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Pathogenesis of liver lesions caused by experimental infection with *Piscirickettsia salmonis* in juvenile Atlantic salmon, *Salmo salar* L

Felipe E. Almendras, I. Carmen Fuentealba, R F. Frederick Markham, David J. Speare

Abstract. *Piscirickettsia salmonis*, the etiologic agent of salmonid rickettsial septicemia (SRS), or piscirickettsiosis, causes substantial economic losses to the salmon industry. The pathogenesis of the disease has not been fully characterized. The aim of this study is to describe the hepatic lesions associated with experimental *P. salmonis* infection in Atlantic salmon juveniles. Fish were maintained in fresh water and inoculated intraperitoneally (IP), orally, or on the gill surface with *P. salmonis*. A group of uninfected fish was kept as control. Liver samples from 5 fish in each inoculated group and 3 controls were collected weekly and processed for histological and immunohistochemical examination. Thickening of the liver capsule by inflammatory cells was a characteristic histologic feature of IP inoculated fish. Three weeks post-IP inoculation, 8 fish had died and 2 fish were sampled. Histological changes at this time consisted of vasculitis, presence of fibrin thrombi, vacuolated hepatocytes and focal areas of necrosis. Leukocytes containing intracytoplasmic basophilic microorganisms were seen within hepatic sinusoids. Vasculitis and intracytoplasmic vacuoles were prominent features in fish inoculated orally and on the gill surface. The presence of *P. salmonis* within hepatocellular vacuoles, endothelial cells, and leukocytes was confirmed by immunohistochemistry. The intracellular location of *P. salmonis* and the vascular damage seen in infected fish are characteristic of rickettsial infections. Histological lesions induced by experimental infection with *P. salmonis* using the oral and gill surface routes were similar to those observed in natural outbreaks of piscirickettsiosis. The tropism of *P. salmonis* for endothelial cells explains the vascular lesions observed in SRS, whereas hepatic lesions are due to ischemic necrosis and direct injury by intracytoplasmic organisms.

Salmonid rickettsial septicemia (SRS), or piscirickettsiosis, is a systemic disease caused by the intracellular gram-negative pathogen *Piscirickettsia salmonis*.⁹ This disease causes high mortality rates and significant economic losses to the salmon industry.¹⁰ Salmonid rickettsial septicemia was first reported in coho salmon, *Oncorhynchus kisutch* Walbaum, in Chile² and has since been observed in other salmonid species, including rainbow trout, *Oncorhynchus mykiss* Walbaum; Chinook salmon, *Oncorhynchus tshawytscha* Walbaum; and Atlantic salmon, *Salmo salar* Linnaeus.⁷ Similar rickettsia-like organisms (RLO) have been reported in Atlantic salmon held in salt water in western Canada,⁴ Ireland,¹⁷ and Norway.¹⁶ Outbreaks in fresh water have recently been reported in rainbow trout and coho salmon.¹¹

Although the lesions of terminal cases from naturally infected fish have been described,^{1,6} little is known about natural routes of infection, mechanisms of transmission, and pathogenesis of SRS. The most characteristic macroscopic lesion is the presence of

multifocal subcapsular hepatic nodules.^{1,2,4,6} Specific histological changes in the liver include multifocal to generalized coagulative necrosis and presence of fibrin thrombi within small blood vessels with necrosis of the endothelium and infiltration by inflammatory cells.^{1,7} The disease has been experimentally reproduced in coho and Atlantic salmon by intraperitoneal inoculation.^{7,12}

The objective of this work was to study the pathogenesis of hepatic lesions associated with experimental inoculation of juvenile Atlantic salmon with *P. salmonis* using 3 routes of infection.

Material and methods

Atlantic salmon weighing approximately 20 g were acclimatized in fresh water at 11 C. Fish were in an environment of 8 hr of artificial light and 16-h dark cycles. Fish were fed twice a day with an Atlantic salmon #2 granular dry diet^a at 2% of body weight per day. After 1 mo, the fish were anesthetized in a solution of benzocaine at a concentration of 50 mg/liter and were inoculated with *P. salmonis* either by the intraperitoneal (IP), oral (PO), or gill surface (GS) route.

