STUDIES ON RENIBACTERIUM SALMONINARUM

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A thesis submitted for the Degree of Doctor of Philosophy

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CONTENTS

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SYNOPSIS

The cultural, serological and biochemical characteristics of laboratory maintained strains of R.salmoninarum were compared with recently isolated strains from rainbow trout (Salmo-gairdneri Richardson) which showed clinical signs of Bacterial Kidney Disease (BKD). The strains were serologically homogeneous and biochemically similar.

A smooth-rough transition during culture occurred in some strains but no specific differences between the two colony forms were detected by immunogel diffusion tests or electron microscope examination.

The cell wall amino-acid composition of the peptido glycan of R.salmoninarum was shown to be similar to that of C.pyogenes, but the organisms were completely different in other characteristics.

A challenge system was developed which allowed reliable reproduction of the disease in the target species. Parallel post-mortem and histological findings were found in both the natural and artificial infections.

Diagnostic procedures to detect the organism were evaluated and the indirect fluorescent antibody technique consistently gave the best results.

Following intraperitoneal injection of the organisms, sequential examinations, using conventional bacteriological techniques were carried out to determine subsequent growth of the organisms and development of lesions.

This work was expanded to detect S35 labelled organisms in whole body longitudinal sections by autoradiography. Radioactivity in various tissues was determined quantitatively by counting in a liquid scintillator.

The liver and spleen were found to be primary target organs, with subsequent spread to the kidney and increased localization in the gastro-intestinal mucosae. Initial high concentration of activity in the gut and swimbladder may have been the result of adherence rather than localisation. The initial uptake into the blood circulation accounts for the high count found in the heart with the decrease at 4 hrs which can be associated with the clearance of organisms from the blood.

Histological studies carried out over a similar time course did not reveal many early diagnostic changes. However, small discrete lesions were observed in the liver at 24 hours post inoculation in some fish.

The role of the inflammatory cells in the distribution of organisms in the peritoneum was examined together with the local cellular response to intramuscular exposure. Organisms rapidly spread from the peritoneal cavity, and an uptake by macrophages was demonstrated.

Intramuscular injection produced extremely rapid and progressive infiltration by inflammatory cells, predominantly macrophages and subsequent uptake of organisms by 48 hours post-injection.

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AIMS

i) To investigate the cultivation of R.salmoninarum and diagnosis of Bacterial Kidney Disease (BKD) in Salmonids, to study the variation in colonial morphology, and to carry out a comparative study between known BKD cultures and C.pyogenes.

ii) To establish a method which reliably reproduces the disease artificially in rainbow trout, and to compare the histopathological changes with those occurring in natural infection.

iii) To investigate the initial stages of the disease in order to obtain information of possible use in vaccine development.

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GENERAL INTRODUCTION

History of Bacterial Kidney Disease

Bacterial Kidney Disease (BKD) of Salmonid fish caused by R.salmoninarum has become one of the most significant diseases of farmed and wild trout and salmon, and is recognised as such in Great Britain by it's inclusion as a notifiable disease in the Diseases of Fish Act 1983.

The disease has been recognised, but unfortunately not controlled since the early days of salmonid farming.

The first reported outbreak of recognisable BKD occurred in Atlantic salmon (Salmo salar) in the rivers of Aberdeenshire, Scotland in 1930 and appeared in the second interim report of the Furunculosis Committee (Mackie, Arkwright & Pryce-Tannatt, 1933). Although the disease was first recognised at that time, it was not until 1934 that it attained serious proportions.

The results of preliminary investigations suggested that the disease was a chronic infection of bacterial origin, with an affinity for the kidney. However, fish inoculated with the suspected aetiological agent, a Gram positive bacterium, failed to produce typical signs of the disease. There was thus no evidence to indicate whether the bacteria isolated were of primary aetiological significance, secondary to some pre-existing metabolic disease, or in fact, the aetiological agent.

