TRANSMISSION AND PATHOGENESIS OF *PISCIRICKETTSIA SALMONIS* IN ATLANTIC SALMON (*SALMO SALAR*)

A Thesis

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- Teacher Training 0530
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- Language
  - General 0679
  - Ancient 0289
  - Linguistics 0290
  - Modern 0291
- Literature
  - General 0401
  - Classical 0294
  - Comparative 0295
  - Medieval 0297
  - Modern 0298
  - African 0316
  - American 0591
  - Asian 0305
  - Canadian (English) 0352
  - Canadian (French) 0355
  - English 0593
  - Germanic 0311
  - Latin American 0312
  - Middle Eastern 0315
  - Romance 0313
  - Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
- Philosophy 0422
- Religion
  - General 0318
  - Biblical Studies 0321
  - Clergy 0319
  - History of 0320
  - Philosophy of 0322
- Theology 0469

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- American Studies 0323
- Anthropology
  - Archaeology 0324
  - Cultural 0326
  - Physical 0327
- Business Administration
  - General 0310
  - Accounting 0272
  - Banking 0770
  - Management 0454
  - Marketing 0338
- Canadian Studies 0385
- Economics
  - General 0501
  - Agricultural 0503
  - Commerce-Business 0505
  - Finance 0508
  - History 0509
  - Labor 0510
  - Theory 0511
- Folklore 0358
- Geography 0366
- Gerontology 0351
- History
  - General 0578
  - Ancient 0579
  - Medieval 0581
  - Modern 0582
  - Black 0328
  - African 0331
  - Asia, Australia and Oceania 0332
  - Canadian 0334
  - European 0335
  - Latin American 0336
  - Middle Eastern 0333
  - United States 0337
- History of Science 0585
- Law 0398
- Political Science
  - General 0615
  - International Law and Relations 0616
  - Public Administration 0617
- Recreation 0814
- Social Work 0452
- Sociology
  - General 0626
  - Criminology and Penology 0627
  - Demography 0938
  - Ethnic and Racial Studies 0631
  - Individual and Family Studies 0628
  - Industrial and Labor Relations 0629
  - Public and Social Welfare 0630
  - Social Structure and Development 0700
  - Theory and Methods 0344
- Transportation 0709
- Urban and Regional Planning 0999
- Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
- Agriculture
  - General 0473
  - Agronomy 0285
  - Animal Culture and Nutrition 0475
  - Animal Pathology 0476
  - Food Science and Technology 0359
  - Forestry and Wildlife 0478
  - Plant Culture 0479
  - Plant Pathology 0480
  - Plant Physiology 0817
  - Range Management 0777
  - Wood Technology 0746
- Biology
  - General 0306
  - Anatomy 0287
  - Biostatistics 0308
  - Botany 0309
  - Cell 0379
  - Ecology 0329
  - Entomology 0353
  - Genetics 0369
  - Limnology 0793
  - Microbiology 0410
  - Molecular 0307
  - Neuroscience 0317
  - Oceanography 0416
  - Physiology 0433
  - Radiation 0821
  - Veterinary Science 0778
  - Zoology 0472
- Biophysics
  - General 0786
  - Medical 0760

EARTH SCIENCES
- Biogeochemistry 0425
- Geochemistry 0996
- Geodesy 0370
- Geology 0372
- Geophysics 0373
- Hydrology 0388
- Mineralogy 0411
- Paleobotany 0345
- Paleoecology 0426
- Paleontology 0418
- Paleozoology 0985
- Palynology 0427
- Physical Geography 0368
- Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
- Environmental Sciences 0768
- Health Sciences
  - General 0566
  - Audiology 0300
  - Chemotherapy 0992
  - Dentistry 0567
  - Education 0350
  - Hospital Management 0769
  - Human Development 0758
  - Immunology 0982
  - Medicine and Surgery 0564
  - Mental Health 0347
  - Nursing 0569
  - Nutrition 0570
  - Obstetrics and Gynecology 0380
  - Occupational Health and Therapy 0354
  - Ophthalmology 0381
  - Pathology 0571
  - Pharmacology 0419
  - Pharmacy 0572
  - Physical Therapy 0382
  - Public Health 0573
  - Radiology 0574
  - Recreation 0575
  - Speech Pathology 0460
  - Toxicology 0383
- Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences
- Chemistry
  - General 0485
  - Agricultural 0749
  - Analytical 0486
  - Biochemistry 0487
  - Inorganic 0488
  - Nuclear 0738
  - Organic 0490
  - Pharmaceutical 0491
  - Physical 0494
  - Polymer 0495
  - Radiation 0754
- Mathematics 0405
- Physics
  - General 0605
  - Acoustics 0986
  - Astronomy and Astrophysics 0606
  - Atmospheric Science 0608
  - Atomic 0748
  - Electronics and Electricity 0607
  - Elementary Particles and High Energy 0798
  - Fluid and Plasma 0759
  - Molecular 0609
  - Nuclear 0610
  - Optics 0752
  - Radiation 0756
  - Solid State 0611
- Statistics 0463

Applied Sciences
- Applied Mechanics 0346
- Computer Science 0984
- Engineering
  - General 0537
  - Aerospace 0538
  - Agricultural 0539
  - Automotive 0540
  - Biomedical 0541
  - Chemical 0542
  - Civil 0543
  - Electronics and Electrical 0544
  - Heat and Thermodynamics 0348
  - Hydraulic 0545
  - Industrial 0546
  - Marine 0547
  - Materials Science 0794
  - Mechanical 0548
  - Metallurgy 0743
  - Mining 0551
  - Nuclear 0552
  - Packaging 0549
  - Petroleum 0765
  - Sanitary and Municipal 0554
  - System Science 0790
- Geotechnology 0428
- Operations Research 0796
- Plastics Technology 0795
- Textile Technology 0994

PSYCHOLOGY
- General 0621
- Behavioral 0384
- Clinical 0622
- Developmental 0620
- Experimental 0623
- Industrial 0624
- Personality 0625
- Physiological 0989
- Psychobiology 0349
- Psychometrics 0632
- Social 0451

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SIGNATURE PAGES

iii-iv

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ABSTRACT

Piscirickettsia salmonis is the etiological agent of Salmonid Rickettsial Septicaemia (SRS), a major disease affecting several species of salmonids cultured in salt water in Southern Chile. Little is known about its epidemiology and transmission mechanisms. Previous reports have demonstrated this organism to grow and produce a characteristic cytopathic effect (CPE) in several fish cell lines but not those from species such as Brown bullhead *Ictalurus nebulosus* L. (BB) and Bluegill *Lepomis macrochirus* R. (BF-2). In a first experiment, Chinook salmon embryo (CHSE-214) and BB cells were inoculated with *P. salmonis*, incubated at 15 °C for 78 days, and studied by light microscopy (LM) and transmission electron microscopy (TEM). Cytopathic effect appeared after 6 days post-infection in CHSE-214, and after 45 days post-infection in BB cell line. Transmission electron microscopy (TEM) examination of BB cells after 78 days post-infection, revealed *P. salmonis* within membrane-bound vacuoles or free within the cytoplasm and the extracellular space. These results indicate that BB cells are susceptible to infection with *P. salmonis* but exhibit a slower pattern of invasion compared to other cell lines described in the literature. In a second experiment, two susceptible Atlantic salmon fibroblasts (ASF) and CHSE-214, and one non-susceptible (BB) fish cell lines were infected with *P. salmonis* and studied by LM and TEM at 1, 7, and 14 days post infection. Results showed differences in the appearance of CPE and in the intracellular infection mechanisms of the pathogen among cell lines. Infected CHSE-214 cells had vacuoles containing *P. salmonis* whereas ASF cells had empty vacuoles and BB cells had empty vacuoles and *P. salmonis* free in the cytosol. An *in vivo* experiment was designed to compare three routes of inoculation (oral, gill surface and intraperitoneal), and to study the effect of physical contact as a risk factor in the horizontal transmision of SRS in Atlantic salmon raised in fresh water. Tissue samples (liver, kidney, spleen, gill, and brain) were collected weekly to study the sequential infection of SRS using indirect fluorescent-antibody technique (IFAT). The pathogen was transmitted horizontally to fish with and without physical contact; however, physical contact appeared as an important risk factor for horizontal transmission of SRS. The sequential study using IFAT indicated that fish inoculated by oral and gill routes as well as naturally infected cohabitant fish, presented a similar hematogenous pattern of infection, different from the capsular (serosa) infection pattern observed in intraperitoneally inoculated fish. *Piscirickettsia salmonis* was also observed within the cytoplasm of leukocytes and renal tubules indicating that elimination of this pathogen in urine may be possible. *Aeromona salmonicida* was also detected (by IFAT) in some of the fish exposed to *P. salmonis.* This finding, the leukocytic infection by *P. salmonis* and the presence of mortalities in fish exposed to *P. salmonis* but not in the controls, suggest an immunosuppressor effect of *P. salmonis* that may increase the susceptibility of the host to other pathogens.

Key words: *Piscirickettsia salmonis*, piscirickettsiosis, salmonid rickettsial septicaemia, inoculation route, transmission, pathogenesis, *in vitro*, *in vivo*.

DEDICATION

To my parents Carmen y José, for their love, encouragement and support.

To Kerry, for her permanent friendship and love.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

TABLE OF ABBREVIATIONS

As	: *Aeromonas salmonicida*
ASF	: Atlantic salmon (*Salmo salar*) fibroblast cell line
BB	: Brown Bullhead (*Ictalurus nebulosus*) cell line
BF-2	: Blue gill *(Lepomis macrochirus)* cell line
CHSE-214	: Chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line
cm	: Centimeter
DFO	: Department of Fisheries and Oceans, Canada
ELISA	: Enzyme linked immuno sorbent assay
EPC	: Epithelioma papulosum cyprini (*Cyrinus carpio*) cell line
Fc	: Fraction C of an antibody
FHM	: Fat Head Minnow cell line
FITC	: Fluorescein-5-isothiocianate
g	: gram
GS	: Gill surface
h	: Hour
IFAT	: Indirect fluorescent antibody technique
IgG	: Immunoglobulin G
IP	: Intraperitoneal
kg/m^3	: Kilograms per cubic meter
L	: Liter
LM	: Light microscopy
min	: Minute
mm	: Millimeter
mm^3	: Cubic millimeter
NCR 1010	: New Chilean rickettsia strain 1010
PAb	: Polyclonal antibody
PBS	: Phosphate buffered saline
pH	: Hydrogen ion activity (neg. log)
PO	: Oral route (*Per os*)
ppm	: Parts per million
Ps	: *Piscirickettsia salmonis*
RLO	: Rickettsia-like organism
rpm	: revolutions per minute
RT	: Room temperature
RTG-2	: Rainbow trout (*Oncorhynchus mykiss*) gonad cell line.
SAS	: Statistical analysis software
SRS	: Salmonid Rickettsial Septicaemia
$TCID_{50}$	: 50 % tissue culture infectious dose endpoint
TEM	: Transmission electron microscopy
wk	: Week

°C	: degree Celsius
%	: Percent
μl	: Microliter
μm	: Micrometer

1. GENERAL INTRODUCTION

1.1 Introduction

Rickettsial infections have been reported in several species of salmonid and non-salmonid fish in fresh water and salt water since 1939. However, these organisms were not considered economically important until 1989, when a new disease of unknown etiology killed approximately 1.5 million market-sized (2 kg) coho salmon, *Oncorhynchus kisutch* cultured near Calbuco, southern Chile (Bravo and Campos 1989; Fryer *et al.*, 1990). Losses were extensive with mortalities of up to 90% (Bravo and Campos, 1989). The disease was later described as affecting Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss* Walbaum), and Chinook salmon (*Oncorhynchus tshawytscha*) (Cvitanich *et al.*, 1991).

The causative organism of the Chilean outbreaks was identified as *Piscirickettsia salmonis*, a new species and genus in the order Rickettsiales, family Rickettsiaceae, and tribe Ehrlichieae (Fryer *et al.*, 1992). It was the first rickettsial agent to be implicated in the etiology of a fish disease, affecting several species of salmonids cultured in salt water (Cvitanich *et al.*, 1991). The systemic character of the disease motivated the proposed name salmonid rickettsial septicaemia, SRS (Cvitanich *et al.*, 1991). Today this disease, also known as Piscirickettsiosis, is probably the most important disease affecting the salmon industry in Chile (Cassigoli, 1994).

1.2 Rickettsial organisms in mammals

Nearly all of the tick-borne rickettsial diseases in mammals are endemic to tropical and semi-tropical regions of the world. Although some of the rickettsial diseases, such as bovine anaplasmosis (*Anaplasma marginale*) and Rocky Mountain spotted fever (*Rickettsia rickettsii*), have been well known and intensely studied, other rickettsiae are relative newcomers to the field. Equine monocytic ehrlichiosis or Potomac Horse fever (*Ehrlichia risticii*) and canine infectious cyclic thrombocytopenia *(Ehrlichia platys)* have been discovered more recently (Woldehiwet and Ristic, 1993).

The diseases caused by rickettsial organisms are of great economical significance and some cause serious zoonoses (Q fever). Organisms like *Coxiella burnetti* affect humans, ruminants, cats, dogs, wild and domestic birds, mice and ticks. Since most of these microorganisms use intermediate hosts (ticks), the global distribution of animal rickettsiosis is dictated by the ecology of their arthropod vectors. For example, Heartwater disease (*Cowdria ruminantium*) transmitted by *Amblyomma spp.*, and Tick-borne fever *(Cytoecetes phagocytophila*) transmitted by *Ixodes spp.*, are limited to areas where the intermediate host resides. In contrast, anaplasmosis (*Anaplasma marginale)* is widely distributed (Woldehiwet and Ristic, 1993).

Morphological characteristics

Members of the genus *Rickettsia* have morphologic and biochemical

properties typical of Gram-negative bacteria, including a distinctive bilaminate cell wall. They are short, rod-shaped or coccobacillary organisms, ranging from 0.8 to 2.0 μm long and 0.3 to 0.5 μm wide (Ris and Fox, 1949). Although rickettsiae usually occur as obligate intracellular parasites (Carter *et al.*, 1995), they can occasionally be found extracellularly.

Some *Rickettsia sp.* exhibit a great deal of pleomorphism, whereas others are quite uniform in size and shape. Most organisms occur in groups in the cytoplasm of the parasitized cells although they have also been observed within the nucleus (Timoney *et al.*, 1988). They stain reasonably well with Giemsa, Castañeda, Giménez, and Macchiavello stains, but stain poorly with Gram stains (Carter *et al.*, 1995). With Macchiavello and Giménez stains they appear bright red against a blue (Macchiavello) or green (Giménez) background (Carter *et al.*, 1995).

Isolation and growth media

Rickettsiae can only be cultivated outside the host in living tissues such as embryonated chicken eggs, and rarely, in media containing body fluids (Timoney *et al.*, 1988). Rickettsiae have been successfully cultivated in cell culture, which is a good medium to study their metabolic requirements. A few species including *Rickettsia melophagi*, a non-pathogenic form found in the sheep ectoparasite, *Melophagus ovinus*, have been cultivated in special acellular laboratory media. The pathogenic forms can readily be propagated in chicken

embryos and in cell cultures (Timoney *et al.*, 1988).

Rickettsiae are exceptionally unstable outside the host cell (Baron *et al.*, 1994), and are readily inactivated at 56° C and by standard disinfectants (McDade and Fishbein, 1988). Rickettsiae require eukaryotic host cells for their propagation and they are susceptible to certain antibiotics. Therefore, antibiotics cannot be used to prevent the occurrence of adventitious agents that might be present in the tissue specimen (McDade and Fishbein, 1988) and to avoid contamination of cell cultures.

Infection and intracellular survival

Rickettsiae infect leucocytes or erythrocytes in both vertebrates and invertebrates (Woldehiwet and Ristic, 1993). Rickettsiae appear to be well-established parasites of the intestinal cells of arthropods (Huff, 1938), and usually transmission is from arthropod to vertebrate. Some are transmitted transovarially in ticks and are not pathogenic to them (Timoney *et al.*, 1988).

Rickettsiae apparently enter cells by phagocytosis (Walker and Winkler, 1978; Woldehiwet and Ristic, 1993). Entry depends on both the metabolic activity of the rickettsiae and the phagocytic ability of the host cell (Winkler, 1982). Rickettsiae possess many of the metabolic functions of bacteria but require exogenous cofactors from animal cells. Rickettsiae have cytochromes, and their metabolic reactions are aerobic (Carter *et al.*, 1995). They can generate their own energy, although they also depend on their host for some

energy.

Although members of the genus *Rickettsia* grow only in the presence of eukaryotic host cells, they possess considerable synthetic capabilities and generate their own ATP via the tricarboxylic acid cycle (Weiss and Moulder, 1984). Rickettsiae possess an ADP-ATP translocator system that enables them to exchange their own adenosine diphosphate (ADP) for host adenosine triphosphate (ATP) by an unusual transport system similar to that of mitochondria (Winkler, 1976; Timoney *et al.*, 1988). Presumably, the rickettsiae use host cell ATP when available and may switch to endogenous ATP when that of the host becomes depleted (Phibbs and Winkler, 1982; Krause *et al.*, 1985).

Upon entry into cells, there are several mechanisms by which rickettsia can survive. The rickettsia may persist and multiply inside the phagocytic vacuole, avoiding the effect of lysosomes. This mechanism suggests that these intracellular organisms may use intricate survival mechanisms (like production of acid phosphatase) to neutralize the oxygen radicals produced by neutrophils, since many bacteria would not survive the low pH generated by the fusion of lysosomes within phagosomes (Woldehiwet and Ristic, 1993). Other proposed mechanisms include inhibiting partially or totally the fusion of lysosomes, or escaping from the phagosome and multiplying in the cytoplasm of the host cell by transverse binary fission (Ris and Fox, 1949; Woldehiwet and Ristic, 1993). Subsequently, host cells are lysed during release of mature rickettsiae (Baron *et*

al., 1994).

Pathogenesis of rickettsial infection

Rickettsial organisms proliferate in endothelial and phagocytic cells. Steps involved in cellular invasion include adherence, endocytosis, and phagosome destruction. Adherence is facilitated by surface receptors on host cells. After engulfment, rickettsiae destroy phagosomal membranes with phospholipase and then multiply within the cytoplasm, or in certain cases (such as spotted fever) in the nucleus also (Carter *et al.*, 1995).

Members of the genus *Rickettsia* display a unique tropism for the endothelial cells of the microcirculatory system, especially capillaries. The subsequent damage to the vasculature is the basis for similarities in the pathogenic, pathologic and pathophysiologic features of many rickettsial diseases (Walker and Mattern, 1980).

Damage to cells results from vascular changes and the influx of inflammatory cells, which increase the host's immune response and increase overall morbidity (Baron *et al.*, 1994). The primary cellular lesion results from dilation and destruction of intracellular membranes, particularly the rough endoplasmic reticulum (Silverman, 1984). As endothelial cells die, necrosis of the intima and media of blood vessels causes formation of hyaline thrombi composed of fibrin and cellular debris (Carter *et al.*, 1995). These hyaline thrombi cause microinfarcts and extravasation of blood; changes are manifested

grossly by petechial lesions, which are usually seen in rickettsial diseases (Walker and Mattern, 1980). The clinical consequences produced by infarcts in affected tissues are much greater than those produced by vasculitis (McDade and Fishbein, 1988). In animals, gross and microscopic lesions are found in brain, kidneys, lung, and heart (McDade and Fishbein, 1988).

Vascular damage can also occur in rickettsial infection from other mechanisms of cell injury. Non-infectious (ultraviolet irradiated) rickettsiae contain toxins which are injurious to mice when administered intravenously (Woldehiwet and Ristic, 1993). Death is caused by damage to capillary endothelial cells, producing loss of plasma, decrease in blood volume, and shock (Woldehiwet and Ristic, 1993).

Immunological aspects

The immunosuppressive role of rickettsia infections has been suspected since several rickettsiae can invade cells involved in the immune response of the host (Woldehiwet and Ristic, 1993). Additionally, the immune responses directed at the rickettsia might cause collateral damage to the host defense (Woldehiwet and Ristic, 1993). Experimental infections with some rickettsiae are often followed by severe infections by other agents, due to reduced capacity of infected neutrophils to phagocytose and kill the secondary agents (Woldehiwet and Ristic, 1993).

Rickettsial infections result in transient suppression of the proliferative

responses of lymphocytes to other unrelated antigens or mitogens (Zachary and Smith, 1984; Jerrels, 1985; Rikihisa *et al.*, 1987; Woldehiwet, 1987). Lymphocytopenia, affecting all types of lymphocytes, occurs during tick-borne fever in ruminants, caused by *Cytocetes (Ehrlichia) phagocytophila* (Woldehiwet and Ristic, 1993).

Among the rickettsia-induced immunosuppressive mechanisms described are the down regulation of cellular and humoral responses to other antigens by suppressor cells (Jerrels, 1985), and the production of prostaglandins by macrophages and polymorphonuclear cells (Koster *et al.*, 1985; Walker and Hoover, 1991).

Control of rickettsial agents of humans and animals

Prevention and control of rickettsial diseases in humans are primarily accomplished by interrupting transmission of the infective agents from its vector or reservoir. This can often be achieved by simply avoiding vector-infested areas or areas where infected animals are kept, and by vector control. In some cases, recovery from rickettsial disease usually confers solid lasting immunity (Holland, 1990; Ristic, 1990).

Presently, immunization plays a minimal role in prevention of rickettsial diseases, because with some exceptions, safe and effective vaccines are generally unavailable (Brezina, 1985; Wisseman and Ordoñez, 1986). A killed adjuvant-fortified vaccine (PHF-VAX; Schering-Plough, NJ, USA) against

Ehrlichia risticii, the cause of Potomac horse fever or equine monocytic ehrlichiosis, was commercially developed and received approval from the United States Department of Agriculture (USDA) in 1988, only three years after the discovery of its etiological agent (Holland *et al.*, 1985; Holland and Ristic, 1993). The vaccine has been successfully used in over 1.5 million horses (Ristic *et al.*, 1988; Goetz *et al.*, 1992). This achievement resulted from early understanding of the mechanisms of protective immunity of the disease (Woldehiwet and Ristic, 1993). Experimental recombinant vaccines have also been produced against *R. rickettsii*, and against *R. conorii* and successfully tested in guinea pigs (McDonald *et al.*, 1988; Vishwanath *et al.*, 1990). Vaccines are not available for prevention of other rickettsial diseases of animals.

Antibiotics such as tetracyclines or chloramphenicol are highly effective against rickettsial diseases (McDade and Fishbein, 1988). Prompt institution of antibiotic therapy is the single most effective measure for preventing morbidity and mortality due to rickettsial infections, with the occasional exception of fulminant or complicated cases (McDade and Fishbein, 1988). Although they are rickettsiostatic rather than rickettsiacidal (Spicer *et al.* 1981; Wisseman and Ordoñez, 1986), tetracyclines and chloramphenicol remain the only proven therapies for rickettsial diseases in humans (McDade and Fishbein, 1988).

There are no controlled data suggesting that any specific derivative of tetracycline is superior to the others in terms of clinical response, but the high blood and tissue levels and long half-life of the lipophilic tetracyclines

(minocycline, doxycycline) give them a theoretical advantage (McDade and Fishbein, 1988).

1.3 Aquatic rickettsial o . .sms

The rickettsia-like organisms (RLO) found in aquatic organisms have been recently summarised by Bower *et al.* (1994), and Fryer and Lannan (1994). Some reports described a pathogenic effect in the host (Fryer *et al.*, 1990, Brocklebank *et al.*, 1992; Olsen *et al.*, 1993; Cvitanich *et al.*, 1995), while others suggest that RLO can infect some aquatic organisms without causing disease or damage to the host (Harshbarger *et al.*, 1977; Meyers, 1979; Morrison and Shum, 1982; Comps, 1983; Elston, 1986; Bower and Figueras, 1989; Le Gall *et al.*, 1992). Diagnosis is usually based on histological findings, characterized by intracytoplasmic inclusions (Bower *et al.*, 1994). Many of these microorganisms have been included in the order Rickettsiales; however, further taxonomic characterizations have not been made (Bower *et al.*, 1994; Fryer and Lannan, 1994). Some RLO have been reported as being pathogenic to the host, but no control methods or treatments are known (Bower *et al.*, 1994).

Rickettsial organisms in shellfish

Rickettsia-like intracellular organisms belonging to the order Rickettsiales infect *Crassostrea angulata* and several other species of oysters in many parts

of the world (Comps and Deltreil, 1979; Bower *et al.*, 1994). Microcolonies are frequently observed in the cytoplasm of epithelial cells of the gills and digestive glands. Infections are usually light and not associated with disease; most species are probably non-pathogenic (Bower *et al.*, 1994).

Rickettsia-like organisms have been described in different mussel species such as *Mytilus trossulus*, *M. galloprovincialis*, *M. californianus* and *M. edulis* (Harshbarger *et al.*, 1977; Comps, 1983; Bower and Figueras, 1989), and a wide variety of other marine bivalves, with an ubiquitous distribution. Clams such as *Mercenaria mercenaria*, *Ruditapes philippinarum*, *Donax trunculus*, and *Tapes japonica* are also infected with RLO (Meyers, 1979; Comps, 1983; Elston, 1986).

An intracellular bacterium belonging to the order Rickettsiales has also been described in scallops such as *Placopecten magellanicus*, *Patinopecten yessoensis*, *Pecten maximus*, and *Argopecten irradians* (Morrison and Shum, 1982; Comps, 1983; Getchell, 1991; Le Gall *et al.*, 1992). The parasite has a global geographic distribution and it is commonly found in epithelial cells of the gills, digestive gland, or kidney. This organism rarely causes disease. However, at least on one occasion it has been linked to mass mortalities in *P. maximus* and *P. magellanicus* (Bower *et al.*, 1994).

Rickettsial organisms in crustaceans

Rickettsia-like organisms occur in prawns *Pandalus platiceros*, producing the so-called stained prawn disease, reported in one prawn from Sabine Channel in the Strait of Georgia, British Columbia, Canada (Bower *et al.*, 1994). The organism infects fixed macrophages on the surface of the digestive gland, producing an intense focal melanotic response (Bower *et al.*, 1994). These compact melanotic whorls of cells then migrate towards the cuticle and are supposedly shed at the moult (Bower *et al.*, 1994). In laboratory assays, mortalities reach 84% in orally infected prawns in comparison to 14% in the controls. The organisms can be transmitted through feeding (orally), and horizontally in the water without physical contact (Bower *et al.*, 1994).

Another rickettsial organism affects the shrimp *Penaeus monodon* (Malaysia and Indonesia), *P. vannamei* (Texas), *P. merguiensis* (Singapore), *P. marginatus*, and experimentally *P. stylirostris* (Hawaii) (Brock and Lightner, 1990; Lightner *et al.*, 1992). The disease has been called Texas necrotizing hepatopancreatitis (TNHP) (Bower *et al.*, 1994). The microorganisms measure about 0.2-0.7 x 0.8-1.6 μm, are basophilic with haematoxylin and eosin (HE), gram-negative, Feulgen positive, and are located in specific target cells filling cytoplasmic vacuoles (Bower *et al.*, 1994). These microorganisms usually infect epithelial and somatic cells of the hepatopancreas, producing hypertrophy, inflammation, necrosis and multiple granulomatous lesions (Bower *et al.*, 1994). Interestingly, infections in *P. monodon* occurred concurrently with a Gram-

negative bacterium, *Monodon baculovirus*, and a reo-like virus (Bower *et al.*, 1994).

Crab species such as *Carcinus mediterraneous, Paralithodes platypus,* and *Cancer magister*, also host rickettsia-like and chlamydia-like organisms in the Mediterranean coast of France, the Bering Sea coast of Alaska and off the Washington State coast in the United States (Bower *et al.*, 1994). Experimental infection produced death within 15 days (Bower *et al.*, 1994), but the impact and distribution in wild populations is unknown.

In *C. magister* seasonal occurrence of the disease was correlated with low water temperatures. Multiplication of the organism is intracellular and apparently by binary fission, with membrane-bound colonies displacing cytoplasmic organelles in epithelial cells of the hepatopancreas (Bower *et al.*, 1994). Massive systemic infection produces swelling (described as hypertrophy by authors) of fixed tissue phagocytes due to heavy intracellular proliferation of the organism (Bower *et al.*, 1994). Cell necrosis and moderate to dense accumulation of haemocytes may also be observed (Bower *et al.*, 1994).

Crayfish (*Cherax quadricarinatus)* inhabiting freshwater streams in Queensland and northern Australia are parasitized by RLO (0.5 x 0.16 μm in size) (Ketterer *et al.*, 1992). The pathogen produces a systemic infection and high mortalities in commercial farms. It is usually observed within a thin-walled vacuole, and evokes prominent hyperplasia and hypertrophy of endothelial and interstitial cells of gills and other organs, with large granular basophilic

cytoplasmic inclusion bodies (Ketterer *et al.*, 1992). Apparently, poor water quality predisposes crayfish to epibiont fouling and contributes to the severity of the disease (Ketterer *et al.*, 1992).

Rickettsial organisms in finfish

Rickettsial infections have been reported in several freshwater (Table 1.1) and saltwater (Table 1.2) fish. Recently, a rickettsia-like organism (0.86 x 0.63 μm) was the cause of an outbreak with mass mortalitiy among pond-reared *Tilapia* in Taiwan (Chern and Chao, 1994). Affected species included *Oreochromis mossambicus, Oreochromis niloticus, Oreochromis aureus, Tilapia zillii,* and *Tilapia hornorum* and some hybrids. Fish had hepatic lesions characterized by diffuse necrotizing hepatitis, severe vasculitis, fibrin thrombi, and granuloma formation. The RLOs were observed inside macrophages near or in the centre of granulomas, especially in the spleen and kidney. Effacement of haematopoietic (spleen and head kidney) and renal tissue by inflammatory cells was often observed. In gills, proliferation of epithelial cells caused fusion of lamellae. Vacuolated macrophages containing the RLO were often seen. Low haematocrits and increased numbers of macrophages containing RLO were also observed (Chern and Chao, 1994).