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.^b The isolate was identified as *P. salmonis* by the use of a rabbit polyclonal antibody produced

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against *P. salmonis* strain FL-89, American Type Culture Collection (ATCC) VR1361.^c

The inoculum consisted of 100 μ l 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 (TCID₅₀) in a 96-well plate seeded with CHSE-214,¹³ was 1.48×10^2 TCID₅₀/ml.

Experimental infection. Intraperitoneal injections were performed using a 1-ml syringe and a 26 G \times 3/8 inch needle.^d 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^e was connected to the needle to perform oral and gill inoculations. For oral inoculations, the fish were gently intubated by inserting the tube about 1–2 cm into the esophagus. Gill infections were performed by dispensing the inoculum over the lamellae between the first and second branchial arch of the left gill. Control fish were inoculated with saline solution. Experimental procedures used in this study were performed according to the guidelines of the Canadian Council on Animal Care.

Sampling. Five fish from each of the 3 experimental groups and 3 from the controls were sampled weekly for 4 wk. Fish were removed from the tank, sedated in benzocaine hydrochloride solution at a concentration of 50 mg/liter, and killed by spinal severance. The tail was cut behind the adipose fin and blood was collected from the dorsal aorta using an heparinized (2 USP units ammonium heparin) microhematocrit capillary tube sealed with a commercial sealant. Blood samples were kept on ice until analyzed. Capillary tubes containing blood samples were centrifuged for 5 min using a hematocrit centrifuge,^f and the packed cell volume was measured in a hematocrit table. Hematocrit values obtained from fish exposed to *P. salmonis* and controls were compared by paired *t*-test at each sampling point. Statistical significance was set at $P \leq 0.05$.

The peritoneal cavity was exposed from the left flank. The liver was removed and fixed in 10% neutral buffered formalin. Paraffin-embedded tissue sections were sectioned at 5–6- μ m thickness and stained with hematoxylin and eosin (HE) for histological and immunohistochemical examination. The gills, kidney, spleen, and brain were also examined (results not reported here). An indirect fluorescent-antibody technique with a rabbit anti-*P. salmonis* polyclonal antibody was used for specific detection of the etiologic agent in tissue sections.¹⁵ As concurrent infection with *Aeromonas salmonicida* was suspected in some fish, tissues from all fish were also evaluated using an immunofluorescent antibody test (IFAT) with rabbit anti-*A. salmonicida* (1/400) serum^g to detect the presence of this organism in the tissues.

Tissue was also processed for immunohistochemistry. Sections 5–6 μ m thick were applied to 0.1% w/v poly L-lysine coated slides.^h All reagents were prepared in phosphate buffered saline (PBS) and used at room temperature. The wash buffer used between steps was 0.05 M Tris-HCl buffer (pH 7.6) containing 0.15 M sodium chloride (TBS). Tissues were deparaffinized, hydrated to 95% ethanol, and then treated with 5% hydrogen peroxide in absolute methanol overnight (approximately 15 hr). The rehydration resumed first with 95% ethanol, then 70% ethanol and distilled

water. Nonspecific tissue binding sites were blocked with 5% normal goat serumⁱ for 30 min. The primary antibody, polyclonal (rabbit) antibody against *Piscirickettsia salmonis*,^g was incubated for 1 hr. The secondary antibody, horse-radish peroxidase-goat antirabbitⁱ at 1:400, was also incubated for 1 hr. The substrate, 3,3-diaminobenzadine tetrahydrochloride (DAB),^h prepared as 10 mg in 17 ml of PBS with 7 μ l of hydrogen peroxide, was incubated for 4 min. The DAB reaction was stopped by placing the slides in distilled water for 5 min. Slides were counterstained with aqueous 4.7% hematoxylin for 20 sec, dehydrated in graded alcohol series, cleared with xylene, mounted with mounting media,^j and examined with a Zeiss light microscope.

Results

Significant gross lesions were not observed in either the inoculated or control fish. Immunohistochemical demonstration of etiologic organisms correlated well with IFAT results. Thus, only immunohistochemical results are described in detail.

