

AN ABSTRACT OF THE THESIS OF

Prasad S.D. Turaga for the degree of Doctor of Philosophy in Microbiology presented on September 15, 1989.

Title: Physical and Functional Characterization of Soluble Protein Antigen(s) produced by *Renibacterium salmoninarum*

Redacted for Privacy

Abstract approved: _____

Dr. Stephen L. Kaattari

Renibacterium salmoninarum produces bacterial kidney disease (BKD) in salmonid fish. Soluble antigen(s) (SA) produced by the bacterium was characterized by physicochemical, functional and immunological methods. The molecular weight determination by SDS-PAGE analysis of SA yielded 14 different molecular weight species. Seven of these species were prominent, 57 kd protein being the major one. All these proteins reacted with rabbit-anti SA in a Western blot analysis. The pI of these proteins was found to be below 5.2, indicating their acidic nature. The SA preparation was demonstrated to possess protease activity, which was more pronounced at 37°C and not at lower temperatures. The protease exhibited its activity on SA itself resulting in almost complete disappearance of the proteins on SDS-PAGE analysis. This protease activity was inhibited by phenyl methynyl sulphonyl fluoride (PMSF), alpha 2-macroglobulin, L-1-chloro-3-[4-tosylamido]-4-phenyl-2 butane (TPCK) and 50% concentrations of ethanol and methanol.

The detection of SA was possible, both qualitatively and quantitatively, by Western blot analysis and an enzyme linked immunosorbent assay (ELISA) sera from infected fish. SA can, thus, be used as a diagnostic marker in monitoring the progression of the disease. It was also shown that SA suppresses in vitro antibody responses of salmon lymphocytes and was found to be associated with decreasing hematocrit values in vivo during infection. The presence of immune complexes was demonstrated in infected fish serum, involving SA and salmon anti-SA. The identification of such complexes may explain some of the pathology associated with the disease.

A protocol was developed to characterize the antibody responses to SA, from three species of fish, Oncorhynchus kisutch, Oncorhynchus tshawytscha and Oncorhynchus mykiss, both qualitatively and quantitatively. Preliminary trials in the development of a vaccine for bacterial kidney disease, demonstrated that Freund's complete adjuvant (FCA) and killed Mycobacterium chelonii may be useful candidates. This indicates that stimulation of cell mediated immunity (CMI) may be the necessary route of immunization to protect the fish from BKD.

PHYSICAL AND FUNCTIONAL CHARACTERIZATION OF SOLUBLE
PROTEIN ANTIGEN(S) PRODUCED BY RENIBACTERIUM
SALMONINARUM

by

Prasad S. D. Turaga

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed September 15, 1989

Commencement June, 1990

APPROVED:

Redacted for Privacy

Associate Professor of Microbiology in charge of major

Redacted for Privacy

Chairman of department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented September 15, 1989

Typed by Prasad Turaga for Prasad S. D. Turaga

TO MY FATHER
SRI. APPA RAO

TO MY MOTHER
SMT. SRI LAKSHMI

TO MY BROTHER
VIJAY

TO MY SISTER
VALLI

ACKNOWLEDGEMENTS

I would like to acknowledge and thank my major professor Dr. Steve Kaattari for his invaluable guidance and encouragement throughout the course of this study. He has been a great teacher and a good friend. Thank you very much, Steve!

I would also like to thank Greg (the "BJ") Wiens for his cooperation and technical assistance in this project.

I am happy to have associated with a bunch of nice people such as Mike, Ralph, Mary, Nancy, Jenefer, Dan the "DK", Don and Leslie.

I am thankful to my parents, my brother and sister for their constant encouragement and support, without which this work would never have materialized.

This work was supported by Bonneville Power Administration, Contract No. DEAI79-87BP16480.

CONTRIBUTION OF AUTHORS

Gregory D. Wiens has contributed the western blot analysis in chapter IV (Analysis of Renibacterium salmoninarum) antigen production in situ) and the in vitro culture work in chapter V (Bacterial kidney disease : the potential role of soluble protein antigen(s)).

Dr. Alberto Villena has contributed the immunoperoxidase staining section in chapter VI (Presence of serum immune complexes in salmon with bacterial kidney disease).

TABLE OF CONTENTS

<u>Chapter</u>	<u>Page</u>
1. Introduction	1
Literature review	3
References	24
2. Characterization of soluble protein antigen(s) produced by the fish pathogen <u>Renibacterium</u> <u>salmoninarum</u>	34
Abstract	35
Introduction	35
Materials and methods	37
Results	43
Discussion	45
References	53
3. Identification and characterization of protease activity associated with a soluble antigen preparation of <u>Renibacterium salmoninarum</u>	55
Abstract	56
Introduction	56
Materials and methods	58
Results	65
Discussion	68
References	79
4. Analysis of <u>Renibacterium salmoninarum</u> antigen production <u>in situ</u>	81
Abstract	82
Introduction	82
Materials and methods	83
Results	89
Discussion	90
References	97

5.	Bacterial kidney disease: the potential role of soluble protein antigens	99
	Abstract	100
	Introduction	100
	Materials and methods	101
	Results	101
	Discussion	102
	References	108
6.	Presence of serum immune complexes in salmon with Bacterial kidney disease	110
	Abstract	111
	Introduction	111
	Materials and methods	113
	Results	118
	Discussion	119
	References	135
7.	Characterization of the antibody response of three salmonid species to soluble antigen(s) secreted by <u>Renibacterium salmoninarum</u>	137
	Abstract	138
	Introduction	138
	Materials and methods	140
	Results	145
	Discussion	146
	References	158
8.	Examination of prophylaxis against bacterial kidney disease induced by various immunogens	162
	Abstract	163
	Introduction	164
	Materials and methods	166
	Results	175
	Discussion	177
	References	193
	BIBLIOGRAPHY	197

LIST OF FIGURES

	<u>Page</u>
<u>Chapter 2</u>	
Fig. 2.1 SDS-PAGE and Western blot analysis of SA	48
Fig. 2.2 Molecular exclusion chromatography of SA	50
Fig. 2.3 SDS-PAGE on SAS preparations of SA	51
Fig. 2.4 IEF analysis on SA	52
<u>Chapter 3</u>	
Fig. 3.1 SDS-PAGE on SA incubated at 4°C and 37°C	71
Fig. 3.2 SDS-PAGE on SA incubated at different temperatures	72
Fig. 3.3 SDS-PAGE on SA protease kinetics	73
Fig. 3.4 Effects of protease inhibitors on SA protease	75
Fig. 3.5 Substrate characterization of SA protease	76
Fig. 3.6 Effects of SA on PFC response	77
Fig. 3.7 Effects of temperature on SA on its PFC response	78
<u>Chapter 4</u>	
Fig. 4.1 ELISA standard curve	94
Fig. 4.2 Quantification of SA during experimental infection	95
Fig. 4.3 Western blot of infected fish sera	96
<u>Chapter 5</u>	
Fig. 5.1 <u>In vitro</u> immunosuppression due to SA	105
Fig. 5.2 Suppressed <u>in vitro</u> PFC responses of infected fish	106
Fig. 5.3 Association of SA with decreasing hematocrits	107

Chapter 6

Fig. 6.1	Estimation of immune complexes by ELISA	124
Fig. 6.2	Determination of immune complexes prepared <u>in vitro</u>	125
Fig. 6.3	Determination of immune complexes in sera from infected fish	126
Fig. 6.4	Immunoperoxidase staining of mesonephros with anti-SA monoclonal	128
Fig. 6.5	Immunoperoxidase staining of mesonephros with anti-fish Ig monoclonal antibody	130
Fig. 6.6	Immunoperoxidase staining of pronephros with anti-SA monoclonal antibody	132
Fig. 6.7	Immunoperoxidase staining of pronephros with anti-fish Ig monoclonal antibody	134

Chapter 7

Fig. 7.1	Standardization of hyperimmune serum	150
Fig. 7.2	Western blot analysis of SA using hyperimmune serum	154
Fig. 7.3A	Western blot analysis of SA using immune sera from Rainbow trout	155
Fig. 7.3B	Western blot analysis of SA using immune sera from Coho salmon	156
Fig. 7.3C	Western blot analysis of SA using immune sera from Chinook salmon	157

Chapter 8

Fig. 8.1	Antibody titration of vaccinated fish sera	181
Fig. 8.2	Vaccine trial with heat killed <u>R. salmoninarum</u>	183
Fig. 8.3	Vaccine trial with soluble antigen	184
Fig. 8.4	Vaccine trial with SA coupled with <u>Vibrio</u> cells	185
Fig. 8.5	Vaccine trial with HKDB plus <u>Vibrio</u> extract	186
Fig. 8.6	Vaccine trial with FCA alone	187

Fig. 8.7	Vaccine trial with heat killed <u>M. chelonii</u> (MC)	188
Fig. 8.8	Vaccine trial with MC plus SA	189
Fig. 8.9	Vaccine trial with polysaccharide from <u>R. salmoninarum</u>	190

LIST OF TABLES

	<u>Page</u>
<u>Chapter 7</u>	
Table 7.1A. Antibody titers of sera from Rainbow trout	151
Table 7.1B. Antibody titers of sera from Coho salmon	152
Table 7.1C. Antibody titers of sera from Chinook salmon	153
<u>Chapter 8</u>	
Table 8.1. Agglutination titers of vaccinated fish sera	182
Table 8.2. RPS values for preliminary screening	183
Table 8.3. RPS values of formal screening	184

PHYSICAL AND FUNCTIONAL CHARACTERIZATION OF SOLUBLE ANTIGEN(S) PRODUCED BY *RENIBACTERIUM SALMONINARUM*

1. INTRODUCTION

The causative agent of Bacterial Kidney Disease (BKD) is *Renibacterium salmoninarum*. This disease is one of the major bacterial disease problems of salmonid fish, causing enormous losses of both hatchery-reared and wild salmonid populations. Currently, there is no vaccine available for prophylaxis. Thus research was initiated with the eventual goal of producing vaccine(s) for this disease. Most of the studies focused on the isolation and characterization of soluble products or soluble antigen (SA) which could be used as possible vaccine candidates. Simultaneously, studies were conducted on expression of soluble antigen during the disease, its relationship to the pathology, and its physicochemical characterization. These studies yielded the following results and observations:

1. Sensitive diagnostic assays, both qualitative and quantitative, were developed using soluble antigen as the diagnostic marker.
2. The discovery of the tremendous antigenic load produced during the infection.
3. Soluble antigen concentrations in infected fish were inversely proportional to the hematocrit values.

4. The in vitro antibody producing cell (plaque forming cell) response was suppressed by soluble antigen and this suppressive activity was temperature sensitive.

5. Soluble antigen is found in immune complexes which, in turn, may be responsible for a portion of the pathology observed.

6. Antibody responses to the soluble antigen in coho, (Oncorhynchus kisutch), chinook salmon, (Oncorhynchus tshawytscha) and rainbow trout, (Oncorhynchus mykiss) were qualitatively similar.

7. The cellular arm of the immune response may play an important role in the protection of fish from BKD.

Initial attempts to develop a vaccine involved an admixture of soluble antigen and/or the killed bacterial cells with immunostimulating agents (adjuvants). Later studies incorporated chemical coupling methods of the SA directly to the adjuvant material.

LITERATURE REVIEW

History

The first published case of BKD was described in 1930 (1). This case occurred in Atlantic salmon (Salmo salar) in Aberdeenshire Dee (and hence occasionally termed Dee disease) and the river Spey in Scotland . The first report of its occurrence in the United States was by Belding and Merrill (3) in brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri) from a hatchery in Massachusetts.

The disease occurs in a variety of species of the Salmonidae family including: coho salmon (Oncorhynchus kisutch), chinook salmon (Oncorhynchus tshawytscha), sockeye salmon (Oncorhynchus nerka) (4), lake trout (Salvelinus namaycush) (5), pink salmon (Oncorhynchus gorbuscha) (6), yamabe (Oncorhynchus masou) and cutthroat trout (Salmo clarkii) (1).

The organism has also been isolated from non-salmonid fish also. The bacterium was isolated from Pacific herring (Clupea harengus pallasii L.) present in cages in which coho salmon with clinical BKD were being held (7). Hicks et al., (8) have experimentally infected fathead minnows (Pimephales promelas) and the common shiner (Notropis cornutus). Bell working with Pacific lamprey (Lampetra tridentata) attempted to infect this non-salmonid host without success (9). Lamprey were chosen for these studies because these cyclostomes are among the species that live in relatively close association with

salmon in fresh water and prey upon salmon in fresh and salt water. In other studies, Bell et al. (72) demonstrated that sablefish (Anoplopoma fimbria) can also be infected by intraperitoneal injection of Renibacterium salmoninarum resulting in death within 50-71 days. Bacterial kidney disease is now routinely reported in hatchery salmonid populations from the United States, Canada, Spain, Italy, France, Iceland, England, Scotland, Yugoslavia, Japan and Chile (10). The foregoing information indicates that salmonid fishes are the primary natural host for the bacterium and that other species of fish may only be infected artificially.

Characteristics of Renibacterium salmoninarum

Renibacterium salmoninarum is a small (0.3-1.5 μm by 0.1-1.0 μm), non-motile, non-encapsulated, strongly gram-positive rod that usually occurs in pairs (1). The organism is fastidious in its requirement for L-cysteine and possesses a slow growth rate on even the best of defined media, Kidney Disease Medium-2 (KDM-2) (11). Approximately 10-14 days are required for maximal growth of the bacteria and up to 3-4 weeks for primary isolation of the bacterium from infected fish. The optimum temperature for growth of the bacterium ranges from 15 - 18°C (13). The bacterium is aerobic and the culture becomes more basic during the growth (1). KDM-2 medium contains cysteine and 20% serum along with peptone and yeast extract. Evelyn (11) indicated that serum is an essential

ingredient for culture of the bacterium. In addition to KDM-2, charcoal agar (KDM-C) (12), cysteine blood agar (13), cysteine serum agar (CSA) (1) and Mueller Hinton medium supplemented with cysteine (14) have been used to culture R. salmoninarum. In charcoal agar, the addition of 0.1 % activated charcoal replaces the requirement for serum supplementation. Daly and Stevenson (12), who developed this medium postulated that both serum and charcoal may serve to adsorb toxic substances from the medium. An advantage of using KDM-C in a broth culture is the elimination of the possibility of serum protein contamination in the culture supernatant. This is of particular importance when the supernatant is processed for production of extracellular products/antigens derived from the bacterium. Regardless of the medium used, optimum growth of the bacterium requires cysteine supplementation in conjunction with the addition of serum. Another medium of use is cysteine blood agar (CBA) which is a modification of Dorset's egg medium that contained cysteine and human blood (20%). Fryer and Sanders (1) have found that the human blood requirement in CBA can be replaced with either fetal bovine or calf serum.

Ordal and Earp isolated the bacterium directly from a lesion of an infected fish onto CBA and thereby were able to complete Koch's postulates, thus demonstrating R. salmoninarum to be the causative agent of this disease (13). Selective isolation of R. salmoninarum from an infected animal was enhanced by the development of a selective KDM-2 (SKDM-2) medium which incorporates the antibiotics such as D-cycloserine, polymyxin B

sulfate and oxolinic acid (15). These compounds were found to be antibacterial for most of the common contaminating organisms, while R. salmoninarum was insensitive. The development of a selective medium has been of particular interest since the slow growth of R. salmoninarum allows other, contaminating bacteria to overgrow the culture quite readily. Another means of overcoming this problem is the drop plate method as developed by Evelyn (11). This method was recommended over the spread plate method because the later technique allows the contaminating bacteria to overgrow R. salmoninarum. Of all the media that are available, the commonly used ones are KDM-2, KDM-C and Mueller Hinton medium with cysteine supplementation.

The G+C content of the DNA of R. salmoninarum ranges from 52.47 to 53.55, averaging 53 (18). Studies have been conducted on the cell wall composition and reveal interesting, and as yet controversial observations. Fryer and Sanders (1) have reported that the major amino acids found in the peptidoglycan were lysine, glutamic acid, glycine and alanine and the sugars were rhamnose, mannose, glucose and arabinose; glucose being the principal sugar. Kusser and Fiedler (19) and Fiedler and Draxl (20) confirmed the amino acid composition reported by Fryer and Sanders, but differed in their analysis of the polysaccharide composition. They reported the constituents of the cell wall polysaccharide as being galactose, rhamnose (6-deoxy-galactose), N-acetyl-fucosamine (2-acetamido-2,6-dideoxy-galactose) and N-acetyl-glucosamine; galactose being

the principal sugar. One interesting feature is the presence of N-acetyl fucosamine, which is rarely found in the cell walls of gram-positive bacteria. They also showed that the cell walls of R. salmoninarum do not possess teichoic acids, contrary to the observations of Fryer and Sanders, who suggested the presence of low amounts of teichoic acids (1). The cell wall polysaccharide was reported to comprise 60-70% of the dry weight of the cell walls (20).

Goodfellow et al. (21) and Fryer and Sanders (1), both reported the absence of mycolic acids in the cell walls. Embley et al. (22) demonstrated that the fatty acid profiles consisted of methyl-branched fatty acids and the polar lipid pattern was that of diphosphatidyl glycerol with two major, six or seven minor glycolipids, and two unidentified minor phospholipids. They also reported that there are menaquinone components consisting of unsaturated menaquinones with nine isoprene units.

Taxonomy

Originally Ordal and Earp (13) and Smith (16) suggested that the bacterium be placed in the genus Corynebacterium, based on morphological studies. Bullock et al. (23) felt that the resemblance between the bacterium and members of the genus, Listeria should place it within this genus. Sanders and Fryer (18) placed the bacterium, based on the G+C content and cell wall composition, within the Coryneform group as a new genus and species. In more recent studies, Stackebrandt et al. (24)

have utilized a 16S ribosomal RNA cataloguing approach to classify R. salmoninarum. Evaluation of more than 165 gram-positive organisms from 50 genera revealed that R. salmoninarum is a member of the actinomycetes subdivision, having closest similarities to Arthrobacter and Micrococcus. Confirmatory studies, using reverse transcript sequencing of 16S ribosomal RNA, were also performed which, again, demonstrated similarities between R. salmoninarum and Arthrobacter (S. Gutenberger and Dr. S. Giovannoni, Dept. of Microbiology, Oregon State University, personal communication). They performed studies, as described above, with Bacillus subtilis, Listeria monocytogenes, Brochothrix thermosphactum, Bacillus brevis, Clostridium innocuum, Arthrobacter globiformis and Streptococcus violaceoruber, and were convinced that the bacterium has the closest similarity with Arthrobacter globiformis.

Pathology

Belding and Merrill (3) were the first to describe the pathology associated with BKD. Externally it is characterized by exophthalmia, a distended abdomen, and occasionally pustules on the skin. The infection is systemic and has a marked affinity for the kidney tissue. Internally, the infected animal presents extensive petechial hemorrhages, splenomegaly, a swollen kidney covered with a grayish white membrane, accumulation of peritoneal fluid and pustular lesions on the kidney. The lesions

characteristically contain a thick white fluid consisting of leucocytes, bacteria and cellular debris (1,6,16,30). Smith (16) described the nature of the grayish white 'false' membrane histologically and showed that there are three distinct layers. The outermost layer appears to consist of fibrin and nucleated cells, the next contains leucocytes with visible nuclear fragmentation and are in the process of being phagocytized by histiocytes, and the innermost layer is comprised of fibroblasts and histiocytes. Bruno and Munro (39) found that the infection often resulted in a significant decrease in the hematocrit, red cell diameter, hemoglobin and an increase in the number of monocytes, thrombocytes and neutrophils. A significant increase in bilirubin, blood urea nitrogen, and potassium were also found in infected fish, along with decreases in total serum protein, cholesterol and sodium (40). Bruno (40) has also demonstrated that the fast-migrating serum proteins (albumins) were primarily affected in infected fish. He proposed that death of infected fish was due to the obliteration of normal kidney and liver structure by the dissemination of large granulomatous lesions. Impaired renal function and heart failure was also considered to be a contributing factor to mortality due to invasion of myocardium by phagocytic cells containing the bacterium (38).

Epizootiology

Disease Transmission: Mitchum and Sherman (25) demonstrated that natural, horizontal transmission of BKD from infected wild brook trout (Salvelinus fontinalis) to newly stocked hatchery brook trout, brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri) can occur within a small lake or stream system. The stocked trout, thus exposed to this infected population, died in nine months or less. Ordal and Earp (13), however, felt that BKD was less severe in trout than salmon. Renibacterium salmoninarum is known to be transmitted horizontally (23,25), however there is increasing evidence that it is vertically transmitted (27,28,29).

Currently there is no experimental evidence that infection occurs by water-borne transmission of the bacteria, but it is likely that the primary reservoir for the bacteria is infected or carrier fish. Austin and Rayment (26) studied the survival of R. salmoninarum in water and sediment of 56 freshwater fish farms in England and Wales, which had a history of BKD, and could not demonstrate the presence of the organisms using a fluorescent antibody technique (26). In the same study they demonstrated the presence of the bacteria in fecal matter of experimentally infected fish and found that they survived for only 21 days after all the experimental fish had succumbed to the disease. Published reports on water-borne challenge of fish with R. salmoninarum are not available except for the attempts reported by McCarthy et al. (66). In these experiments

undiluted broth cultures of the bacteria were used to challenge juvenile rainbow trout (Salmo gairdneri) both by intraperitoneal (i.p) injection and by immersion. The i.p. injection resulted in 75% mortality (within 35 days) and the immersion challenge produced 14% mortality (within 45 days), by which time there was complete mortality in the i.p. group. Belding and Merrill (3) artificially infected fish by injecting purulent material from kidney abscesses of infected fish. Experimentally infecting fish by feeding infected fish tissues has yielded varying results. One hundred percent transmission of BKD was accomplished by feeding infected flesh and viscera of adult chinook salmon to young chinook salmon (30). Smith (16), however, found that fish could not be infected, if the infected fish tissues were stored at -17 to -20°C for more than four months. This finding suggested that the bacterium retains its virulence for less than four months after freezing. Parasites have been suggested as being vectors in the transmission of R. salmoninarum (23,31). Bullock et al., (23) artificially infected brook trout by introducing the bacteria by first abrading or pricking the skin. They, thus, proposed that parasites which cause skin lesions may be involved in the transmission of the bacterium. These observations were concurred by Cusack and Cone (31) who suggested that skin-penetrating ectoparasites might be involved in the transmission of R. salmoninarum.

Effects of Environmental and Genetic Factors on Bacterial Kidney Disease: A number of environmental factors have been found to exert an influence on the severity of BKD including

water temperature, diet, and entrance into salt water. Belding and Merrill (3) noted the seasonal occurrence of the disease and its association with warm water. They found that high mortalities were confined to spring and early summer, and tends to parallel the rise in water temperature. They also observed that hatchery epizootics routinely occurred at water temperatures above 11°C, which is also associated with an earlier onset of the disease. Fryer and Sanders (1) reported that the mean day to death (MTD) was approximately 25 at water temperatures between 15-20.5°C, while it was 70 days at 4°C, indicating an inverse correlation of mortality with water temperature.

The affect of dietary factors on BKD has been of interest to researchers (Ref. 1). Diets high in fluorine and iodine have been observed to be associated with a lower prevalence of BKD compared to diets high in calcium, magnesium, zinc, iron, copper and cobalt (32,33). Paterson et al., (32) conducted a nutritional study for two consecutive years with Atlantic salmon reared under natural conditions at Margaree Hatchery (Canada), where a high incidence of natural BKD exists. They concluded that incorporating high concentrations of fluorine and iodine was important in the control of clinical BKD. They also felt that the dietary requirements and metabolism of the trace elements are influenced by the mineral concentration in the feed, surrounding water, as well as the environmental conditions and physiological stage of the animal.

Suzumoto et al., have demonstrated that the genetic make up of juvenile coho salmon may be responsible for their resistance to BKD (34). They demonstrated that among the three transferrin genotypes of coho salmon (e.g., AA, AC and CC), the AA genotype was the most susceptible while the CC genotype was the most resistant to experimental infection. Although the sample size used in this study (six per group) was quite small, the implication of this observed phenomenon is of interest. It suggests that certain stocks of fish may, to some degree, be genetically resistant to BKD.

Studies were undertaken to investigate the occurrence of BKD during the ocean phase of the salmon life cycle. Banner et al. demonstrated that BKD is an important factor effecting survival in experimental saltwater conditions and in the ocean (35, 36). It may be that the fish are infected in the hatchery and harbor the bacteria internally during their migration into the ocean. Banner et al.,(35) have collected salmon exhibiting clinical signs of BKD from the coastal waters of Washington and Oregon, and have confirmed the presence of the bacterium using a fluorescent antibody technique. Their earlier work (36) confirmed that mortalities due to BKD can increase in a saltwater environment.

Mechanisms of Pathogenicity

Possible Virulence Factors: There are an increasing number of recent reports describing the existence of

extracellular factors produced by R. salmoninarum and their possible roles in pathogenicity. Some of these observed activities were the ability to agglutinate rabbit erythrocytes (41), leukoagglutinating and macrophage inducing properties (42), hemolytic activity against rabbit erythrocytes (17), DNAase and protease activities (17). None of the above-mentioned factors, however, have been shown to be responsible for any pathological manifestations of the disease. This is in direct contrast to other salmonid pathogens such as Aeromonas salmonicida, which have proteolytic (78), leukolytic (79) and hemolytic (80) activities that can reproduce the characteristic histopathological effects when injected into rainbow trout .

Bruno and Munro (39) have postulated that the decreased hematocrit values seen during BKD may be due to the sequestering of erythrocytes in the spleen. This sequestering, they postulated, may be precipitated by the elaboration of soluble factor(s) derived from R. salmoninarum possessing affinity for erythrocytes. Although these researchers did not identify the soluble factor(s), they felt that such a cell-bound factor operating by this mechanism could explain the splenomegaly and the decreased hematocrit values observed in infected animals. Bruno (38) also proposed that direct damage to soft tissues may be due to products liberated from the bacterium, or from disrupted macrophages, as well as the release of hydrolytic and oxidizing enzymes of macrophage origin. Although many properties were observed associated with the bacterium, none of them was definitively demonstrated to act as

virulence factors in vivo, with the possible exception of Bruno's studies. In these studies it was demonstrated that there was a direct relationship between auto-agglutination, hydrophobicity and virulence among various isolates of R. salmoninarum (43). The strains that were virulent in test animals were sticky, auto-agglutinating and possessed a hydrophobic cell surface compared to non-adherent, non-agglutinating low-virulent strains.

Hypersensitivity Reactions: A number of researchers have studied the structural and histopathological aspects of this disease (37,38). Young and Chapman (37) proposed that the pathological changes in the fine structure of the glomeruli and renal tubules during experimental and natural infections resembled those of mammalian renal diseases, glomerulonephritis and nephrotic syndrome. Thickening due to subendothelial proteinaceous deposits and irregularities found in the basement membrane of the glomeruli indicated that these structures were damaged. The renal tubule cells were also seen to have ruptured mitochondria and multiple vacuolar spaces adjacent to the endoplasmic reticulum, indicating irreversible cellular injury. The induction of a host immune and/or inflammatory response appeared evident during both these experimental and natural infections. Host cells resembling macrophages or polymorphonuclear leucocytes with phagocytosed bacterial cells often appeared in lesions. Electron microscopy of these cells showed the morphological integrity of the phagocytosed bacterial cells and evidence of their active division, indicating intracellular survival. They proposed that

the observed subendothelial deposits resembled antigen-antibody complexes that accumulated during nephrotic serum nephritis, thus indicating a type III hypersensitivity reaction. Bruno (38) also defined BKD as a diffuse, chronic granulomatous inflammatory reaction. He conducted studies with rainbow trout and Atlantic salmon in which he performed histopathological examination of various organs from infected fish and confirmed the observations of Young and Chapman. In more recent histological examinations of kidney tissues from naturally infected chinook salmon, revealed protein deposits which resulted in a thickening of the endothelium of glomeruli, indicating possible antigen-antibody complex formation (personal communication, Dr. R. Hedrick, University of California, Davis).

Detection and diagnosis

Early studies devoted to the detection and diagnosis of R. salmoninarum were based on gram-staining (44), culturing of the tissue isolates on selective media, and observation of the actual clinical signs of the disease (46). Lillies Allochrome was also used for the detection of the bacterial cells which resulted in better clarity when compared to the gram stain (50). Subsequently, sensitive immunological methods were developed, incorporating specific antisera in various types of assays. The first such assays was a method of immunodiffusion (53). In this test specific precipitin lines were formed when rabbit antiserum

was reacted with tissue homogenates of infected fish. Bullock and Stuckey (48) were the first to develop the fluorescent antibody technique (FAT) for the detection of R. salmoninarum. They used both goat and rabbit antisera on kidney smears from infected fish and showed that the technique was more sensitive than the gram stain. Laidler (45) has also confirmed the results of Bullock and Stuckey. Paterson et al. (46) have also used an indirect FAT analysis for detection of R. salmoninarum in the wild salmonid populations of the Marjaree River system in Canada. Elliott and Barila (47) have also used IFAT method to detect and quantify the bacterium in the coelomic fluid of spring chinook salmon by first concentrating the bacteria onto polycarbonate filters.

A staphylococcal co-agglutination test was developed that employed rabbit antisera coupled to staphylococcal protein A. This reagent was used to produce antigen-specific agglutination with heat extracted antigens from infected fish kidney tissues (51). This method proved to be highly specific and sensitive as seen when 758 fish from 23 farms were tested. The rate of detection in this study was found to be as high or higher than that seen with gram staining or clinical examinations. Counter immunoelectrophoresis (52,58) has also been employed to detect heat extracted antigen from infected fish kidney tissues. The modified peroxidase and anti-peroxidase procedure developed by Sakai et al., (54) basically followed the form an enzyme linked immunosorbent assay (ELISA) method. In this method the antigen is coated on cellulose ester membranes and

incubated with rabbit antiserum, followed by goat anti-rabbit serum, and, finally, horseradish-peroxidase coupled anti-goat IgG. This assay was tested in the field and appeared to be superior to FAT analysis without crossreactions with other fish pathogens. ELISAs (56,57) represent the latest development in diagnostic strategies for the detection of BKD. Pascho and Mulcahy (56), in particular, have studied various parameters of the method such as selection of suitable assay plates, incubation temperatures, times of incubation for each reagent, and different dilution buffers. They have shown that the method can be highly specific, without crossreactions with heat extracted antigens of selected species of bacteria. The sensitivity of the assay ranged from 2-20 ng of soluble antigens. A summary of the various diagnostic procedures have been reviewed by Cipriano et al.,(57) and Pascho et. al. (58).

Increased specificity and sensitivity can also be afforded by the development of monoclonal antibodies to R. salmoninarum antigens (59,60). Wiens and Kaattari (59) have demonstrated the efficacy of monoclonal antibodies in identifying soluble protein antigens from infected fish sera, both by ELISA and Western blot analysis. They also tested the reactivity of the monoclonal antibodies against ten different isolates of the bacteria and found that the monoclonal antibodies reacted similarly with all ten isolates. It was further demonstrated that the monoclonals reacted consistently with a 57-58 kd protein doublet presence on the cell surface of all the isolates tested. The two monoclonal antibodies did not crossreact

with other species of gram-positive or gram-negative fish pathogens.

Control of Bacterial Kidney Disease

Bacterial Kidney Disease is one of the most difficult bacterial fish diseases to control. One plausible reason for this may be that the bacterium is a facultative intracellular parasite (37) which can survive and multiply within phagocytic cells. This intracellular nature could result in the protection of the bacterium from humoral antibody and antibiotic treatment.

Initial attempts at prophylaxis were reported by Belding and Merrill (3). In this first attempt they tried a form of nutritional prophylaxis. They felt that the disease was of a metabolic nature and, thus, they incorporated cod-liver oil, iodine, clam meal, and other vitamin supplements in the form of green vegetation in the diet for extended periods of time. This treatment met with no success.

Attempts at chemoprophylaxis and therapy have been more rigorously pursued. Initially, incorporation of sulfadiazine in the diet, resulted in decreased mortality during the administration period and for a short time thereafter (61). A few weeks after the discontinuance of the treatment, the mortality rate, once again, increased. Although there have been reports on the use of other sulfa drugs, none were found to be capable of eliminating the infectious agent from the host. In one detailed study, Wolf and Dunbar (62) tested 34 therapeutic

agents on 16 strains of R. salmoninarum in vitro and, based on antibiotic feeding trials, concluded that erythromycin fed for 21 days gave the best protection. The application of erythromycin is also supported by studies (63). Intraperitoneal injection of antibiotics such as erythromycin and oxytetracycline have yielded variable results (1). The most current practice is to inject mature females with erythromycin prior to spawning, and then to feed the offspring erythromycin for 21 days (Tony Amandi, personal communication). Use of erythromycin in preventing vertical transmission also has been studied by Evelyn et al. (70). They have reported that when brood fish were injected with the antibiotic, 30 to 56 days prior to spawning, bactericidal concentrations of the antibiotic were found in the eggs which protected them from a subsequent artificial infection. It is of importance to note that all the above-mentioned trials indicate that certain antibiotic treatments can be helpful in breaking the infection cycle.

Immunology and Protective Immunity

The development of antisera to various isolates of R. salmoninarum has revealed that there is serological homogeneity among different isolates from a worldwide distribution (20,59,64). This may be of practical benefit in the preparation of vaccine(s), in that one preparation may be able to cross-protect animals infected by other isolates. At present, however, there is no immunoprophylactic agent available for use with hatchery-

reared fish. A likely reason for this is related to the bacterium's intracellular nature. This property could facilitate the escape of the bacterium from both antibiotics and antibody. However, even though this may be occurring, no reports of infected fish possessing circulating antibody subsequent to a clinical episode of BKD have been forthcoming (65). Therefore, at this point, several attempts to stimulate a specific humoral immune response to protect fish against R. salmoninarum have been reported using different forms of antigen and adjuvants (32,66,67,68). These trials did not yield a protective preparation, although some of the tests did demonstrate a reduction of the incidence of lesions of BKD and the number of bacteria. McCarthy et al., (66) have used formalin-killed bacteria (bacterin) without adjuvant to immunize rainbow trout and found that the intraperitoneal injection of pH-lysed bacterial cell preparation was protective. No protection was observed when the fish were vaccinated by immersion or by hyperosmotic infiltration (69). Paterson et al., (67) used formalin-killed R. salmoninarum in Freund's complete adjuvant (FCA) to immunize Atlantic salmon post-yearling parr and demonstrated a reduced prevalence of BKD lesions compared to control animals smolts were examined 1 year after vaccination. Two species of Pacific salmon, coho and sockeye were used to test anti-BKD vaccines (74), and were not found to be protected following natural or experimental (injected) challenge with live pathogen. Evelyn et al., (74) concluded that Oncorhynchus species may not benefit from vaccination to the degree seen with Salmo species (66,67).

All the preparations studied thus far have been effective in reducing lesions of BKD, but have not been found to be effective in completely eradicating the bacteria. Although the published attempts at vaccination of fish against BKD are not numerous, there are some factors that must be addressed in the evaluation of vaccine efficacy. These include the appropriate dosage per fish depending on size and/or age, route of immunization (intraperitoneal injection or immersion or hyperosmotic infiltration or feeding), establishment of suitable challenge procedures (intraperitoneal injection or immersion), and determination of the length of time needed before challenge after vaccination.

