure. Every column represents an individual fish. Samples are aligned on the chart from the left to the right with increasing OD values in pELISA. Samples that were positive in nPCR are marked specially on the chart with an arrow pointing to the positive individuals.

4.3.3 Sample group 2c: Brown trout in Lake Ellidavatn in 2009

The pELISA detected antigens of *R. salmoninarum* in 74% of all samples (Figure 13). The average OD value was 0.632. Culture on SKDM was negative. The nPCR method gave positive results in 2 samples (7.1%). When using the qPCR method developed by Chase *et al.* (2006), 17.8% of the samples were positive but the qPCR methods developed by Powell *et al.* (2005) and Rhodes *et al.* (2006) did not show positive reactions in any of the samples (data not shown). Significantly more positive samples were detected by pELISA than all other methods tested ($p<0.001$). pELISA, nPCR and qPCR all agreed on one sample being positive, (no.9), and on 5 samples being negative. The relationship between OD values in pELISA and Ct values in qPCR showed random distribution, being 0.177 (Ct: 34.2), 0.205 (Ct: 32.7), 0.305 (Ct: 33.1), 0.482 (Ct: 34.4) and 1.357 (Ct: 37.99) for samples 1, 2, 9, 16 and 18 respectively. The prevalence of positive samples in pELISA in sample group 2c (2009) had decreased significantly ($p<0.05$) compared to the brown trout samples in sample group 2b (2008).



Pcl ELISA	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SKDM culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
nPCR	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
qPCR (C)	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-
Fish no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27

Figure 13. Results for the detection methods in sample group 2c.

OD values for pELISA are shown on the figure. Every column represents an individual in the sample group. Samples are aligned on the chart from the left to the right with increasing OD values. The results for the SKDM culture, nPCR and qPCR as in Chase (2006) for every individual are shown below the columns.

4.4 Sample group 3: Atlantic salmon treated with ECP

4.4.1 Silver staining and Western blot of ECP

Silver staining and Western blot were done on ECP, harvested from a broth culture of *R. salmoninarum*, used for injection or bathing of fry. Silver staining reveals the major proteins of the ECP. The thickest band was estimated to be 59 kDa with the duplex below being 51 and 48 kDa respectively. In the Western blot, using the monoclonal antibody 1A1, raised against the MSA protein of *R. salmoninarum*, the presence of the MSA protein is confirmed in bands estimated to be of sizes 58 and 50 kDa (Figure 14).

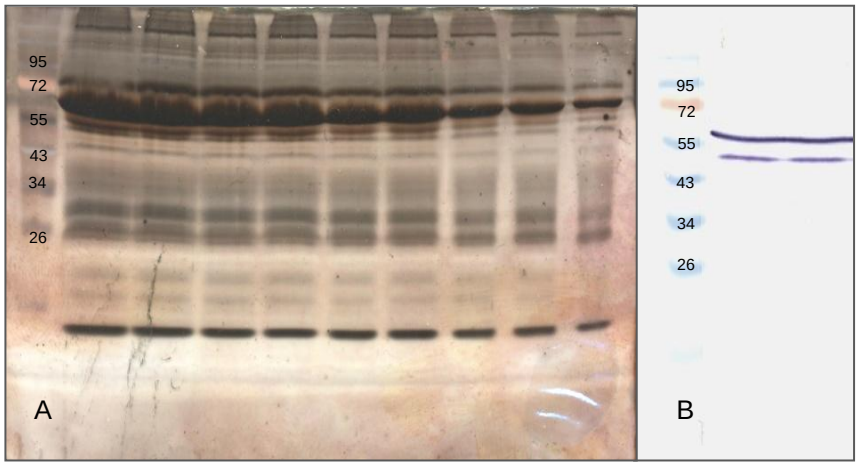


Figure 14. Silver staining and western blot on the ECP of R. salmoninarum.