IP inoculated fish

Week 1. The capsule of the liver was slightly thickened due to mild inflammatory cell infiltration in 1 out of 5 fish. Otherwise significant morphologic changes were not seen in the liver. *Piscirickettsia salmonis* was detected using immunohistochemistry in the liver capsule in 1 out of 5 fish.

Week 2. Diffuse thickening of the capsule due to inflammatory cell infiltration was seen in the livers of all fish sampled at this time. Multifocal to locally extensive areas of coagulative necrosis, characterized by loss of cell detail, hypereosinophilia of the cytoplasm, pyknosis, and karyolysis, were observed in hepatocytes beneath the capsule. Basophilic microorganisms were seen in the cytoplasm of leucocytes, the wall of blood vessels, cytoplasm of hepatocytes, and adjacent to necrotic hepatocytes. These organisms were easily detected with immunohistochemistry in all fish sampled at this time (Fig. 1).

Week 3. Five fish died 17 days postinoculation and 3 fish died 18 days postinoculation. Two fish were available for sampling at week 3, at which point the study concluded for this inoculation route. Lesions in the liver were morphologically similar in sampled and dead fish. Vasculitis was characterized by endothelial cell hypertrophy, vacuolation of endothelial cells, presence of leukocytes within the wall of the blood vessels, and fibrin thrombi within the lumen of affected blood vessels. Vacuolated hepatocytes were numerous, and small multifocal areas of subcapsular hepatocellular necrosis were seen. *Piscirickettsia salmonis* was detected in the livers of all fish (dead and sampled) examined at this time. Organisms were located in the cytoplasm of leucocytes, the wall of blood vessels, and within vacuoles in the cytoplasm of hepatocytes. *Aero-*

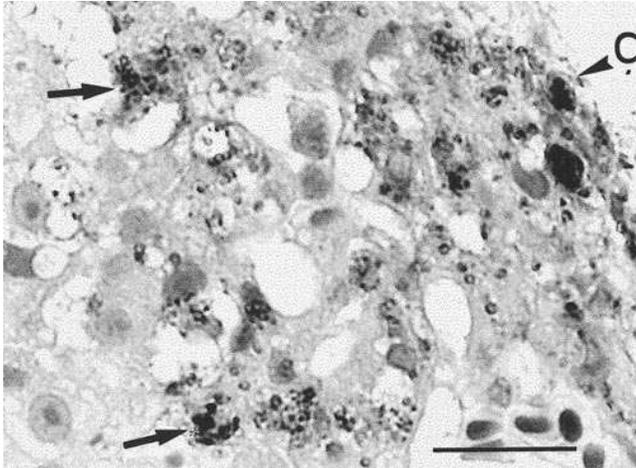


Figure 1. Liver: Atlantic salmon sampled 2 weeks post-IP inoculation. Subcapsular (C = capsule) area of coagulative necrosis, marked hepatocellular vacuolation, and intralesional *P. salmonis* (arrows). Immunohistochemical stain using polyclonal antibody against *P. salmonis* and hematoxylin counterstain. Bar = 25 μm .

monas salmonicida was detected in the liver of 3 out of 10 fish (2 out of 8 found dead and 1 out of 2 sampled fish).

Oral inoculation

Week 1. Histological lesions were not observed. However, *P. salmonis* was detected by immunohistochemistry in a few leucocytes located within liver sinusoids. *Aeromonas salmonicida* was detected in 1 out of 5 fish.

Week 2. Sinusoids were distended with red blood cells and a few leucocytes. *Piscirickettsia salmonis* was detected by immunohistochemistry within a few leukocytes.

Week 3. Blood vessels contained fibrin and numerous vacuolated mononuclear cells. Vasculitis, as evidenced by thickening of the vessel walls and infiltration of leukocytes through the wall, was commonly seen in small blood vessels. Numerous perivascular necrotic hepatocytes, characterized by slight hypereosinophilia, pyknosis, and karyolysis, were also present. Intracytoplasmic vacuoles were often observed in individual hepatocytes. *Piscirickettsia salmonis* was detected using immunohistochemistry in 3 out of 5 livers.