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In 1935 Belding and Merrill reported an outbreak of disease occurring in Salvelinus fontinalis and Salmo trutta at a Massachusetts State Fish Hatchery. The range of externally detectable signs varied from the inapparent to severe skin lesions including "blister" formation and haemorrhagic fins. The most striking feature observed was a bilateral exophthalmos referred to colloquially as "Pop-eye". The predilection site for internal lesions was the kidney and 53% of lesions were located there. These varied from small circumscribed abscesses to massive tissue damage. The liver, spleen, skeletal muscle and heart were also commonly involved and became covered with a false membrane, easily seen at necropsy. Microscopically, the kidneys showed acute and chronic lesions with extensive destruction of glomeruli and supporting structures. Examination of smears prepared from affected organs revealed large numbers of short Gram positive coccobacilli less than 1μ in length. Attempts at culture were not successful.

The disease was again recognised in Washington in 1946 and spread to New Hampshire and New York where it involved S.fontinalis, Salmo trutta and Salmo gairdneri.

During the period between 1946 and 1953 increasing numbers of outbreaks were reported, and in 1953 Earp, Ellis and Ordal referred to it as "Kidney Disease". Stocks of young salmon in hatcheries in the Pacific Northwest and trout in the Eastern United States were seriously affected. In many cases the death rates were catastrophic.

Further epizootics in wild Atlantic salmon in the River Dee in Scotland were described by Smith (1964) as "Dee Disease". She also noticed the similarity between "Dee Disease" and "Kidney Disease", and tentatively identified the organism as "Corynebacterium".

Ordal and Earp (1956) were the first to cultivate a Gram positive coccobacillus in suspensions of minced chick embryo tissue and to subculture it. They subsequently cultured the bacterium on Dorset's egg medium with and without added cysteine, and achieved greater success using a tryptose base blood agar with added cysteine (Ordal & Earp, 1956). The organism grew slowly over 7-10 days at 17°C. These workers were encouraged to test Koch's Postulates and proved that their isolate was indeed the causative agent of B.K.D. Suspensions of the organism were injected intraperitoneally into healthy yearling salmon. Death occurred 12-24 days later and characteristic Gram positive coccobacilli were seen in large numbers in smears from affected organs.

The strains isolated from trout were also shown to be indistinguishable from those isolated from salmon in which they were similarly virulent.

Many epizootics of the disease have been reported since the early 1950s.

Ajmal and Hobbs (1967) reported an outbreak of the disease in trout and salmon in the United Kingdom and isolated an organism which differed significantly in cultural characteristics from those described by Ordal and Earp (1956) and Smith (1964). At first the organism was considered to be a Listeria spp, but after intensive investigation the organism was classified as an unidentified species of Corynebacterium.

A study of the bacteria isolated from American char and rainbow trout was made by Vladik, Vitovec, Cervinka (1974) who attempted to characterize the morphological and biochemical properties of the Corynebacteria which they believed caused B.K.D. To some extent their findings corresponded with those of Ajmal and Hobbs who isolated bacteria with similar cultural, morphological and biochemical characteristics.

In 1973, B.K.D. was detected for the first time in Yugoslavia (Fijan 1977) and caused severe mortalities in one year old rainbow trout. In that year the disease was also detected and reported from Italy by Ghittino, Andruetto & Vighan (1977) (where it followed importation of coho salmon eyed eggs from North America), and from Japan by Kimura & Awakura (1977) in a severe epizootic in juvenile chinook salmon (Oncorhynchus tschawytscha, Kokanee salmon O.nerka, pink salmon O.gorbuscha and masou salmon O.masou at the Hokkaido Fish Hatchery.

De Kinkelin reported on outbreak in France in 1974, and in 1977 Millan recognised it for the first time in Spain when young salmon were moved from fresh to salt water.

Most confirmed cases of B.K.D. have been in cultured salmonids. The disease has rarely been reported in wild salmonids, but it was diagnosed and confirmed by isolation by Wood and Wallis (1955), in North America, by Smith (1964), in Scotland and by Evelyn, Hoskins & Bell (1973) in wild rainbow trout Salmo gairdneri in British Columbia.

The above reports were of fish in fresh water, but the first report of B.K.D. in wild salmonids in salt water was by Ellis, Novotny & Harrell (1978) from an advanced case of the disease in a fish taken from the sea.