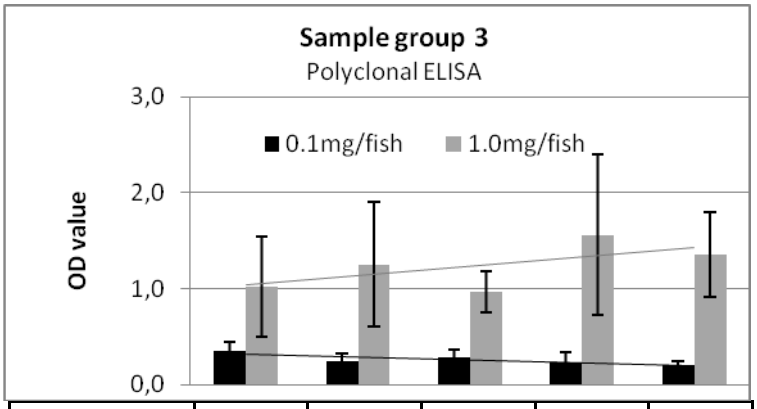
Picture A: Silver stained serial dilutions of ECP. Lane 1: ladder. Lanes 2 and 3: 24 µg ECP. Lanes 4 and 5: 18 µg ECP. Lanes 6 and 7; 12 µg ECP. Lanes 8 and 9: 6 µg EPC. Lane 10: 2.4 µg ECP.

Picture B: Western blot on ECP confirming the presence of the MSA protein with monoclonal antibody. Lane 1: ladder. Lanes 2 and 3: 12 µg of ECP. The bands are approximately 58 and 50 kDa.

4.4.2 ECP challenge i.p.: results in pELISA

In fish, injected i.p. with ECP, the kidney samples were positive in pELISA while samples from control groups were negative. A dose dependent response was evident when comparing pELISA results from fish injected with 0.1 versus 1.0 mg of ECP (Figure 15). All samples from untreated fish on day 0, as well as from fish injected with KDM broth or PBS were negative in pELISA.

The difference between the lowest and highest OD values in each injected sampling group was in most cases big, especially in the higher dose experiment. Therefore, the standard deviation of OD readings was high.



0.1 mg/fish	2dpi	7dpi	14dpi	28dpi	42dpi
Min	0.247	0.103	0.152	0.116	0.137
Average	0.35	0.237	0.279	0.23	0.2
Max	0.521	0.356	0.387	0.404	0.26
Std. dev.	0.09	0.081	0.081	0.102	0.048
1.0 mg/fish	**2dpi**	**7dpi**	**14dpi**	**28dpi**	**42dpi**
Min	0.225	0.671	0.656	0.238	0.857
Average	1.02	1.25	0.97	1.56	1.350
Max	1.678	2.480	1.228	2.798	2.255
Std. dev.	0.519	0.648	0.212	0.841	0.444

Figure 15. Accumulation of ECP in the fry kidneys in sample group 3 measured with pELISA.

The black columns represent the average OD value for each sampling day in fry injected with 0.1 mg of ECP.

The grey columns represent the average OD value for each sampling day in fry injected with 1.0 mg of ECP.

The lowest, highest, average values per group are listed below the columns along with the standard deviation values.

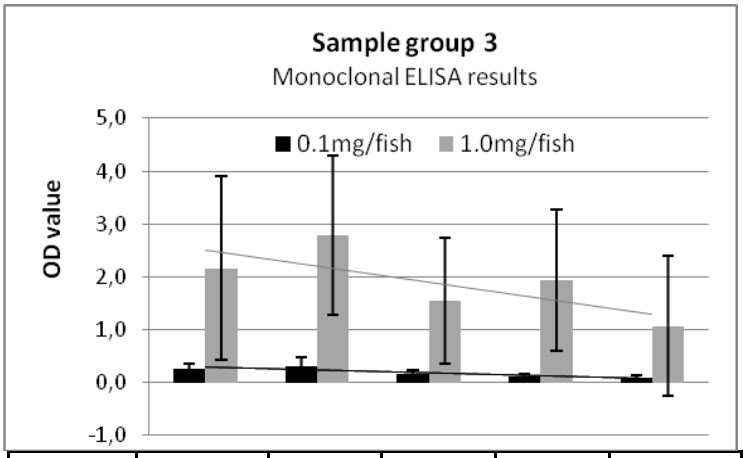
Regression line was calculated for both dosage experiments to evaluate if an increase or decrease of ECP was being measured.