The RLO causing outbreaks in Tilapia was isolated in the epithelioma papulosum ciprini (EPC) carp cell line. The disease was experimentally reproduced by intramuscular inoculation, and by cohabitation with eight *Tilapia*

species and the Cichlidae *Cichlasoma managuense.* Experimental inoculations produced higher mortalities at 15° C than at 30° C. No infection or disease was produced in eight other cichlid species (Chern and Chao, 1994). Oral administration of oxytetracycline at 30-50 mg/kg of body weight for 10-14 days controlled the disease.

A rickettsia-like organism (RLO) was described from the blue-eyed plecostomus (*Panaque suttoni),* a pet fish imported to the U.S.A. from Colombia (Khoo *et al.*, 1995). Numerous intracytoplasmic rickettsial organisms were observed in the cells of the mononuclear phagocytic system, and in macrophages of the heart, spleen, kidney, and liver (Khoo *et al.*, 1995). The microorganisms were within membrane-bound vacuoles, measured about 0.5 μm, and possessed a trilaminar cell wall. Tufts of fibrin indicative of endothelial damage were present within the interstitium of the kidney and spleen. Granulomatous lesions, necrosis and effacement of the haematopoietic elements were frequently observed. Other parasites such as trypanosomes, nematodes and embryonated eggs were also observed in liver, kidney, spleen and heart (Khoo *et al.*, 1995).

Recently, a rickettsia-like organism infecting juvenile sea-bass, *Dicentrarchus labrax,* reared in floating sea cages at 12-15 °C in the Mediterranean coast of France was described (Comps *et al.*. 1996). Focal necrosis of mesencephalic tissues associated with inflammatory reaction, and rickettsia-like organisms contained within vacuoles were seen in affected fish

(Comps *et al.*, 1996).

Table 1.1: Rickettsial organisms reported in freshwater finfish.

Host species	Country	M-I	Reference
Tetrodon fahaka	Egypt	M	Mohamed, 1939
Oreochromis mossambicus	Taiwan	M-I	Chern & Chao, 1994
Oreochromis niloticus	Taiwan		Chern & Chao, 1994
Oreochromis aureus	Taiwan		Chern & Chao, 1994
Tilapia zillii	Taiwan		Chern & Chao, 1994
Tilapia hornorum	Taiwan		Chern & Chao, 1994
Cichlasoma managuense [a]	Taiwan		Chern & Chao, 1994
Panaque suttoni	Colombia[b]	M	Khoo *et al.*, 1995
Oncorhynchus mykiss	Germany	M	Ozel & Schwanz-Pfitzner, 1975
	Chile	M	Bravo, 1994a,b
	Chile	M-I	Gaggero *et al.*, 1995
Oncorhynchus kisutch	Chile	M-I [a]	Cvitanich *et al.*, 1991
	Chile	M-I	Gaggero *et al.*, 1995
Salmo salar	Chile	M-I [a]	Garcés *et al.*, 1991
	Chile	M-I	Cvitanich *et al.*, 1995

[a] Susceptible in experimental inoculation
[b] Fish imported from Colombia, diagnosed in U.S.A
M Microscopic diagnosis of the organism
I Isolation of the organism in cell lines

Table 1.2: Rickettsial organisms reported in saltwater finfish.

Host species	Country	M-I	Reference
Callionenymus lyra	Great Britain	M	Davies, 1986
Dicentrarchus labrax	France	M	Comps *et al.*, 1996
Oncorhynchus kisutch	Chile	M	Bravo & Campos, 1989
		M-I	Fryer *et al.*, 1990, 1992
		M-I	Cvitanich *et al.*, 1991
		M	Branson & Nieto, 1991
		M-I	Garcés *et al.*, 1991
		M	Lannan *et al.*, 1991
Oncorhynchus mykiss	Chile	M	Cvitanich *et al.*, 1991
		M	Fryer *et al.*, 1992
Oncorhynchus tshawytscha	Chile	M	Cvitanich *et al.*, 1991
		M	Fryer *et al.*, 1992
	Canada	M	Evelyn, 1992
		M	Brocklebank *et al.*, 1993
Oncorhynchus gorbuscha	Canada	M	Evelyn, 1992
Salmo salar	Chile	M	Cvitanich *et al.*, 1991
		M	Garcés *et al.*, 1991
		M	Fryer *et al.*, 1992
	Canada	M	Brocklebank *et al.*, 1992
		M	Evelyn, 1992
		M-I	Brocklebank *et al.*, 1993
	Norway	M	Olsen *et al.*, 1993
	Ireland	M	Rodger & Drinan, 1993

M Microscopic diagnosis of the organism
I Isolation of the organism in cell lines

1.4 Salmonid rickettsial septicaemia (SRS).

Isolation and growth of *Piscirickettsia salmonis*

Piscirickettsia salmonis is an obligate intracellular Gram-negative, coccoid, often pleomorphic, non-motile, non-encapsulated organism ranging in size from 0.5 to 1.8 μm in diameter (Cvitanich *et al.*, 1991). *P. salmonis* is periodic acid-Schiff (PAS), acid-fast and Giménez-negative, but stains well with HE, Giemsa and methylene blue (Cvitanich *et al.*, 1991; Branson and Nieto, 1991).

Piscirickettsia salmonis has been cultured in six fish cell lines maintained in buffered minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). Typically, piscirickettsial growth is determined by the gradual appearance of cytopathic effect (CPE) in cell monolayers. Characteristic CPE includes cell rounding and the development of one or more large vacuoles within the cytoplasm (Cvitanich *et al.*, 1991; Garcés *et al.*, 1991). Four susceptible fish cell lines have been derived from salmonid species: Chinook salmon embryo (CHSE-214); chum salmon (*Oncorhynchus keta* Walbaum) heart (CHH-1); coho salmon embryo (CSE-119); and rainbow trout gonad (RTG-2). The other two susceptible cell lines are from warm water fish species, epithelioma papulosum ciprini (EPC) from *Cyprinus carpio L.*, and the fathead minnow (*Pimephales promelas* Rafinesque) line of epithelial cells (FHM), exclusive of the caudal fin (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). Conversely, two cell lines, brown bullhead (*Ictalurus nebulosus* Lesueur) posterior trunk (BB), and bluegill (*Lepomis*

macrochirus Rafinesque) fry caudal trunk cells (BF-2), did not develop CPE after infection with *P. salmonis* and incubation at 15 °C for 30 days (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). The reasons for the observed differences in susceptibility among cell lines have not been studied, although such studies may reveal important information regarding intracellular survival mechanisms of the pathogen.

Presentation of SRS

Natural SRS outbreaks in salt water

The first outbreaks appeared in 1988 in the Huito channel, Calbuco, Chile, caused massive mortalities (up to 90%) and affected only coho salmon cultured in the area (Bravo and Campos, 1989). Due to the unusual characteristics of this new disease, it was initially named U.A. (unknown agent), "coho salmon syndrome", or "Huito disease" (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). This disease causes substantial economic losses to the salmon aquaculture industry of southern Chile (Bravo and Campos, 1989; Cassigoll, 1994). Epidemics typically occur 10-12 weeks after fish are introduced into seawater, usually from March through August (Fall-Winter), and may last up to 10 weeks before subsiding (Cvitanich *et al.*, 1991). The disease has also been described in several other species of salmonids cultured in salt water in southern Chile, including Atlantic salmon (Cvitanich *et al.*, 1991), rainbow trout, and Chinook salmon (Lannan and Fryer, 1993).

The geographical distribution of this condition may be wider than previously estimated since similar pathogenic rickettsial organisms have been reported in salmonids cultured in saltwater sites in Canada (Brocklebank *et al.*, 1992, 1993), Ireland (Rodger and Drinan, 1993), and Norway (Olsen *et al.*, 1993). Mortalities in these countries did not reach the importance and magnitude of the Chilean outbreaks, and it is not clear whether the agent producing these outbreaks is truly *P. salmonis*. However, isolates from the Canadian and Irish outbreaks reacted positively with a polyclonal antibody made against *P. salmonis* (Brocklebank *et al.*, 1993; Alday-Sanz *et al.*, 1994).

Natural SRS outbreaks in fresh water

Although piscirickettsiosis was originally reported in fish raised in salt water (Bravo and Campos, 1989; Fryer *et al.*, 1992), outbreaks of SRS have since been reported in Chilean rainbow trout and coho salmon cultured in a freshwater lake (Bravo, 1994a b; Gaggero *et al.*, 1995). Lesions observed in affected fish and the *in vitro* growth characteristics of a freshwater isolate were similar to those observed previously from saltwater outbreaks (Gaggero *et al.*, 1995).

Rickettsial outbreaks in freshwater-reared salmonids are limited to those occurring in Chile. Although the geographical distribution of freshwater outbreaks of RLO is not restricted to salmonids (Chern and Chao, 1994; Khoo *et al.*, 1995), no information is available comparing *P. salmonis* with the RLO

affecting non-salmonid fish (*Tilapia sp.*, and *Plecostomus sp.*). An apparently new RLO was recently reported from Atlantic salmon held in fresh water, salt water and an estuary in Chile (Cvitanich *et al.*, 1995). Whether the etiologic agent of these new outbreaks is *P. salmonis* remains uncertain. This pathogen, known as UA2 (unknown agent 2), differed in several important respects from *P. salmonis*: it did not react with a rabbit anti-*P. salmonis* polyclonal antibody; it occurred either extra or intracellularly; and it grew in BB and BF-2 cell lines, both of which were previously reported as non-susceptible to *P. salmonis* (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991; Cvitanich *et al.*, 1995).

Pathology

Clinical signs and gross external lesions

The clinical signs and gross lesions described for naturally and experimentally infected fish are similar (Cvitanich *et al.*, 1991). Clinical signs include lethargy, anorexia, darkening of the skin, respiratory distress, and surface swimming (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). Skin lesions include perianal and periocular haemorrhages, petechiae in the abdomen, shallow haemorrhagic ulcers varying in size from 0.5 cm to 2 cm in diameter, and firm white nodules up to 1 cm in diameter (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). Bilateral exophthalmia and ulcerative stomatitis have also been described. However, clinical signs and external lesions are occasionally absent in affected fish (Branson and Nieto, 1991; Cvitanich *et al.*,

1991).

Gross internal lesions and histological changes

In natural outbreaks, the most characteristic lesions observed in heavily infected fish are off-white to yellow subcapsular nodules, up to 2 cm in diameter, scattered throughout the liver (Cubillos *et al.*, 1990; Branson and Nieto, 1991; Cvitanich *et al.*, 1991; Garcés *et al.*, 1991; Brocklebank *et al.*, 1993). Other macroscopic changes include ascites, peritonitis, general pallor and diffuse swelling and presence of multifocal pale areas in the kidney and spleen (Bravo and Campos, 1989; Cubillos *et al.*, 1990; Branson and Nieto, 1991). In coho salmon, renal lesions have been interpreted as chronic damage characterized by fibrosis (Cubillos *et al.*, 1990). Petechiae and ecchymoses on the serosal surfaces of the pyloric ceca, swim bladder and caudal intestine also occur in Atlantic salmon (Brocklebank *et al.*, 1993).

Histological changes (detected by light microscopy) have usually been classified broadly as necrosis and inflammation (Garcés *et al.*, 1991). Commonly affected organs are liver, spleen, intestine and haematopoietic tissue of the kidney (Garcés *et al.*, 1991). Specific lesions include multifocal to generalized coagulative necrosis, presence of fibrin thrombi within small blood vessels with necrosis of the endothelium and infiltration by inflammatory cells (Branson and Nieto, 1991; Cvitanich *et al.*, 1991).

Microscopic lesions have also been described in brain, skeletal muscle,

skin, heart, intestine, gills and ovary (Cvitanich *et al.*, 1991). Specific lesions include mild to severe pericarditis, mild endocarditis and focal hyaline necrosis of the myocardium (Cvitanich *et al.*, 1991). Necrosis and inflammation of the lamina propria of the intestine, epithelial hyperplasia of the gill, fusion of secondary lamellae, and presence of RLO within blood spaces of the secondary lamella have also been detected (Branson and Nieto, 1991). The latter described renal tubular degeneration, pyogranulomatous myositis with ulceration of the overlying epidermis. Additionally, meningoencephalitis, focal pyogranulomatous branchitis, and pyogranulomatous splenitis with acute vasculitis and haemorrhage were detected in the Canadian outbreak (Brocklebank *et al.*, 1993).

The rickettsial organism infects various cells, including circulating monocytes, in which they replicate within variably sized, membrane-bound, intracytoplasmic vacuoles (Fryer *et al.*, 1990). Although varying numbers of organisms are frequently observed within these intracytoplasmic vacuoles, *P. salmonis* has also been found extracellularly, presumably as a result of cell lysis (Branson and Nieto, 1991; Cvitanich *et al.*, 1991).

Mixed infections of *P. salmonis* with *Renibacterium salmoninarum*, the causative agent of Bacterial Kidney Disease (BKD), and a pathogenic microsporidian protozoa have affected seawater cultured salmonids in Chile (Cvitanich *et al.*, 1991; Smith *et al.*, 1995). Recently, the microsporidian *Enterocytozoon salmonis*, has been reported as occurring in some fish of the

atypical freshwater SRS outbreaks (Cvitanich *et al.*, 1995).

Infection and intracellular survival

The location and distribution pattern of *P. salmonis* have been observed by electron microscopy in cell lines such as CHSE-214 (Chinook salmon embryo) by Fryer *et al.* (1990) and Cvitanich *et al.* (1991). Using scanning electron microscopy, 24 h after inoculation, microorganisms of 1 μm in diameter were observed attached to the exterior surfaces of CHSE-214 cells, and after 8 days of incubation, the organisms were released from ruptured host cells or were observed in the intercellular spaces (Fryer *et al.*, 1990). Tissues from naturally and experimentally infected fish had either individual or paired coccoid, often pleomorphic organisms, usually enclosed within membrane-bound cytoplasmic vacuoles, with some cellular debris in addition to the RLO (Cvitanich *et al.*, 1991). Organisms undergoing binary fission were also observed (Cvitanich *et al.*, 1991).

Piscirickettsia salmonis is surrounded by two membrane layers: a closely apposed inner or plasma membrane, and an external layer (cell wall), usually separated from the plasma membrane and occasionally observed with rippled appearance (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). Many organisms contained one or more electron-lucent spherical structures (Fryer *et al.*, 1990). Electron-dense areas containing ribosome-like structures dispersed throughout the cell, and fibrillar DNA-like material was localized in the central region

(Cvitanich *et al.*, 1991). Small electron-lucent vacuoles, variable in size, and apparently lacking any membrane, were also observed in some cells (Cvitanich *et al.*, 1991). The mechanisms by which *P. salmonis* infects cells, avoids the intracellular killing activity, and survives inside host cells are not clear.

Pathogenesis of SRS

The pathogenesis of piscirickettsiosis, or any other aquatic rickettsial organism, has yet to be clarified. Histopathological findings of naturally and experimentally infected fish have been described in advanced stages of the disease (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). No description of the sequence of histological changes and systemic dissemination of *Piscirickettsia salmonis* has been published.

Transmission of SRS

Despite the high impact of RLOs on the Chilean salmon industry, the mode of invasion into the host and the mechanisms of transmission in the natural environment are unclear (Cvitanich *et al.*, 1991; Garcés *et al.*, 1991; Bravo, 1994b).

Route of infection

The IP route is the only one which has been reported to be effective in experimentally infecting coho and Atlantic salmon (Cvitanich *et al.*, 1991;

Garcés *et al.*, 1991). Examination of tissues from experimentally IP infected coho salmon showed similar lesions to naturally infected fish (Cvitanich *et al.*, 1991). This route while producing infection, does not provide information regarding establishment of natural infections.

Natural outbreaks of SRS typically occur a few weeks after smolts are transferred to the sea (Fryer *et al.*, 1990; Branson and Nieto, 1991; Cvitanich *et al.*, 1991), which suggests that the oral transmission route might be especially important in the case of *P. salmonis*. It is not known if infection routes such as gill, skin or rectum, commonly used by other fish pathogens (Kanno *et al.*, 1989), are important in SRS transmission.

Horizontal transmission

Natural horizontal transmission in sea water has been reported in stocks of salmon showing mortalities two weeks after their introduction into infected sites, although the mechanisms of transmission are unknown (Bravo, 1994b). Elucidation of the natural mode(s) of infection and transmission of *P. salmonis* is a requirement to establish control methods for SRS (Lannan and Fryer, 1993).

Studies in aquaria regarding horizontal transmission have shown different results. Garcés *et al.* (1991), did not observe horizontal transmission in a group of non-inoculated coho salmon held in the same tank with IP injected fish. However, Cvitanich *et al.* (1991) showed that horizontal transmission of the RLO can occur in coho salmon held in sea water or fresh water without parasite

vectors. The presence of the pathogen was demonstrated using light microscopy and isolation of the RLO from mortalities of experimentally inoculated, and non-inoculated cohabitant fish.

Abundant RLO-laden cells occur in the large intestine of coho salmon, suggesting that the agent could be released in the feces, and survive long enough to infect other fish (Cvitanich *et al.*, 1991). However, viable *P. salmonis* could not be detected immediately after exposure to fresh water. Conversely, when suspended in salt water, infectious particles of *P. salmonis* were detected for 10 to 15 days (Lannan and Fryer, 1994). The capacity of *P. salmonis* to persist in salt water could be important epidemiologically.

Whether transmission is direct or through an intermediate host is unclear (Garcés *et al.*, 1991; Cvitanich *et al.*, 1991). Vectors may also play an important role in the natural transmission of SRS; as it occurs in mammals where rickettsial diseases are mainly transmitted by ticks (Woldehiwet and Ristic, 1993). External parasites, such as the haematophagous isopod *Ceratothoa gaudichaudii,* or sea lice are commonly found affecting cultured salmonids in Chile (Inostroza *et al.*, 1993; Sievers *et al.*, 1995). Recently, *P. salmonis* was detected by indirect fluorescent antibody technique (IFAT) in histologic preparations of *C. gaudichaudii* (Garcés *et al.*, 1994). However, the importance of this finding in the transmission of the disease requires further study.

Vertical transmission

Recently, vertical transmission of *P. salmonis* was reported in coho salmon by Bustos *et al.* (1994). Results of IFAT showed that 98.3% of the fingerlings from RLO-positive broodstock were positive to *P. salmonis*; in contrast, only 26.7% of the fingerlings from RLO-negative broodstock were positive (Bustos *et al.*, 1994). Due to the risk of congenital or true vertical transmission, the Chilean salmon farming industry screens broodstock as a preventive measure (Cassigoli, 1994).

Diagnostic methods for SRS

Salmonid rickettsial septicemia is usually diagnosed by gross lesions, and by the use of histochemical stains such as haematoxylin and eosin, Gram, Giemsa, acridine orange and Machiavello to detect the pathogen in smears or tissue sections (Fryer *et al.* 1990; Branson and Nieto, 1991; Cvitanich *et al.*, 1991; Lannan and Fryer, 1991). Although these techniques are nonspecific, they are relatively fast, and widely used for diagnosis of SRS.

The isolation of *P. salmonis* in cell lines is possible (Lannan and Fryer, 1991). However, the technique is time consuming, requires cell culture capabilitiy, and is difficult since the culture has to be performed without antibiotics, which increases the possibility that adventitious agents may proliferate and contaminate the culture (Lannan *et al.*, 1991; Bustos *et al.*, 1994).

Immunofluorescent antibody technique (IFAT) and immunohistochemistry with peroxidase-antiperoxidase (PAP) using a rabbit anti-*P. salmonis* polyclonal antibody, are alternative procedures to detect this pathogen (Lannan *et al.*, 1991; Alday-Sanz *et al.*, 1994). Enzyme-linked immunosorbent assay (ELISA) is also available (Cassigoli, 1994). These techniques are relatively fast, and more specific than histochemical stains, however, they are also more expensive, require special equipment, and are not practical for on-site diagnosis.

Control of SRS

Chemotherapy

In vitro, P. salmonis is sensitive to streptomycin, gentamicin, tetracycline (Fryer *et al.*, 1990), chloramphenicol, erythromycin, oxytetracycline, clarithromycin, and sarafloxacin (Cvitanich *et al.*, 1991), and resistant to penicillin (Fryer *et al.*, 1990), penicillin G, and spectinomycin (Cvitanich *et al.*, 1991). In practice, variable results occur with oral antibiotics (Cassigoli, 1994). The number of drugs used and losses due to this pathogen have increased progressively, apparently due to resistance to antibiotics (Bustos *et al.* 1994; Cassigoli, 1994). Among the available antibiotics, the quinolones, including oxolinic acid and flumequine are the most widely used to control outbreaks (Cassigoli, 1994). Quinolones act by interference with the bacterial DNA gyrase, inhibiting the supercoiling of DNA (Björklund, 1991).

Although the possibility of resistance to quinolones cannot be completely

eliminated, there are other factors that may influence the efficacy of the treatments. Several aquaculture antibacterials are antagonized by seawater cations (Barnes *et al.,* 1995). The *in vitro* minimum inhibitory concentrations of oxolinic acid, fluoroquinolones, and oxytetracycline were increased 40 to 60 fold when exposed to Mg^{2+}. Hence, the inactivity of antibiotics in the intestines of salmon held in the marine environment may be especially important due to the large amounts of sea water ingested (Barnes *et al.*, 1995). Differences regarding recommended effective dosages and periods of treatment have also been observed among pharmaceutical companies (Cassigoli, 1994).

Other quinolones such as enrofloxacine (Bayer®) and danofloxacine (Pfizer®) are used as IP injections. Good results have been achieved with these procedures, possibly because every fish receives a therapeutic dose (Cassigoli, 1994). Oxytetracycline, spiramicine, and florfenicol, and unspecified sulpha combinations have also been used (Cassigoli, 1994).

Other strategies have been used to decrease the vertical or congenital transmission of *P. salmonis*. Intraperitoneal injection of broodstock with antibiotics 30 to 60 days before spawning, and incorporation of antibiotics in the water during hardening of the eggs after fertilization, have also been used prophylactically (Bustos *et al.*, 1994).

Vaccines

There is general agreement that a vaccine would be the only solution to control SRS (Cassigoli, 1994), and several groups have established research programs towards a vaccine in Chile (Cassigoli, personal communication) and Canada. Only one vaccine trial using a bacterin has been published and the authors reported an immunoprotective effect in vaccinated fish compared to controls (Smith *et al.*, 1995). Although the results obtained are encouraging, they must be taken cautiously since the naturally low challenge. Additionally, *Renibacterium salmoninarum* was also detected infecting the experimental fish, and the trial was not long enough to determine whether a protective effect remained active in larger fish in which economic losses are more significant (Smith *et al.*, 1995).

Other methods

Another important factor predisposing development of SRS is stress (Branson and Nieto, 1991; Cassigoli, 1994). Fish positive to *P. salmonis* are observed in sea cages with neither clinical signs nor mortalities (Branson and Nieto, 1991). Outbreaks occur after smolt transfer (an osmotic stress), water temperature changes, and severe storms (Branson and Nieto, 1991). In salmonid populations with a high prevalence of *P. salmonis* infection, the reduction of stressful general husbandry practices such as grading, sampling, and net changes, are effective in preventing outbreaks (Cassigoli, 1994).

Early removal of mortalities and clinically diseased fish, appropriate disposal of blood from harvested fish, reducing fish stocking densities, decreasing biomass per site and region, and providing periods of site fallowing, are management practices suggested by the Salmon and Trout Growers Association of Chile (Cassigoli, 1994). Strategic measures such as examination and diagnosis of infected broodstock, rejection of eggs from positive broodstock, and individual batch incubation of eggs, are management practices proposed by Bustos *et al.* (1994) based on their observations of vertical transmission.

1.5 Conclusions

Recently, reports of rickettsial diseases affecting several finfish species have been increasing around the world. Due to the intracellular location of these organisms, control of these pathogens via antibiotics has been variable. *Piscirickettsia salmonis* continues to cause large losses in the Chilean salmon industry. This increases the production cost of the fish as a result of the mortalities and costs associated with repeated use of antibiotics.

No comparisons between isolates from different countries have been reported. Further information regarding horizontal and vertical transmission, pathogenesis, intracellular survival, and immunity is needed in order to establish control strategies for SRS. Identification of risk factors in the production cycle may help to avoid unnecessary management practices that may induce

outbreaks in populations at risk.

1.6 Objectives

The objectives of this study are to describe the pathogenesis of *P. salmonis*, compare transmission by IP, oral, and gill inoculation routes, and evaluate the importance of physical contact in the transmission of *P. salmonis* in Atlantic salmon, *Salmo salar*, held in fresh water. *In vitro* studies using fish cell lines will be utilized to study the infection process comparing susceptible and non-susceptible cell lines.

2. INFECTION OF SALMONID AND NON-SALMONID FISH CELL LINES WITH *PISCIRICKETTSIA SALMONIS:* LIGHT AND TRANSMISSION ELECTRON MICROSCOPIC STUDY.

2.1 Summary

Piscirickettsia salmonis is the etiological agent of Salmonid Rickettsial Septicaemia (SRS). This organism grows and produces a characteristic cytopathic effect (CPE) in several fish cell lines but not in those from species such as brown bullhead *Ictalurus nebulosus* Lesieur, posterior trunk (BB), and bluegill *Lepomis macrochirus* Rafinesque, fry caudal trunk (BF-2). Chinook salmon embryo (CHSE-214) and BB cells were inoculated with *P. salmonis*, incubated at 15 °C for 78 days, and studied by light microscopy (LM) and transmission electron microscopy (TEM). Cytopathic effect (CPE) appeared after 6 days post-infection in CHSE-214, and after 45 days post-infection in BB cell line. Transmission electron microscopic examination of BB cells after 78 days post-infection, revealed *P. salmonis* within membrane-bound vacuoles or free within the cytoplasm and the extracellular space. Subsequently, two susceptible cell lines, Atlantic salmon fibroblast (ASF) and CHSE-214, and one less susceptible (BB) fish cell line were infected with *P. salmonis* and studied by LM and TEM at one, seven, and fourteen days post-infection. Cytopathic effect was detected in ASF and CHSE-214 by seven days post infection. Rickettsiae within intracytoplasmic vacuoles and free in the cytosol were observed in CHSE-214 and ASF cell lines. Rickettsiae were observed free in the cytosol of BB

cells from one day post-infection, but CPE was not observed. The BB cells are susceptible to infection with *P. salmonis* but exhibit a slower pattern of invasion with this organism compared to other cell lines described in the literature.

2.2 Introduction

Piscirickettsia salmonis is an obligate intracellular Gram-negative, spherical to coccoid, often pleomorphic, non-motile, non-encapsulated organism ranging from 0.5 to 1.8 μm in diameter (Cvitanich *et al.*, 1991; Branson and Nieto, 1991). The microorganism causes the economically important disease Salmonid Rickettsial Septicemia (SRS) (Cvitanich *et al.*, 1991). Although the etiologic agent has been identified and characterized (Fryer *et al.*, 1990), little is known about the mechanisms of cell entry and intracellular survival, and about the pathogenesis of the disease. In salmonid species, the organism infects various cells in which it replicates within one or more variably sized, membrane-bound, intracytoplasmic vacuoles (Fryer *et al.*, 1990). A higher susceptibility of coho salmon to SRS over other salmonid species has also been observed (Cassigoli, 1994). However, the reasons for this are unknown.

In vitro models have been widely used as substitutes for *in vivo* models to study the infection, replication and pathogenic mechanisms of intracellular microorganisms, thus reducing the need for animal experimentation. *Piscirickettsia salmonis* has been grown in six fish cell lines (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). Four of these fish cell lines are derived from salmonid species including Chinook salmon, *Oncorhynchus tshawytscha* Walbaum, embryo (CHSE-214); chum salmon, *Oncorhynchus keta* Walbaum, heart (CHH-1); coho salmon, *Oncorhynchus kisutch* Walbaum, embryo (CSE-119); and rainbow trout, *Oncorhynchus mykiss* Walbaum, gonad (RTG-2). The other two

cell lines are from non-salmonid fish species: epithelioma papulosum cyprini, *Cyprinus carpio* Linnaeus (EPC), and fathead minnow, *Pimephales promelas* Rafinesque (FHM). Typically, rickettsial growth *in vitro* is determined by the gradual appearance of cytopathic effect (CPE) in cell monolayers. Characteristic CPE includes cell rounding and the development of one or more large intracytoplasmic vacuoles which may or may not contain the agent (Cvitanich *et al.,* 1991; Lannan *et al.*, 1984).

Two cell lines, brown bullhead, *Ictalurus nebulosus* Lesueur, posterior trunk (BB), and bluegill, *Lepomis macrochirus* Rafinesque, fry caudal trunk (BF-2), have failed to develop a CPE after infection with *P. salmonis* and incubation at 15 °C for 30 days (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). It was not determined whether *P. salmonis* was able to infect these cell lines, nor were the mechanisms of intracellular survival studied.

The aim of the present study was to compare the infectivity of *P. salmonis* in permissive and non-permissive fish cell lines and to characterize some of the morphological events associated with this infection.

2.3 Materials and Methods

Cell culture and inoculation of the monolayers

Antibiotic-free minimal essential medium (MEM) (Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) was buffered with sodium bicarbonate (2.2 g/L) (Sigma Chemical Co., St Louis, MO, USA), adjusted to pH 7.0, filtered in a SFCA 0.2 μm Nalgene bottle top filter (Nalge Company, Rochester, NY, USA), and stored at 4 °C. Cultured cells were suspended in sterile MEM plus 2% sterile fetal bovine serum (Cansera, Rexdale, ON, Canada), seeded into 75 cm^2 culture flasks (Corning Glass Works, Corning, NY, USA), and incubated at 15 °C.

The cells were inoculated 24 h after seeding by adding 1 ml of supernatant from an infected CHSE-214 cell line showing 100% CPE. When possible, the final infectious dose was estimated by calculating the 50% tissue culture infection dose ($TCID_{50}$) according to the method described by Hsiung (1994). Infected and non-infected negative controls were incubated in similar conditions at 15 °C. Development of CPE was routinely assessed and photographed with a Nikon TMS (Japan) phase contrast inverted microscope. Acridine orange staining was also used to identify the organism. Thin smears were made from supernatant from infected culture on cleaned glass slides. Smears were dried at 35 °C for 15 minutes, fixed in 100% methanol until evaporation, stained with 1% acridine orange aqueous solution for 30 seconds, rinsed and dried at 35 °C. Smears were observed under oil immersion at 1000x

in a Zeiss Axioplan universal epifluorescent microscope. Confirmation of the presence of *P. salmonis* in the cells was assessed by indirect fluorescent antibody technique (IFAT) according to the method of Lannan and Fryer (1993) (Appendix A) using a rabbit polyclonal antibody produced against *P. salmonis* strain FL-89 (ATCC, VR1361) and donated by Dr. J. L. Fryer (Department of Microbiology, Hatfield Marine Science Center, Oregon State University, U.S.A.).