Immunopotentiating Agents and Vaccine Development

Certain substances, when administered along with an antigen will enhance the immune response to that particular antigen. These substances are commonly termed adjuvants or immunopotentiating agents. There are reports on the use of such substances in treating fish diseases. Freund's complete adjuvant (FCA) is the most commonly used adjuvant and is composed of a killed preparation of Mycobacteria suspended in mineral oil. It has been shown that FCA stimulates antibody production against an antigen when injected in the form of an emulsion. This adjuvant also stimulates delayed type hypersensitivity (DTH), which can play an important role in the production of protective immunity against intracellular microbial and parasitic infections

(77). Studies have demonstrated that FCA (modified by incorporating M. butyricum) alone can protect fish from infection due to Aeromonas salmonicida (70). Other adjuvants tested in the same study were levamisole and MDP (N-acetyl-muramyl-L-alanyl-D-isoglutamine). MDP is the smallest component of the Mycobacteria capable of replacing the whole cell in FCA (78). Derivatives of MDP, such as FK-156 and FK 565 have found use as adjuvants in some prophylactic preparations used for fish diseases (76).

The intracellular nature of R. salmoninarum and the lack of a correlation of circulating antibody with protection, suggests that stimulation of cell-mediated immunity may be the prophylactic method of choice for BKD. Induction of cell-mediated immune (CMI) responses results in the activation of phagocytic cells and digestion of the pathogens residing within (77). It is possible to enhance the CMI response with adjuvants such as FCA, although such immunization procedures would be impractical for large scale vaccine programs. In such instances, it may be possible to physically link antigens from Renibacterium salmoninarum to the active component of FCA, MDP, and use this material in bath immunizations or hyperosmotic infiltration methods. Future research towards developing an effective prophylactic treatment for BKD, may require the effective generation of specific CMI.

REFERENCES

1. Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. *Ann. Rev. of Microbiology.* 35:273-298.
2. Mitchum, D. L., Sherman, L. E., and G. T. Baxter. 1979. Bacterial kidney disease in feral populations of brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri). *J. Fish. Res. Board Can.* 36:1370-1376.
3. Belding, D. L., and B. Merrill. 1935. A preliminary report upon a hatchery disease of the salmonidae. *Trans. Am. Fish. Soc.* 65:76-84.
4. Awakura, T. 1978. Bacterial kidney disease in mature salmonid. *Fish Pathology* 13:53-54.
5. Ehlinger, N. F. 1963. Kidney disease in lake trout complicated by lymphosarcoma. *Progressive Fish Culturist.* 25:3-7.
6. Bell, G. R. 1961. Two epidemics of apparent kidney disease in cultured pink salmon (Oncorhynchus gorbuscha). *J. of Fish Res. Board Can.* 18:559-562.
7. Munro, A. L. S., and D. W. Bruno. 1988. Vaccination against bacterial kidney disease. In *Fish Vaccination*. Ed. A. E. Ellis. Academic Press. pp 125.
8. Hicks, B. D., Daly, J. G., and V. E. Ostland. 1986. Experimental infection of minnows with the bacterial kidney disease bacterium. *Aquaculture Association of Canada meeting, University of Guelph, Canada. Abstracts: p 3-7.*
9. Bell, G. R., and G. S. Traxler. 1986. Resistance of the pacific lamprey, Lampetra tridentata (Gairdner), to challenge by Renibacterium salmoninarum, the causative agent of kidney disease in salmonids. *J. Fish Dis.* 9: 277-279.

10. Sanders, J. E., and M. J. Barros. 1986. Evidence by the fluorescent antibody test for the occurrence of Renibacterium salmoninarum among salmonid fish in Chile. *J. of Wildlife Diseases*. 22:255-257.
11. Evelyn, T. P. T. 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. *Bull. Off. Int. Epiz.* 87:511-513.
12. Daly, J. G., and Stevenson, R. M. W. 1984. Charcoal agar: a new growth medium for the fish disease bacterium, Renibacterium salmoninarum. *Appl. and Env. Microb.* 50:868-871.
13. Ordal, E. J., and B. J. Earp. 1956. Cultivation and transmission of the etiological agent of kidney disease in salmonid fishes. *Proc. Soc. Exp. Biol. Med.* 92:85-88.
14. Wolf, K., and Dunbar, C. E. 1959. Methods of infecting trout with kidney disease and some effects of temperature on experimental infections. U. S. Department of the Interior. Fish and Wildlife Serv. Spec. Sa. Rep. Fish. No. 286. 8 p.
15. Austin, B., Embley, T. M., and M. Goodfellow. 1983. Selective isolation of Renibacterium salmoninarum. *FEMS Microbiology Letters*. 17:111-114.
16. Smith, I. W. 1964. The occurrence and pathology of Dee disease. *Fresh Water Salmon Fish. Res.* 34:1-12.
17. Bruno, D. W., and A. L. S. Munro. 1986. Uniformity in the biochemical properties of Renibacterium salmoninarum isolates obtained from several sources. *FEMS Microbiology Letters*. 33:247-250.
18. Sanders, J. E., and J. L. Fryer. 1980. Renibacterium salmoninarum gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. *Int. J. Syst. Bacteriology*. 30:496-502.

19. Kusser, W., and F. Fiedler. 1983. Murein type and polysaccharide composition of cell walls from Renibacterium salmoninarum. FEMS Microbiology Letters. 20:391-394.
20. Fiedler, F., and R. Draxl. 1986. Biochemical and immunochemical properties of the cell surface of Renibacterium salmoninarum. J. of Bact. 168:799-804.
21. Goodfellow, M., Collins, M. D., and D. E. Minnikin. 1976. Thin-layer chromatographic analysis of mycolic acids and other long chain components in whole organism methanolysates of coryneform and related taxa. J. of Gen. Micro. 96:351-358.
22. Embley, T. M., Goodfellow, M., Minnikin, D. E., and B. Austin. 1983. Fatty acid, isoprenoid quinone and polar lipid composition in the classification of Renibacterium salmoninarum. J. Appl. Bact. 55:31-37.
23. Bullock, G. L., Stuckey, H. M., and Wolf, K. 1975. Bacterial kidney disease of salmonid fishes. Fish and Wildlife Service, Fish Disease Leaflet No. 41. U. S. Department of the Interior, Washington, D.C.
24. Stakebrandt, E., Wehmeyer, U., Nader, H., and F. Fiedler. 1988. Phylogenetic relationship of the fish pathogenic Renibacterium salmoninarum to Arthrobacter micrococcus and related taxa. FEMS Microbiology Letters. 50:117-120.
25. Mitchum, D. L., and L. E. Sherman. 1981. Transmission of bacterial kidney disease from wild to stocked hatchery trout. Can. J. Fish Aquat. Sci. 38:547-551.
26. Austin, B., and J. N. Rayment. 1985. Epizootiology of Renibacterium salmoninarum, the causal agent of bacterial kidney disease in salmonid fish. J. Fish Dis. 8:505-509.
27. Allison, L. N. 1958. Multiple sulfa therapy of kidney disease among brook trout. Prog. Fish. Cult. 20:66-68.

28. Evelyn, T. P. T., Ketcheson, J. E., and L. Prosperi-Porta. 1984. Further evidence for the presence of Renibacterium salmoninarum in salmonid eggs and for the failure of poridone-iodine to reduce the intra-ovum infection rate in water hardened eggs. J. of Fish Dis. 7:173-182.
29. Bullock, G. L., Stuckey, H. M., D. Mulcahy. 1978. Corynebacterial kidney disease: Egg transmission following iodophore disinfection. Fish Health News. 7:51-52.
30. Wood, J. W., and J. Wallis. 1955. Kidney disease in adult chinook salmon and its transmission by feeding young chinook salmon. Res. Briefs, Fish. Comm. Oregon. 6:32-40.
31. Cusak, R., and D. K. Cone. 1986. A review of parasites as vectors of viral and bacterial diseases of fish. J. of Fish Dis. 9:169-171.
32. Paterson, W. D., Lall, S. P., Airdrie, D., Greer, P., Greenham, G., and M. Poy. 1985. Prevention of disease in salmonids by vaccination and dietary modification. Fish Path. 20:427-434.
33. Lall, S. P., Paterson, W. D., Hines, J. A., and N. J. Adams. 1985. Control of bacterial kidney disease in Atlantic salmon, Salmo salar L., by dietary modification. J. of Fish Dis. 8:113-124.
34. Suzumoto, B. K., Schreck, C. B., and J. D. McIntyre. 1977. Relative resistances of three transferrin genotypes of coho salmon (Oncorhynchus kisutch) and their hematological responses to bacterial kidney disease. J. Fish. Res. Board Can. 34:1-8.
35. Banner, C. R., Long, J. J., Fryer, J. L., and J. S. Rohovec. 1986. Occurrence of salmonid fish infected with Renibacterium salmoninarum in the Pacific ocean. J. of Fish Dis. 9:273-275.
36. Banner, C. R., Rohovec, J. S., and J. L. Fryer. 1983. Renibacterium salmoninarum as a cause of mortality among chinook salmon in salt water. J. World Maricul. Soc. 14:236-239.

37. Young, C. L., and G. B. Chapman. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 35:1234-1248.
38. Bruno, D. W. 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L., with reference to naturally infected fish. J. Fish Dis. 9:523-537.
39. Bruno, D. W., and A. L. S. Munro. 1986. Hematological assessment of rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L., infected with Renibacterium salmoninarum. J. Fish Dis. 9:195-204.
40. D. W. Bruno. 1986. Changes in serum parameters of rainbow trout, Salmo gairdneri, Richardson, and Atlantic salmon, Salmo salar L., infected with Renibacterium salmoninarum. J. of Fish Dis. 9:205-211.
41. Daly, J. G., and R. M. W. Stevenson. 1987. Hydrophobic and hemagglutinating properties of Renibacterium salmoninarum. J. Gen. Micro. 133:3575-3580.
42. Wiens, G. W., and S. L. Kaattari. 1989. Characterization of a leukoagglutinating and macrophage inducing protein produced by Renibacterium salmoninarum. Fish Health Section. International Fish Health Conference. Annapolis, MD. July 17-20. Abstracts.
43. Bruno, D. W. 1988. The relationship between autoagglutination, pathogen Renibacterium salmoninarum. FEMS Microbiology Letters. 51:135-140.
44. Pippy, J. H. C. 1969. Kidney disease in juvenile Atlantic salmon (Salmo salar) in the Mangaru River. J. Fish. Res. Board Can. 26:2535-2537.
45. Laidler, L. A. 1980. Detection and identification of the bacterial kidney disease (BKD) organism by the indirect fluorescent antibody technique. J. Fish Dis. 3:67-69.

46. Paterson, W. D., Gallant, C., Desautels, D., and L. Marshall. 1979. Detection of bacterial kidney disease in wild salmonids in the Margaree river system and adjacent waters using an indirect fluorescent antibody technique. *J. Fish. Res. Board Can.* 26:1464-1468.
47. Elliott, D. G., and T. Y. Barila. 1987. Membrane filtration fluorescent antibody staining procedure for detecting and quantifying Renibacterium salmoninarum in coelomic fluid of chinook salmon (Oncorhynchus tshawytscha). *Can. J. Fish. Aquat. Sci.* 44:206-210.
48. Bullock, G. L., and H. M. Stuckey. 1975. Fluorescent antibody identification and detection of the Corynebacterium causing kidney disease of salmonids. *J. Fish. Res. Board Can.* 32:2224-2227.
49. Banner, C. R., Rohovec, J. S., and J. L. Fryer. 1982. A rapid method for labeling rabbit immunoglobulin with fluorescein for use in detection of fish pathogens. *Bull. Eur. Assoc. Fish Path.* 2:35-37.
50. Bruno, D. W., and A. L. S. Munro. 1982. Detection of the causative agent of bacterial kidney disease. *Bull. Eur. Ass. Fish Path.* 2:10-12.
51. Kimura, T. and M. Yoshimizu. 1981. A coagglutination test with antibody sensitized staphylococci for rapid and simple diagnostics of bacterial kidney disease (BKD). *Dev. Biol. Standard.* 49:135-148.
52. Groman, D. B. 1981. The detection of precipitating antibody to Renibacterium salmoninarum in chinook salmon by counter immunoelectrophoresis. *Dev. Biol. Stand.* 49:189-192.
53. Chen, P. K., Bullock, G. L., Stuckey, H. M., and A. C. Bullock. 1974. serological diagnosis of corynebacterial kidney disease of salmonids. *J. Fish. Res. Board Can.* 31:1939-1940.

54. Sakai, M., Koyama, G., and S. Atsuta. 1987. Detection of Renibacterium salmoninarum by a modified peroxidase-antiperoxidase (PAP) procedure. Fish Path. 22:1-5.
55. Pasco, R. J., and D. Mulcahy. 1987. Enzyme-linked immunosorbent assay for a soluble antigen of Renibacterium salmoninarum, the causative agent of salmonid bacterial kidney disease. Can. J. Fish. Aquat. Sci. 44:183-191.
56. Dixon, P. F. 1987. Detection of Renibacterium salmoninarum by the enzyme-linked immunosorbent assay (ELISA). J. Appl. Ichthyol. 3:77-82.
57. Pasco, R. J., Elliott, D. G., Mallett, R. W., and D. Mulcahy. 1987. Comparison of five techniques for the detection of Renibacterium salmoninarum in adult coho salmon. Trans. Am. Fish. Soc. 116:882-890.
58. Cipriano, R. C., Starliper, C. E., and J. H. Schachte. 1985. Comparative sensitivities of diagnostic procedures used to detect bacterial kidney disease in salmonid fishes. J. Wild. Dis. 21:144-148.
59. Wiens, G. W., and S. L. Kaattari. 1989. Monoclonal antibody analysis of common surface protein(s) of Renibacterium salmoninarum. Fish Path. 24:1-7.
60. Arakawa, C. K., Sanders, J. E., and J. L. Fryer. 1987. Production of monoclonal antibodies against Renibacterium salmoninarum. J. Fish Dis. 10:249-253.
61. Rucker, R. R., Bernier, A. F., Whipple, W. J., and R. G. Burrows. 1951. Sulfadiazine for kidney disease. Prog. Fish Cult. 13:135-137.
62. Wolf, K., and C. E. Dunbar. 1959. Test of 34 therapeutic agents for control of kidney disease in trout. Trans. Amer. Fish. Soc. 88:117-124.
63. B. Austin. 1985. Evaluation of antimicrobial compounds for the control of bacterial kidney disease in rainbow trout, Salmo gairdneri Richardson. J. Fish. Dis. 8:209-220.

64. Getchell, R. G., Rohovec, J. S., and J. L. Fryer. 1985. Comparison of Renibacterium salmoninarum isolates by antigenic analysis. *Fish Path.* 20:149-159.
65. Banowetz, G. M. 1974. Certain aspects of the immunology and chemotherapy of bacterial kidney disease in juvenile coho salmon (Oncorhynchus kisutch). M. S. Thesis. Oregon State University, Corvallis, OR. U.S.A. 53 pp.
66. McCarthy, D. H., Croy, T. R., and D. F. Amend. 1984. Immunization of rainbow trout, Salmo gairdneri Richardson, against bacterial kidney disease: preliminary efficacy evaluation. *J. of Fish Dis.* 7:65-71.
67. Paterson, W. D., Desautels, D., and J. M. Weber. 1981. The immune response of Atlantic salmon, Salmo solar L., to the causative agent of bacterial kidney disease, Renibacterium salmoninarum. *J. Fish Dis.* 4:99-11.
68. Evelyn, T. P. T. 1971. The agglutinin response in sockeye salmon vaccinated intraperitoneally with a heat-killed preparation of the bacterium responsible for salmonid kidney disease. *J. Wildl. Dis.* 7:328-335.
69. Croy, T. R., and D. F. Amend. 1977. Immunization of sockeye salmon (Oncorhynchus nerka) against vibriosis using hyperosmotic in filtration. *Aquaculture.* 12:317-325.
70. Olivier, G., Evelyn, T. P. T., and R. Lalher. 1985. Immunity to Aeromonas salmonicida in coho salmon (Oncorhynchus kisutch) induced by modified Freund's complete adjuvant: its nonspecific nature and probable role of macrophages in the phenomenon. *Dev. Comp. Immunol.* 9:419-432.
71. Evelyn, T. P. T., Ketcheson, J. E., and L. Proserpi-Porta. 1986. Use of erythromycin as a means of preventing vertical transmission of Renibacterium salmoninarum. *Dis. Aqua. Organ.* 2:7-11.

72. Bell, G. R., Hoffmann, R. W., and L. L. Brown. 1988. Pathology of experimental infections of the sablefish (Anoploma fimbria) with Renibacterium salmoninarum, the agent of bacterial kidney disease in salmonids. International Fish Health Conference, Vancouver, B. C., Canada. Abstracts: p. 31.
73. Evelyn, T. P. T., Ketcheson, J. E., and L. Prosperi-Porta. 1988. Trials with anti-bacterial kidney disease vaccines in two species of pacific salmon. International Fish Health Conference, Vancouver, B. C., Canada. Abstracts: p. 38.
74. Ellouz, F., Adam, A., Ciorbaru, R., and E. Lederer. 1974. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem. Biophys. Res. Commun. 59:1317-1325.
75. Kitao, T., Yoshida, T., Anderson, D. P., Dixon, O. W., and A. Blanch. 1987. Immunostimulation of antibody-producing cells and humoral antibody fish bacterins by a biological response modifier. Fish Immunology Symposium, Plymouth Polytechnique, Plymouth, England. Abstracts.
76. Mackness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vitro. J. Exp. Med. 129:973-979.
77. Liew, F. Y. 1982. Regulation of delayed type hypersensitivity to pathogens and alloantigens. Immunology Today. 3:18-23.
78. Shieh, H. S., and J. R. MacLean. 1975. Purification and properties of an extracellular protease of Aeromonas salmonicida, the causative agent of furunculosis. Int. J. Biochem. 6: 653-656.
79. Fuller, D. W., Pilcher, K. S., and J. L. Fryer. 1977. A leukocytolytic factor isolated from cultures of Aeromonas salmonicida. J. Fish. Res. Board Can. 34: 1118-1125.

80. Nomura, S., and H. Saito. 1982. Production of the extracellular hemolytic toxin by an isolated strain of Aeromonas salmonicida. Bull. Jpn. Soc. Fish. 48: 1589-1597.

2. CHARACTERIZATION OF SOLUBLE PROTEIN ANTIGEN(S)
PRODUCED BY THE FISH PATHOGEN, RENIBACTERIUM
SALMONINARUM

PRASAD S.D. TURAGA and STEPHEN L. KAATTARI

Department of Microbiology
Oregon State University
Corvallis, OR - 97331

ABSTRACT

We report the physicochemical characterization of soluble protein antigen(s) (SA) produced by Renibacterium salmoninarum. Supernatants from bacterial cultures were processed for the preparation of SA and characterized by the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, molecular exclusion chromatography, saturated ammonium sulfate (SAS) precipitation, and isoelectric focusing (IEF). Approximately 14 molecular weight species were routinely observed in the SA preparation which were capable of reacting with rabbit-anti SA. A prominent 57 kd protein fraction could be isolated by gel filtration and SAS precipitation. Isoelectric focusing indicated that the pI of all proteins was below 5.2.

INTRODUCTION

Renibacterium salmoninarum is the causative agent of bacterial kidney disease (BKD), one of the most devastating diseases to salmonid culture (Sanders and Fryer, 1980). It

causes a chronic, systemic infection with granulomatous lesions primarily occurring on the kidney, spleen, liver (Fryer and Sanders, (1981). One of the more interesting characteristics of the bacterium is its ability to survive and multiply within the in phagocytic cells of the host (Young and Chapman, 1978; Bruno, 1986; Gutenberger, S., personal communication).

Renibacterium salmoninarum is a gram-positive, diplobacillus, requiring cysteine for its growth (Evelyn, 1977). There has been relatively little work performed on the characterizaton of the extracellular products elaborated by this bacterium. The work performed in this laboratory and that of others (Getchell et al., 1985; Daly and Stevenson, 1987) has yielded information on the properties of these extracellular products. These studies have indicated that the products possess hydrophobic and hemagglutinating (Daly and Stevenson, 1987), leukoagglutinating (Wiens and Kaattari, in preparation), immunosuppressive (Turaga et al. 1987b) and protease (Turaga and Kaattari, in preparation) activities and have been associated with decreasing hematocrit values in infected fish (Turaga et al. 1987b).

These foregoing studies have indicated that the 57 kd protein may be responsible for most of these activities. It has also been shown that the 57 kd protein is a common antigen found in all isolates tested (Getchell et al. 1985). Although the 57 kd protein has been implicated as being responsible for these biological activities with the exception of protease activity, there

are no published reports on the characterization of the soluble proteins produced by the bacterium.

This study, therefore, employed a variety of biochemical methods to isolate and characterize the soluble product(s) and/or antigen(s) (SA). To date, no single factor has been identified as being sufficient to produce the disease in vivo, suggesting that virulence may be multifactorial. The studies performed here are essential in that, they may help delineate the activities associated with different molecular species of the SA.

MATERIALS AND METHODS

Growth of *Renibacterium salmoninarum*

Renibacterium salmoninarum (ATCC 33209) was grown in modified KDM-2 medium (Evelyn, 1977) without the serum supplementation. Cultures were grown in 250 ml shake flasks at 17°C until the late log phase of the culture (about 10-12 days). The culture stock was then aliquoted in sterile snap cap tubes (Falcon) and stored at -70°C.

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH₂PO₄ and 0.809 g of Na₂HPO₄ in 1 liter of distilled water. Tris-buffered

saline (TBS), pH 8.2 was prepared by dissolving Tris base 6.07 g, EDTA 0.3 g and NaCl 8.7 g in 1 liter of distilled water.

Preparation of Soluble Antigen (SA) of Renibacterium salmonianum

The preparation of soluble antigen was basically after that of Turaga et al. (1987a). Briefly, five hundred ml of the sterile KDM-2 medium in 2.8 L Erlenmeyer flasks were inoculated with a 5% (v/v) inoculum of the bacteria which had been grown to late log stage. The culture flask was then incubated on a reciprocating shaker at 17°C for 10-14 days. When the culture reached late log phase, they were centrifuged at 4000 x g for 30 min. The supernatant was removed and concentrated by passage through a PTGC 10000 NWML filter packet in a Minitan Ultrafiltration apparatus (Millipore Corp., Bedford, MA). Typically a ten-fold reduction of the original volume was achieved. This supernatant was further concentrated by ammonium sulphate (Sigma, MO) precipitation. The salt was added gradually over a period of one hour to a final concentration of 313 g/L with constant stirring, while holding the supernatant in an ice bath. This solution was then stirred for an additional 3 hr in the ice bath. The precipitate was removed by centrifugation at 400 x g for 30 min at 4°C, and resuspended in 10 ml of PBS. Using a saturated ammonium sulfate (SAS) solution (Campbell et al, 1970), two additional 50% precipitations were performed. The final precipitate was then resuspended

into 10 ml of PBS and dialyzed at 4°C against three changes of 3 liters of PBS over a period of 16 hr. The preparation was then filter-sterilized using a 0.45µm filter (Corning, Corning, NY). The protein content of the preparation was determined by the method of Lowry et al. (1951).

Rabbit anti-Soluble Antigen Antibody Preparation

Female New Zealand white rabbits were injected subcutaneously between the scapulae (1.4 ml) and in the footpads (0.4 ml each) with a 1:1 emulsion of SA (1 mg/ml of PBS) and Freund's complete adjuvant (FCA) (Difco, MI). The rabbits were rested 30 days then bled weekly. Seven weeks post-immunization the rabbits were boosted using the same protocol. The serum was distributed into aliquotes and stored at -70°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The method followed was basically that of Laemmli (1970). Separating gels of 10% polyacrylamide and a stacking gels of 3% polyacrylamide were used in all the experiments. The apparatus used was a mini gel electrophoresis unit (Idea Scientific, Corvallis, OR) and the power source was an EC 600 (E-C apparatus Corporation, St. Petersburg, FL). The samples were mixed with the sample buffer at a 1:1 ratio and placed in boiling

water bath for 3 minutes prior to electrophoresis. The samples were usually loaded in a duplicate fashion so that one half could be stained for protein while the other half was used for Western blotting. Samples containing 5 μg of protein were applied to each lane. During the electrophoresis a constant current of 10 mA was applied during migration through the stacking gel and 20 mA during migration through the separating gel.

Transblotting

After the electrophoresis, the protein bands were transferred from the gel onto nitrocellulose (NC) paper (Bio Rad, Richmond, CA), using a transblot apparatus (Bio Rad, Richmond, CA) (Towbin et al. 1983; manufacturers instructions). The protein transfer was done in transblotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) and by applying 100 V for one hour (with cooling of the buffer by placing a unit with ice in the transfer chamber). After transblotting the nitrocellulose paper was divided in half for protein staining and for Western blotting.

Protein Staining

After transblotting, the NC paper was incubated in 0.3% tween 20 in PBS for 30 minutes at 30°C. The NC paper was then washed in the same buffer with three changes of the buffer over 15 minutes, while under constant agitation at room temperature.

After brief rinsing with distilled water, Aurodye forte (Janssen Life Sciences Products, Olen, Belgium) protein staining reagent was added to the NC paper with constant shaking at room temperature till dark purple bands became visible. This usually occurred in about 2-3 hours. The blot was then briefly rinsed with distilled water and blotted dry.

Western Blotting

The transblotted proteins were alternatively examined for the presence of antigens by Western blot analysis and the method was basically that of Towbin et. al. (1979). The NC paper was incubated in 3% bovine serum albumin (BSA) in T-TBS (0.1% tween-20 in TBS) solution at 37°C for one hour. The paper was then washed with three changes of T-TBS for a period of 15 minutes, while under constant shaking. Biotinylated rabbit anti-SA (Turaga et. al., 1987a) was used at a concentration of 5 µg in 10 ml of T-TBS, was incubated with the NC paper for one hour at room temperature. The NC paper was then washed as described above. A 1/100 dilution of streptavidin-peroxidase (Sigma, St. Louis, MO) in T-TBS was incubated with the NC paper for 30 minutes at room temperature. After washing the NC paper as above with T-TBS, the antigen bands were stained by incubating the NC paper with a substrate solution at 37°C until the bands were darkly stained. The substrate solution consisted of 10 ml of PBS, 2 ml of 4-chloronaphthol (3 mg/ml in methanol; Bio Rad, Richmond, CA) and 10 µl of hydrogen peroxide.

Molecular Exclusion Chromatography of Soluble Antigen

Soluble antigen was fractionated, with respect to the molecular weight, by the use of gel filtration. Briefly, one ml (3 mg/ml) of soluble antigen was chromatographed on a P-150 column (polyacrylamide gel column, Bio-Rad, Richmond, CA) using PBS as the elution buffer. The flow rate was 7 ml/hour and 2.0 ml fractions were collected the O. D. of the fractions was measured (Spectronic 21) at a wavelength of 280 nm. The peak fractions were subjected to SDS-PAGE analysis.

Saturated Ammonium Sulfate (SAS) Precipitation of Soluble Antigen

Soluble antigen was subjected to differential SAS precipitations (10%, 20%, 30%, 40%, 50% and 80%). Each precipitation was performed by adding the necessary quantity of SAS to attain a concentration of 10% in the soluble antigen solution. This mixture was then incubated for 30 minutes on an ice bath with constant stirring. The preparation was then centrifuged at 4000 x g for 15 minutes at 4°C to pellet the precipitate and the supernatant was subjected to similar treatment upon addition of a sufficient quantity of SAS to yield the next higher SAS concentration. This process was continued until all the precipitations were performed. Thus, each precipitation contained only proteins capable of being precipitated within a range of SA concentration (i.e., 50-80%; 40-

50%; 30-40% etc.). The pellet from each precipitation was resuspended in 0.5 ml of PBS and dialysed against PBS with three changes of the buffer at 4°C. SDS-PAGE analysis was performed on these different SAS precipitated preparations.

RESULTS

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis of Soluble Antigen (SA)

SDS-PAGE, a technique which separates proteins depending on their mass and charge, revealed the existence of approximately 14 different molecular weight species within the soluble antigen preparation. The molecular weights of these species ranged from 22 kilodaltons to 72 kilodaltons. The prominent bands were of 58, 57, 49, 39, 37, 30 and 27 kd molecular weight. The total number of bands varied slightly between different batches of SA, but the prominent band pattern remained the same. In duplicate gels, which were processed for western blotting, all the 14 protein bands reacted with the rabbit anti-SA (Fig 2.1).

Molecular Exclusion Chromatography of Soluble Antigen

In these studies a polyacrylamide gel matrix was employed to further characterize the different fractions of soluble antigens produced by chromatographic separation. Fig.

2.2 demonstrates the distribution of proteins as they are eluted from the column. Fractions from the peak shown in Fig. 2.2.a. were analysed by SDS-PAGE to determine the separation of different molecular weight fractions. The fractions tested were 11 through 16, 18, 20, 24 and 30 (Fig. 2.2 b). The prominent 57 kd protein band appears in fraction in 11 and trace amounts of it appear in later fractions where the lower molecular weight fractions become more prominent. The chromatography thus proved to be a useful tool in characterizing different molecular weight fractions of the soluble antigen.

Sequential SAS Precipitation of Soluble Antigen

Analysis of differential SAS precipitates by SDS-PAGE (Fig. 2.3) indicated that the different molecular weight antigenic species can be resolved by this method. Isolation of the prominent 57 kd band occurs optimally at 30% SAS precipitation, while 50-80% precipitation for isolation of smaller molecular weight fractions of the soluble antigen.

Isoelectric Focusing

Isoelectric focusing was performed on the soluble antigen preparations from cultures of R. salmoninarum grown in KDM-2 and ultrafiltered KDM-2 medium. The gel shown in the figure 2.4 has a pH range of 3-10. The standards have a pI range of 5-10.5, the top-most being β -lactoglobulin (pI 5.2). Antigen

preparations from both KDM-2 and ultrafiltered KDM-2 were observed to have pIs below the 5.2. The gel suggests that both preparations contain eight distinguishable proteins, two major and six minor and no bands in the KDM-2 medium control.

DISCUSSION

There are an increasing number of recent reports describing the extracellular factors expressed by R. salmoninarum and their putative functions (Daly and Stevenson, 1987; Turaga et al., 1987a & b; Turaga and Kaattari, in preparation; Wiens and Kaattari, in preparation). None of these studies utilized purified molecular weight species of soluble antigen (SA). In addition, there are no reports on the physical characterization of these extracellular products.

This report, therefore, describes the physicochemical characteristics of SA produced by R. salmoninarum. The molecular weight analysis performed by SDS-PAGE (Fig. 2.1) indicated that there were approximately 14 molecular weight species of which 7 were distinctly prominent. In other experiments, differences in the number of bands obtained with different batches of SA have been observed, but the prominent bands remained the same. One possible reason for this variability appears to be the existence of a protease activity associated with the SA preparation (Turaga and Kaattari, in preparation). Proteolytic cleavage of the major protein(s) may result in a number of breakdown products with lower molecular

weight species. Western blot analysis indicates that all the protein bands were antigenic since they react with rabbit-anti SA. Monoclonal antibodies developed against SA (Wiens and Kaattari, 1989) have also shown to recognize the same major protein bands as are seen here. They also demonstrated that the 58 & 57 kd proteins were also found on R. salmoninarum cells.

Molecular exclusion chromatography (MEC), using a polyacrylamide gel column, facilitates the separation of different molecular weight species of SA (Fig. 2.2) The 57 kd molecular weight molecule and others in this weight range were effectively isolated by this procedure. Another biochemical approach which was effective in separating the different molecular weight species of SA was saturated ammonium sulfate (SAS) precipitation.

Isoelectric focusing (IEF) demonstrated that the proteins found in SA have a pI below 5.2, indicating that they are acidic proteins. Again, the variation in number of bands observed by IEF as well as by SDS-PAGE may be due to the generation of breakdown products due to the protease activity. An explanation for the restricted pI pattern for all the protein bands in SA may be that they may be the products of a single parent molecule which was partially digested. They, thus, may possess some core region which contained the bulk of the functional groups that contribute to generation of the common pI value observed with all molecules.

The foregoing methods were designed to evaluate methods by which different molecular weight species of SA may be

isolated and used in future experiments to assess their biological activity. Such purified moities could also be used to generate specific antisera and possible candidates for vaccines. Purified bacterial products have been used in identification of their biological activities such as, the role of toxins of Vibrio in molluscs (Nottage and Birkbeck, 1987); the isolation of an immunosuppressive substance produced by Streptococcus mutans (Santarem et al., 1987), purification of hemolytic toxin from a fish pathogen, Aeromonas salmonicida (Nomura et al., 1988) and purification of an extracellular-protease from Pseudomonas cepacia (McKevitt et al., 1988).

ACKNOWLEDGEMENTS

This work was supported by Bonneville Power Administration contract No. DEAI79-87BP16480.

Best scan
available.

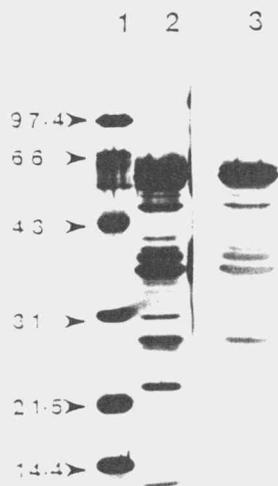
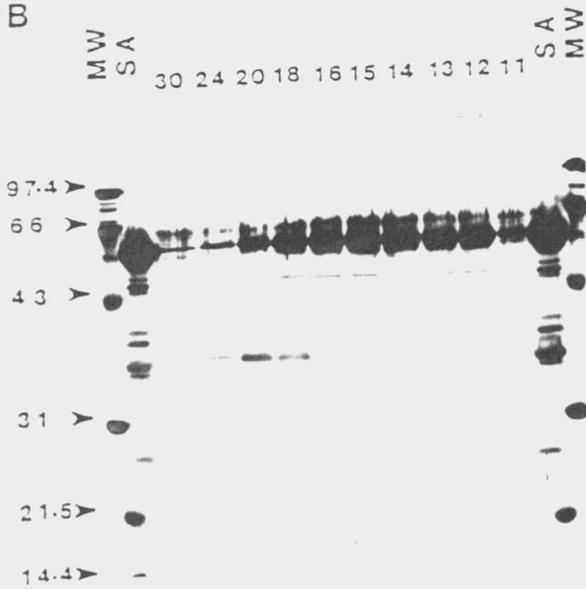
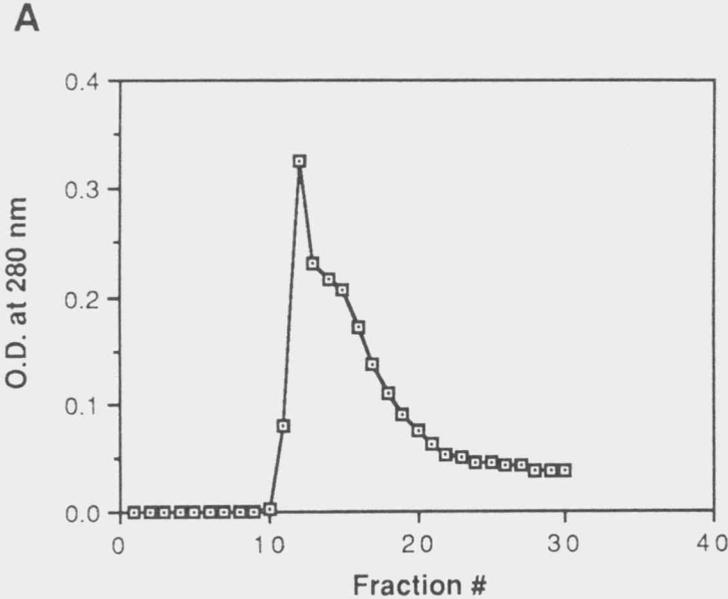


Fig. 2.1. SDS-PAGE and western blot analysis of soluble antigen: A. Protein staining (Aurodye^R forte) of electrophoretically separated protein bands of molecular weight markers (lane 1) and SA (lane 2). B. Western blotting of soluble antigen indicating the antigenic nature of all the bands (lane 3) stained for protein.