At the first sampling, two days post injection, the ECP antigens had reached the kidney, (Figure 15). Calculations of the regression lines reveal the value for the lower dose experiment to be -0.031, which indicates decrease over time, but it was not significant when using ANOVA for statistical analysis. The value of the regression line for the group receiving 1.0 mg of ECP was 0.097, indicating increase over time that was not significant in an ANOVA analysis.

4.4.3 ECP challenge i.p.: results in mELISA

All kidney samples from fish, injected i.p. with ECP, were positive in mELISA, showing a clear dose response. Samples from all three control groups were negative.

In both groups, receiving 0.1 and 1.0 mg, the antigen had reached the kidney on day two after injection, but the average OD readings were highest on day 7 after injection. As demonstrated in figure 16, there was a tenfold difference between min and max OD readings on day 7 in the sample groups receiving the lower dose. Within all sample groups given the higher dose there was a 10-25 fold difference between OD readings. Consequently, the standard deviation value was high for most sample groups. Calculated regression line for both groups showed negative values, indicating a decrease in OD values over time. For the 0.1mg dose group this value was -0.053 and significant in an ANOVA analysis with ($p<0.05$). Similar value for the higher dose group was -0.3035, insignificant in ANOVA analysis.



0.1 mg/fish	2dpi	7dpi	14dpi	28dpi	42dpi
Min	0.184	0.053	0.073	0.080	0.056
Average	0.266	0.310	0.174	0.120	0.096
Max	0.441	0.547	0.258	0.203	0.152
Std. dev.	0.097	0.162	0.071	0.044	0.033
1.0 mg/fish	2dpi	7dpi	14dpi	28dpi	42dpi
Min	0.194	0.387	0.218	0.150	0.209
Average	2.164	2.781	1.54	1.93	1.072
Max	5	4.899	3.217	3.882	3.447
Std. dev.	1.735	1.506	1.193	1.337	1.315

Figure 16. Accumulation of MSA in the fry kidneys in sample group 3 measured with mELISA.

The black columns represent the average OD value for each sampling day in fry injected with 0.1 mg of ECP.

The grey columns represent the average OD value for each sampling day in fry injected with 1.0 mg of ECP.

The lowest, highest, average values per group are listed below the columns along with the standard deviation values.

Regression line was calculated for both dosage experiments to evaluate if an increase or decrease of MSA was being measured.

4.4.4 Bath challenge

All kidney samples from fish in the bath challenge trial were negative in ELISA.

5 Discussion

5.1 Semi-nested PCR and FTA minicard

A semi-nested PCR, snPCR, for detection of the msa gene of *R. salmoninarum* was developed and compared to nPCR, the method recommended by OIE (OIE, 2006). Both methods share an important feature, i.e. reduced possibilities of false positive results due to amplification of products from unexpected primer binding sites in comparison to conventional PCR. Including UNG enzyme treatment in the protocol for snPCR, to eliminate the risk of contamination, was tested. Although the enzyme was effective in degrading the DNA template containing dUTP instead of dTTP in the PCR reaction, the treatment diminished the sensitivity of the PCR test, as has previously been reported (Ritzler *et al.*, 1999). Consequently it was not included in the final protocol.

In the snPCR method, three primers, chosen with different optimum annealing temperature, can form two different primer pairs which are controlled by change of temperature in the thermal cycling machine. The primer pair that produces the second fragment, or the fragment within the first fragment, has lower annealing temperature than the primer pair that produces the first fragment. snPCR also minimizes the risk of contamination because the reaction takes place in a single tube and the template is not transferred between tubes, as needs to be done in nPCR. snPCR has other advantages over nPCR. Only half of all the plastic and reagent materials needed for nPCR is used in snPCR and snPCR is less time consuming than nPCR, both in preparation and in the thermal cycling machine. Single-tube nPCR and snPCR protocols have previously been described (Chan *et al.*, 1996; Berg *et al.*, 2001; Gookin *et al.*, 2002; Tao *et al.*, 2004; Saini *et al.*, 2009), some by using heat controlled primers as is described in this current study (Chan *et al.*, 1996; Gookin *et al.*, 2002; Saini *et al.*, 2009).