In summary, B.K.D. has been found in North America, Canada, France, Italy, Yugoslavia, Turkey, Norway, Iceland, Great Britain, Spain and Japan, and has caused particularly severe losses in cultured fish. The species of salmonids affected by B.K.D. to date are:

1) All species of Pacific salmon Oncorhynchus spp;
2) Atlantic salmon Salmo salar;
3) Rainbow trout Salmo gairdneri;
4) Brook trout Salvelinus fontinalis; and
5) Brown trout Salmo trutta.

AETIOLOGY

The Causative Agent

The causal organism of B.K.D. is a very small (0.3μ - 0.5μ x 0.5μ - 1.0μ) Gram positive coccobacillus. It is non-motile, non-sporulating, non-capsulate and non-acid fast. Although the organisms tend to lie at acute angles to each other they do not display the pleomorphism and metachromatic granules which early workers reported which first led to the suggestion that the organisms should be placed in the genus Corynebacterium, and classified as C.salmoninus (Sanders and Fryer 1978). The organism has now been designated Renibacterium salmoninarum as a result of recent taxonomic data. (Sanders and Fryer 1980).

Following isolation from fish tissues on specialised medium for growing the kidney disease bacterium (KDB) such as "KDM_2", (Evelyn 1977) the isolate may not appear for up to 26 days and discrete colonies are only formed if culture time is in excess of 30 days.

Even in subculture, Renibacterium salmoninarum is a slow growing organism. The optimum temperature for growth is between 15 and 17°C. Growth is very slow at 5°C and 22°C and no growth will occur at 37°C. Maximum cell yield can be obtained after 20-30 days incubation. Cysteine is an essential growth requirement and incorporated in tryptose blood agar. The growth of the organism can appear between 7-10 days and well developed colonies will be observed after 14 days.

It has been noted that a considerable variation in colony size occurs on most culture media used. In 1964 Smith described the colonial characteristics of the organism on several culture media. On Ordal and Earp's medium (KDM_1), the colonies are creamy, white, circular, raised and shiny. Prolonged incubation produces a creamy yellow growth. On Mueller Hinton agar plus 0.1% cysteine the colonies are white, shiny and translucent. On Dorset's egg medium and sea water egg medium the growth appears as a raised, smooth, shiny, creamy-yellow layer. In Ordal and Earp's broth the creamy-yellow growth initially appears as a powdery sediment, adheres to the sides of the container and eventually produces a pellicle. Evelyn et al. in 1973 described creamy-white, circular, convex, entire and moderately shiny colonial characteristics on KDM_2 (containing serum instead of whole blood as in KDM_1 and adjusting the pH to pH6.5). He stated that on ageing the colonies changed from a butyrous to a sticky consistency, and that on crowded plates, colony size was usually less than 1mm in diameter and conferred a "ground glass" effect to the agar surface. On uncrowded plates a maximum colony size of 2.5mm could be found after about two months.

In biochemical terms, the organism is proteolytic, produces catalase and is non-fermentative. The average guanine plus cytosine content of the DNA of isolates studied by Sanders and Fryer (1980) was 53 $\pm$ 0.46 mol%. The major amino acids in the cell wall peptido glycan were lysine, glutamic acid, glycine and alanine in the molar ratios 1:1:1:3.5 respectively. The cell walls contain glucose, arabinose, rhamnose and mannose.

The primary structure of the murein of R.salmoninarum and the composition of the polysaccharide covalently attached to the murein were determined by Kusser and Fiedler (1983). The results confirmed that R.salmoninarum is significantly different from Listeria denitrificans, which had previously been presented as a possible relation (Collins, Feresu and Jones, 1983).

Embley, Goodfellow, Minnikin and Austin (1983) examined the lipid composition of R.salmoninarum and the resultant data served to distinguish Renibacterium from Corynebacterium and related mycolic acid containing bacteria, and also lent further support to the establishment of the new genus Renibacterium (Sanders and Fryer, 1980).

Studies by Bullock, Stuckey & Chen (1974) indicated that the strains of KD bacteria from different geographic locations of North America were antigenically homogeneous by the immunodiffusion technique.