Week 4. Two fish died 23 days postinoculation, 1 fish died 27 days postinoculation, and 2 fish were sampled at this time. Vasculitis was detected in all fish examined. *Piscirickettsia salmonis* was detected in all the livers of fish examined at this time. *Aeromonas salmonicida* was detected in the 3 fish found dead, but bacterial colonies were not associated with an inflammatory response.

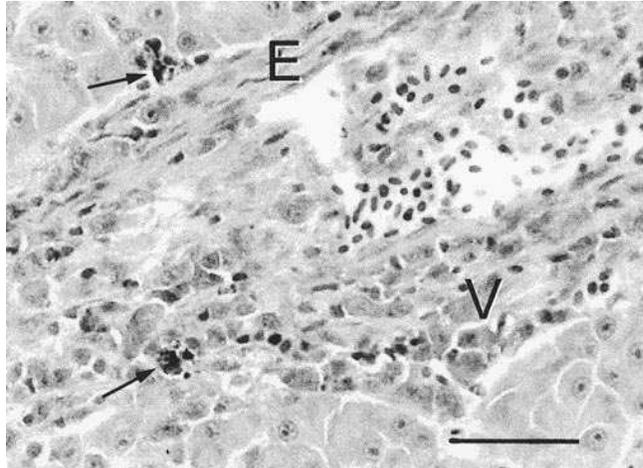


Figure 2. Liver: Atlantic salmon found dead 3 weeks post-gill-surface inoculation. Vasculitis (V) and perivascular hepatocellular necrosis with intralesional *P. salmonis* (arrows). An area of unremarkable endothelium (E) is also seen. Immunohistochemical stain using polyclonal antibody against *P. salmonis* and hematoxylin counterstain. Bar = 50 μm .

Gill inoculation

Week 1. Liver lesions were not detected at this time. Etiologic agents were not observed in the livers.

Week 2. Histological changes were mild and consisted of presence of intracytoplasmic vacuoles in perivascular hepatocytes. Blood vessels were distended with fibrin and leucocytes. *Piscirickettsia salmonis* was detected by IFAT in the liver of 3 out of 5 fish. Immunohistochemistry revealed the presence of occasional organisms within leucocytes. *Aeromonas salmonicida* was detected in 2 of 5 fish.

Week 3. One fish died at 19 days postinoculation, 1 fish died 18 days postinoculation, 2 fish died 19 days postinoculation, and 1 fish died 20 days postinoculation. The remaining 5 fish were sampled at this time and the experiment concluded for this inoculation route. Vasculitis and perivascular hepatocellular necrosis and inflammatory infiltration, associated with presence of numerous *P. salmonis*, were detected in the livers of all fish found dead (Fig. 2). In contrast, a few organisms and mild lesions were detected in the liver of sampled fish. *Aeromonas salmonicida* was detected in 5 of 5 found dead fish and 1 of 5 sampled fish.

Control fish

Morphologic changes and etiologic agents were not observed in the livers of control fish.

Hematocrit. Hematocrit was measured as the percentage (%) of cells in the whole blood. Hematocrit values were measured from week 1 in fish exposed to *P. salmonis* and week 2 in the control group (Table 1). No differences were observed in the hematocrit among fish inoculated with *P. salmonis* through IP, oral, or

Table 1. Hematocrit (%)* of fish inoculated with *P. salmonis* and controls sampled at different days postinfection.

Weeks postinoculation†	Hematocrit (%)	
	Inoculated fish (mean ± SEM)	Control fish (mean ± SEM)
1	56.8 ± 1.7	ND
2	42.1 ± 0.9 ^a	54 ± 0.1 ^b
3	37.6 ± 1.1 ^a	50 ± 3.2 ^b
4	37.6 ± 1.8 ^a	50 ± 2.1 ^b

* Hematocrit is expressed as the mean and standard error of the mean (SEM). Values not sharing the same letter are significantly different at $P < 0.05$.

† In each sampling point, $n = 15$ for inoculated fish and 3 for control fish.

‡ ND = not determined.

gill routes at any point of the sampling periods. A statistically significant decrease ($P < 0.05$) for the hematocrit values was detected at 2, 3, and 4 weeks postinoculation in all fish exposed to *P. salmonis* when compared with the controls.