A new method for DNA isolation using FTA minicard, was also tested in the current study and compared to a common method using a commercial DNA kit. FTA minicard is a fairly new product for isolation of nucleic acids and storing samples. The use of FTA for DNA isolation has numerous advantages over the commonly used DNA kit. Above all it is easier in use and less time consuming. According to the manufacturer, samples on FTA can be stored at room temperature for years without affecting the quality of the DNA. In this study the dilution of kidney samples on the FTA minicard was the same as in the sample preparation for ELISA so the samples could be taken from the ELISA homogenate and placed on the FTA minicard.

Treating the samples with achromopeptidase was examined because this enzyme has been reported to rupture the rigid cell wall of *R. salmoninarum* more effectively than proteinase K (Magnusson *et al.*, 1994). Since the achromopeptidase treatment did not enhance the PCR efficiency when using FTA, this step was not included in the FTA minicard DNA isolation protocol. However, achromopeptidase replaced proteinase K in the DNA kit isolation protocol and was as well included in the RNA isolation protocol using TRIzol.

5.2 Sample group 1: Farmed Atlantic salmon brood fish

This group was chosen for testing of all detection methods, as an active natural infection was escalating in the group. Such studies have most commonly been carried out in naturally infected groups (Cipriano *et al.*, 1985; Griffiths *et al.*, 1991; Gudmundsdottir *et al.*, 1991; Hsu *et al.*, 1991; Gudmundsdottir *et al.*, 1993; Meyers *et al.*, 1993a; Olea *et al.*, 1993; Griffiths *et al.*, 1996; Elliott & McKibben, 1997; Pascho *et al.*, 1998; Bruno *et al.*, 2007), although there are exceptions (White *et al.*, 1995; Miriam *et al.*, 1997; Jansson *et al.*, 2008; Halaihel *et al.*, 2009). A collection of diagnostic methods were tested and compared to the novel snPCR and the use of FTA minicard for DNA isolation. The results showed that there was no statistical difference between the sensitivity of snPCR and nPCR, so the conclusion was made that these two methods were equally sensitive. For both methods, the use of FTA minicard gave more positive results than using the kit for DNA isolation although the difference was only significant when using nPCR. Therefore the use of FTA for DNA isolation can be recommended for further use in detecting *R. salmoninarum*. The comparison between pELISA and mELISA in this study underlines the difference of sensitivity between those two methods. pELISA detected significantly more positive samples than mELISA. Previous comparison studies have been conducted and the pELISA has been shown to be either more sensitive (Jansson *et al.*, 1996; Jansson *et al.*, 2008) or comparable to mELISA (Bandin *et al.*, 1996).

Figure 9 demonstrates that as the OD value for pELISA gets higher, more and more detection methods are in agreement. OD value of approximately 0.75 seems to be a critical point. pELISA detected more positive samples than all the PCR methods tested in sample group 1. Several previous studies have compared ELISA to PCR with various results. One study concludes that ELISA is the more sensitive method (Elliott *et al.*, 2009) but others stated that PCR is more sensitive (Miriam *et al.*, 1997; Pascho *et al.*, 1998; Bruno *et al.*, 2007; Halaihel *et al.*, 2009). In the comparison studies conducted by Bruno *et al.* (2007) and Halaihel *et al.* (2009), where ELISA was considered less sensitive than the PCR, mELISA was used in the comparison but not pELISA. In the comparison study conducted by Pascho *et al.* (1998), pELISA was used for the comparison but the samples used were ovarian fluid.

Using ovarian fluid and gill tissue for the detection of *R. salmoninarum* would be ideal if results for these samples were as informative as the kidney samples, because sampling would then be possible without killing the brood fish. Here, the use of ovarian fluid and gill tissue for the detection of *R. salmoninarum* gave poor results when compared to the kidneys in sample group 1 and are therefore not feasible for detection of the bacterium in Atlantic salmon. Inconsistency between kidney tissue and ovarian fluid using different diagnostic methods has previously been reported (Magnusson *et al.*, 1994; Griffiths *et al.*, 1996) and the kidney tissue has been proven to give more positive samples than ovarian fluid (Griffiths *et al.*, 1996; Miriam *et al.*, 1997). In the current study, PCR detected more positive samples than pELISA in ovarian fluid although the difference was not statistically significant. In one previous study, PCR detected significantly more positive samples than pELISA in Pacific salmon (Pascho *et al.*, 1998).