Fig. 2.2. Molecular exclusion chromatography of SA. A. O. D. values of 2 ml fraction of a P-150 column fractionated preparation of soluble antigens are depicted. B. SDS-PAGE analysis of chromatographic fractions 11 through 16, 18, 20, 24 and 30. Lanes containing unfractionated soluble antigen (SA) and molecular weight (M. W.) are also shown.

Fig. 2.2. Molecular exclusion chromatography of SA.



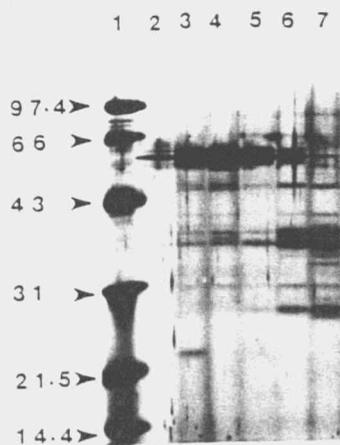


Fig. 2.3. SDS-PAGE analysis of the preparations obtained by sequential SAS precipitation of soluble antigen. Lane 1 (mol. wt. markers), lane 2 (10%), lane 3 (20%), lane 4 (30%), lane 5 (40%), lane 6 (50%) and lane 7 (80%) precipitations.

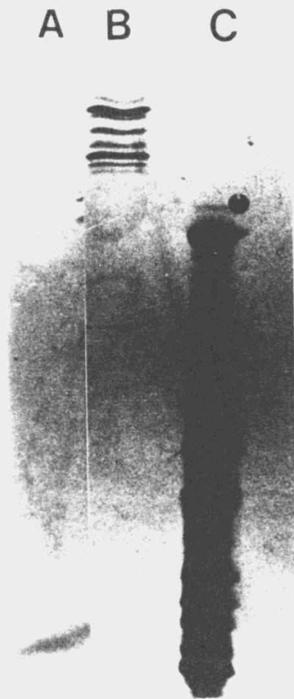


Fig. 2.4. Isoelectric focusing of soluble antigen. Lane A, KDM-2 medium control; lane B soluble antigen; lane C, pI markers. The pI marker of β -lactoglobulin (pI value of 5.2) is identified.

REFERENCES

- Daly, J. G., and R. M. W. Stevenson. 1987. Hydrophobic and hemagglutinating properties of Renibacterium salmoninarum. J. Gen. Micro. 133: 3575-3580.
- Evelyn, T. P. T. 1977. An improved growth medium for the kidney disease bacterium and some notes in using the medium. Bull. Off. Int. Epiz. 87: 511-513.
- Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. of Microbiology. 35: 273-298.
- Getchell, R. G., Rohovec, J. S., and J. L. Fryer. 1985. Comparison of Renibacterium salmoninarum isolates by antigenic analysis. Fish Path. 20: 149-159.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.
- McKevitt, A. I., Bajaksouzian, S., Klinger, J. D., and D. E. Woods. 1989. Purification and characterization of an extracellular protease from Pseudomonas cepacia. Infect. Immun. 57: 771-778.
- Nomura, S., Fujino, M., Yamakawa, M., and E. Kawahara. 1988. Purification and characterization of salmolysin, an extracellular hemolytic toxin from Aeromonas salmonicida. J. of Bact. 170: 3694-3702.
- Nottage, A. S., and T. H. Birkbeck. 1987. The role of toxins in Vibrio infections of bivalve molluscs. Aquaculture. 67: 244-246.
- Sanders, J. E., and J. L. Fryer. 1980. Renibacterium salmoninarum gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. Int. J. Syst. Bact. 30: 496-502.

Santarem, M. M. G., Porto, M. T., Ferreira, P., Soares, R., and M. P. Arala-chaves. 1987. Semi-purification of an immunosuppressor substance secreted by Straptococcus mutans that plays a role in the protection of the bacteria in the host. Scand. J. Immunol. 26: 755-761.

Towbin, H., Staehelin, T., and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 75: 4350-4354.

Young, C. L., and G. B. Chapman. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 35: 1234-1248.

3. IDENTIFICATION AND CHARACTERIZATION OF PROTEASE
ACTIVITY ASSOCIATED WITH A SOLUBLE ANTIGEN
PREPARATION OF RENIBACTERIUM SALMONINARUM

PRASAD S.D. TURAGA and STEPHEN L. KAATTARI

Department of Microbiology
Oregon state University
Corvallis, OR - 97331

ABSTRACT

A protease activity associated with a soluble antigen preparation of Renibacterium salmoninarum was identified and partially characterized. It was shown that the protease activity against the soluble antigen was more pronounced at a temperature of 37°C and above and not at 4°C, 17°C and 23°C. Phenyl methane sulfonyl fluoride (PMSF), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butane (TPCK), alpha 2-macroglobulin (alpha-2M) and 50%, but not 10% ethanol or methanol were found to be inhibitors of the protease activity. Phosphorylase B, bovine serum albumin and soybean trypsin inhibitor were also found capable of serving as substrates. We also demonstrate that this protease activity can act to neutralize the immunosuppression induced by the soluble antigen.

INTRODUCTION

Renibacterium salmoninarum is the causative agent of one of the most devastating diseases of salmonid fish, bacterial kidney disease (BKD) (Fryer and Sanders, 1981). The bacterium

is a gram positive, pleomorphic, diplobacillus and is classified as a unique species and genus (Sanders and Fryer, 1980). It requires cysteine for growth (Evelyn, 1977) and is a slow growing bacterium even on the best of the defined media. It has been shown that there is uniformity of biochemical properties among the different isolates tested (Bruno and Munro, 1985; Fiedler and Draxl, 1986). A unique peptidoglycan structure as well as an unusual cell wall polysaccharide incorporating N-acetyl fucosamine as one of the constituents has been identified (Fiedler and Draxl, 1986). Smith (1961) has shown that protease and catalase activities have been associated with the bacterium and Bruno and Munro (1986) have demonstrated both DNAase and hemolysin activities. In more recent studies, hydrophobic and hemagglutinating properties have been demonstrated (Daly and Stevenson, 1987) as well as a relationship between auto-agglutination, cell surface hydrophobicity and the virulence of the bacterium (Bruno, 1988). Except for the studies of Bruno (1988), none of the biochemical factors or activities described for the bacterium have been shown to be associated with the virulence of the bacterium.

This study represents the first report on the identification and characterization of protease activity associated with a soluble antigen preparation of R. salmoninarum. We also show the relationship of protease activity and its biological activity, in vitro.

MATERIALS AND METHODS

Preparation of Soluble Antigen

Renibacterium salmoninarum was cultured in KDM-2 (Evelyn, 1977) medium without serum supplementation, for 10-14 days in shake flasks at 17°C. The culture was then centrifuged at 4000 x g for 30 min at 4°C and the supernatant was processed for the isolation of soluble antigen (SA) according to the method of Turaga et al. (1987a).

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH₂PO₄ and 0.809 g of Na₂HPO₄ in 1 liter of distilled water. The PBS used in protein staining was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.2 g of KH₂PO₄ in 1 liter of distilled water. Tris base 6.07 g, EDTA 0.3 g and NaCl 8.7 g were dissolved in distilled water to prepare tris buffered saline (TBS), pH 8.2.

Lymphocyte Culture Media

Different media preparations used for lymphocyte culture and plaque assay were prepared as described by Kaattari and

Yui (1987). Tissue culture medium (TCM) consisted of RPMI 1640 containing L-glutamine and sodium bicarbonate and was supplemented with 10% fetal calf serum, 50 $\mu\text{g/ml}$ gentamicin, 50 μM 2-mercaptoethanol and 10 $\mu\text{g/ml}$ each of adenosine, uracil, cytosine and guanine (Sigma, St. Louis, MO). Holding medium consisted of 100 $\mu\text{g/ml}$ gentamicin, 10% fetal calf serum (Whittaker M.A. Bioproducts) in RPMI 1640 (Gibco, Grand Island, NY) with bicarbonate supplementation. All the components were purchased from Whittaker M. A. Bioproducts, unless otherwise noted.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The method followed was basically that of Laemmli (1970). Separating gels of 10% polyacrylamide (Sigma, St. Louis, MO) and stacking gels of 3% polyacrylamide were used in all the experiments. The apparatus used was a mini gel electrophoresis unit (Idea Scientific, Corvallis, OR) and the power source was an EC 600 (E-C apparatus Corporation, St. Petersburg, FL). The samples were mixed with the sample buffer (120 mM tris base, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% w/v glycerol, and 3mM bromophenol blue) at a 1:1 ratio and placed in boiling water bath for 3 minutes prior to electrophoresis. The samples were usually loaded in a duplicate fashion so that one half of the gel could be stained for protein while the other half was used for Western blotting. Samples containing about 5 μg of protein were

applied to each lane. During the electrophoresis a constant current of 10 mA was applied during migration through the stacking gel and 20 mA during migration through the separating gel.

Transblotting

After the electrophoresis, the protein bands were transferred from the gel onto nitrocellulose (NC) paper (Bio Rad, Richmond, CA), using transblot apparatus (Bio Rad, Richmond, CA) (manufacturers instructions; Towbin et al., 1979). The transfer of proteins was done in transblotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) and by applying 100 V for one hour. The transblot buffer was continuously cooled by placing a cooling unit in the transblot chamber while constantly stirring the buffer. After transblotting the nitrocellulose paper was processed for protein staining and/or Western blotting.

Protein Staining

After transblotting, the NC paper was incubated in 0.3% tween 20 in PBS for 30 min at 30°C. The NC paper was washed in the same buffer for 15 min with three changes of the buffer, while under constant agitation at room temperature. After a brief rinsing with distilled water, Aurodye^R forte (Janssen Life Sciences Products, Olen, Belgium) protein staining reagent was applied to the NC paper with constant shaking at room

temperature until dark purple bands became visible. This usually occurred in about 2-3 hr. The blot was then rinsed with distilled water and blotted dry.

Western Blotting

The transblotted proteins were alternatively examined for the presence of antigens by Western Blot analysis and the method was basically that of Towbin et. al. (1979). The NC paper was incubated in 3% bovine serum albumin (BSA) in T-TBS (0.1% tween-20 in TBS) solution at 37°C for one hour. The paper was then washed with three changes of T-TBS during a period of 15 min, while under constant shaking. Rabbit anti-SA-biotinylated antibody (Turaga et. al., 1987a) was used at a concentration of 5 µg in 10 ml of T-TBS, was incubated with the NC paper for one hour at room temperature. The NC paper was washed as before. A 1/100 dilution of streptavidin-peroxidase (Sigma, St. Louis, MO) in T-TBS was incubated with the NC paper for 30 min at room temperature. After washing the NC paper as described with T-TBS, the antigen bands were stained by incubating the NC paper with the substrate solution at 37°C. The substrate solution consisted of 10 ml of PBS, 2 ml of 4-chloronaphthol (3 mg/ml in methanol; Bio Rad, Richmond, CA) and 10 µl of hydrogen peroxide.

Identification of Substrate for Protease Activity

Identification of alternate substrates for the protease was performed by incubating soluble antigen with both high and low molecular weight markers used in SDS-PAGE analysis (Bio-Rad, Richmond, CA) at 37°C. This facilitated the testing of a number of proteins as potential substrates. The high molecular weight markers were: myosin (200,000 d), β galactosidase (116,250 d), phosphorylase B (97,400 d), bovine serum albumin (BSA) (66,200 d) and ovalbumin (43,000 d). The low molecular weight markers were: phosphorylase B, BSA, ovalbumin, carbonic anhydrase (31,000 d), soybean trypsin inhibitor (21,500 d) and lysozyme (14,400 d). After overnight incubation of the soluble antigen with these markers at 37°C, the samples were subjected to SDS-PAGE analysis (Laemmli, 1970). Molecular weight markers were prepared by diluting the concentrate (1/30) in nanopure water. Soluble antigen (1 mg/ml in PBS) was mixed 1:1 with the prepared molecular weight markers and incubated at 37°C .

Characterization of the Nature of the Protease Activity

The characterization was accomplished by using the protease inhibitors: phenyl methane sulfonyl fluoride (PMSF), L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butane (TPCK), α -2 macroglobulin (α -2 M). Different starting concentrations of inhibitors and soluble antigen were: PMSF at 100 mM in

methanol, TPCK at 20 mg/ml in absolute ethanol, alpha-2 M at 25 units/ml in sterile distilled water and soluble antigen at 1 mg/ml in PBS. For the incubation, 1 μ l each of PMSF, TPCK and alpha-2 M was added to 10 μ l of soluble antigen along with methanol and ethanol controls. These preparations were then subjected to SDS-PAGE analysis.

Lymphocyte Cell Culture

Fish were sacrificed by an anesthetic overdose in a benzocaine bath. The benzocaine bath was prepared by diluting 2 ml of a stock solution [10% (w/v) of benzocaine (Sigma, Mo) in methanol] into 15 liters of water. After the fish were fully sedated the caudal peduncle was severed and the fish were exsanguinated (this procedure reduces the number of red blood cells during the subsequent isolation of leukocytes from the anterior kidney). Anterior kidney tissue was aseptically removed, placed in a sterile tube with holding medium and held on ice. A single cell suspension was prepared by gently passing the tissue repeatedly through a one ml syringe. Tissue aggregates were then allowed to settle. The supernatant, containing single cells, was then washed twice by centrifugation at 500 x g for 10 minutes at 4°C using the holding medium. The cellular pellet was resuspended in TCM. Viable cell counts were performed utilizing the trypan blue exclusion procedure (Phillips, 1973). The cell suspension was adjusted to a final concentration of 2×10^7 cells/ml TCM. From this cell suspension,

0.1 ml was then aliquoted into each well of a 24 well, flatbottom, tissue culture plate (Corning, NY) containing appropriate quantities of antigen (TNP-LPS) (Jacobs and Morrison) or TNP-LPS plus soluble antigen from R. salmoninarum. Prior to addition to the culture, the soluble antigen was dialysed in RPMI 1640 (with bicarbonate) overnight at 4°C (2 ml of 3 mg/ml soluble antigen was dialysed in one liter of the medium). The plates were then incubated at 17°C in Incubator Culture Chamber (C.B.S. Scientific, Del Mar, CA, model #624) under an atmosphere of 10% CO₂, 10% O₂ and the balance N₂. The cultures were maintained by adding 50 µl of nutritional supplement (cocktail medium) (Tittle and Rittenberg, 1978) on alternate days.

Plaque Forming Cell (PFC) Assay

Cells secreting anti-trinitrophenyl (TNP) antibodies upon in vitro antigenic stimulation were detected by a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). Cells from each well were harvested on day nine of the culture and washed twice with RPMI. The pellet from the final wash was resuspended into 0.2 ml of RPMI 1640 and were held on ice until plated. One hundred µl of the cell suspension, 20 µl of a 10% suspension of TNP-sheep red blood cells (TNP-SRBC) (Rittenberg and Pratt, 1969) in modified barbital buffer (MBB) and 20 ul steelhead serum (diluted in MBB) as the source of complement were mixed in individual wells of a 96 well

microtiter plate (Linbro, McLean, VA). The contents of each well was then pipetted into a slide chamber, sealed with paraffin and incubated for 1-2 hours at 17°C. Plaques, clear zones of lysis within the TNP-SRBC lawn, were then enumerated under low power using a dissecting microscope.

RESULTS

Identification of Protease Activity

The normal storage temperature of soluble antigen was at 4°C and demonstrated a consistent band pattern on SDS-polyacrylamide gel electrophoresis. It was a fortuitous observation that when SA was incubated at 37°C, there was a disappearance of most of the protein bands when tested on SDS-PAGE (Fig. 3.1).

Effect of Temperature on Protease Activity

Soluble antigen was incubated at a variety of temperatures and the resultant preparations were examined by SDS-PAGE analysis. The temperatures used were 4°C, 17°C, 23°C, 37°C and 56°C. The presence of protease activity was demonstrated by loss of prominent bands on a SDS-PAGE gel compared to the original preparation. Fig. 3.2 indicates that incubation at 37°C and 56°C results in demonstrable protease

activity. For all subsequent protease assays, incubation of soluble antigen at 37°C overnight was used.

Kinetics of Proteolysis

The kinetics associated with the proteolysis of soluble antigen was studied by incubating the SA at 37°C and aliquots were withdrawn at 0, 2, 4, 8, 12 and 24 hr. The aliquots were mixed with an equal volume of sample buffer and placed in boiling water bath for 3 minutes. They aliquots used immediately for SDS-PAGE analysis or stored at -20°C until the analysis was performed. Most of the proteolysis was observed to occur within the initial 2 hours of incubation (Fig. 3.3). This proteolytic activity had a particularly pronounced effect on the 57 kd protein band. Most of the proteolysis occurred within 24 hours of incubation, except that the band at 52 kd remained throughout the incubation period and the bands that appeared at lower molecular weights are probably due to products of the proteolysis.

Charaterization of the Protease

Soluble antigen was incubated at 37°C with the protease inhibitors such as PMSF, TPCK and alpha-2 M. These inhibitors demonstrated the inhibition of the protease activity (Fig. 3.4). It was also found that ethanol and methanol at a concentration of 50%, but not 10%, also protected soluble antigen from

proteolysis. Western blot analysis of these samples indicated that inhibition of proteolysis protected the antigenic nature of the soluble antigen (Fig. 3.4 B).

Identification of Substrate for the Protease

To identify alternate substrates for the protease, soluble antigen preparation was incubated with the molecular weight markers, both high and low (Bio-Rad, Richmond, CA). These marker preparations possessed the following proteins: myosin, β galactosidase, phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. SDS-PAGE analysis of these samples after incubation with the soluble antigen preparation (Fig. 3.5) demonstrated that phosphorylase B (97,000 d), BSA (66,000 d) and soybean trypsin inhibitor (21,500 d) were digested and thus can serve as substrates for the protease activity.

Effect of Protease on Biological Activity of the Soluble Antigen

In our previous studies we demonstrated that SA had a suppressive effect on the in vitro antibody responses of coho salmon lymphocytes to a defined antigen TNP-LPS (Turaga et al., 1987). Figure 3.6 demonstrates this dose-dependent suppressive effect of SA when co-cultured with TNP-LPS and lymphocytes.

Soluble antigen (0.5 mg/ml in RPMI 1640) was incubated at 37°C and periodic samples were withdrawn periodically. These aliquots were tested in the in vitro PFC assay. Disappearance of the protein bands by incubation at this temperature correlated with the loss of immunosuppressive nature of SA (Fig. 3.7) .

DISCUSSION

The only proteolytic activity described for R. salmoninarum was the gelatin liquifaction (Smith, 1964) and this protease activity was never characterized. Data contained in this report provides identification and characterization of protease activity associated with a soluble antigen (SA) preparation of R. salmoninarum.

Initial studies indicated that the substrate for the protease(s) was soluble antigen itself. In an attempt to identify other more defined substrates, soluble antigen was incubated with molecular weight markers used in SDS-PAGE analysis. This facilitated the search by allowing a number of proteins to be studied in a single digestion. It was revealed that phosphorylase B, BSA and soybean trypsin inhibitor were susceptible to the proteolytic activity of the soluble antigen. It must be cautioned, however, that commercial kits of molecular weight markers incorporate glycerol along with a denaturing agent such as dithiothriitol. This is of importance since if the substrate assay is performed with native protein substrates, no proteolysis will

be evident (data not shown). This indicates that these particular substrates must be in at least a partially denatured condition in order to facilitate the proteolysis.

The protease activity was inhibited by PMSF, alpha-2 M, TPCK and in concentrations of 50% ethanol and methanol, while the inhibitors such as zinc sulfate, EDTA did not inhibit the proteolysis (data not shown). These studies demonstrate that the protease associated with soluble antigen may be a serine protease and possibly chymotrypsin-like protease. Other studies in our laboratory have indicated that the protease may have molecular weight of $> 200,000$ d (Rockey, D.D. and Kaattari, S.L., manuscript in preparation).

Examination of the protein blots indicated that the protease activity has pronounced effect on the 57 kd protein (Figs. 3.1 and 3.2). Most of the 57 kd protein was degraded within two hours of incubation of SA at 37°C and was almost completely digested by 12 hours of incubation. The protease activity associated with SA indicates that its storage conditions should have a dramatic influence in its antigenic quality. Thus, assays which call for the incubation of SA at 37°C (i.e., ELISA) may result in a significant loss of antigen.

We have previously demonstrated that soluble antigen suppressed in vitro antibody responses to TNP-LPS as assessed by the plaque forming cell (PFC) assay (Turaga et al., 1987b). It is of interest to note that this suppressive effect was almost completely neutralized when the preparation of SA that was pre-incubated at 37°C for 24 hours (Fig. 3.6). The kinetics of the

degradation of the 57 kd protein band paralleled the progressive decrease in suppression by the soluble antigen (Fig. 3.6). This may indicate that the 57 kd protein is responsible for the in vitro toxicity of the soluble antigen.

Daly and Stevenson (1987) have proposed that the 57 kd protein may be a hemagglutinin for rabbit erythrocytes, and Getchell et al. (1985) have shown that the 57 kd protein is the major, common surface antigen of R. salmoninarum. among seven different isolates of the pathogen. Other studies conducted in our laboratory have demonstrated that incubation at 37°C also neutralized another biologic activity of SA, namely a leukocoagglutinating activity (Wiens and Kaattari, manuscript in preparation).

ACKNOWLEDGEMENTS

This work was supported by Bonneville Power Administration contract DEAI79-87BP16480.

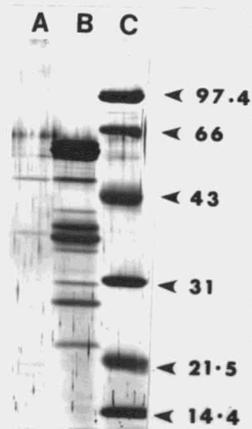


Fig. 3.1. SDS-PAGE analysis of soluble antigen (5 μ g) incubated overnight at different temperatures. Lane A (37°C), lane B (4°C) and lane C (mol. wt. markers). The nitrocellulose paper was stained for protein with Aurodyer^R forte.

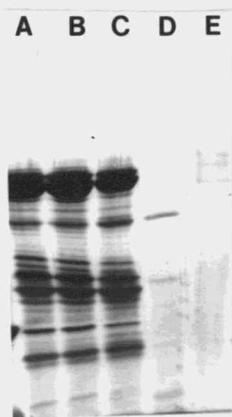


Fig. 3.2. SDS-PAGE analysis of soluble antigen (5 μ g) incubated overnight at different temperatures: Lane A (4°C), lane B (17°C), lane C (23°C), lane D (37°C) and lane E (56°C). The nitrocellulose paper was stained for protein with Aurodye^R forte.

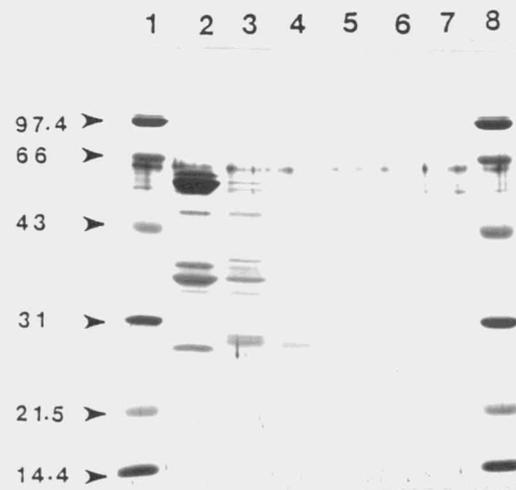
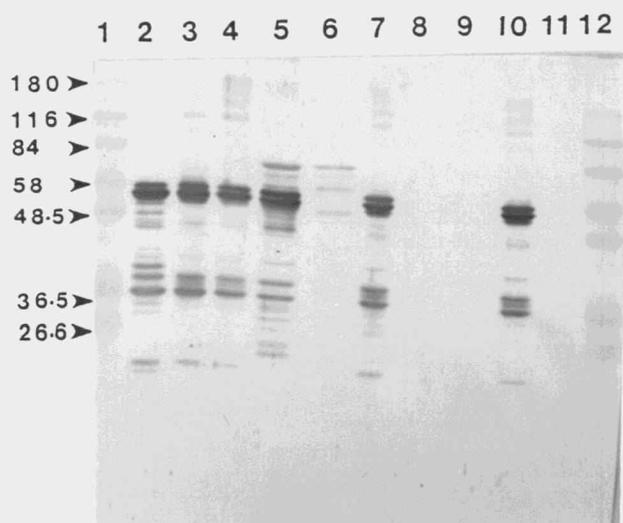
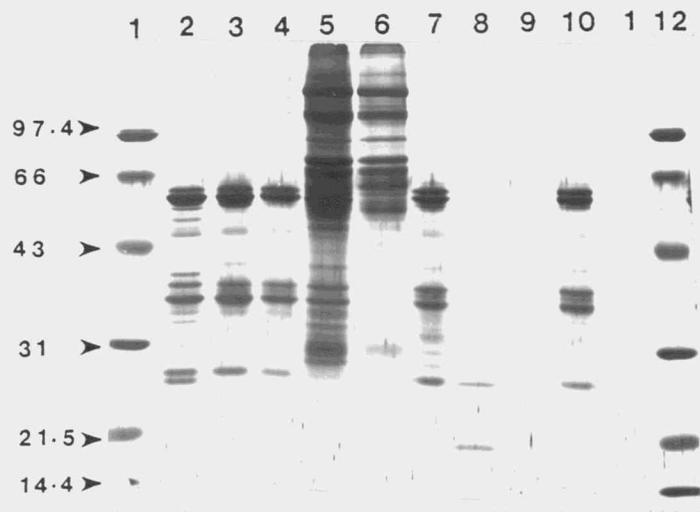


Fig. 3.3. SDS-PAGE analysis of soluble antigen incubated for various lengths of time at 37°C. Each lane was loaded with 5 μ g of SA. lane 1: mol. wt. markers, lane 2: 0 hr, lane 3: 2 hr, lane 4: 4 hr, lane 5: 8 hr, lane 6: 12 hr and lane 7: 24 hr incubation. The nitrocellulose paper was stained for protein by Aurodyer^R forte.

Fig. 3.4. Effect of protease inhibitors on SA protease activity. SDS-PAGE analysis of preparations of SA with PMSF, TPCK, alpha-2 macroglobulin and 50% concentration of ethanol and methanol and the diluent controls including alcohols and PBS. A. protein staining of the nitrocellulose paper with AurodyerR forte and B. Western blotting with rabbit-anti-SA.

Fig. 3.4. Effect of protease inhibitors on SA protease



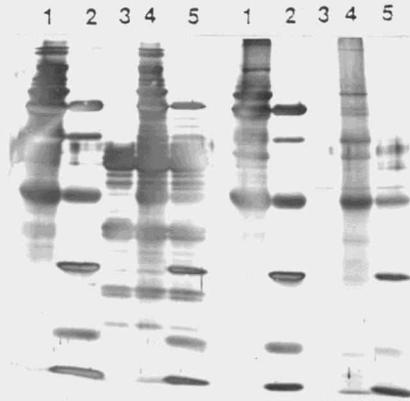


Fig. 3.5. Sustrate characterization of the protease. Protein staining of SDS-PAGE of samples containing only high low molecular weight markers (HMW-1) or low molecular weight markers (LMW-2) or soluble antigen (KDSA-3) or combinations thereof (1+3; 2+3). A. the samples at 0 hr and B. the samples incubated at 37°C overnight.

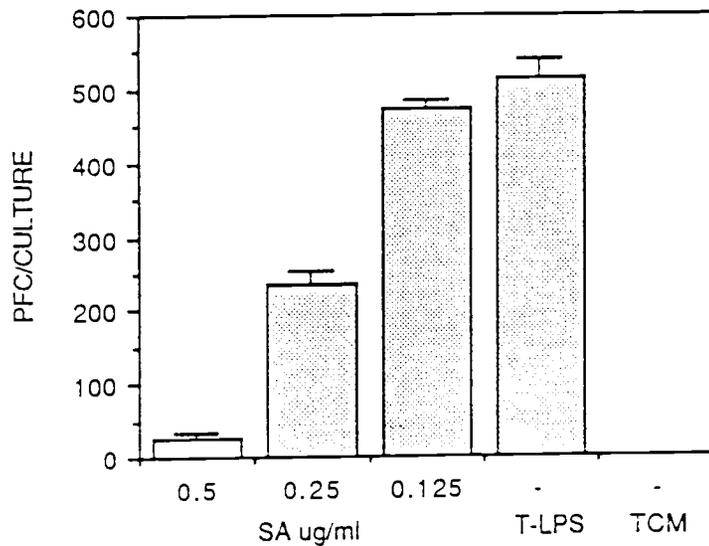


Fig. 3.6. Effect of soluble antigen on the plaque-forming cell response to TNP-LPS. Anterior kidney leukocytes were either co-cultured with TNP-LPS in the presence of various concentrations of soluble antigen (125, 62.5 and 31.25 $\mu\text{g/ml}$). A control consisting of leukocytes in tissue culture medium (TCM) only is depicted. Each histogram represents the mean of 4 cultures and the error bar represents one S.E.

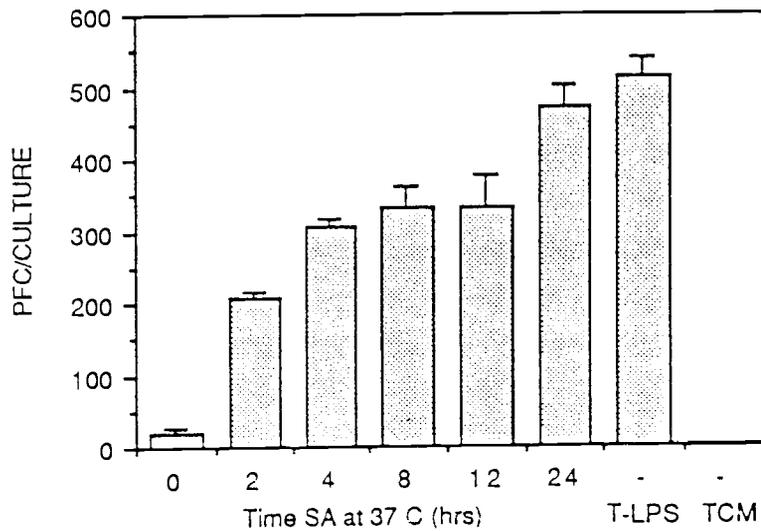


Fig. 3.7. The effect of preincubated soluble antigen on the *in vitro* PFC assay. Soluble antigen was preincubated for 0, 2, 4, 8, 12 and 24 hours at 37°C before co-culture with anterior kidney leukocytes. The bar graphs shows the number of PFCs/culture when the soluble antigen preparations were co-cultured with TNP-LPS, leukocytes cultured with TNP-LPS alone and TCM alone. Each histogram represents the mean of 4 cultures and the error bar represents one S.E.

REFERENCES

- Bruno, D. W. 1988. The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen Renibacterium salmoninarum. FEMS Microbiology Letters. 51: 135-140.
- Bruno, D. W., and A. L. S. Munro. 1986. Uniformity in the biochemical properties of Renibacterium salmoninarum isolates obtained from several sources. FEMS Microbiology Letters. 33: 247-250.
- Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology. 14: 599.
- Daly, J. G., and R. M. W. Stevenson. 1987. Hydrophobic and haemagglutinating properties of Renibacterium salmoninarum. J. Gen Micro. 133: 3575-3580.
- Evelyn, T. P. T. 1977. An improved growth medium for the Kidney disease bacterium and some notes on using the medium. Bull. Off. Int. Epiz. 87: 511-513.
- Fiedler, F., and R. Draxl. 1986. Biochemical and immunochemical properties of the cell surface of Renibacterium salmoninarum J. Bact. 168: 799-804.
- Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. Microbiology. 35: 273-298.
- Getchell, R. G., Rohovec, J. S., and J. L. Fryer. 1985. Comparison of Renibacterium salmoninarum isolates by antigenic analysis. Fish Path. 20: 149-159.
- Jacobs, D. M., and D. C. Morrison. 1975. Stimulation of T-independent primary anti-hapten response *in vitro* by TNP-lipopolysaccharide (TNP-LPS). J. Immunol. 144: 360-364.
- Kaattari, S. L., and M. A. Yui. 1987. Polyclonal activation of salmonid B lymphocytes. Dev. Comp. Immun. 11: 155-165.

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

Phillips, H. J. 1973. Dye exclusion tests for cell viability. In *Tissue culture: Methods and applications*. Ed. Kruser, P. F., and M. K. Patterson, JR. Academic Press. p 406-408.

Rittenberg, M. B., and K. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* 132: 575-581.

Sanders, J. E., and J. L. Fryer. 1980. Renibacterium salmoninarum gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. *Int. J. Syst. Bact.* 30: 496-502.

Smith, I. W. 1964. The occurrence and pathology of Dee Disease. *Freshwater Salmon Fish. Res.* 34: 1-8.

Tittle, R. V., and M. B. Rittenberg. 1978. Expression of IgG memory response in vitro to thymus-dependent and thymus-independent antigens. *Cell. Immunol.* 35: 180

Towbin, H., Staehelin, T., and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350-4354.

Turaga, P. S. D., Wiens, G. D., and S. L. Kaattari. 1987a. Analysis of Renibacterium salmoninarum antigen production in situ. *Fish Path.* 22: 209-214.

Turaga, P. S. D., Wiens, G. D., and S. L. Kaattari. 1987b. Bacterial kidney disease: the potential role of soluble protein antigen(s). *J. Fish Biol.* 31: 191-194.

4. ANALYSIS OF RENIBACTERIUM SALMONINARUM ANTIGEN
PRODUCTION IN SITU

PRASAD S.D. TURAGA, GREGORY D. WIENS,
and STEPHEN L. KAATTARI

Department of Microbiology
Oregon State University
Corvallis, OR - 97331

Published in: Fish Pathology, volume 20: 209-214

ABSTRACT

This study represents the first report of the development and use of an enzyme-linked immunosorbent assay (ELISA) and western blot analysis to monitor the production of Renibacterium salmoninarum soluble antigens (SA) in infected salmon. The sensitivity of the ELISA permitted detection of soluble antigens in sera samples to concentrations of 0.1 µg/ml. This assay demonstrates a clear resolution between infected and non-infected fish in experimental infections. The ELISA and western blot systems were used to assess the temporal progress of the disease in a quantitative and qualitative manner.

