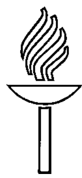

Copyright © , by University of Jyväskylä

To Harri, Henri and Olga

ABSTRACT

Päivi Pylkkö
Atypical Aeromonas salmonicida -infection as a threat to farming of Arctic charr (Salvelinus alpinus L.) and European grayling (Thymallus thymallus L.) and putative means to prevent the infection
Jyväskylä: University of Jyväskylä, 2004, 46 p.
(Jyväskylä Studies in Biological and Environmental Science,
ISSN 1456-9701; 142)
ISBN 951-39-1842-4

This review summarizes the results of studies on atypical *Aeromonas salmonicida* (aAS) -infection among farmed Arctic charr (*Salvelinus alpinus*) and European grayling (*Thymallus thymallus*) (later referred as charr and grayling, respectively). Specifically, I aimed to characterize the causative agent of aAS -infection, to describe putative virulence factors of the bacteria and cross pathology of the infection in charr and grayling. I also aimed to describe some of the immune defense functions in charr. I also described a concomitant presence of a common fresh water fish parasite, *Diplostomum spathaceum*, and aAS -bacteria in manifestation of the bacterial infection. The experimental juvenile charr and grayling, originated from Vuoksi, Kymijoki and Inari watercourses in Finland. Several (n=66) aAS isolates from 7 watercourses in Finland and from the Baltic sea were characterized in the study. These were compared to 11 Swedish, 1 Icelandic aAS isolates, to 3 *A. salmonicida* subsp. *salmonicida* (ASS) isolates and to ATCC 33659, a type strain for *A. salmonicida* subsp. *achromogenes*. Classical biochemical analyzes and modern molecular tools (macrorestricted genome and plasmid profiles) were utilized in characterizing. AAS was detected from fish tissue (intestine, lens and heart) by a combination of bacterial cultivation and polymerase chain reaction (PCR) techniques. Pathology of aAS infection in charr and grayling was described both in natural outbreaks at fish farms and after experimental challenge. The effects of oil-adjuvanted vaccine on the immune system and on the growth of charr at different post vaccination (p.v.) temperatures was studied. Interactions between bacteria and parasites were studied using grayling subjected to concomitant exposure of aAS bacteria and *D. spathaceum* parasites. Genetically identical aAS isolates within the Vuoksi and the Kymijoki watercourse were found, whereas genetic differences between aAS isolates from various river basins were observed. A major virulence factor in Vuoksi aAS -isolates need to be studied further. In charr and grayling aAS caused systemic infection. Vaccination induced in charr a significant anti-aAS specific antibody response and increased plasma lysozyme activity at all p.v. temperatures. The vaccination had no ultimate negative effects on the growth of charr. AAS bacteria was successfully detected from a heart tissue of grayling by the combination of bacterial cultivation and PCR techniques. The detection level was 17 aAS cells per 100 mg sampled tissue. *D. spathaceum* enhanced bacterial infections in fish. Diplostomids also caused serious eye ruptures in grayling.

Key words: Atypical Aeromonas salmonicida; Salvelinus alpinus; Thymallus thymallus; Diplostomum spathaceum; pathology; characterization; virulence, prevention; farming; PCR; PFGE

P. Pylkkö, Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FI-40014, University of Jyväskylä, Finland

Author's address Päivi Pylkkö
University of Jyväskylä,
Department of Biological and Environmental Sciences,
P.O. Box 35,
40014 University of Jyväskylä,
Finland
e-mail: paivi.pylkko@mtt.fi

Supervisor Professor E. Tellervo Valtonen
Department of Biological and Environmental Sciences
University of Jyväskylä
P.O. Box 35
FI-40014 University of Jyväskylä

Professor Sinikka Pelkonen
National Veterinary and Food Research Institute,
Kuopio Department
PL 92
70701 Kuopio
Finland

Reviewers Dr. Bjarnheiður K. Guðmundsdóttir,
Institute for Experimental Pathology,
University of Iceland,
Keldur v/Vesturlandsveg,
IS-112 Reykjavík.
Iceland

Research Professor Kurt Buchmann
Royal Veterinary and Agricultural University
Department of Veterinary Pathobiology
Section of Fish Diseases
Stigbøjlen 4
DK-1870 Frederiksberg C.
Denmark

Opponent Professor Alexandra Adams
The Aquatic Vaccine Unit
Institute of Aquaculture
University of Stirling
FK9 4LA
Scotland

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

INTRODUCTION ... 9
Endangered fish species ... 9
Infectious diseases – a threat to stocking program and genetic diversity ... 9
Detection and identification methods for pathogens ... 11
Putative virulence factors of *A. salmonicida* ... 11
Means to prevent infections at fish farms ... 12

OBJECTIVES ... 14

MATERIALS AND METHODS ... 15
Experimental fish and facilities ... 15
In vivo experiments using bacteria and parasites ... 16
Detection and characterization of bacteria ... 17
Isolation and characterization of bacterial products ... 17
Measurements of immune defense function ... 19
Vaccination experiments ... 20
Statistical analyses ... 20

RESULTS AND DISCUSSIONS ... 21
Characterization and virulence factors of atypical *Aeromonas salmonicida* ... 21
Atypical *Aeromonas salmonicida* -infection in charr and grayling ... 24
Evoking immune defence function in charr ... 26
Putative preventive means against aAS bacterial infection – vaccination and control of parasite pressure ... 28

CONCLUSIONS ... 31

Acknowledgements ... 33

YHTEENVETO (Resumé in Finnish) ... 35

REFERENCES ... 37

LIST OF ORIGINAL PUBLICATIONS

I Pylkkö P, Pohjanvirta, T, Madetoja J, Pelkonen P. Characterization of atypical *Aeromonas salmonicida* infection in Arctic charr *Salvelinus alpinus* and European grayling *Thymallus thymallus*. Submitted.

II Pylkkö P, Suomalainen L-R, Tiirola M, Valtonen T. Evidence of enhanced bacterial invasion during *Diplostomum spathaceum* infection in European grayling (*Thymallus thymallus* L.). Manuscript.

III Pylkkö P, Lyytikäinen T, Ritola O, Pelkonen S, Valtonen ET 2002. Temperature effect on the immune defence functions of vaccinated and unvaccinated Arctic charr *Salvelinus alpinus*. Diseases of Aquatic Organisms 52: 47-55.

IV Madetoja J, Pylkkö P, Pohjanvirta T, Schildt L, Pelkonen S 2003. Putative virulence factors of atypical *Aeromonas salmonicida* isolated from Arctic charr, *Salvelinus alpinus* (L.) and European grayling, *Thymallus thymallus* (L.). Journal of Fish Diseases 26: 349-359.

V Pylkkö P, Lyytikäinen T, Ritola O, Pelkonen S 2000. Vaccination influences growth of Arctic charr. Diseases of Aquatic Organisms 43: 77-80.

INTRODUCTION

Endangered fish species

When Lake Saimaa in the The Vuoksi watercourse, in southeastern Finland, was formed after the last glacial period some 10 000 years ago (Saarnisto *et al.* 1995, Saarnisto & Saarinen 2001), several salmonid species were isolated from the sea. At present Arctic charr (*Salvelinus alpinus* L.), European grayling (*Thymallus thymallus* L), landlocked salmon (*Salmo salar* m. *sebago* G.) and landlocked trout (*Salmo trutta* m. *lacustris* L.) still occur in the area.

Charr is classified either as a critically endangered (in Lake Saimaa in the Vuoksi watercourse) or threatened (in Inari) fish species in Finland (International Union for Conservation of Nature and Natural Resources 2000, IUCN). Grayling is also one of the near threatened fish species (IUCN 2000). Spawning of charr and grayling in nature does not maintain the natural stocks, which are either dependent on, or are supported by, stocking. Brood fish, juveniles and eggs of grayling are conserved by government fish farms.

To preserve the genetic background, Finnish government policy advises stocking fish that originate from the same water body where they are released. Restocking of charr and grayling is carried out in four watercourses in Finland. These are Vuoksi, draining eastwards via Lake Ladoga to the Gulf of Finland; Kymijoki, draining southwards to the Gulf of Finland; Oulujoki, draining to the northern Gulf of Bothnia, and Inari, draining to the Arctic Ocean.

Infectious diseases – a threat to stocking program and genetic diversity

Cultivation of fish at high densities in fish farms attracts a variety of parasites, most of which are protozoans or other ectoparasites (e.g. Rintamäki-Kinnunen & Valtonen 1996, 1997; Hakalahti & Valtonen 2003). Some endoparasitic trematode species such as larvae of *Diplostomum spathaceum,* migrating through fish skin and tissues, can also cause outbreaks (e.g. Crowden & Broom 1980; Lafferty & Morris 1996).

While parasitic diseases are the most common cause of mortality in northern fish farms, bacterial diseases are also prevalent (Rintamäki-Kinnunen 1997). In northern latitudes the open water period lasts 4 to 5 months. During summer, lake water temperature increases from 4 to as high as 20ºC. At this time bacterial and parasitic pathogens are concomitantly present at farms and fishes are exposed to both pathogens.

The most common bacterial diseases creating problems at Nordic fish farms are "typical" *Aeromonas salmonicida* (ASS), the causative agent of furunculosis , several "atypical" *A. salmonicida* subspecies (Griffin et al 1953, Rintamäki & Valtonen 1991), *Listonella anguillarum* (Pedersen *et al.* 1999), *Pseudomonas anguilliseptica* (Rintamäki & Koski 1987, Wiklund & Lönnstrom 1994), *Renibacter salmoninarum* (Wiklund & Lönnstrom 1994), *Flavobacterium columnare* (Koski *et al.* 1993), *Flavobacterium psychrophilum* (Wiklund *et al.* 1994) and *Yersinia ruckeri* (Valtonen et. al 1992). At Finnish fish farms, charr and grayling are most susceptible to atypical *Aeromonas salmonicida* -infection and are annually affected by this disease.

Atypical *Aeromonas salmonicida* (aAS) has been isolated in several places in Finland, most often however in the Vuoksi and Kymijoki watercourses and mainly from diseased charr and grayling. Rintamäki & Valtonen (1991) have isolated aAS from brown trout in the Kymijoki and Oulujoki watercourses. In addition, aAS has occasionally been isolated from diseased wild fish such as roach (*Rutilus rutilus* L.), vendace (*Coregonus albula* L.) and dace (*Leuciscus leuciscus* L.) (National Veterinary and Food Research Institute, EELA, annual statistics) and cyprinid fish and flounder in the Archipelago Sea of the Baltic Sea (Wiklund et al. 1999).

In Iceland, aAS-infection is considered endemic in wild salmon (Guðmundsdóttir 1997). In Sweden, aAS infection has been a severe threat to the grayling stocking program (Carlstein 1996). Atypical *A. salmonicida* disease has also been described from salmonids in Canada and Chile (Olivier 1990, Bravo 2000). However, the pathogenesis of the disease has not been thoroughly described.

Atypical AS outbreaks in charr and grayling stocks at Finnish fish farms are usually followed by a high mortality rate, exceeding 60% (paper I). Mortality of 30 % of the total salmon production has been estimated in Iceland (Guðmundsdóttir 1997). Rintamäki & Valtonen (1991) reported mortality from 4% to 20% among grayling stocks at a northern Finnish farm. The disease affects equally fish from fingerlings to brood fish. In addition, outbreaks are an annual nuisance at the fish farms. Therefore, aAS disease is really a severe threat to charr and grayling restocking programs and to the genetic diversity of these vulnerable fish species. The aAS disease also causes considerable economic losses to commercial farming of salmonid fishes.

Detection and identification methods for pathogens

Isolation of bacteria on nutrient agar plates combined with biochemical analyzes of utilization of sugars by bacteria have been commonly used in fish disease diagnostics. However, a low number of viable bacteria, as is the case at the beginning of the disease already creates a problem in this kind of diagnostics. It has been shown that less than 1% of aquatic and terrestrial bacteria can be isolated on agar plates (Jannasch & Jones 1959). The percentage may be higher for fast-growing organisms such as fish pathogens, but may still hamper the diagnosis.

Polymerase chain reaction (PCR) is reported to be a useful tool for the identification of microbiological pathogens from fish (Hänninen & Hirvelä-Koski (1997, 1999). Several PCR based methods are available. Species-specific PCR has previously been used to detect *A salmonicida* from mixed cultures (Høie et al. 1997, Austin et al 1998, O'hIci et al 2000). In addition, microbial communities can be analyzed by PCR amplification of 16S ribosomal DNA (rDNA) with universal bacterial primers to reveal the pathogen. This approach can also be applied to fish diseases, such as flavobacterial diseases (Tiirola et. al. 2003).

Macrorestriction of genomic DNA (MRP) and plasmid profile analyzes have been successfully applied to epidemiological studies of fish diseases (Whittington et al. 1995, Sørum et al. 2000, Madetoja et al. 2001). These methods have also greatly improved the characterization of *Salmonella* subtypes in diagnostic use (Lukinmaa et al. 1999).

Putative virulence factors of *A. salmonicida*

The pathogenic mechanism of aAS infection in charr and grayling is unresolved. Virulence factors of ASS have been intensively studied.

A cell surface A-layer and secreted extracellular products (ECP) containing enzymatic and toxic activities, have been associated with the pathogenicity of the bacterium. Two secreted toxins, a cytolytic toxin, glycerophospholipid-cholesterol acyltransferase (GCAT), and a toxic 70 kDa serine protease have been implicated as major factors in the pathogenesis of furunculosis (Sheeran & Smith 1981; Lee & Ellis 1990; Ellis 1997). However, it has been suggested that the exotoxic activities are different in typical and aAS bacteria (Guðmundsdóttir et al. 1990, Guðmundsdóttir 1996, Austin *et al.*1998).