Many gill tissue and ovarian fluid samples were semi-positive in snPCR and nPCR. The most likely explanation is that the amount of bacteria in those samples was close to the detection limits of the tests. Semi-positive results are by definition inconclusive so the need for retesting arises, which is inconvenient in a brood stock culling program as well as being expensive. In kidney tissue, few samples were semi-positive and when they were retested to get conclusive results, most of them turned out negative.

In several samples in sample group 1, pELISA detected bacterial antigens while the PCR methods produced negative results. One possible explanation for the negative PCRs is that inhibitory components in kidney tissue repressed the reaction as reported in some studies (Magnusson *et al.*, 1994; Konigsson *et al.*, 2005). Further, there is some concern that pELISA might detect antigens from other bacteria (Bandin *et al.*, 1993b; Brown *et al.*, 1995; Wood *et al.*, 1995). The pELISA used in the present study has been run for two decades without trouble. Originally it was shown that samples made from rich growth of *Mycobacterium* sp. and an unidentified Gram-negative rod could result in low positive OD values (Gudmundsdottir *et al.*, 1993). Similar observation has since been made with rich growth of *Williamsia* sp., also isolated from the kidney of Atlantic salmon. Cross-reactions have never been shown to result in high OD values, such as observed in sample group 1. Finally, it has been suggested that antigens from a previous infection could reside in the host or even that fish may absorb antigens from the environment. The time it takes for the fish immune system to get rid of deposited antigen, after the fish may have gained control over the infection, is not known. Positive ELISA with negative PCR results might also arise when the antigen, which is secreted abundantly by the bacterium and circulates with the blood, has reached and accumulated in the tissue that is being tested, ahead of the bacterium (Austin & Rayment, 1985; Pascho *et al.*, 1987; Magnusson *et al.*, 1994; Rhodes *et al.*, 1998). In few samples the opposite was observed, i.e. PCR detected bacterial DNA but ELISA was negative. That sort of inconsistency has been reported before (Pascho *et al.*, 1998; Bruno *et al.*, 2007; Jansson *et al.*, 2008; Halaihel *et al.*, 2009). In those samples the bacterium itself seems to have reached the tissue that is being tested but the levels of the antigens are below the sensitivity of the ELISA test. The bacterium may also be inactive or even not viable in such cases and therefore antigens are not produced or secreted. The same scene might explain the difference between the PCR methods using genomic DNA (gDNA) as the template for the PCR reaction (snPCR, nPCR and qPCR) compared to the PCR method using complementary DNA (cDNA) as the template (RT-qPCR). Prior to RT-qPCR test, the mRNA transcribed by a viable bacterium is converted into cDNA via reverse transcription. If an amplification of the target gene is succeeded in RT-qPCR, the conclusion can be made that the bacterium was alive at the time of sampling because mRNA is a very unstable molecule and has a short lifetime in tissue samples. The PCR methods using gDNA as template could be amplifying DNA in the samples from an dead or inactive bacterium (Josephson *et al.*, 1993).

Using qPCR or RT-qPCR targeting both the msa and 16S rRNA genes did not detect as many positive samples as nPCR and snPCR using FTA minicard for DNA isolation or pELISA. The qPCR and RT-qPCR are also more expensive than snPCR. The difference between the two target genes in qPCR and RT-qPCR was not statistically significant. *R. salmoninarum* has two copies of the rRNA operon, as many other slow growing bacteria (Grayson *et al.*, 2000) and 2-3 copies of the msa gene.

Both genes are expressed constituently during active infection and growth of the bacterium (O'Farrell & Strom, 1999; Grayson *et al.*, 2002). It has been suggested that the inconsistency between different diagnostic methods may form a pattern that represents a probable stage along the course of natural *R. salmoninarum* infection and from studying such patterns, one can determine the course of the infection in a particular population (Faisal & Eissa, 2009).

5.3 Sample group 2: Wild Atlantic salmon in River Ellidaár and wild Arctic charr and brown trout in Lake Ellidavatn.

Here, the inconsistency between pELISA and PCR results were noticeable and most probably due to the reasons discussed above. In pELISA, high OD values were few and the average OD value was low compared to the samples in sample group 1. Symptomatic fish were not observed in any group and in fact symptoms are rarely observed in wild fish.