INTRODUCTION

Bacterial kidney disease (BKD) is one of the most widespread and devastating salmonid diseases in North America, Europe and Asia (Fryer and Sanders, 1981). The causative agent of this disease, Renibacterium salmoninarum, is a gram-positive, pleomorphic, diplobacillus which has been classified as a unique species as well as genus (Sanders and Fryer, 1980).

Furthermore, this disease is not only a serious problem for hatchery reared-salmonids, but it has also been demonstrated to occur in wild populations (Mitchum, et al., 1979). Currently, no commercial vaccine exists for BKD, and diagnostic procedures are primarily limited to detection using gram stain (Pippy, 1969) or fluorescent antibody techniques (Bullock and Stuckey, 1975; Laidler, 1980).

An alternate means that may be used to follow the progress of this disease would be the monitoring of soluble antigens, secreted by R. salmoninarum in the host. Previous studies have identified specific molecular weight antigens produced in broth culture (Getchell et al., 1985), and we have now found that these specific molecular weight antigens are also produced in infected salmon. ELISA and western blot techniques have been employed here to quantitatively and qualitatively monitor the serum levels of the soluble antigen (SA) during the disease process. This form of in situ antigen analysis can facilitate our understanding of the pathogenic process of BKD and could be adapted for use in diagnostic procedures.

MATERIALS AND METHODS

Animals

Coho salmon (Oncorhynchus kisutch), aged 6-12 months, were maintained at the Oregon State University Fish Disease

Laboratory. The fish were held in 12°C, pathogen-free well water and fed Oregon Moist Pellet.

Adult, female New Zealand white (NZW) rabbits were maintained by the Laboratory Animal Resource Center at Oregon State University.

Bacterial strain

R. salmoninarum, isolate Lea-1-74 (ATCC 33209), was obtained from Dr. J.S. ROHOVEC, Dept. of Microbiology, Oregon State University and used throughout this study. The bacteria were grown in a modified Kidney Disease Medium (M-KDM-2) without ferric sulphate supplementation as described by Getchell et al. (1985).

Fish were injected, intraperitoneally, with 0.1 ml of a 1.0 O.D. (500 nm) broth culture in the exponential phase of growth.

Preparation of soluble antigen

SA of R. salmoninarum were prepared from the supernatants of spent cultures. After growth of the organisms to an O.D. of approximately 1.0 (500 nm), the cells were removed by centrifugation at 6000 x g for 30 min at 4°C. The supernatant fluid was filtered through a "minitan" ultrafiltration apparatus with a PTGC-10,000 NMWL filter packet (Millipore, Bedford, MA), held at 4°C. This filtration removed low molecular weight molecules (<10 kd), and reduced the volume 10-fold. The

retentate, or high molecular weight fraction, was concentrated by 50% saturated ammonium sulfate (SAS) precipitation. This mixture was stirred for 3-4 h at 4°C, and the precipitate was retrieved by centrifugation at 6000 x g for 15 min at 4°C. This precipitate was dissolved in a volume of 0.01 M, phosphate buffered saline (PBS), pH 7.2, equivalent to 10% the original retentate volume. The solution was then reprecipitated twice as described above, and dialysed extensively against PBS at 4°C. The solution was then filter sterilized (0.45 µm) and the protein concentration was measured by the method of Lowry et al. (1951).

Antibody preparation

The anti-SA serum was prepared by injecting a 1:1 mixture of SA (1 mg/ml PBS) and Freund's complete adjuvant (Difco, Detroit, MI, USA) subcutaneously into rabbits, between the scapulae (1.4 ml) and in the hind footpads (0.4 ml each). The rabbits were allowed to rest for 30 days, then bled weekly. Seven weeks post-immunization, the rabbits were boosted and subsequently bled using the same protocol. The serum was aliquoted and stored at -70°C.

Biotinylation of antibody

Immunoglobulin was isolated from an aliquot (1-2 ml) of the anti-SA serum by 50% saturated ammonium sulfate

precipitation. The pellet was resuspended in 0.01 M PBS and extensively dialysed against the same buffer at 4°C. The protein content was estimated by the method of Lowry et al. (1951). Biotinylation of this antibody fraction was performed as described by Kendall et al. (1983). Biotin-N-hydroxysuccinimide (BNHS) (Calbiochem, La Jolla, CA, USA) was used to covalently bind biotin to the antibody. The reaction mixture was prepared by adding 1 ml of a 10 mg/ml solution of antibody in 0.1 M NaHCO₃ to 57 µl of a 0.1 M solution of BNHS dissolved in distilled dimethyl formamide (Sigma, St. Louis, MO, USA). After incubation for one hour at 22°C, the reaction mixture was dialysed for 24 h at 4°C against three changes of PBS. After dialysis an equal volume of glycerol was added and the biotinylated antibody was stored at -20°C.

ELISA procedure

The detection of SA in the serum of infected fish was accomplished utilizing an antibody capture technique. The method and buffers are modifications of that described by Voller et al. (1976). Individual wells of a 96-well EIA flat-bottom plate (Costar, Cambridge, MA, USA) were coated with 0.1 ml of unconjugated anti-SA antibody (5 µg/ml in carbonate-bicarbonate coating buffer, pH 9.6) and incubated overnight at 17°C. Following three rinses of the plates with 0.1% Tween-20 in tris-buffered saline, pH 8.2, (TTBS) and three rinses with tris-buffered saline (TBS) alone, the well surfaces were then blocked

by incubation with 3% bovine serum albumin (BSA) in TBS. At this point all incubations were performed at room temperature in a humid chamber and the same wash procedure followed each incubation. After rinsing, 100 μ l of test serum dilutions were incubated, in parallel, with standards consisting of 5, 1, 0.5, 0.125, 0.1 μ g/ml SA in a diluent consisting of normal salmon serum diluted 1/20 in TTBS. The samples were incubated for one hour at room temperature. The plates were then washed and 100 μ l of the appropriate dilution of the biotinylated antibody in 1% BSA in TBS was added to each well and allowed to incubate for another hour. After rinsing, 100 μ l of an appropriate concentration streptavidin-horseradish peroxidase (S-HRPO) (Sigma) was incubated in each well for 30 min. After the last rinse, 100 μ l of substrate solution was added and color development was monitored spectrophotometrically at 405 nm on an EIA autoreader (Model EL 310, Biotek Instruments, Burlington, VT, USA). The substrate solution was a mixture of : 10 ml of citrate buffer (pH 4.0), 10 μ l of hydrogen peroxide and 75 μ l of a 10 mg/ml solution 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) in distilled water. A standard curve was then plotted and used to determine the concentration of SA in the test sera (Fig. 1).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Serum samples from infected and normal coho salmon were stored at -20°C . Immediately prior to use, samples were mixed 1:1 in sample buffer (120 mM tris base, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% w/v glycerol, and 3mM bromophenol blue), then placed in a boiling water bath for 2-3 min. Samples were then immediately run on 83x103 mm mini-slab gels (Idea Scientific, Corvallis, OR, USA) under conditions similar to those of Laemmli (1970), using a 12% acrylamide separating gel and a 3.7% acrylamide stacking gel, for approximately 1.5 hours with a constant current (20 mAmps).

Western blotting

Protein transfer from gel to nitrocellulose paper (Bio-Rad) was performed according to a modified method of Towbin et al. (1979). Briefly, proteins were transferred by overnight electrophoresis at 30 V and 4°C in a transblot apparatus (Bio-Rad). The nitrocellulose was blocked for one hour with 3% BSA in TBS at 37°C . The nitrocellulose bound kproteins were probed with biotinylated rabbit anti-SA diluted 1/1000 in 0.1% tween-TBS for one hour. After a 30 min wash in 0.1% tween-TBS, a 1/200 dilution of S-HRPO in 0.1% tween-TBS was applied for 25 min. Visualization of the protein bands, was achieved using a substrate solution consisting of 2 ml of 4-chloro-naphthol (Bio-

Rad) (3 mg/ml in methanol stock), 10 ml of 0.1 M phosphate buffered saline and 10 μ l of hydrogen peroxide incubated for 15-30 min. Blots were rinsed in double distilled water for approximately 3 min and dried.

RESULTS

Quantitative Analysis of Soluble Antigen (SA) Production in Experimental Infections of Salmon

Quantitative assessment of soluble antigen levels in the serum of normal and infected salmon was accomplished by using the ELISA to compare dilutions of various test sera with a standard curve (Fig. 4.1). As can be seen in figure 1 the sensitivity of our assay is such that concentrations as low as 0.1 μ g SA/ml of serum can be detected.

To determine if the progress of this disease could be monitored by quantification of antigen levels using the ELISA, salmon were injected with a standard concentration of live R. salmoninarum and the sera from these fish were tested at five day intervals. Fig. 4.2. demonstrates a gradual increase in SA over the course of the disease. Mortalities routinely occurred between day 20 and 40 with this form of experimental infection.

Western Blot Analysis of SA Production in Experimentally Infected Fish

Serum samples from the experimentally infected fish (described above) were also qualitatively analysed by western blotting (Fig. 4.3). Three distinct observations were made using this form of analysis : 1) crossreactive antigens were found in control salmon serum that reacted with the rabbit anti-SA. However, these antigens are readily distinguishable from the SA on the basis of molecular weight. 2) Four major SA bands were identified; I=70 kd, II=60 kd, III=33-37 kd, IV=26 kd (Fig. 4.3, lane G). A 95 kd antigen appears in infected serum (Fig. 4.3, lanes B-E) which is not found in purified SA preparation (Fig. 4.3, lane G). 3) A gradual increase in band density of major SA (I, II, III) was observed over the time of the infection. The most prominent band was antigen II.

DISCUSSION

These studies demonstrate the value of an antibody capture ELISA based on the detection of soluble antigens secreted by Renibacterium salmoninarum. This assay was used to trace the progress of experimental BKD infections by quantifying the increase of antigen in the serum (Fig. 4.2). Furthermore, utilization of western blot analysis for the detection of R. salmoninarum antigens in infected animals demonstrated a high degree of specificity which easily resolved

many antigens of R. salmoninarum as well as false positive reactions.

Control of bacterial kidney disease is a problem of primary concern wherever salmon culture exists. This disease can easily become a chronic problem within a hatchery, since the bacteria are slow growing and infected animals may harbor the organism for long periods before manifesting gross pathology (Fryer and Sanders, 1981). Furthermore, infected brood stock may serve as a reservoir, infecting gametes and thus fry via vertical transmission (Evelyn et al., 1984). Unfortunately, control cannot be achieved through immunization at this time, since no commercial vaccine is available. The rapid diagnosis of this disease could aid tremendously in its control by the identification of infected brood stock or gametes, and their subsequent culling or treatment.

Diagnosis of this disease thus far, however, has primarily been through the identification of organisms by the Gram stain (Pippy, 1969), fluorescent antibody techniques (Bullock and Stuckey, 1975; Laidler, 1980) and coagglutination (Kimura and Yoshimizu, 1981; Yoshimizu and Kimura, 1985; Cipriano et al., 1985). Pascho and Mulcahy (1987) have developed a procedure to detect a heat-stable soluble antigen of R. salmoninarum using an ELISA, but as yet this technique has not been applied to the analysis of infected fish.

ELISAs, such as described here and by Pascho and Mulcahy (1987), are not capable of distinguishing between the various R. salmoninarum antigens that may be present, nor can false positive reactions be identified. Therefore, use of a western blot assay, as we describe, is extremely useful in resolution of these potential shortcomings. The western blot (Fig. 4.3) demonstrates at least four different R. salmoninarum antigens with molecular weights of 70 kd (I), 60 kd (II), 33-37 kd (III), and 26 kd (IV). Control salmon serum reveals two cross reactive antigens (lane I), which are easily distinguished from the soluble antigens (lane G). Therefore, R. salmoninarum specific antigens can be distinguished from the cross-reactive antigens.

During the course of the infection an increase in the soluble antigens are not only seen in the ELISA (Fig. 4.2) but also in the western blot (Fig. 4.3). Antigen II was detectable in the serum from day 5, while I and III became detectable at later times. Antigen IV, however, did not appear in the serum. Other investigators have identified soluble antigens in infected fish samples by alternate means of detection such as coagglutination (Kimura and Yoshimizu, 1981; Yoshimizu and Kimura, 1985), counterimmunoelectrophoresis (Cipriano et al., 1985) and peroxidase-antiperoxidase systems (Sakai et al., 1987). However, these previous studies relied on antiserum directed toward whole R. salmoninarum cells. This investigation was conducted with antibodies specifically directed to the soluble products released by R. salmoninarum, which reacted only with distinct molecular weight species (Fig. 4.3).

Investigators have previously identified R. salmoninarum antigens of distinct molecular weights. Getchell, et al. (1985) described an antigen F (57 kd) as a heat stable major surface antigen, common to several isolates of R. salmoninarum. Fiedler and Draxl (1986), however, have reported the major surface antigen has a molecular weight of 70 kd. At present, the biochemical relationship of the soluble antigens reported here to other reported antigens is the object of further analysis.

ACKNOWLEDGEMENTS

This work was supported by Bonneville Power Administration contract DE-AI79-87BP16480. The authors thank Dr. J.L. FRYER, DR. J.S. ROHOVEC, M. ARKOOSH, R. TRIPP, and J. DEKONING for their critical review of the manuscript, and Ms. G. TAJWALL for her typing of the manuscript. Oregon Experiment Station Technical Paper Number 8230.

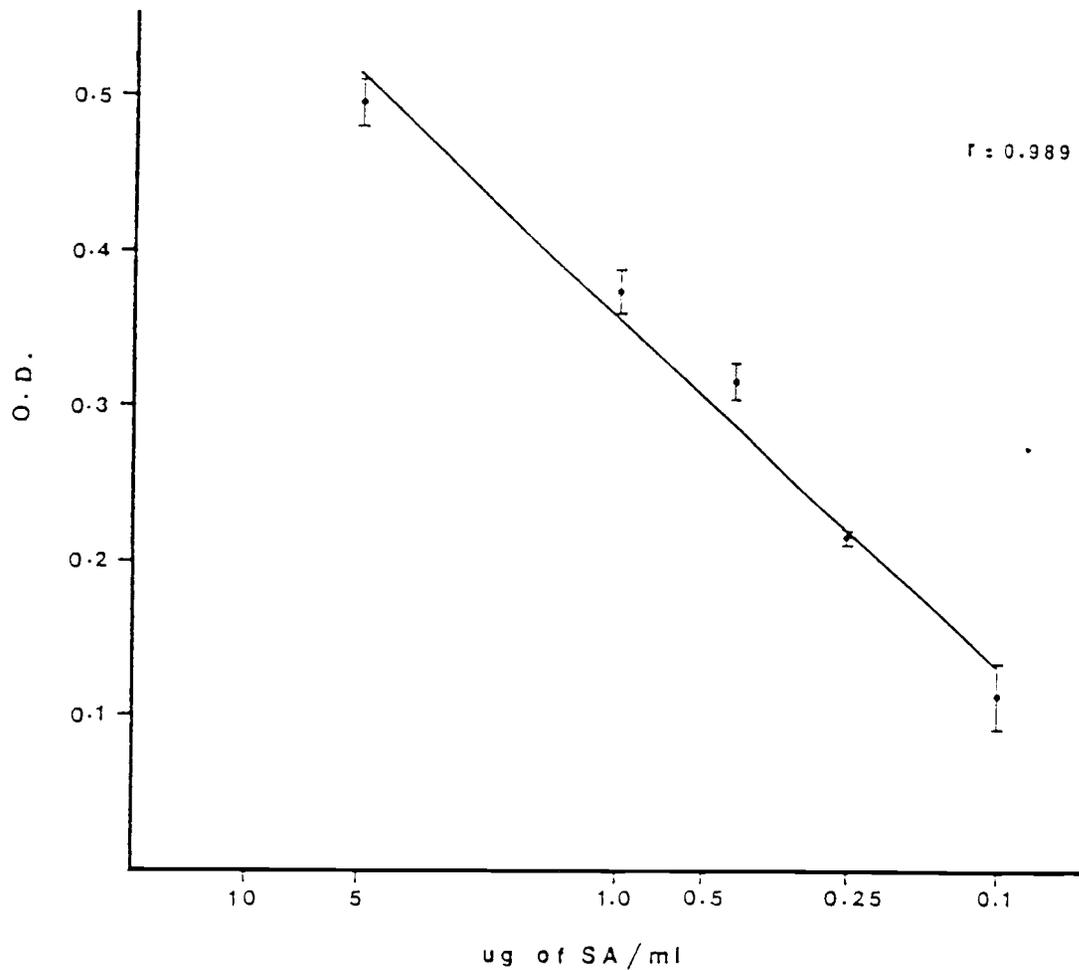


Fig 4.1. ELISA standard curve: an antibody capture method was employed by coating rabbit anti - R. salmoninarum antibody onto the wells of an ELISA plate. Soluble antigen dilutions were made in normal, non-infected control serum to produce concentrations of 5, 1, 0.5, 0.125, 0.1 $\mu\text{g/ml}$ of serum. Each point represents an average of triplicates \pm one standard error.

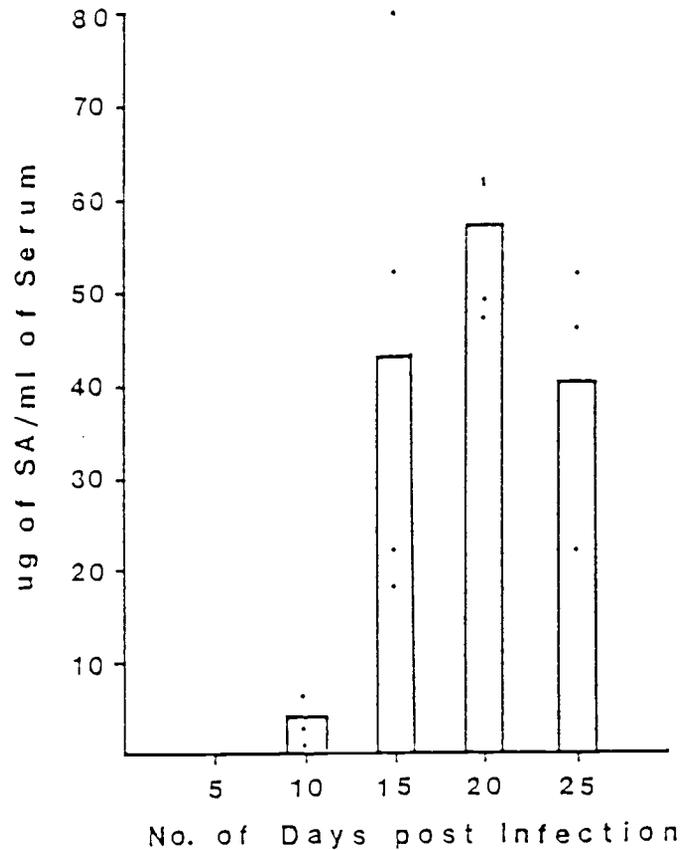


Fig. 4.2. Quantification of soluble antigen during experimental infection. Each point represents the value of the soluble antigen concentration in the serum of one infected animal, sacrificed on day 5, 10, 15, 20, or 25 following injection of live R. salmoninarum on day 0. Each histogram represents the mean of all the points at that concentration.

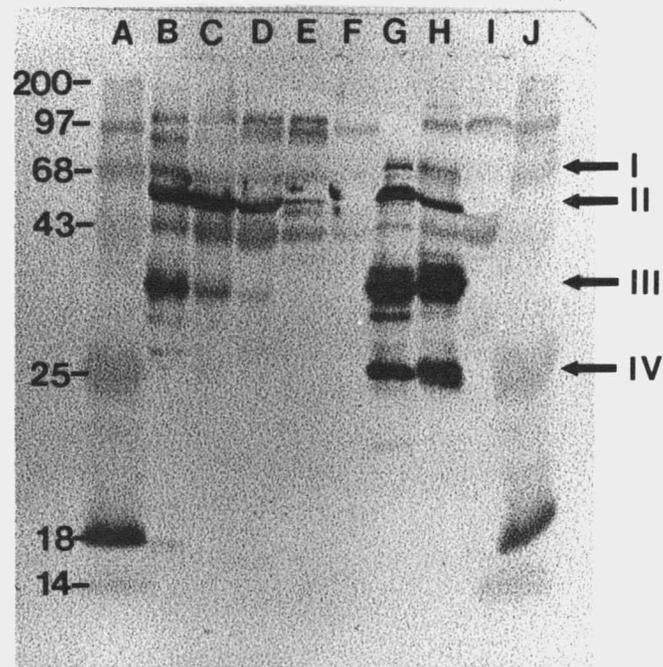


Fig. 4.3 Western blot of infected fish sera. Protein concentration in the lanes are as follows; A) Prestained molecular weight markers (9 μ l), B) Day 25 infected coho sera (1.5 μ l), C) Day 20 infected coho sera (1.5 μ l), D) Day 15 infected coho sera (1.5 μ l), F) Day 5 infected coho sera (1.5 μ l), G) BKD soluble antigen 1.25 μ g, I) Control coho sera, 1.5 μ l, J) Prestained molecular weight markers (Bethesda Research Laboratories, Bethesda, MD, USA), 9 μ l. Arrows indicate the four major BKD soluble antigens.

REFERENCES

- Bullock, G.L., and H.M. Stuckey. 1975. Fluorescent antibody identification and detection of the Corynebacterium causing kidney disease of salmonids. J. Fish. Res. Board Can., 32: 2224-2227.
- Cipriano, R. C., Starliper, C. E., and J. H. Schachte. 1985. Comparative sensitivities of diagnostic procedures used to detect bacterial kidney disease in salmonid fishes. J. Wildlife Dis. 21: 144-148.
- Evelyn, T. P. T., Ketcheson, J. E., and L. Prosperi-Porta. 1984. Further evidence for the presence of Renibacterium salmoninarum in salmonid eggs and for the failure of povidone-iodine to reduce the intra-ovum infection rate in water-hardened eggs. J. Fish Dis. 7: 173-182.
- Fiedler, F., and R. Draxl. 1986. Biochemical and immunochemical properties of the cell surface of Renibacterium salmoninarum. J. Bacteriol. 168(2): 799-804.
- Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. Microbiol. 35: 273-298.
- Getchell, R. G., Rohovec, J. S., and J. L. Fryer. 1985. Comparison of Renibacterium salmoninarum isolates by antigenic analysis. Fish Pathology. 20(2/3): 149-159.
- Kendall, C., Ionesca-Matiu, I., and R. G. Dressman 1983. Utilization of the Biotin/Avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). J. Imm. Methods 56: 329-339.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
- Laidler, L. A. 1980. Detection and identification of the bacterial kidney disease (BKD) organism by the indirect fluorescent antibody technique. J. Fish Diseases. 3: 67-69.

Lowry, O. H., Rosebrough, N. J., Farr, D. L., and R. J. Randall 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 205-276.

Mitchum, D. L., Sherman, L. E., and G. T. Baxter. 1979. Bacterial kidney disease in feral populations of brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*) and rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.*, 36, 1370-1376.

Pascho, R. J., and D. Mulcahy. 1987. Enzyme-linked immunosorbent assay for a soluble antigen of *Renibacterium salmoninarum*, the causative agent of salmonid bacterial kidney disease. *Can. J. Fish. Aquat. Sci.* 44: 183-191.

Pippy, J. H. C. 1969. Kidney disease in juvenile Atlantic salmon (*Salmo salar*) in the Mangaru River. *J. Fish. Res. Board Can.* 26: 2535-2537.

Sakai, M., Koyama, G., and S. Atsuta. 1987. Detection of *Renibacterium salmoninarum* by a modified peroxidase-antiperoxidase (PAP) procedure. *Fish Pathology.* 22: 1-5.

Sanders, J. E., and J. L. Fryer 1980. *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease of salmonid fish. *Inter. J. Syst. Bacteriol.* 30: 496-502.

Towbin, H., Staehelin, T., and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76: 4350-4354.

Voller, A., Bidwell, D. E., and A. Bentlett. 1976. Enzyme immunology in diagnostic medicine (Theory and Practice). *Bull. World Health Organ.* 53: 55-65.

Yoshimizu, M., and T. Kimura. 1985. A coagglutination test with antibody sensitized staphylococci for rapid and simple diagnosis of bacterial and viral diseases in fish. *Fish Pathology.* 20: 243-261.

5. BACTERIAL KIDNEY DISEASE : THE POTENTIAL ROLE OF
SOLUBLE PROTEIN ANTIGEN(S)

PRASAD S.D. TURAGA, GREGORY D. WIENS,
and STEPHEN L. KAATTARI

Department of Microbiology
Oregon State University
Corvallis, OR - 97331

Published in: Journal of Fish Biology,
volume 31 (supplement A): 191-194.

ABSTRACT

Soluble protein antigens, isolated from the supernatants of Renibacterium salmoninarum cultures, have been found to suppress the in vitro antibody responses of coho salmon, Oncorhynchus kisutch, lymphocytes and to be associated with decreasing haematocrit values in vivo.

INTRODUCTION

Renibacterium salmoninarum is responsible for one of the most devastating salmonid bacterial diseases in the world (Fryer & Sanders, 1981), bacterial kidney disease (BKD). Of major epidemiologic importance is the fact that this disease is not only a big problem in hatchery-reared salmonids, but also in wild populations (Mitchum et al., 1979).

Unfortunately, little information has been forthcoming as to the possible molecular mechanisms of the pathogenesis of BKD. This study, therefore, focuses on the possible toxigenic role(s) that R. salmoninarum soluble proteins (SP) may have in BKD pathology.

MATERIALS AND METHODS

Renibacterium salmoninarum soluble antigens were prepared as described by Getchell et al. (1985). The effects of SP on the in vitro antibody responses of normal and infected coho salmon to trinitrophenylated-lipopolysaccharide (TNP-LPS; Jacobs & Morrison, 1975) were examined using in vitro tissue culture medium (TCM) and techniques (Kaattari & Yui, 1987). Prior to culture addition, SP was diluted in tissue culture medium and filter sterilized (0.45 μm). Haematocrit values were assessed using heparinized blood samples taken from 5 fish at 10 intervals after intraperitoneal injection of 0.1 ml of 1 O.D. (500 nm) live R. salmoninarum. The plasma portions of these samples were analyzed for the concentration of SP by use of an ELISA procedure (P. Turaga et al., in prep.).

RESULTS

In vitro cultures of anterior kidney lymphocytes, stimulated with an optimal concentration of TNP-LPS, were suppressed by 10 and 100 $\mu\text{g ml}^{-1}$ SP (Fig. 5.1). Comparable concentrations of the control protein, chicken ovalbumin, were not suppressive. This suppression was not due to a toxic effect, since control and suppressed cultures expressed equivalent cellular viability as assessed by trypan blue exclusion staining.

Anterior kidney lymphocytes from normal and infected fish were cultured with TNP-LPS (Fig. 5.2). Cultures of lymphocytes from infected fish (possessing 30-80 $\mu\text{g ml}^{-1}$ serum SP) demonstrated a marked suppression as compared to lymphocytes from normal fish. This suppression was comparable to the suppression seen when normal lymphocytes are co-cultured with 100 $\mu\text{g ml}^{-1}$ SP.

Examination of experimentally infected salmon at various times post-injection revealed a distinct association of decreasing haematocrit with increasing levels of SP antigen in the serum (Fig. 5.3).

DISCUSSION

These studies demonstrated that soluble antigens produced by R. salmoninarum are capable of suppressing the in vitro antibody response, and are associated in vivo with decreasing haematocrit values.

Studies concerning the mechanisms of pathogenesis for R. salmoninarum have primarily been limited to the analysis of the histopathology (Wood & Yasutake, 1956; Hendricks & Leek, 1975; Lester & Budd, 1979; Young & Chapman, 1978) and the appearance of abnormal clinical indices (Hunn, 1964). Although the initial focus of the infection appears to be the kidney (Wood & Yasutake, 1956) with subsequent haematological dysfunction

(Hunn, 1964), the disease eventually becomes systemic, with lesions occurring in many organs and tissues.

The identification of R. salmoninarum toxins has not been forthcoming, except for the detection of an haemolysin-like activity found in formalinized cells (Bruno & Munro, 1986). Those authors have suggested that a putative toxin may be responsible for the decreases in haematocrit values and for indices of splenomegaly associated with the disease. Our observations of increasing serum SP levels associated with decreasing haematocrits lend support for that pathogenic mechanism.

The in vitro antibody assay revealed that a non-cytotoxic antigen(s) was capable of suppressing the production of the antibody response. Of particular interest were the observations in the present study of a decrease in the number of adherent (e.g. macrophage) cells upon culture with SP. Recent studies with catfish (Miller et al., 1985) and with coho salmon lymphocytes (Tripp & Kaattari, in prep.) reveal that antibody responses to TNP-LPS require adherent cell function. Since antibody responses from lymphocytes from infected fish appear suppressed (Fig.2), it may be possible that immune dysfunction in vivo could be mediated by the elaboration of these antigens. Further studies, however, will be required to determine the role of alternative in vivo mechanisms (e.g. bacterial destruction of tissues, or contamination due to live R. salmoninarum) involved in the reduction of antibody-producing cells from infected fish.

ACKNOWLEDGEMENTS

This research was supported by funds derived from Bonneville Power Administration contract number DE-AI-87BP16480. The authors thank Ms G. Tajwall for typing the manuscript.

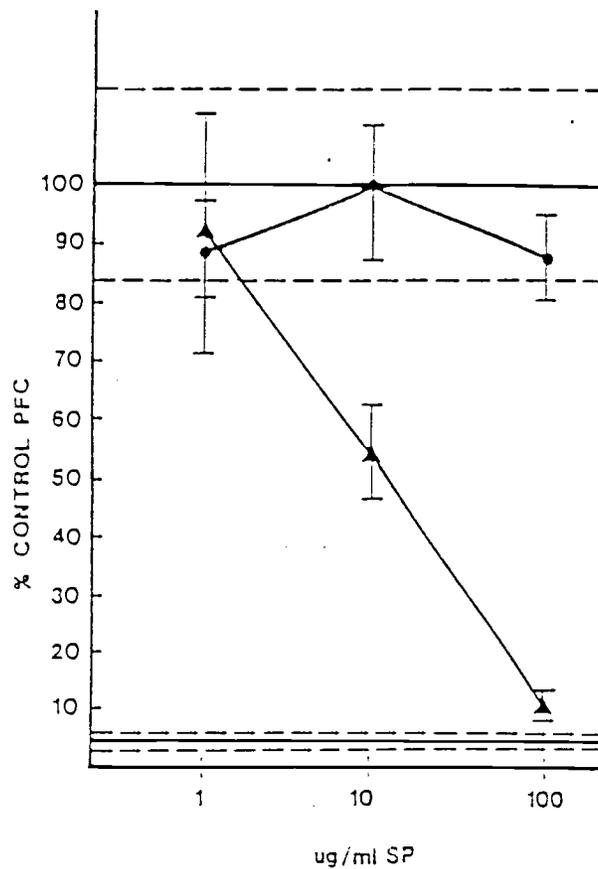


Fig. 5.1. Lymphocytes from anterior kidney were cultured with TNP-LPS in the presence of 1, 10, 100 ug SP () or ovalbumin (). The control response (without protein addition) was equal to 431 ± 21 antibody-producing cells per 10^6 lymphocytes. Each point represents the mean of triplicate cultures and bars ± 1 S.E.

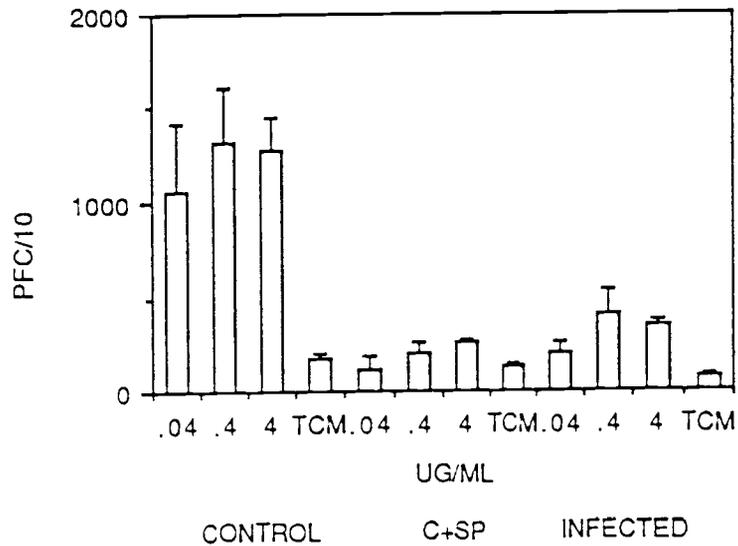


Fig. 5. 2. Lymphocytes from the anterior kidneys of normal and infected salmon were cultured with 0.04, 0.4, and 4.0 $\mu\text{g ml}^{-1}$ of TNP-LPS concentrations. A portion of the normal lymphocytes were also cultured with 100 $\mu\text{g ml}^{-1}$ SP (a concentration comparable to that seen in infected fish). Each histogram represents the mean of triplicate cultures and + 1 S.E. are indicated.

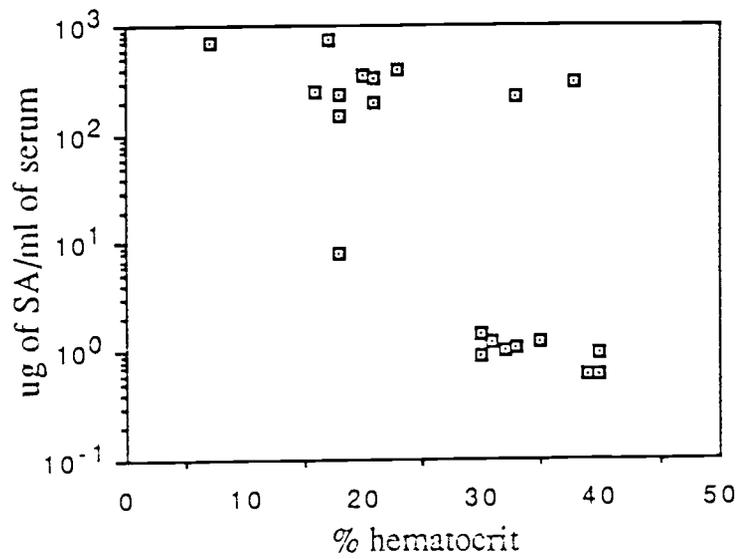


Fig. 5. 3. Haematocrit values, expressed as percentage packed red cell volume, plotted versus the corresponding SP serum concentration for individuals sampled at various stages of infection.