Guðmundsdóttir et al. (1990) and Guðmundsdóttir et al. (1995) have characterized a toxic protease, from the ECP of the pigment producing aAS. This protease, characterized as a metallocaseinase, AsaP1, has been suggested to play an important role in the pathogenesis of the disease in Atlantic salmon (Guðmundsdóttir 1996). Injection of the AsaP1 toxoid in Atlantic salmon induced protection against aAS infection (Guðmundsdóttir & Magnadóttir 1997).

However, there are great differences in the activities of the ECP between atypical isolates originating from different geographical locations and different fish species (Guðmundsdóttir 1996; Gunnlaugsdóttir & Guðmundsdóttir 1997; Austin et al. 1998) which apparently relate to the heterogeneity of aAS bacteria. Furthermore, Austin et al. (1998) had earlier observed no caseinase production and very poor gelatinase production by the aAS isolate originated from Finnish grayling. Guðmundsdóttir et al. (2003) classified the analyzed Finnish strains as non-toxic according to their exotoxin secretion. Braun et al. 2002 have characterized an ADP-ribosyltransferase toxin (AexT) from ASS extra cellular products. The AexT have been associated with the virulence of ASS via a type III secretion pathway of Gram negative bacteria (Salmond & Reeves 1993, Hueck 1998, Lee 1997) by translocation of the toxin directly from bacterial cell into the target cell in a host (Braun et al. 2002, Burr et al. 2003).

Virulence factors of aAS isolates are indeed a complicated matter and not yet thoroughly understood.

Means to prevent infections at fish farms

Pathogens usually have their virulence components located on their surface and in the cell wall. Protection by the host is based on inactivation of such components. In vertebrates, lysozyme is one of the enzymes in the first-line of defense against invading pathogens (see e.g. Roitt 1994, Janeway et al. 1999). The defense response is directed against the peptidoglycan layer, especially in the cell wall of Gram-positive bacteria, that is suspected to be weakened by the alternative pathway of the complement system before enzymatic (lysozyme) attack (see e.g. Janeway et al. 1999). Activation of the complement system, the classical pathway, is based on specific recognition of an invading pathogen while activation of the alternative pathway needs no specific recognition (see e.g. Roitt 1994, Janeway et al. 1999).

Antibody responses against ASS and aAS after vaccination have been studied in Atlantic salmon (*Salmo salar)*, brown trout (*Salmo trutta* L.*)*, and various *Oncorhynchus* species. (Thuvander et al. 1987, Guðmundsdóttir & Magnadóttir 1997). Humoral antibodies and neutralization of bacterial toxins are important protective mechanisms in fishes (Guðmundsdóttir & Magnadóttir 1997). The variety of virulence factors and mechanisms of aAS and typical *Aeromonas salmonicida* (ASS) bacteria indicates that they possess many different means to evade the immune system of fish (Garrote et al. 1992, Merino et al. 1994).

Vaccination against furunculosis and vibriosis (*Listonella anguillarum*) is a well-established practice in fish farming. These vaccines have been successful in protecting brown trout, (*Salmo trutta* m. *lacustris* L.), Atlantic salmon, and rainbow trout, (*Oncorhynchus mykiss* W.) against the diseases (Thuvander et al. 1987, 1993, Midtlyng et al. 1996). Injection of the AsaP1 toxoid into Atlantic salmon induced protection against infection (Guðmundsdóttir & Magnadóttir 1997). An autogenous oil-based vaccine against aAS (Iceland Bioject.OO,

IBOO) has been developed and first used in Iceland (Gunnlaugsdóttir & Gudmundsdóttir 1997). This autogenous aAS vaccine (later referred as IBOO) gives good protection; twelve weeks after vaccination the relative percentage of survival (RPS) value of challenged vaccinated Atlantic salmon was 100 and the RPS among non vaccinated group was 60 (Guðmundsdóttir et al. 1997). It was the first vaccine against aAS that was commercially available. However, the IBOO vaccine has never been tested in Finland. At the time of this study was the vaccine was not yet available. Arctic charr vaccinated with commercial, monovalent or multivalent furunculosis vaccines, have died due to aAS infection (P. Pylkkö unpubl.). This observation is supported by Guðmundsdóttir & Guðmundsdóttir (1997) who reported better protection against aAS infection with IBOO than commercial furunculosis vaccine.

Among salmonid fishes the Arctic charr is well adapted to cold waters (Johnson 1980). Temperature is an important factor in immunological and physiological processes in poikilothermic animals (Fry 1971). Intraperitoneal (i.p.) vaccination with oil adjuvanted vaccines has been reported to result in either enhanced (Buchmann et al. 1997) or reduced fish growth (Lillehaug 1991, Lillehaug et al. 1992, Midtlyng 1997, Midtlyng & Lillehaug 1998). Knowledge about the effects of oil-based vaccination on growth of Arctic charr is limited. The optimum temperature for growth of farmed charr is from 13 to 14°C (Swift 1964, Jobling et al. 1993, Lyytikäinen 1998). Little is known about the immune responses of charr and the effect of temperature on its immune system how the immune system of charr reacts to increasing temperatures after vaccination is also unknown. Atypical AS infection has been reported to occur typically at water temperatures of 15°C in Finland, 8-10°C in Iceland or 11-17 °C in Chile (Rintamäki & Valtonen 1991, Gunnlaugsdóttir B. & Gudmundsdóttir 1997, Bravo 2000). Fish vaccinations in Finland generally take place either from October to November and/or from February to April when the water temperature ranges from 1 to 3°C. The temperature in which fish are cultured after vaccination may influence the protection induced by vaccine.

OBJECTIVES

The questions asked in this study were connected to farming of Arctic charr (*Salvelinus alpinus*) and European grayling (*Thymallus thymallus*) for supporting their restocking of them into the Vuoksi watercourse. It was also the purpose to increase generally applicable knowledge about preventing fish diseases at fish farms. Healthy fish mean better quality of harvested fish and enhance economically sustainable fish farming.
Specifically I aimed:

1. To characterize the causative agent of atypical *Aeromonas salmonicida* (aAS) and to describe putative virulence factors and cross pathology of the infection in Arctic charr and European grayling.
2. To describe some of the immune defense functions in Arctic charr in order to gain knowledge for development of preventive means, such as vaccination, against aAS infection.
3. To study the possible role of concomitant presence of a common fresh water parasite and aAS bacteria in the onset of the aAS disease.

MATERIALS AND METHODS

Experimental fish and facilities

One year old fish, Arctic charr, *Salvelinus alpinus* (n=30), originating from Lake Kuolimo stock (mean weight of 49.8 g ±4.81 g) and 53 European grayling, *Thymallus thymallus*, fingerlings (0+) originating from Lake Puruvesi stock (mean weight of 22.1 ± 8.5g) from a government fish farm at The Vuoksi watercourse were challenged by atypical *Aeromonas salmonicida* (aAS) strain n:o 9 (Table 1). The fish were transferred for the challenge experiment to EELA, Kuopio Department, experimental fish facilities. The facilities provided by 140 l fibreglass tanks with flow trough lake water. The rearing density was adjusted to 8 kg/m^3 (I).

A group of 0+ European grayling, , originating from Lake Päijänne stock (totally n=480, mean weight 52.9 g) were transferred from rearing to experimental facilities at a government fish farm located at the Kymijoki watercourse. The fish were exposed to aAS bacteria and *Diplostomum spathaceum* parasite.

The taxonomy of the genus *Diplostomum* spp. is problematic and not yet fully resolved (Valtonen & Gibson 1997). Fish in northern Finland carry two species of *Diplostomum* in the lens (Valtonen & Gibson 1997) and the majority of those resemble *D. indistictum*, referred to as *D. spathaceum* by Niewiadomska (1986). The cercariae, that bend their body into 90° angle while swimming which is typical of *D. spathaceum*, and were selected for the experiment (II). However, it was recognized that other species might also be present.

The experimental fish were kept in 0.138 m^3 fibreglass tanks at ambient water temperature with a continuous flow of lake water. Dissolved oxygen in the inflow remained at 8.3 mg/ml throughout the experiment. Fish density in the tanks was adjusted to 15 kg/m^3 (II).

A group of Arctic charr, (average weight = 25.2 g, SD= 7.6 g), originating from Lake Inari stock, was vaccinated intraperitoneally (i.p.) at a water temperature of 2.9°C with an experimental vaccine (0.2 ml/fish) prepared for the study by Alpharma AS, Norway. Fish were reared at a government fish

farm at the Kymijoki watercourse. The charr at the farm were individually marked 25 days post vaccination with passive integrated transponder (PIT) tags. Water temperature was 6°C at the time of tagging and during the following 7 days the water temperature increased naturally to 10°C. Water temperature was then adjusted to, 10.3, 14.1 and 18.1°C to meet the experimental design. Fish were held in two 4m^2 tanks with continuous flow of lake water and received commercial feed (III, V).

The lethal effect of ECP on fish was studied by challenge tests with charr fingerlings (mean weight 37 g) from Lake Kuolimo stock from the government fish farm at the Vuoksi watercourse. The fish were transferred for the challenge experiment to EELA, Kuopio Department, experimental fish facilities. The water temperature during the experiments was 13 ± 1°C (IV).

Dissolved oxygen in the inflow remained at 7.5 to 8.5 mg/l in all the experiments if not specifically stated otherwise. The fish were handled for blood samples, tagging, vaccination and in i.p. challenge experiments under tricaine sulphonate (MS-222, 0.15 g/l) anaesthetic. Depending on the experimental design, anaesthetized fish were killed by a blow on the head.

All the *in vivo* experiments were carried out by authorized personnel in accordance with Finnish national legislation concerning the protection of live animals used for experimental and other scientific purposes. Each *in vivo* experiment was authorized by a license granted by the responsible authority.

In vivo experiments using bacteria and parasites

Bacterial challenge strains and parasitic pathogens. Atypical strains were originally isolated at the National Veterinary and Food Research Institute, Kuopio Research unit, EELA, Finland from diseased grayling (see Table 1).

The bath challenge method was used when introducing live pathogen into the host. This is supposed to mimic as naturally as possible the route of pathogens to induce infections. The bath challenge method was used in papers I, II and also when efficacy of experimental aAS vaccine was tested (P. Pylkkö unpubl.). A high dose (10^7 cfu/ml) was utilized when severe signs of infection and in efficacy tests of experimental aAS vaccines were studied (I, P. Pylkkö unpubl.). An intermediate dose (10^5 cfu/ml) was utilized when the interaction of the bacteria and the parasite was studied and a highly lethal bacterial exposure was to be avoided (II). The parasite dose of *D. spataceum* was 150 cercariae per fish (II).

The intraperitoneal (i.p) challenge method was used when lethality of ECP produced by aAS strains in fish was studied (IV). Fish were injected i.p. with 100 µl of ECP. Control fish were injected with 100 µl of sterile 0.9% NaCl. Positive control fish were injected with the ECP from strain 265-87 (Table 1).

Detection and characterization of bacteria

Bacterial culture from tissue samples (eye, intestine or heart) or pure bacterial cultures on nutrient agar were needed for further studies (I, II, IV). Aliquots of serial 10-fold dilutions of the samples or pure aAS bacterial cultures were streaked on TS, TS supplemented 5% bovine blood or brain heart infusion (BHI) agar. The bacterial density on a plate was expressed as colony forming unit (CFU) (II).

Biochemical characterization of the isolates was made according to the methods described by Hirvelä-Koski et al.(1994) and Hänninen & Hirvelä-Koski (1997). Pigment production of the isolates was analyzed after incubation for 7 days on L-tyrosine-containing furunculosis agar at 15°C (Hirvelä-Koski, Koski & Niiranen 1994). (I, II, IV)

Cross pathology. Fish were dissected and sampled for histopathological examination. Samples from skin, muscle, intestine, kidney, spleen, liver and heart were fixed in phosphate buffered 4 % formaldehyde and subsequently embedded in paraffin (I). Embedded 5 µm sections were stained with haematoxylin-eosine (H&E) and Gram stain, as routinely used in veterinary diagnostics (I).

Macrorestricted genome (MRP) analyses by pulsed field electrophoresis (PFGE) was used for more precise grouping of aAS bacteria since the method usage has added more information to diagnosis of *Salmonella* subtypes in mammalian diseases (Lukinmaa et al. 1999). This method has also been suggested for the atypical *Aeromonas salmonicida* grouping (Hänninen & Hirvelä-Koski 1999). Bacterial cells were lysed in order to extract the genomic DNA for analyses. DNA was digested with restriction enzymes *Spe*I or *Xba*I or *Not*I, *Sfi*I and *Sma*I (New England Biolabs). The digested genome was treated by electrophoresis in Bio-Rad CHEF DRIII apparatus with linear pulse ramp from 2.5 to 25 s for *Spe*I and 1-16 s for *Xba*I angle 120° and voltage 6 V/cm. (I)

Plasmid profiles. Plasmid DNA was isolated in paper I. Plasmids were visualized in a 0.9 % agarose gel (SeaKem LE, BMA) after horizontal electrophoresis at 4.5 V/cm. Plasmids from *E. coli* isolates V517 and 39R861 served as molecular weight markers (Macrina et al. 1978, Threlfall 1986) (I).