TREATMENT

Having been recognised as a serious threat to many species of the Salmonidae, several attempts have been made to control Bacterial Kidney Disease.

The intracellular nature of Renibacterium salmoninarum raises problems for treatment of the disease. As intracellular organisms they are found within phagocytic cells whilst growing in vivo, but may also multiply within extracellular spaces. Such organisms are referred to as facultative intracellular parasites. Secondary characteristics used to differentiate these organisms include; the relative inability of the humoral response to control infection; the development of a delayed hypersensitivity; the establishment of an ongoing carrier state and a lack of susceptibility to antibiotics in vivo.

However, experiments using a wide range of antibiotics including aureomycin, chloramphenicol, gantrisin (3,4-Dimethyl-5-Sulphanilamido-isoxazole), sulphadiazine, sulphamerazine, sulphanilamide, sulphathiazole and terramycin were carried out by Snieszko and Griffin (1955), which showed that some control was obtained with all sulphonamides, although a degree of growth retarding effect was noted. Treatment was effected orally mixed with food on a w/w basis (grams of drug per 100 pounds of fish). A temporary protection only was achieved, as small numbers of the bacteria remained in situ which produced epizootics of the disease once treatment had ceased. This temporary effect of the sulphonamide therapy was also observed by Allison (1958). Prophylactic use of sulpha-drugs for this disease is, therefore, not considered useful.

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The lack of success in producing a lasting effective control resulted in further work being carried out by Wolf and Dunbar (1959) which involved testing sixteen strains of the kidney disease bacterium in vitro to determine their reaction to thirty four therapeutic agents, ten of which were chosen for experimental therapy in eastern brook trout (Salvelinus fontinalis). Of the ten, erythromycin at a dose of 10mg/kg per day for 21 days gave optimum results. Approximately 50% of treated fish survived as opposed to a mortality rate of 82.5% in untreated fish. Erythromycin, therefore, has since been frequently employed in attempts to control BKD.

Immunology

A naturally acquired immunity in the form of circulating antibodies in fish exposed to BKD has not been reported. Carrier fish, however, have been found to have circulating agglutinins and perhaps precipitins. An active immune response has been studied by some workers. Evelyn (1971) investigated the immune response by exposing immature sockeye salmon (Oncorhynchus nerka) to a heat killed, adjuvanted preparation of R.salmoninarum. He introduced the vaccine by intraperitoneal injection, (a method which might be feasible on commercial establishments), and induced an antibody response specific for the pathogen detected by a tube agglutination technique for at least 16 months following a single injection. A second injection given 13 months after the first elicited an anamnestic response. Ninety days following primary and secondary vaccination, maximum agglutinating titres were 1:2,560 and 10,240 respectively. No studies on the protection afforded by these antibodies were carried out, but the experiments demonstrated that immature sockeye salmon were immunologically competent.

Paterson, Desautels and Weber (1981) demonstrated some protective immunity in Atlantic salmon Salmo salar following an intraperitoneal injection of killed R.salmoninarum emulsified in Freund's complete adjuvant. Protective immunity was demonstrable for 2 years. A lack of humoral antibody response to cells injected in saline suggested that R.salmoninarum is less antigenic in fish than Gram negative pathogens, e.g. Aeromonas salmonicida.

This work, however, did indicate that a system of vaccination to reduce the effects of BKD could be considered.

Section I. THE NATURAL INFECTION

Isolation and Characterisation of Renibacterium salmoninarum.

Introduction and Literature Review.

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Following the initial report by Mackie et al (1933), bacterial involvement in BKD was also reported by Belding and Merrill (1935) in an outbreak of the disease in brook and brown trout at a Massachusetts State fish hatchery. They described a Gram positive coccobacillus which was found in very large numbers in smears prepared from affected viscera. Although apparently similar organisms were cultured on hormone agar after 2 days at room temperature, later work on their cultural and morphological characteristics has shown that this isolate was not the causative organism.