5.3.1 Sample group 2a

In sample group 2a, all the smolts were positive for the antigen before migrating to sea. The smolting period is one of the most stressful period in the salmonid fish life cycle and has probably been instrumental in the BKD progression in the smolts (Mesa *et al.*, 1999). Following a one year dwelling in the sea, most of the returning adults tested negative for the antigen in pELISA. In their marine phase, the fish gains weight and increases dramatically in size and can be expected to experience less stressful periods than in their freshwater phase. The differences between the migrating smolts and the returning adults indicate that some of the infected fish either died from the infection, eliminated the bacterium, or that the bacterium entered a latent or inactive stage in the fish as is for example observed in the related *Mycobacterium tuberculosis* infection in humans (Nuermberger *et al.*, 2004). The longer the returning adults stayed in the river water the higher percentage became positive for the antigen and additionally the OD readings got higher, indicating increased amount of *R. salmoninarum* antigens in the fish. The returning adults were either getting infected the second time via horizontal transmission and/or latent bacteria were activated because of increased stress and immunosuppression accompanying sexual maturation. In fact, a persistent, usually non-lethal infection that is activated during spawning, may secure the continued survival of the bacterium through vertical transmission (Dale, 1994). A possible increase in *R. salmoninarum* concentration in the river may as well induce the BKD progression observed in sample group 2a. In a recent study, cultured Atlantic salmon smolts, of River Ellidaár-origin, testing free of *R. salmoninarum* antigens in pELISA, were released into a releasing pond fed by the river water. After one month stay in the pond, 76% of the fish were positive in pELISA (Kristmundsson, personal communication). These results might indicate that there is an increase in concentration of *R. salmoninarum* cells in the river but additionally the

transportation from the farm to the pond may have induced stress response in the fish and aided in the increased prevalence observed.

5.3.2 Sample group 2b

The Arctic charr and brown trout from Lake Ellidavatn, in sample group 2b, showed high prevalence using the pELISA test or 89% and 98% respectively. These numbers are much higher than observed in 1993, in a thorough study conducted by Jónsdóttir *et al.* (1998). Then, the prevalence was 16% in Arctic charr and 27% in brown trout in Lake Ellidavatn. The average amount of antigens in the fish was also much greater than observed in 1993. The increase in prevalence is not restricted to this lake and it's river system. Similar observations are being made in other rivers around Iceland (Kristmundsson *et al.*, 2009). From 1988 to 2006, a long term study on environmental factors in Lake Ellidavatn was conducted. During these 18 years, the mean water temperature has increased significantly (Malmquist *et al.*, 2009). This change in water temperature may be the main factor explaining the increase in prevalence of *R. salmoninarum* in the fish population over the last few years as has also been postulated for proliferative kidney disease (PKD) that was diagnosed for the first time in Lake Ellidavatn in the autumn of 2008 (Kristmundsson *et al.*, 2010b).

The difference in prevalence between Arctic charr and brown trout in sample group 2b was not statistically different. Both these fish species are considered fairly resistant to *R. salmoninarum* along with Atlantic salmon. Comparing the percentage of positive samples in ELISA between the anadromous Atlantic salmon in sample group 2a to the freshwater lake inhabitants in sample group 2b is not feasible due to differences in environmental circumstances and stage of life during sampling. The Arctic charr and brown trout in Lake Ellidavatn do not smoltify because of their constant stay in freshwater, and that might diminish their susceptibility to *R. salmoninarum* infection. Definition of the word "resistant" in relation with BKD susceptibility can be a bit confusing. Some state that the more resistant salmonid species are able to mount an effective immune response and therefore can maintain the infection in a more manageable state (Dale, 1994) or that the more resistant species may be more adapted to *R. salmoninarum* than the more susceptible species. The resistance to *R. salmoninarum* may have evolved in the nonanadromous species due to their continual existence in freshwater where natural exposure and transmission of *R. salmoninarum* is most likely to occur (Meyers *et al.*, 1999). Jónsdóttir *et al.* (1998) speculates that the bacterium may be a normal low density resident in wild Arctic charr and brown trout. The pathological lesions seen in severe infection may largely be due to the host's immune response (Young & Chapman, 1978), therefore it is possible that the immune system of more "resistant" salmonid species are not responding to the bacterial infection. The bacterium is then able to reside and maybe even replicate inside the host without an effective immune response from the host. If that is the case, the word "resistant" is not the appropriate word to use.