Isolation and characterization of bacterial products

Extra cellular products (ECP) of the isolates were produced by the cellophane overlay method as described by Liu 1957 (IV). ECP of aAS isolates and from reference strains were used for more detailed analysis by substrate sodium dodecyl-sulphate polyacrylamide gel electrophoresis, SDS-PAGE, (IV), *in vitro* cell toxicity test (IV) and challenge experiments *in vivo* (IV).

Assays used for detection of proteolytic, haemolytic and cytotoxic activities in ECP. Proteolytic activities of the ECP were studied by gelatinase, caseinase and phospholipase assays and by substrate SDS-PAGE (IV). The gelatinase activity was detected by the radial diffusion method using agar plates supplemented

with 3% gelatine (Difco) as described by Hastings & Ellis (1985) (IV). Caseinase activity was determined as an increase in absorbance using azocasein (Sigma, A-2765) as a substrate (Guðmundsdóttir 1996) (IV). Phospholipase activity was determined by the radial diffusion method on agar plates, supplemented with 1% Lα -lecithin (Sigma) (Guðmundsdóttir et al. 1990) (IV). The molecular weights of native proteases present in the ECP were analysed by substrate SDS-PAGE modified from the method of Heussen & Dowdle (1980). Gelatine or casein were used as substrates in SDS-PAGE for proteolytic activitites (IV). The gels were stained with Coomassie brilliant blue (CBB) R-250 and destained until the clear bands were visible (IV). Molecular weights of the proteases were estimated as in SDS-PAGE. *Haemolytic activity* was determined using red blood cells (RBC) from rainbow trout as target cells (Hastings & Ellis 1985) (IV). *The cytotoxic activity* of ECP from selected isolates was tested using two cultured cell lines, Chinook salmon embryo (CHSE) and Epithelioma papillosum cyprini (EPC). The reaction systems were incubated with ECP of aAS isolates at 15°C and cells were examined after 2, 20 and 40 h (IV).

Whole cell lysates of the bacteria were prepared as described by Hitchcock & Brown (1983), but without proteinase K treatment and used in SDS-PAGE and Western blotting analyzes (IV).

SDS-PAGE was carried out as described by Laemmli (1970) (IV. P. Pylkkö unpubl.). The samples were boiled (100°C, 10 min) in sample buffer. The protein bands were visualized by CBB G-250 staining (Sambrook et al. 1989) or were transferred onto nitrocellulose membrane for Western blotting (IV, P.Pylkkö unpubl.). The molecular weight of the purified A-layer protein of AAS has been indicated to be 48.1 kDa (Maurice et al. 1999). A pre-stained marker from 6.8 to 205 kDa (BioRad) was used for estimation of the molecular weight of the proteins (IV).

Autoagglutination and binding of Coomassie brilliant blue The A-layer protein has been observed to be the most abundant cell surface protein in strongly autoagglutinating *A. salmonicida* (Evenberg et al. 1985). Thus, the capability of the isolates to bind (CBB) dye was tested on tryptone soy agar (Oxoid, CM 131) as described by Evenberg et al. (1985) (IV).

Western blotting. Cell lysates obtained by electrophoresis were transferred onto nitrocellulose membrane (Sambrook et al. 1989). The blots were incubated either with plasma from fish or anti-A-layer mouse monoclonal antibody 182 (kindly provided by C. Syvertsen, ALPHARMA Ltd.) For the enzymatic detection of bound antibodies, the membranes were incubated with alkaline phosphatase conjugated anti-mouse Ig antibody (A-2179 Sigma). The membranes were treated with the substrate buffer containing BCIP (5-bromo-4-chloro-3-indonyl phosphate p-toluidine salt) and NBT (nitroblue tetrazolium chloride) (IV, P. Pylkkö unpubl.).

Polymerase chain reaction (PCR) was used to detect virulence factor genes in the genome of aAS strains, including the gene *satA* for glyserophospholipid: cholesterol acyltransferase (GCAT), the gene *aspA* for 70 kDa serine protease and the gene for *vapA*, coding for an additional outer layer (A-layer) in bacteria

(IV). Species-specific PCR was used in paper II for detecting aAS from the DNA extractions from eye, heart and gut tissue. Universal PCR combined with Length Heterogeneity analysis of PCR products (LH-PCR), were performed from heart samples for microbial diversity analysis in paper II. The PCR products were run in 6% denaturating polyacrylamide sequencing gel in a Li-Cor DNA 4200 automated sequencer. The data received from the run was analysed with Quantity One 4.1.0. Software (Bio-Rad Laboratories, CA) based on length standards generated from LH-PCR-product of *Sphingomonas subarcticum* (470bp), *Yersinia ruckeri* (527 bp) and *Lactococcus casei* (553 bp).

Measurements of immune defense function

Methods for measuring immune defense functions in fish are widely utilized and described. However, in most cases they have been developed for species such as rainbow trout (*Onchorhyncus mykiss* L.), cod (*Gadus morphua* L.) or other economically important fish species. The main problem was to find antibodies that are able to recognize charr IgM molecules. Therefore, anti-charr-IgM-rabbit polyclonal antibodies were produced for the study by the Department of Applied Zoology and Veterinary Medicine, University of Kuopio, Finland. In addition, I produced anti-sheep red blood-cells -charr for measuring the activity of the complement system activation via the classical pathway. (III)

Plasma samples Blood was drawn from the dorsal vein into a heparinized syringe and immediately centrifuged. Plasma was then divided to aliquots and immediately frozen in liquid nitrogen. The samples were later stored at –80 °C. (III)

The specific antibody response The specific response towards atypical *Aeromonas salmonicida* was analyzed by double-sandwich, enzyme-linked, immunosorbent assay (ds-ELISA) as described by Thuvander et al. (1987) and Magnadóttir & Guðmundsdóttir (1992) but the microtitre-plate wells were precoated with poly-L-lysine (Graves 1988). Precoated microplates were then coated with 10^{-5} cfu ml^{-1} of sonicated aAS-cells (Sorensen & Brodbeck 1986, Kuen et al. 1993).

Anti-charr IgM-rabbit polyclonal antibodies were produced, since antibodies against Atlantic salmon (*Salmo salar*) produced for the present work by Alpharma and commercially available rainbow trout immunoglobulins (SIGMA) did not cross-react with charr immunoglobulin. The secondary antibody was anti-rabbit IgG peroxidase conjugate (SIGMA, A-9169).

Lysozyme activity in plasma was determined by a turbidometric assay as described by Parry *et al.* (1965). *Micrococcus lysodeikticus* lyophilised cells were used as targets for the lysozyme enzyme. The decline in absorbance at 550 nm was measured over 5 min at 1 min intervals immediately after addition of the bacterial suspension (Multiskan® MCC/340, Labsystems Inc., Finland). One unit of lysozyme activity was expressed as the amount of lysozyme that caused a decrease in absorbance of 0.001 min^{-1} (Lie et al. 1989).

Activity of the complement system via the classical pathway was measured using sensitized sheep's red blood-cells (SRBC) as a substrate (Reid & Porter

1981, Joiner et al. 1984, Yano 1992). Antibodies were produced by immunizing charr by injecting SRBC intarperitoneally. Haemolysis caused by the classical pathway of the complement system was expressed as the percentage of hemolysis, and was calculated as the ratio of observed absorbance of lysed SRBC and the absorbance of buffer at 540 nm multiplied by 100.

Activity of the complement system via the alternative pathway was studied by direct haemolysis of rabbit red blood cells (RaRBC) as described by Yano (1992). Absorbance was read at 414 nm, and the percentage of haemolysis caused by the alternative pathway of the complement system was calculated as (Abs_{414nm} Obs/Abs_{414nm} buffer) x 100.

Total plasma protein concentration was measured by Bradford method (Bradford 1976) using a Bio-Rad protein assay, Bio-Rad Laboratories Ltd with bovine serum albumin as a standard (III, IV).

Vaccination experiments

Intraperitoneal (i.p.) vaccination followed the procedure described by manufacturer AlPharma AS, Norway (III, V). The vaccine included metabolizable oil-adjuvanted, bivalent vaccine (0.2 ml/fish) containing, formalin (1% solution) killed aAS ($3x10^9$ cells/ml) and ASS ($6x10^9$ cells/ml) whole cell bacteria prepared for the study by Alpharma AS, Norway. The same vaccine was used in papers III and V.

Statistical analyses

Fisher's Exact Test and Kruskall-Wallis test were used to determine the statistical significance of concomitant exposure of parasite and bacteria (II). The tests were performed by SPSS for Windows version 9.0 (SPSS Inc, Chicago, IL, USA) (II).

The data in paper III, except the aAS-specific antibody level and the total plasma protein concentration, were not normally distributed. Full factorial 3-way analysis of variance was performed from rank-transformed data to analyze the effects of temperature, vaccination and sampling time (Conover & Iman 1981). A statistically significant difference was considered to exist if $p < 0.05$.

In paper V the influence of vaccination on specific growth rate was tested according to a nested-factorial design (Montgomery 1997). In the model, the effect of temperature was nested within tanks and vaccination was a factorial factor (Montgomery 1997). Within a temperature treatment group, the influence of vaccination was tested by simple contrasts (SYSTAT, 1997).

RESULTS AND DISCUSSIONS

Characterization and virulence factors of atypical *Aeromonas salmonicida*

Heterogeneity is a typical feature of *Aeromonas salmonicida* bacteria (Rintamäki & Valtonen 1991, Rockey et al. 1991, Whitby et al. 1992, Hellberg et al. 1996, Hänninen & Hirvelä-Koski 1997, Austin et al. 1998, O'hIci et al. 2000). The current taxonomy of *Aeromonas salmonicida* includes five subspecies – *A. salmonicida* subspecies *salmonicida* (ASS), subsp. *achromogenes* (aAS), subsp. *smithia,* subsp. *masoucida* (Popoff 1984) and subsp. *pectinolytica* (Pavan 2000). Heterogeneity creates problems in characterization, in understanding putative virulence factors, in reliable and rapid isolation methods during disease outbreaks and in diagnostics based on antigen-antibody complex (among others Adams & Thompson 1990, Olivier 1990, Lund et al. 1991, Rockey et al. 1991, Hellberg et al. 1996, Høie et al. 1997, Austin et al. 1998, O'hIci et al. 2000, Guðmundsdóttir et al. 2003). These problems also concern Finnish aAS isolates (IV, Rintamäki &Valtonen 1991, Hirvelä-Koski et al. 1994, Hänninen & Hirvelä-Koski 1997). Bearing in mind the heterogeneity of aAS isolates, I focused my attention on characterization and on analyzing the virulence factors of the causative agent of aAS infection among charr and grayling in the the Vuoksi and the Kymijoki watercourses.

Hirvelä-Koski et al. (1994) concluded that aAS isolates are difficult to classify based on biochemical analyzes. Thus, pigment production, tested under stringent conditions (at 15°C for 7 days on furunculose agar) has been suggested to be the most suitable phenotyping character for differentiating the major pigment and non-pigment groups producing of aAS (Hänninen & Hirvelä-Koski 1997). However, pigment production of aAS is not generally accepted as a major character for grouping aAS isolates (Popoff 1984). In the present study various methods from classical biochemical analyzes of aAS (I, II, III, IV) to modern molecular tools (I, II, IV) as well as the pigment production (I, II, IV) were utilized for characterization, for grouping and on analyzing aAS isolates.

The causative agent of aAS infection in the Vuoksi watercourse analyzed according to biochemical characteristics as well as to features of its genome (I). The examined Vuoksi isolates originated from diseased charr and grayling (n=21) and from wild fish (n=7). These were compared to 33 aAS isolates from other Finnish and Swedish watercourses, one Icelandic aAS isolate, and also to 3 ASS isolates and the American type culture collection (ATCC) 33659 -type strain (Table 1, I, IV). Pigment production of altogether 66 *A. salmonicida* isolates at 15°C for 7 days on furunculose agar was reported in papers I and IV. It was observed that the Vuoksi watercourse aAS isolates from farmed or from wild fish, were oxidase positive and non-pigment producing throughout the study period from 1988 to 1995 (I, IV). Hänninen & Hirvelä-Koski (1997) described ribopatterns and plasmid profiles of oxidase-positive aAS isolates by cluster analyzes. However, the putative connection between lack of pigment production and virulence of the bacteria is not understood nor studied in the present paper.

MPR genome analyzes were suggested by Hänninen & Hirvelä-Koski (1997) and Sørum et al. (2000) to be applicable in epidemiological studies. Atypical AS isolates in the Vuoksi watercourse present a one MPR genome fragment type (restricted by *Spe*I endonuclease) (I). In addition, one type of plasmid profile, typically with three plasmids (147kb, 42kb and 23kb) was also found (I). The MPR and the plasmid profiles of aAS isolates from the Vuoksi watercourse differ from other Finnish watercourses isolates (I). The profiles were also different from those of reference strains (ATCC 33659, Baltic sea, Icelandic) (I).

The MPR profiles of aAS isolates from goldfish (*Carasius auratus*), silver perch (*Bidyanus bidyanus*) and greenback flounder (*Rhombosolea tapirina*) indicated, that aAS isolates were more connected to the fish farm than to the fish species (Whittington et al. 1995). Similarly, the aAS isolates from the Vuoksi and Kymijoki watercourses were connected to the watercourse not to the fish species (I). However, Hänninen & Hirvelä-Koski (1999) reported a distinguishable MPR genomic group of aAS isolates associated with diseased charr and grayling in Finland.