Transmission electron microscopy (TEM)

The monolayer was washed using filtered Hanks' balanced salt solution (Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) with trypsin (0.5 g/l) and ethylene diaminetetraacetic acid (EDTA) (0.2 g/l) at pH 7.0, to remove all non-adherent material. Cells were separated in trypsin-EDTA solution for 1-3 min and washed twice with phosphate buffered saline (PBS) in 1.7 ml polypropylene conical micro-centrifuge tubes (VWR Scientific, London, ON, Canada). The cell pellet was fixed in place with 2% glutaraldehyde (Sigma Co., USA) in 0.1 M Sorensen's phosphate buffer pH 7.4 for 90 min at room temperature, washed three times in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 60 min at room temperature (20-22 °C). Pellets were washed in 0.1 M phosphate buffer for 60 min and dehydrated through a graded ethanol series: 50% (1 x 10 min), 70% (2 x 10 min), 95% (2 x 10 min), 100% (2 x 15 min). Following two changes (10 min each) of propylene oxide (PO), the tissues were infiltrated in 50:50

(Epon/Araldite : PO) for 60 min, 75:25 (Epon/Araldite : PO) for 60 min, and 100% Epon/Araldite overnight under vacuum in a desiccator. Samples were then embedded directly in the same polypropylene tubes, labelled, and polymerized under vacuum for 15 h at 65-70 °C.

Semi-thin (0.3-0.5 μm) sections were obtained using an Ultracut E Microtome (Reichert-Jung, Austria), stained with 1% toluidine blue in a 1% borax solution for 1-2 min, covered with a coverslip mounted with FLO-TEXX® mounting medium (Lerner Laboratories, New Haven, CT, USA) and examined using a Nikon 104 Labophot (Japan) light microscope. Ultrathin sections were cut at 60-90 nm, collected on 200 mesh copper grids, stained with 5% uranyl acetate in 50% ethanol for 30 min, rinsed in distilled water and post stained with Sato's lead stain for 2 min. Ultrathin sections were examined using Hitachi H-600 and H-7000 transmission electron microscopes (TEM). Exposures were obtained using 3¼" x 4" ESTAR thick base 4489 Kodak electron microscope film (Eastman Kodak Inc., NY, USA), and the film was processed routinely. Prints were obtained on F grade Kodak polycontrast III RC glossy paper.

Susceptibility to *Piscirickettsia salmonis* infection

This experiment was designed as a preliminary effort to characterize the morphological events associated with the apparent resistance of some cell lines to infection with *P. salmonis.* Chinook salmon embryo (CHSE-214) (ATCC CRL 1681), and brown bullhead (BB) (ATCC CCL 59) cells were inoculated with *P.*

salmonis at a 50 fold ($2x10^{-2}$) final dilution. The selected strain of *P. salmonis* was a coho salmon isolate, obtained from the American Type Culture Collection (ATCC VR 1361) and was previously designated as the type strain (Fryer *et al.*, 1992). Uninoculated CHSE-214 and BB cells were maintained as controls. Cells were incubated at 15 °C, routinely observed by light microscopy (LM), and processed for TEM 78 days post-infection (DPI).

Infection mechanisms of *Piscirickettsia salmonis*

In a second experiment, two susceptible salmonid cell lines, Atlantic salmon, *Salmo salar* Linnaeus, fibroblasts (ASF), produced in the Department of Fisheries and Oceans (DFO) Canada and donated by Dr. R. Cawthorn (Atlantic Veterinary College, UPEI, PE, Canada), and Chinook salmon embryo cells (CHSE-214) were inoculated with *P. salmonis.* A non-susceptible, non-salmonid cell line, brown bullhead (BB), was also inoculated with *P. salmonis.* The cell lines were given a dose of 25 $TCID_{50}$/ml, incubated at 15 °C, and studied by LM and TEM 1, 7, and 14 DPI. Uninoculated CHSE-214, ASF and BB cells were maintained as controls, observed weekly by LM and processed for TEM 14 DPI.

The strain of *P. salmonis* used in this experiment was isolated from naturally infected Atlantic salmon reared in salt water in southern Chile, donated by Dr. Enrique Madrid (Fish Pathology Laboratory, Marine Harvest McConnell, Puerto Montt, Chile) and was named strain NCR 1010. It was identified as *Piscirickettsia salmonis* by the use of the rabbit polyclonal antibody.

2.4 Results

Susceptibility to *P. salmonis* infection

Cytopathic effect in CHSE-214 cells was first detected at 6 days post-infection; 100% CPE characterized by vacuolation and a partially detached monolayer was observed 20 days post-infection. In BB cells, CPE first appeared 45 days post-infection and 100% CPE was evident at 78 days post-infection. The CPE was similar to that seen in CHSE-214 cells. Acridine-orange stained smears of infected BB cell cultures showed rickettsial organisms within the cytoplasm, as well as free in the extracellular space adjacent to destroyed cells. Non-infected BB cells (controls) did not showed CPE (no vacuolation or cell lysis) as observed in infected cells.

Transmission electron microscopy revealed that non-infected BB cells (controls) had normal cellular integrity, with a characteristic fibroblast-like morphology and presence of pseudopodia (Figure 2.1 a). Infected BB cells were vacuolated and contained a variable number of typically coccoid organisms usually enclosed within membrane-bound vacuoles located randomly throughout the cytoplasm (Figure 2.1 b). Some vacuoles contained what appeared to be cellular debris in addition to *P. salmonis*. Small electron-lucent empty vacuoles of variable size were also observed throughout the cytoplasm in some cells (Figure 2.1 b,c).

Piscirickettsia salmonis was also seen free within the cytoplasm (Figure 2.1 c) or outside the cell (Figure 2.1 d). A double cell wall was frequently

observed around rickettsia present in the cytoplasm of BB cells (Figure 2.1 c). The rickettsial cell wall was occasionally separated from the plasma membrane (Figure 2.1 b) or had a rippled appearance (Figure 2.1 c,d).

The rickettsial cytoplasm often contained an abundance of electron dense ribosomes usually dispersed throughout the cell. Single or multiple nucleoid regions, often with electron-dense central areas believed to be coagulated DNA, were located peripherally or eccentrically within the cell (Figure 2.1 e).

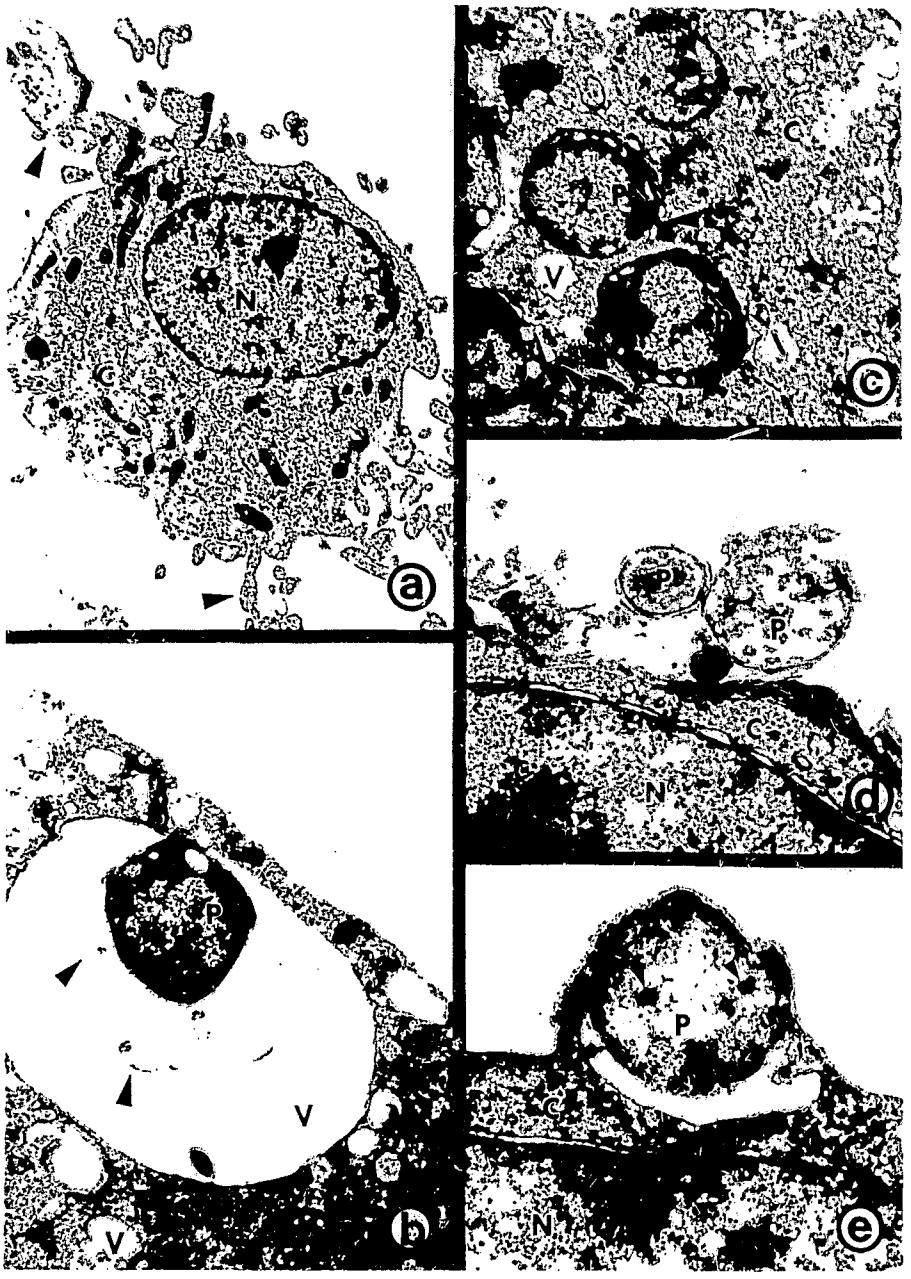

Figure 2.1: Electron micrographs of Brown Bullhead (BB) cell lines. **a:** Uninfected BB cell line (control) after 78 days of incubation showing nucleus (N), cytoplasm (C) and pseudopodia (arrowhead) (x 8,000); **b:** *Piscirickettsia salmonis* (P) enclosed within a membrane bound vacuole (V) in a BB cell. The bilaminated rickettsial cell wall is separated from the plasma membrane (arrowhead). Other small vacuoles (V) can be observed throughout the cytoplasm (x 21,000); **c:** *Piscirickettsia salmonis* (P) within the cytoplasm (C) of a BB cell. Note the rippled appearance (arrowhead) of the bilaminated cell wall (x 25,000); **d:** *Piscirickettsia salmonis* (P) close to a BB cell, showing the host cytoplasm (C) and nucleus (N) (x 25,000); **e:** *Piscirickettsia salmonis* apparently being phagocytised and included into the cytoplasm (C) of a BB cell. Note nucleoid regions with electron-dense central areas (arrowhead) inside *P. salmonis*. Nucleus (N) (x 30,000).

Infection mechanisms of *P. salmonis*

The comparison of salmonid and non-salmonid cell lines showed important differences in the appearance of the CPE by LM, and in the cellular infection pattern observed by TEM (see Table 2.1).

Monolayers of the two infected salmonid cell lines were confluent and showed clear CPE (characterized by several foci of vacuolated cells) at 7 DPI. By 14 DPI the CPE had spread throughout the monolayer with some areas of detached cells in CHSE-214 (Figure 2.2 a) and large numbers of vacuolated ASF cells (Figure 2.2 e). Cytopathic effect was not observed in the BB cell line during the 14 days of observation (Figure 2.2 c), but infected cells did not reach 100% confluency as observed in uninfected controls 14 DPI (Figure 2.2 d). Uninfected CHSE-214, ASF, and BB cells were confluent and maintained their morphologic appearance during the 14 days of culture (Figure 2.2 b, d, f).

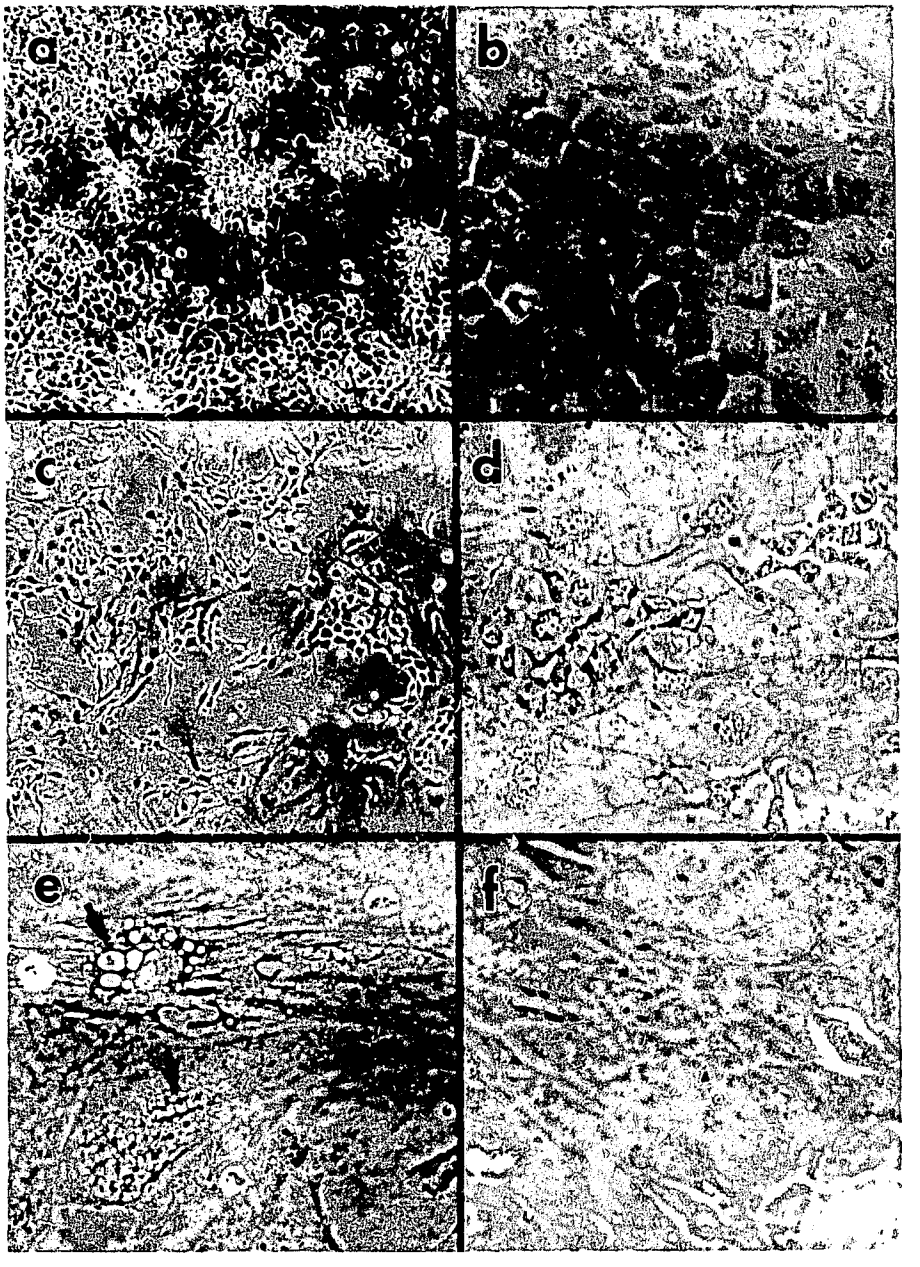

Figure 2.2: Cell lines observed by inverted light microscopy. **a:** CHSE-214 cell line 14 DPI with *Piscirickettsia salmonis*. Several foci (arrows) of CPE are observed in the monolayer (x 100). **b:** Uninoculated control CHSE-214 cell line 14 DPI, showing a confluent monolayer (x 400). **c:** BB cells showing lack of confluency, absence of CPE, and normal appearance 14 DPI with *P. salmonis* (x 100). **d:** Uninoculated control BB cells 14 DPI, showing a confluent monolayer (x 400). **e:** ASF cell line 14 DPI with *P. salmonis*. Cytopathic effect (arrows) is observed in a group of cells (x 400). **f:** Confluent uninoculated control ASF cells 14 DPI (x 400).

TEM examination revealed that non-infected ASF cells (controls) demonstrated normal morphology (Figure 2.3 a). Infected CHSE-214 and ASF cells revealed free intracytoplasmic rickettsia after 1 DPI. However, the cells maintained their general integrity and shape. At 1 DPI of CHSE-214 and ASF cells, *P. salmonis* was also observed within intracytoplasmic vacuoles (phagosome), with the vacuolar membrane closely attached to the rickettsia. By 7 days post infection, salmonid cell lines showed *P. salmonis* inside vacuoles with some separation between the vacuolar membrane and the rickettsia. Organisms free in the cytoplasm were also observed in CHSE and ASF cell lines by 7 DPI. The cytoplasm of ASF cells 14 DPI showed extensive vacuolation (Figure 2.3 b), and mitochondrial disruption (Figure 2.3 c). Large dilated vacuoles sometimes containing rickettsia and cellular debris were commonly observed in CHSE-214 cells at 14 DPI (Figure 2.4 a, b). *Piscirickettsia salmonis* was also observed free in the cytoplasm within CHSE-214 cells 14 DPI (Figure 2.4 b,c). Electron-lucent and electron-dense areas were more easily distinguished in the rickettsial organisms at 14 DPI whereas this differentiation was more difficult to make at 1 or 7 DPI.

The BB cells 1 DPI had numerous empty vacuoles throughout the cytoplasm (Figure 2.5 a, b). Extracellular rickettsiae were also observed (Figure 2.5 c). Cells containing free intracytoplasmic rickettsiae but maintaining their general integrity and shape were commonly observed (Figure 2.5 d). BB cells containing abundant rickettsiae free in the cytoplasm were observed 7 and 14

DPI (Figure 2.5 e). At higher magnification the rickettsiae had electron-lucent areas and a double membrane (Figures 2.6 a, b).

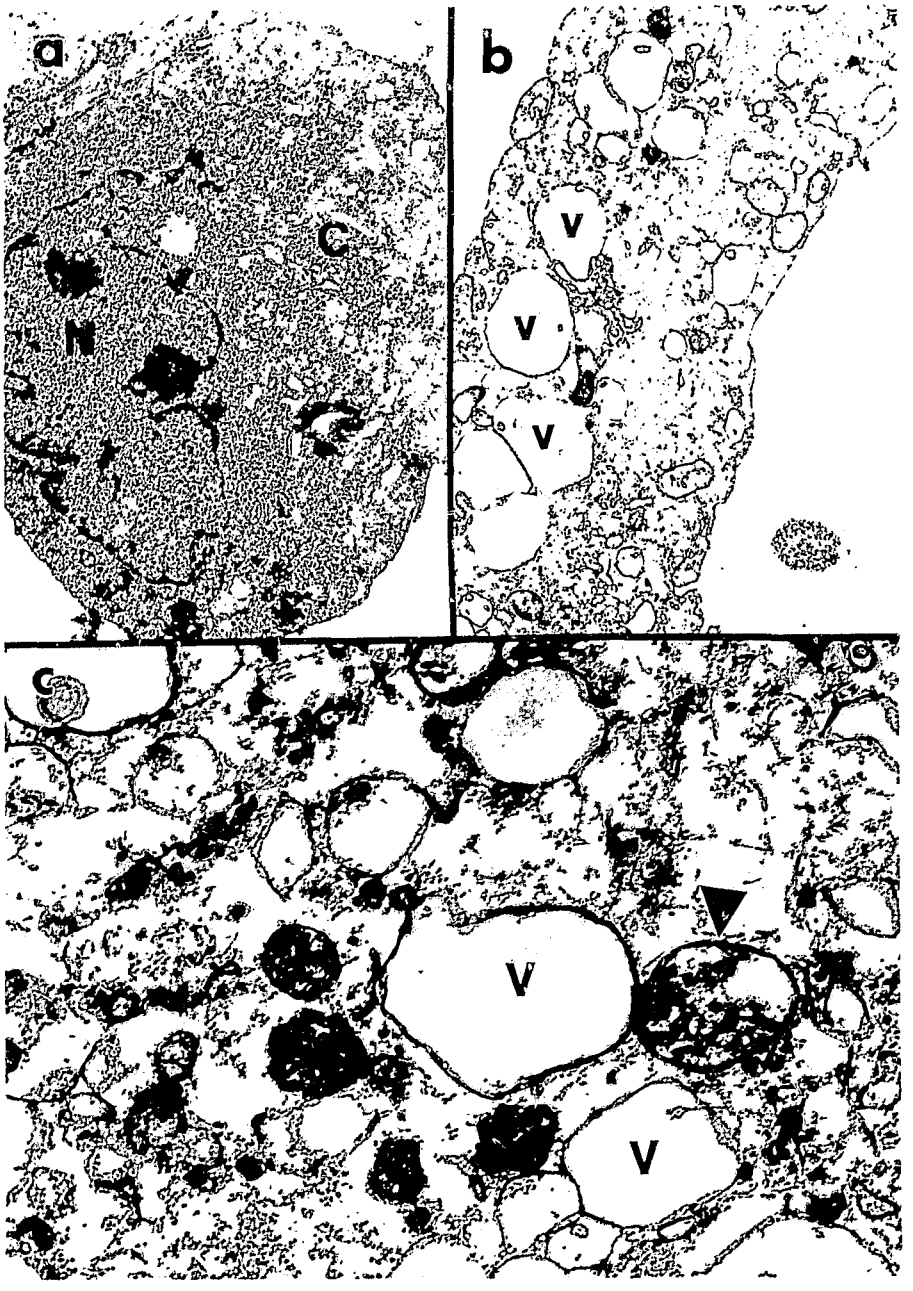

Figure 2.3: Electron micrographs of Atlantic salmon fibroblast (ASF) cell line infected with *Piscirickettsia salmonis*. **a:** Uninoculated (control) ASF cells with normal morphology showing nucleus (N) and cytoplasm (C) 14 days after incubation (x 5,000). **b:** ASF cell 14 DPI with *P. salmonis* showing extensive vacuolation (V) throughout the cytoplasm (x 7,800). **c:** Empty vacuoles (V) and destroyed mitochondria (arrowheads) are also observed inside an ASF cell 14 DPI (x 15,000).

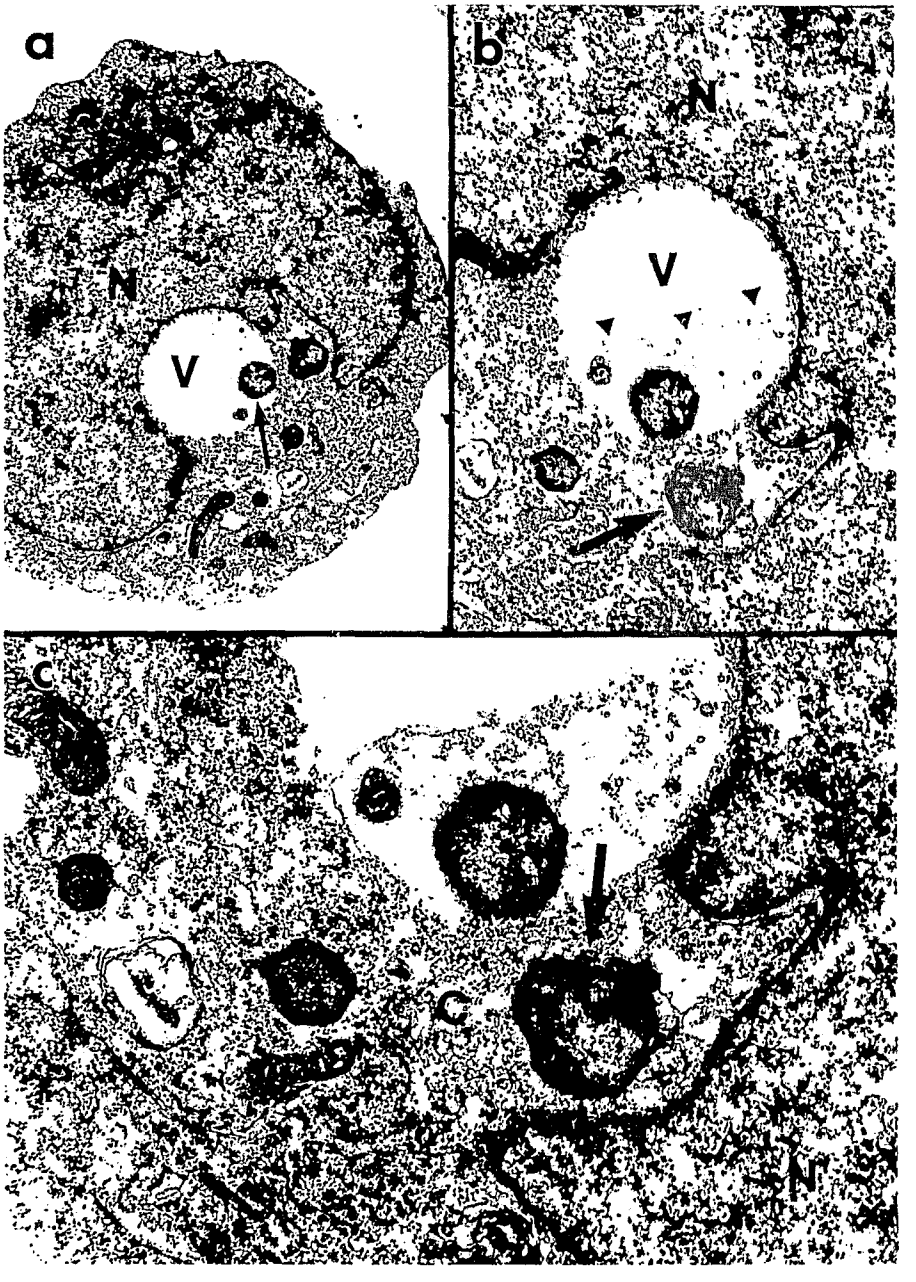

Figure 2.4: Electron micrographs of chinook salmon cells (CHSE-214) infected with *Piscirickettsia salmonis*. Nucleus (N). **a:** CHSE-214 cell line showing a large vacuole (V) containing the rickettsial organism (arrow) 14 DPI with *P. salmonis* (x 5,000). **b:** Higher magnification showing *P. salmonis* free in the cytosol (arrow) and inside an intracytoplasmic vacuole (V) close to the nucleus within a CHSE-214 cell 14 DPI (x 12,500). Shrinkage of the vacuole membrane (arrowheads) can also be observed. **c:** Still higher magnification showing *P. salmonis* free in the cytosol (arrow) within a CHSE-214 cell 14 DPI. Electron-lucent and electron-dense areas are easily identified inside *P. salmonis* (x 23,500). Mitochondria (M), cytoplasm (C).

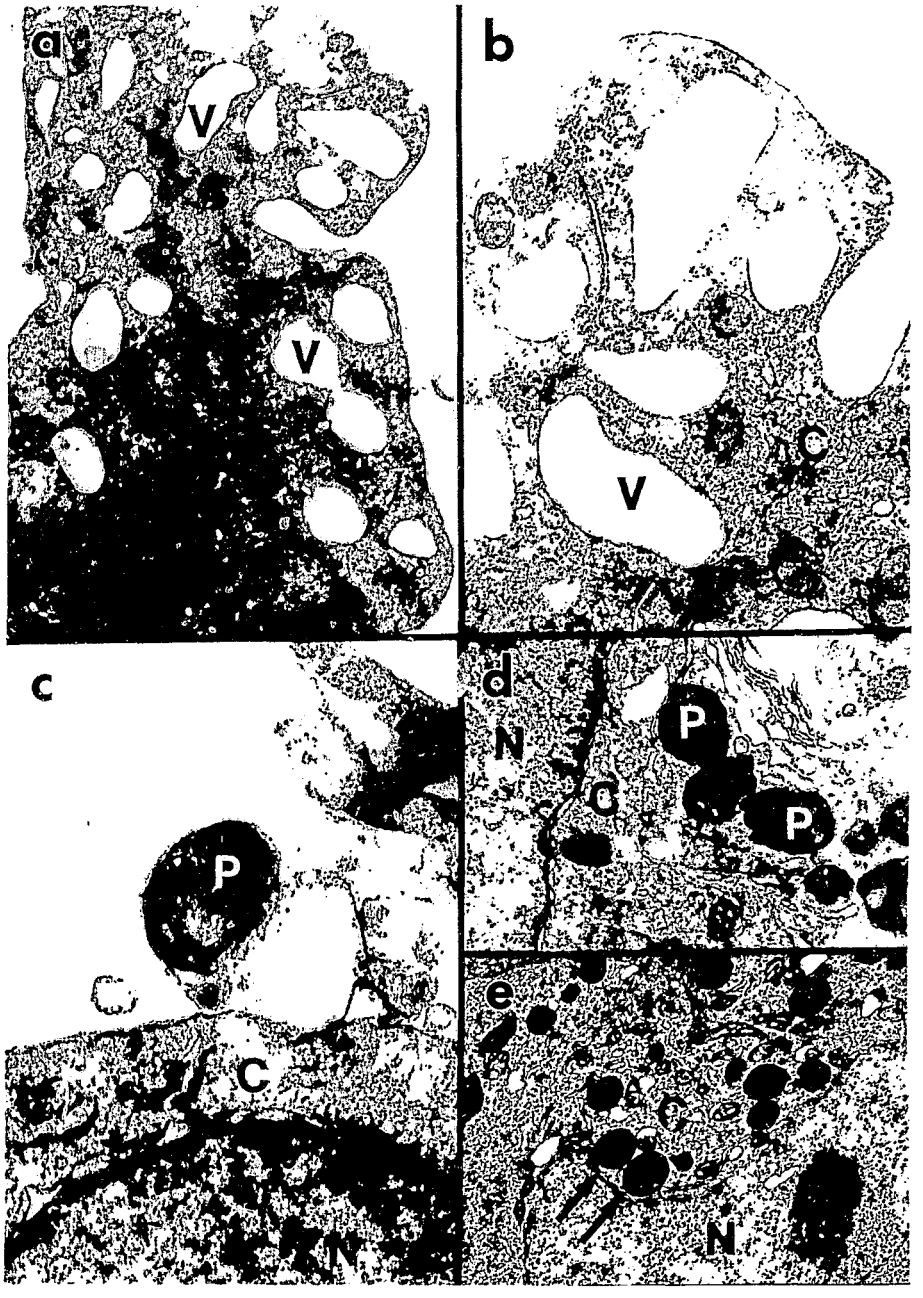

Figure 2.5: Electron micrographs of non-salmonid fish (BB) cell line infected with *Piscirickettsia salmonis*. Nucleus (N) and cytoplasm (C). **a:** BB cell line showing numerous empty vacuoles (V) 1 DPI with *P. salmonis* (x 14,400). **b:** Higher magnification of vacuolated (V) BB cell 1 DPI (x 30,000). **c:** *Piscirickettsia salmonis* (P) occurring extracellularly close to a BB cell 1 DPI (x 50,000). **d:** *P. salmonis* (P) free in the cytosol of a BB cell 1 DPI (x 15,000). **e:** *P. salmonis* (arrows) free in the cytosol and close to the nucleus of a BB cell 14 DPI (x 7,000).