The first successful isolation of R.salmoninarum was achieved by Ordal and Earp (1956) using minced chick embryo tissues incubated at 15°C. This medium was superceded by a modified Dorset's egg medium fortified with cysteine, tryptone and yeast autolysates. Good growth was obtained by direct inoculation with infected tissues. Still better growth occurred within 7-10 days incubation at 17°C on a blood agar medium containing 10-20% human blood and 0.05 to 0.1% cysteine, which was

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proved to be an essential growth requirement. This cysteine blood agar was also found to be satisfactory for the isolation of the organism from fish in the early stages of the disease, but more prolonged incubation was necessary. Considerable variation in colony size was noted, but the morphological characteristics of the bacteria were constant. The cells ranged from 0.3 to 0.5μ by 0.5 to 1.0μ in size and chains sometimes developed after prolonged culture. Pleomorphism was noted in some cultures and resembled that seen in the intracellular forms in infected tissues.

Between 1930-32 fourteen Scottish salmon from the Aberdeenshire Dee and the River Spey were reported to have small necrotic lesions of the spleen (cited by Smith, 1964) from which a small Gram positive bacillus could be demonstrated in smears, but not cultured. The fish were described as suffering from "Dee Disease" during ensuing years. Smith (1964) noted "Dee Disease" was similar to the Kidney Disease of salmonids in North America, described by Earp, Ellis & Ordal (1953). Both showed higher mortalities in low water temperatures and the associated organisms were Gram positive diplobacilli between 0.5 to 0.7μ by 0.8 to 1.0μ (Bell 1961) and 0,6 to 0.9μ by 1.0 to 2.8μ (Smith 1964). The organisms grew on Ordal and Earp's medium and Mueller Hinton agar (Wolf and Dunbar 1959) with similar cultural characteristics and were catalase positive, proteolytic and non spore-bearing. They were tentatively classified as Corynebacteria.

No further studies on the classification of the BKD organism were reported until Sanders and Fryer (1978) proposed the name Corynebacterium salmoninus on the basis of their taxonomic work, in which

they determined the cell wall peptidoglycan, DNA ratios and the principal sugars in cell wall hydrolysates.

They later proposed the establishment of a new genus Renibacterium salmoninarum and suggested that Renibacterium was closely related to members of the Coryneform group of bacteria (Sanders & Fryer 1980).

With the development of new diagnostic techniques, more positive and rapid identification of the Kidney Disease bacterium became possible. Using an immuno-diffusion test (Bullock et al., 1974), detection of the soluble kidney disease antigen in homogenised infected tissues was possible within 24 hours. A heat stable antigen may be extracted from infected kidney tissue which allows long distance transport of specimens for diagnosis (Kimura, Ezura, Tajima & Yoshimizu, 1978). Although this technique eliminated the considerable time needed to culture the organism, doubt was expressed on the possibility of detecting the KD bacterium from covertly infected fish.

The development of an indirect fluorescent antibody technique (IFAT) allowed identification of the aetiological agent of BKD within hours. Using fresh, frozen or formalin fixed tissue Bullock & Stuckey (1975) identified the KD bacterium within 2 hours. The IFAT technique proved to be far superior in detecting low numbers of the KD bacteria and also revealed covert or asymptomatic disease. Kidney smears were examined by culture, IFAT and immunodiffusion, and IFAT was positive in twice as many samples as the other techniques.

Due to the escalation of BKD incidence, work proceeded to find a culture medium to establish beyond doubt the classification of the Gram positive coccobacillus. Media used with most success had been cysteine blood agar (Ordal and Earp 1956), and a cysteine supplemented Mueller Hinton medium (Wolf and Dunbar 1959). Evelyn (1977) developed a new growth medium which contained 0.1% cysteine and 20% foetal calf serum, designated KDM_2 (Kidney Disease Medium 2) which gave superior results over all other media previously used, and has become the accepted medium for the culture of Renibacterium salmoninarum.

This section reports work mainly concerned with the isolation and characterization of the causative organism of BKD. The work started in 1979 when the organism was unclassified, but had tentatively been placed in the genus "Corynebacterium".