Sørum et al. (2000) found different plasmid profiles within one fish species. This is not in an agreement with the plasmid profiles found in this work, because those found among Vuoksi and Kymijoki isolates did not differ between charr and grayling (I). Sørum et al. (2000) offered two explanations for epidemiological information based on plasmid profiles of aAS. They suggested that each plasmid group represents an aAS clone spread from one farm to another by fish transfer or through water. They also considered the possibility that the same bacterial clone inhabits different geographical areas and act as an opportunistic pathogen at fish farms. Virulent clones have been described also among other opportunistic fish pathogens such as waterborne fungi, *Saprolegnia* sp. (Bangyeekhun et al. 2003).

The results in paper I indicate that the Vuoksi and Kymijoki watercourses each have one aAS clone present. However, more isolates need to be studied before a firm conclusion about virulent aAS clone can be drawn. The same

applies to MPR and/or plasmid profiles before they can be accepted as tools for epidemiological studies.

Bacterial pathogens secrete extracellular products (ECP) containing enzymatic and toxic activities or have other virulence components located on the cell surface (A-layer) and in the cell wall (see e.g. Janeway et al. 1999). The virulence factors of ASS have been intensively studied. A cell surface A-layer and two secreted toxins, a cytolytic toxin, glycerophospholipid-cholesterol acyltransferase (GCAT) and a toxic 70 kDa serine protease, have been implicated as major factors in the pathogenesis of furunculosis (Sheeran & Smith 1981, Lee & Ellis 1990, Ellis 1997, Garduno et al. 2000). A toxic 70 kDa serine protease is an expression of the *aspA* gene. However, it has been suggested that the exotoxic activities are different in ASS and aAS (Guðmundsdóttir et al. 1990, Guðmundsdóttir 1996, Austin et al.1998, Guðmundsdóttir et al. 2003).

Guðmundsdóttir *et al.* (1990), Guðmundsdóttir (1996), Guðmundsdóttir & Magnadóttir (1997) and Gunnlaugsdóttir & Guðmundsdóttir (1997) have characterized a toxic protease from the ECP of an aAS isolate which is grouped with the type strain (ATCC 33659) (IV). This protease, characterized as a metallocaseinase, AsaP1, has been suggested to play an important role in the pathogenesis of the aAS infection in Atlantic salmon (Guðmundsdóttir 1996). The characters of ECP differ between aAS isolates originating from different geographical locations and different fish species (Guðmundsdóttir 1996, Austin et al. 1998). This feature is apparently related to the heterogeneity of these bacteria. ECP produced by the non-pigment producing aAS isolates from the Vuoksi watercourse did not show gelatinolytic, caseinolytic and/or haemolytic activities (IV). The ECP showed low cytotoxic activity against cultured cells and did not induce mortality in challenge experiments with charr (IV). The results in paper IV indicate that toxic components like ECP secreted by aAS bacterium may not be the major virulence factor in aAS-infection in charr and grayling in the Vuoksi watercourse. However, it also has to be considered that the low cytotoxic activity may be a result of the ECP isolation method at room temperature (20 to 22°C) as was done in paper IV (Stuber et al. 2003). Stuber et al. (2003) suggested *A. salmonicida* to be unable to secrete AexT toxin when grown at temperatures between 22 to 28°C. The AexT have been associated with the virulence of ASS via a type III secretion pathway of Gram negative bacteria by translocation of the toxin directly from bacterial cell into the target cell in a host (Braun et al. 2002, Burr et al. 2003).

In vertebrates, serine proteases perform important functions in blood clotting and the complement system (see Janeway et al. 1999). Interestingly, none of the Vuoksi aAS isolates had the *aspA* gene whereas all the Kymijoki aAS isolates had the gene (I, IV). The aAS outbreaks occur annually among charr and grayling in both the Vuoksi and the Kymijoki watercourses (I, Pylkkö et al. 1993). These issues indicate only a minor role for 70 kDa serine protease among aAS virulence factors. In addition to virulence studies the presence of the *aspA* gene has been suggested by Whitby et al. (1992) to be a criterion for

grouping ASS and aAS isolates. This would be an acceptable and useful tool among Vuoksi and Kymijoki aAS isolates (I, IV).

The A-layer as an important virulence factor is not universally accepted (Olivier 1990, Wiklund et al. 1994). Non-virulent A-layer positive aAS isolates have been reported by Olivier (1990) and Wiklund et al. (1994). The bacterial A-layer is expressed by the *vapA* gene. The A-layer is connected to virulent ASS and aAS (among others Evenberg et al. 1988, Austin & Austin 1993). The examined Finnish aAS isolates possess a *vapA* gene and express the A-layer (IV). However, when the Vuoksi aAS isolates from diseased grayling, charr and roach (*Rutilus rutilus*) were used to challenge charr and grayling, mortality among them was induced only by one aAS isolate originating from diseased grayling (P. Pylkkö unpubl.). Therefore, the results in the present paper do not support the role of the A-layer as a virulence factor. The A-layer might have other features that can be utilized in evoking the immune system of a fish host when new vaccines against aAS are developed (see later).

Atypical *Aeromonas salmonicida* -infection in charr and grayling

Atypical *Aeromonas salmonicida* bacteria is able to infect a variety of wild and cultured fish species, the symptoms varying from mild ulcerative disease to more severe systemic infection (Boomker et al. 1984, Rintamäki & Valtonen 1991, Carlstein 1996, Hellberg et al. 1996, Wiklund & Dalsgaard 1998, Bravo 2000). In the reported infections, the bacterial agents vary because the isolated agent may denote all *Aeromonas salmonicida* subspecies, designated either as "typical" or "atypical". It was illustrated in paper I, that the manifestation of aAS disease clearly differs between charr and grayling even at a single fish farm on the Vuoksi watercourse. However, aAS isolates at the farm show homogeneity in genome since 1988 (IV). No other fish species are affected at the farm even though landlocked salmon (*Salmo salar* m. *sebago*) and trout (*Salmo trutta* m. *lacustris*) are cultivated. However, it has been reported that elsewhere in Finland salmon, and especially sea trout and brown trout, stocks have suffered aAS infection (Rintamäki & Valtonen 1991). All age groups (e.g. brood fish and fingerlings) of both charr and grayling are affected annually and mortality rate can be high during outbreaks (I). Carlstein (1996) have described natural achromogenic aAS infection in grayling fingerlings. Bravo (2000) described aAS infection in juvenile and adult Atlantic salmon (in average weight 0.9 to 4 kg).

In non-salmonids, aAS infection often creates a skin ulceration without subsequent septicaemia. Carp erythodermatitis, goldfish ulcer disease and ulcer disease of flounder in the Baltic and Northern Sea are the best described diseases of this sort. In histopathological descriptions mononuclear inflammatory cells around the ulcers are often described (for references see Wiklund and Dalsgaard 1998). However, a systemic aAS infection in non-salmonids has also been described (among others Guðmundsdóttir et al. 2002, Lund et al. 2002).

The pathology of aAS infection is not so well described in salmonids. Rintamäki and Valtonen (1991) stated that shallow lateral ulcers on the skin were most typical in non-pigment producing aAS infection, and often no other macroscopic changes were seen. The gross signs in furunculosis and in pigment-producing atypical AS infection were similar: fin base haemorrhages and erosions and sometimes small furuncles were found (Rintamäki and Valtonen 1991). Carlstein (1996) described natural non-pigment producing aAS infection in grayling fingerlings which had skin haemorrhages and ulcerations. Boomker et al. (1984) described skin ulcerations that varied from small vesicles to large shallow ulcers in rainbow trout infected by an achromogenic *Aeromonas salmonicida* that shared characteristics with all three subspecies: *salmonicida, masoucida* and *achromogenes*. Bacterial colonies were present only in the sections of skin. Slight enlargement of spleen and kidney and discoloration of liver were observed. Cellular infiltration and considerable tissue destruction were seen in ulcerations in histological studies. The spleen and liver were congested and in the kidney slight vasculitis and degeneration of tubular epithelium were encountered. The findings described by Boomker et al. (1984) are similar to the pathological changes reported in grayling (I).

During natural aAS outbreaks at the farm located in the Vuoksi watercourse, grayling were observed to develop deep skin lesions extending to muscular tissue and haemorrhagia in the intestine, often accompanied by anus relapse (I). Infected charr, in contrast, had only slight if any haemorrhages in the internal organs (I). The aAS bacteria from charr and grayling were isolated on blood agar mainly from kidney or skin lesion, but also from intestine (I). Atypical *A. salmonicida* bacteria were detected from lenses and intestine of grayling in 30% of the cases by PCR (II). In experimentally challenged grayling, tissue damage in the epidermis was severe necrosis extending to deep muscular fibers (I). No cellular defense, such as macrophages around lesions, was observed (I). In charr the disease manifested more slowly than in grayling, and severe necrotic tissue was rare (I). In histopathology, thickening of the skin mucus layer and gram negative bacterial invasion of scale pockets was seen in charr epidermis during the first few days after exposure. In the outer most layer of epidermis, bacterial colonies surrounded by some macrophages and lymphocytic cell types were observed (I). These findings indicate that in charr immune defense functions have been activated during the bacterial invasion of tissue. Even at a later stage of infection no histopathological changes in pancreas, liver, kidney or intestine could be detected in either charr or grayling. However, in the hearts of grayling sampled during the last days of the experiment, gram negative bacterial colonies could be seen either in the pericardium or the ventriculum (I). The presence of aAS bacteria in grayling's heart tissue was also confirmed in paper II.

The intact mucus layer is penetrated by aAS (Svendsen & Bøgwald 1997). This is in an agreement with the findings in paper II in which the role of parasitic invasion in enhancement of aAS infection was studied experimentally. It was observed that *Diplostomum spathaceum* significantly enhances invasion of aAS to grayling’s heart tissue in two weeks time post exposure (II). However,

aAS bacteria were also found in those fish which did not receive the parasitic exposure. Atypical AS was also detected in control fish all through the experiment but the incidence of aAS was lower than that in the concomitant bacterial and parasitic treatment (II).

Carlstein (1996) reported challenged grayling to develop cloudy and partly eroded cornea. Buchman & Bresciani (1997) and Karvonen et al. (2004a) reported *D. spathaceum* infection to cause cataracts in previously healthy eyes of rainbow trout, but severe damage and eye rupture has not been reported. The most severe eye damage such as eye rupture and nuclear extrusion was observed in grayling concomitantly exposed to *D. spataceum* and aAS or *D. spathaceum* alone (II). This type of severe eye damage has been previously reported in wolf-fish (*Anarchicas* spp.) after a rapid decrease in water salinity (Bjerkås et al. 1998).

The present study appears to be the first description of gross signs of aAS infection, with histological changes in internal organs of charr and grayling (I). The presence of aAS bacteria was detected in heart of grayling both by traditional Gram staining in histological tissue sections and by molecular tools (I, II). This work is one of the first known experimentally proven reports about the parasitic enhanced bacterial invasion in grayling tissue.

Evoking immune defense function in charr

The variety of virulence factors and mechanisms of aAS and ASS bacteria, indicates that they possess many different means to evade the immune system of fish (among others Evenberg et al. 1985, Garrote et al. 1992, Austin & Austin 1993, Merino et al. 1994, Guðmundsdóttir 1996, Austin et al.1998, Guðmundsdóttir et al. 2003). Protection by the host is based on inactivation of virulence components of pathogen.

Lysozyme in vertebrates is one of the enzymes in the first-line of defense against invading pathogens (see e.g. Roitt 1994, Janeway et al. 1999). The defense response is directed against the peptidoglycan layer, especially in the cell wall of Gram-positive bacteria, that is suspected to be weakened by the alternative pathway of the complement system before lysozyme attack (see e.g. Janeway et al. 1999). In paper III it was observed that a significant increase in the plasma lysozyme activity in vaccinated charr, when compared to unvaccinated charr, indicates the activation of the nonspecific immune defense functions (see e.g. Roitt 1994, Janeway et al. 1999). Similar findings are reported in Atlantic salmon after injection of M-glycan (Engstad et al. 1992). This increased lysozyme activity is possibly induced by emulsification oil used in vaccine.

The effector functions of the complement system can be activated through three pathways: classical (III), alternative (III) and lectin binding. The complement consists of some 30 proteins circulating in the blood plasma (designated by the letter C with an extension number). Most of these are inactive until either they are cleaved by a "foreign" protease such as 70 kDa

serine protease (IV) which, in turn, converts complement system proteins into a protease. In mammal blood plasma the principal serine protease inhibitor, α1-antiproteinase, represents 18% of total plasma volume (see e.g . Harfenist & Murray 1993). For example, the binding of an antibody to its antigen often triggers the complement system through the classical pathway. The complement system can also be triggered without antigen-antibody complexes - alternative pathway (see e.g. Roitt 1994, Janeway et al. 1999)

Cell lysis is only one function of the complement system. The complement system acts in several ways to mobilize defense mechanisms; for example, opsonization by C3b targets foreign particles for phagosytosis, chemotaxis by C5a attracts phagocytic cells to the site of damage. This is aided by the increased permeability of the capillary beds mediated by C3a and C5a. The early complement components are also important for solubilizing antigen-antibody complexes assisting in their catabolism and elimination from the body (see e.g. Roitt 1994, Janeway et al. 1999).