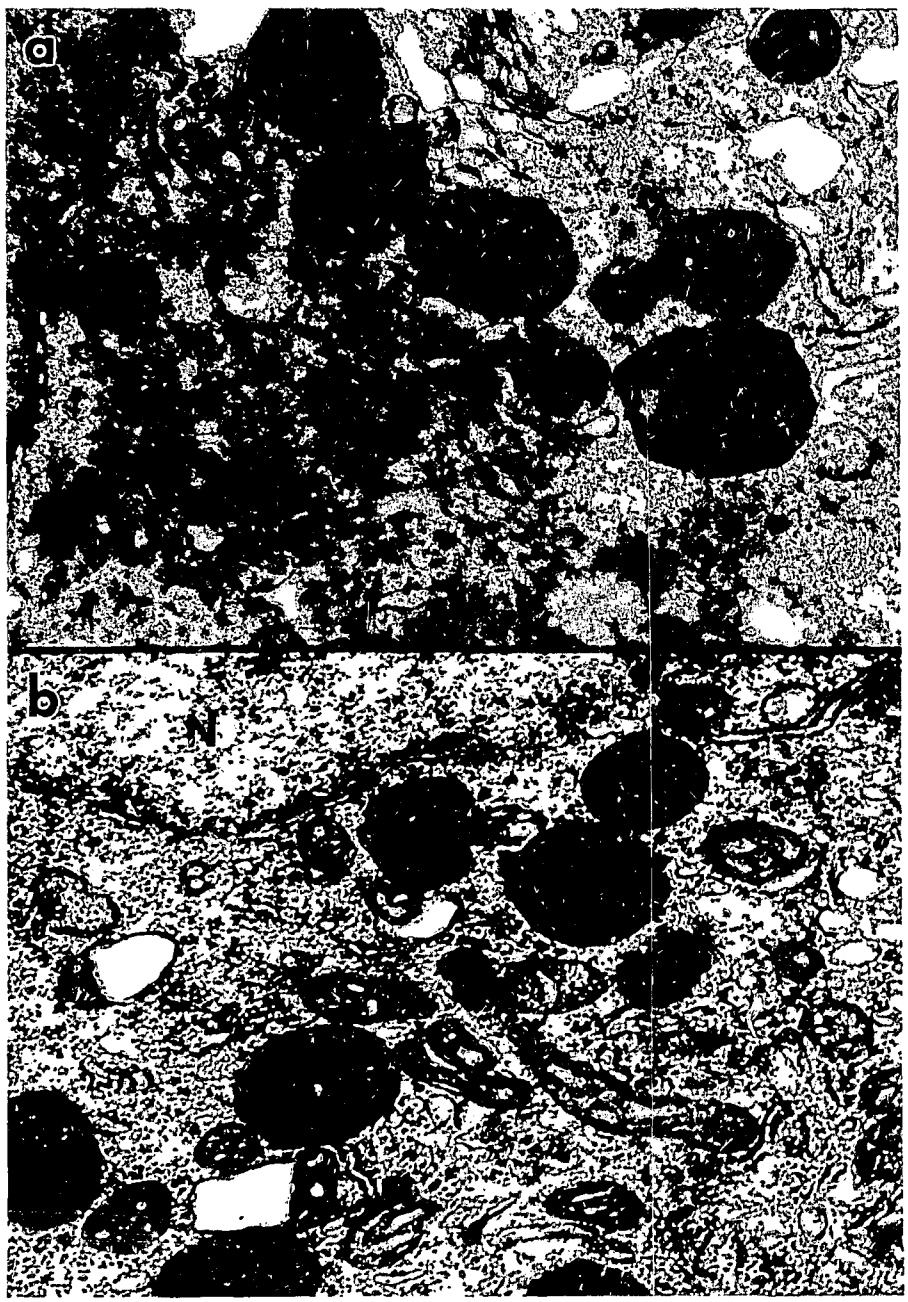

Figure 2.6: Electron micrographs of non-salmonid fish (BB) cell line infected with *Piscirickettsia salmonis*. Nucleus (N) and cytoplasm (C). a: *P. salmonis* (P) free in the cytosol of a BB cell 1 DPI (x 28,000). b: *P. salmonis* (arrows) free in the cytosol and close to the nucleus of a BB cell 14 DPI (x 25,000).

Table 2.1: Production of cytopathic effect (CPE) and location of *Piscirickettsia salmonis* within different fish cell lines after 1, 7 and 14 days post inoculation (DPI).

Cell line	DPI[a]	CPE[b]	Vacuole[c]	Free[d]
CHSE-214	1	no	yes	yes
	7	yes	yes	yes
	14	yes	yes	yes
ASF	1	no	no	yes
	7	yes	yes	yes
	14	yes	yes	yes
BB	1	no	no	yes
	7	no	no	yes
	14	no	no	yes
	78	yes	yes	yes

a : Days post infection with *P. salmonis*
b : Cytopathic effect detected by inverted light microscopy
c : Intravacuolar location of *P. salmonis*, TEM
d : *P. salmonis* free in the cytoplasm, TEM

2.5 Discussion

A characteristic CPE in the form of clusters of rounded cells was observed in the CHSE-214 salmonid cell line 7 DPI with *P. salmonis*. The intravacuolar location, distribution and morphological features of *P. salmonis* within CHSE-214 infected cells 14 days post infection described in this study are similar to those previously seen in salmonid cell lines infected with *P. salmonis* (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). Similar features have recently been described for rickettsia-like organisms (RLO) in Atlantic salmon (Rodger and Drinan, 1993), and Tilapia (Chern and Chao, 1994).

The ASF cell line was shown for the first time, to be susceptible to *P. salmonis* infection. Using light microscopy, ASF cells infected with *P. salmonis* had a pattern of cytopathic effect (CPE), characterized by extensive vacuolation of the cytoplasm. The intracellular microorganism produced extensive vacuolation of infected cells and evidently spread to adjacent cells. Electron microscopy revealed the presence of large numbers of empty vacuoles or vacuoles containing cellular debris inside ASF cells, which may have been the result of the escape of rickettsiae into the cytoplasm and intravacuolar destruction of the rickettsia, respectively.

In the present study, no CPE was observed in the non-salmonid BB cell line after 14 DPI with *P. salmonis*. Light microscopy revealed clear CPE in BB cells by 45 days post-infection with *P. salmonis*. Examination of BB cells by TEM 78 DPI, revealed *P. salmonis* within membrane-bound vacuoles or free in

the cytoplasm and extracellular space. Occasionally, the cell wall was separated from the plasma membrane or had a rippled appearance. Either condition may have been caused by shrinkage of the cytoplasm during fixation and embedding procedures (Cvitanich *et al.* 1991).

Previous reports have shown that cell lines from two warm water fish species, brown bullhead (BB) and bluegill (BF-2) failed to develop a CPE after infection with *P. salmonis* (Fryer *et al.,* 1990; Cvitanich *et al.,* 1991). However, the BB cell line is susceptible to infectious pancreatic necrosis virus (IPNV), and channel catfish virus (Bureau of Sport Fisheries and Wildlife, 1970; American Type Culture Collection, 1988). The BB cell line was originally isolated from posterior trunk tissue (not including fins) of two-year old brown bullheads by Cerini and Malsberger (1969), and submitted to the American Type Culture Collection (ATCC) at the 100th passage level. These cells have fibroblast-like characteristics. a temperature tolerance range from 4 to 34 °C and an optimal temperature for proliferation of between 25 and 30 °C (ATCC, 1988). Data presented here refute an earlier claim that BB cells are non-susceptible to *P. salmonis* (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). However, this cell line did not show the characteristic CPE observed in salmonid cell lines when examined 7 and 14 days post-infection. This study demonstrates that the BB cell line is susceptible to infection with *P. salmonis*, even though the incubation period is much longer than that described for other cell lines.

Persistent intracellular infections by rickettsial organisms in the absence

of CPE similar to that observed in BB cells between 14 and 45 days following infection with *P. salmonis,* has also been previously described. Studies with *Rickettsia prowazekii* showed that although large numbers of organisms accumulated in the cytoplasm of infected cells, there was no evidence of cellular injury (Silverman *et al.*, 1980). *Coxiella burnetti*, a rickettsial organism of mammals, persistently infects several cultured cell lines including L929 mouse fibroblast cells and several macrophage lines (Baca, 1989). These persistently infected cell populations have been maintained in continuous culture for months, and in the case of L929 cells, for over 3 years (Baca, 1989). After entry into cells and proliferation, *C. burnetti* were shown to be sequestered within phagolysosomal vacuoles whose pH is approximately 5.2 (Baca, 1989). Furthermore, heavily infected cells containing hundreds of rickettsiae are capable of mitosis and cytokinesis (Baca, 1989).

Piscirickettsia salmonis was observed being phagocytosed by BB cells in culture 78 DPI. Rickettsial penetration into host cells is considered to be mediated by induced phagocytosis, since drugs that block phagocytosis, i.e. cytochalasins, also block rickettsial entry (Walker and Winkler, 1978; Walker, 1984; Silverman, 1989). Induced phagocytosis occurs in other intracellular pathogens such as *Shigella sp.* and *Listeria sp.* (Clerc and Sansonetti, 1987; Gaillard *et al.,* 1987). *Rickettsia conorii* enters Vero cells via induced phagocytosis (Teysseire *et al.*, 1995). Cell entry occurs within 3 min after the bacterium contact the cell, when, *R. conorii* was observed in the process of

engulfment, within a phagocytic vacuole, or free in the cytosol (Teysseire *et al.*, 1995). These researchers demonstrated that a transient phagocytic vacuole exists, and escape from the phagosome is a very rapid step since phagosome lysis was only occasionally observed (Teysseire *et al.*, 1995).

There are at least three types of post-phagocytic behaviour that are favourable to the survival of intracellular bacteria: microbial resistance to digestive enzymes; escape from the phagosome into the cytoplasm; and evasion of phagosome-lysosome fusion (Colston, 1989). Transmission electron microscopic observations revealed that different intracellular survival mechanisms are used by *P. salmonis* when infecting salmonid or non-salmonid cell lines.

Because *Piscirickettsia salmonis* is able to survive inside vacuoles within CHSE-214 cells, this bacterium may be able to prevent lysosome fusion. Similar intravacuolar location was also observed in BB cells after 78 days post infection. Inhibition of lysosomal fusion to the vesicles in which intracellular pathogens grow is a (known) survival mechanism (Janeway and Travers, 1994). After entry in cells, chlamydiae occur within vesicles and these vesicles do not fuse with host cell lysosomes (Friis, 1972). The inhibition of lysosome fusion is not a generalized process, but rather restricted to vesicles which contain chlamydiae (Eissenberg and Wyrick, 1981), which allows the host cell to continue using lysosomes to degrade other products. The integrity of the chlamydial surface is important in both ensuring efficient uptake (Byrne and

Moulder, 1978) and inhibiting lysosome fusion (Friis, 1972). In chlamydiae, the capacity to inhibit lysosome fusion appears to be host-cell related (Byrne, 1989). A similar mechanism may explain differences in susceptibility to *P. salmonis* among salmonid and non-salmonid cell lines.

The presence of large numbers of empty vacuoles, and the observation of *P. salmonis* free in the cytoplasm in CHSE-214, ASF, and BB cell lines, suggests that *P. salmonis* may also escape from the phagosome and exist free in the cytoplasm. Anderson *et al.* (1965), reported that *Rickettsia rickettsii* lies free in the cytosol and is not surrounded by a limiting host membrane. After entry into phagocytic or non-phagocytic cells, some rickettsiae escape from the phagosomes (Teysseire *et al.*, 1995). Furthermore, in certain species of rickettsia, the presence of phagocytic vacuoles containing rickettsiae have never been demonstrated (Teysseire *et al.*, 1995). Once free within the cytoplasm, rickettsiae can grow, multiply, acquire actin-based motility, and exit the cell (Wisseman *et al.*, 1976; Teysseire *et al.*, 1992).

Intact rickettsia were present inside large intracytoplasmic vacuoles in CHSE-214 cell lines by 14 DPI and in BB cell lines by 78 DPI. After phagocytosis, *P. salmonis* remains inside the vacuole within CHSE-214 cells. This is further supported by the capacity of *P. salmonis* for intravacuolar reproduction by binary fission in salmonid fish and cell lines (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). After a few days these vacuoles increase in size and finally destroy the host cell; *P. salmonis* is released and infects a new cell. The

final effect is a fast-spreading cytopathic effect in the monolayer.

With BB cells *P. salmonis* escapes the vacuole and remains free in the cytoplasm for long periods without production of vacuolation of the host and lysosome fusion. The observation of *P. salmonis* being phagocytized by BB cells and inside large vacuoles by 78 days post infection may indicate that after an extended period *P. salmonis* may be released from BB cells and infect new cells. *Piscirícketsia salmonis* in newly infected cells may not be able to escape the phagosome and remains in the vacuole producing the vacuolation and cytophatic effect similar to those observed in CHSE-214, 5 to 7 days post infection.

Final remarks

Ultramicroscopic observations revealed that different intracellular survival mechanisms are used by *P. salmonis* when infecting salmonid or non-salmonid cell lines. The Atlantic salmon fibroblast (ASF) cell line was shown to be susceptible to *P. salmonis* infection, producing a cytopathic effect (CPE) as early as the CHSE-214 cell line.

Although the BB cell lines, previously reported as non-susceptible, did not show the characteristic CPE observed in salmonid cell lines after a few days post-infection, there was clear ultrastructural evidence that they were infected by this pathogen. Differences in appearance of CPE among different cell lines infected with *P. salmonis* may be due to survival mechanisms such as early

escape to the cytosol, inhibition of phagosome-lysosome fusion and resistance to degradation. The fact that some fish cell lines are less permissive to infection may explain the differences in susceptibility to *P. salmonis* observed among salmonid species. Perhaps non-salmonid native fish play a role in the persistence and transmission of the disease in the natural environment. The possibility of reservoirs of *P. salmonis* existing among transient and resident non-salmonid fish and shellfish has been proposed based on the finding of rickettsia-like organisms among marine molluscs, crustacea and non-salmonid fish (Cvitanich *et al.*, 1991).

Further morphological, immunocytochemical and molecular studies of the pattern of infection in this particular cell line may give valuable insight into the mechanisms involved in cell resistance and susceptibility to *P. salmonis* infection.

3. INOCULATION ROUTE AND HORIZONTAL TRANSMISSION OF *PISCIRICKETTSIA SALMONIS* IN FRESHWATER RAISED ATLANTIC SALMON, *SALMO SALAR*

3.1 Summary

Salmonid rickettsial septicaemia (SRS) is a systemic disease caused by *Piscirickettsia salmonis.* In spite of its economic impact on the Chilean salmon industry, little is known about its epidemiology and transmission mechanisms. An experiment was designed to compare three routes of experimental transmission (oral, gill surface and intraperitoneal injection), and to study the effect of physical contact as a risk factor in the horizontal transmission of SRS in Atlantic salmon raised in fresh water. Tissue samples (liver, kidney, spleen, gill, and brain) were collected weekly to study the sequential infection of SRS using IFAT. The pathogen was transmitted horizontally to fish with and without physical contact. However, transmission of *P. salmonis* occurred significantly faster among fish with physical contact. The sequential study using IFAT indicated that a similar haematogenous pattern of infection occurred among fish inoculated by oral and gill routes, and in cohabitants. This was different from the capsular (serosa) infection pattern observed in intraperitoneally inoculated fish. *Piscirickettsia salmonis* was also observed within the cytoplasm of leucocytes and renal tubules, the latter indicating that elimination of this pathogen through the urine may be possible. *Aeromonas salmonicida* was also detected (by IFAT) in some of the fish exposed to *P. salmonis. Piscirickettsia*

salmonis may cause immunosuppression which increases the susceptibility of the host to other pathogens.

3.2 Introduction

Salmonid rickettsial septicaemia (SRS) or piscirickettsiosis, is a systemic disease caused by the intracellular pathogen *Piscirickettsia salmonis* (Fryer *et al.*, 1992), which causes high mortalities (Fryer *et al.*, 1990), and significant economic losses to the salmon industry (Cassigoli, 1994). This disease was first reported in coho salmon *Oncorhynchus kisutch* Walbaum in Chile (Bravo and Campos, 1989), and has since been observed in other salmonid species including rainbow trout, *Oncorhynchus mykiss* Walbaum, Chinook salmon *Oncorhynchus tshawytscha* Walbaum, Atlantic salmon *Salmo salar* Linnaeus (Cvitanich *et al.*, 1991). Similar rickettsia-like organisms (RLO) have been reported in Atlantic salmon held in salt water in western Canada (Brocklebank *et al.*, 1992, 1993), Ireland (Rodger and Drinan, 1993), and Norway (Olsen *et al.*, 1993).

Very little is known about natural routes of infection, mechanisms of horizontal transmission, and rate of progress of the infection in the host. Identification of the source of infection and elucidation of the mode of transmission of the agent under natural conditions are important factors to be clarified (Garcés *et al.*, 1991).

Salmonid rickettsial septicaemia has been experimentally reproduced in

coho and Atlantic salmon by intraperitoneal inoculation (Cvitanich *et al.*, 1991; Garcés *et al.*, 1991). Horizontal transmission occurred in salt water from experimentally (IP) inoculated coho salmon to non-inoculated cohabitants (Cvitanich *et al.*, 1991). Horizontal transmission in fresh water is still controversial (Cvitanich *et al.*, 1991; Garcés *et al.*, 1991), however, in natural cases of SRS, mortalities usually appear a few weeks after transfer of smolts to sea water (Fryer, *et al.*, 1990). Atlantic salmon maintained in sea water drink large amounts of water for osmoregulatory purposes (Usher *et al.*, 1988). However, the possibility of oral or gill infection by *P. salmonis* has not been studied yet.

Chile is the third largest world producer of Atlantic salmon (F.A.O., 1994), and this production is likely to increase, as the importation of eyed eggs for this species increased 58% from 1993 to 1994 (Méndez, 1995). The study of the pathogenesis and transmission of the SRS in Atlantic salmon is important in developing methods for controlling the disease and ensuring a sustained growth of the industry.

The main objectives of this project were to compare experimental infections via intraperitoneal, oral and gill routes, and to clarify the importance of physical contact in the horizontal transmission of *P. salmonis* in Atlantic salmon.

3.3 Material and Methods

Culture and identification of the organism

Isolates of *P. salmonis* used in this experiment were obtained from naturally infected Atlantic salmon reared in salt water in southern Chile, with clinical SRS (donated by Dr. Enrique Madrid, Fish Pathology Laboratory, Marine Harvest McConnell, Puerto Montt, Chile). The isolate was named strain NCR 1010, and identified as *P. salmonis* by the use of a rabbit polyclonal antibody produced against *P. salmonis* strain FL-89 (American Type Culture Collection, VR1361) and generously donated by Dr. J. L. Fryer (Department of Microbiology, Hatfield Marine Science Center, Oregon State University, U.S.A.).

Maintenance of the fish

The fish used were under-yearling Atlantic salmon parr, wildstock La Have, from the Department of Fisheries and Oceans (D.F.O.), Cold Brook hatchery (Nova Scotia, Canada), kept at 11 °C. Three hundred and eighty fish weighing 20 ± 2.3 g were separated and kept in fresh water at 11 °C. After one month, the fish were anesthetized in a solution of benzocaine at a concentration of 50 mg/L, separated into three groups and dye-tagged on the caudal (Group A), pectoral (Group B), or pelvic fins (Group C) and acclimatised for 10 days at 11 °C. Dye-tagging was done with 0.1 ml of pressure injected methylene blue using a Madajet XL (Mada, Carlstadt, NJ, U.S.A.) injection system.

Experimental infections

One week after tagging, fish from group A (n = 108) were arbitrarily assigned to three subgroups to be inoculated with *P. salmonis*. Thirty-six fish were anesthetized by placing them in a solution of benzocaine at a concentration of 50 mg/L, and inoculated either by intraperitoneal (IP), oral (PO), or gill surface (GS) route. Inoculated fish were placed separately in six plastic square tanks of approximately 60 L, using two tanks per inoculation route, and allocating 18 inoculated fish per tank (Figure 3.1). The inoculum consisted of 100 μl of diluted supernatant from infected chinook salmon embryo (CHSE-214) cell line (ATCC CRL 1681), showing 100% cytopathic effect (CPE). The final infectious dose, estimated by calculating the 50% tissue culture infectious dose endpoint ($TCID_{50}$) in a 96-well plate seeded with CHSE-214 (Hsiung, 1994), was $1.48x10^2$ $TCID_{50}$/ml.

Intraperitoneal injections were performed using a one-ml syringe and a 26 3/8 G needle (Becton Dickinson & Co., Rutherford, NJ, U.S.A.). A 10 cm long piece of flexible PVC Tygon tubing with 0.1 mm internal diameter, 0.1 mm wall thickness, and 0.3 mm external diameter (Norton Co., Akron, OH, U.S.A.), was connected to the needle to perform oral and gill inoculations. For oral inoculations the fish were gently intubated by inserting the tube about one to two centimetres into the oesophagus. Gill infections were performed by spreading the rickettsial solution over the lamella, between the first and second branchial arch of the left gill.

Cohabitants

In each of the six tanks, groups of 18 uninoculated fish were placed in cohabitation with (group B) or without (group C) direct physical contact with inoculated (group A) fish (Figure 3.1). Fish in groups C were separated from the other two groups by a one-inch-thick double plastic mesh and occupied a third of the water volume in order to maintain a stocking density equal to that of the other groups (27 kg/m^3). The combination of three routes and three modes of contact with the pathogen provided nine experimental groups. Two replicates (tanks) were used for each experimental group. Fifty-six fish were allocated into a separate tank to be used as uninfected controls.

The fish were kept in fresh water at 11 °C, with a flow of 2.0 L/min in a flow-through system. The water had 10 ppm of dissolved oxygen concentration, 140% oxygen, 90% nitrogen, and 101% of total gas pressure. All experimental and control fish were exposed to 8 hours of artificial light daily, and fed twice a day with an Atlantic salmon # 2 granular dry diet (Corey Feed, NB, Canada) at 2% of body weight per day. Experimental procedures used in this study were performed according to the guidelines of the Canadian Council on Animal Care (Olfert *et al.*, 1993).

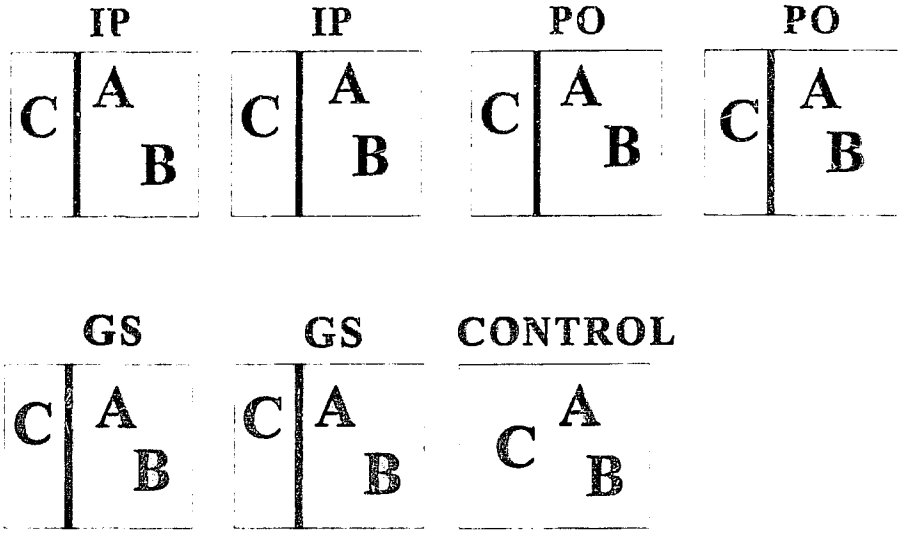

Figure 3.1: Experimental design. Inoculation routes used in Groups: IP = intraperitoneal, PO = oral, GS = gill surface, and a non-inoculated control group. Three sources of contact with the pathogen: A = directly inoculated fish, B = cohabitants in contact with inoculated groups, C = non-contact cohabitants. Each subgroup had 18 fish (54 fish per tank).

Sampling

Five fish from each of the 9 experimental groups, and three from the control group, were sampled weekly for 5 weeks. During the sampling process no attempt to select fish showing clinical signs was made. Fish were removed from the tank, sedated in 50 ppm of benzocaine hydrochloride solution and euthanised by spinal severance. The carcass was weighed (Appendix B) and the peritoneal cavity was exposed from the left flank for post-mortem examination. Samples of liver, spleen, head kidney, posterior kidney, brain and the second left branchial arch were removed and fixed in 10% neutral buffered formalin for morphologic and immunohistochemical examination by light microscopy (LM). The tail was cut behind the adipose fin and blood was collected from the dorsal aorta using a heparinised (2 usp units ammonium heparin) microhaematocrit capillary tube (Fisher Scientific, Pittsburgh, PA, U.S.A.) sealed with a commercial sealant (Critoseal™). Blood samples were kept on ice for further studies not included in this chapter.

Tissues from fish that died during the study period were sampled between 24 h of death and processed as described above.

Light microscopy (LM)

Tissues samples were fixed and stored in 10 % buffered formalin at room temperature (20-22 °C) for 1-3 months, trimmed and transferred to coded plastic histology cassettes (Tissue-Tek, Miles, Etobicoke, ON, Canada). Tissues were dehydrated in a graded series of ethanol baths (1 h in each reagent): 2x 70% ethanol, 2x 95% ethanol, 2x 100% ethanol, and 2x 100% xylene, using a Fisher Histomatic Tissue Processor, under vacuum, at room temperature (RT). Tissues were then embedded in an initial wax bath (Tissue Prep II paraffin, Fisher Scientific Ltd., Canada) for 1 h at 60° C, followed by a second wax bath for 2 h. The final embedding step was made using a Brinkman Embedding Centre in the same paraffin. Coded blocks were picked randomly and renumbered in the order in which they were picked to allow blind reading of the tissues. Blocks were then trimmed, chilled on ice, and sectioned at 6 μm thickness on an American Optical 820 rotary microtome (American Optical, U.S.A.). Sections were dried in a gravity convection oven (Precision Scientific Inc., Chicago, Illinois, U.S.A.) for 1 h at 55° C.

Tissue sections were deparaffinized in xylene (10 min), hydrated through 100% ethanol (10 min), 95% ethanol (5 min), 70% ethanol (5 min), and water.

Giemsa stain

Histological sections obtained from samples taken from live or dead fish were stained with Giemsa stain (Appendix C) to detect microorganisms. Sections were dehydrated from water to xylene, mounted under glass cover slips with Flo-texx® mounting medium (Lerner Laboratories, Pittsburgh, PA, U.S.A.) and observed using a Nikon Labophot microscope (Nikon Canada, Mississauga, ON, Canada).

Immunohistochemistry

An indirect fluorescent-antibody technique (IFAT)with the rabbit anti-*P. salmonis* polyclonal antibody (see Appendix A) was used for specific detection of the etiologic agent (Lannan *et al.*, 1991). Briefly, formalin fixed tissue sections were incubated with a rabbit anti-*P. salmonis* polyclonal antibody (1/800) for 30 min, followed by three washes in PBS, incubation with a fluorescein-labelled goat anti-rabbit secondary antibody (1/80) for 30 min, and three washes in PBS. Tissues were then coverslipped, and sealed with nail polish. For each assay, a positive control using supernatant of CHSE-214 infected cells and a negative control using infected CHSE-214 cells incubated with PBS instead of the first antibody were also included. Smears of *Aeromonas salmonicida* subspecies *salmonicida* (Aqua Health Ltd., P.E.I., Canada), and tissues of rainbow trout infected with *A. salmonicida* were included to assess the specificity of the reaction. Immunostained samples were

stored in the dark at 4 °C and analysed within 24 h. For each sampled fish, every organ available was thoroughly examined, using an epifluorescent Zeiss microscope (Zeiss, Germany). Results were reported using a 0 to 4+ scale employed for routine IFAT diagnosis of *P. salmonis* (Bustos *et al.*, 1994). This scale is based on the number of rickettsial organisms per fifty microscopic fields (400 x total magnification) (Table 3.1). An IFAT with rabbit anti-*A. salmonicida* (1/400) serum (Aqua Health Ltd., P.E.I., Canada) was also used in some samples to assess the specificity of the IFAT, using the protocol described previously.

Statistical analysis

The effect of exposure by various routes and type of contact was assessed in SAS (SAS Institute, 1988) by survival analysis. Relative risk as a measure of association was used to compare rates of death and evaluate the effect of different risk factors. Relative risk of mortality and cumulative probability of survival was calculated using the Cox proportional hazards model (Lee, 1980). Decisions on statistical significance were based on $p \leq 0.05$.

Table 3.1: Criteria to establish degree of infection using immunohistochemistry (IFAT) in tissue samples from fish infected with *Piscirickettsia salmonis* (Bustos *et al.*, 1994).