The IFAT and immuno-gel diffusion test were investigated as confirmatory methods for the identification of isolates; and the cultural characteristics were studied in some detail, in particular after repeated subculture. This led to an examination of the ultrastructure of the organisms from the colony forms encountered.

Biochemical properties were studied in an endeavour to differentiate the BKD species from Corynebacterium pyogenes. This was followed by the examination of the amino-acid composition of the cell wall layer.

Section I

MATERIALS AND METHODS

1) Strains

The strains used in this study are listed in Table I below.

TABLE 1

A) BKD Strains (Renibacterium salmoninarum)

Strain No.	Source	History
WS01	B.Spacey	Isolated from kidney, liver, heart and spleen of rainbow trout (Salmo gairdneri) 1978 commercial trout farm; Northern England.
WS02	B.Spacey	Isolated from kidney, spleen of rainbow trout. 1979 - History as above.
WS03	B.Spacey	Isolated from kidney, spleen, liver, heart of rainbow trout 1979.
WS04	NCMB	NCMB1111 - I.W.Smith 1962; K.Wolf SUHD; isolated from Salmo sp. Massachusetts State Hatchery, Massachusetts, USA.
WS05	NCMB	NCMB1112 - I.W.Smith 1962; K.Wolf CHAT isolated from Salvelinus fontinalis. Chatahoochee Forest National Hatchery, Georgia, USA.
WS06	NCMB	NCMB1113 - I.W.Smith 1962; K.Wolf CRAW; isolated from Salmo gairdneri. Crawford National Fish Hatchery, Nebraska, USA.
WS07	NCMB	NCMB1114 - I.W.Smith 1962. 1085 isolated from Salmo salar, River Dee, Aberdeenshire, Scotland.
WS08	NCMB	NCMB1115 - I.W.Smith 1962; 1086 isolated from Salmo salar, River Dee, Aberdeenshire, Scotland.
WS09	NCMB	BCMB1116 - I.W.Smith 1962. Strain T530 isolated from Salmo trutta after passage with isolate from salmon (Salmo salar), River Dee, Aberdeenshire, Scotland.
WS10	Dr.B.Austin	Isolated from Sockeye salmon. Ex Maraino, British Columbia.
WS11	Dr.B.Austin	Ex Hood River, Canada.

TABLE 1 (continued)

(B) Corynebacterium pyogenes strains

Strain No.	Source	History
WS12	Culture Collection (WRL)	CN 1858 - Chemotherapy Department "C strain Alexander".
WS13	Culture Collection (WRL)	CN 1859 - Chemotherapy Department "D strain Greenlands", Dr.Kelly.
WS14	Culture Collection (WRL)	Dr.Roberts. Isolated from ox liver abscess obtained from Canada.

2) Media

KDM_2; This medium contains peptone 1% (W/V), yeast extract 0.05% (W/V), cysteine HCl 1% (W/V), agar 1.5% (W/V and foetal calf serum 20% (V/V) described by Evelyn (1977).

Fungizone : Gibco Laboratories. Used at 25µg/ml medium. Incorporated in the medium for direct isolation work only.

Muellor Hinton Agar : Oxoid Laboratories (Beef infusion, Casein hydrolysate, Starch, Agar No.1, 35g/litre autoclaved at 15lb/sq.in for 15 minutes).

Blood Agar: Prepared from nutrient agar (Wellcome Research Laboratories) by the addition of 5% v/v defibrinated sheep's blood.

Loeffler's Serum Slopes: Wellcome Medium Production Unit (Normal Horse Serum No.5, 1.0 litre, Nutrient Broth No.2 (Oxoid CM67)) 8.5gms, Glucose B.P. 1.25gms).

Dorset's Egg Medium: Wellcome Medium Production Unit (Beaten infertile hen's egg fluid 500mls, Veal Broth, 400mls).

Hank's Balanced Salt Solution: Wellcome Medium Production Unit (Method described by Hanks & Wallis, 1949).

3) Isolation and maintenance of strains

Isolation of Renibacterium salmoninarum from infected Rainbow trout was achieved on KDM_2 (Evelyn 1977) containing fungizone (25µg/ml medium). Homogenised tissues were plated onto dry agar plates which were sealed with plastic film and incubated at 15°C for up to 20 days.