Vaccination did not stimulate the haemolytic activity of either the classical or alternative pathways of the complement system (III). The hemolytic activity of the both pathways changed from April to August regardless the vaccination status of charr. The haemolytic activity of the classical and alternative pathways of the complement system was low in April (4.5 and 1.5%, respectively), but increased later in the summer (III). The complement system via the classical pathway reached 50% haemolysis in August while the alternative complement pathway displayed the highest activity in June (over 50% haemolysis) and a lower haemolytic activity (10%) in July and August. Rintamäki & Valtonen (1991) have reported aAS outbreaks in Finland to occur at water temperatures higher than 15°C, which indicates an increase in the number of aAS or related *Aeromonas* bacteria in incoming water in July and August. This assumed increase of the aAS cells in inflow water correlates to the activity increase of the classical pathway of the complement system in August. The activation of the classical complement system requires specific recognition of invading pathogen (see Janeway et al. 1999). Possibly, the utilization of the complement system via classical pathway is an efficient defense mechanisms for charr when the number of pathogen cells increases.

I reported that pathological tissue changes and lethality of non-pigment producing aAS infection differ between charr and grayling at the farm in the Vuoksi watercourse (I). This infection is also different than an infection caused by a pigment producing aAS-infection in Atlantic salmon (Guðmundsdóttir 1997). I also reported that in the Vuoksi watercourse aAS bacteria are genetically homogeneous (I). These results indicate that immune response against aAS bacteria differs between fish species.

Putative preventive means against aAS bacterial infection – vaccination and control of parasite pressure

An immune response against an invading pathogen involves opsonization of pathogens by the complement system resulting to phagocytosis by macrophages or B and/or T cell actions followed by a humoral response (see e.g. Roitt 1994, Janeway et al. 1999). In vertebrates a typical secondary response resulting from previous immunization (natural or induced by vaccination) follows the same pattern as the primary response but with more rapid kinetics and a stronger response (see e.g. Roitt 1994, Janeway et al. 1999). This basic knowledge has been utilized when preventive means against fish diseases are developed. The main results in paper III indicate that aAS-specific immune responses of charr can be evoked with oil-based vaccine containing ASS and aAS bacterins.

Atypical AS infection is an increasing problem in commercial halibut farming in Norway (Ingilæ et al. 2000) and in Atlantic salmon farming in Chile (Bravo 2000). The problems with aAS infection seem to arise when either new fish species are taken to commercial farming or the fish farming industry is spreading to new locations. Increasing problems with bacterial diseases usually create a need to find an effective vaccination. Vaccine developments against aAS infection in Atlantic salmon and Atlantic halibut (*Hippoglossus hippoglossus*) and spotted wolffish (*Anarchichas minor*) have been conducted at least in Iceland and Norway (Guðmundsdóttir et al. 1997, Ingilæ et al. 2000, Lund et al. 2002). Injection of the AsaP1 toxoid in Atlantic salmon induced protection against infection (Guðmundsdóttir & Magnadóttir 1997).

In Iceland the autogenous oil-based vaccine, IBOO, gives good protection against aAS. In challenged Atlantic salmon 12 weeks after vaccination all fish were protected (relative percentage of survival, RPS, value 100) (Guðmundsdóttir et al. 1997). Gunnlaugsdóttir & Guðmundsdóttir (1997) reported a protection against aAS bacteria also gained by Atlantic salmon vaccinated with commercial furunculosis vaccine. However, such preventive means have not been used against aAS disease in Finnish charr and grayling farming.

I have observed good protection in charr vaccinated i.p. with 4 oil-based, autogenous (containing formalin killed aAS isolates from the Vuoksi watercourse), and monovalent (containing one aAS isolate as an antigen) experimental vaccines (prepared for the experiment by Alpharma AS, Norway). The charr (2-year old) were cross bath challenged with 4 Vuoksi watercourse aAS isolates. RPS value 88 was achieved with one autogenous aAS vaccine and RPS value 60 with the two commercial furunculosis vaccines (P. Pylkkö unpubl.). Similarly, vaccinated and challenged grayling were poorly protected by all the vaccines (RPS values less than 40, unpublished data). No correlation between specific antibody level and protection was observed (P. Pylkkö unpubl.). These results suggest the immune defense functions against aAS bacteria differ between charr and grayling in the Vuoksi watercourse.

A-layer proteins from typical and atypical *A. salmonicida* were observed to evoke a specific immune response in Atlantic salmon and rainbow trout (Bjørnsdottir et al. 1992, Lund et al. 1991, Hastings & Ellis 1990). Maurice et al (1999) have produced recombinant A-layer protein from acromogenic aAS isolated from diseased goldfish. The recombinant protein is fused to cellulose binding domain (Maurice et al. 2003). The cellulose recombinant protein successfully evoked the immunological memory of goldfish when challenged with aAS bacteria. Thus, no correlation was observed between specific antibody level and intensity of ulceration (Maurice et al. 2003). In preliminary studies, charr were i.p. vaccinated with experimental aAS vaccine (produced by Alpharma, Norway) and plasma collected several weeks from vaccination (data not shown). The A-layer was recognized in Western blotting of aAS whole cell lysate (see paper IV) probed with plasma from vaccinated charr, whereas when probed with plasma from non vaccinated charr no A-layer recognition occurred (P.Pylkkö unpubl.). I suggest that the role of A-layer from Finnish aAS isolates as an antigen evoking immune response of should be studied further.

Both enhancement of fish growth (Buchman et al. 1997) or reduced growth (Midtlyng et al. 1996, Sørum & Damsgard 2004) have been reported after i.p. vaccination. Such discrepancies in growth rate in fish after i.p. vaccination may result from fish species, time scale of the follow up, or methods used to evaluate growth. The effect of i.p. vaccination on growth of charr was studied in paper V. Compensation of growth suppression in charr after vaccination indicates that modern oil adjuvant vaccines have no ultimate negative effect on growth (V).

This compensatory growth in suitable conditions might indicate that passively induced activation (e.g. vaccination) of immune defense functions demands energy. After sufficient activation of the immune defense system is reached the fish is again able to put energy into growing. This suppression of growth is therefore a sign of an activated immune system after vaccination (III, IV) and not a sign of vaccine induced poor growth.

Vaccinations with good efficacy against furunculosis have improved the economics of salmonid fish farming in general and reduced usage of antimicrobial medication. The common interest in fish meat production is ecologically sustainable fish production and good quality of fish. Good fish meat quality for consumers is guaranteed by rearing healthy fish with minimal need for antimicrobial drugs. In conservation of endangered fish stocks it is critically important that the fish stocked are healthy, fit to survive and are able to avoid predators efficiently after stocking. Vaccinations with good protection against bacterial diseases could improve the farming of endangered fish species and help conservation of the genetic diversity of fish stocks.

Rearing conditions at fish farms are important in introducing pathogens to fish hosts. This topic was studied in paper II. Cultivation of fish at high densities at fish farms attracts a variety of parasites, most of which are protozoans or other ectoparasites (e.g. Rintamäki-Kinnunen & Valtonen 1996, 1997; Hakalahti & Valtonen 2003). *Diplostomum spathaceum* is a common parasite of freshwater and brackish water fishes (Valtonen & Gibson 1997; Valtonen et al. 1997, 2003) and utilizes three hosts in completing its life cycle: fish-eating

birds, aquatic snails and fish. Occurrence of diplostomids at fish farms is dependent on several factors, especially on the infrastructure of the farm and on its water intake systems. Some parasites (*Trichodina* spp., *Riboscyphidia sp., Apiosoma sp.*) are tolerated at fish farms to some extent (Rintamäki-Kinnunen & Valtonen 1997). *D. spathaceum* larvae which migrate through fish skin and tissues, can cause outbreaks at fish farms (e.g. Crowden & Broom 1980; Lafferty & Morris 1996). Karvonen et al. (2004 b) have reported that fish previously exposed to a low level (as used in paper II) of *D. spathaceum*, cercaria penetrating fish epidermis decrease by even 80 to 90% in further parasite infections. In nature fish can recognize the parasites and avoid further infections (Karvonen et al. 2004). Therefore, at the fish farms the immune defense functions of fish have to be fit to respond to random pathogen load that is introduced through inflow water. The environment can, and even should, never be sterile but would it be necessary to regulate parasite populations at fish farms? The optimal level of skin-penetrating parasites at high density fish populations may help prevent the problem also caused by bacterial fish diseases.

CONCLUSIONS

– Homogeneity in macrorestricted genome and plasmid profiles indicated narrow genetic variation in atypical *Aeromonas salmonicida* strains isolated from the Vuoksi and the Kymijoki watercourse. Therefore, preventive measures against aAS –infection can potentially be developed. Possibly one autogenous bacterial strain included in a vaccine is sufficient to evoke immune defense responses against aAS infection in Arctic charr (*Salvelinus alpinus*) and European grayling (*Thymallus thymallus*). However, the MRP genome and plasmid profiles need to be studied more in order to be put to use in epidemiological studies.

– The detection method of aAS latent carrier fish by combination of bacterial cultivation and PCR techniques was successfully carried out and offers a potential new method for fish disease diagnostics. A heart as an organ for aAS isolation among latent carrier salmonids could be added to traditional bacterial isolation from kidney tissue of fish.

– Symptoms and lethality of the aAS disease vary greatly even between charr and grayling. The reason for this variation could not be properly explained in this study. Do the symptoms vary because of the different immune defense response between the two species? Therefore, I suggest that activation of the immune defense functions of these two fish species needs to be studied in more detail.

– The studies of fish complement system activation and functions in the inactivation of pathogens should be continued. The factors in the innate immune system, especially activation of the complement system, would be most beneficial in understanding parasite-bacteria interactions in fish hosts. The lectin pathway during a parasite infection in fish may have an important role as a defense mechanism in fish. Studies in this area should be conducted among farmed Arctic charr and European grayling.

– Infectious diseases both bacterial and parasitic among farmed fish should not be ignored in aquaculture management. Both pathogen types cause severe damage to the farmed Arctic charr and European grayling such as high mortality and eye rupture. These types of damage narrow the genetic diversity and may reduce the survival of fish stocks. However, an optimal level of skin penetrating parasites in farmed fish might activate immune responses against all pathogenic exposures. This possibility needs to be studied further.

Acknowledgements

I would like to express my warmest gratitude to my supervisor and mentor Professor E. Tellervo Valtonen. Her encouragement has been the key to unlock the doors to the world of scientific thinking and writing. She is exceptionally talented in asking "in your own words, please, just say it!" without those questions this paper would have been never written. I am also thankful to my another supervisor Professor Sinikka Pelkonen for providing me essential financial support for the work and excellent laboratory facilities and equipment. While working with you I have learned a lot on many aspects of life and science.

I am especially grateful for the reviewers of this work Dr. Bjarnheidur Guðmundsdóttir and Professor Karl Buchmann for valuable comments. These comments have greatly improved and clarified the text. I also greatly appreciate Professor Roger Jones for checking the English of this work.

I am thankful to Professor Asko Mäki-Tanila and Dr. Teppo Rekilä at MTT, Agrifood Research Finland, for kindly providing me this opportunity to finalize the PhD thesis. You have shown a broadminded attitude towards me since my present work at Kannus with mammals greatly differs from the issues involved in this thesis.

The Finnish Game and Fisheries Research Institute, Saimaa and Laukaa Aquaculture Stations have greatly contributed the experimental work. The skilful staff at the stations are to be thanked for excellent maintenance of the experimental fish and facilities. I also am grateful to Maria Kananen, Riitta Liimatainen and Tiina Kortelainen for skilful laboratory assistance.

I would also like to thank my co-authors, Tapani Lyytikäinen, Jari Madetoja, Tarja Pohjanvirta, Ossi Ritola, Lotta-Riina Suomalainen and Marja Tiirola. It has been a pleasure to get to know you.

The contribution of Christian Syvertsen and Rune Wiulsrød from Alpharma Inc., Norway, has been essential in providing the experimental vaccines and in consulting in methodology in vaccination procedures with oil-based vaccines. I would also like to thank my colleagues and friends Mikael Carlstein, Jere Lindén and Pia Vennerström for sharing the mutual interest of fish well being.

I am thankful to my fellow workers at Kannus for making an everyday work to be an enjoyable event.

I am also thankful to Jorma Piironen who gave the very first idea about connecting microbiology and fishes when he pointed out the problems with aAS outbreaks among farmed Arctic charr and European grayling.

I owe my dearest friends a debt of gratitude for being with me all through these years. Thank you, Marjatta Huittinen, Jyrki Jauhianen, Nita Nenonen, Minna Rahkola-Sorsa, Riitta Seppinen, Jyrki Suikkanen, Markku Vaajala, Heljä Väisänen. It has been "a long and winding road". I would have not made it without your never-ending encouragement and the laughs we shared. Hopefully, I will be there for you all in the days to come.

I also express my warmest gratitude my parents Irja and Pentti Huotari and my parents-in-law Aini and Aarne Pylkkö for giving their helping hand whenever needed. My brother Juha Huotari and his wife Ritva and their lovely daughters Heini, Soile and Maija have been fun to be with and always offered me a place to stay at their home in Jyväskylä. I am also thankful to my brothers-in-law and their families Jukka, Eija and Mikko Pylkkö and Jorma and Pirkko Pylkkö for their support and kindness.

My husband Harri and children Henri and Olga have been the most patient with a week-end-mother and -wife, occasionally visiting home. You have never lost your faith in my doings and never given up your support. I love you.

Financial support was obtained from the Nordic Council of Ministers and the Board of the Environmental and Natural Resources of Academy of Finland.