Degree of Infection	Rickettsial organisms in 50 fields (400x)
0	none
1+	1 to 10
2+	11 to 25
3+	26 to 50
4+	50 and up

3.4 Results

Weekly sampled fish and mortalities

A total of 174 fish were sampled over a period of 35 days post inoculation (DPI). At this time, the only group with live fish remaining was the control group. All fish had been sampled from the IP and GS groups by 28 DPI, whereas the PO and control groups were sampled for 35 DPI.

A total of 162 fish (48%) died during the 35 days of experimental trial with 60 of these (37%) coming from the IP injected and contact groups, 57 (35%) from the gill inoculated and contacts, and 45 (28%) from the orally inoculated and contacts. No statistical differences in weight were detected among groups during the 5 weeks of experimental period.

In each of the three inoculation routes used, every exposed group, whether by inoculation, physical contact or by water cohabitation only, had mortalities starting 12 DPI (Table 3.2). None of the control fish died during the 35 day experimental period. Mortalities were compared using life tables for cumulative survival analysis. When comparing routes of inoculation, the oral route showed a pattern of mortality that was significantly delayed ($\propto \leq 0.05$) from IP and gill routes (Figure 3.2). However, significant differences among replicates of the orally inoculated tanks were also detected.

Table 3.2: Mortalities observed after *P.salmonis* exposure in each of the experimental groups. The groups are identified according to the exposure to *Piscirickettsia salmonis*, indicating inoculation route used and contact with the pathogen.

ROUTE	CONTACT	n	FIRST MORT (DPI)	LAST MORT (DPI)	DAYS WITH MORTS	DEAD / TOTAL (%)
Intraperitoneal	Direct	36	17	23	7	57
	Contact	36	17	27	11	59
	Water	36	19	28	10	50
Oral	Direct	36	12	35	24	41
	Contact	36	18	30	13	42
	Water	36	25	35	11	41
Gill Surface	Direct	36	14	24	11	45
	Contact	36	12	27	16	64
	Water	36	19	27	9	46
CONTROLS	--	54	0	0	0	0
TOTAL	--	378	--	--	24	48

Direct: Direct inoculation with *P. salmonis* (IP, oral or Gill routes).
Contact: Contact cohabitants with inoculated fish.
Water: Non-contact cohabitants with water contact only with inoculated fish.
FIRST MORT: First event of mortality in days post inoculation (DPI).
LAST MORT: Last event of mortality in days post inoculation (DPI).
DAYS WITH MORTS: Days between first and last morta;ity event.
DEAD/TOTAL: Percentage of dead fish in the group (%).

In either IP, GS, or PO inoculated groups, non-contact cohabitants (group C), showed a longer ($P<0.05$) survival time (delayed pattern of mortality) when compared with directly inoculated fish (groups A) or contact cohabitants fish (group B) (Figure 3.3).

The estimates of the relative risk (RR) of dying for each predictor variable are shown in Table 3.3. In the present study, the lowest risk group corresponded to non-contact cohabitants in water contact with orally inoculated fish (route PO, group C). The analysis showed that fish inoculated IP and by GS had a significantly higher probability of dying than fish inoculated orally. Contact cohabitants with inoculated fish had a higher probability of death than non-contact cohabitants ($P< 0.05$). In addition, there was interaction between route of inoculation and type of contact. The risk of death in fish inoculated IP or GS (as opposed to orally inoculated fish) and those in direct contact with fish inoculated IP or GS, was significantly higher than fish with water contact only with orally inoculated fish (Table 3.3).

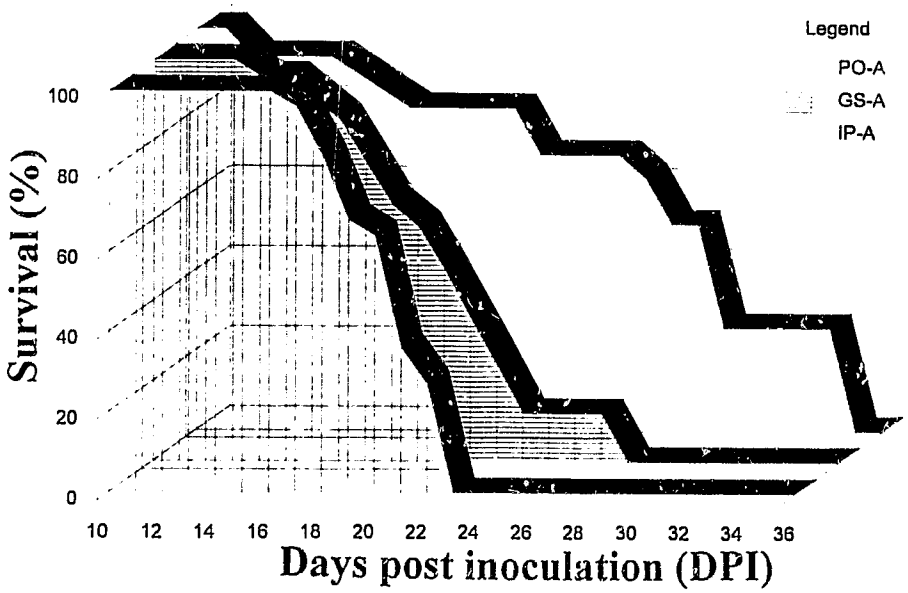

Figure 3.2: Cumulative probability of survival of fish comparing inoculation routes (directly inoculated fish only). Inoculations are intraperitoneal (IP-A), gill surface (GS-A), and oral route (PO-A) with *Piscirickettsia salmonis*.

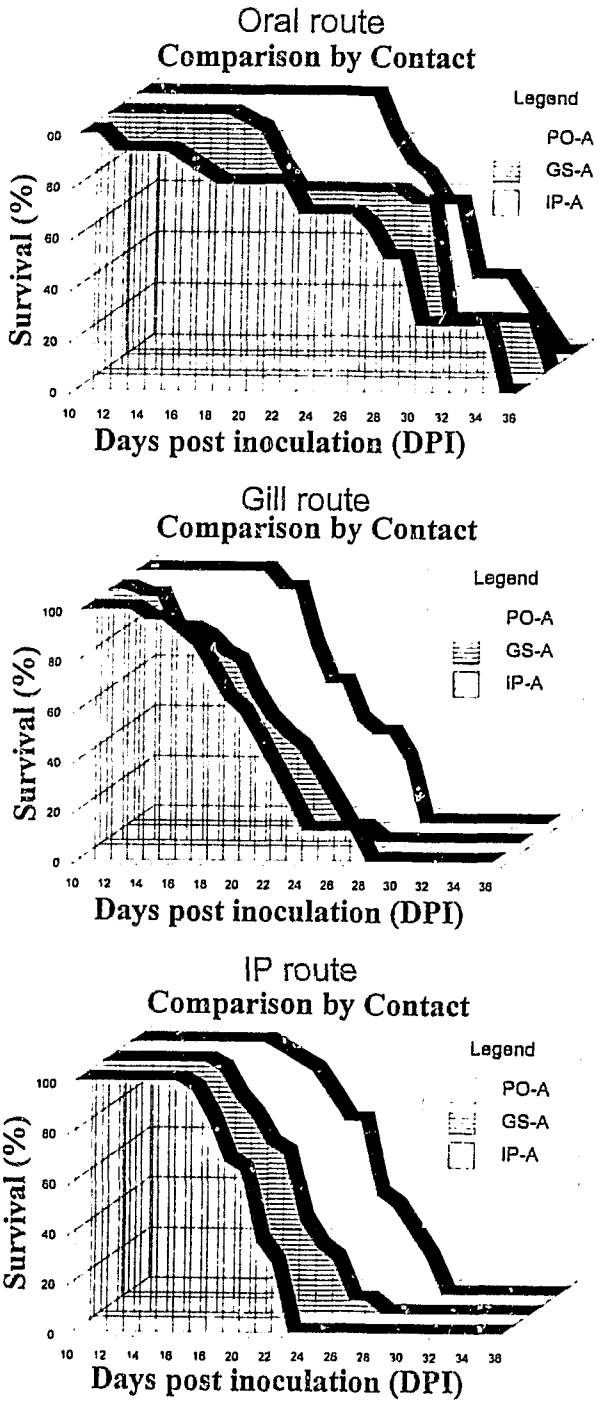

Figure 3.3: Cumulative probability of survival of fish comparing contacts in orally (PO) inoculated (Top), gill (GS) inoculated (Center), and intraperitoneally (IP) inoculated (bottom) groups with *Piscirickettsia salmonis*. In each of the three graphs fish are directly inoculated (-A), contact cohabitants with inoculated fish (-B), and non-contact cohabitants with inoculated fish (-C).

Table 3.3: Relative risk (RR) of dying at a given time for each predictor variable (route and contact).

Predictor variables	Relative Risk	P value
Non-contact cohabitants with orally inoculated fish	1.00	
Contact cohabitants with orally inoculated fish	1.09	0.790
Orally inoculated fish (PO)	1.09	0.790
Non-contact cohabitants with IP or GS inoculated fish	2.57	0.005*
Contact cohabitants with fish inoculated IP or GS	6.82	0.021*
Fish inoculated IP or GS.	6.82	0.021*

* significantly different from RR = 1.0 ($p \leq 0.05$).

Giemsa stain

Rickettsial organisms infecting cells of different tissues were difficult to identify using Giemsa stain. In some cases, small blue stained RLO were observed within intracytoplasmic vacuoles in hepatocytes or renal tubular epithelial cells. Additionally, blue stained extracellular rod-shaped microorganisms were observed usually in variably sized clusters in the liver, head kidney, posterior kidney, spleen, or gills of some fish in all the experimental groups. The colony formation of these organisms was similar to those found in typical furunculosis. Subsequently, these microorganisms were identified as *Aeromonas salmonicida* using IFAT. The presence of *A. salmonicida* in tissues was classified in three different levels:

1 = few bacteria

2 = small bacterial colonies

3 = large bacterial clusters displacing normal tissue

IFAT in sampled fish

In each batch of slides used for IFAT the positive control showed red CHSE-214 cells with green fluorescing rickettsial organisms within the cytoplasm. Negative controls had red CHSE-214 without green fluorescence. Smears and tissues of fish infected with *A. salmonicida* did not fluoresce when incubated with the rabbit anti-*P. salmonis* polyclonal antibody. There was evidence for small fluorescent microorganisms in weekly sampled tissues from

all groups exposed to *P. salmonis* (Table 3.4). By 7 DPI, 29% (13/45) of sampled fish had *P. salmonis* infection with levels 1 to 4. By 14 DPI, 47% (21/45) of the fish showed level 1 to 4 in the tissues, but predominantly level 4. By 21 DPI 47% (21/45) of the fish showed infection with levels 2 to 4. Fish sampled 28 DPI showed 75% (18/24) of the fish with level 2 to 4 of infection with *P. salmonis*.

Table 3.4: Levels of infection with *Piscirickettsia salmonis* in tissues of five Atlantic salmon sampled weekly from each experimental group. Levels of infection are given as 1+ to 4+ (see Table 3.1 for definition). Levels reported from organ showing the highest infection level by IFAT.

Group	7 DPI				14 DPI				21 DPI				28 DPI			
	1+	2-3+	4+	T	1+	2-3+	4+	T	1+	2-3+	4+	T	1+	2-3+	4+	T
IP-A		2/5	2/5	4/5		2/5	3/5	5/5		1/5	4/5	5/5			N/A	N/A
IP-B	1/5		1/5	2\5			1/5	1/5		1/5	2/5	3/5		1/1		1/1
IP-C				0/5			1/5	1/5			2/5	2/5				0/2
PO-A	1/5	1/5		2/5	2/5		1/5	3/5		1/5	2/5	3/5			5/5	5/5
PO-B				0/5			1/5	1/5			3/5	3/5		1/5	3/5	4/5
PO-C	1/5		1/5	2/5	1/5			1/5			2/5	2/5			5/5	5/5
GS-A	1/5			1/5		1/5	2/5	3/5			1/5	1/5				0/1
GS-B				0/5		1/5	2/5	3/5				0/5			N/A	N/A
GS-C		2/5		2/5	1/5	1/5	1/5	3/5			2/5	2/5		1/5	2/5	3/5
TOTAL (%)	9	11	9	29	9	11	27	47	0	7	40	47	0	12	63	75
CONTROL							0/3				0/3				0/3	

Routes: Intraperitoneal (IP), orally (PO), and gill surface (GS)
Contact: Direct infection (A), contact cohabitant (B), non-contact cohabitant (C)
Empty spaces: all fish IFAT negative
N/A: No fish available
T: Total positive fish
DPI: days post infection with *Piscirickettsia salmonis*

Comparison of fish directly inoculated by the three different routes revealed a distinct pattern of *P. salmonis* infection. Intraperitoneally infected fish showed capsular (serosa) infection of liver and spleen by *P. salmonis* from 7 DPI (Figure 3.4), and in later weeks, *P. salmonis* was also observed infecting leucocytes and other cells of the spleen and other organs. In the PO and GS inoculated fish, and in contact cohabitants (group B) or non-contact cohabitants (group C), the infection pattern observed was haematogenous with the organism invading the organs from the blood vessels to the surrounding tissues. No evidence of capsular infection by *P. salmonis* was observed in these groups. In the liver, leucocytes carrying *P. salmonis* in the cytoplasm were frequently observed within hepatic sinusoids and blood vessels (Figure 3.5). In some cohabitant fish (Groups B or C), the presence of *P. salmonis* was evident as early as 7 DPI (Table 3.4).

The sequence of infection of *P. salmonis* in the different organs from the weekly sampled Atlantic salmon is summarized in Table 3.5. The pathogen was commonly observed in liver (Figure 3.5), posterior kidney, head kidney and spleen. *Piscirickettsia salmonis* was occasionally observed in gills and brain (Table 3.5). By 7 DPI *P. salmonis* was more frequently observed infecting posterior kidney (17 %), followed by the liver (14%). However, the liver appeared as the organ most frequently infected at 14, 21, and 28 DPI respectively (Table 3.5).

Some fish from each of the nine experimental groups also had bacterial colonies ranging from 3 to 100 μm in diameter, composed of coccobacillary organisms counterstained red (with Evans blue), and sometimes displacing the tissue. These bacteria were identified as *A. salmonicida* using IFAT.

Analysis by IFAT of tissues from all (174) sampled fish from groups experimentally exposed to *P. salmonis* showed that 44% of the sampled fish presented piscirickettsial infection, 27% alone and 17% associated with *A. salmonicida* infection.

The degree of infection with *P. salmonis* during the sampling period was as follows: 5% showed level 1+, 6% showed level 2+, 3% showed level 3+, and 30% showed level 4+ of infection. From all the control fish sampled (12), none had evidence of piscirickettsial infection or mortalities, and only one fish, sampled 35 DPI had evidence of *A. salmonicida* infection.

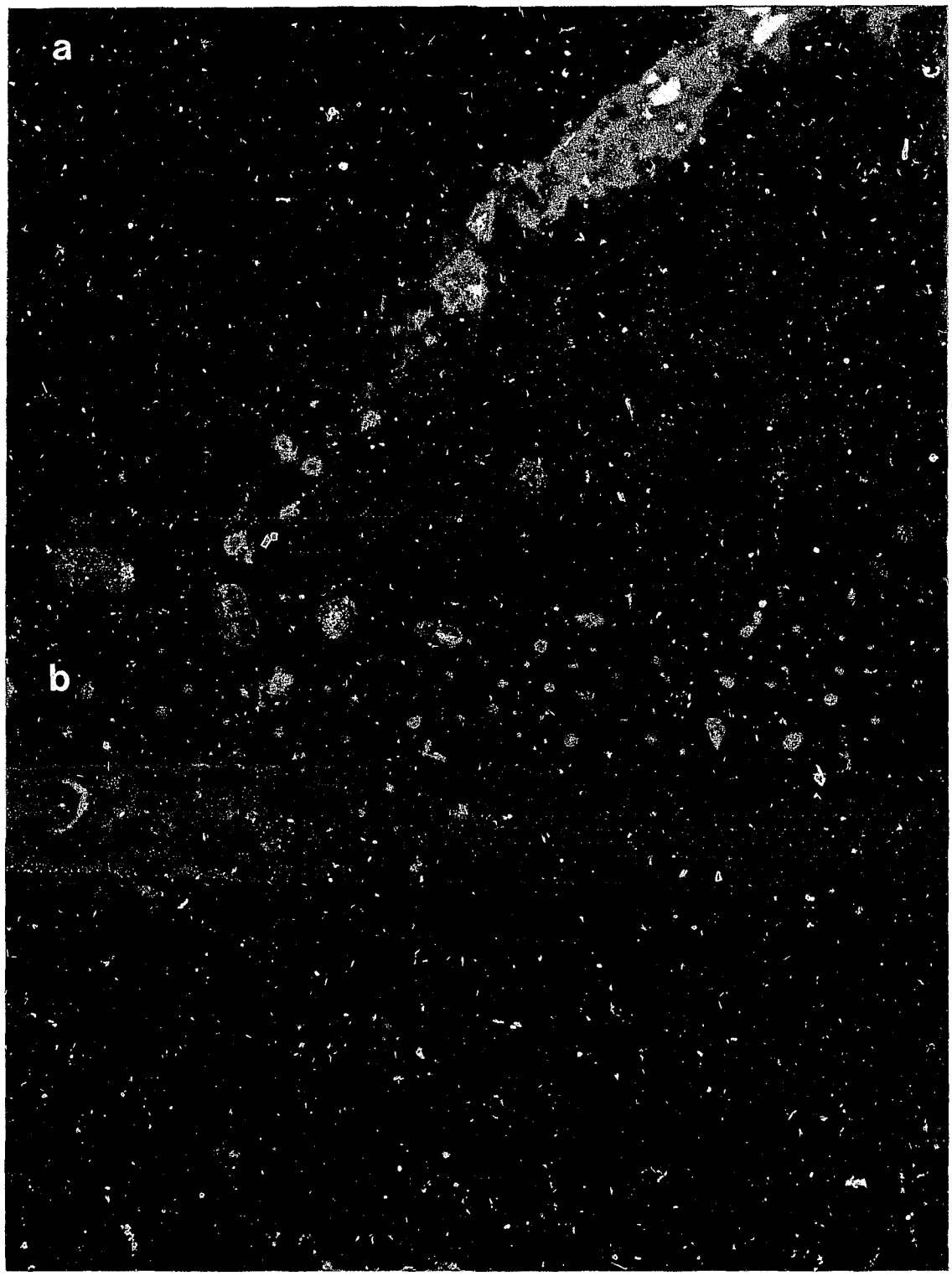

Figure 3.4: Atlantic salmon spleen showing capsular infection by *Piscirickettsia salmonis* 7 (a) and14 (b) days post IP inoculation (IFAT x 400).

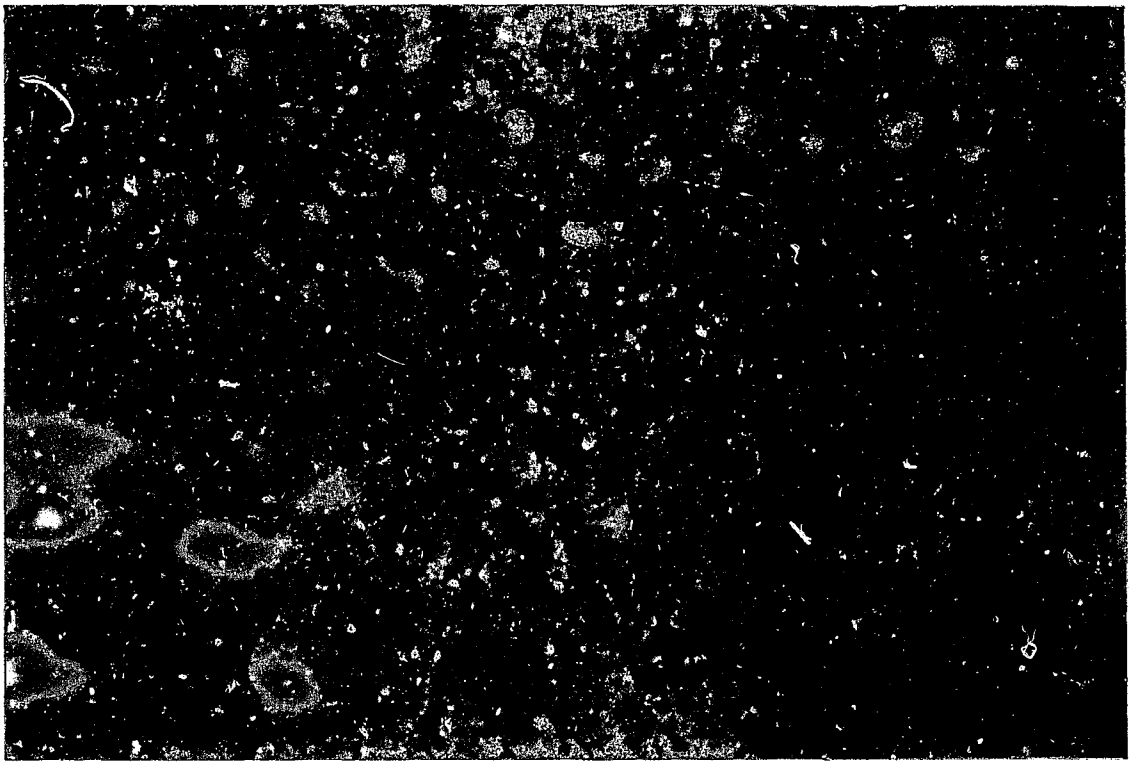

Figure 3.5: Atlantic salmon liver showing infection by *Piscirickettsia salmonis* 14 days post oral inoculation. The rickettsiae can be observed within the cytoplasm of leucocytes in the hepatic sinusoids (IFAT, a: x 100; b: x 1,000).

Table 3.5: Presence of infection with *Piscirickettsia salmonis* in tissues from Atlantic salmon sampled weekly from each experimental group. Infections were reported according to organs regardless of level of infection (1 + to 4+) detected by IFAT.

GROUP	7 DPI						14 DPI						21 DPI						28 DPI					
	L	HK	PK	S	G	B	L	HK	PK	S	G	B	L	HK	PK	S	G	B	L	HK	PK	S	G	B
IP-A	1/5	2/4	3/5	2/3		1/5	5/5	3/4	2/5	3/3	2/4	1/5	5/5	3/5	4/5	3/4	1/4	2/5	N/A					
IP-B				1/5			1/5		1/5				2/5	1/5	1/4	1/4			1/1					
IP-C							1/5						2/5	1/4					0/2					
PO-A	4	2/4							2/5		1/4		3/5	1/5	1/5				5/5		1/5			
PO-B											1/4		2/5	1/4	2/5	1/2			2/5	1/5	3/5			
PO-C	2/5	2/2						1/5					2/5		1/4				5/5		1/5			
GS-A			1/5				3/5	2/4	2/5	1/4			1/5		1/5									
GS-B							2/5	2/4	2/3										N/A					
GS-C	2/5						1/3	2/4					2/5	1/5	1/5		1/5		3/5	2/5	1/5			
TOTAL(%)	14	17	9	20	0	3	36	29	24	22	12	3	42	20	26	18	5	5	64	12	24	0	0	0
CONTROL							0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Routes: Intraperitoneal (IP), orally (PO), and gill surface (GS).
Contact: Direct infection (A), physical contact (B), water contact only (C).
Liver (L), head kidney (HK), posterior kidney (PK), spleen (S), gill (G), and Brain (BN).
Empty spaces: all fish IFAT negative
N/A: Not available
DPI: Days post infection with *Piscirickettsia salmonis*

IFAT in mortalities

Foci of fluorescing microorganisms were seen in the liver and spleen serosa of intraperitoneally inoculated fish, and these occasionally extended into the parenchymal tissue. Mortalities usually showed high reactivity with IFAT for *P. salmonis* in different organs.

From all the mortalities, 88% of the fish had detectable infection by *P. salmonis*, with 83% of these presenting high levels of infection (4+). However, only 12% of the fish had piscirickettsiosis alone, 76% of the fish had mixed infection of *P. salmonis* and *A. salmonicida*, 8% of the fish had evidence of *A. salmonicida*, and 4% of the observed mortalities showed no evidence of microorganisms (Table 3.6). When comparing dead fish from the inoculated groups, 10/19 of the IP inoculated fish showed presence of *P. salmonis* alone, whereas the fish inoculated by PO and GS routes showed only mixed infections of *P. salmonis* and *A. salmonicida* (Table 3.6).

In fish showing mixed infections with *P. salmonis* and *A. salmonicida,* it was possible to observe leucocytes infected with *P. salmonis* surrounding clusters of *A. salmonicida* (Figure 3.6)

Table 3.6: Presence of *Piscirickettsia salmonis* and *Aeromonas salmonicida* in tissues of Atlantic salmon which died during the experimental period. The number of fish with no infection (no), with *P. salmonis* infection alone (Ps), *A. salmonicida* alone (As), or association of *P. salmonis* and *A. salmonicida* (Ps-As) are indicated. Fish were reported as positive when showing any level of infection.

GROUP	Fish (n)	*P. salmonis*					*A. salmonicida*				Total infection			
		0	1+	2+	3+	4+	0	1	2	3	Ps	As	Ps-As	no
IP-A	19/20	0	0	0	0	19	10	3	0	6	10	0	9	0
IP-B	18/22	1	1	0	2	14	2	1	0	15	2	1	15	0
IP-C	18/18	2	0	0	0	16	2	0	0	16	1	1	15	1
PO-A	9/14	0	0	0	1	8	0	0	1	8	0	0	9	0
PO-B	16/17	4	1	0	1	10	5	0	1	10	4	3	8	1
PO-C	14/14	3	0	0	0	11	3	0	0	11	0	0	11	3
GS-A	8/13	0	0	0	0	8	0	0	1	7	0	0	8	0
GS-B	16/27	2	1	0	0	13	0	0	0	16	0	2	14	0
GS-C	17/17	4	0	0	0	13	0	0	1	16	0	4	13	0
TOTAL (%)	83	12	2	0	3	83	16	3	3	78	12	8	76	4

Routes: Intraperitoneal (IP), orally (PO), and gill surface (GS).
Contact: Direct infection (A), physical contact (B), water contact only (C).
Fish (n): Ratio of analyzed fish from the total dead.

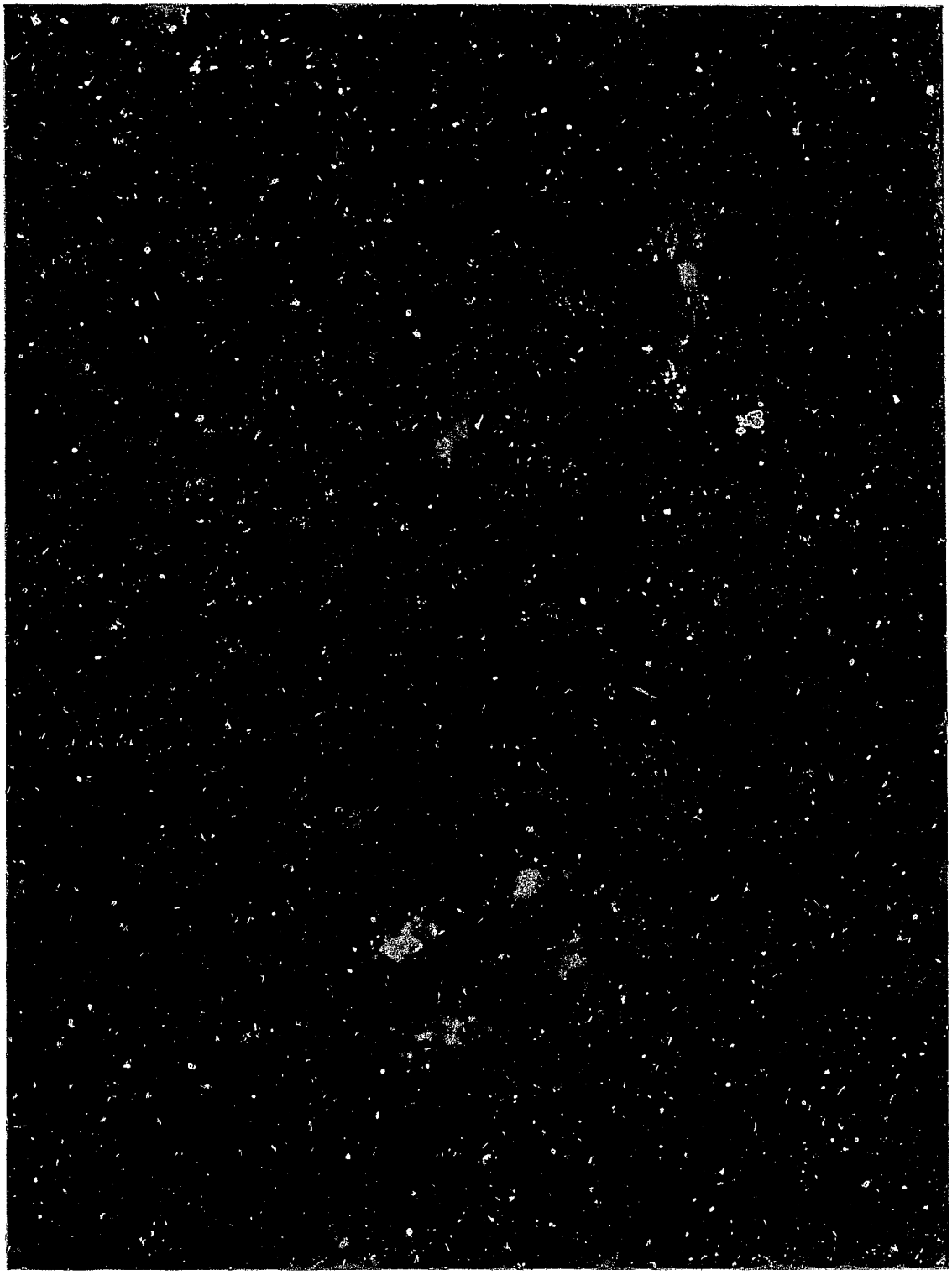

Figure 3.6: Atlantic salmon spleen showing a large cluster of *Aeromonas salmonicida* (A) surrounded by leucocytes infected by *Piscirickettsia salmonis* (arrows) (IFAT, **a:** x 400; **b:** x 1,000).