REFERENCES

- Bruno, D.W. & Munro, A.L.S. 1986. Haematological assessment of rainbow trout, Salmo gairdneri Richardson and Atlantic salmon, Salmo salar L., infected with Renibacterium salmoninarum. J. Fish Dis. 9, 195-204.
- Fryer, J.L. & Sanders, J.E. 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. Microbiol. 35, 273 -298.
- Getchell, R.G., Rohovec, J.S. & Fryer, J.L. 1985. Comparison of Renibacterium salmoninarum isolates by antigenic analysis. Fish Pathol. 20, 149 -159.
- Hendricks, J. & Leek, S.L. 1975. Kidney disease pastorbital lesions in spring chinook salmon (Oncorhynchus tshawytscha). Trans. Am. Fish. Soc. 104, 805-807.
- Hunn, J.B. 1964. Some patho-physiologic effects of bacterial kidney disease in brook trout. Proc. Soc. exp. Biol. Med. 117, 383-385.
- Jacobs, D.M. & Morrison, D. C. 1975. Stimulation of a T-independent primary anti-hapten response in vitro by TNP-lipopolysaccharide (TNP-LPS). J. Immun. 114, 360-364.
- Kaattari, S.L. & Yui, M.A. 1987. Polyclonal activation of salmonid B lymphocytes. Dev. Comp. Immun. 11, 155-165.
- Lester, R.J.G. & Budd, J. 1979. Same changes in the blood cells of diseased coho salmon. Can. J. Zool. 57, 1458-1464.
- Miller, N.W., Sizemore, R.G. & Clem, L.W. 1985. Phylogeny of lymphocyte heterogeneity: the cellular requirements for in vitro antibody responses of channel catfish leukocytes. J. Immun. 134, 2884-2888.
- Mitchum, D.L., Sherman, L.E. & Baxter, G.T. 1979. Bacterial kidney disease in feral populations of brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri). J. Fish. Res. Bd Can. 36, 1370-1376.

Wood, E.M. & Yasutake, W.T. 1956. Histopathology of kidney disease in fish. *Am. J. Path.* 32, 845-857.

Young, C.L. & Chapman, G.B. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). *J. Fish. Res. Bd. Can.* 35, 1234-1248.

6. PRESENCE OF SERUM IMMUNE COMPLEXES IN SALMON WITH
BACTERIAL KIDNEY DISEASE

PRASAD S.D. TURAGA¹, ALBERTO VILLENA²
and STEPHEN L. KAATTARI¹

¹Department of Microbiology
Oregon State University
Corvallis, OR., USA

and ²University of Leon, Leon, Spain

ABSTRACT

This is the first report which demonstrating the generation of soluble immune complexes (ICs) in fish infected with Renibacterium salmoninarum. An antibody capture ELISA method was employed to detect the presence of salmonid anti R. salmoninarum-soluble antigen immune complexes. Indirect, yet corroborative evidence of this phenomenon was demonstrated by immunohistochemical staining of kidney tissue from infected fish, for both antigen and antibody.

INTRODUCTION

Renibacterium salmoninarum, is the causative agent of bacterial kidney disease (BKD) in salmonid fish. The bacterium is a gram-positive, pleomorphic, diplobacillus (Sanders and Fryer, 1980), which can survive and multiply intracellularly within phagocytic cells (Young and Chapman, 1978). Bacterial kidney disease is one of the most devastating diseases in hatchery-

reared (Fryer and Sanders, 1981) and wild salmonid populations (Mitchum et al., 1979).

The disease produced is the result of a chronic, systemic and granulomatous infection. External signs of the disease are exophthalmia, a distended abdomen and pustules. Internally the disease is characterized by petechial hemorrhages, splenomegaly and a swollen kidney covered with a greyish white membrane (Smith, 1964). In advanced stages of the disease, the peritoneal cavity is filled with serous fluid and pustular lesions can be found on the kidney. In earlier studies, we have demonstrated that the bacterium elaborates large quantities of soluble antigen (SA), reaching concentrations of 1 mg/ml in serum (Turaga et al 1987a). It has been observed that in naturally infected fish, which present clinical signs of the disease, there usually were no detectable antibody titers (Banowetz, 1974). A possible explanation for this phenomenon may be that the quantity of SA produced in vivo is large enough to mask any antibody that is produced and thereby making it unavailable for subsequent detection. It is reasonable to assume that if such a situation were occurring, the resultant antigen-antibody complexes would be soluble and saturated with antigen.

An ELISA method is described which can detect the presence of antibody within immune complexes (ICs) formed to a specific antigen of R. salmoninarum. This study demonstrates the existence of such immune complexes in serum from infected fish.

MATERIALS AND METHODS

Animals

Fingerling coho salmon (50-100 g) were kept in ambient (12°C) pathogen-free well water in 800 liter circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL), Corvallis, Oregon. Experimental infections were performed using 100 liter tanks with the same water source. All the fish were fed Oregon Moist Pellet (OMP).

Growth of *Renibacterium salmoninarum*

Renibacterium salmoninarum was grown in modified KDM-2 medium (Evelyn, 1977) without serum supplementation. Cultures were grown in 250 ml shake flasks at 17°C until late log phase of the culture (about 10-12 days). The culture stock was then aliquoted in sterile snap cap tubes (Falcon) and stored at -70°C.

Preparation of Soluble Antigen of *Renibacterium salmoninarum*

Five hundred ml of the sterile KDM-2 medium in 2.8 L Erlenmeyer flasks were inoculated with 5% (v/v) of an inoculum which was in the late log phase of growth. The culture flask was incubated on a reciprocating shaker at 17°C for 10-14 days. As these cultures grew to late log phase they were then centrifuged

at 4000 x g for 30 min. The supernatant was removed and concentrated by passage through a PTGC 10000 NWML filter packet in a Minitan Ultrafiltration apparatus (Millipore Corp., Bedford, MA). Typically, ten-fold reductions of the original volume were achieved. This supernatant was further concentrated by ammonium sulphate (Sigma, MO) precipitation (313 g of ammonium sulphate/1000 ml of concentrated supernatant). The salt was added gradually over a period of one hour with constant stirring, while holding the supernatant in an ice bath. This solution was stirred for an additional 3 hr at 4°C. The precipitate was removed by centrifugation at 400 x g for 30 min at 4°C, and resuspended in 10 ml of PBS [pH 7.2, 0.01 M (7.5 g of NaCl, 0.245 g of KH₂PO₄ and 0.809 g of Na₂HPO₄ dissolved in one liter of distilled water)]. Using a saturated ammonium sulfate (SAS) solution, two additional 50% precipitations were performed. The final precipitate was then resuspended in 10 ml of PBS and dialyzed extensively at 4°C against 3 liters of PBS with three changes of the buffer over a period of 16 hr. The preparation was then filter sterilized using a 0.45 µm filter (Corning, Corning, NY). The protein content of the preparation was determined by the method of Lowry et al. (1951).

Artificial Infection

The D6 isolate of R. salmoninarum was grown in KDM-2 with 10% calf serum (Hyclone) in shake flasks at 17°C for about 10 days. The culture suspension was then adjusted to specific

concentration (1 O.D., 0.5 O.D. and 0.01 O.D. at 500 nm) with sterile PBS (pH 7.2, 0.01 M). One hundred μ l of this suspension was injected intraperitoneally into each fish anesthetized with benzocaine (Sigma) (10% stock in alcohol, 1 ml/4 liters of water). Five fish from each group, which had survived 7 weeks post-challenge, were sacrificed and their sera collected, pooled within the same group and stored at -20°C . These pools were designated as # 1 for the pool of sera from five fish from the group of fish injected with the 0.01 O.D. bacterial suspension, #2 for the 0.5 O.D. injected group and #3 for the 1.0 O.D. injected group.

Immune Complex Immunoassay

Soluble immune complexes (ICs) in the serum samples were detected using an antibody capture ELISA method (Fig 5.1). In this method, EIA plates were coated with 5 μ g/ml of a monoclonal antibody against the soluble antigen of R. salmoninarum, 4D3 (Wiens and Kaattari, 1989) in coating buffer and incubated at 17°C overnight. The plates were then washed with three rinses of 0.1% Tween-20 in tris-buffered saline, pH 8.2, (TTBS) and three rinses with tris-buffered saline (TBS) alone (6.07 g of Tris base, 0.3 g of EDTA and 8.7 g of NaCl dissolved in 1 liter of distilled water). The well surfaces were then blocked by incubation with 3% bovine serum albumin (BSA) in TTBS. The wells were washed as above and the test and control sera samples were added at a 1 : 4 dilution in T-TBS to the wells and

incubated for one hour at room temperature. After washing the wells biotinylated (Kaattari and Yui, 1987) anti-fish Ig antibody, 1-14 was added at an appropriate dilution in T-TBS to the wells and incubated for an additional hour. After washing the wells, horseradish peroxidase labeled streptavidin (Sigma, MO), suitably diluted in T-TBS was added to the wells and incubated for 30 min. The wells were washed and the substrate solution (100 μ l/well) was added and spectrophotometric readings at 405 nm on EIA autoreader (Model 310, Biotek Instruments, Burlington, VT, USA) were monitored. The substrate solution was a mixture of 10 ml of citrate buffer (pH 4.0), 10 μ l of hydrogen peroxide and 75 μ l of a 10 mg/ml solution 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) (Sigma, St. Louis, MO) in distilled water.

Preparation of Synthetic Immune Complexes

In order to test the efficacy of the ELISA to detect immune complexes, synthetic complexes were prepared in vitro. Soluble antigen (0.2 ml) diluted in PBS to concentrations of 2, 1, 0.5, 0.25 and 0.125 mg/ml were mixed with 0.2 ml of a 1/50 dilution of hyperimmune coho anti-SA in PBS. The tubes containing these mixtures were incubated for one hour at room temperature and then for 48 hr at 17°C. The tubes were then centrifuged at 2000 x g for 10 min. The supernatants were carefully removed and tested in the ELISA as described.

Immunoperoxidase Staining

The method followed was basically that of Ridley and Ridley (1986). Cryostat sections of pronephros and mesonephros were prepared (7-8 microns in thickness) from infected fish and normal controls and were allowed to dry for at least one hour at room temperature. The sections were fixed in acetone for five minutes and allowed to dry for half hour to one hour. The endogenous peroxidase was inhibited by incubation of the sections in methanol with 0.3% hydrogen peroxide for 10 minutes at room temperature. The sections were given three, five minute washes with phosphate buffered saline, pH 7.2, 0.01 M. An appropriate dilution of the primary antibody was prepared in PBS (either 3H1, an anti-SA monoclonal or 1-14, an anti-fish Ig monoclonal antibody), overlaid onto the sections and incubated for one hour at room temperature in a humid chamber. The sections were washed with PBS as described above. The secondary antibody (goat anti-mouse IgG-peroxidase was diluted 1:1000 in PBS and incubated with the sections for 30 minutes at room temperature in a humid chamber. The sections were again washed as above. The sections were incubated with the developing solution for approximately 20 minutes at room temperature and the staining intensity was periodically checked by observation at 25x using a light microscope.

The developing solution was prepared as follows: DAB (3,3' diaminobenzidine; Sigma, MO.) was prepared at a 0.5%

concentration in Tris-Cl buffer (0.05 M, pH 7.6) and 0.5 ml aliquots were stored at -20°C. Immediately prior to use, one 0.5 ml aliquot of the DAB solution was added to 4.5 ml of the Tris-Cl buffer, to this solution a mixture of 1 ml of nanopure water and 30 µl of hydrogen peroxide was added. After staining, the slides were dried in ethanol by immersion in increasing concentrations of 70, 95 and 99%, dipped in xylene twice and mounted.

RESULTS

Detection of Synthetic Immune Complexes

Using the ELISA, it was possible to demonstrate the antigen-antibody complexes generated *in vitro*. It is evident from the figure (Fig. 6.2) that the *in vitro* complexes exhibit significant titers compared to the controls of soluble antigen only, hyperimmune coho salmon serum only and normal coho salmon serum only.

Determination of Immune Complexes in Infected Fish Serum

The results depicted in Fig. 6.3 clearly demonstrate the presence of immune complexes in infected coho sera. These immune complex ELISA titers increase significantly with increasing amounts of the bacterial inoculum. No reactivity was found in control serum.

Immunoperoxidase Staining

Tissue sections of both pronephros and mesonephros stained with anti-soluble antigen and anti-fish Ig antibody, clearly indicate that a uniform deposition of antigen and antibody is occurring. As can be seen in the Figs. 6.4 - 6.7, the tissue sections from infected fish show a diffuse staining pattern for both the fish immunoglobulin (fish Ig) and the soluble antigen. In the section of mesonephros stained for soluble antigen (Fig. 6.4), the endothelial staining of the glomerulus is clearly seen (arrow). When stained for antibody with anti-fish-Ig there was a diffuse staining indicating deposition of antibody. In comparison, tissue sections from non-infected fish showed no staining reaction for either antigen or fish immunoglobulin.

DISCUSSION

The involvement of immune system in the pathogenesis of bacterial kidney disease (BKD) was originally proposed by Smith (1964), based on his observation of an inflammatory response in kidney tissue of infected fish. Belding and Merrill (1935) were the first to observe extensive destruction of glomeruli and the supporting structures suggesting the possibility of glomerulonephritis. Bruno (1986) has proposed that direct damage to soft tissues occurs due to products liberated from free bacteria, or from infected and disrupted macrophages, as well as

the release of hydrolytic and oxidizing enzymes of macrophage origin. There have been only two detailed studies devoted to the histopathology of BKD (Young and Chapman, 1978; Bruno, 1986), which indicated that the infection appeared to be granulomatous, resulting in inflammatory reactions. They also found that a large accumulation of macrophages, fibroblasts and polymorphonuclear leukocytes (PMNs) occur around these lesions.

The presence of granulomatous lesions, subendothelial deposits of protein in the kidneys (Young and Chapman, 1978), large quantities of soluble antigen (Turaga et al., 1987) and apparent absence of antibody during BKD (Banowetz, 1974) has prompted us to investigate the possibility of the presence of immune complexes (ICs) in infected fish.

This study demonstrates the presence of soluble immune complexes in the serum of fish which were artificially infected with Renibacterium salmoninarum. Using two different antibodies, one directed against the specific antigen and the other against the salmonid immunoglobulin, facilitated the precise identification of such complexes. The ELISA method described is a tool which can be used to detect antibodies, the binding sites of which are blocked by large quantities of SA. The anti-SA monoclonal antibody functions to bind the soluble antigen portion of the complex onto the plate. When the anti-fish Ig monoclonal antibody is added it recognizes the antibody bound to the plate via the soluble antigen. This method proved to be highly sensitive in recognizing immune complexes.

Previous studies examining the presence of viral antibody and associated immune complexes utilized a polyclonal antibody against the viral antigen to coat the ELISA plate and a monoclonal antibody to recognize the antigen-associated antibody (Izui and Lange, 1988). Problems of cross-reactivity between the polyclonal and monoclonal antibodies found in their ELISA were avoided by the use of two murine monoclonal antibodies and biotinylating only one (anti-fish Ig) of the two antibodies used.

The immunoperoxidase staining also lends indirect support for the existence of immune complexes in infected fish, in that it demonstrates the uniform deposition of both antigen and antibody in the infected kidney tissues. Histochemical staining of tubercular lesions from patients, indicated similar diffused pattern of antigen deposition (Ridley and Ridley, 1986). They also proposed that antigen excess would render the antibody undetectable in circulation. Whether these deposits of antigen and antibody are in the form of immune complexes cannot be deduced from these studies, however the concurrent presence of both antigen and antibody would be a consequence of the deposition of immune complexes.

In a normal immune response, immune complexes are processed and eliminated by phagocytosis. Some of the immune complexes escape this process and eventually are deposited in intravascular spaces and tissues such as the kidney (Williams, 1981; Dick and Kirkwood, 1984). If these complexes were to act in a similar manner as seen in human infections, they would

release chemotactic factors and induce local inflammatory changes resulting in vessel wall destruction, vasculitis and edema (Dick and Kirkwood, 1984). Sites such as the glomeruli would be damaged by these such processes and the tissues would be infiltrated by polymorphonuclear leukocytes (PMNs), eosinophils and neutrophils leading to granuloma formation (Cochrane and Koffler, 1973). All the phenomenon mentioned above are consequences of immune complexes or type III hypersensitivity reactions.

Electron microscopy studies by Young and Chapman (1978) indicated that the pathological changes in the fine structure of glomerulus and renal tubules found during bacterial kidney disease are similar to mammalian renal diseases inducing glomerulonephritis and nephrotic syndrome. Examination of kidney tissues from naturally infected chinook salmon, Oncorhynchus tshawytscha, (Dr. R. P. Hedrick, University of California at Davis, personal communication) revealed thickening of the basement membranes of glomeruli with proteinaceous deposits. Demonstration of soluble immune complexes in this study corroborates the electron microscopy studies and the histochemical analyses of Dr. Hedrick and others, which together indicate that there are immune complexes may be involved in the pathology of the BKD.

ACKNOWLEDGEMENTS

This work was supported by Bonneville Power Administration contract DEAI79-87BP16480.

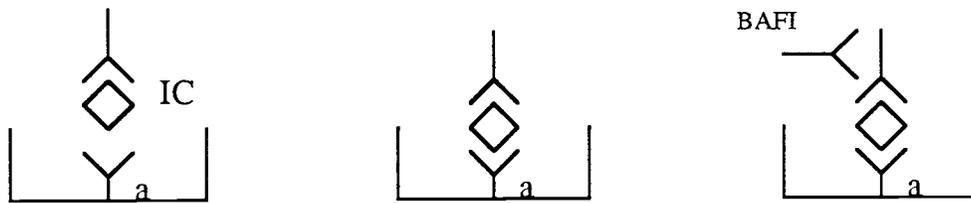


Fig. 6.1. Estimation of immune complexes by antibody capture ELISA. A. anti-soluble antigen monoclonal antibody (a) was coated on to the plate, B. the test sera containing immune complexes (IC) were added and C. biotinylated anti fish-Ig monoclonal antibody (BAFI) was then added.

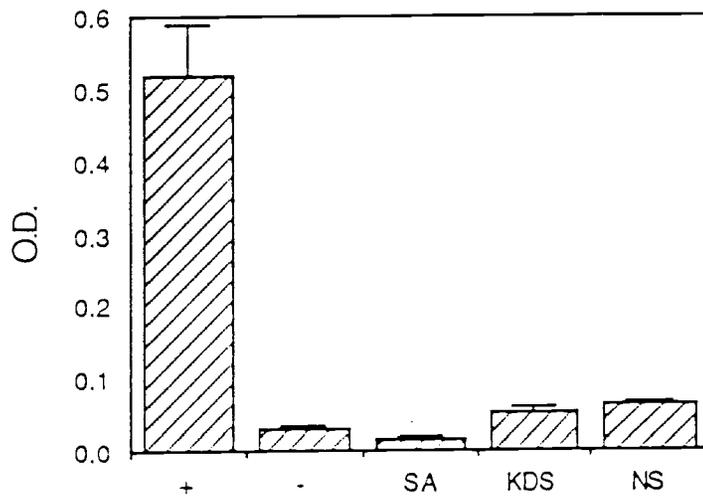


Fig. 6.2. Determination of immune complexes prepared in vitro. ELISA titers are expressed in terms of O.D. at 405 nm. + is the value obtained by mixing SA and hyperimmune serum, - is with by mixing SA and normal serum and the controls include soluble antigen alone (SA), hyperimmune coho serum alone (KDS) and normal serum alone (NS). Histograms represent the mean of 3 wells in the ELISA with 1 S.E.

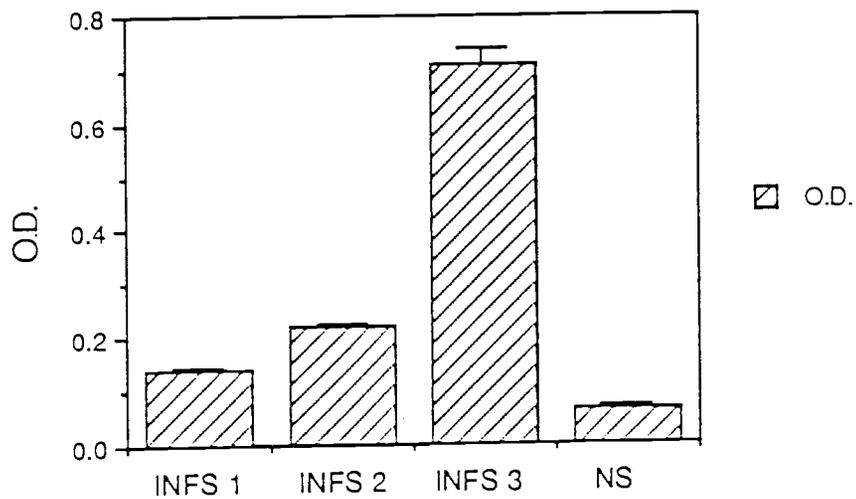


Fig. 6.3. Determination of immune complexes in sera from infected fish. ELISA titers are expressed in terms of O.D. at 405 nm. Infected fish sera (INFS) samples were derived from fish injected with different concentrations of a bacterial suspension [(#1 0.01 O.D.; #2 0.5 O.D.; and #3 1.0 O.D. (500 nm)]. Each histogram represents the mean of 3 wells in the ELISA. The error bar represents one S.E.

Fig. 6.4. Immunoperoxidase staining of mesonephros with anti-soluble antigen monoclonal antibody. Tissue section of infected fish (A). The diffused staining pattern around glomerulus is significant compared to tissue section from normal fish (B).

Fig. 6.4. Immunoperoxidase staining of mesonephros with anti-SA monoclonal antibody.

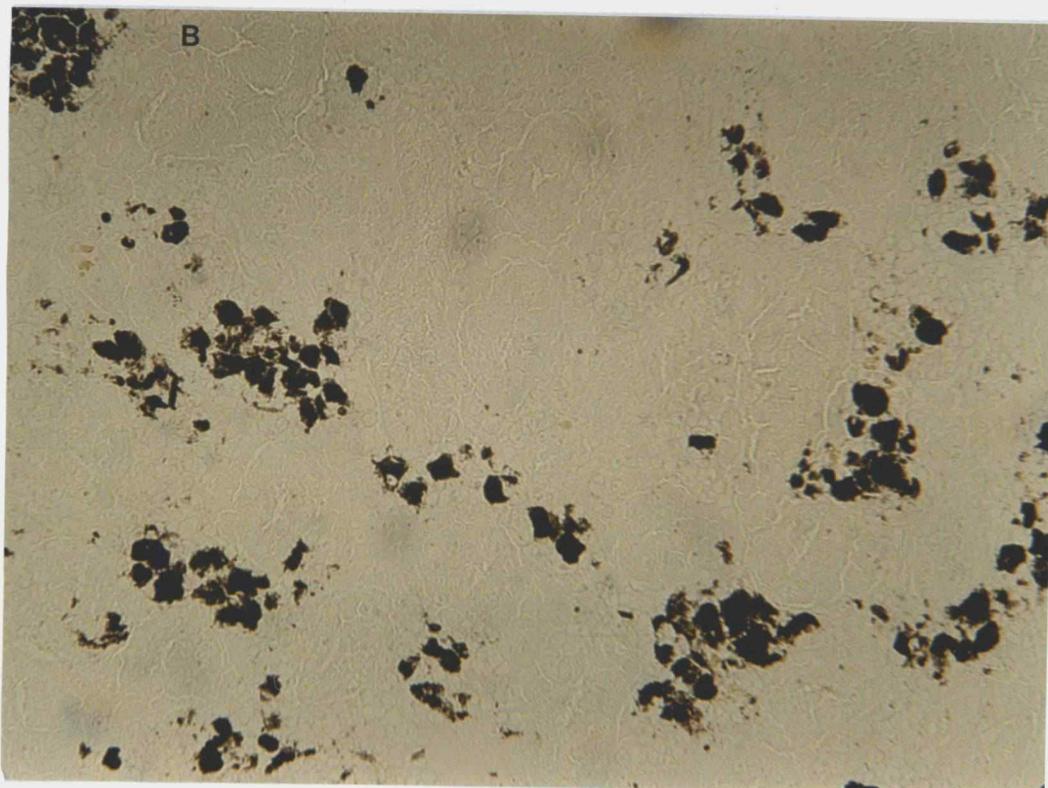
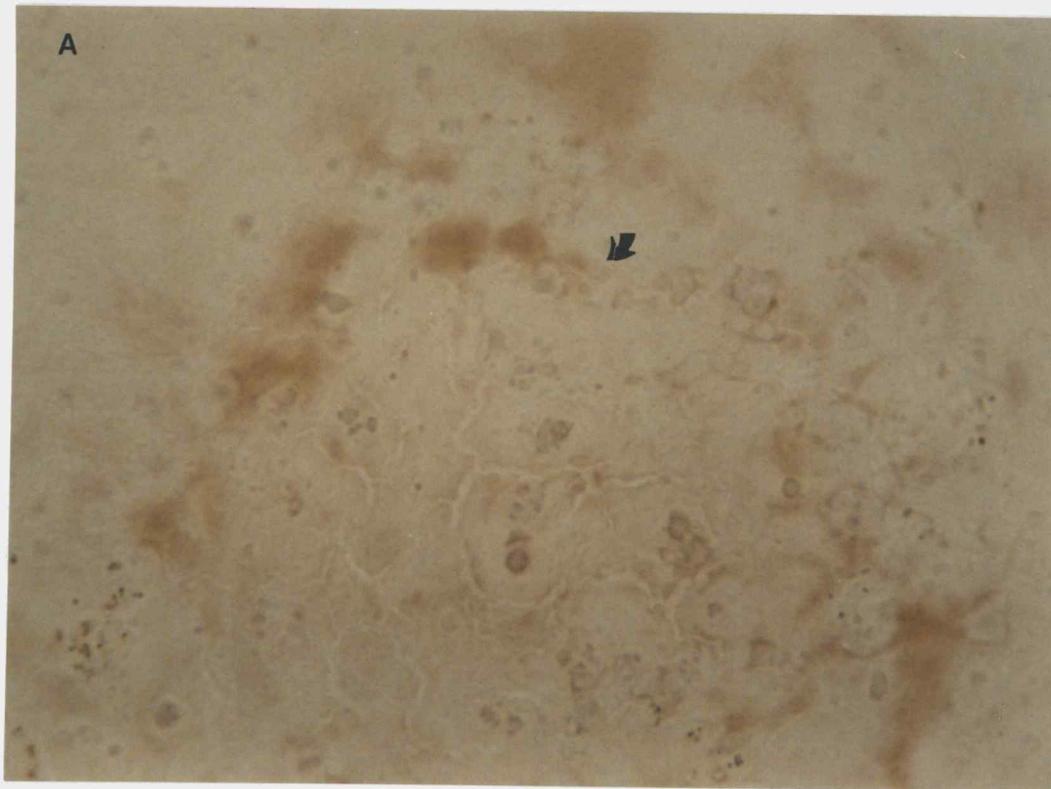


Fig. 6.5. Immunoperoxidase staining of mesonephros with anti fish-Ig monoclonal antibody. Tissue section of the infected fish (A). Along with the diffused staining pattern of antibody, some antibody producing cells are also stained. Staining of tissue from normal fish (B). The black cells are the melanomacrophages.

Fig. 6.5. Immunoperoxidase staining of mesonephros with anti-fish Ig monoclonal antibody.

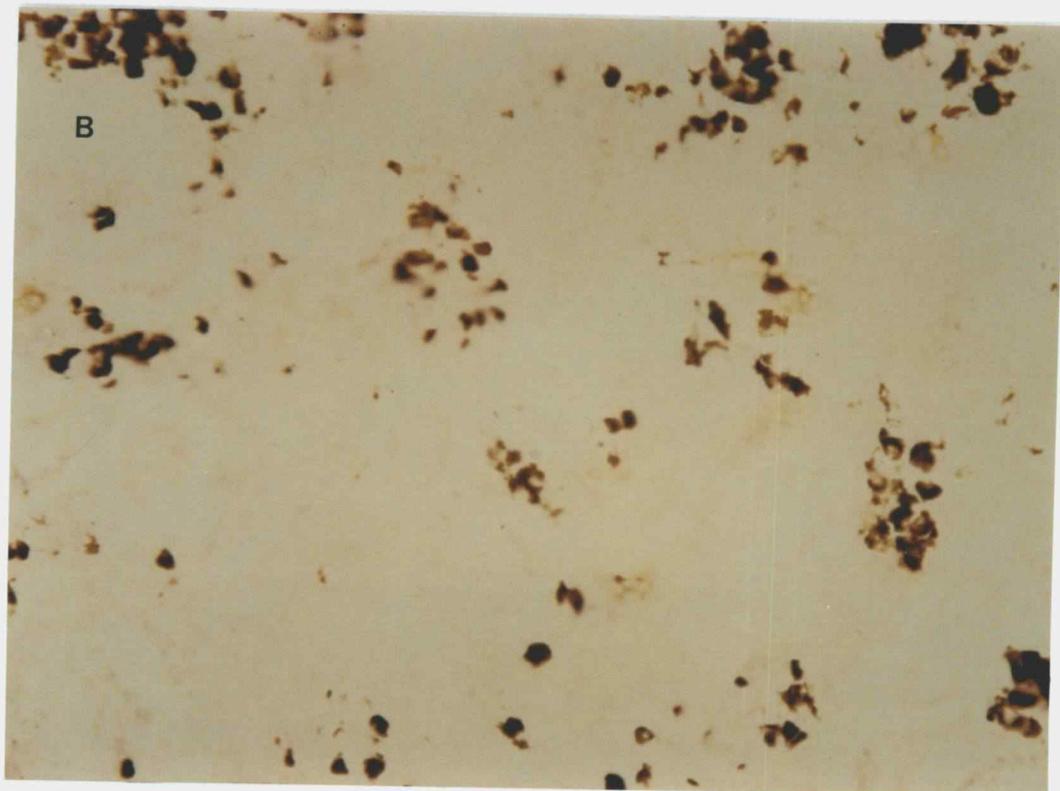
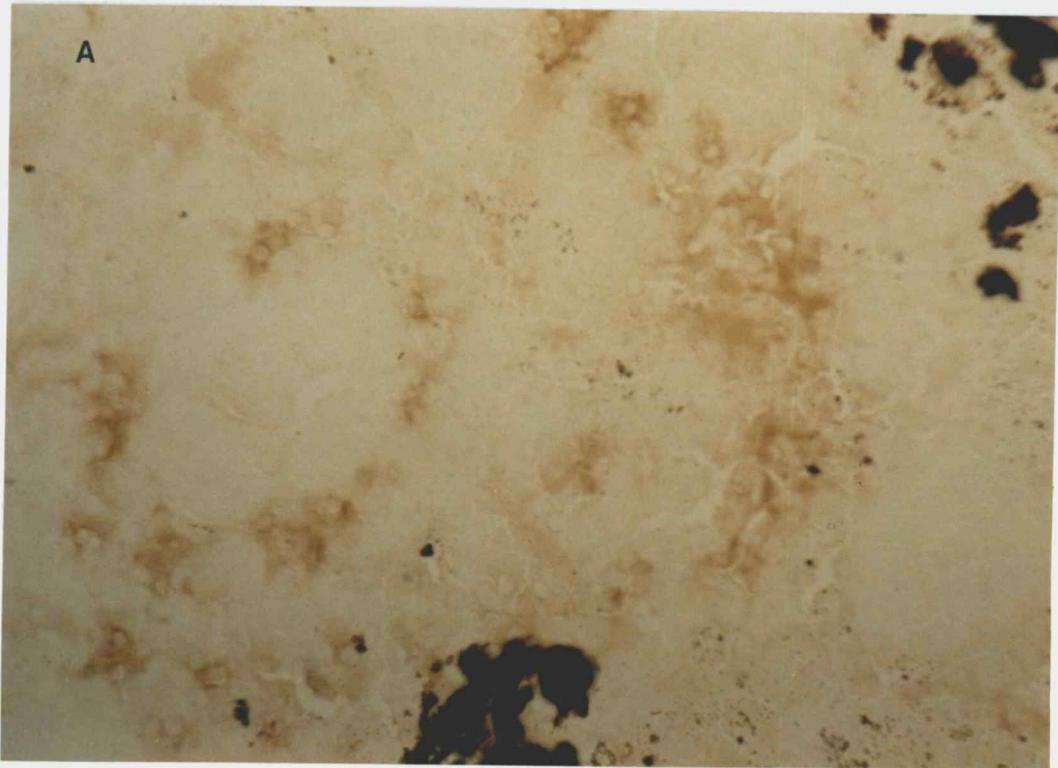


Fig. 6.6. Immunoperoxidase staining of pronephros with anti-soluble antigen monoclonal antibody. The staining pattern is similar to that of mesonephros. Infected fish tissue section (A) and normal fish tissue section (B).

Fig. 6.6. Immunoperoxidase staining of pronephros with anti-SA monoclonal antibody.