YHTEENVETO (Résumé in Finnish)

Epätyyppinen Aeromonas salmonicida -bakteeritartunta uhkana harjuksen (Thymallus thymallus L.) ja nieriän (Salvelinus alpinus L.) laitoskasvatukselle ja mahdollisia keinoja tartunnan ennaltaehkäisyyn

Tämä työ on yhteenveto viljellyn harjuksen (*Thymallus thymallus* L.) ja nieriän (*Salvelinus alpinus* L.) vakavaan sairastumiseen johtavasta bakteeritartunnasta, jonka aiheuttaa epätyyppinen *Aeromonas salmonicida* -bakteeri (aAS). Työtä varten tutkin useiden suomalaisten aAS -kantojen (66 kpl) perimää ja niiden taudinaiheutuskykyyn liittyviä ominaisuuksia. Tutkittuja aAS -kantoja vertasin 1 islantilaiseen, 11 ruotsalaiseen aAS -kantaan ja 3 *Aeromonas salmonicida* subsp. *salmonicida* -kantaan ja *A. salmonicida* subsp. *achromogenes* tyyppikantaan (ATCC 33659). Tutkin myös kokeellisesti aAS -bakteerille altistetuissa harjuksissa ja nieriöissä syntyneitä kudosvaurioita, joita vertasin luonnollisissa tartunnoissa todettuihin aAS -bakteerin aiheuttamiin viljelykalojen kudosvaurioihin. Lisäksi selvitin kahdeksan vuoden ajalta yhden Vuoksen vesistön alueella sijaitsevan kalaviljelylaitoksen diagnosoidut aAS -tartunnat, niihin kuolleet kalalajit, kalojen määrän ja tartunnan hoitoon annetut antibioottilääkinnät. Työssäni tutkin myös nieriän immuunipuolustuksen aktivoitumista rokotuksen jälkeen (mm. anti-aAS-spesifisiä vasta-ainetaso, plasman lysotsyymiaktiivisuus) ja rokotuksen vaikutusta nieriän kasvuun. Selvitin kokeellisesti makeassa vedessä yleisen *Diplostomum spathaceum* -kalaloisen ja aAS -bakteerin vuorovaikusta aAS -tartunnan puhkeamisessa. Selvitin myös bakteerien rikastusviljelyn ja geenimonistusmenetelmän (PCR) yhdistelmän käyttökelpoisuutta bakteerien tunnistamiseen kalan kudoksista (sydän, suoli ja mykiö). Koekaloina käytin eri-ikäisiä Vuoksen, Kymijoen ja Inarin vesistöjen harjuksia ja nieriöitä. Vuoksen ja Kymijoen vesistöalueiden sisällä aAS -kannat olivat perimältään (genomi- ja plasmidiprofiili) samanlaiset. Mutta Vuoksen, Kymijoen ja muiden vesistöalueiden aAS -kantojen perimät erosivat toisistaan. Yhtä selkeää aAS - bakteerin taudinaiheutuskykyyn vaikuttavaa ominaisuutta en tässä työssä löytänyt. Kokeellinen aAS -bakteerialtistus aiheutti viimeistään 15 vrk altistuksesta kuolemaan johtavan yleistulehduksen harjukselle ja nieriälle. Harjukselle muodostui nopeammin ihon pinnalta (epidermis) lihaskudokseen ulottuvat vauriot kuin nieriälle, jonka ihomuutokset rajoittuivat usein epidermiksen alueelle. Harjuksen sydänkudoksessa näkyi bakteerisoluja ja sydänlihas tuhoutui näiltä alueita. Kalanviljelylaitokselta koottujen tietojen perusteella harjus ja nieriä osoittautuivat alttiiksi aAS -tartunnalle. Harjus ja nieriä olivat ainoat kalalajit, joista eristettiin aAS -bakteeria kahdeksan vuoden aikana vaikka samalla laitoksella kasvatettiin samanaikaisesti mm. järvilohta (*Salmo salar* m. *sebago*) ja järvitaimenta (*Salmo trutta* m. *lacustris*). Rokotus nosti merkittävästi nieriän plasman anti-aAS-spesifisten vasta-aineiden tasoa ja tehosti merkittävästi lysotsyymi-entsyymin aktiivisuutta. Rokote ei alentanut nieriän kasvua pysyvästi vaan 52 päivää rokottamisen jälkeen rokotetut kasvoivat nopeammin kuin rokottamattomat nieriät. Harjukseen, ihon läpi,

tunkeutuneet *D. spathaceum* -loisen toukat lisäsivät sydänkudoksesta PCR -menetelmällä tunnistettujen aAS bakteerisolujen määrää. Yhdistetyn bakteeririkastusviljelyn ja PCR -menetelmän avulla kyettiin tunnistamaan 17 aAS -bakteerisolua 100 mg:ssa tutkittua kudosta. AAS -bakteeri tunnistettiin tehokkaimmin oireettomien kantajakalojen sydänkudoksesta. *D. spathaceum* -loinen aiheutti harjukselle tulehduksen mykiöön, mistä seurasi silmän puhkeaminen ja mykiön menetys. Lois- ja bakteeritaudit haittaavat kalanviljelyä ja niitä ei voida eikä pidäkään kokonaan poistaa mutta niiden halittu säätely on kalojen laitoskasvatuksen onnistumiselle ja kalojen hyvinvoinnille ensiarvoista. Loisten ja bakteerien vuorovaikutus tautien puhkeamisessa vaatii vielä lisätutkimuksia mutta näiden vuorovaikutusten ymmärtäminen voi lisätä tehokkaita ennaltaehkäisykeinoja rokotusten rinnalle tulevaisuuden kalanviljelyyn.

REFERENCES

Adams, A. & Thompson, K. 1990. Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of Aeromonas salmonicida in fish tissue. Journal of Aquatic Animal Health 2: 281-288.

Austin, B. & Austin, D. A. 1993. Aeromonadaceae representatives (Aeromonas salmonicida). In: Laird, L.M (ed.), Bacterial Fish Pathogens: Diseases in Farmed and Wild Fish 2nd edn, p., 86–170. Ellis Horwood, London.

Austin, B., Austin, D. A., Dalsgaard, I., Guðmundsdóttir, B. K., Høie, S., Thornton, J. M., Larsen, J. L., O´Hici, B. & Powell, R. 1998. Characterisation of atypical Aeromonas salmonicida by different methods. Systematic and Applied Microbiology 21: 50–64.

Bangyeekhun, E., Pylkkö, P., Vennerström, P., Kuronen, H. & Cerenius, L. 2003. Prevalence of a single fish-pathogenic Saprolegnia sp. clone in Finland and Sweden. Diseases of Aquatic Organisms 53: 47-53.

Bjerkås, E., Bjerkås, I. & Moksness E. 1998. An outbreak of cataract with lens rupture and nuclear extrusion in wolf-fish (Anarhicas spp.). Veterinary Ophtalmology 1(1): 9-15.

Bjørnsdottir, R., Eggset, G., Nilsen, R. & Jørgensen, T. Ø. 1992. The A-layer protein of Aeromonas salmomonicida: further characterization and a new isolation procedure. Journal of Fish Diseases 15(2): 105-118.

Boomker, J., Henton, M. M., Naudé, T. W. & Huchzermeyer, F.W. 1984. Furunculosis in rainbow trout (Salmo gairdneri) raised in sea water. Onderstepoort Journal of Veterinary Research 51: 91-94.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Bravo, S. 2000. Occurence of atypical furunculosis in Chile. Bulletin of the European Association of Fish Pathologists 20: 209-211.

Braun, M., Stuber, K., Schlatter, Y., Wahli, T., Kuhnert, P. & Frey, J. 2002. Characterization of an ADP-ribosyltransferase toxin (AexT) from Aeromonas salmonicida subsp. salmonicida. Journal of Bacteriology 7 (184): 1851-1858.

Buchmann, K., Dalsgaard, I., Nielsen, M. E., Pedersen, K., Uldal, A., Garcia, J. A. & Larsen, J. L. 1997. Vaccination improves survival of Baltic salmon (Salmo salar) smolts in delayed release sea ranching (net-pen period). Aquaculture 156: 339-351.

Buchmann, K. & Bresciani, J. 1997. Parasitic infections in pond-reared rainbow trout Oncorhynchus mykiss in Denmark. Diseases of Aquatic Organisms 28(2): 125-138.

Burr, S. E., Wahli, T., Segner, H., Pugovkin, D. & Frey, J. 2003. Association of type III secretion genes with virulence of Aeromonas salmonicida subsp. Salmonicida. Diseases of Aquatic Organisms 57: 167-171.

Carlstein, M. 1996. Feeding, growth and survival of European grayling in culture and after stocking. PhD dissertation Acta Universitatis

Agriculturae Sueciae, Agraria 7, Swedish University of Agricultural Sciences, Umeå, Sweden.

Conover, W. J. & Iman, R. L. 1981. Rank transformation as a bridge between parametric and nonparametric statistics. The Am Statistn 35: 124-129.

Crowden, A. E. & Broom, D. M. 1980. Effects of the eyefluke, Diplostomum spathaceum, on the behaviour of dace (Leuciscus leuciscus). Animal Behavior 28: 287-294.

Ellis, A. E. 1997. The extracellular toxins of Aeromonas salmonicida subsp. salmonicida. In: Bernoth, E-M., Ellis, A. E., Midtlyng, P. J., Olivier, G. & Smith, P. (eds.), Furunculosis. Multidisciplinary fish Diseases research, p. 248–268. Academic Press.London.

Evenberg, D., Versluis, R. & Lugtenberg, B. 1985. Biochemical and immunological characterisation of the cell surface of the fish pathogenic bacterium Aeromonas salmonicida. Biochimica et Biophysica Acta 815: 233–244.

Evenberg, D., De Graaf, P., Lugtenberg, B. & Muiswinkel, W. B. 1988. Vaccine induced protective immunity against Aeromonas salmonicida tested in experimental carp erythrodermatitis. Journal of Fish Diseases. 11: 337–350.

Engstad, R. E., Robertsen, B. & Frivold, E. 1992. Yeast glucan induces increase in lysozyme and complement mediated haemolytic activity in Atlantic salmon blood. Fish and Shellfish Immunology 2: 287-297.

Fry, F. E. J. 1971. The effect of environmental factors on the physiology of fish. In: Hoar, W.S. & Randall, D.J. (eds), Fish Physiology. Vol. VI: 1-98. Academic Press, London.

Garrote, A., Bonet, R., Merino, S., Simon-Pujol, M. D. & Congregado, F. 1992. Occurrence of capsule in A. salmonicida. FEMS Microbiology Letters 95: 127-132.

Garduno, R. A., Moore, A. R., Olivier, G., Lizama, A. L., Garduno, E. & Kay, W. W. 2000. Host cell invasion and intracellular residence by Aeromonas salmonicida: role of the S-layer. Canadian Journal of Microbiology 46: 660-668.

Graves, H. C. B. 1988. The effect of surface charge on non-specific binding of rabbitimmunoglobulin G in solid-phase immunoassay. Journal of Immunological Methods 111: 157-166.

Griffin, P. J., Snieszko, S. F. & Friddle, S. B. 1953. A more comprehensive description of Bacterium salmonicida. Transactions of the American Fisheries Society 82: 129-138.

Guðmundsdóttir, B.K., Hastings, T.S. & Ellis, A.E. 1990. Isolation of a new toxic protease from a strain of Aeromonas salmonicida subspecies achromogenes. Diseases of Aquatic Organisms 9: 199–208.

Guðmundsdóttir, B. K. 1996. Comparison of extracellular proteases produced by Aeromonas salmonicida strains, isolated from various fish species. Journal of Applied Bacteriology 80: 105–113.

Guðmundsdóttir, B. K. 1997. Aeromonas salmonicida subsp achromogenes – A study of extracellular virulence factors and immunity in Atlantic salmon

(Salmo salar L.) PhD dissertation Institute for Experimental Pathology, Keldur, Faculty of Medicine, University of Iceland, Iceland.

Guðmundsdóttir, B. K. & Magnadóttir, B. 1997. Protection of Atlantic salmon (Salmo salar L.) against an experimental infection of Aeromonas salmonicida ssp achromogenes. Fish and Shellfish Immunology 7: 55-69.

Guðmundsdóttir, B. K., Jónsdóttir, H., Steinthórsdóttir,V., Magnadóttir, B. & Guðmundsdóttir, S. 1997. Survival and humoral antibody response of Atlantic salmon (Salmo salar L.) vaccinated against Aeromonas salmonicida ssp achromogenes. Journal of Fish Diseases 20: 351-360.

Guðmundsdóttir, B. K., Hvanndal, Í., Björnsdóttir, B. & Wagner, U. 2003. Analysis of exotoxins produced by atypical and typical Aeromonas salmonicida, by enzymatic and serological methods. Journal of Fish Diseases 26: 15-29.

Guðmundsdóttir, S., Lange, S., Magnadóttir, B. & Guðmundsdóttir, B. K. 2003. Protection against atypical furunculosis in Atlantic halibut, Hippoglossus hippoglossus (L.); comparison of a commercial furunculosis vaccine and an autogenous vaccine. Journal of Fish Diseases 26: 331-338.

Gunnlaugsdóttir B. & Guðmundsdóttir B. K. 1997. Pathogenicity of atypical Aeromonas salmonicida in Atlantic salmon compared with protease production. Journal of Applied Microbiology 83: 542–551.

Hakalahti, T. & Valtonen, E.T. 2003. Population structure and recruitment of the ectoparasite Argulus coregoni Thorell (Crustacea: Branchiura) on a fish farm. parasitology 127: 79-85.