3.5 Discussion

Infection routes

In this work *P. salmonis* was transmitted to freshwater maintained Atlantic salmon by intraperitoneal injection, oral intubation and by application to gill surface. *Piscirickettsia salmonis* was also detected in contact and non-contact cohabitants of fish belonging to each inoculation group. In inoculated fish, as in cohabitants, the presence of *P. salmonis* was easily detected by IFAT in organs such as liver and kidney, but was not very frequent in the gills or brain. Furthermore, the presence of *A. salmonicida* in some of the fish did not interfere with detection of *P. salmonis* by IFAT.

The pattern of infection in gill and orally infected fish resembled that observed in natural outbreaks of SRS (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). The IP route was previously reported as being effective in reproducing piscirickettsiosis in experimentally inoculated coho salmon and Atlantic salmon (Cvitanich *et al.*, 1991; Garcés *et al.*, 1991). Although the IP route does produce infection and IP infection through skin wounds is possible in nature, the oral and gill routes are perhaps more likely to represent the infection route in naturally infected fish. Experimental infection by oral and gill routes suggests that they may occur in nature and this may have important implications in the epizootiology of this systemic disease.

The first requisites for virulence in horizontally transmitted pathogens are the ability to survive in the aquatic environment and the ability to enter the host

(Trust, 1986). Fish pathogens probably enter via the gills and across the skin, and on occasion via the oral and gastrointestinal routes (Trust,1986). The biological interface between the aqueous environment and the fish (gills, skin, and gut) is the mucous coat. The clearance kinetics of the mucous coat, and the presence of antimicrobial protective factors such as lysozyme, agglutinins and local antibodies are some of the barriers that have to be crossed by any pathogen (Speare and Mirsalimi, 1992; Magariños *et al.,* 1995).

The oral route might be especially important in the case of *P. salmonis,* since natural outbreaks of SRS typically occur a few weeks after smolts are transferred to the sea (Fryer *et al.,* 1990; Branson and Nieto, 1991; Cvitanich *et al.,* 1991). Smolts ingest and absorb large amounts of water to balance the osmotic loss of water after they are transferred to the marine environment (Evans, 1993), offering a port of entry for pathogens in suspension. Experimental infection of Ayu *(Plecoglossus altivelis)* with *Vibrio anguillarum* in fresh water, by oral and anal intubation have been previously reported (Kanno *et al.,* 1989). Based on the present results, the gills may also be important in the natural transmission of SRS. Evidence that gills provide an effective and fast infection route has been demonstrated for several fish pathogens such as spring viraemia of carp virus (SVCV), viral haemorrhagic septicaemia virus (VHSV), and *A. salmonicida* (Anhe, 1978; Chilmonczyk, 1980; Effendi and Austin, 1995). The pathogen *A. salmonicida* was also present in some of the fish of the present work. This bacterium can enter Atlantic salmon through a

range of sites including the oral route, gills, anus, flank, ventral surface, and lateral line (Effendi and Austin, 1995).

The IFAT results of the present report showed that the pattern of infection observed in the IP inoculated fish is very different from the one observed in orally, gill or cohabitant infected fish. After IP inoculation, *P. salmonis* invaded the capsule (serosa) of the spleen and liver. A similar pattern of capsular infection has been shown after IP inoculation of Atlantic salmon and rainbow trout with the intracellular pathogen *Renibacterium salmoninarum* (Bruno, 1986). Although systemic piscirickettsiosis eventually developed in IP inoculated fish, the process of colonization of the host does not appear to resemble natural infection. Conversely, fish inoculated orally or on the gill surface showed a systemic pattern of infection, with infected leucocytes commonly observed in blood vessels.

In the present study, mortalities of Atlantic salmon held in fresh water at 11 °C began after 12, 14 and 17 DPI in the orally, gill and IP inoculated fish, respectively. Garcés *et al.,* (1991) reported mortalities from 14 DPI in IP inoculated coho and Atlantic salmon held in fresh water at 10.5 °C. Another report showed that in IP inoculated coho salmon held at 15 °C, mortalities started after 7 and 9 DPI in salt water and after 10 and 11 DPI in freshwater aquaria (Cvitanich *et al.,* 1991). Differences in the onset of mortalities may be explained by the water temperature, species used, size of the fish, dosage of the inoculum used, and the presence of *A. salmonicida.*

In the Cox proportional hazards survival analysis model, IP and GS infected fish had a lower cumulative probability of survival than PO infected fish ($P<0.05$). No differences in cumulative probability of survival were observed between the IP and GS infected fish, and no mortalities were observed in the uninoculated control group. Differences in the onset of mortalities resulted in higher numbers of fish available for sampling 28 and 35 DPI for the orally inoculated fish. Previously, other authors have reported mortalities reaching 100% in IP inoculated Atlantic salmon (Garcés *et al.,* 1991). However, these authors used a higher infectious dose, and did not sample live fish during the experiment.

Horizontal transmission

This study is the first that tests physical contact as a risk factor for transmission of SRS in fresh water. Piscirickettsial infection and mortalities developed in all contact and non-contact cohabitants with IP, PO or GS inoculated Atlantic salmon. Relative risk analysis of cohabitant fish showed in the IP and GS infected groups, a statistically higher risk of dying for cohabitants with physical contact (6.8) than for those without (2.5).

Although initially piscirickettsiosis was only described as occurring in sea water (Cvitanich *et al.,* 1991; Branson and Nieto, 1991), natural freshwater outbreaks of SRS were recently reported in rainbow trout, coho, and Atlantic salmon (Bravo, 1994 a b; Gaggero *et al.,* 1995; Cvitanich *et al.,* 1995).

Previous studies in aquaria regarding horizontal transmission have shown different results. Cvitanich *et al.* (1991) reported horizontal transmission of *P. salmonis* from IP inoculated coho salmon to cohabitants held in sea water or fresh water. However, Garcés *et al.* (1991), reported that horizontal transmission did not occur in uninoculated cohabitants held in fresh water with IP inoculated coho salmon. Furthermore, *in vitro* experiments regarding extracellular survival of purified *P. salmonis* (coho salmon isolate), showed that no infectious particles could be detected immediately after suspension in fresh water. However in salt water, infectious particles of *P. salmonis* were detected 10 to 15 days later (Lannan and Fryer, 1994). The poor capacity of rickettsial particles to survive in fresh water may explain the differences in the relative risk analysis due to physical contact. Physical contact is likely to be an important risk factor in the transmission of SRS in fresh water.

Within a tank of fish there are several factors promoting horizontal spread of established infections. For some diseases such as *R. salmoninarum* infection, an important epizootiologic factor is the close proximity between animals held for long periods (Murray *et al.,* 1992). Close contact with live or dead fish is also important in transmission of external parasites (Bakke *et al.,* 1991, 1992). Necrophagic behaviour could also be important for transmission of SRS, thus early extraction and appropriate disposal of the mortalities, might be important in the control of this disease.

In non-contact cohabitants, transmission of the disease in fresh water is

not easily explained. Cvitanich *et al.* (1991) reported abundant RLO-laden cells in the large intestine of coho salmon, and the authors hypothesised that the agent could be released through the faeces and survive long enough to infect other fish. Furthermore, if viable rickettsia are present in the faeces, coprophagic behaviour may also be an alternative mechanism of transmission.

Based on the favourable intravacuolar microenvironment created by rickettsial organisms contained inside the cell (Woldehiwet and Ristic, 1993), a possible survival mechanism could be that the intracellular rickettsiae are surrounded by cellular debris and mucous material and are protected from the hypotonic environment created by the fresh water. Cells were observed carrying *P. salmonis* in the kidney, tubular epithelial cells, and sometimes in the glomeruli. Thus this organism could also be released into the urine, and consequently represent another factor involved in the horizontal transmission of SRS.

In natural outbreaks other factors such as intermediate host or vectors may also play an important role in the transmission of SRS, as occurs in mammals where rickettsial diseases are mainly transmitted by ticks. Recently, a rickettsia antigenically similar to P. *salmonis* was detected by IFAT in sections of *Ceratothoa gaudichaudii* (Garcés *et al.,* 1994), a common haematophagus ectoparasite of cultured salmonids in Chile (Sievers *et al., 1995).*

Double infection

In the present experiment, microorganisms other than *P. salmonis* were also detected in a number of fish. The organism was identified as *A. salmonicida* by morphologic characteristics, Giemsa stain, and IFAT. *Aeromonas salmonicida* did not show cross-reactivity with the rabbit anti-*P. salmonis* antibody used in the analysis, which allowed an easy identification. *Aeromonas salmonicida is* the causative agent of furunculosis, a rapidly disseminating disease of salmonids at all stages of their development and is abundant in fresh, estuarine and salt water (McCarthy and Roberts, 1980; Garduño *et al.,* 1993 b).

It was not po ssible to establish the source of the *A. salmonicida* infection. This pathogen could have been carried by some of the fish used in the present study. However, previous to initiation of the experiments a group of 60 fish from the stock were injected with prednisolone acetate to produce immunosuppression and detect carriers of common fish pathogens. None of the treated fish showed clinical signs of disease, mortality, or growth or bacteria (Aqua Health Ltd., personal communication). The 60 fish sampled reacted negatively to a carrier-stress test which can be interpreted that there is a 95% confidence that the presence of culturable *Aeromonas salmonicida* is 5% or less (Martin *et al.*, 1987). Another possibility is the contamination of the water with *A. salmonicida*. However, mortalities only occurred in fish infected with *P. salmonis* and *A. salmonicida* was detected in 1/12 control fish.

Mixed infections of *P. salmonis* with other pathogens have been reported previously (Cvitanich *et al.,* 1991; Brocklebank *et al.,* 1993). The presence of *Renibacterium salmoninarum,* the causative agent of Bacterial Kidney Disease (BKD), along with *P. salmonis* infection has been described in sea water and fresh water outbreaks (Cvitanich *et al.,* 1991; Gaggero *et al.,* 1995; Smith *et al.,* 1995). The Chilean Association of Salmon and Trout Growers reported that 58% of the coho salmon outbreaks are associated with *Renibacterium salmoninarum* (Cassigoli, 1994). Additionally, a microsporidean protozoa *(Enterocytozoon salmonis)* has been described in salmonids with piscirickettsiosis (Cvitanich *et al.,* 1991; 1995). Recent outbreaks of a RLO in salmon on the west coast of Canada have also been associated with microsporidian infections (Dr. David Groman, 1995, personal communication).

Rickettsial organisms infecting leucocytes usually produce an immunosuppressor effect on the host that may allow other pathogens to proliferate (Woldehiwet and Ristic, 1993). The presence of *P. salmonis* within leucocytes and the double infection observed in some inoculated fish suggest an immunosuppressive effect of *P. salmonis* that may have played a role in the capacity of *Aeromonas salmonicida* to infect and proliferate in the host.

Final remarks

In this study oral and gill routes were found to be viable portals of entry for *P. salmonis* infection. The importance of physical contact as a risk factor

for the horizontal transmission of SRS was also confirmed, although, physical contact was not necessary for horizontal transmission in fresh water. The sequential IFAT study showed that orally and gill inoculated fish, and infected cohabitants showed a similar systemic pattern of infection, different from the capsular (serosa) infection pattern observed in IP inoculated fish. The presence of *P. salmonis* in the cytoplasm of epithelial cells of the renal tubules indicates that elimination of this pathogen through urine may be possible. Additionally, the presence of *P. salmonis* within leucocytes, and the double infection (*P. salmonis* - *A. salmonicida)* observed in some of the fish exposed to this pathogen suggests an immunosuppressive effect of *Piscirickettsia salmonis* that may predispose the host to infections with other pathogens.

4. PATHOGENESIS OF *PISCIRICKETTSIA SALMONIS* IN FRESHWATER REARED ATLANTIC SALMON, *SALMO SALAR* L..

4.1 Summary

Piscirickettsia salmonis, the causative agent of salmonid rickettsial septicemia (SRS) or piscirickettsiosis, is the most important pathogen affecting the Chilean salmon industry. The pathogenesis of lesions associated with *P. salmonis* infection was studied in Atlantic salmon juveniles (n = 108). Fish were maintained in fresh water and inoculated IP, orally, or on the gill surface with *P. salmonis* (36 fish per route). Inoculated fish were separated into six tanks (two tanks of 18 fish per route). To each tank was added 18 contact-cohabitant and 18 non-contact cohabitants. A group of uninfected fish held at similar stocking densities, was kept as control. Liver, spleen, kidney, brain and gill samples were collected weekly at necropsy and processed for routine histological examination. The presence of *P. salmonis* in tissues was confirmed by IFAT. All infected fish showed histological changes, from 7 days post inoculation which indicated a systemic infection characterized by vasculitis, fibrin thrombi, foci of necrosis and occasional vacuolated hepatocytes and tubular epithelial cells. Leucocytes containing intracytoplasmic basophilic microorganisms were often seen. Gill changes consisted of thickening of the endothelium with presence of intracytoplasmic vacuoles in endothelial cells. Histological changes were not seen in the brain. Another microorganism, identified by IFAT as *Aeromonas salmonicida*, was also observed in some of the

sampled fish. Although *A. salmonicida* may increase the severity of the observed lesions, the intracellular location of *P. salmonis* and the vascular damage seen in infected fish are characteristic of rickettsial infections. Histological lesions were similar to those observed in natural outbreaks of piscirickettsiosis, revealing a relative tropism of *P. salmonis* for endothelial cells.

4.2 Introduction

The growth of the Chilean salmon industry has been challenged recently by the emergence of new diseases such as salmonid rickettsial septicaemia (SRS), a systemic disease caused by *Piscirickettsia salmonis*. The disease has been experimentally reproduced in coho and Atlantic salmon by intraperitoneal inoculation (Cvitanich *et al.*, 1991; Garcés *et al.*, 1991). However, very little is known about natural routes of infection, and the sequence of pathological changes produced during SRS. The lesions of terminal cases from naturally infected fish have been described (Cvitanich *et al.*, 1991, Branson and Nieto, 1991), but a sequential development of the lesions has not been described. Furthermore, very few studies have examined experimental *P. salmonis* infection in Atlantic salmon, *Salmo salar* L., although this species is becoming increasingly important in the Chilean aquaculture industry (Méndez, 1995). The main objectives of this work were to describe the pathogenesis of *P. salmonis* in Atlantic salmon using experimental inoculations via IP, oral and gill routes, and cohabitation to clarify the nature of the lesions observed in natural outbreaks.

4.3 Material and Methods

Experimental design

Isolates of *P. salmonis* used in this experiment were obtained from naturally infected Atlantic salmon reared in salt water in southern Chile, with clinical SRS (generous gift of Dr. Enrique Madrid, Fish Pathology Laboratory, Marine Harvest McConnell, Puerto Montt, Chile).

Three hundred and eighty fish weighing 20 ± 2.3 g were maintained in fresh water at 11 °C. A total of 108 fish were inoculated with cultured *P. salmonis*, either by intraperitoneal (IP), oral (PO), or gill surface (GS) route and separated in six tanks. Cohabitant fish, with and without physical contact with inoculated fish were also included in each tank. An uninoculated control group was also included in a seventh tank. For details on the experimental design refer to Figure 3.1 (page 67).

Five fish from each of the 9 experimental groups, and three from the control group, were sampled weekly for 5 weeks following infection. During the sampling process no attempt to select fish showing clinical signs was made. Fish were removed from the tank, sedated in 50 ppm of benzocaine hydrochloride solution, and euthanised by spinal severance. The carcass was weighed and the peritoneal cavity was exposed from the left flank for post-mortem examination.

The tail was cut behind the adipose fin and blood was collected from the dorsal aorta using an heparinised (2 usp units ammonium heparin) micro-

haematocrit capillary tube (Fisher Scientific, Pittsburgh, PA, U.S.A.) sealed with a commercial sealant (Critoseal™). Blood samples were kept on ice. The liver, spleen, head kidney, posterior kidney, brain and the second branchial arch of the left gill were removed and placed in 10% neutral buffered formalin. Tissues from fish that died during the study period were similarly sampled and fixed.

Light microscopy (LM)

Tissues were fixed and processed for light microscopy as described in Chapter 3 (page 69). Sections were stained for routine LM with haematoxylin and eosin (HE) (Appendix D) to examine light microscopic changes. Finally, sections were dehydrated from water to xylene, mounted with Flo-texx® mounting medium (Lerner Laboratories, Pittsburgh, PA, U.S.A.), cover slipped, observed using a Nikon Labophot microscope (Nikon Canada, Mississauga, ON, Canada), and photographed using a Leitz microscope and Kodak Gold 100 film.

Haematocrits

Capillary tubes containing blood samples were centrifuged for 5 min at 7000 rpm in a Haemofuge® (Canlab, Heraeus Christ GmbH, Osterode Model 1301, Germany) and the packed cell volume was measured in a haematocrit table. Haematocrit values obtained from fish exposed to *P. salmonis* and uninfected controls were compared by paired t test at each sampling day. Statistical significance was set at $p \leq 0.05$.

4.4 Results

Clinical signs and gross lesions

Clinical signs including swimming at the edge of the tank and at the water surface, lethargy, darkening of the skin, increased opercular frequency and anorexia were observed from 12 DPI to the end of the experimental period. Clinical signs were first detected in 2 fish from group IP-A (inoculated IP), 2 fish from IP-B group (contact cohabitants with IP inoculated), and one fish from group PO-A (orally inoculated). Clinical signs culminating with death developed very rapidly (within 12 h) in fish that died during the sampling period.

A gross external lesion of abdominal distension was first observed 14 DPI in two fish from group IP-A (inoculated IP), and one fish from group PO-A (orally inoculated). Petechiae and small ulcers (up to 2 mm in diameter) were first observed 28 DPI, in the skin of the abdomen in one fish from IP-B group (contact cohabitant with IP inoculated). External lesions were not observed at any sampling time in non-contact cohabitants.

Post-mortem examination of sampled fish revealed swelling of the kidney and spleen, and occasional petechiae. Foci of pale tan discolouration measuring about 1 mm in size were also observed on the capsular surface of the liver. At 14 DPI , one fish from IP-B group (contact cohabitants with IP inoculated) had off-white foci measuring up to 1 mm in diameter, on the capsular surface of the liver. Petechiae in the liver were observed 28 DPI, in one fish from group PO-A (inoculated PO), one fish from PO-B group (contact cohabitants with PO

inoculated), and one fish from GS-C group (non-contact cohabitants with GS inoculated), and 35 DPI, in one fish from group PO-B (contact cohabitants with PO inoculated). Post-mortem examination of fish that died during the experiment revealed splenomegaly and petechiae in the liver and spleen. However, lesions were not always evident since mortalities were collected and examined up to 24 h after death. None of the control fish sampled showed clinical signs or gross lesions.

Haematocrits

Haematocrit or packed cell volume (PCV) were measured as the percentage (%) of cells in the whole blood. No differences were observed in the haematocrit among fish inoculated through IP, oral or gill routes at any point of the sampling periods. Similarly, no differences were observed between inoculated versus non-inoculated (cohabitants) fish. Haematocrit values were measured from 7 DPI in fish exposed to *P. salmonis* and 14 DPI in the control group. A statistically significant decrease ($p<0.05$) for the haematocrit values was detected in all fish exposed (i.e., inoculated and cohabitants) to *P. salmonis* (42.1, 37.6, 37.6) when compared with the unexposed controls (54, 50, 50) at 14, 21, and 28 days post inoculation (DPI) respectively (Figure 4.1).

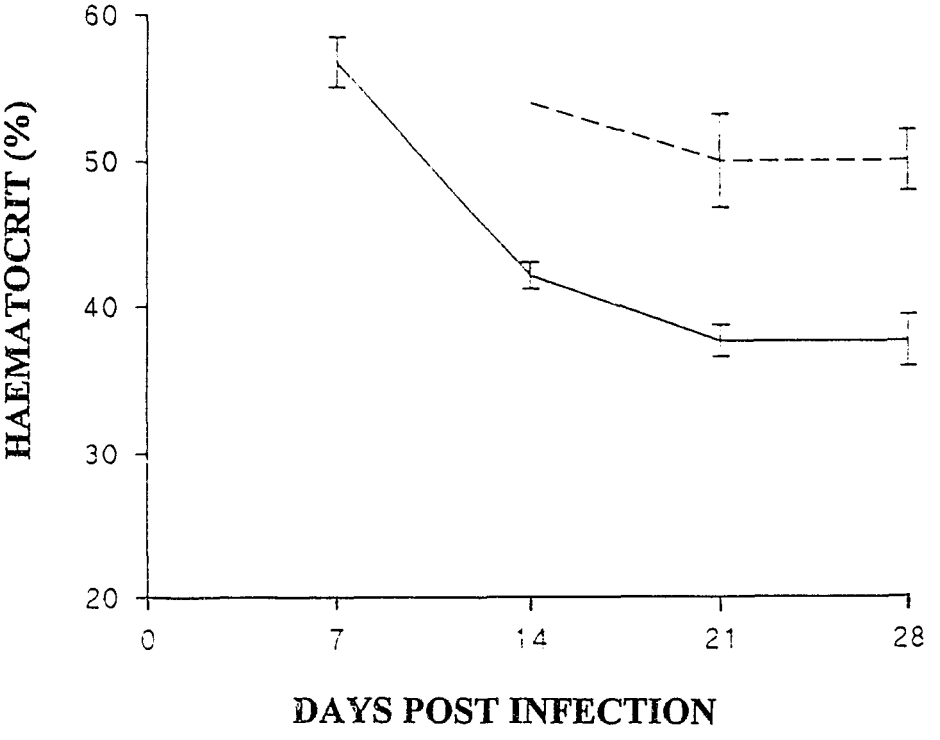

Figure 4.1: Mean and standard error of the mean of the haematocrit (%) of fish sampled at different days post infection from nine groups exposed to *Piscirickettsia salmonis* (——) and from the unexposed control (— —) group.

Histological Examination of sampled fish

Histological changes were described at the light microscopic level using HE stained specimens from sampled and dead fish from all groups. Morphologic changes were not observed in the brain of fish in experimental or control groups.

IP inoculated fish

Sampled fish from the IP inoculated group had lesions in several organs. The capsule of the liver had locally extensive areas of thickening due to inflammatory cell infiltration (Figure 4.2) in 1/5 fish by 7 DPI, progressing to diffuse capsulitis in 5/5 fish by 14 DPI, and in 4/5 fish by 21 DPI. Moderate vasculitis characterized by the presence of leukocytes and fibrin within the wall of the blood vessels were also observed in 4/5 fish by 7 DPI and 14 DPI, and in 2/5 by 21 DPI. Multifocal to locally extensive areas of coagulative necrosis, characterized by loss of cell detail, pyknosis, and karyolysis were observed close to the capsule of the liver in 4/5 fish by 7 DPI, 5/5 fish by 14 DPI, and 3/5 fish by 21 DPI (Figure 4.3). Vacuoles containing basophilic microorganisms interpreted as *P. salmonis* were seen in the cytoplasm of leucocytes, the wall of blood vessels and cytoplasm of hepatocytes.

The spleen of the IP inoculated fish also had locally extensive to diffuse areas of serosal inflammation, with vacuolation, and necrosis of the cells close to the capsule (Figure 4.2) in 2/5 fish by 7 DPI, in 5/5 fish by 14 DPI, and in

4/5 fish by 21 DPI. Multifocal to locally extensive areas of accumulation of leucocytes with vacuolated cytoplasm were present in 3/5 fish by 7 DPI, in 2/5 fish by 14 DPI and 21 DPI. Vasculitis with thickened and vacuolated endothelia were observed in 3/5 fish at 14 DPI, and 21 DPI. Focal to multifocal granulomas were observed in 3/5 fish 7 DPI. Granulomas had a core of caseous necrosis characterized by loss of tissue architecture and cellular detail surrounded by fibrous connective tissue and numerous mononuclear cells containing abundant intracytoplasmic eosinophilic granules (Figure 4.4).

Multifocal to diffuse areas of interstitial nephritis with cytoplasmic vacuolation and necrosis of tubular cells were observed in the posterior kidney in 2/5 IP inoculated fish by 7 DPI, in 5/5 fish by 14 DPI and in 4/5 fish by 21 DPI. Glomerulitis and vacuolation of cells of the glomerular tuft along with presence of intravacuolar basophilic microorganisms were observed 7 DPI in 3/5 fish, and in 1/5 fish 14 and 21 DPI. Fibrin thrombi within small vessels were also observed 7 and 14 DPI in 2/5 fish. Focal to multifocal areas of vasculitis with presence of vacuoles in the endothelium were observed in 4/5 fish, and progressed to a multifocal to diffuse distribution in 4/5 fish by 14 and 21 DPI. Granulomas similar to those observed in the spleen were observed in the renal interstitium of 1/5 fish by 7 DPI. Multifocal vacuolation and necrosis of white blood cells was observed in 1/5 fish by 7 DPI, in 2/5 by 14 DPI, and in 1/5 by 21 DPI.

The haematopoietic head kidney of IP inoculated fish had focal to diffuse

areas of leucocyte accumulation. Organisms were present within vacuoles in the cytoplasm of leucocytes in 4/5 fish by 7 DPI, in 3/5 by 14 DPI, and in 2/5 by 21 DPI. Fibrin thrombi and areas of coagulative necrosis were observed in 3/5 fish by 7 DPI, and in 4/5 fish by 14 DPI. Granulomas were present in 1/5 fish by 7 DPI, and presence of intravacuolar microorganisms in the thickened vascular endothelium of large vessels was observed in 1/5 fish by 7 and 14 DPI, and in 2/5 fish by 21 DPI. Haematopoietic cells were vacuolated in 2/5 fish by 7 DPI.

No lesions were observed in the gill and brain of the fish sampled at any time from the IP injected group.

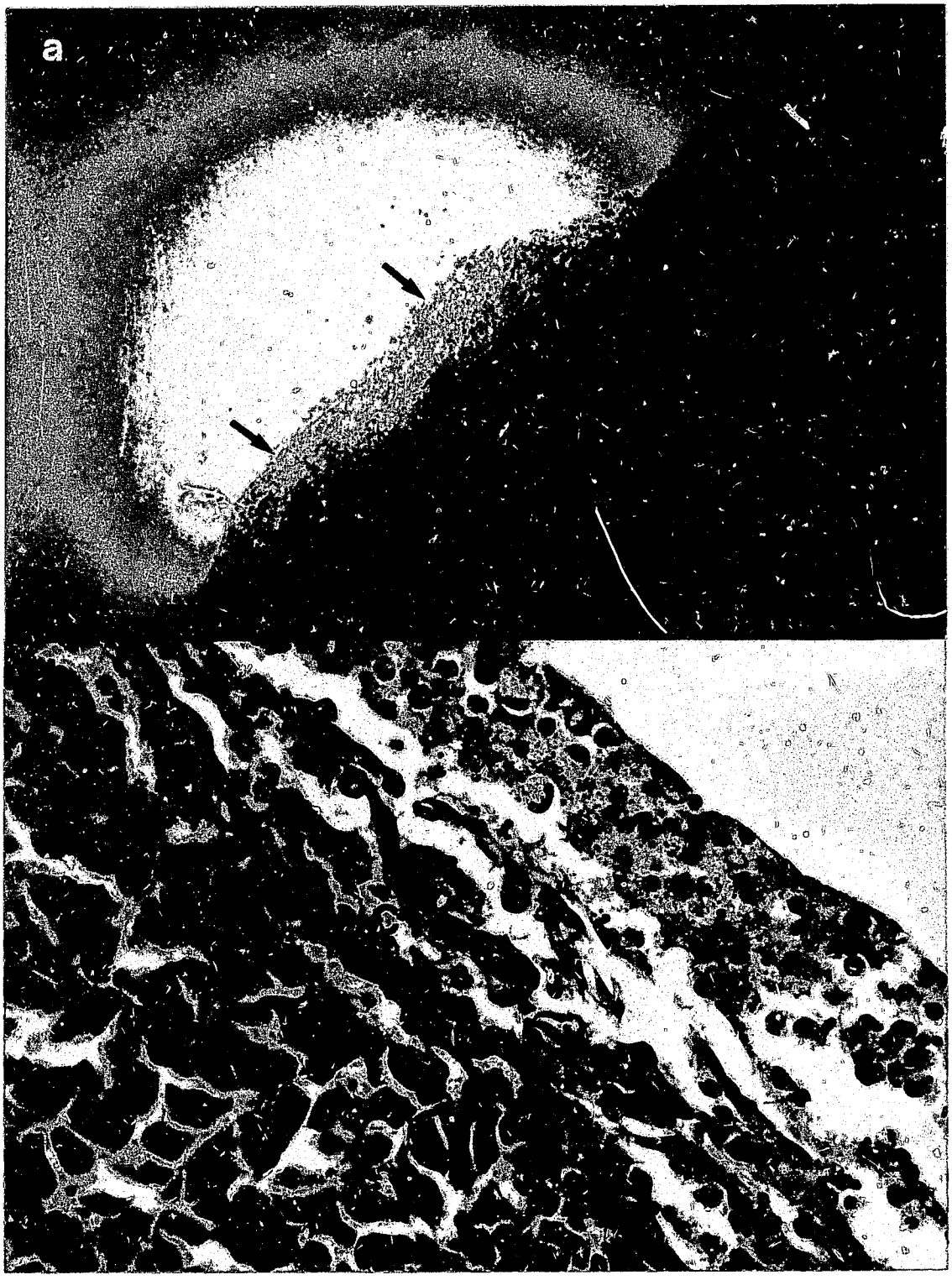

Figure 4.2: Lesions observed in tissues sampled from Atlantic salmon inoculated intraperitoneally with *Piscirickettsia salmonis*. **a:** Locally extensive serosal inflammatory infiltration in spleen (arrows) 14 days post inoculation (x 100); **b:** Thickening of hepatic capsule (C) due to infiltration by leukocytes (arrows) 14 days post inoculation (x 400).