Discussion

In this work, juvenile Atlantic salmon were experimentally infected with *P. salmonis* using intraperitoneal injection, oral intubation, and application to gill surface. The pattern of infection in gill and orally infected fish resembled that observed in natural outbreaks of SRS.^{1,7} In addition to being effective routes to reproduce the natural disease, successful 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.

In spontaneous cases of SRS, heavily infected fish have off-white to yellow subcapsular nodules, measuring up to 2 cm in diameter, scattered throughout the liver.^{1,7,12} In contrast, hepatic lesions observed in an outbreak in British Columbia were umbilicated and consisted of focal necrosis surrounded by a hyperemic border.⁴ Gross lesions in the liver were not observed in the present study, which is not an unexpected outcome of the experimental infection since the liver lesions, although regarded as fairly diagnostic of SRS, are not always observed.¹⁵ Additionally, the presence of hepatic subcapsular nodules in spontaneous SRS may indicate a higher degree of chronicity, which was not duplicated within our experimental infections.

Although pale organs have been described in natural cases of SRS¹ and a low hematocrit value is commonly found in SRS,^{9,14} it is not clear whether anemia is a characteristic of the disease. In the present work, a decrease of the hematocrit to 37.5% was observed 3 weeks postinoculation with *P. salmonis*. Low hematocrit values, ranging from 4 to 34%, have been reported in blood collected from moribund naturally in-

fectured coho salmon, while hematocrit values from naturally exposed but apparently healthy coho salmon ranged from 35 to 50%.⁷ The cause or causes of decreased hematocrit value have not been established. The organism is not associated with red blood cells, erythrocytes are typically normochromic-normocytic, and immature erythrocytes are rare.⁷ It must be clarified that a low hematocrit in fish does not necessarily indicate a decreased number of erythrocytes or anemia. A decreased number of white blood cells, macrophages, or changes in the fluid control capabilities of the fish may cause a low hematocrit.¹⁸ In fish, due to the relative disproportion of blood and lymph volume, a small change in the volume of one can lead to large changes in the other, and these changes would be reflected in the hematocrit.⁸ Low hematocrit observed in an outbreak of a disease caused by a rickettsia-like organism in tilapia was interpreted as a result of destruction of hematopoietic tissue by the etiologic agent.⁶ Therefore, phagocytosis by macrophages, endothelial damage, and destruction of hematopoietic tissue by *P. salmonis* may have produced the low hematocrit observed in the present study.

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.^{7,12} In the present work, the hepatic lesions observed were milder than those described in natural outbreaks.^{1,7} 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. Lesions observed in the present study are consistent with the initial stages of the disease, which are characterized by multifocal areas of necrosis secondary to vasculitis and 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.^{1,7,12} On the other hand, the commonly described raised nodules in the liver and kidney¹ are consistent with a chronic inflammatory lesion (such as a granuloma) or repair by fibrosis. Thickening and inflammation of the capsule of the liver were commonly observed in the IP inoculated fish. Intraperitoneal inoculation of many *Rickettsia* species results in infection and inflammatory reaction of the peritoneal lining.²⁰ Therefore, capsular lesions observed in this study may be the result of direct injury or may be due to macrophages infiltration after IP inoculation of *P. salmonis*. In contrast, the lesions observed in fish inoculated orally and on the gill had features of systemic infection characterized by vasculitis (as evidenced by endothelial cell hypertrophy and intimal thickening), fibrin thrombi, endothelial cell vacuolation, and necrosis of blood vessel walls. Mem-

bers of the genus *rickettsia* have tropism for endothelial cells.¹⁹ Damage to endothelial cells with subsequent necrosis of intima and media and secondary thrombosis have been described in rickettsial infections in mammals.⁵ Previously, histological changes in SRS have often been classified in the broad category of necrosis and inflammation.^{1,12} Commonly affected organs are liver, spleen, intestine and hematopoietic tissue of the kidney.¹² 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.^{1,7} These histologic lesions correlate well with some gross lesions such as skin ulceration and widespread hemorrhage observed in the abdominal serosal surfaces in SRS.³ However, the formation of raised hepatic nodules observed by other authors cannot be interpreted as a result of thrombosis and necrosis.