Harfenist. E. J. & Murray, R. K. 1993. Plasma proteins, immunoglobulins, & blood coagulation. In Murray, R. B., Granner, D. K., Mayes, P. A. & Rodwell, V. W. (eds), Harper's Biochemistry: 665-688. Prentice-Hall International Inc., New Jersey, USA.

Hastings,T.S. & Ellis, A. E. 1985. Differences in production of haemolytic and proteolytic activities by various isolates of Aeromonas salmonicida. In: Ellis, A. E. (ed). Fish and Shellfish Pathology p. 69–77. Academic Press, London.

Hastings,T.S. & Ellis, A.E. 1990. Detection of antibodies induced in rainbow trout by different Aeromonas salmonicida vaccine preparations. Journal of Aquatic Animal Health 2: 135-140.

Hellberg, H., Moksness, E., & Høie, S. 1996. Infection with atypical Aeromonas salmonicida in farmed common wolfish, Anarchias lupus L. Short communication. Journal of Fish Diseases 19: 329-332.

Heussen, C. & Dowdle, E. B. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerized substrates. Analytical Biochemistry 102: 196–202.

Hirvelä-Koski, V., Koski, P. & Niiranen, H. 1994. Biochemical properties and drug resistance of Aeromonas salmonicida in Finland. Diseases of Aquatic Organisms 20: 191–196.

Hitchcock, P. J. & Brown, T. M. 1983. Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver stained polyacrylamide gels. Journal of Bacteriology 154: 269–277.

Hueck, C. J. 1998. Type III protein secretion system in bacterial pathogens of animals and plants. Microbiology and Molecular Biology Reviews 62(2): 379-433.

Høie, S., Heum, M. & Thoresen, O. F. 1997. Evaluation of polymerase chain reaction-based assay for the detection of Aeromonas salmonicida ss salmonicida in Atlantic salmon Salmo salar. Diseases of Aquatic Organism 30: 27-35.

Hänninen, M. L. & Hirvelä-Koski, V. 1997. Molecular and phenotypic methods for the characterisation of atypical Aeromonas salmonicida. Veterinary Microbiology 56: 147–158.

Hänninen, M. L. & Hirvelä-Koski, V. 1999. Genetic diversity of atypical Aeromonas salmonicida studied by pulsed-field gel electrophoresis. Epidemiology and Infection 123: 299–307.

Ingilæ, M., Arnesen, J. A., Lund, V. & Eggset, G. 2000. Vaccination of Atlantic halibut Hippoglossus hippoglossus L., and spotted wolfish Anarchas minor L., against atypical Aeromonas salmonicida. Aquaculture 183: 31-44.

Jannasch, H. W. & Jones G. E. 1959. Bacterial populations in sea water as determined by different methods of enumeration. Limnology and Oceanography 4: 128-139.

Janeway, C. A., Travers, P., Walport, M. & Capra J. D. (eds) 1999. Immunobiology – the immune system in health and disease 4th edn. Elsevier Science, New York.

Jobling, M., Jørgensen, E. H., Arnesen, A.M., Ringø, E. 1993. Feeding, growth and environmental requirements of arctic char: a review of aquaculture potential. Aquac Int 1: 20-46.

Johnson, L. 1980. The Arctic charr (Salvelinus alpinus). In: Balon E. K. (ed) Charrs, salmonid fishes of genus salvelinus 15-98. Dr W. Junk Publishers, The Hague, p 15-98.

Joiner, K. A., Brown, E. J. & Frank, M. M. 1984. Complement and bacteria: chemistry and biology in host defence. Annual Review of Immunology 84: 461-491.

Karvonen, A. Seppälä, O. & Valtonen, E. T. 2004 a). Eye fluke-induced cataract formation in fish: quantitative analysis using a ophtalmological microscope. Parasitology, in press.

Karvonen, A. Hudson, P., Seppälä, O. & Valtonen, E. T. 2004 b). Transmission of a trematode parasite to individual hosts: relationships between acquired resistance and parasite aggregation. Parasitology Research 92: 183-188.

Koski, P., Hirvelä-Koski, V. & Bernardet, J-F. 1993. Flexibacter columnaris infection in Arctic char (Salvelinus alpinus L.). First isolation in Finland. Bulletin of the European Association of Fish Pathologists 13: 66-69.

Kuen, S.K., Ming, C. H. & Yap, S. F. 1993. Background noise in ELISA procedures. Influence of the pH of the coating buffer and correlations with serum IgM concentration. Journal of Immunological Methods 163: 277- 278.

Lafferty, K. D. & Morris, A. K. 1996. Altered behavior of parasitized killifish increases susceptibility to predation by bird final hosts. Ecology 77: 1390-1397.

Laemmli, U. K. 1970. Cleveage of structural proteins during assembly of the head of the bacteriophage T4. Nature 227: 680–685.

Lee, K. K. & Ellis , A. E. 1990. Glycerophospholipid: cholestrol acyltransferase complexed with LPS is a major lethal exotoxin and cytolysin of Aeromonas salmonicida: LPS stabilizes and enhances toxicity of the enzyme. Journal of Bacteriology 172: 5382–5393.

Lee, C. A. 1997. Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells?. Trends in Microbiology 5(4): 148-156.

Lie, Ø., Evensen, Ø., Sørensen, A. & Frøysadal, E. 1989. Study on lysozyme activity in some fish species. Diseases of Aquatic Organisms 6: 1-5.

Lillehaug, A. 1991. Vaccination of Atlantic salmon (Salmo salar L.) against cold-water vibriosis - duration of protection and effect on growth rate. Aquaculture 92: 99-107.

Lillehaug, A., Lunder, T. & Poppe, T. T. 1992. Field testing of adjuvanted furunculosis vaccines in Atlantic salmon, Salmo salar L. Journal of Fish Diseases 15: 485-496.

Liu, P. V. 1957. Survey of hemolysin production among species of Pseudomonads. Journal of Bacteriology 74: 718–727.

Lukinmaa, S., Schildt, R., Rinttilä, T. & Siitonen A. 1999. Salmonella Enteritidis phage types 1 and 4: phenol- and genotypic epidemiology of recent outbreaks in Finland. Journal of Clinical Microbiology 37(7): 2176-2182.

Lund, V., Jørgensen, T., Holm, K. O. & Eggset, G. 1991. Humoral immune response in Atlantic salmon, Salmo salar L., to cellular and extracellular antigens of Aeromonas salmonicida. Journal of Fish Diseases 14(4): 443-452.

Lund, V., Arnesen, J. A. & Eggset, G. 2002. Vaccine development for atypical furunculosis in spotted wolfish Anarchichas minor O.: Comparison of efficacy of vaccines containing different strains of atypical Aeromonas salmonicida. Aquaculture 204: 33-44.

Lyytikäinen T. 1998. Thermal biology of underyearling Lake Inari Arctic charr Salvelinus alpinus. Ph. D. thesis. University of Jyväskylä, Jyväskylä Biological Research Reports from the University of Jyväskylä.

Macrina, F. L., Kopecko, D. J., Jones, K. R., Ayers, D. J. & McCoven, S. M. 1978. A multiple plasmid-containing Escherichia coli strain: Convenient source of size reference plasmid molecules. Plasmid 1: 417-420.

Madetoja, J. Hänninen, M-L., Hirvelä-Koski, V., Dalsgaard, I. & Wiklund, T. 2001. Phenotypic and genotypic characterization of Flavobacterium psychrophilum from Finnish fish farms. Journal of Fish Diseases 24: 469-479.

Magnadóttir, B. & Guðmundsdóttir, B. K. 1992. A comparison of total and specific immunoglobulin levels in healthy Atlantic salmon (Salmo salar L.) and in salmon naturally infected with Aeromonas salmonicida ssp. achromogenes. Veterinary Immunology and Immunopathology 32: 179-189.

Magnadóttir, B., Guðmundsdóttir, S. & Guðmundsdóttir, B. K. 1995. Study of the humoral response of Atlantic salmon (Salmo salar L.), naturally infected with Aeromonas salmonicida ssp. achromogenes. Veterinary Immunology and Immunopathology 49: 127–142.

Maurice, S., Hädge, D., Dekel, M., Friedman, A., Gertler, A. & Shoseyov, O. 1999. A-protein from achromogenic atypical Aeromonas salmonicida: molecular cloning, expression, purification, and characterization. Protein Expression and Purification 16: 396–404.

Maurice, S., D., Dekel, M., Shoseyov, O. & Gertler, A. 2003. Cellulose beads bound to cellulose binding domain-fused recombinant proteins; an adjuvant system for parenteral vaccination of fish. Vaccine 21: 3200-3207.

Merino, S., Albert, S. & Tomas, J. M. 1994. Aeromonas salmonicida resistance to complement-mediated killing. Infection and Immunity: 5483-5490.

Midtlyng, P. J. 1997. Vaccinated fish welfare: protection versus side-effects. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F (eds) Fish Vaccinology. Developments in Biological Standardization 90: 371-379.

Midtlyng, P. J., Lillehaug, A. 1998. Growth of Atlantic salmon Salmo salar after intraperitoneal administration of vaccines containing adjuvant. Diseases of Aquatic Organisms 32: 91-97.

Midtlyng, P. J., Reitan, L. J. & Speilberg 1996. Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (Salmo salar L.) against furunculosis. Fish and Shellfish Immunology 6: 335-350.

Miyata, M., Inglis, V. & Aoki, T. 1996. Rapid identification of Aeromonas salmonicida subspecies salmonicida by the polumerase chain reaction. Aquaculture 141: 13-24.

Montgomery, D.C. 1997. Design and analysis of experiments. 4th edn. John Wiley & Sons, USA, p., 519-521.

Niewiadomska, K. 1986. Verification of the life-cycles of Diplostomum spathaceum (Rudolphi 1891) and D. pseudospathaceum Niewiadomska 1984 (Trematoda, Diplostomidae). Systematic Parasitology 8: 23-31.

O'hIci, B., Olivier, G., Powell, R. 2000. Genetic diversity of the fish pathogen Aeromonas salmonicida demonstrated by random amplified polymorphic DNA and pulsed field gel electrophoresis analyses. Diseases of Aquatic Organisms 39: 109-119.

Olivier, G. 1990. Virulence of Aeromonas salmonicida: lack of relationship with phenotypic characteristics. Journal of Aquatic Animal Health 19: 119-127.

Parry, R. M., Chandan, Jr R. C. & Shahani, K. M. 1965. A rapid and sensitive assay of muramidase. Proceedings of the Society for Experimental Biology and Medicine 119: 384-386.

Pavan, M. E., Abbott, S.L., Zorzópulos, J. & Janda, J. M. 2000. Aeromonas salmonicida subsp. pectinolytica subsp nov., a new pectinase-positive subspecies isolated from a heavily polluted river. International Journal of Systemic and Evolutionary Microbiology 50: 1119-1124.

Pedersen, K., Grisez, L., van Houdt, R., Tiainen, T., Ollevier, F. & Larsen, J. L. 1999. Extended serotyping scheme for Vibrio anguillarum with the

definition and characterization of seven provisional O-serogroups. Current Microbiology 38: 183-189.

Popoff, M. 1984. Genus III. Aeromonas Kluyver and van Niel 1936, 398AL. In: Krieg, N. R. & Holt, J. G. (eds) Bergey's manual of systematic bacteriology, Vol 1, p. 545–548. Williams and Wilkins, Baltimore.

Pylkkö P. 1993. The effect of feeding and rearing density on the susceptibility to ASA-disease of grayling (Thymallus thymallus L.) and Arctic charr (Salvelinus alpinus L.). Kalatutkimuksia-Fiskundersökningar 74. Finnish Game and Fisheries Research Institute, (abstract in English).

Reid, B. M., Porter, R. R. 1981. The proteolytic activation systems of complement. Annual Review of Biochemistry 50: 433-464.

Rintamäki-Kinnunen, P. & Valtonen, E. T. 1996. Finnish salmon resistant to Gyrodactylus salaris: a long term study at fish farms. International Journal for Parasitology 26: 723-732.

Rintamäki-Kinnunen, P. & Valtonen, E. T. 1997. Epizootiology of protozoans in farmed salmonids at northern latitudes. International Journal for Parasitology 27: 89-99.

Rintamäki-Kinnunen, P. 1997. Parasitic and bacterial diseases at salmonid fish farms in northern Finland. Ph.D. thesis, University of Oulu, Oulu

Rintamäki, P. & Koski, P. 1987. Outbreaks of furunculosis in northern Finland. In Stenmark A & Malmberg G (eds) Parasites and Diseases in natural waters and aquaculture in Nordic countries. Naturhistoriska Riksmuseet, Stockholm, p. 121-128.

Rintamäki, P. & Valtonen, E. T. 1991. Aeromonas salmonicida in Finland: pathological problems associated with atypical and typical strains. Journal of Fish Diseases 14: 323–331.

Rockey, D. D., Dunghan, C. F., Lunder, T. & Rohovec, J. S. 1991. Monoclonal antibodies against Aeromonas salmonicida lipopolysaccharide identify differences among strains. Diseases of Aquatic Organisms 10: 115-120.

Roitt, I. 1994. Essential immunology, 8th edn Blackwell Science, Paris

Saarnisto, M., Grönlund, M. & Ekman, I. 1995. Lateglacial of Lake Oneega - Contribution to the history of the eastern Baltic basin. Quaternary International 27: 111-120.

Saarnisto, M. & Saarinen, T. 2001. Deglaciation chronology of the Scandinavian Ice Sheet from east Lake Onega to the Salpauselkä end moraines. Global and Planetary Change 31: 387-405.