5.3.3 Sample group 2c

In sample group 2c pELISA was again the most sensitive method and attempts to isolate the bacterium by culture on SKDM were unsuccessful. Culture has been compared to ELISA in previous comparison studies. ELISA was considered more sensitive in some studies using pELISA (Pascho *et al.*, 1987; Gudmundsdottir *et al.*, 1993; Olea *et al.*, 1993) but culture proved to be more sensitive than ELISA in one study but there, mELISA was used (Griffiths *et al.*, 1996). There is no evidence of viable bacteria being present in the samples in sample group 2c. The only method determining the presence of viable bacteria in this sample group, i.e. culture on SKDM agar, gave negative results. There was no correlation between OD values in pELISA and Ct values in qPCR targeting the msa gene in sample group 2c. The fact that the pELISA is detecting other antigens from *R. salmoninarum* besides MSA may explain the difference but good correlation between the OD value in both mELISA and pELISA to Ct value in qPCR has been reported in rainbow trout (Jansson *et al.*, 2008). A significant difference in prevalence of pELISA positive samples between the years 2008 and 2009 was observed in brown trout in Lake Ellidavatn.

5.4 Sample group 3: Atlantic salmon fry, treated with ECP of *R. salmoninarum*

The most prominent band with silver staining was determined to be 59 kDa and 58 kDa in Western blotting, probed with monoclonal antibodies against MSA so it can safely be said to be an MSA component. The monoclonal antibody also detected a band of 50 kDa, but since smaller bands were not detected, it can be assumed that the antigen was not extensively proteolyzed (Wiens & Kaattari, 1991). Further, the ECP tested positive in pELISA as well as mELISA but had to be diluted 1:33 before testing in pELISA to get similar OD values as observed for undiluted samples in mELISA. The explanation is that pELISA detects MSA as well as other products produced by the bacterium and presumably many epitopes on the larger antigens. The average amount of antigen in the fish kidney declined slowly over time. The decrease was significant when using mELISA. The bathing experiment in this sample group was conducted to evaluate the absorption of antigens from the surrounding environment. The fish did not absorb enough of the antigens during the immersion to give positive reaction in pELISA. Possibly, longer or repeated immersion might result in absorption.

6 Concluding remarks

The semi-nested PCR method developed in the current study equaled the nPCR method recommended by OIE in sensitivity as confirmed using three different approaches. Samples prepared for snPCR and nPCR on FTA minicard, a new product for DNA isolation, increased the number of positive samples in comparison to isolations done with a DNA kit. A higher number of positive samples were detected using FTA isolation and those two PCR methods, than when using a number of qPCR methods targeting either msa or 16S rRNA gene.

The pELISA detected the highest number of positives in all groups. Further, as observed in the brood fish, it becomes positive in presumably the earlier stages of the disease, when other methods are giving negative results. Ovarian fluid and gill tissue were compared to kidney samples *for R. salmoninarum* detection and the results show that these tissues can not replace kidney as the organ of choice when screening for *R. salmoninarum*. The disagreement between pELISA and PCR in wild fish underlines the necessity to study the situation more closely.

Accumulation of bacterial ECP in the fish kidney was studied and the ability of the fish to dispose of the antigens evaluated. The average amount of MSA antigen detected by mELISA declined slowly in the fish kidney over 6 weeks, while results in pELISA showed no change. Another study, with longer follow up time, needs to be conducted to determine how long the antigens can persist in the kidney.

Results obtained from wild fish revealed a significant increase in the prevalence of *R. salmoninarum* positive fish, when compared to earlier studies. There are indications that rising water temperature is an important factor in this change.

Screening and diagnosis of *R. salmoninarum* is very important for the fish farming industry as well as restocking programs, and consequently the choice of detection methods, to base the brood stock culling or segregation programs on, is very important. “False” positive results are costly, instigating culling of uninfected ova, but “false” negative results would definitely be more costly in the long run. The conclusion from this study is in accordance with previous studies: There is really no single method that can be considered “the golden standard” for all fish and all situations to determine the true prevalence of the bacterium. Dr. Diane G. Elliott has described the situation quite fittingly by stating that:

“There is no silver bullet”

7 References

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