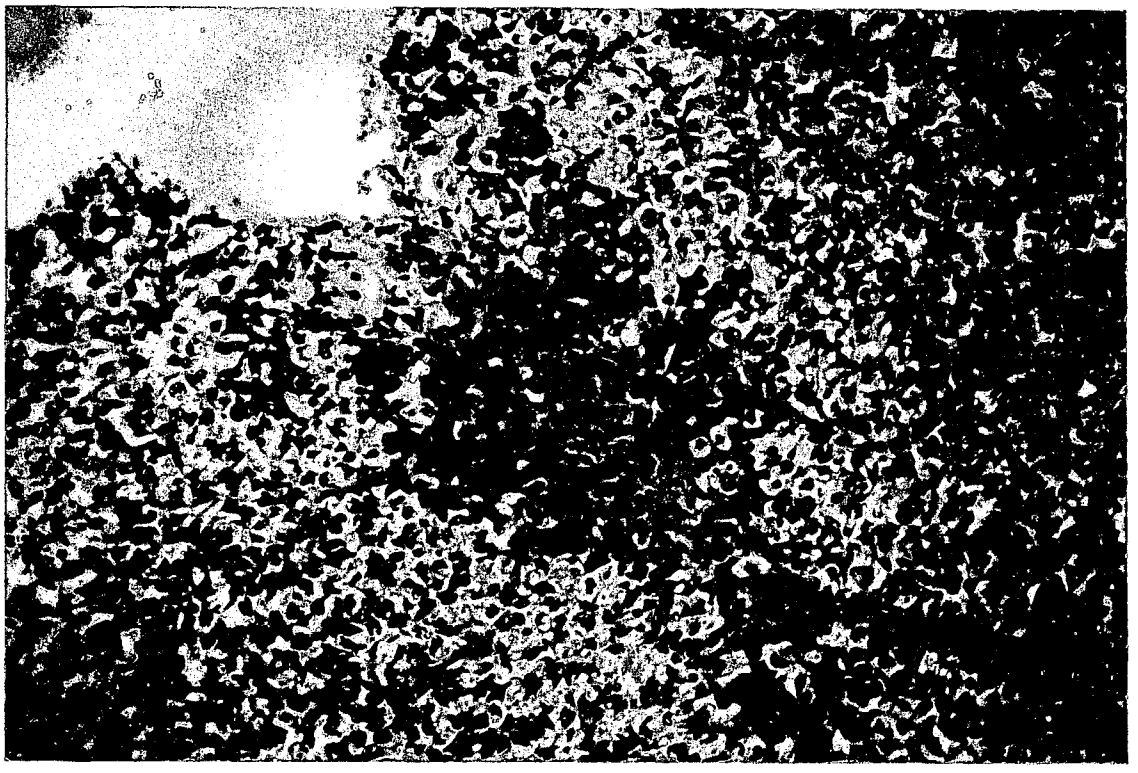

Figure 4.3: Lesion observed in hepatic tissue sampled from Atlantic salmon inoculated intraperitoneally with *Piscirickettsia salmonis*. Focal area of coagulative necrosis (arrows) in liver 14 days post inoculation (x 250).

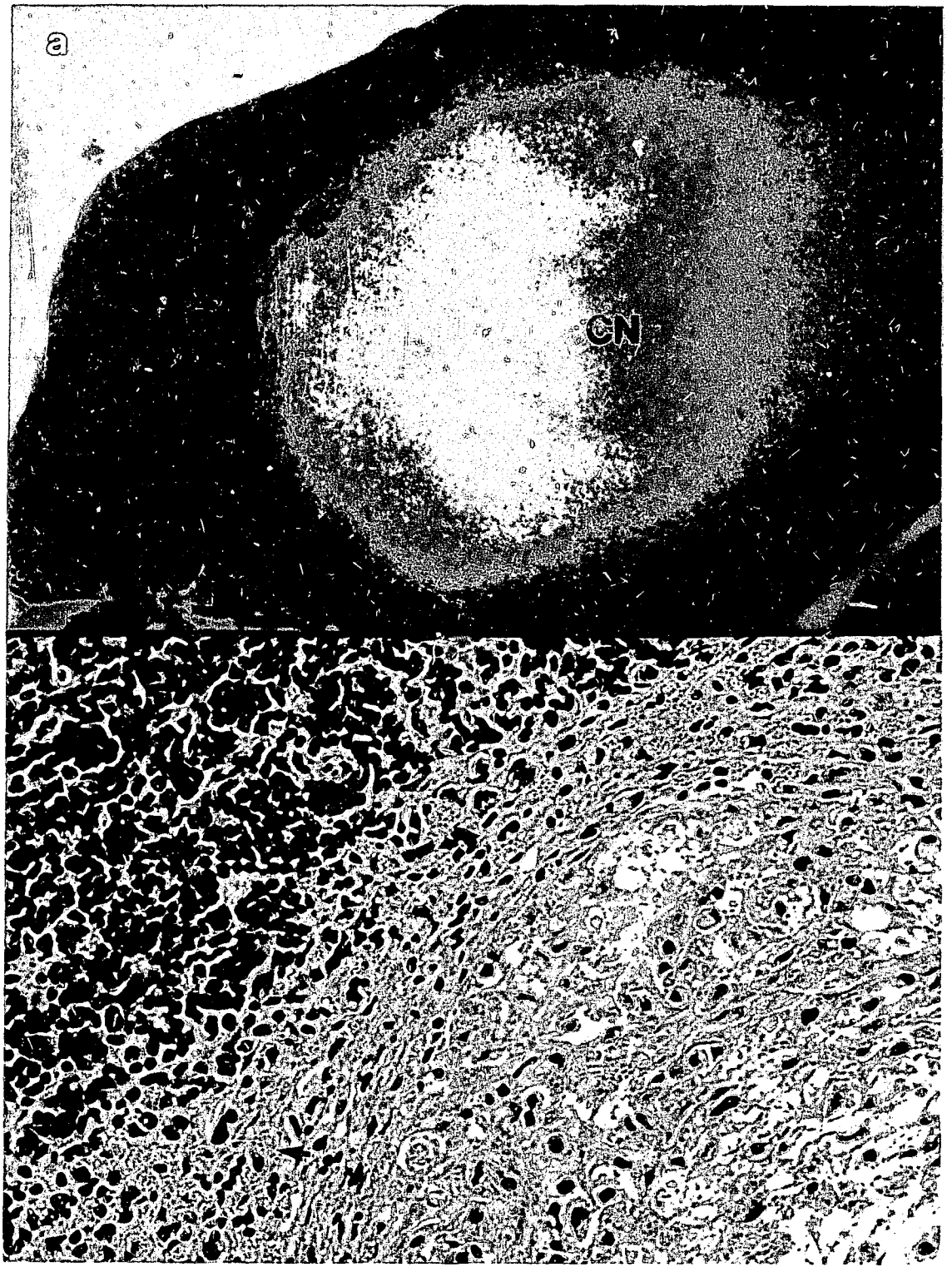

Figure 4.4: Large granuloma in spleen of Atlantic salmon 7 days after intraperitoneal inoculation with *Piscirickettsia salmonis*. **a:** Granuloma in the spleen with a center of caseous necrosis (CN) and a distinct capsule (C) (x 100); **b:** Periphery of splenic granuloma with numerous cells containing eosinophilic granules (arrows) (x 400).

PO inoculated, GS inoculated, and cohabitant fish

The histological changes in the liver and spleen capsule described for the IP injected group, were not observed in the orally and gill inoculated fish, or in the cohabitant groups. In these groups the lesions in liver and spleen indicated a systemic infection rather than a serosal infection. Liver lesions characterized by vasculitis with infiltration of leucocytes through the wall of major blood vessels, thickened vessel walls caused by accumulation of eosinophilic fibrillar and granular material and presence of fibrin within the lumen of the vessels (Figure 4.5) were observed from 7 DPI. Numerous perivascular necrotic hepatocytes, characterized by slight hypereosinophilia, pyknosis and karyolysis were a common feature starting at 7 DPI (Figure 4.6). Intracytoplasmic vacuoles were often observed in individual hepatocytes (Figure 4.7).

In the posterior kidney, multifocal to diffuse areas of mild interstitial nephritis (Figure 4.8), cytoplasmic vacuolation and necrosis of the tubular cells (Figure 4.9) were observed by 7 and 14 DPI. Vacuolation of basal and apical portions of the cytoplasm of the tubular cells and separation of the basement membrane of the renal tubules was observed from 7 DPI (Figure 4.9). Intracytoplasmic eosinophilic droplets were usually observed in the cytoplasm of tubular cells undergoing necrosis (Figure 4.9). Glomerulitis and presence of intracytoplasmic vacuoles were also observed from 14 DPI (Figure 4.8). Vacuolation and necrosis of leucocytes was also observed from 7 DPI. Leucocytes containing intravacuolar microorganisms were observed in the head

kidney from 7 DPI.

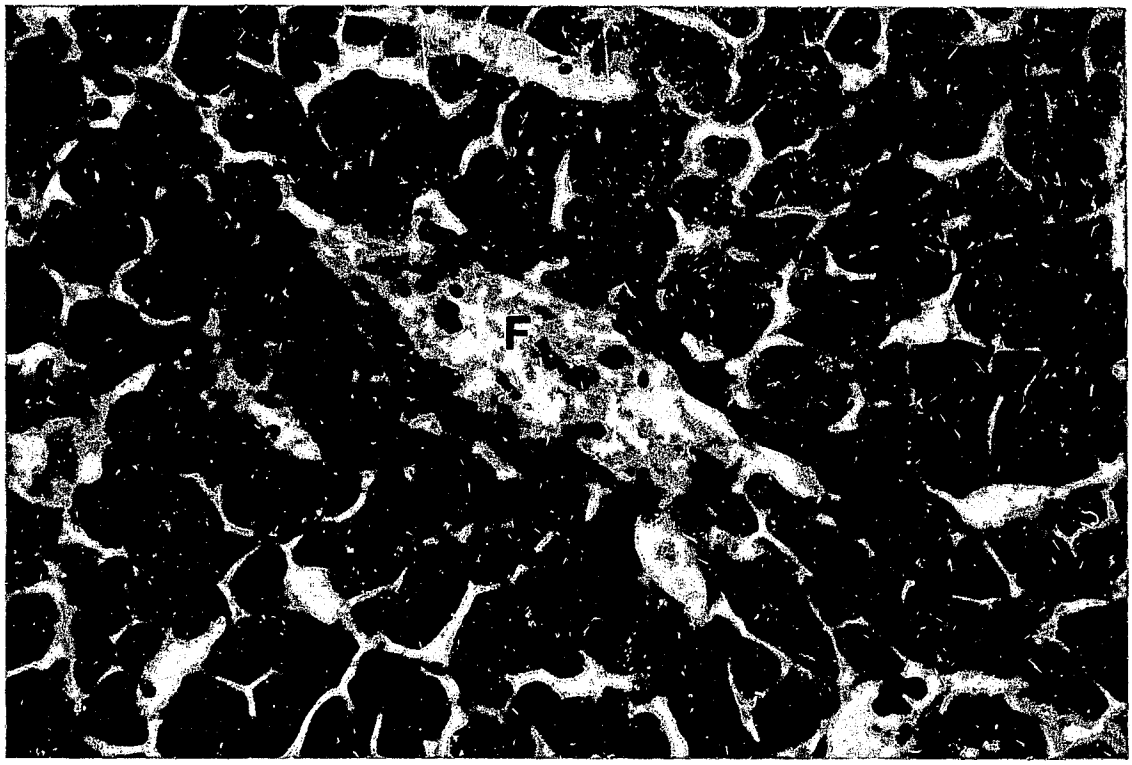

Figure 4.5: Histological changes in liver of Atlantic salmon liver orally inoculated with *Piscirickettsia salmonis* 7 dpi. Fibrin-like material (F) within the lumen of blood vessels (x 400).

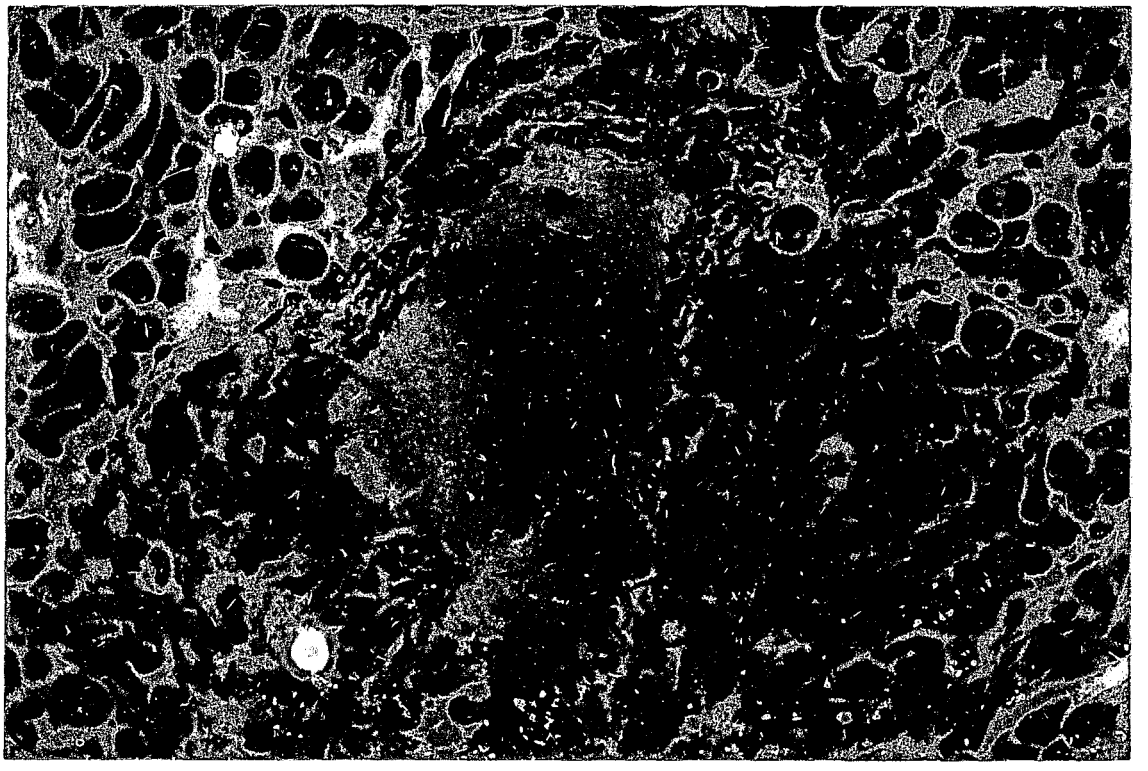

Figure 4.6: Lesions in liver of Atlantic salmon infected by contact-cohabitation with *Piscirickettsia salmonis* 7 dpi. Numerous perivascular necrotic hepatocytes (arrows) (x 400).

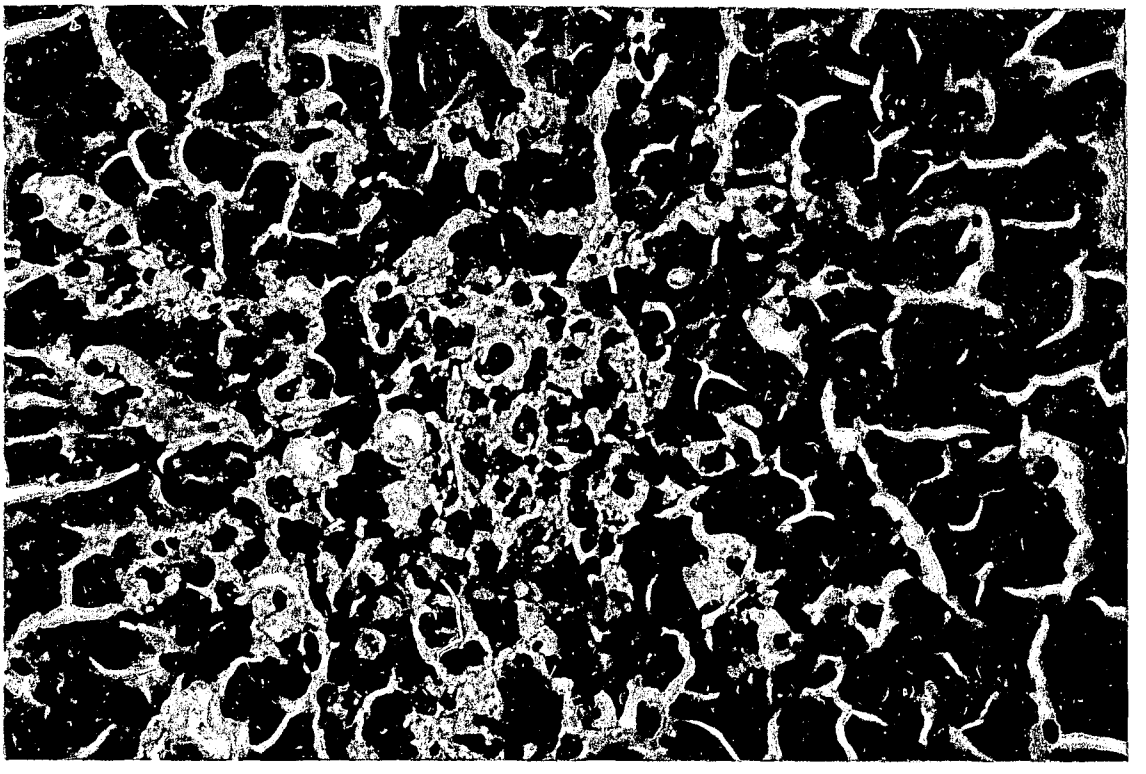

Figure 4.7: Histological changes in liver of Atlantic salmon orally inoculated with *Piscirickettsia salmonis,* 21 dpi. Focus of coagulative necrosis of hepatocytes (thick arrows) and intracytoplasmic vacuoles (curved arrows) (x 400).

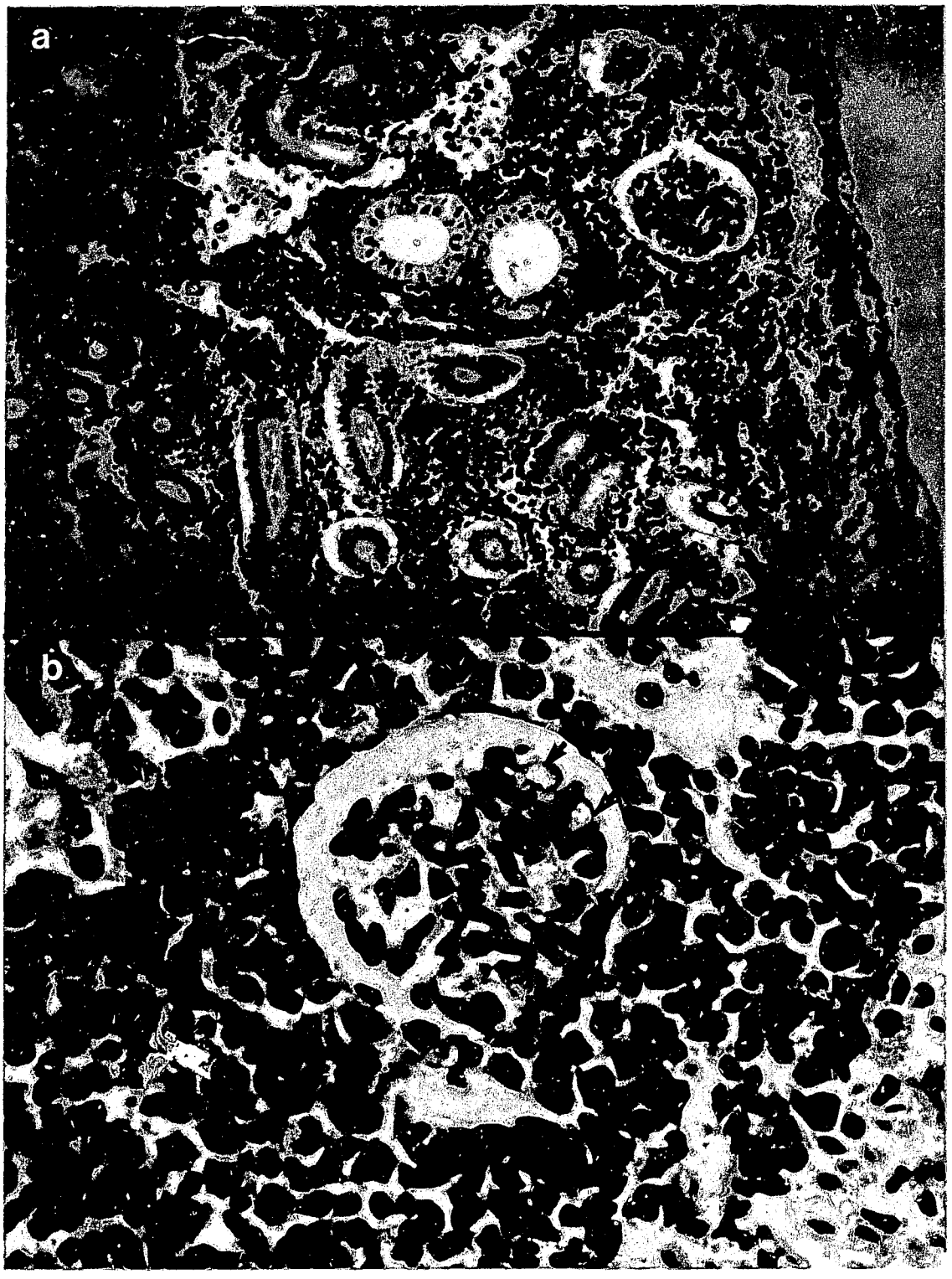

Figure 4.8: Histological changes in posterior kidney of Atlantic salmon orally inoculated with *Piscirickettsia salmonis*. **a:** Mild interstitial nephritis with numerous melanomacrophages in posterior kidney (x 100). **b:** Hypercellularity of glomerular tufts and presence of intracytoplasmic vacuoles (arrows) (x 400).

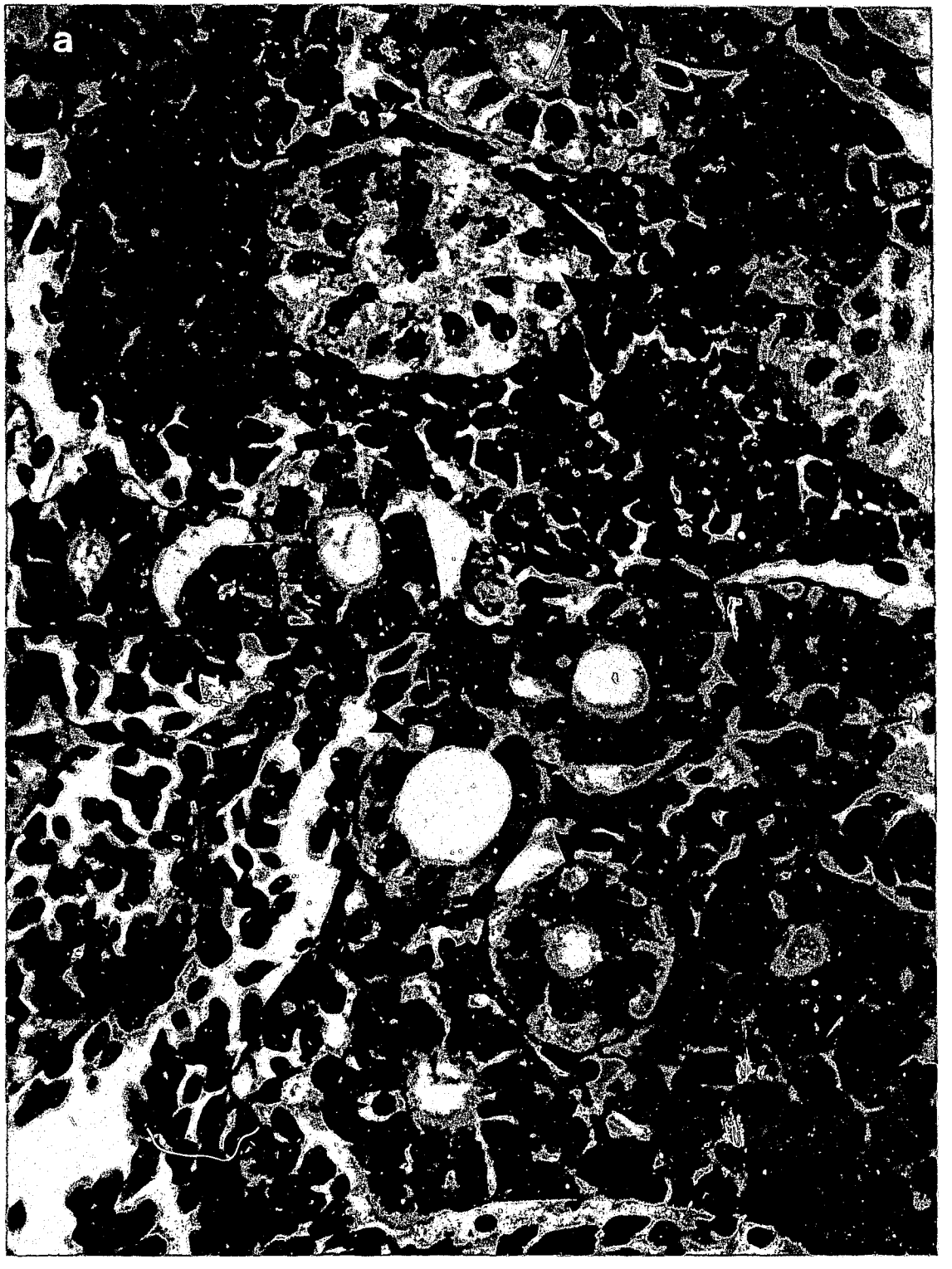

Figure 4.9: Histological changes in posterior kidney of Atlantic salmon infected with *Piscirickettsia salmonis* by contact-cohabitation. **a:** Vacuolation of tubular epithelial cells (arrowheads) and presence of a vacuolated cell inside the tubular lumen (arrow) (x 400). **b:** Eosinophilic droplets within the cytoplasm of tubular epithelial cells (arrows) and numerous vacuolated tubular cells (arrowheads) (x 400).

The spleen had locally extensive to diffuse areas of inflammatory cell infiltration and vasculitis from 7 DPI (Figure 4.10). Granulomas were also observed in the spleen at 21 and 28 DPI.

Increased numbers of eosinophilic granule cells (Figure 4.11), thickening, vacuolation and occasional disruption of the endothelium of the central vessels of the gill filament, were occasionally observed from 14 DPI. Increased number of eosinophilic granule cells were usually observed adjacent to the endothelium and epithelium of the lamella from 21 DPI.

Numerous clusters of basophilic microorganisms, interpreted as *A. salmonicida*, were observed in the liver, posterior kidney, head kidney, spleen, and gills of some fish from 21 DPI (see chapter 3).

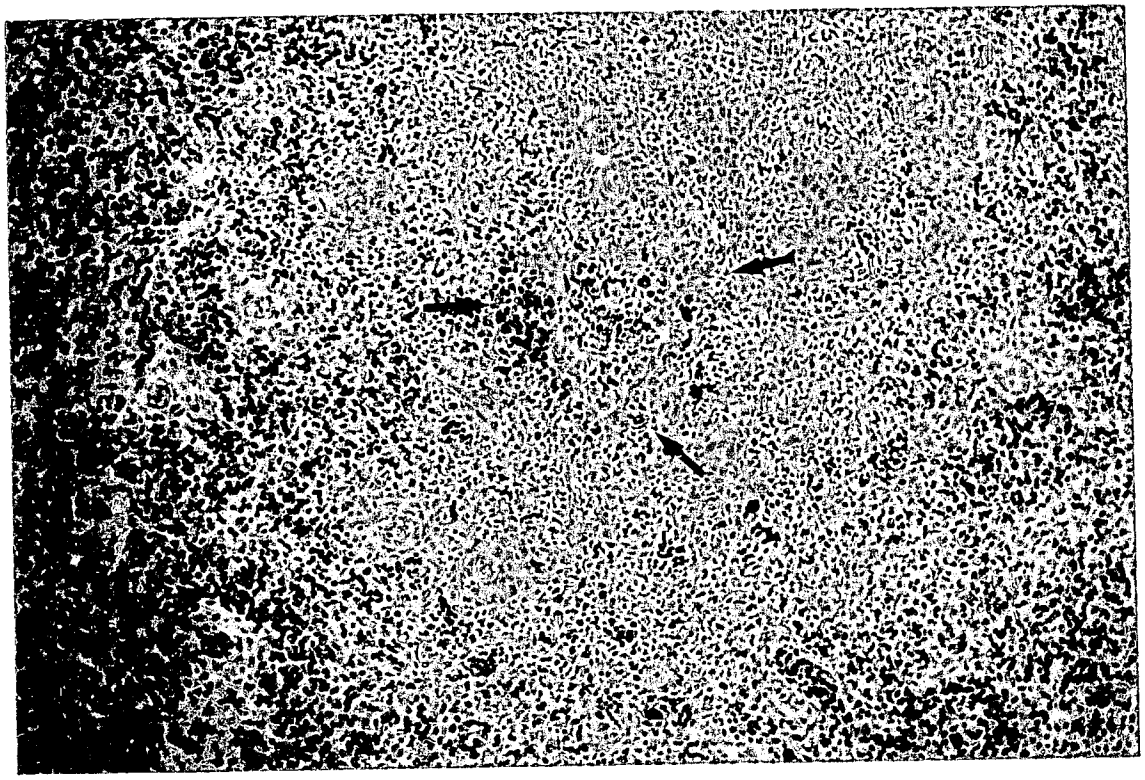

Figure 4.10: Histological changes in Atlantic salmon spleen infected with *Piscirickettsia salmonis* by non-contact cohabitation. Vasculitis showing perivascular inflammatory cells (arrows) (x 100).