Salmond, G. P. & Reeves, P. J. 1993. membrane traffic wardens and protein secretion in gram-negative bacteria. Trends in Biochemical Sciences 18(1): 7-12.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, (2nd ed.), . Cold Spring Harbour Press, Cold Spring Harbour, NY.

Sheeran, B. & Smith, P. R. 1981. A second extracellular proteolytic activity associated with the fish pathogen Aeromonas salmonicida. FEMS Microbiology Letters 11: 73–76.

Sorensen, K. & Brodbeck, U. 1986. Assessment of coating-efficiency in ELISA plates by direct protein determination. Journal of Immunological Methods 95: 291-293.

Sørum, H., Holstad, G., Lunder, T. & Håstein, T. 2000. Grouping by plasmid profiles of atypical Aeromonas salmonicida isolated from fish, with special reference to salmonid fish. Diseases of Aquatic Organisms 41: 159–171.

Sørum, H. & Damsgard, B. 2004. Effect of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (Salmo salar L.). Aquaculture 1-4: 333–341.

Stuber, K., Burr, S. E., Braun, M., Wahli, T. & Frey, J. 2003. Type III secretion genes in Aeromonas salmonicida subsp. Salmonicida are located on a large thermolabile virulence plasmids. Journal of Clinical Microbiology (41)8: 3854-3856.

Svendsen, Y. S. & Bøgwald, J. 1997. Influence of artificial wound and non-intact mucus layer on mortality of Atlantic salmon Salmo salar L. following a bath challenge with Vibrio anguillarum and aeromonas salmonicida. Fish and Shellfish Immunology 7(5): 317-325.

Swift, D. R. 1964. The effect of temperature and oxygen on the growth rate of the Windemere char Salvelinus alpinus willughbii. Comparative Biochemistry and Physiology 12: 179-183.

SYSTAT 1997. SYSTAT® 7.0 for Windows: statistics, SPSS Inc., Chicago

Tiirola, M. A., Suvilampi, J. E., Kulomaa, M. S. & Rintala, J. A. 2003. Microbial diversity in a thermophilic aerobic biofilm process: analysis by length heterogeneity PCR (LH-PCR). Water Research 37: 2259-2268.

Threlfall, E. J., Rowe, B., Ferguson, J. L. & Ward, L. R. 1986. Characterization of plasmids conferring resistance to gentamycin and ampramycin in strains of Salmonella typhimurium definitive type 204c isolated in Britain. Journal of Hygiene 97: 419-426.

Thuvander, A., Hongslo, T., Janson, J. & Sundquist, B. 1987. Duration of protective immunity and antibody titres measured by ELISA after vaccination of rainbow trout, Salmo gairdneri Richardson, against vibriosis. Journal of Fish Diseases 10: 479-486.

Thuvander, A., Wichard, U. P. & Reitan, L. J. 1993. Humoral antibody response of brown trout Salmo trutta vaccinated against furunculosis. Diseases of Aquatic Organisms 17: 17-23.

Valtonen, E. T., Rintamäki, P. & Koskivaara, M. 1992. Occurrence and pathogenicity of Yersinia ruckeri at fish farms in northern and central Finland. Journal of Fish Diseases 15: 163-171.

Valtonen, E. T. & Gibson, D. I. 1997. Aspects of the biology of diplostomid metacercarial (Digenea) populations occurring in fishes in different localities of northern Finland. Annales Zoologie Fennici 34: 47-59.

Valtonen, E. T., Holmes, J. C. & Koskivaara, M. 1997. Eutrophicatio, pollution and fragmentation: effects on parasite communities in roach (Rutilus rutilus) and perch (Perca fluviatilis) in four lakes in central Finland. Canadian Journal of Fisheries and Aquatic Sciences 54: 572-585.

Valtonen, E. T., Holmes, J. C., Aronen, J. & Rautalahti, I. 2003. Parasite communities as indicators of recovery from pollution: parasites of roach (Rutilus rutilus) and perch (Perca fluviatilis) in Central Finland. Parasitology 126: S43-S52.

Whitby, P. W., Landon, M. & Coleman, G. 1992 a). The cloning and nucleotide sequence of the serine protease gene (aspA) of Aeromonas salmonicida ssp. salmonicida. FEMS Microbiology Letters 99: 65–72.

Whitby, P. W., Delany, S. G., Coleman, G. & Munro, A. L. S. 1992 b). The occurrence of 70-kDa serine protease gene in typical and atypical strains of Aeromonas salmonicid. Journal of Fish Diseases 15: 529-535.

Whittington, R. J., Djordjevic, S. P., Carson, J. & Callinan, R. B. 1995. Restriction endonuclease analysis of atypical Aeromonas salmonicida isolates from goldfish Carassius auratus, silver perch Bidyanus bidyanus, and greenback flounder Rhombosolea tapirina in Australia. Diseases of Aquatic Organisms 22: 185-191.

Wiklund, T. & Lönnström, L. 1994. Occurrence of Pseudomonas anguilliseptica in Finnish fish farms during 1986-1991. Aquaculture 126: 211-217.

Wiklund, T., Kaas, K., Lönnström, L. & Dalsgaard, I. 1994. Isolation of Cytophaga psychrophila (Flexibacter psychrophilus) from wild and farmed rainbow trout (Onchorhynchus mykiss) in Finland. Bulletin of the European Association of Fish Pathologists 14: 44-46.

Wiklund, T. & Dalsgaard, I. 1998. Occurrence and significance of a atypical Aeromonas salmonicida in non-salmonid and salmonid fish species: a review. Diseases of Aquatic Organisms 32: 49–69.

Wiklund, T., Tabolina, I. & Bezgachina, T. V. 1999. Recovery of atypical Aeromonas salmonicida from ulcerated fish from Baltic Sea – short communication 56: 175-179.

Yano, T. 1992. Assays of hemolytic complement activity. In: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Kaatari, S.L. & Rowley, A.F. (eds), Techniques in Fish Immunology 2: 131-141. SOS Publications, USA.

TABLE 1. *Aeromonas samonicida* strains analyzed in the studies. The original paper is referred by Roman numerals. The isolates used in challenge experiments are shown in bold.

Atypical AS non-pigm.producing Research number	**Fish species**	**Location**	**Year of isolation**	**Farmed/Wild**	**paper**	**Reference**
8	*Thymallus thymallus*	Vuoksi	1989	F	I, IV	EELA, Kuopio
9	***Thymallus thymallus***	**Vuoksi**	**1989**	**F**	**I, IV**	**EELA, Kuopio**
111	*Thymallus thymallus*	Vuoksi	1991	F	I, IV	EELA, Kuopio
113	*Thymallus thymallus*	Vuoksi	1992	F	I, IV	EELA, Kuopio
115	*Thymallus thymallus*	Vuoksi	1992	F	IV	EELA, Kuopio
117	*Thymallus thymallus*	Vuoksi	1992	F	I, IV	EELA, Kuopio
127	*Thymallus thymallus*	Vuoksi	1993	F	I, IV	EELA, Kuopio
150	*Thymallus thymallus*	Vuoksi	1994	F	IV	EELA, Kuopio
153	*Thymallus thymallus*	Vuoksi	1994	F	IV	EELA, Kuopio
156	*Thymallus thymallus*	Vuoksi	1994	F	I, IV	EELA, Kuopio
200	*Thymallus thymallus*	Vuoksi	1995	F	IV	EELA, Kuopio
205	*Thymallus thymallus*	Vuoksi	1995	F	I, IV	EELA, Kuopio
7	*Salvelinus alpinus*	Vuoksi	1988	F	I, IV	EELA, Kuopio
112	*Salvelinus alpinus*	Vuoksi	1992	F	IV	EELA, Kuopio
116	*Salvelinus alpinus*	Jänisjoki	1992	F	I, IV	EELA, Kuopio
128	*Salvelinus alpinus*	Vuoksi	un	F	IV	EELA, Kuopio
143	*Salvelinus alpinus*	Vuoksi	1994	F	IV	EELA, Kuopio
154	*Salvelinus alpinus*	Vuoksi	1994	F	I, IV	EELA, Kuopio
206	*Salvelinus alpinus*	Vuoksi	1995	F	I, IV	EELA, Kuopio
203	*Salvelinus alpinus*	Vuoksi	1995	F	I, IV	EELA, Kuopio
121	*Coregonus albula*	Vuoksi	un	F	IV	EELA, Kuopio
125	*Coregonus albula*	Vuoksi	1993	F	I, IV	EELA, Kuopio
129	*Thymallus thymallus*	Vuoksi	1993	F	I, IV	EELA, Kuopio
2	*Rutilus rutilus*	Vuoksi	1988	Wild	I, IV	EELA, Kuopio
6	*Leucius leucius*	Vuoksi	1988	Wild	I, IV	EELA, Kuopio
114	*Rutilus rutilus*	Vuoksi	1992	Wild	I, IV	EELA, Kuopio
130	*Rutilus rutilus*	Vuoksi	1993	Wild	I, IV	EELA, Kuopio
131	*Rutilus rutilus*	Vuoksi	1993	Wild	I, IV	EELA, Kuopio
1	*Thymallus thymallus*	Kymijoki	1990	F	I, IV	EELA, Kuopio
118	*Thymallus thymallus*	Kymijoki	1992	F	I, IV	EELA, Kuopio
129	*Thymallus thymallus*	Kymijoki	1993	F	IV	EELA, Kuopio
132	*Thymallus thymallus*	Kymijoki	1993	F	I, IV	EELA, Kuopio
155	*Thymallus thymallus*	Kymijoki	1994	F	I, IV	EELA, Kuopio
157	*Thymallus thymallus*	Kymijoki	1994	F	IV	EELA, Kuopio
211	***Thymallus thymallus***	**Kymijoki**	**1995**	**F**	**II**	**EELA, Kuopio**
201	*Thymallus thymallus*	Kymijoki	1995	F	I, IV	EELA, Kuopio
16	*Salvelinus alpinus*	Kymijoki	un	F	IV	EELA, Kuopio
122	*Salvelinus fontinalis*	Kymijoki	un	F	IV	EELA, Kuopio
15	*Onchorhynchus mykiss*	Kymijoki	1988	F	I, IV	EELA, Kuopio
60	*Onchorhynchus mykiss*	Kymijoki	1988	F	I, IV	EELA, Kuopio
61	*Onchorhynchus mykiss*	Kymijoki	1988	F	I, IV	EELA, Kuopio
100	*Rutilus rutilus*	Kymijoki	1988	Wild	I, IV	EELA, Kuopio
44	*Salmo trutta f. fario*	Kokemäenjoki	1988	F	I, IV	EELA, Kuopio
64	*Salmo trutta f. lacustris*	Kokemäenjoki	1988	F	I, IV	EELA, Kuopio
20	*Salmo trutta* m. *lacustris*	Oulunjoki	un	F	IV	EELA, Kuopio
26	*Onchorhynchus mykiss*	Iijoki	1988	F	I, IV	EELA, Kuopio
31	*Rutilus rutilus*	Kemijoki	1988	Wild	I, IV	EELA, Kuopio
29	*Salmo trutta m. lacustris*	Oulunjoki	un	F	IV	EELA, Kuopio
63	*Onchorhynchus mykiss*	Baltic sea	un	F	IV	EELA, Kuopio
181	*Salvelinus alpinus*	Sweden	1991	unknown	I, IV	SVA, Sweden
182	*Salvelinus alpinus*	Sweden	un	unknown	IV	SVA, Sweden
183	*Salvelinus alpinus*	Sweden	1994	unknown	I, IV	SVA, Sweden
186	*Salvelinus alpinus*	Sweden	1988	unknown	I, IV	SVA, Sweden
189	*Salvelinus alpinus*	Sweden	1992	unknown	I, IV	SVA, Sweden
196	*Salvelinus alpinus*	Sweden	1985	unknown	I, IV	SVA, Sweden

184	*Salvelinus fontinalis*	Sweden	1986	unknown	I, IV	SVA, Sweden
188	*Thymallus thymallus*	Sweden	1994	Wild	I, IV	SVA, Sweden
191	*Salvelinus fontinalis*	Sweden	un	unknown	IV	SVA, Sweden
195	*Thymallus thymallus*	Sweden	1985	Wild	I, IV	SVA, Sweden
198	*Salvelinus alpinus*	Sweden	un	unknown	IV	SVA, Sweden
Atypical AS pigment producing						
202	*Salvelinus alpinus*	Inari	1995	F	I, IV	EELA, Kuopio
Reference strains						
*ATCC 33659					I, IV	
**ASS MT423	*Salmo trutta*	Finland	nk	unknown	IV	Hirst & Ellis 1994[1]
265-87	*Salmo salar*	Iceland	nk	unknown	IV	Gudmunsdottir et al. 1990[2]
ASS P110A/91	*Salmo trutta m. lacustris*	Finland	1991	unknown	IV	Wiklund et al. 1993[3]
ASS P131A/91	*Oncorhynchus mykisss*	Finland	1991	unknown	IV	

[1] Hirst I.D. & Ellis A.E. 1994. Fish and Shellfish Immunology 4: 29-45.

[2] Gudmundsdóttir B.K., Hastings T.S. & Ellis A.E. 1990. Diseases of Aquatic Organisms 9:199-208.

[3] Wiklund T., Lönnström L. & Niiranen H. 1993.Disease of Aquatic Organisms 32: 65-72.

* American Type Culture Collection, type strain for *Aeromonas salmoicida achromogenes*

** *Aeromonas salmonicida* subsp. *salmonicida,* A-layer positive isolate