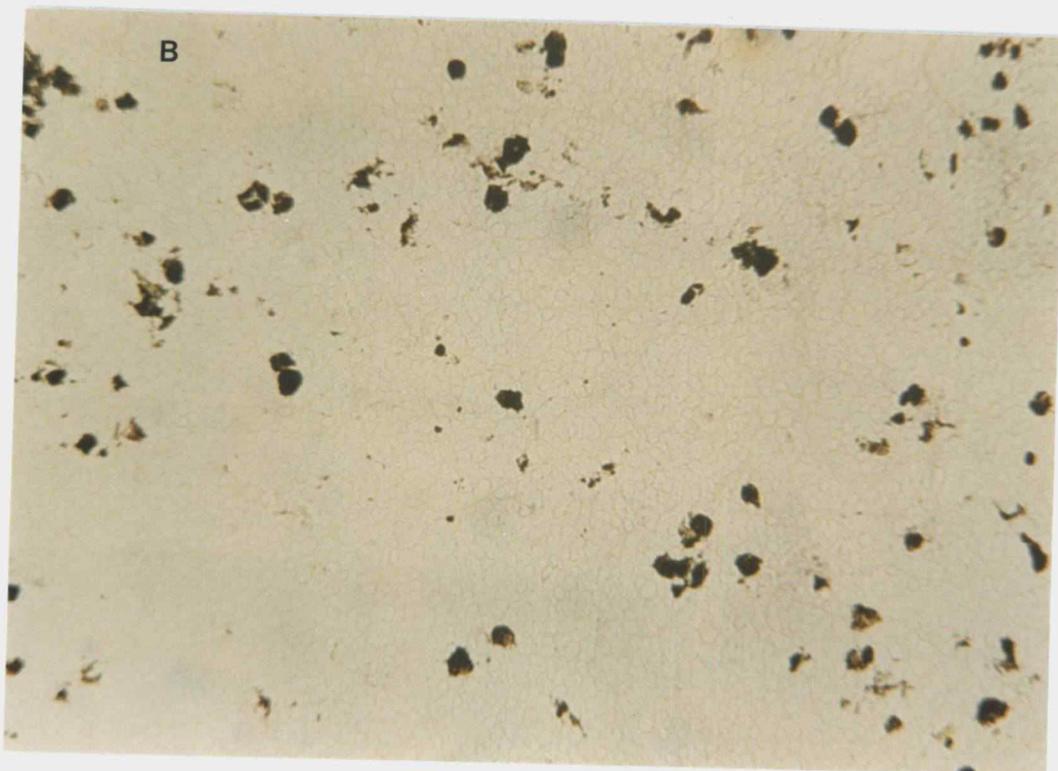
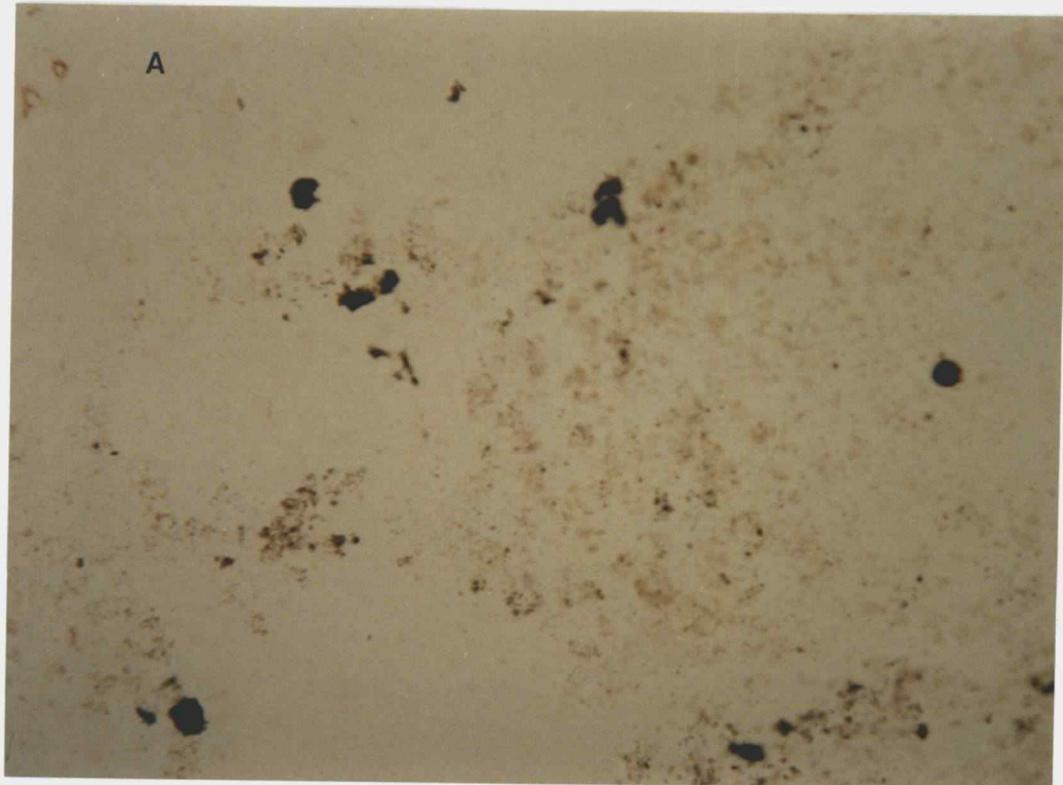
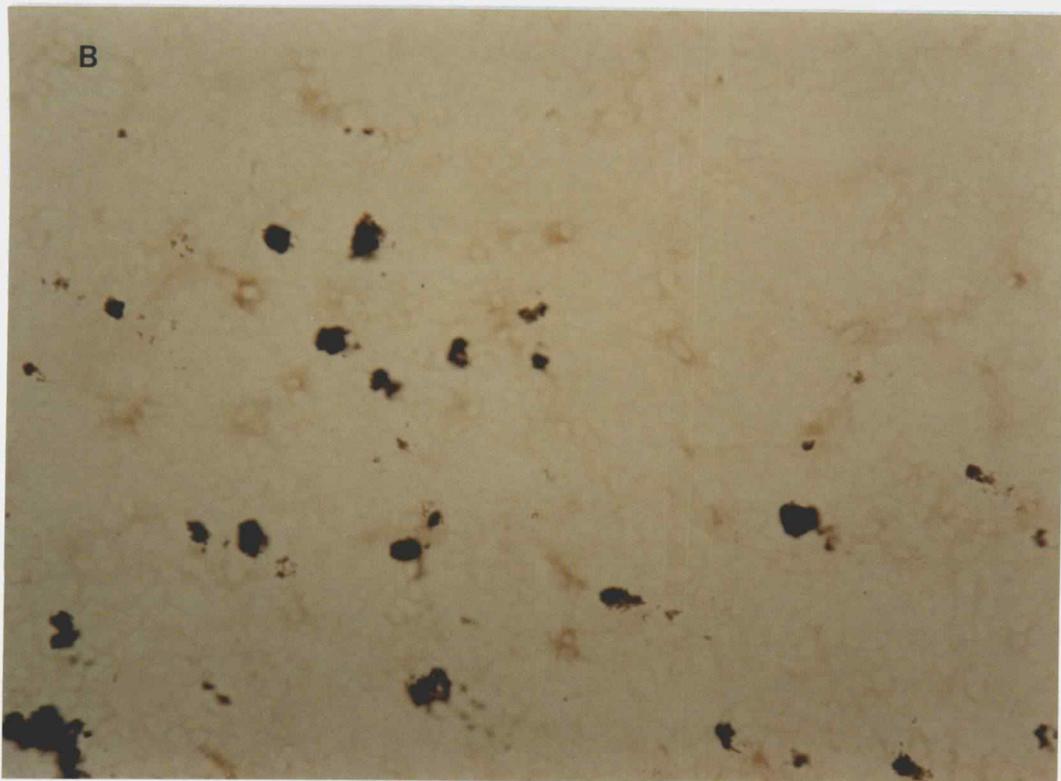
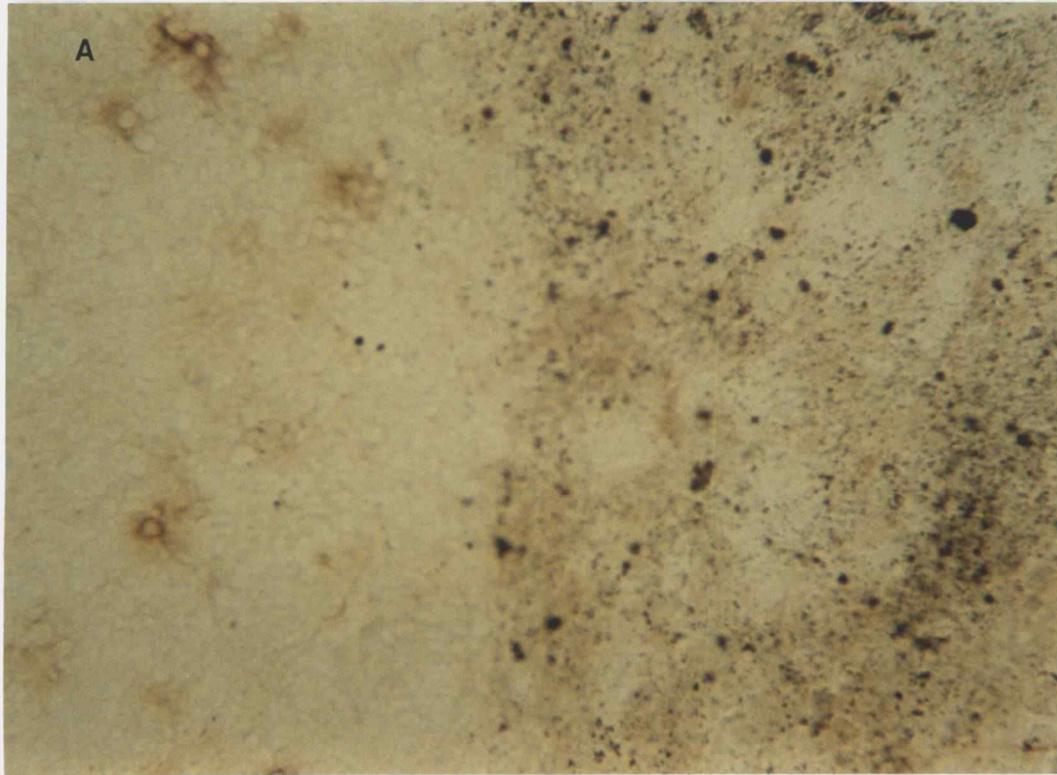


Fig. 6.7. Immunoperoxidase staining of pronephros with anti fish-Ig monoclonal antibody. The staining pattern is similar to that of mesonephros. Infected fish tissue section (A) and normal fish tissue section (B).

Fig. 6.7. Immunoperoxidase staining of pronephros with anti-fish Ig monoclonal antibody.



REFERENCES

- Banowetz, G. M. 1974. Certain aspects of the immunology and chemotherapy of bacterial kidney disease in juvenile coho salmon (Oncorhynchus kisutch). M. S. Thesis. Oregon State University. Corvallis, OR. U.S.A. 53 pp.
- Belding, D. L., and B. Merrill. 1935. A preliminary report upon a hatchery disease of the salmonidae. Trans. Am. Fish. Soc. 65: 76-84.
- Bruno, D. W. 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L., with reference to naturally infected fish. J. Fish Dis. 9: 523-537.
- Cochrane, C. G. and D. Koffler. 1973. Immune complex Disease in Experimental Animals and Man. Adv. Immunol. 16: 185-264.
- DeLuca, D., Wilson, M. R., and G. W. Warr. 1983. Lymphocyte heterogeneity in the trout Salmo gairdneri, defined with monoclonal antibodies to IgM. Eur. J. Immunol. 10: 546-551.
- Dick, H. M., and E. Kirkwood. 1984. Immunity to infection-hypersensitivity states and infection. In Topley and Wilson's Principles of Bacteriology, Virology and Immunity. Volume I, 7 Ed. Williams and Wilkins, Baltimore. p 374-401.
- Evelyn, T. P. T. 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. Bull. Off. Int. Epiz. 87: 511-513.
- Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. of Microbio. 35: 273-298.
- Izui, S., and G. Lange. 1988. Enzyme-linked immunosorbent assay for detection of retroviral gp70 and gp70-anti-gp70 immune complexes in sera from SLE mice. Clin. Exp. Immunol. 71: 45-49.

- Mitchum, D. L., Sherman, L. E., and G. T. Baxter. 1979. Bacterial kidney disease in feral populations of brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri). J. Fish. Res. Board Can. 36: 1370-1376.
- Ridley, M. J., and D. S. Ridley. 1986. Histochemical demonstration of mycobacterial antigen, specific antibody and complement in the lesions of tuberculosis. Histochem. J. 18: 551-556.
- Sanders, J. E., and J. L. Fryer. 1980. Renibacterium salmoninarum gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. Int. J. Syst. Bact. 30: 496-502.
- Smith, I. W. 1964. The occurrence and pathology of Dee disease. Fresh Water Salmon Fish. Res. 34: 1-12.
- Turaga P. S. D., Wiens, G. W., and S. L. Kaattari. 1987a. Analysis of Renibacterium salmoninarum antigen production in situ. Fish Path. 22: 209-214.
- Wiens, G. W., and S. L. Kaattari. 1989. Monoclonal antibody analysis of common surface protein(s) of Renibacterium salmoninarum. Fish Path. 24: 1-7.
- Williams, R. C. 1981. Immune complexes: A clinical perspective. Am. J. Med. 71: 743-745.
- Young, C. L., and G. B. Chapman. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 35: 1234-1248.

7. CHARACTERISTICS OF THE ANTIBODY RESPONSE OF THREE
SALMONID SPECIES TO SOLUBLE ANTIGEN(S) SECRETED BY
RENIBACTERIUM SALMONINARUM

PRASAD S.D. TURAGA and STEPHEN L. KAATTARI

Department of Microbiology
Oregon State University
Corvallis, OR - 97331

ABSTRACT

Antibody responses of coho salmon (Oncorhynchus kisutch), chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Oncorhynchus mykiss) to soluble antigen from Renibacterium salmoninarum were examined. A protocol was developed for the comparison of antiserum specificities from different species and individuals of the genus Oncorhynchus, using both ELISA and Western blot analysis. Variable titers of antibody were found both within and between the three species tested. The ELISA titers of the sera and their specificities for soluble antigen as assessed by Western blot analysis, mostly demonstrated a strong recognition of the 57 kd protein of soluble antigen, however some variation in overall specificity was observed.

INTRODUCTION

Bacterial kidney disease (BKD) of salmonids is caused by Renibacterium salmoninarum (Sanders and Fryer, 1980). The disease is responsible for devastating losses both of hatchery-reared (Fryer and Sanders, 1981) and wild salmon populations

(Mitchum et al., 1979). The organism is a slow-growing, gram-positive, diplobacillus which has been classified into a unique genus and species (Sanders and Fryer, 1980). A number of methods have been developed to detect the organism in diseased fish, they include: the use of selective medium for isolation (Pippy, 1969), fluorescent antibody techniques (Bullock and Stuckey, 1975; Laidler, 1980; Elliott and Barila, 1987), counter immunoelectrophoresis (Groman, 1975), co-agglutination (Kimura and Yoshimizu, 1981), enzyme linked immunosorbent assays (ELISAs) (Pascho and Mulcahy, 1987; Dixon, 1987; Turaga et al., 1987) and Western blot analysis (Turaga et al., 1987). Estimation of agglutination titers and precipitin titers (Evelyn, 1971; Banowetz, 1974; Bruno, 1987) have also been used to detect antibody in infected fish. However, detection of antibody levels has not correlated well with survival of infected fish. It has also been suggested that there can be false-positive reactions in agglutination assays, due to serum components such as lectins or crossreacting antibodies (Bruno, 1987).

Most of the studies, thus far have utilized either rabbit antisera or mouse monoclonal antibodies, to characterize R. salmoninarum antigens. Antigens characterized by these antibodies may not represent those that may be recognized in fish, which is the host system. Such serological characterization utilizing fish serum will aid in developing better vaccine candidates for prophylactic treatment of the disease. Therefore, one should examine the specificities of immune responses to salmonid pathogen in salmonid host. We demonstrate, in this

study, a protocol to characterize the specificity of antibodies to the soluble products of R. salmoninarum which are found in fish serum. This characterization first requires the standardization of the individual serum samples by an ELISA titration protocol. Specificity analysis can then be performed on equivalent concentrations of each individual serum through Western blot analysis.

MATERIALS AND METHODS

Animals

Yearling coho salmon (Oncorhynchus kisutch), chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Oncorhynchus mykiss) weighing 500-600 g, were used in this study. The salmon were maintained in 220 gallon circular tanks at Oregon State University-Fish Disease Laboratory (OSU-FDL) in ambient (12°C) pathogen-free well water. Rainbow trout were maintained under identical conditions at Environmental Protection Agency, Goodnight facility, Corvallis, OR. All fish were fed Oregon moist pellets (OMP).

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH₂PO₄ and

0.809 g of Na_2HPO_4 in 1 liter of distilled water. Tris-buffered saline (TBS), pH 8.2 was prepared by dissolving Tris base 6.07 g, EDTA 0.3 g and NaCl 8.7 g in 1 liter of distilled water.

Immunization and collection of antisera

Soluble antigen (SA) (2 mg/ml in PBS), prepared according to the method of Turaga et al. (Turaga et al., 1987a), was emulsified in Freund's complete adjuvant (FCA) (Difco, Detroit, MI) at a 1:1 ratio (v/v). Each fish was immunized intraperitoneally with 0.1 ml of this preparation. The fish were bled one month post immunization through the caudal vein. Sera was prepared and stored at -20°C until use.

Determination of Serum Antibody Titers

The ELISA method and the buffers were modified version of Voller et al. (1975). Briefly, one hundred μl of soluble antigen (5 $\mu\text{g}/\text{ml}$ in carbonate/ bicarbonate buffer, pH 9.6) was coated on the wells of Enzyme Immuno Assay (EIA) plate by overnight incubation at 17°C . After blocking the wells with 1% bovine serum albumin (BSA) in 0.1% tween 20 in tris-buffered saline, (TTBS) (100 $\mu\text{l}/\text{well}$) for one hour, wells were washed three times with TTBS followed by TBS. One hundred μl of hyperimmunized coho anti-SA serum dilutions prepared in TTBS (1/100, 1/1000, 1/5000, 1/10000 and 1/20000) were incubated for one hour in the wells along with dilutions of the experimental

fish sera (1/30, 1/150, 1/300 and 1/900). The dilutions of hyperimmune serum were used to standardize values obtained from the various ELISA tests. The plate was washed as described above and then an appropriate dilution of biotinylated (Kendall et al., 1983) anti-fish immunoglobulin monoclonal antibody, 1-14 (DeLuca et al., 1983) in TTBS (100 μ l/well) was added and incubated for one hour at room temperature. After washing the plate, 100 μ l of a 1/100 dilution of streptavidin-horseradish peroxidase (Sigma, St. Louis, MO) was added and incubated for 45 minutes. The wells were washed again and 100 μ l of the substrate solution was added, the color development was measured spectrophotometrically at 405 nm on an EIA autoreader (model EL 310, Biotek Instruments, Burlington, VT). The substrate solution was a mixture of: 10 ml of citrate buffer (pH 4.0), 10 μ l of hydrogen peroxide and 75 μ l of a 10 mg/ml solution 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) in distilled water. The amount of antibody activity in each test serum was determined by comparison of the volume required for 50% of the maximum O. D. (1 unit of antibody activity) with the volume required for the same 50% response. Thus according to Arkoosh and Kaattari (1989),

Units of activity (test serum) =

$$\text{Units of activity(standard)} \times \frac{\text{Volume of standard serum required for 50\%}}{\text{Volume of test serum required for 50\%}}$$

Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method followed was basically that of Laemmli (1970). Separating gels of 10% polyacrylamide and a stacking gels of 3% polyacrylamide were used. The apparatus used was a mini gel electrophoresis unit (Idea Scientific, Corvallis, OR) and the power supply was an EC 600 (E-C apparatus Corporation, St. Petersburg, FL). Instead of using a toothed comb in the stacking gel, a preparative gel comb was used. Twenty μ l containing 20 μ g of SA was mixed with equal volume of sample buffer and placed in boiling water bath for 3 minutes before electrophoresis. During the electrophoresis a constant current of 10 mA was applied during migration through the stacking gel and 20 mA during migration through the separating gel.

Transblotting

After the electrophoresis, the protein bands were transferred from the gel onto nitrocellulose (NC) paper (Bio Rad, Richmond, CA), using transblot apparatus (Bio Rad, Richmond, CA) (Towbin et al.; manufacturers instructions). The transblotting was conducted in transblotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) by applying 100 V for one hour. The buffer was cooled by placing a cooling unit with ice in the chamber. After transblotting the nitrocellulose paper was processed for Western blotting.

Western Blot analysis

Western blot analysis was done basically after the method of Towbin et al. (1979). Upon transfer of SA proteins, the nitrocellulose (NC) paper was placed in a blocking solution consisting of 3% BSA in TTBS for one hour at 37°C. The NC paper was then washed with three changes of TTBS over a period of 15 minutes. The NC paper was then placed in a miniblotted apparatus (Immunelect, Boston, MA), which consists of multiple slots, each with a capacity of 100 µl. The fluid placed in these slots comes into direct contact with the NC paper. The dilutions of the test sera were applied to the slots and incubated for one hour in a humid chamber at room temperature. The NC paper was then washed with TTBS and an appropriate dilution of biotinylated anti-fish immunoglobulin (1-14) was applied and incubated for one hour. The paper was washed and horseradish peroxidase labelled streptavidin was added to the NC paper and incubated for 45 minutes. The paper was washed again and the bands of soluble antigen which were bound by salmonid antibody were stained by placing the NC paper in the substrate solution. The substrate solution consisted of 2 ml of 4-chloro-naphthol (Bio-Rad) (3 mg/ml in absolute methanol), 10 ml of 0.1 M phosphate buffered saline (pH 7.2) and 10 µl of hydrogen peroxide.

RESULTS

Standardization of the Sera for Western Blot Analysis

A. Determination of Hyperimmune Serum antibody activity

The titer determination of the standard (hyperimmune) serum was performed to allow for determination of the amounts of each test serum which would yield comparable concentrations. The titration was first performed on a pool of hyperimmune serum, using the ELISA method and a standard curve was generated as shown in Fig. 7.1. The number of anti-SA antibody units of the hyperimmune serum was calculated to be 5500 units/100 μ l from the standard curve (i.e., 50% (1 unit) of the maximum O. D. is produced by 0.018 μ l of the serum, thus the number of units in 100 μ l is 5500).

Determination of minimal number of these antibody units required for production of a positive reaction on Western blot was performed by using different dilutions of the titered hyperimmune serum. An adequate amount of antiserum which produced a detectable reaction was found to be the equivalent of 67 units, or 100 μ l of a 1/81 dilution of hyperimmune serum (Fig 7.2). The determination of this quantity of activity was essential for the comparison of specificities of the different sera by Western blot.

B. Standardization of the Sera from Different Species

Each individual antiserum was titrated and the amount of antibody activity and dilution volumes required to produce 67

units of activity were determined (Table 7.1 A, B, C). These dilutions of the sera were used in a Western blot analysis to compare their soluble antigen recognition specificity.

Additionally it is of interest to note the large variation of the titers within and between the species of fish tested. Chinook salmon, especially showed no or low titers of anti-soluble antigen antibody compared to coho salmon or rainbow trout.

Western Blot analysis of different test sera

The dilution volumes as shown in table 7.1 were used in Western blot analysis. All the antisera recognized the 57 kd protein band of SA (Figs. 7.3 A-C), and to different degrees of intensity and most of the sera also recognized the 58 kd protein band. Other lower molecular weight bands of soluble antigen were not detected by the sera at the dilutions used.

DISCUSSION

Attempts to generate immune responses to R. salmoninarum antigens and correlation of these responses to protection from bacterial kidney disease (BKD) has been of interest to many researchers (McCarthy et al., 1984; Paterson et al., 1985; Evelyn et al., 1988). The production of agglutination titers to various immunogens was routinely used to monitor the efficacy of immunization. Analysis of antibody levels, however, were not found to correlate well with the survival of fish

infected with R. salmoninarum. One confounding aspect of such studies was the use of unpurified antigen preparations, such as heat or formalin killed , sonicated or lysed bacterial suspensions.

Earlier studies incorporated polyclonal or monoclonal antibodies (Getchell et al., 1985; Turaga et al., 1987a; Wiens and Kaattari, 1989) in characterizing antigens produced by R. salmoninarum. It is, however, also important to identify the antigens recognized by salmonid host if protective immune responses induced by vaccination are to be evaluated. Hastings and Ellis (1988) performed such studies with Aeromonas salmonicida, another fish pathogen, in an attempt to determine the antigenicity of its extracellular products (ECPs) in rainbow trout and rabbits. They demonstrated that the rabbit developed antibodies to the virulence factors, hemolysin and protease, whereas the trout did not. When the fish were passively immunized with the rabbit antiserum and subsequently challenged with the bacteria, they were found to be protected. This finding suggests that certain fish vaccines may not be efficacious because the fish are not capable of producing antibodies to crucial antigens. One alternative to this dilemma may be through modification of the extracellular products to improve their immunogenicity in fish.

This report demonstrates a means of characterizing the antibody response of three species of salmonid fish to the extracellular soluble antigen(s) produced by R. salmoninarum. A quantitative ELISA procedure was used to determine the antibody activity. The antibody titration utilized the anti-fish Ig

monoclonal antibody (1-14), thus it was necessary to develop a means whereby only comparable amounts of 1-14 recognizable antibodies were being detected. If this is not done different reactivity patterns could simply be due to differential amounts of antibody activity within each sample. The development of such a method was facilitated by calibration of the test sera using a standard preparation of hyperimmune serum. Thus, equivalent, calibrated volumes of test sera were used in the qualitative Western blot analysis.

In other studies, two species of Pacific salmon, coho (*Oncorhynchus kisutch*) and sockeye (*Oncorhynchus nerka*) were used to test anti-BKD vaccines (killed bacterial preparations in Freund's complete adjuvant) and were not found to be protected following natural or experimental (injected) challenge with live pathogen (Evelyn et al. 1988). Evelyn et al., concluded that *Oncorhynchus* species may not benefit from similar vaccination procedure to the degree seen with *Salmo* species. It would appear from these studies that any difference in susceptibility to BKD among these species may not be dependent on differential recognition of soluble antigen. Thus it would be important to examine specificities of different species to the bacterial antigen and determine if a lack of recognition may be contributory to any difference in susceptibility. Knowledge of the antigens that are recognized by the fish species should aid in the development of methods to assess the effectiveness of antigen preparation procedures or the incorporation of various immunomodulating substances into vaccines.

ACKNOWLEDGEMENTS

This work was supported by Bonneville Power Administration contract DEAI79-87BP16480.

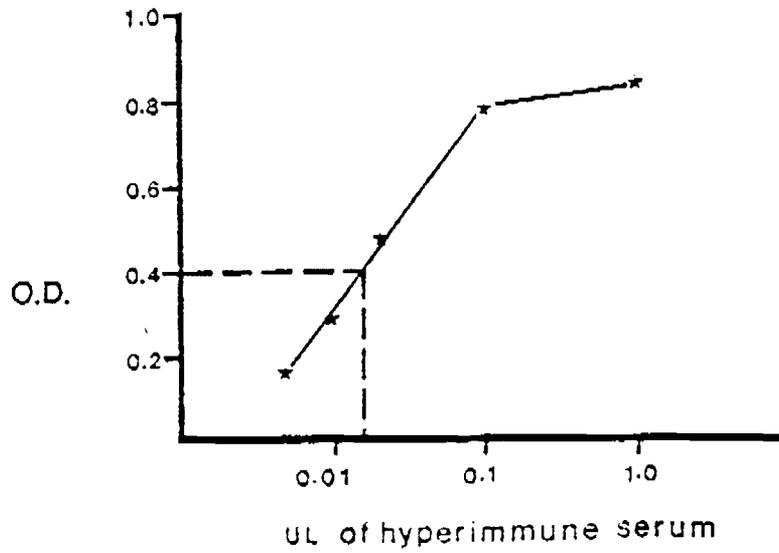


Fig 7.1. Standardization of hyperimmune serum. The ELISA values are plotted as O. D. vs the dilution of the serum.

Table 7.1. A. Antibody titration of sera from Rainbow trout.

Fish Number	# of Units ¹	Dilution ²
1	NT ³	-----
2	71	1.05
3	102	1.52
4	2857	42
5	NT	-----
6	156	2.3
7	NT	-----
8	14	DNR ⁴
9	156	2.3
10	1136	16.9

1 = No. of units/0.1 ml of the serum

2 = Dilution of serum required to produce 67 units of activity

3 = no titer

4 = dilution not required

Table 7.1. B. Antibody titration of sera from Coho salmon.

Fish Number	# of Units ¹	Dilution ²
1	NT ³	-----
2	370	5.5
3	NT	-----
4	114	1.7
5	55	DNR ⁴
6	55	DNR
7	270	4.0
8	370	5.5
9	43	DNR
10	143	2.1

1 = No. of units/0.1 ml of the serum

2 = Dilution of serum required to produce 67 units of activity

3 = no titer

4 = dilution not required

Table 7.1. C. Antibody titration of sera from Chinook salmon.

Fish Number	# of Units ¹	Dilution ²
1	277	4.1
2	16	DNR ⁴
3	19	DNR
4	NT ³	----
5	NT	----
6	NT	----
7	66	DNR
8	27	DNR
9	37	DNR
10	3571	53

1 = No. of units/0.1 ml of the serum

2 = Dilution of serum required to produce 67 units of activity

3 = no titer

4 = dilution not required

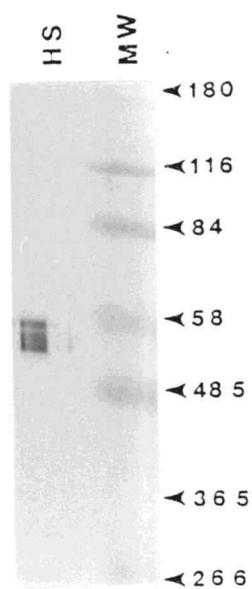


Fig. 7.2. Western blotting of SA using hyperimmune serum (HS). The serum was diluted so as to give 67 units of activity. Mol. wt. markers (MW) are also depicted.

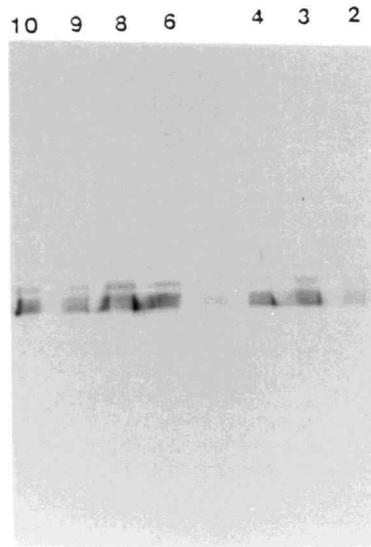


Fig. 7.3. A. Western blot analysis of SA using immune sera from Rainbow trout. The sera were diluted according to the values from table 6.1 A. and the sera from fish number 2, 3, 4, 6, 8, 9 and 10 were employed. All the sera recognize the 57 kd protein band of SA with different intensities.

10 9 8 7 6 5 4 2



Fig. 7.3. B. Western blot analysis of SA using immune sera from Coho salmon. The sera were diluted according to the values from table 6.1 B. and the sera from fish number 2, 4 through 10 were employed. All the sera recognize the 57 kd protein band of SA with different intensities.

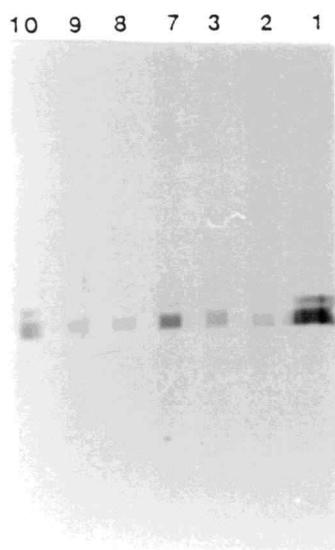


Fig. 7.3. C. Western blot analysis of SA using immune sera from Chinook salmon. The sera were diluted according to the values from table 6.1 C. and the sera from fish number 1, 2, 3, 7, 8, 9 and 10 were employed. All the sera recognize the 57 kd protein band of SA with different intensities.

REFERENCES

- Banowetz, G. M. 1974. Certain aspects of the immunology and chemotherapy of bacterial kidney disease in juvenile coho salmon (Oncorhynchus kisutch). M. S. Thesis. Oregon State University, Corvallis, OR. U.S.A.
- Bruno, D. W. 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L., with reference to naturally infected fish. J. Fish Dis. 9: 523-537.
- Bruno, D. W. 1987. Serum agglutinating titres against Renibacterium salmoninarum the causative agent of bacterial kidney disease, in rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L. J. Fish Biol. 30: 327-334.
- Bullock, G. L., and H. M. Stuckey. 1975. Fluorescent antibody identification and detection of the Corynebacterium causing kidney disease of salmonids. J. Fish. Res. Board Can. 32: 2224-2227.
- Daly, J. G., and R. M. W. Stevenson. 1987. Intracellular survival of Renibacterium salmoninarum. Fish Immunology Symposium, Plymouth polytechnique, Plymouth, England. Abstracts.
- Dixon, P. F. 1987. Detection of Renibacterium salmoninarum by the enzyme-linked immunosorbent assay (ELISA). J. Appl. Ichthyol. 3: 77-82.
- Elliott, D. G., and T. Y. Barila. 1987. Membrane filtration fluorescent antibody staining procedure for detecting and quantifying Renibacterium salmoninarum in coelomic fluid of chinook salmon (Oncorhynchus tshawytscha). Can. J. Fish. Aquat. Sci. 44: 206-210.
- Evelyn, T. P. T. 1971. The agglutinin response in sockeye salmon vaccinated intraperitoneally with a heat-killed preparation of the bacterium responsible for salmonid kidney disease. J. Wild. Dis. 7: 328-335.

- Evelyn, T. P. T., Ketcheson, J. E., and L. Prospero-Porta. 1988. Tirals with anti-bacterial kidney disease vaccines in two species of pacific salmon. International Fish Health Conference, Vancouver, B. C., Canada. Abstracts: p 38.
- Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. *Ann. Rev. of Microbiol.* 35: 273-298.
- Getchell, R. G., Rohovec, J. S., and J. L. Fryer. 1985. Comparison of Renibacterium salmoninarum isolates by antigenic analysis. *Fish Path.* 20: 149-159.
- Groman, D. B. 1981. The detection of precipitating antibody to Renibacterium salmoninarum in chinook salmon by counter immunoelectrophoresis. *Dev. Biol. Stand.* 49: 189-192.
- Hastings, T. S., and A. E. Ellis. 1988. The humoral immune response of rainbow trout, Salmo gairdneri Richardson, and rabbits to Aeromonas salmonicida extracellular products. *J. Fish Dis.* 11: 147-160.
- Kendall, C., Ionesca-Matiu, I., and R. G. Dressman. 1983. Utilization of the biotin/avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). *J. Imm. Methods.* 56: 329-339.
- Kimura, T., and M., Yoshimizu. 1981. A coagglutination test with antibody sensitized staphylococci for rapid and simple diagnostics of bacterial kidney disease (BKD). *Dev. Biol. Standard.* 49: 135-148.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature.* 227: 680-685.
- Laidler, kL. A. 1980. Detection and identification of the bacterial kidney disease (BKD) organism by the indirect fluorescent antibody technique. *J. Fish Dis.* 3: 67-69.
- McCarthy, D. H., Croy, T. R., and D. F. Amend. 1984. Immunization of rainbow trout, Salmo gairdneri Richardson, against bacterial kidney disease: preliminary efficacy evaluation. *J. Fish Dis.* 7: 65-71.

Mitchum, D. L., Sherman, L. E., and G. T. Baxter. 1979. Bacterial kidney disease in feral populations of brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri). J. Fish. Res. Board Can. 36: 1370-1376.

Pascho, R. J., and D. Mulcahy. 1987. Enzyme-linked immunosorbent assay for a soluble antigen of Renibacterium salmoninarum, the causative agent of salmonid bacterial kidney disease. Can. J. Fish. Aquat. Sci. 44: 183-191.

Paterson, W. D., Lall, S. P., Airdrie, D., Greer, P., Greenham, G., and M. Poy. 1985. Prevention of disease in salmonids by vaccination and dietary modification. Fish. Path. 20: 427-434.

Pippy, J. H. C. 1969. Kidney disease in juvenile Atlantic salmon (Salmo salar) in the Mangaru River. J. Fish. Res. Board Can. 26: 2535-2337.

Sanders, J. E., and J. L. Fryer. 1980. Renibacterium salmoninarum gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. Int. J. Syst. Bact. 30: 496-502.

Towbin, H., Staehelin, T., and J. Gordon (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76: 4350-4354.

Turaga, P. S. D., Wiens, G. W., and S. L. Kaattari. 1987. Analysis of Renibacterium salmoninarum antigen production in situ. Fish Path. 22: 209-214.

Voller, A., Bidwell, D. E., and A. Bentlett. 1976. Enzyme immunology in diagnostic medicine (Theory and Practice). Bull. World Health Organ. 53: 55-65.

Wiens, G. W., and S. L. Kaattari. 1989. Monoclonal antibody analysis of common surface protein(s) of Renibacterium salmoninarum. Fish Path. 24: 1-7.

Young, C. L., and G. B. Chapman. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 35: 1234-1248.