Hepatocytes in the vicinity of the areas of vasculitis were necrotic and had increased numbers of intracytoplasmic vacuoles with intralesional organisms. Necrosis of parenchymal cells may occur as a result of thrombosis or may represent a toxic effect of rickettsial lipopolysaccharide.²³ The formation of hepatic granulomas in SRS may be due to the presence of vacuoles and intralesional organisms within the cytoplasm of hepatocytes. The capacity of other rickettsial organisms to infect hepatocytes *in vivo* has been previously demonstrated.²⁰ Furthermore, it has been suggested that hepatic granulomas observed in human Boutonneuse fever are due to infection of hepatocytes by rickettsial organisms resulting in hepatocellular necrosis, influx of macrophages to form a transient granuloma, and subsequent infiltration by lymphocytes associated with rickettsial clearance.²¹ Therefore, hepatic lesions observed in natural outbreaks of SRS may develop as the result of 2 different mechanisms: endothelial damage to hepatic blood vessels, thrombosis, and hepatocellular ischemic necrosis and/or direct damage to hepatocytes with resulting inflammatory reaction culminating with the formation of hepatic granulomas.

The histological changes and immunohistochemical demonstration of etiologic agents suggest that, after *P. salmonis* locally enters and infects the host, it infects circulating leukocytes, 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 hepatic sinusoids and blood vessels of the liver. Vasculitis produces endothelial damage, leakage and release of prothrombotic

factors, formation of fibrin thrombi, and eventually areas of perivascular ischemic 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.⁷

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.⁹ 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.^{1,7}

In the present study, microorganisms other than *P. salmonis* were also detected in some fish. The organism was identified as *A. salmonicida*, but it was not possible to establish its source. Although it can be argued that the presence of *A. salmonicida* in some of the fish may be partially responsible for the histological changes observed, the intracellular location of *P. salmonis* and the vascular damage seen in the sampled fish are characteristic of rickettsial infections. Mixed infections with *R. salmoninarum*, the causative agent of bacterial kidney disease, and with the microsporidian protozoa *Nucleospora (Enterocytozoon) salmonis* have been described in salmonids with piscirickettsiosis.⁷ Exposure to *P. salmonis* was likely an important factor for *A. salmonicida* to proliferate, and immunosuppression due to infection of mononuclear cells by *P. salmonis* could be a reasonable mechanism for the development of furunculosis. Rickettsial infection results in a transient suppression of the proliferative responses of lymphocytes to other unrelated antigens or mitogens.²³ Rickettsia-induced immunosuppressive mechanisms include the down-regulation of cellular and humoral responses to other antigens by suppressor cells and the production of prostaglandins by macrophages and polymorphonuclear cells.²³ Although the stimulatory effect of *A. salmonicida* on macrophage chemotaxis has been reported, this chemotactic response does not guarantee that macrophages will be able to eliminate the bacteria.²² Vascular lesions with disseminated intravascular coagulation and fibrin thrombi, as well as perivascular inflammation, are seen in other bacterial fish diseases. 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.⁸

Based on the histological changes and immunohistochemical demonstration of etiologic agents, we postulate that, after *P. salmonis* locally infects the host, it is carried and disseminated through the circulatory system within leucocytes 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 are the result of ischemic necrosis and direct injury by intracytoplasmic organisms.

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Sources and manufacturers

- a. Corey Feed, NB, Canada.
- b. Dr. E. Madrid, Marine Harvest McConnell, Puerto Montt, Chile.
- c. Dr. J. L. Fryer, Oregon State University, Corvallis, OR.
- d. Becton Dickinson and Co., Rutherford, NJ.
- e. Norton Co., Akron, OH.
- f. Canlab, Mississauga, ON, Canada.
- g. Aqua Health Ltd., PE, Canada.
- h. Sigma, St. Louis, MO.
- i. Dako Corp., Carpinteria, CA.
- j. Lerner Lab, Pittsburgh, PA.

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