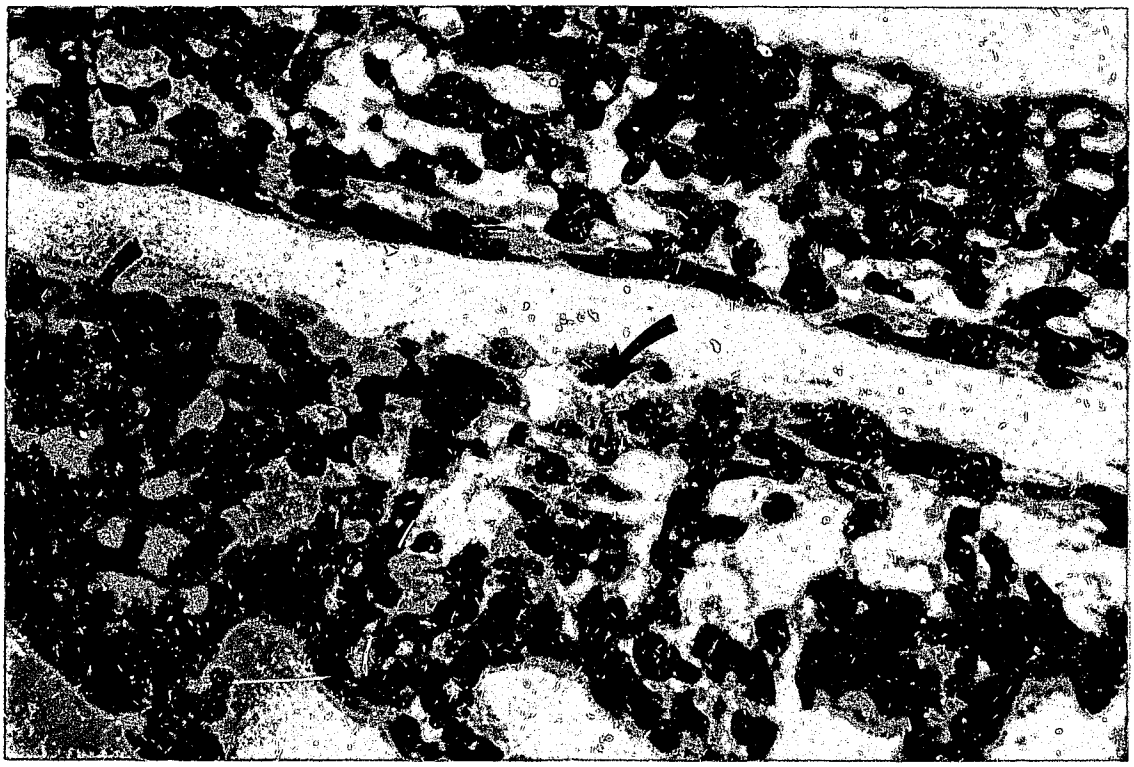

Figure 4.11: Gill of Atlantic salmon orally inoculated with *Piscirickettsia salmonis,* 14 dpi. Eosinophilic granule cells (arrows) (x 400).

Control fish

Fish from the control group showed mild thickening of vascular endothelium in large vessels of the liver of 4/12 fish and accumulation of melanomacrophage in 1/12 fish. The posterior kidney showed small focal areas of mild interstitial nephritis in 1/12 fish.

Splenic changes included presence of a small focal area of inflammatory cell accumulation and melanomacrophages in 2/12 fish. Large clusters of basophilic microorganisms interpreted as *Aeromonas salmonicida* were observed in posterior kidney, head kidney, and in the lamella of 1/12 fish.

Histology in dead fish

Mortalities showed similar changes as those noted for sampled fish. Focal areas containing clusters of basophilic microorganisms (*A. salmonicida*), accompanied by extensive necrotic debris, were also observed displacing the normal tissue of the posterior kidney, head kidney, spleen, and from 14 DPI. Vacuolation of the cytoplasm of leucocytes was also observed in liver, kidney and spleen.

4.5 Discussion

Sequential pathogenesis

The clinical signs and gross lesions described in the present work are similar to those described for natural SRS and in experimentally infected fish (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). In the present experiment, most moribund fish were lethargic, dark in colour and swam near the surface. However, some fish were normal. Previously reported clinical signs included lethargy, anorexia, darkening of the skin, respiratory distress, and surface swimming (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). These clinical signs are considered non-specific as they are described in several fish diseases (Shotts and Nemetz, 1993). Skin petechiae and swollen abdomens were observed in the present work. Similarly skin lesions including perianal and periocular haemorrhages, petechia in the abdomen, shallow haemorrhagic ulcers varying in size from 0.5 cm to 2 cm in diameter, and firm white nodules measuring up to 1 cm in diameter have been described in natural cases (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). Bilateral exophthalmia and ulcerative stomatitis have been previously reported in natural cases (Branson and Nieto, 1991; Cvitanich *et al.*, 1991), but were not observed in the present study. Differences in clinical signs and the lack of severe macroscopic lesions in this study may be due to the chronicity of the lesions described for the natural cases and differences among fish species infected by *P. salmonis*.

In the present work, external signs such as petechiae in skin and swollen

abdomen were closely related to the internal gross lesions observed. Internal gross lessions in experimentally infected Atlantic salmon were mild petechiation in the liver and spleen, splenomegaly and swollen kidney, ascitis and focal areas of pale tan discolouration in the liver capsule. Petechiae and ecchymoses on the serosal surfaces of the pyloric ceca, swim bladder and caudal intestine have also been reported in Atlantic salmon outbreaks of an RLO in Canada (Brocklebank *et al.*, 1993). Enlarged spleen, ascites, peritonitis, pale gills, and presence of multifocal pale areas in the kidney have also been described in natural infections (Bravo and Campos, 1989; Cubillos *et al.*, 1990; Branson and Nieto, 1991; Cvitanich *et al.*, 1991; Garcés *et al.*, 1991).

The presence of multifocal hemorrages on viceral organs (petechiae and ecchymoses) and enlarged spleen, such as those observed in the present study and in natural outbreaks, indicate vascular damage. In the present work, multifocal pale areas, presence of necrotic foci and formation of granuloma were also observed. In natural cases, heavily infected fish have off-white to yellow subcapsular nodules, measuring up to 2 cm in diameter, scattered throughout the liver (Cubillos *et al.*, 1990; Branson and Nieto, 1991; Cvitanich *et al.*, 1991; Garcés *et al.*, 1991; Brocklebank *et al.*, 1993). The presence of subcapsular nodules indicate a higher degree of chronicity of the lesions that may be present in naturally infected salmon but not in short-term experimental infections. In coho salmon, the renal lesions observed in natural cases have been interpreted as chronic damage characterized by fibrosis (Cubillos *et al.*, 1990). Although

pale organs have been described (Branson and Nieto, 1991), it is still not clear whether anaemia is a characteristic of SRS. In the present work, a decrease of the haematocrit to 37.6 % was observed 21 DPI in all the fish exposed to *P. salmonis*, but not in the controls (50 %). Cvitanich *et al.* (1991), reported low haematocrits in blood collected from moribund naturally infected coho salmon, ranging from 4% to 34%, while those from apparently healthy coho salmon (but also naturally exposed to *P. salmonis*) ranged from 35% to 50%. These authors proposed that the anaemia associated with this disease appears to be haemolytic, however, the organism was not found to be associated with RBCs, erythrocytes were typically normochromic-normocytic, and immature RBCs were rare. A low haematocrit does not necessarily indicate a decreased number of RBCs or anaemia. A decreased number of WBC or macrophages or changes in the fluid control capabilities of the fish may explain the lower haematocrit (Stoskopf, 1993) associated with piscirickettsiosis. In fish, due to the relative disproportions of blood and lymph volume a small change in one can lead to large changes in the other, and the changes would be reflected in the haematocrit (Ferguson, 1989). Invasion of macrophages, endothelial damage, and destruction of haematopoietic tissue in the spleen, head kidney and posterior kidney may have produced a lower haematocrit.

The Atlantic salmon isolate of *P. salmonis* used in the present work caused histological lesions similar to those produced by the coho salmon isolate previously described by other authors (Cvitanich *et al.*, 1991; Garcés *et al.*,

1991).

Thickening and inflammation of the capsule of the liver and spleen were consistently observed in the IP inoculated fish. These capsular lesions may be the result of direct injury or due to a response by peritoneal and circulating macrophages after IP inoculation of *P. salmonis*. These type of lesions were not observed in fish inoculated orally, on the gill, nor in the infected cohabitants. Furthermore, the lesions observed in these groups had characteristics of systemic infection characterized by vasculitis, as evidenced by thickening of the endothelium and intima, fibrin thrombi, endothelial cell vacuolation and necrosis of blood vessel walls. Previously, histological changes detected by light microscopy have been usually classified in the broad category of necrosis and inflammation (Garcés *et al.*, 1991). Commonly affected organs are liver, spleen, intestine and haematopoietic tissue of the kidney (Garcés *et al.*, 1991). Specific lesions in these organs include multifocal to diffuse coagulative necrosis, presence of fibrin thrombi within small blood vessels with necrosis of the endothelium and infiltration by inflammatory cells (Branson and Nieto, 1991; Cvitanich *et al.*, 1991). Members of the genus Rickettsia have tropism for endothelial cells (Walker and Mattern, 1980). Damage to endothelial cells with subsequent necrosis of intima and media, and secondary thrombosis have been discribed in rickettsial infections in mammals (Carter *et al.*, 1995). Parenchymal cells in the vicinity of the areas of vasculitis were necrotic and had increased numbers of intracytoplasmic vacuoles. Necrosis of parenchymal cells may be

due to local ischemia secondary to thrombosis or may be the result of toxic effect of rickettsial LPS (Woldehiwet and Ristic, 1993). The glomerular lesions observed in this study may also be explained by a tropism of *P. salmonis* for endothelial cells of the microcirculatory system (Walker and Mattern, 1980). Vacuolation and degeneration of renal tubules were previously described in natural outbreaks (Branson and Nieto, 1991).

Epithelial hyperplasia of the gill, fusion of secondary lamella, and presence of RLO within the secondary lamella blood spaces have also been reported (Branson and Nieto, 1991). Additionally, focal pyogranulomatous branchitis, pyogranulomatous splenitis with acute vasculitis and haemorrhage were detected in the Canadian outbreak (Brocklebank *et al.*, 1993).

Lesions observed in the present study are consistent with the initial stages of the disease, which is characterized by multifocal areas of necrosis secondary to vasculitis and possibly direct cell injury due to intracytoplasmic localization of the organism. These histological lesions may explain the presence of multifocal pale areas scattered throughout the liver observed in natural cases (Branson and Nieto, 1991; Cvitanich *et al.*, 1991; Garcés *et al.*, 1991). On the other hand, the commonly described raised nodules in the liver and kidney (Branson and Nieto, 1991) are consistent with a chronic inflammatory lesion (granuloma) or repair by fibrosis (Slauson and Cooper, 1990).

In the present work, the hepatic, renal, splenic and gill lesions observed

were milder than those described in natural outbreaks (Branson and Nieto, 1991; Cvitanich *et al.,* 1991). Differences in the severity of the lesions may be due to variations in the host species, age, mode of infection, chronicity of the infections, and water temperature.

Based on the histological changes and IFAT results, it is possible to postulate that after *P. salmonis* locally enters and infects the host, it infects circulating leucocytes producing local inflammation. The organism is then carried and disseminated through the circulatory system within intracytoplasmic vacuoles, and reaches the main organs by infecting the endothelial cells of blood vessels. Evidence to support this includes the presence of mononuclear cells carrying *P. salmonis* observed in the spleen, kidney, gill, and sinusoids and blood vessels of the liver. Infection of vessel walls produces endothelial damage, induces vasculitis, leakage and release of prothrombotic factors, formation of fibrin thrombi, and eventually areas of perivascular necrosis, evidenced by the gross lesions and histological changes observed in the present experiment. In naturally infected fish, the presence of numerous leucocytes containing degenerated cellular debris or organisms within cytoplasmic vacuoles has also been reported in peripheral blood smears and in tissues closely associated with blood vessels (Cvitanich *et al.,* 1991).

In salmonids, neutrophils (recognized by their multilobulated nucleus) have a relatively poor phagocytic activity, especially obvious when compared with macrophages (Ferguson, 1989). However, in the present study it was not

possible to differentiate at the light microscopy level whether macrophages, neutrophils or other mononuclear cells were the predominant cell type carrying *P. salmonis* in the blood. The rickettsial organism infects a wide variety of cells, including circulating macrophages, in which they replicate within variable sized, membrane-bound, intracytoplasmic vacuoles (Fryer *et al.*, 1990). Although varying numbers of organisms are frequently observed within these intracytoplasmic vacuoles, *P. salmonis* has also been found extracellularly as a result of cell lysis (Branson and Nieto, 1991; Cvitanich *et al.*, 1991).

Double infection

The presence of *A. salmonicida* in some fish may be partially responsible for the histological changes observed. Some histological changes described in the present report and by previous authors, especially the vascular lesions with disseminated intravascular coagulation and fibrin thrombi, as well as perivascular inflammation (Branson and Nieto, 1991; Cvitanich *et al.*, 1991; Garcés *et al.*, 1991), are similar to those described for other fish pathogens. In many bacteremias, especially those associated with *A. salmonicida* and *R. salmoninarum*, careful examination will frequently reveal septic thrombosis and occasional infarcts in a variety of tissues (Ferguson, 1989). Furthermore, both *A. salmonicida* and *R. salmoninarum* infection produce decreased haematocrit, erythrocyte count and haemoglobin levels (Stoskopf, 1993).

Prior to the current experiment a group of fish from the stock used in the

trial were immunosuppressed to test for carriers of *Aeromonas salmonicida*, and no disease was observed. *Aeromonas salmonicida* infection may have been produced by proliferation of the pathogen in carrier fish after *P. salmonis* infection. The presence of asymptomatic carrier fish, even in vaccinated ones, is a widely recognized problem that makes difficult the eradication of *A. salmonicida* from fish stocks (Hiney, 1995). *Aeromonas salmonicida* infection from the water source should also be considered as a possibility. However, although all fish were exposed to the same water supply, only groups exposed to *P. salmonis* presented mortalities. The control group had no mortalities, no presence of *P. salmonis*, and only one fish positive to *A. salmonicida* was observed the last day of the experimental trial (35 DPI).

Exposure to *P. salmonis* was likely an important factor for *A. salmonicida* to proliferate, and that immunosuppression due to infection of mononuclear cells by *P. salmonis* could be a reasonable mechanism for the development of lesions compatible with furunculosis. Rickettsial infection results in a transient suppression of the proliferative responses of lymphocytes to other unrelated antigens or mitogens (Woldehiwet and Ristic, 1993). Among the rickettsia-induced immunosuppressive mechanisms described are the down-regulation of cellular and humoral responses to other antigens by suppressor cells and the production of prostaglandins by macrophages and polymorphonuclear cells (Woldehiwet and Ristic, 1993).

Mononuclear cells carrying *P. salmonis* were frequently observed

surrounding clusters of *A. salmonicida* in different organs. This observation suggests that although leucocytes were infected by *P. salmonis*, their capacity for chemotaxis was not affected. The stimulatory effect of *A. salmonicida* on macrophage chemotaxis has been recently reported (Weeks-Perkins and Ellis, 1995). However, this chemotactic response does not guarantee that macrophages will be able to eliminate the bacteria (Weeks-Perkins and Ellis, 1995).

Although *Aeromonas salmonicida* has not been reported in Chilean salmon, mixed infections between *P. salmonis* and a microsporidian or *R. salmoninarum*, the causative agent of bacterial kidney disease (BKD), have been reported (Cvitanich *et al.*, 1991; Cassigoli *et al.*, 1994). Recently, the microsporidian *Enterocytozoon salmonis* has been described from fish involved in atypical freshwater outbreaks of a rickettsia in Chile (Cvitanich *et al.*, 1995). Recent outbreaks of rickettsial disease in salmonids raised in salt water in the west coast of Canada, have also shown mixed infection with a microsporidean and, apparently, with plasmacytoid leukemia virus (PCLV) (Dr. A. Forsythe; Dr. D. Groman, 1995; personal communications).

The presence of *Renibacterium salmoninarum* along with *Piscirickettsia salmonis* infection has been reported in outbreaks occurring in sea water and fresh water (Cvitanich *et al.*, 1991; Gaggero *et al.*, 1995; Smith *et al.*, 1995).

The Association of Chilean Salmon and Trout Farmers reported that according to a survey including saltwater data from 1992 and 1993, the

percentage of *P. salmonis* outbreaks associated with BKD was 58% in coho salmon, 8% in rainbow trout, and 0% in Atlantic salmon (Cassigoli, 1994). The high prevalence of BKD in the Chilean coho salmon population might explain the higher prevalence of SRS and mixed infections in this species.

Stress is another important factor apparently necessary for the development of SRS. Several stressors have been shown to increase the susceptibility of teleost fish to disease (Ferguson, 1989). In populations of salmonids at risk to *P. salmonis* infection, the reduction of stressful husbandry practices such as grading, sampling, change of nets, have proved to be a very effective preventive measure to avoid outbreaks (Dr. V. Palma, 1996, personal communication). Stress may also be a particularly important factor for the presentation of SRS in fish populations with high prevalence of mixed infections.

Fish carrying *P. salmonis* but not showing clinical signs or presenting mortalities are often seen in sea cages. Additional factors are needed to precipitate the massive losses initially reported (Branson and Nieto, 1991). Outbreaks have been reported to occur after transfer of smolt (osmotic stress), fluctuation of temperatures in the water, and severe storms (Branson and Nieto, 1991).

Final remarks

The sequential histopathologic study showed that fish infected orally, by

gill inoculation and naturally infected fish had a similar pattern of infection, different from the local (capsular) infection pattern observed in early stages of infection in IP inoculated fish.

Fish infected by oral and gill inoculation route, and by cohabitation showed histological changes from 7 days post inoculation indicating a systemic infection characterized by vasculitis, fibrinous thrombi and foci of necrosis. Intracytoplasmic basophilic microorganisms were observed within vacuoles in hepatocytes, tubular epithelial cells, endothelial cells and leucocytes.

Another microorganism, *Aeromonas salmonicida,* was also observed in some of the sampled fish and mortalities. Although *A. salmonicida* in some of the fish may have played a role in the pathogenesis of the observed lesions and mortalities, the intracellular location of *P. salmonis* and the vascular damage seen in the sampled fish are characteristic of rickettsial infections. With the exception of the early lesions in IP inoculated fish, the histological changes observed were similar to those reported in natural outbreaks of piscirickettsiosis.

Based on the histological changes and IFAT results it is possible to postulate that after *P. salmonis* locally infects the host, it is carried and disseminated through the circulatory system inside leucocytes, protected by the vacuole from the intracellular bactericidal mechanisms of the mononuclear cells and the extracellular complement system, and reaches the main organs by infecting the endothelial cells of blood vessels. The endothelial damage induces vasculitis, leakage through the endothelia and formation of fibrin thrombi. Focal

areas of necrosis in liver and kidney are the result of ischemic necrosis and direct injury by intracytoplasmic organisms.

The presence of *P. salmonis* in the cytoplasm of epithelial cells of the renal tubules indicates that elimination of this pathogen through urine may be possible. Additionally, the presence of *P. salmonis* within white blood cells, and the double infection (*P. salmonis* - *A. salmonicida*) observed in some of the fish exposed to this pathogen suggest an immunosuppressor effect of *P. salmonis* in the host that may facilitate other pathogens to proliferate.

5. GENERAL DISCUSSION

The present work involved *in vitro* and *in vivo* studies of infection by *P. salmonis* to obtain insight into the pathogenesis and transmission of this microorganism. The *in vitro* study showed that a fish cell line previously reported as non-susceptible to *P. salmonis* infection (BB) is susceptible to infection by this pathogen. Differences were observed in the appearence of cytopathic effect and intracellular multiplication of *P. salmonis* in BB cells, when compared with two susceptible salmonid cell lines (CHSE-214 and ASF). The use of an *in vitro* model showed that in some cases important information may be obtained, and further studies using this approach are encouraged to reduce the amount of animals used in pathogenic studies.

Ultrastructural observations revealed that different intracellular survival mechanisms are used by *P. salmonis* when infecting salmonid or non-salmonid cell lines. The Atlantic salmon fibroblast (ASF) cell line was shown to be susceptible to *P. salmonis* infection showing signs of cytopathic effect as early as the CHSE-214 cell line.

Although the BB cell line, previously reported as non-susceptible, did not show the characteristic CPE observed in salmonid cell lines after a few days post-infection, there was clear ultrastructural evidence that BB cells were infected by this pathogen. Differences in appearance of CPE among different

cell lines infected with *P. salmonis* may be due to survival mechanisms such as early escape to the cytosol, inhibition of phagosome-lysosome fusion and resistance to degradation. Some fish cell lines are less permissive to infection which may explain the differences in susceptibility to *P. salmonis* observed among salmonid species. Non-salmonid native fish may play a role in the persistence and transmission of the disease in the natural environment. The possibility of reservoirs of *P. salmonis* existing among transient and resident non-salmonid fish and shellfish has been proposed based on the finding of rickettsia-like organisms among marine molluscs, crustacea and non-salmonid fish (Cvitanich *et al.*, 1991).

Further morphological, immunocytochemical and molecular studies of the pattern of infection in this particular cell line may give valuable insight into the mechanisms involved in cell resistance and susceptibility to *P. salmonis* infection.

The pathogenesis of *P. salmonis* infection using three inoculation routes and the importance of physical contact on the horizontal transmission of the disease in fresh water was studied using Atlantic salmon. Oral and gill routes were shown to be viable portals of entry for *P. salmonis* infection. The importance of physical contact as a risk factor for the horizontal transmission of SRS was also identified, although physical contact was not necessary for

horizontal transmission in fresh water. Since it has been reported that *P. salmonis* cultured *in vitro* do not survive in fresh water, further work is needed to clarify the mode of infection of *P. salmonis* to non-contact cohabitants. It is possible that mucus present in the skin, gills or gut gives *P. salmonis* an extended survival in fresh water.

The sequential histopathologic and IFAT study showed that the pattern of infection in orally and gill inoculated fish, and in infected cohabitants was systemic and different from the capsular (serosa) infection pattern observed in IP inoculated fish.

Fish infected by oral and gill inoculation routes, and by cohabitation showed histological changes from 7 days post inoculation characterized by vasculitis, fibrinous thrombi and foci of necrosis. Intracytoplasmic basophilic microorganisms were observed within vacuoles in hepatocytes, tubular epithelial cells, endothelial cells and leucocytes.

Another microorganism, identified as *Aeromonas salmonicida,* was also observed in some of the sampled fish and mortalities. Although *A. salmonicida* in some of the fish may have played a role in the severity of the observed lesions and mortalities, the intracellular location of *P. salmonis* and the vascular damage seen in the sampled fish are characteristic of rickettsial infections. Fish with *P. salmonis* only presented mortalities and lesions indicative of vascular

damage. With the exception of the early lesions in IP inoculated fish, the histological changes observed were similar to those reported in natural outbreaks of piscirickettsiosis. Based on the histological changes and IFAT results it is possible to postulate that after *P. salmonis* locally infects the host, it is carried and disseminated through the circulatory system inside leucocytes, protected by the vacuole from the intracellular bactericidal mechanisms of the mononuclear cells and the extracellular complement system, and reaches the main organs by infecting the endothelial cells of blood vessels. The endothelial damage induces vasculitis, leakage through the endothelia and formation of fibrin thrombi. Focal areas of necrosis in liver and kidney are the result of ischemic necrosis and direct injury by intracytoplasmic organisms.

The presence of *P. salmonis* in the cytoplasm of epithelial cells of the renal tubules indicates that elimination of this pathogen through urine may be possible. Additionally, the presence of *P. salmonis* within white blood cells, and the double infection (*P. salmonis* - *A. salmonicida*) observed in some of the fish exposed to this pathogen suggest an immunosuppressor effect of *P. salmonis* in the host that may facilitate other pathogens to proliferate.

Despite the increasing appearance of rickettsial organisms affecting salmonid and non-salmonid finfish during the past few years, very little scientific information has been produced. Further knowledge regarding mechanisms of

intracellular survival *in vivo* will be necessary to understand the approach to be used to effectively control the presentation of Piscirickettsiosis in farmed salmon. So far only one *P. salmonis* isolate from coho salmon in Chile has been characterized. Other rickettsial organisms have been recently described and isolated, but no comparative studies exist among isolates. The number and amount of antibiotics being used by the Chilean salmon industry and the problems of microbial resistance to antibiotics are increasing, and will not be reduced unless solid alternative control strategies are designed. No studies have been developed to evaluate treatments, and vaccination trials have not shown convincing evidence of protection.

On-site diagnosis of SRS is still based on non-specific fast stains and no specific diagnostic tests (such as ELISA) are available for on-site diagnosis of SRS. Production of monoclonal antibodies would be a valuable tool to compare antigenicity of different isolates, to develop more sensitive and specific techniques for on-site diagnosis and to study the presence of potentially immunogenic antigenic sites of this Gram-negative bacteria.

Upon infection of a new host, *Piscirickettsia salmonis* appears to be disseminated inside the white blood cells of the infected host. Since some rickettsial organisms are immunosuppresive, further laboratory studies are needed to confirm that *P. salmonis* causes immunosuppression in salmon. In

addition, further research regarding humoral and cellular immune response to *P. salmonis* is needed to design appropriate vaccination strategies before good results are to be achieved with bacterines and vaccines.

The observation of different intracellular survival mechanisms among salmonid and non-salmonid fish cell lines opens the possibility that non-salmonid reservoirs of *P. salmonis* could exist in the natural environment. Due to the importance of physical contact in the transmission of SRS and the delayed appearance of cytopathic effect in non-salmonid fish cell lines, non-salmonid native fish species may represent an important epidemiological factor in the natural transmission of SRS. Differences in susceptibility among salmon species and ages are usually observed and there is some evidence in the literature to propose that ectoparasites may also act as vectors for the horizontal transmission of SRS.

Year class and species separation by geographic areas, coordinated control strategies of ectoparasites, early elimination and appropriate disposal of moribund and dead fish, presence of a second net surrounding the cages to avoid physical contact of cultured salmon with native resident fish, and reduction of fish handling and stressors, may also be important management strategies to prevent or control the transmission of this bacterium among fish. Similar strategies have been successfully employed to improve the health status

of cultured salmon in Norway.

APPENDIX A

Indirect fluorescent-antibody technique (IFAT)

Dry, dewax and rehydrate sections.

Blot slides carefully around tissues.

Incubate sections with 1/800 Rabbit anti-*P. salmonis* polyclonal antibody for 30 min.

Wash sections in phosphate buffered saline (PBS).

Blot slides carefully around tissues.

Incubate sections with fluorescein-labelled goat anti-rabbit secondary antibody (1/80) for 30 min. in the dark.

Wash sections in phosphate buffered saline (PBS).

Blot slides carefully around tissues.

Mount with 3 drops of water-soluble mounting fluid (Dakko).

Cover slides with 24x50 mm coverslips.

Blot carefully around coverslips and seal with nail polish.

Keep samples in darkness and refrigerated until observed.

APPENDIX B

Individual weights of Atlantic salmon (*Salmo salar*) sampled after inoculation with *Piscirickettsia salmonis*.

	IP			ORAL			GILL			
DPI	A	B	C	A	B	C	A	B	C	CONTROL
	22.6	19.2	21.2	28.2	29.6	17.1	26.9	24.9	13.4	
	17.4	22.4	17.2	22.2	15.2	16.5	21.8	20.6	22.3	
7	26.9	21.1	21.3	21.6	21.4	23.8	21.0	14.8	26.7	
	25.9	20.9	19.2	19.5	20.5	22.7	24.9	19.4	18.9	
	20.5	26.2	16.2	23.3	21.9	16.4	19.4	21.9	22.8	
	25.1	20.2	27.8	23.3	25.6	17.3	24.7	12.9	24.6	30.5
	18.1	15.6	25.0	15.2	21.2	11.6	28.1	10.9	26.9	30.9
14	22.6	21.2	34.1	18.7	18.8	22.5	28.6	14.1	25.9	14.7
	31.2	24.7	27.4	25.8	18.8	29.2	24.0	18.8	23.4	
	22.7	18.4	14.7	20.4	18.7	26.1	22.6	32.7	30.0	
	21.0	16.4	24.2	23.1	24.7	21.0	22.2	22.3	15.9	23.4
	24.1	26.2	18.6	14.3	15.3	18.3	26.0	20.0	23.3	31.2
21	17.9	24.5	19.3	26.1	13.7	16.2	22.0	22.7	25.4	25.4
	22.6	26.4	20.8	20.7	17.5	24.9	24.2	15.2	14.7	
	22.8	23.2	16.3	25.5	24.0	17.1	23.0	25.5	22.0	
			19.5	29.6	31.5	23.3	18.8		18.6	24.4
			17.1	22.2	27.2	26.8			22.5	21.6
28			18.5	22.4	18.2	20.6			17.8	25.4
				26.5	12.7	27.3			16.0	
				21.8	15.6	23.6			22.1	
					24.3					23.1
					20.0					21.0
35					18.3					28.6
X	22.8	21.8	21.0	22.5	20.6	21.1	23.6	19.8	21.7	25.0
sd	3.54	3.33	4.83	3.78	4.83	4.53	2.76	5.49	4.39	4.63
cv	0.16	0.15	0.23	0.17	0.23	0.21	0.12	0.28	0.20	0.18

DPI: Days post inoculation with *Piscirickettsia salmonis*.

A: Fish directly inoculated by either intraperitoneal (IP), oral or gill routes with *Piscirickettsia salmonis*.

B: Contact cohabitant fish with fish inoculated with *Piscirickettsia salmonis*.

C: Non-contact cohabitant fish with fish inoculated with *Piscirickettsia salmonis*.

APPENDIX C

Giemsa stain:

Dewax and hydrate sections to water.

Methyl alcohol, 3 changes for 3 min. each.

Working Jenner solution for 6 min.

Working Giemsa solution for 45 min.

Differentiate in 1% acetic water solution (about 10 sec.).

Rinse in distilled water.

Dehydrate quickly in:

95% ethanol, two changes.

100% ethanol, two changes.

100% xylene, two changes.

Mount with 3 drops of FLO-TEXX.

Cover slides with 24x50 mm coverslips.

Solutions:

Stock Jenner solution:

- Jenner stain	1.0 g
- Methyl alcohol	400 ml

Working Jenner solution:

- Jenner (stock)	20ml
- Distilled water	20 ml

Stock Giemsa solution (*):

- Giemsa	1.0 g
- Glycerin	66 ml
- Methyl alcohol	66 ml

Working Giemsa solution:

- Stock Giemsa	50 drops (1.4 ml)
- Distilled water	50 ml

* Mix Glycerin with Giemsa. Place in 60° C oven for 2 h. Finally add methyl alcohol.

APPENDIX D

Hematoxylin and Eosin (HE) stain:

Dewax and hydrate sections to water.

Stain with hematoxylin solution for 6 to 8 minutes.

Wash in running tap water for 2 minutes.

Check for staining (if desired).

Clear in acid alcohol solution for 30 seconds.

Wash in running tap water for 1 minute.

Immerse in ammonia water (alkaline water) for 10 to 15 seconds.

Wash in running tap water for 10 minutes.

Stain in eosin solution for 4 minutes.

Dehydrate in 95% ethanol for 2 minutes.

100% ethanol for 2 minutes.

100% xylene for 2 minutes.

Mount using 3 drops of FLO-TEXX, and 24x50 mm coverslips.

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