8. EXAMINATION OF PROPHYLAXIS AGAINST BACTERIAL
KIDNEY DISEASE INDUCED BY VARIOUS IMMUNOGENS

PRASAD S.D. TURAGA and STEPHEN L. KAATTARI

Department of Microbiology
Oregon State University
Corvallis, OR - 97331

ABSTRACT

Studies were performed with the intent of developing a prophylactic preparation for the protection of salmon (Oncorhynchus) species against bacterial kidney disease (BKD). The R. salmoninarum antigens used were heat killed bacteria, soluble antigen(s) (SA) produced by the bacteria and a polysaccharide extracted from the bacterial cell walls. These antigens were administered to the fish along with immunomodulating agents such as Vibrio anguillarum extract, Freund's complete adjuvant (FCA), formalin killed Vibrio anguillarum and heat killed Mycobacterium chelonii. Before challenge with the live pathogen, agglutinin titers and enzyme linked immunosorbent assay (ELISA) titers were performed on the sera from vaccinated fish. The percent survival of the fish indicated that FCA alone or a component thereof (i.e., Mycobacteria) may confer protection. This report represents preliminary studies on the prophylactic effectiveness of various forms of the immnogen.

INTRODUCTION

Bacterial kidney disease of salmonid fish is caused by Renibacterium salmoninarum (Sanders and Fryer, 1980). The disease produces devastating losses in hatchery-reared (Fryer and Sanders, 1981) and wild salmon populations (Mitchum, 1979). The disease is characterized by exophthalmia, a distended abdomen, blisters on the skin, and, internally by petechial hemorrhages, splenomegaly, swollen kidney and granulomatous lesions on various internal organs (Belding and Merrill, 1935; Wood and Wallis, 1955; Smith, 1964; Young and Chapman, 1978). The bacterium has been demonstrated to survive and multiply within phagocytic cells (Young and Chapman, 1978; Bruno, 1986; Daly and Stevenson, 1987; S. Gutenberger, Oregon State University, personal communication). This facultative, intracellular nature of the bacterium may protect it from antibiotics and antibodies.

Immunoprophylaxis and chemotherapy, dietary modification and vaccination have been used in attempts to prevent and/or treat this disease. In terms of antibiotic therapy the most current practice is to inject spawning females with erythromycin and then to feed the juveniles with erythromycin for 21 days (Wolf and Dunbar, 1959; T. Amandi, Oregon State University, personal communication). Incorporating high amounts of fluorine and iodine in diets has also resulted in lower prevalences of BKD (Paterson et al. 1985; Lall et al. 1985).

Other studies utilized heat killed or formalin killed R. salmoninarum in attempts to vaccinate (Atlantic salmon, Salmo salar, and Rainbow trout, Salmo gairdneri) against BKD (Paterson et al. 1981; McCarthy et al. 1984). Both studies resulted in a reduced incidence of BKD lesions compared to controls. In more recent studies, Evelyn et al., (1987) employed formalin killed bacteria and used various immunization procedures to vaccinate two species of Pacific salmon (i.e., coho, O. kisutch and sockeye, O. nerka). They found that the vaccinated fish were not protected against challenge and concluded that Oncorhynchus species may not benefit from vaccination to the degree seen with Salmo species.

These studies were conducted with Oncorhynchus kisutch and utilized heat killed R. salmoninarum, soluble antigen(s) (SA) produced by the bacteria, and a polysaccharide extracted from the cell walls of the bacteria as immunogens. These immunogens were used in combination with immunostimulatory agents such as formalin killed Vibrio anguillarum cells, and its cell extract, Freund's complete adjuvant (FCA) (killed M. tuberculosis, H37Rv suspended in mineral oil), and M. chelonii as a replacement for the M. tuberculosis. It has previously been shown that formalin killed V. anguillarum is an effective vaccine in fish culture and an extract of its cell wall is a potent polyclonal activator of salmonid lymphocytes (Yui and Kaattari, 1987). Freund's complete adjuvant has also been used for fish immunization as a non-specific immunostimulatory agent. Soluble antigen(s) were

chosen because they are produced in large quantities (Turaga et al. 1987a) during culture of the bacterium and have been found to exhibit toxigenic properties (Turaga et al. 1987b).

MATERIALS AND METHODS

Animals

Fingerling coho salmon (Oncorhynchus kisutch) (50-100 g) were kept in ambient (12°C) temperature pathogen-free well water in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL), Corvallis, Oregon. The fish were fed on Oregon Moist Pellets. Unless otherwise stated, fish of the above mentioned weight range were used and maintained as described throughout the study. The disease challenges were performed on salmon housed in 30 liter tanks at both OSU-FDL and at the Corvallis Environmental Protection Agency (EPA) facility. The effluent from these tanks was chlorinated.

Buffers

Phosphate buffered saline (PBS), pH 7.2 and 0.01 M, was prepared by dissolving 7.5 g of NaCl, 0.245 g of KH_2PO_4 and 0.809 g of Na_2HPO_4 in 1 liter of distilled water. Tris buffered saline (TBS), pH 8.2, was prepared by dissolving tris-base 6.07 g, EDTA 0.3 g and NaCl 8.7 g in one l of distilled water.

Bacterial Growth

Vibrio anguillarum and Mycobacterium chelonii were grown in tryptic soy broth (Gibco, MI) at room temperature. Formalin fixation of Vibrio cells was accomplished by addition of formaldehyde to achieve a 0.3% concentration in the culture. This was then incubated at room temperature for 24 hr. The bacterial cells were washed 3 times with sterile PBS (2000 x g for 20 min. at 4°C) and resuspended in PBS for use in the conjugation procedures.

Mycobacterium chelonii required constant agitation throughout the culture period to prevent the cells from clumping. Prior to conjugation, the cells were heat-killed by incubating them at 56°C for one hour. The cells were subsequently washed as above, and resuspended in PBS.

Preparation of Soluble Antigen from Renibacterium salmoninarum

Five hundred ml of the sterile KDM-2 medium in 2.8 L Erlenmeyer flasks were inoculated with a 5% (v/v) inoculum of a bacterial culture which was in a late log phase of growth. The culture flask was then incubated on a reciprocating shaker at 17°C for 10-14 days. As these cultures grew to late log phase they were centrifuged at 4000 x g for 30 min. The supernatant was removed and concentrated by passage through a PTGC

10000 NWML filter packet in a Minitan Ultrafiltration apparatus (Millipore Corp., Bedford, MA). Typically ten-fold reductions of the original volume were achieved. This supernatant was further concentrated by ammonium sulphate (Sigma, MO) precipitation (313 g of ammonium sulphate/1000 ml of concentrated supernatant). The salt was added gradually over a period of one hour with constant stirring, while holding the supernatant on an ice bath. This solution was stirred for an additional 3 hours at 4°C. The precipitate was removed by centrifugation at 400 x g for 30 minutes at 4°C, and resuspended in 10 ml of PBS. Using a saturated ammonium sulfate (SAS) solution, two additional 50% precipitations were performed. The final precipitate was then resuspended in 10 ml of PBS and dialyzed extensively at 4°C against 3 liters of PBS with three changes of the buffer over a period of 16 hours. The preparation was then filter sterilized using a 0.45 µm filter (Corning, Corning, NY). The protein content of the preparation was determined by the method of Lowry et al. (1951).

Preparation of *Vibrio anguillarum* Extract

The *Vibrio anguillarum* extract (VE) was prepared (Yui and Kaattari, 1987) from *Vibrio anguillarum* strain SL-174 which had been formalin killed and stored frozen. Fifty ml thawed packed cells were suspended in 10 volumes of 2% saline and placed in a boiling water bath for two hours. Cells were washed three times

in 2% saline, centrifuged at 3,000 x g for ten min at 4°C, resuspended in 95% ethanol, and incubated for 48 hr at 37°C. The cells were then washed two times in acetone, centrifuging at 3000 x g for 10 min and the resulting paste was dried overnight at 37°C. The dried paste was ground to a fine powder with mortar and pestle and stored at 4°C. The soluble VE used for these studies was prepared by resuspending the powder in PBS at 10 mg/ml and placing the tube in a boiling water bath for one hour with frequent agitation. This suspension was then centrifuged at 1000 x g to remove particulates and filter sterilized. Protein concentrations were determined by the method of Lowry et al. (1951).

Polysaccharide Extraction from Renibacterium salmoninarum

The methods followed were basically that of Fiedler and Draxl (1986) and Schleifer and Kandler (1967). Live bacterial cells were obtained from 5 liters of culture medium and stored at -20°C. After thawing, the cells were heat-killed by incubation in a boiling water bath for 30 min. The cells were washed with PBS and finally resuspended in distilled water. Using a Bead-Beater^R Cell disrupter (Biospec Products, Bartlesville, OK) and 0.1 mm diameter glass beads, the cells were disrupted with 20, one minute bursts with a five minute interval between each burst. The preparation was examined under a light microscope to determine if any intact cells remained. This cell wall preparation

was centrifuged at 1500 x g for 20 min. The supernatant, containing the cell walls, was isolated and centrifuged at 12000 x g for 30 min and washed once with distilled water. The final pellet was resuspended to a volume of 10 ml in distilled water. Trypsin (0.5 mg/ml in distilled water) (Sigma, St. Louis, MO.) was added to the suspension and incubated at 37°C for 24 hr. The cell walls were washed four times with distilled water at 12000 x g for 30 min. The preparation was stored at this stage at -20°C until the next step.

Formamide extraction of the cell walls was performed according to the method of Perkin (1965). The frozen cell walls were thawed and washed again with distilled water. The pellet was resuspended to 15 ml with formamide (Sigma, St. Louis, MO.) and heated for 30 min at 170°C, under reflux condensing conditions. The preparation was then cooled on ice and 2.5 volumes of an acid/alcohol solution (5 parts of 2N HCl/95 parts of ethanol) were added to the preparation and centrifuged at 100,000 x g for 30 min. The supernatant was then diluted with 10 volumes of acetone. A white precipitate was allowed to form by incubation at 4°C for 48 hr. The precipitate was pelleted by centrifugation at 1000 x g for 10 min and washed with distilled water. The final pellet was resuspended in 1 ml of distilled water and lyophilized.

Antibody Titration of Sera from Vaccinated Fish

The method used was basically that of Voller et al. (1978). Briefly, 100 μ l of soluble antigen (SA) was coated on the wells of an EIA plate (Corning, New York, NY) and incubated overnight at 17°C. After blocking the wells with 3% bovine serum albumin (BSA) in 0.1% tween 20 in TBS (TTBS) (100 μ l/well) for an hour, the wells were washed three times with TTBS followed by another three washes with TBS. One hundred μ l of different dilutions of hyperimmune anti-SA coho serum in TTBS were incubated for one hour in the wells along with the dilutions of the sera from vaccinated fish diluted in TTBS. The plate was washed as described above, and an appropriate dilution of biotinylated (Kendall et al. 1983) anti-salmonid immunoglobulin monoclonal antibody, 1-14 (DeLuca et al., 1983) in TTBS (100 μ l/well) was added and incubated for an hour at room temperature. After washing the plate, 100 μ l of a 1/100 dilution of streptavidin coupled to horseradish peroxidase (Sigma, St. Louis, MO) was added to each well and incubated for 45 min. The wells were washed again and 100 μ l of the substrate solution was added. The color development was measured spectrophotometrically at 405 nm. The substrate solution consisted of: 10 ml of citrate buffer (pH 4.0), 10 μ l of hydrogen peroxide and 75 μ l of a 10 mg/ml solution of 2,2'-Azinobis, 3-ethyl benzthiazoline sulfonic acid (ABTS) in distilled water.

Bacterial Agglutination Assay

The method used is basically that of Campbell et al. (1970). On a 96 well plate 50 μ l of PBS was placed in each well. Fifty μ l of sera from immunized and normal coho salmon were added to the first two columns of the wells. Serial two fold dilutions of the sera was made in the remaining ten columns. Fifty μ l heat killed R. salmoninarum suspension (1.0 O.D. at 520 nm) in PBS was added to each well from the second column onwards. The plate was allowed to incubate at room temperature for two hours and checked for agglutination. If no agglutination had occurred at this time, the plate was then transferred to a 17°C incubator and checked every six hours for 24 hours.

Conjugation Methods of Antigens from R. salmoninarum to other Adjuvants

The following methods were used for the conjugation of adjuvant material to R. salmoninarum.

A. Conjugation with Tannic Acid: The method was basically that of Campbell et al. (1970). Vibrio anguillarum cells (0.5 ml packed cells) were washed 3 times with PBS (pH 7.2). Phosphate buffered saline (PBS) was prepared by mixing 100 ml of saline with 100 ml of phosphate buffer consisting of 24 ml of 0.15 M KH_2PO_4 and 76 ml of 0.15 M Na_2HPO_4 . The cells were then diluted with 7 ml of PBS (pH 7.2). To 2 ml of this Vibrio

suspension, 2 ml of tannic acid (0.05 mg/ml in saline) solution were added. This suspension was mixed for 10 min in a 37°C water bath. The tanned cells were then washed 3 times in PBS. One ml of soluble antigen (1 mg/ml in saline), 2 ml of the above suspension, and 4 ml of PBS (pH 6.4) were mixed for 10 minutes at room temperature. PBS, pH (6.4), was prepared by mixing 100 ml of saline with 100 of phosphate buffer consisting of 32.2 ml of 0.15 M Na₂HPO₄ and 67.8 ml of 0.15 M KH₂PO₄. To block remaining protein binding sites on the Vibrio cells, the conjugated cells were incubated for 10 min in a gelatin solution (2 mg/ml in saline). Finally, the preparation was washed 3 times with PBS and emulsified in FCA. The preparation of M. chelonii plus SA employed similar protocol was followed except that the V. anguillarum cells were replaced with M. chelonii.

B. Conjugation with Glutaraldehyde: The method followed was that of Avrameas (1969). Washed, pelleted [the pellet obtained after 5 ml of a 1.0 O. D. (500 nm) cell suspension was centrifuged] bacterial cells (heat killed R. salmoninarum) were added to Vibrio anguillarum extract (VE) (5 ml of a 0.260 mg/ml preparation), mixed well and 25% glutaraldehyde (Sigma, St. Louis, MO) was added to the mixture (10 µl/ml of the mixture). The mixture was allowed to incubate for two hours on ice with occasional stirring. The preparation was then washed three times with PBS by centrifuging at 4000 x g at 4°C for 30 min. Finally the pellet was resuspended to the original volume (5 ml) of the bacterial suspension.

Immunization Procedures and Collection of Sera

The primary screening for prophylactic efficiency assessed the effectiveness of SA alone, V. anguillarum cells plus SA, heat killed R. salmoninarum (HKDB), HKDB plus VE and FCA only. The fish (30 animals per group) were injected intraperitoneally with 0.1 ml of each preparation. When Freund's complete adjuvant (FCA) was employed, it was emulsified 1:1 (v/v) with the vaccine preparation. The secondary injections, comprising the same amounts of preparations, were performed by resuspending the materials in PBS only. These preparations were administered 30 days after the primary injection. The formal immunizations incorporated triplicated tanks each containing 35 individual fish for each preparation of M. chelonii alone, M. chelonii plus SA, polysaccharide alone groups, and non-injected controls. Prior to live challenge of these groups, two fish were sacrificed from each group and were bled through caudal vein. The serum was collected and stored at -20°C until titrations were performed.

Bacterial Challenge

Renibacterium salmoninarum was grown in KDM-2 medium (Evelyn, 1977) on shake flasks at 17°C for 10 to 14 days. When the culture attained late log phase, the O. D. (500 nm) of the cells was adjusted to an O. D. of 1.0 unit on a

Spectronic 20 spectrophotometer (Baush & Laumb). Fish were challenged, 30 days after their secondary injection, with an intraperitoneal injection of 0.1 ml of this culture suspension. Mortalities were recorded daily.

RESULTS

Antibody Titration of Sera from Vaccinated Fish

The anti-SA antibody titers of the vaccinated fish sera varied considerably between groups. Fish sera were collected from two fish and the average values obtained for the two fish are depicted in Fig. 8.1. Soluble antigen alone or coupled with formalin killed Vibrio cells demonstrated higher antibody titers compared to the other test immunogens. Uninjected controls and those individuals injected with Freund's complete adjuvant (FCA) alone did not exhibit appreciable antibody titers.

Agglutination Titers of Sera from Vaccinated Fish

The sera used for specific antibody estimation were also screened to estimate bacterial agglutination titers (Table 8.1). No appreciable difference between the vaccinated groups and controls were apparent.

Survival Studies of Vaccinated Fish after Challenge

Fish were challenged 60 days post secondary immunization in the preliminary screening studies, with live R. salmoninarum (suspension at 0.5 O. D. at 500 nm; 0.1 ml/fish). The mortalities began to appear by day 53 after challenge and mortalities were recorded on each subsequent day. The percent survival for each group during the time allotted for the challenge are depicted in Figs. 8.2-8.9. Relative percent survival was calculated (Table 8.2) for these groups as suggested by Amend (1981). A single injection (1°) or two injections (2°) of heat-killed kidney disease bacterium (HKDB) or heat-killed R. salmoninarum alone [RPS of 0 (1°) and 29 (2°) on day 104] and Vibrio anguillarum extract (VE)-coupled HKDB [RPS of 4 (1°) and 5 (2°) on day 104] demonstrated no appreciable affect on survival compared to controls. Soluble antigen (SA) alone [RPS of 29 (1°) and 20 (2°) on day 104], SA plus Vibrio anguillarum cells [RPS of 57 (1°) and 34 (2°) on day 104], and FCA alone [RPS of 67 (1° and 2°) on day 104] demonstrated better protection, compared to controls. One intriguing observation is that FCA alone appears to offer the best protection against the challenge. The formal immunization studies demonstrated equivalent and statistically significant protection given by M. chelonii alone (RPS of 67 at day 35 but of 7 at day 55) and soluble antigen coupled to M. chelonii (RPS of 67 at day 35 but of 6 at day 55) but poor or no protection was

conferred by the polysaccharide alone group (RPS of 22 on day 35 and 2 on day55).

DISCUSSION

This study was designed to assess the effectiveness of various forms of antigen and adjuvants for the prophylactic treatment of BKD. The preliminary screening utilized different immunogens incorporated in Freund's complete adjuvant (FCA) as the vehicle. It was of particular interest to note that FCA alone conferred the best protection (RPS of 67 on day 104 after challenge) (Table 8.2) followed by V. anguillarum cells plus SA and SA alone. Heat killed R. salmoninarum resulted in the least protection. This finding suggests that non-specific cellular immunity may be the most protective form of immunity. Such non-specific prophylactic effects by FCA have previously been observed in salmonid fish (Olivier et al. 1985). Furthermore, the incorporation of SA together with FCA resulted in an obvious decrease in the RPS [67 (FCA 2^o) vs 20 (SA + FCA, 2^o) on day 104] (Table 8.2). This difference suggests that SA may have toxic properties and thus if injected prior to challenge may exacerbate the artificial challenge. Indeed this exacerbation appeared to diminish the protective value of FCA alone as evidenced by the decrease in RPS. Such toxicity does seem likely in view of studies revealing its immunosuppressive effects on salmonid lymphocytes in vitro (Turaga et al. 1987b), its

association with decreasing hematocrit values during infection (Turaga et al. 1987), hemagglutination activity (Daly and Stevenson, 1987) and leukoagglutination properties (Wiens and Kaattari, manuscript in preparation). Alternatively, soluble antigen may also be responsible for such exacerbation by inducing the formation of immune complexes, and thus be responsible for the pathology seen in BKD (Turaga et al., manuscript in preparation). If this is indeed the case, the generation of antibody response to SA may in itself be detrimental to prophylaxis. This may suggest why no correlation of antibody titer with protection was observed. In fact FCA demonstrated the lowest antibody titer by ELISA and yet demonstrated the greatest degree of protection.

Based on the preliminary screening studies M. chelonii (a salmonid mycobacterium) was used in place of the killed M. tuberculosis, H37Rv, more commonly used in FCA. Incorporation of the M. tuberculosis in the mineral oil adjuvant was cost prohibitive and thus M. chelonii, was substituted for M. tuberculosis and was used without the mineral oil vehicle. Use of this preparation in an aqueous suspension would facilitate future large scale immunization procedures such as by bath or hyperosmotic infiltration techniques (Smith,1988). Furthermore, such modifications of FCA preparation protocols have previously been demonstrated to still be prophylactic for other fish diseases (Olivier et al. 1985). When these researchers used M. butyricum in mineral oil alone, it was found to confer protection against

lethal a challenge of Aeromonas salmonicida. In our studies, M. chelonii in aqueous form demonstrated significant protection on day 35, but no difference in RPS after 55 days (Table 8.3). The statistically significant delay in the death rate is felt to be an important indication of protection since intraperitoneal injection of the live pathogen, although considered by many to be an excessive and unnatural challenge procedure, it is currently the only reliable means by which to secure an artificial infection. It is reasonable to assume that because protection is afforded by M. chelonii alone, non-specific cellular immunity may play an important role in controlling BKD.

ACKNOWLEDGEMENTS

This work was supported by Bonneville Power Administration contract No. DEAI79-87BP16480.

Fig. 8.1. Antibody titration of vaccinated fish sera. ELISA titers are depicted as average values of sera from two fish. The sera were diluted 1/20 and 1/80 in TTBS. FCA, Freund's complete adjuvant; KDSA, soluble antigen; Vib. cells, formalin killed Vibrio anguillarum cells; Vib. ext., V. anguillarum extract; HKDB, heat killed kidney disease bacterium (R. salmoninarum) were the immunogens. ^aPrimary injection and ^bsecondary injection.

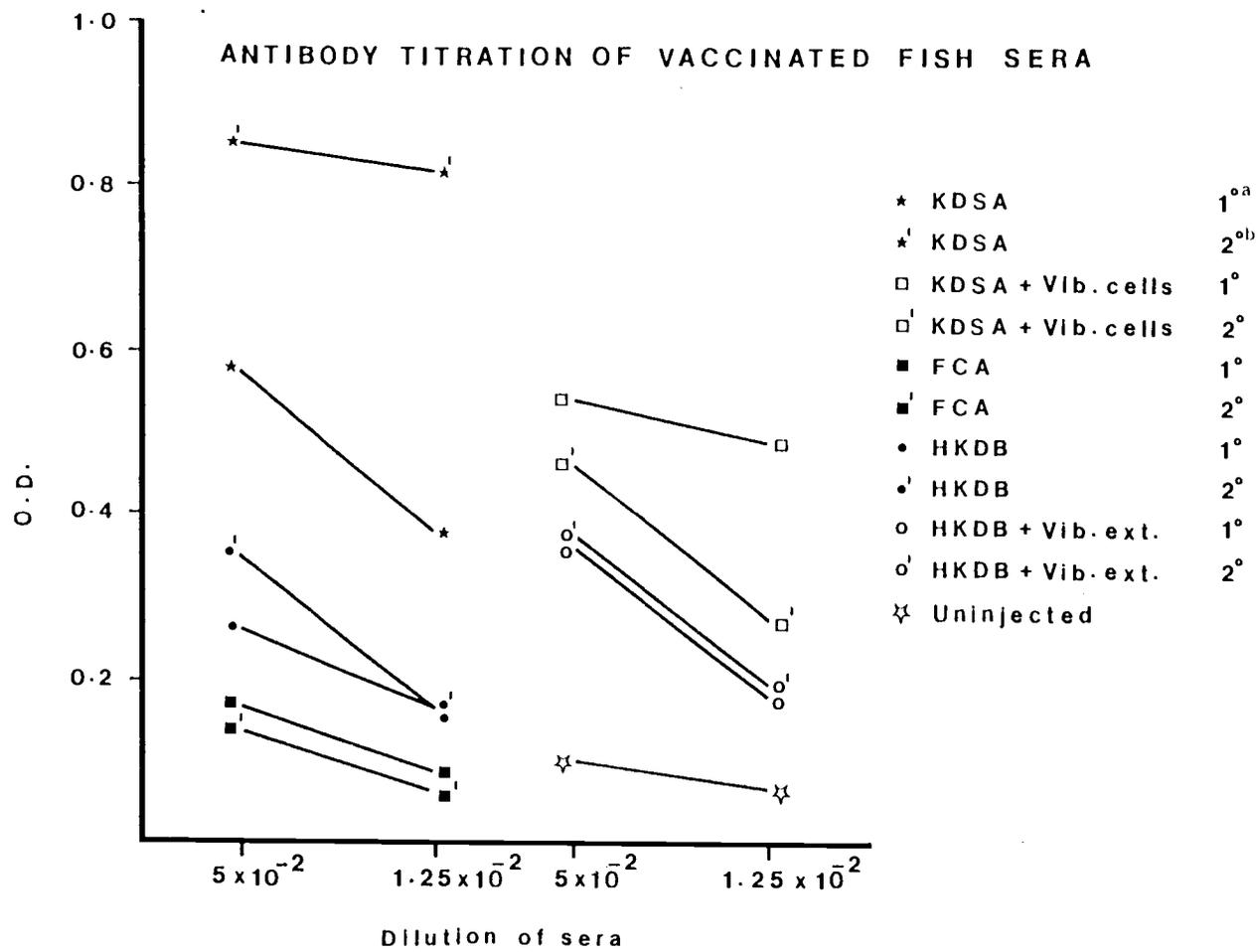


TABLE 8.1. AGGLUTINATION TITERS OF VACCINATED FISH SERA

Serum source	Titer
FCA (1°) ^a	1:64
FCA (1° FCA/ 2° PBS) ^b	1:64
SA (1°)	1:64
SA (2°)	1:128
Vib. cells + SA (1°)	1:128
Vib. cells + SA (2°)	1:128
VE + HKDB (1°)	1:128
VE + HKDB (2°)	1:128
HKDB (1°)	1:128
HKDB (2°)	1:128
Uninjected control	1:64

a = titer, 60 days post primary injection

b = titer, 30 days post secondary injection

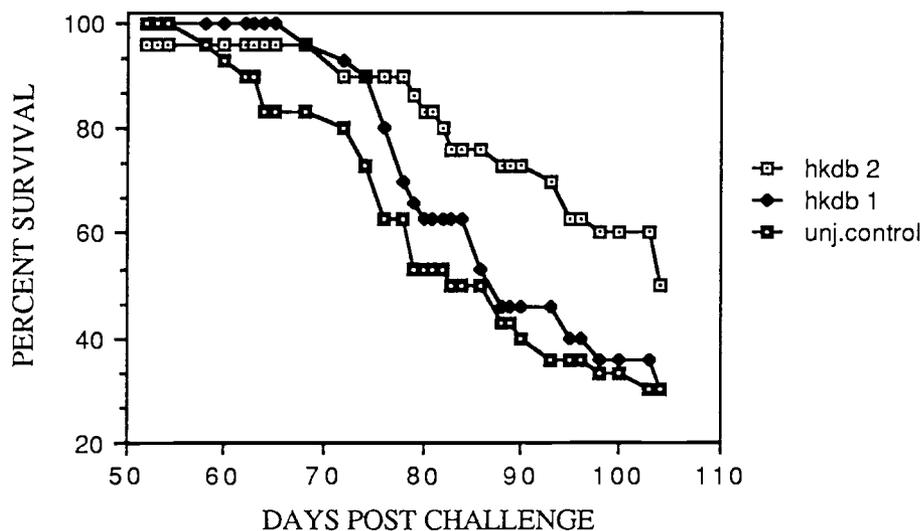
FCA = Freund's complete adjuvant

SA = soluble antigen produced by R. salmoninarum

Vib. cells = formalin killed Vibrio anguillarum cells

VE = V. anguillarum extract

HKDB = heat killed kidney disease bacterium (R. salmoninarum)



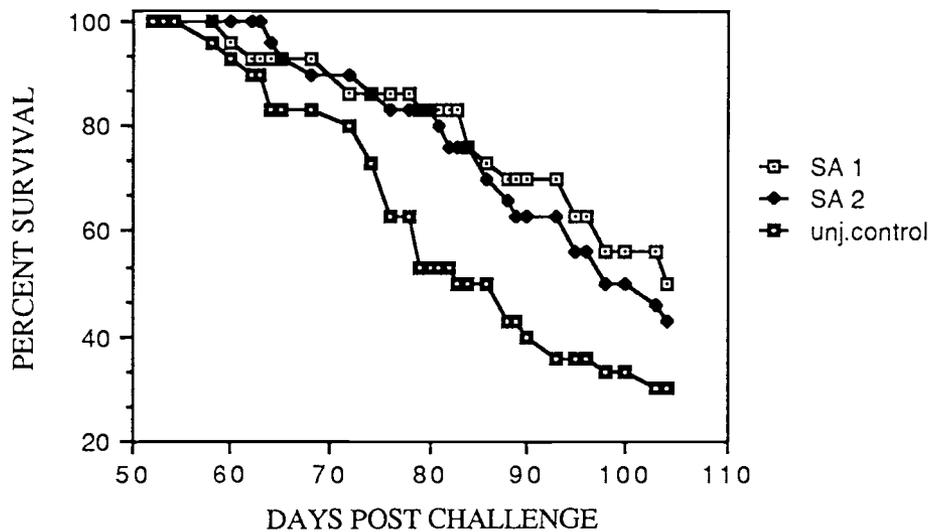


Fig. 8.3. Vaccine trial with soluble antigen (SA). Percent survival vs no. of days post challenge was depicted. Legend 1 and 2 indicate primary and booster injected groups and unj. control is uninjected control group. Mortalities began to appear on day 52, post challenge.

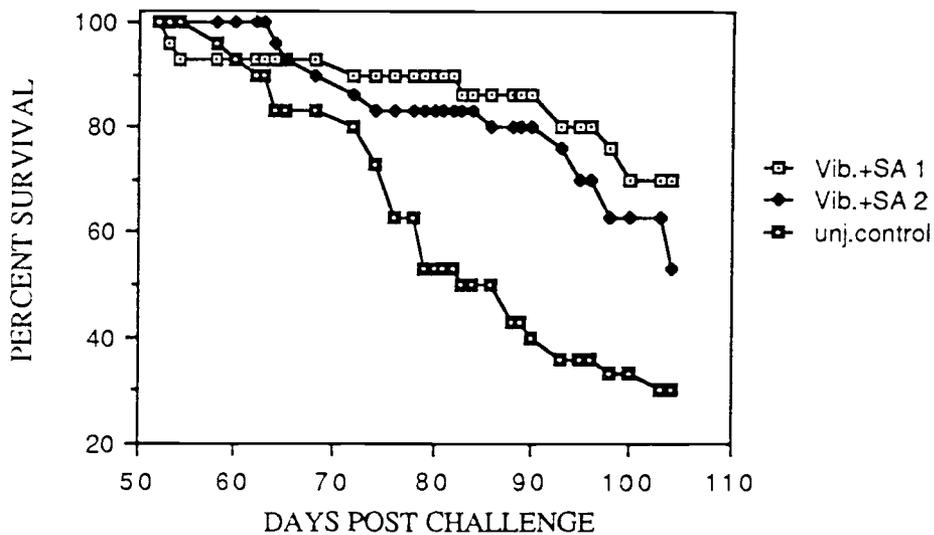


Fig. 8.4. Vaccine trial with *Vibrio* cells conjugated with SA via tannic acid. Percent survival vs no. of days post challenge was depicted. Legends 1 and 2 indicate primary and booster injected groups and unj. control is uninjected control group. Mortalities began to appear on day 52, post challenge.

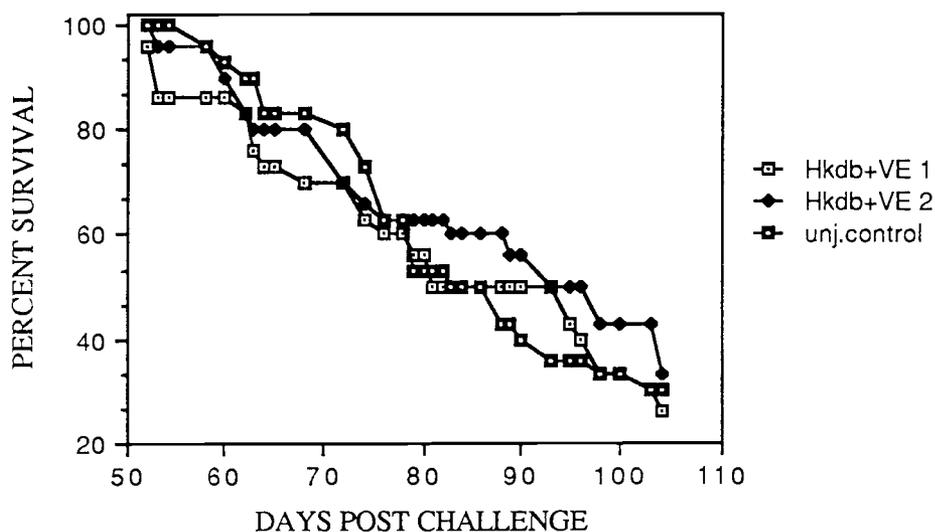


Fig. 8.5. Vaccine trial with heat killed kidney disease bacterium (HKDB) (*R. salmoninarum*) conjugated to *Vibrio* extract (VE) via glutaraldehyde. Percent survival vs no. of days post challenge was depicted. Legends 1 and 2 indicate primary and booster injectioned groups and unj. control is uninjected control group. Mortalities began to appear on day 52, post challenge.

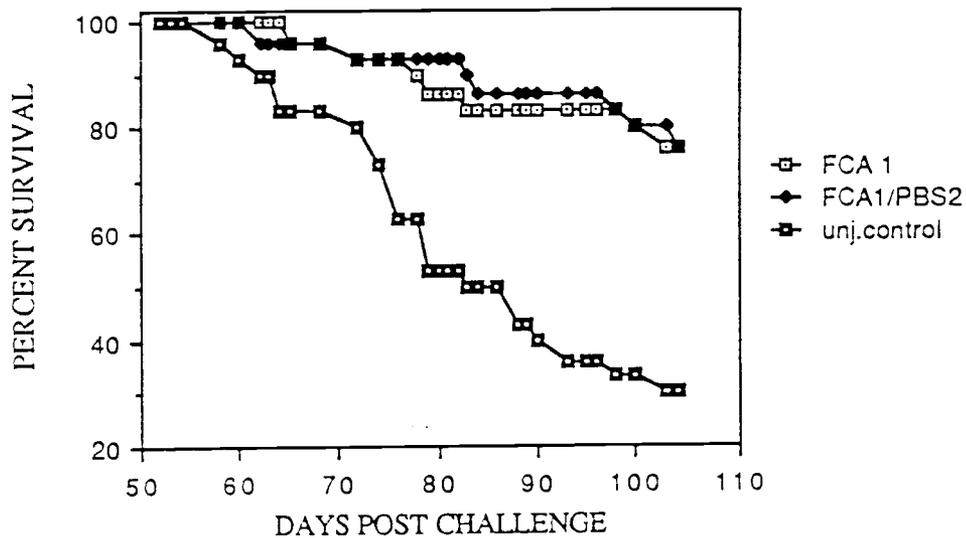


Fig. 8.6. Vaccine trial with Freund's complete adjuvant (FCA) alone. Percent survival vs no. of days post challenge was depicted. Legends 1 and 2 indicate primary and booster injected groups and unj. control is uninjected control group. The booster injection consisted of the buffer, PBS alone. Mortalities began to appear on day 52, post challenge.

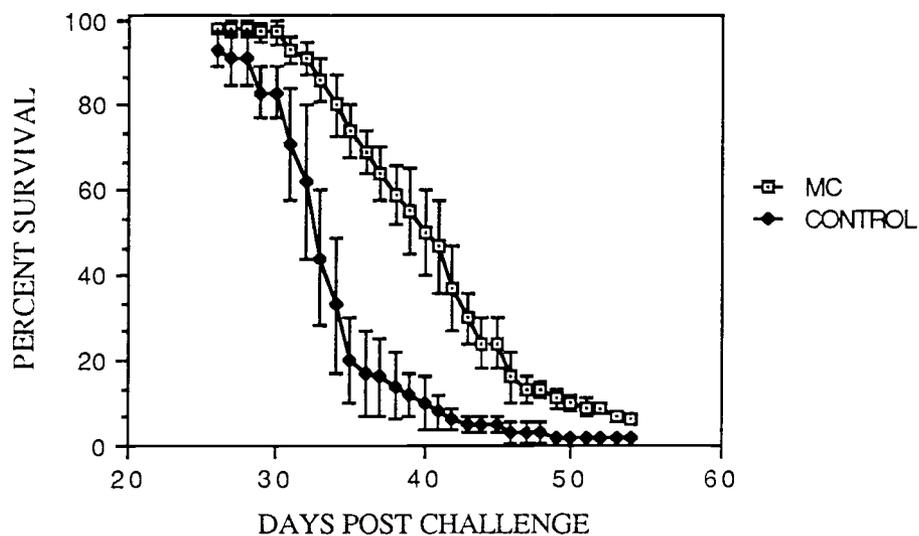


Fig. 8.7. Vaccine trial with heat killed M. chelonii (MC). Percent survival vs no. of days post challenge was depicted. Each point represents an average of triplicate tanks and one S.E. about the mean. Mortalities began to appear on day 26, post challenge.

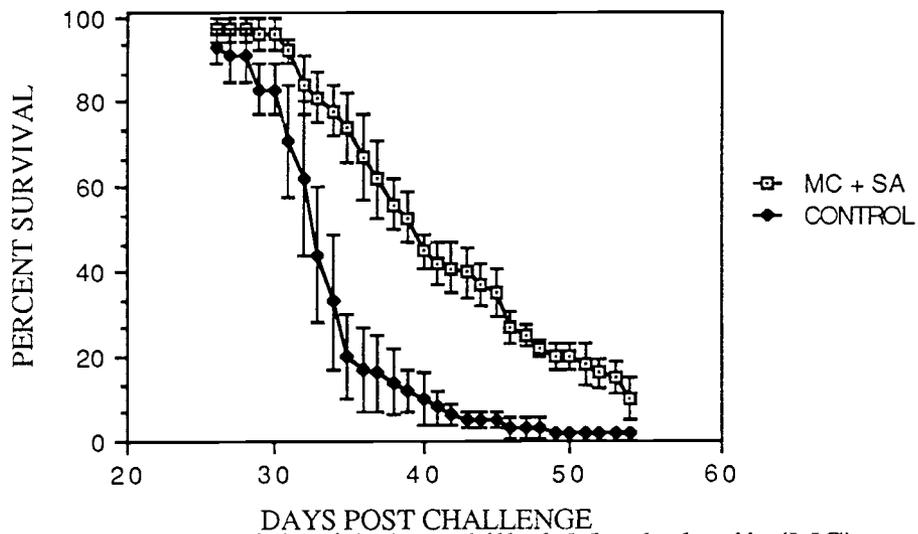


Fig. 8.8. Vaccine trial with heat killed *M. chelonii* (MC) coupled with soluble antigen (SA). Percent survival vs no. of days post challenge was depicted. Each point represents an average of triplicate tanks and one S.E. about the mean. Mortalities began to appear on day 26, post challenge.

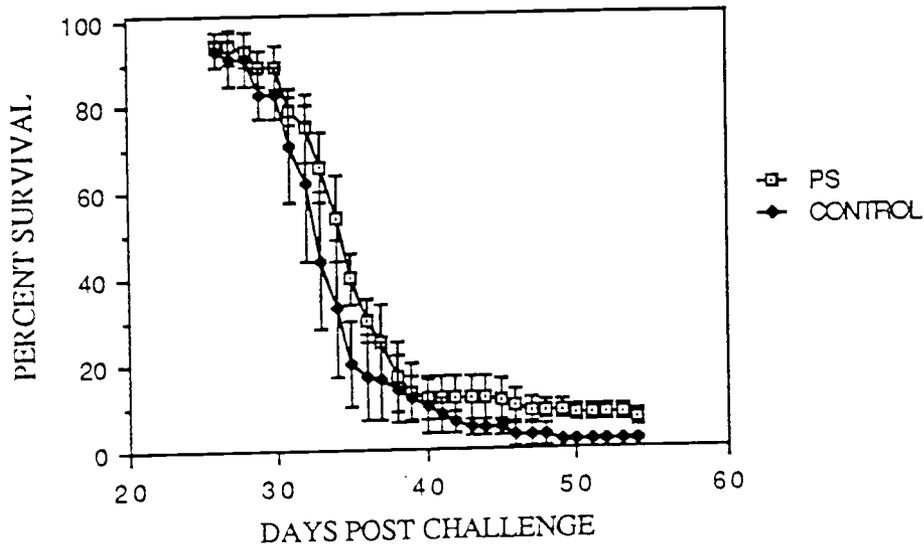


Fig. 8.9. Vaccine trial with polysaccharide (PS) extracted from R. salmoninarum. Percent survival vs no. of days post challenge was depicted. Each point represents an average of triplicate tanks and one S.E. about the mean. Mortalities began to appear on day 26, post challenge.

Table 8.2. RPS¹ values of preliminary screening.

Vaccine group	Day 80 ²	Day104 ³
FCA 1 ^o	85.7	67
FCA 1 ^o /PBS 2 ^o	71.0	67
Vib. cells + SA 1 ^o	78	57
Vib. cells + SA 2 ^o	64	34
SA 1 ^o	64	29
SA 2 ^o	64	20
HKDB 1 ^o	21	0
HKDB 2 ^o	64	29
VE + HKDB 1 ^o	6	4
VE + HKDB 2 ^o	21	5

1. RPS (relative percent survival) = $1 - \frac{\% \text{ mortality in vaccine gr.}}{\% \text{ mortality in control gr.}} \times 100$

2. day 80 post challenge

3. day 104 post challenge

FCA = Freund's complete adjuvant

Vib. cells = formalin killed V. anguillarum cells

SA = soluble antigen produced by R. salmoninarum

VE = V. anguillarum extract

HKDB = heat killed R. salmoninarum

Table 8.3. RPS¹ values for formal immunizations.

Vaccine group	Day 35 ²	Day 55 ³
<u>M. chelonii</u> alone	67	7
<u>M. chelonii</u> + SA	67	6
Polysaccharide	22	2

1. RPS (relative percent survival) = $1 - \frac{\% \text{ mortality in vaccine gr.}}{\% \text{ mortality in control gr.}} \times 100$

2. day 35 post challenge

3. day 55 post challenge

REFERENCES

- Amend, D. F. 1981. Potency testing of fish vaccines. *Dev. Biol. Standard.* 49: 447-454.
- Averameus, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. *Immunochem.* 6: 42-52.
- Belding, D. L., and B. Merrill. 1935. A preliminary report upon a hatchery disease of the salmonidae. *Trans. Am. Fish. Soc.* 65: 76-84.
- Bruno, D. W. 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L., with reference to naturally infected fish. *J. Fish Dis.* 9: 523-537.
- Campbell, D. H., Garvey, J. S., Cremer, N. E., and D. H. Sussdorf. 1970. *Methods in Immunology*. Second Edn. W. A. Benjamin, Inc., Massachusetts.
- Daly, J. G., and R. M. W. Stevenson. 1987. Hydrophobic and hemagglutinating properties of Renibacterium salmoninarum. *J. Gen. Micro.* 133: 3575-3580.
- Daly, J. G., and R. M. W. Stevenson. 1987. Survival of Renibacterium salmoninarum in salmonid macrophages. *Fish Immunology Symposium*, Plymouth Polytechnique, Plymouth, England. Abstracts.
- DeLuca, D., Wilson, M. R., and G. W. Warr. 1983. Lymphocyte heterogeneity in the trout Salmo gairdneri, defined with monoclonal antibodies to IgM. *Eur. J. Immunol.* 10: 546-551.
- Evelyn, T. P. T., Ketcheson, J. E., and L. Prosperi-Porta. 1988. Trials with anti-bacterial kidney disease vaccines in two species of pacific salmon. *International Fish Health Conference*, Vancouver, B. C., Canada. Abstracts.
- Fiedler, F., and R. Draxl. 1986. Biochemical and immunochemical properties of the cell surface of Renibacterium salmoninarum. *J. Bacteriol.* 168: 799-804.

Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. *Ann. Rev. Microbiol.* 35: 273-298.

Kendall, C., Ionesca-Matiu, I., and R. G. Dressman. 1983. Utilization of the biotin/avidin system to amplify the sensitivity of the enzyme linked immunosorbent assay (ELISA). *J. Imm. Methods.* 56: 329-339.

Lall, S. P., Paterson, W. D., Hines, J. A., and N. J. Adams. 1985. Control of bacterial kidney disease in Atlantic salmon, Salmo salar L., by dietary modification. *J. Fish Dis.* 8: 113-124.

Lowry, O. H., N. J. Rosebrough, D. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 205-276.

McCarthy, D. H., Croy, T. R., and D. F. Amend. 1984. Immunization of rainbow trout, Salmo gairdneri Richardson, against bacterial kidney disease: preliminary efficacy evaluation. *J. of Fish Dis.* 7: 65-71.

Mitchum, D. L., Sherman, L. E., and G. T. Baxter. 1979. Bacterial kidney disease in feral populations of brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri). *J. Fish. Res. Board Can.* 36: 1370-1376.

Olivier, G., Evelyn, T. P. T., and R. Laaier. 1985. Immunity to Aeromonas salmonicida in coho salmon (Oncorhynchus kisutch), induced by modified Freund's complete adjuvant: its non specific nature and the probable role of macrophages in the phenomenon. *Dev. Comp. Immunol.* 9: 419-432.

Paterson, W. D., Desautels, D., and J. M. Weber. 1981. The immune response of Atlantic salmon, Salmo salar L., to the causative agent of bacterial kidney disease, Renibacterium salmoninarum. *J. Fish Dis.* 4: 99-111.

Paterson, W. D., Lall, S. P., Airdrie, D., Greer, P., Greenham, G., and M. Poy. 1985. Prevention of disease in salmonids by vaccination and dietary modification. *Fish Path.* 20: 427-434.

Perkins, H. R. 1965. The action of hot formamide on bacterial cell walls. *Biochem. J.* 95: 876-882.

Sanders, J. E., and J. L. Fryer. 1980. Renibacterium salmoninarum gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. *Int. J. Syst. Bact.* 30: 496-502.

Schleifer, K. H., and O. Kandler. 1967. Zur chemischen Zusammensetzung der Zellwand der Streptokokken. I. Zur Aminosäuresequenz des Mureins von *Str. thermophilus* and *Str. faecalis*. *Arch. Mikrobiol.* 57: 335-365.

Smith, P. D. 1988. Vaccination against vibriosis. In *Fish Vaccination*. Ed. A. E. Ellis. Academic Press. pp 67.

Turaga, P. S. D., Wiens, G. W., and S. L. Kaattari. 1987a. Analysis of Renibacterium salmoninarum antigen production in situ. *Fish Path.* 22: 209-214.

Turaga, P. S. D., Wiens, G. W., and S. L. Kaattari. 1987b. Bacterial kidney disease: the potential role of soluble protein antigen(s). *J. Fish. Biol.* 31 (supplement A): 191-194.

Voller, A., Bidwell, D. E., and A. Bentlett. 1976. Enzyme immunology in diagnostic medicine (Theory and Practice). *Bull. World Health Organ.* 53: 55-65.

Wolf, K., and C. E. Dunbar. 1959. Test of 34 therapeutic agents for control of kidney disease in trout. *Trans. Amer. Fish. Soc.* 88: 117-124.

Wood, J. W., and J. Wallis. 1955. Kidney disease in adult chinook salmon and its transmission by feeding young chinook salmon. *Res. Briefs, Fish. Comm. Oregon.* 6: 32-40.

Young, C. L., and G. B. Chapman. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). *J. Fish. Res. Board Can.* 35: 1234-1248.

Yui, M. A., and S. L. Kaattari. 1987. Vibrio anguillarum antigen stimulates mitogenesis and polyclonal activation of salmonid lymphocytes. Dev. Comp. Immunol. 11: 539-549.

BIBLIOGRAPHY

- Allison, L. N. 1958. Multiple sulfa therapy of kidney disease among brook trout. *Prog. Fish. Cult.* 20:66-68.
- Amend, D. F. 1981. Potency testing of fish vaccines. *Dev. Biol. Standard.* 49: 447-454.
- Arakawa, C. K., Sanders, J. E., and J. L. Fryer. 1987. Production of monoclonal antibodies against Renibacterium salmoninarum. *J. Fish Dis.* 10:249-253.
- Austin, B., and J. N. Rayment. 1985. Epizootiology of Renibacterium salmoninarum, the causal agent of bacterial kidney disease in salmonid fish. *J. Fish Dis.* 8:505-509.
- Austin, B., Embley, T. M., and M. Goodfellow. 1983. Selective isolation of Renibacterium salmoninarum. *FEMS Microbiology Letters.* 17:111-114.
- Austin. 1985. Evaluation of antimicrobial compounds for the control of bacterial kidney disease in rainbow trout, Salmo gairdneri Richardson. *J. Fish. Dis.* 8:209-220.
- Averameus, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. *Immunochem.* 6: 42-52.
- Awakura, T. 1978. Bacterial kidney disease in mature salmonid. *Fish Pathology* 13:53-54.
- Banner, C. R., Long, J. J., Fryer, J. L., and J. S. Rohovec. 1986. Occurrence of salmonid fish infected with Renibacterium salmoninarum in the Pacific ocean. *J. of Fish Dis.* 9:273-275.
- Banner, C. R., Rohovec, J. S., and J. L. Fryer. 1982. A rapid method for labeling rabbit immunoglobulin with fluorescein for use in detection of fish pathogens. *Bull. Eur. Assoc. Fish Path.* 2:35-37.
- Banner, C. R., Rohovec, J. S., and J. L. Fryer. 1983. Renibacterium salmoninarum as a cause of mortality among chinook salmon in salt water. *J. World Maricul. Soc.* 14:236-239.

Banowitz, G. M. 1974. Certain aspects of the immunology and chemotherapy of bacterial kidney disease in juvenile coho salmon (Oncorhynchus kisutch). M. S. Thesis. Oregon State University, Corvallis, OR. U.S.A. 53 pp.

Belding, D. L., and B. Merrill. 1935. A preliminary report upon a hatchery disease of the salmonidae. Trans. Am. Fish. Soc. 65:76-84.

Bell, G. R. 1961. Two epidemics of apparent kidney disease in cultured pink salmon (Oncorhynchus gorbuscha). J. of Fish Res. Board Can. 18:559-562.

Bell, G. R., and G. S. Traxler. 1986. Resistance of the pacific lamprey, Lampetra tridentata (Gairdner), to challenge by Renibacterium salmoninarum, the causative agent of kidney disease in salmonids. J. Fish Dis. 9: 277-279.

Bell, G. R., Hoffmann, R. W., and L. L. Brown. 1988. Pathology of experimental infections of the sablefish (Anoploma fimbria) with Renibacterium salmoninarum, the agent of bacterial kidney disease in salmonids. International Fish Health Conference, Vancouver, B. C., Canada. Abstracts: p. 31.

Bruno, D. W. 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L., with reference to naturally infected fish. J. Fish Dis. 9:523-537.

Bruno, D. W. 1988. The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen Renibacterium salmoninarum. FEMS Microbiology Letters. 51: 135-140.

Bruno, D. W., and A. L. S. Munro. 1982. Detection of the causative agent of bacterial kidney disease. Bull. Eur. Ass. Fish Path. 2:10-12.

Bruno, D. W., and A. L. S. Munro. 1986. Hematological assessment of rainbow trout, Salmo gairdneri Richardson, and Atlantic salmon, Salmo salar L., infected with Renibacterium salmoninarum. J. Fish Dis. 9:195-204.

Bruno, D. W., and A. L. S. Munro. 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. *FEMS Microbiology Letters*. 33: 247-250.

Bruno., D. W. 1986. Changes in serum parameters of rainbow trout, *Salmo gairdneri*, Richardson, and Atlantic salmon, *Salmo salar* L., infected with *Renibacterium salmoninarum*. *J. of Fish Dis.* 9:205-211.

Bullock, G. L., and H. M. Stuckey. 1975. Fluorescent antibody identification and detection of the *Corynebacterium* causing kidney disease of salmonids. *J. Fish. Res. Board Can.* 32:2224-2227.

Bullock, G. L., Stuckey, H. M., and Wolf, K. 1975. Bacterial kidney disease of salmonid fishes. Fish and Wildlife Service, Fish Disease Leaflet No. 41. U. S. Department of the Interior, Washington, D.C.

Bullock, G. L., Stuckey, H. M., D. Mulcahy. 1978. Corynebacterial kidney disease: Egg transmission following iodophore disinfection. *Fish Health News*. 7:51-52.

Campbell, D. H., Garvey, J. S., Cremer, N. E., and D. H. Sussdorf. 1970. *Methods in Immunology*. Second Edn. W. A. Benjamin, Inc., Massachusetts.

Chen, P. K., Bullock, G. L., Stuckey, H. M., and A. C. Bullock. 1974. serological diagnosis of corynebacterial kidney disease of salmonids. *J. Fish. Res. Board Can.* 31:1939-1940.

Cipriano, R. C., Starliper, C. E., and J. H. Schachte. 1985. Comparative sensitivities of diagnostic procedures used to detect bacterial kidney disease in salmonid fishes. *J. Wild. Dis.* 21:144-148.

Cochrane, C. G. and D. Koffler. 1973. Immune complex Disease in Experimental Animals and Man. *Adv. Immunol.* 16: 185-264.

- Croy, T. R., and D. F. Amend. 1977. Immunization of sockeye salmon (Oncorhynchus nerka) against vibriosis using hyperosmotic in filtration. *Aquaculture*. 12:317-325.
- Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology*. 14: 599.
- Cusak, R., and D. K. Cone. 1986. A review of parasites as vectors of viral and bacterial diseases of fish. *J. of Fish Dis.* 9:169-171.
- Daly, J. G., and R. M. W. Stevenson. 1987. Hydrophobic and haemagglutinating properties of Renibacterium salmoninarum. *J. Gen Micro.* 133: 3575-3580.
- Daly, J. G., and R. M. W. Stevenson. 1987. Survival of Renibacterium salmoninarum in salmonid macrophages. *Fish Immunology Symposium*, Plymouth Polytechnique, Plymouth, England. Abstracts.
- Daly, J. G., and Stevenson, R. M. W. 1984. Charcoal agar: a new growth medium for the fish disease bacterium, Renibacterium salmoninarum. *Appl. and Env. Microb.* 50:868-871.
- DeLuca, D., Wilson, M. R., and G. W. Warr. 1983. Lymphocyte heterogeneity in the trout Salmo gairdneri, defined with monoclonal antibodies to IgM. *Eur. J. Immunol.* 10: 546-551.
- Dick, H. M., and E. Kirkwood. 1984. Immunity to infection-hypersensitivity states and infection. In Topley and Wilson's *Principles of Bacteriology, Virology and Immunity*. Volume I, 7 Ed. Williams and Wilkins, Baltimore. p 374-401.
- Dixon, P. F. 1987. Detection of Renibacterium salmoninarum by the enzyme-linked immunosorbent assay (ELISA). *J. Appl. Ichthyol.* 3:77-82.
- Ehlinger, N. F. 1963. Kidney disease in lake trout complicated by lymphosarcoma. *Progressive Fish Culturist.* 25:3-7.

Elliott, D. G., and T. Y. Barila. 1987. Membrane filtration fluorescent antibody staining procedure for detecting and quantifying Renibacterium salmoninarum in coelomic fluid of chinook salmon (Oncorhynchus tshawytscha). Can. J. Fish. Aquat. Sci. 44:206-210.

Ellouz, F., Adam, A., Ciorbaru, R., and E. Lederer. 1974. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. Biochem. Biophys. Res. Commun. 59:1317-1325.

Embley, T. M., Goodfellow, M., Minnikin, D. E., and B. Austin. 1983. Fatty acid, isoprenoid quinone and polar lipid composition in the classification of Renibacterium salmoninarum. J. Appl. Bact. 55:31-37.

Evelyn, T. P. T. 1971. The agglutinin response in sockeye salmon vaccinated intraperitoneally with a heat-killed preparation of the bacterium responsible for salmonid kidney disease. J. Wild. Dis. 7: 328-335.

Evelyn, T. P. T. 1977. An improved growth medium for the Kidney disease bacterium and some notes on using the medium. Bull. Off. Int. Epiz. 87: 511-513.

Evelyn, T. P. T., Ketcheson, J. E., and L. Prospero-Porta. 1984. Further evidence for the presence of Renibacterium salmoninarum in salmonid eggs and for the failure of poridone-iodine to reduce the intra-ovum infection rate in water hardened eggs. J. of Fish Dis. 7:173-182.

Evelyn, T. P. T., Ketcheson, J. E., and L. Prospero-Porta. 1986. Use of erythromycin as a means of preventing vertical transmission of Renibacterium salmoninarum. Dis. Aqua. Organ. 2:7-11.

Evelyn, T. P. T., Ketcheson, J. E., and L. Prospero-Porta. 1988. Trials with anti-bacterial kidney disease vaccines in two species of pacific salmon. International Fish Health Conference, Vancouver, B. C., Canada. Abstracts: p. 38.

Fiedler, F., and R. Draxl. 1986. Biochemical and immunochemical properties of the cell surface of Renibacterium salmoninarum. J. of Bact. 168:799-804.

Fryer, J. L., and J. E. Sanders. 1981. Bacterial kidney disease of salmonid fish. *Ann. Rev. Microbiology.* 35: 273-298.

Fuller, D. W., Pilcher, K. S., and J. L. Fryer. 1977. A leukocytolytic factor isolated from cultures of *Aeromonas salmonicida*. *J. Fish. Res. Board Can.* 34: 1118-1125.

Getchell, R. G., Rohovec, J. S., and J. L. Fryer. 1985. Comparison of *Renibacterium salmoninarum* isolates by antigenic analysis. *Fish Path.* 20:149-159.

Goodfellow, M., Collins, M. D., and D. E. Minnikin. 1976. Thin-layer chromatographic analysis of mycolic acids and other long chain components in whole organism methanolysates of coryneform and related taxa. *J. of Gen. Micro.* 96:351-358.

Groman, D. B. 1981. The detection of precipitating antibody to *Renibacterium salmoninarum* in chinook salmon by counter immunoelectrophoresis. *Dev. Biol. Stand.* 49:189-192.

Hastings, T. S., and A. E. Ellis. 1988. The humoral immune response of rainbow trout, *Salmo gairdneri* Richardson, and rabbits to *Aeromonas salmonicida* extracellular products. *J. Fish Dis.* 11: 147-160.

Hendricks, J. & Leek, S.L. 1975. Kidney disease pastorbital lesions in spring chinook salmon (*Oncorhynchus tshawytscha*). *Trans. Am. Fish. Soc.* 104, 805-807.

Hicks, B. D., Daly, J. G., and V. E. Ostland. 1986. Experimental infection of minnows with the bacterial kidney disease bacterium. Aquaculture Association of Canada meeting, University of Guelph, Canada. Abstracts: p 3-7.

Hunn, J.B. 1964. Some patho-physiologic effects of bacterial kidney disease in brook trout. *Proc. Soc. exp. Biol. Med.* 117, 383-385.

Izui, S., and G. Lange. 1988. Enzyme-linked immunosorbent assay for detection of retroviral gp70 and gp70-anti-gp70 immune complexes in sera from SLE mice. *Clin. Exp. Immunol.* 71: 45-49.

- Jacobs, D. M., and D. C. Morrison. 1975. Stimulation of T-independent primary anti-hapten response in vitro by TNP-lipopolysaccharide (TNP-LPS). *J. Immunol.* 144: 360-364.
- Kaattari, S. L., and M. A. Yui. 1987. Polyclonal activation of salmonid B lymphocytes. *Dev. Comp. Immun.* 11: 155-165.
- Kendall, C., Ionesca-Matiu, I., and R. G. Dressman 1983. Utilization of the Biotin/Avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). *J. Imm. Methods* 56: 329-339.
- Kimura, T. and M. Yoshimizu. 1981. A coagglutination test with antibody sensitized staphylococci for rapid and simple diagnostics of bacterial kidney disease (BKD). *Dev. Biol. Standard.* 49:135-148.
- Kitao, T., Yoshida, T., Anderson, D. P., Dixon, O. W., and A. Blanch. 1987. Immunostimulation of antibody-producing cells and humoral antibody fish bacterins by a biological response modifier. *Fish Immunology Symposium*, Plymouth Polytechnique, Plymouth, England. Abstracts.
- Kusser, W., and F. Fiedler. 1983. Murein type and polysaccharide composition of cell walls from Renibacterium salmoninarum. *FEMS Microbiology Letters.* 20:391-394.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.
- Laidler, L. A. 1980. Detection and identification of the bacterial kidney disease (BKD) organism by the indirect fluorescent antibody technique. *J. Fish Dis.* 3:67-69.
- Lall, S. P., Paterson, W. D., Hines, J. A., and N. J. Adams. 1985. Control of bacterial kidney disease in Atlantic salmon, Salmo salar L., by dietary modification. *J. of Fish Dis.* 8:113-124.
- Lester, R.J.G. & Budd, J. 1979. Same changes in the blood cells of diseased coho salmon. *Can. J. Zool.* 57, 1458-1464.

- Liew, F. Y. 1982. Regulation of delayed type hypersensitivity to pathogens and alloantigens. *Immunology Today*. 3:18-23.
- Lowry, O. H., Rosebrough, N. J., Farr, D. L., and R. J. Randall 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 205-276.
- Mackness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vitro. *J. Exp. Med.* 129:973-979.
- McCarthy, D. H., Croy, T. R., and D. F. Amend. 1984. Immunization of rainbow trout, Salmo gairdneri Richardson, against bacterial kidney disease: preliminary efficacy evaluation. *J. of Fish Dis.* 7:65-71.
- McKevitt, A. I., Bajaksouzian, S., Klinger, J. D., and D. E. Woods. 1989. Purification and characterization of an extracellular protease from Pseudomonas cepacia. *Infect. Immun.* 57: 771-778.
- Miller, N.W., Sizemore, R.G. & Clem, L.W. 1985. Phylogeny of lymphocyte heterogeneity: the cellular requirements for in vitro antibody responses of channel catfish leukocytes. *J. Immun.* 134, 2884-2888.
- Mitchum, D. L., and L. E. Sherman. 1981. Transmission of bacterial kidney disease from wild to stocked hatchery trout. *Can. J. Fish Aquat. Sci.* 38:547-551.
- Mitchum, D. L., Sherman, L. E., and G. T. Baxter. 1979. Bacterial kidney disease in feral populations of brook trout (Salvelinus fontinalis), brown trout (Salmo trutta) and rainbow trout (Salmo gairdneri). *J. Fish. Res. Board Can.* 36:1370-1376.
- Munro, A. L. S., and D. W. Bruno. 1988. Vaccination against bacterial kidney disease. In *Fish Vaccination*. Ed. A. E. Ellis. Academic Press. pp 125.
- Nomura, S., and H. Saito. 1982. Production of the extracellular hemolytic toxin by an isolated strain of Aeromonas salmonicida. *Bull. Jpn. Soc. Fish.* 48: 1589-1597.

Nomura, S., Fujino, M., Yamakawa, M., and E. Kawahara. 1988. Purification and characterization of salmolysin, an extracellular hemolytic toxin from Aeromonas salmonicida. J. of Bact. 170: 3694-3702.

Nottage, A. S., and T. H. Birkbeck. 1987. The role of toxins in Vibrio infections of bivalve molluscs. Aquaculture. 67: 244-246.

Olivier, G., Evelyn, T. P. T., and R. Laidler. 1985. Immunity to Aeromonas salmonicida in coho salmon (Oncorhynchus kisutch), induced by modified Freund's complete adjuvant: its non specific nature and the probable role of macrophages in the phenomenon. Dev. Comp. Immunol. 9: 419-432.

Ordal, E. J., and B. J. Earp. 1956. Cultivation and transmission of the etiological agent of kidney disease in salmonid fishes. Proc. Soc. Exp. Biol. Med. 92:85-88.

Pasco, R. J., and D. Mulcahy. 1987. Enzyme-linked immunosorbent assay for a soluble antigen of Renibacterium salmoninarum, the causative agent of salmonid bacterial kidney disease. Can. J. Fish. Aquat. Sci. 44:183-191.

Pasco, R. J., Elliott, D. G., Mallett, R. W., and D. Mulcahy. 1987. Comparison of five techniques for the detection of Renibacterium salmoninarum in adult coho salmon. Trans. Am. Fish. Soc. 116:882-890.

Paterson, W. D., Desautels, D., and J. M. Weber. 1981. The immune response of Atlantic salmon, Salmo solar L., to the causative agent of bacterial kidney disease, Renibacterium salmoninarum. J. Fish Dis. 4:99-11.

Paterson, W. D., Gallant, C., Desautels, D., and L. Marshall. 1979. Detection of bacterial kidney disease in wild salmonids in the Margaree river system and adjacent waters using an indirect fluorescent antibody technique. J. Fish. Res. Board Can. 26:1464-1468.

Paterson, W. D., Lall, S. P., Airdrie, D., Greer, P., Greenham, G., and M. Poy. 1985. Prevention of disease in salmonids by vaccination and dietary modification. Fish Path. 20:427-434.

Perkins, H. R. 1965. The action of hot formamide on bacterial cell walls. *Biochem. J.* 95: 876-882.

Phillips, H. J. 1973. Dye exclusion tests for cell viability. In *Tissue culture: Methods and applications.* Ed. Kruser, P. F., and M. K. Patterson, JR. Academic Press. p 406-408.

Pippy, J. H. C. 1969. Kidney disease in juvenile Atlantic salmon (*Salmo salar*) in the Mangaru River. *J. Fish. Res. Board Can.* 26:2535-2537.

Ridley, M. J., and D. S. Ridley. 1986. Histochemical demonstration of mycobacterial antigen, specific antibody and complement in the lesions of tuberculosis. *Histochem. J.* 18: 551-556.

Rittenberg, M. B., and K. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* 132: 575-581.

Rucker, R. R., Bernier, A. F., Whipple, W. J., and R. G. Burrows. 1951. Sulfadiazine for kidney disease. *Prog. Fish Cult.* 13:135-137.

Sakai, M., Koyama, G., and S. Atsuta. 1987. Detection of *Renibacterium salmoninarum* by a modified peroxidase-antiperoxidase (PAP) procedure. *Fish Path.* 22:1-5.

Sanders, J. E., and J. L. Fryer. 1980. *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. *Int. J. Syst. Bact.* 30: 496-502.

Sanders, J. E., and M. J. Barros. 1986. Evidence by the fluorescent antibody test for the occurrence of *Renibacterium salmoninarum* among salmonid fish in Chile. *J. of Wildlife Diseases.* 22:255-257.

Santarem, M. M. G., Porto, M. T., Ferreira, P., Soares, R., and M. P. Arala-chaves. 1987. Semi-purification of an immunosuppressor substance secreted by *Straptococcus mutans* that plays a role in the protection of the bacteria in the host. *Scand. J. Immunol.* 26: 755-761.

- Schleifer, K. H., and O. Kandler. 1967. Zur chemischen Zusammensetzung der Zellwand der Streptokokken. I. Zur Aminosäuresequenz des Mureins von Str. thermophilus und Str. faecalis. Arch. Mikrobiol. 57: 335-365.
- Shieh, H. S., and J. R. MacLean. 1975. Purification and properties of an extracellular protease of Aeromonas salmonicida, the causative agent of furunculosis. Int. J. Biochem. 6: 653-656.
- Smith, I. W. 1964. The occurrence and pathology of Dee disease. Fresh Water Salmon Fish. Res. 34:1-12.
- Stakebrandt, E., Wehmeyer, U., Nader, H., and F. Fiedler. 1988. Phylogenetic relationship of the fish pathogenic Renibacterium salmoninarum to Arthrobacter micrococcus and related taxa. FEMS Microbiology Letters. 50:117-120.
- Suzumoto, B. K., Schreck, C. B., and J. D. McIntyre. 1977. Relative resistances of three transferrin genotypes of coho salmon (Oncorhynchus kisutch) and their hematological responses to bacterial kidney disease. J. Fish. Res. Board Can. 34:1-8.
- Tittle, R. V., and M. B. Rittenberg. 1978. Expression of IgG memory response in vitro to thymus-dependent and thymus-independent antigens. Cell. Immunol. 35: 180
- Towbin, H., Staehelin, T., and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76: 4350-4354.
- Turaga, P. S. D., Wiens, G. D., and S. L. Kaattari. 1987a. Analysis of Renibacterium salmoninarum antigen production in situ. Fish Path. 22: 209-214.
- Turaga, P. S. D., Wiens, G. D., and S. L. Kaattari. 1987b. Bacterial kidney disease: the potential role of soluble protein antigen(s). J. Fish Biol. 31: 191-194.
- Voller, A., Bidwell, D. E., and A. Bentlett. 1976. Enzyme immunology in diagnostic medicine (Theory and Practice). Bull. World Health Organ. 53: 55-65.

Wiens, G. W., and S. L. Kaattari. 1989. Characterization of a leukoagglutinating and macrophage inducing protein produced by Renibacterium salmoninarum. Fish Health Section. International Fish Health Conference. Annapolis, MD. July 17-20. Abstracts.

Wiens, G. W., and S. L. Kaattari. 1989. Monoclonal antibody analysis of common surface protein(s) of Renibacterium salmoninarum. Fish Path. 24:1-7.

Williams, R. C. 1981. Immune complexes: A clinical perspective. Am. J. Med. 71: 743-745.

Wolf, K., and C. E. Dunbar. 1959. Test of 34 therapeutic agents for control of kidney disease in trout. Trans. Amer. Fish. Soc. 88:117-124.

Wolf, K., and Dunbar, C. E. 1959. Methods of infecting trout with kidney disease and some effects of temperature on experimental infections. U. S. Department of the Interior. Fish and Wildlife Serv. Spec. Sa. Rep. Fish. No. 286. 8 p.

Wood, E.M. & Yasutake, W.T. 1956. Histopathology of kidney disease in fish. Am. J. Path. 32, 845-857.

Wood, J. W., and J. Wallis. 1955. Kidney disease in adult chinook salmon and its transmission by feeding young chinook salmon. Res. Briefs, Fish. Comm. Oregon. 6:32-40.

Young, C. L., and G. B. Chapman. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 35:1234-1248.

Yui, M. A., and S. L. Kaattari. 1987. Vibrio anguillarum antigen stimulates mitogenesis and polyclonal activation of salmonid lymphocytes. Dev. Comp. Immunol. 11: 539-549.