For routine subculturing fresh KDM_2 was prepared to ensure reliable growth. Heavy yields of bacteria were obtained on KDM_2 agar plates by producing confluent growth by subculturing from young cultures (5-8 days old). Static liquid cultures in KDM_2 without the addition of agar were produced in 100ml and 300ml vessels. The inoculum was a suspension of the KD organism in liquid medium harvested from a plate culture and growth was continued at 15°C for up to 20 days.

All strains were stored in a lyophilised state in sealed ampoules at 4°C. Initial attempts at direct freeze drying the organism in sugar solution containing 12% mannitol and 5% sucrose, nutrient broth and saline with and without Horse serum No.3 or 1% Foetal Calf serum, failed. A freeze drying suspending fluid was prepared containing 3 parts of Wellcome Horse Serum No.3 : 1 part nutrient broth (prepared by Wellcome Research Laboratories) with the addition of 7.5% glucose (Fry & Greeves 1951) which allowed good recovery of organisms. Primary culture from freeze dried material was obtained by reconstitution of the pellet in horse serum broth and "drop plating" onto dry KDM plates. Sealed plates were incubated at 15°C for up to 20 days. Growth of the organism from freeze dried (FD) cultures occurred within 5-10 days.

4) Viable Counts

These were performed by a modification of the method of Miles and Misra (1938). Ten fold serial dilutions of cultures, previously diluted to give an optical density (OD) of 0.2 @ 650nm on an SPL600 spectrophotometer (Unicam) were prepared in 0.85% normal saline and 50µl volumes were distributed onto duplicate KDM_2 agar plates.

5) Preparation of Antisera

The strains chosen were all isolated from fish showing clinical signs of BKD and originated from different geographical locations. The cultures were grown on KDM_2 for 12 days at $15^{o}C$. Whole cell antigens were prepared by harvesting the growth from the medium into Hanks balanced salt solution. The cells were washed three times and then re-suspended in Hanks BSS containing 0.4% formalin to a concentration of OD 0.4 @ 650nm on SPL600 spectrophotometer. Equal volumes of cell suspension and Freund's incomplete adjuvant (Wellcome Laboratories) were used to immunize rabbits (New Zealand Whites, Ranch Rabbits) 2ml of vaccine were injected into each flank weekly for 2 weeks. Blood was taken prior to the first injection and 10 days after the last. Subsequent bleeds were taken after booster injections.

6) Serological Procedures

a) Gel Diffusion

This was performed in agar gel containing Oxoid ion agar No.2 dissolved to a concentration of 1% in phosphate buffer pH 7.0 containing EDTA (1.86gms) and sodium azide (0.65 gms) as a preservative. Microscope slides 75 x 25mm holding 2.5ml of agar were used. Wells were cut 3.5mm in diameter, and were 3mm apart holding 10µl of reagent. Slides were incubated in a moist chamber at room temperature, and precipitin patterns

were recorded after 24 and 48 hours. Slides were photographed in oblique light against a dark background.

b) Agglutination Tests

The microtitre agglutination test was employed to determine agglutinin titres of all sera. 25µl serial, two fold, dilutions of the antisera were prepared in 0.85% normal saline in microtitre "U" plates (Wellcome Reagents U.K). The antigen was prepared by suspending growth from plate cultures in Hanks BSS containing 0.4% formalin. After incubation at room temperature for 1 hour the cells were washed three times in Hanks BSS and then re-suspended in Hanks BSS to opacity of 0.9 OD at 420nm on an SPL 600 spectrophotometer. The suspension was then sonicated for 14 seconds to obtain an homogeneous suspension. After addition of the antigen, the plates were incubated overnight at $4^{o}C$. The final serum dilution showing agglutination was recorded as the titre.

c) Slide Agglutination Tests

The slide agglutination test was applied for fast, presumptive identification of the KD organisms from plate culture. The procedure was carried out on an standard glass microscope slide. A drop of 0.85% normal saline was placed on the slide and a small amount of culture was emulsified in it by means of an inoculating loop. A drop of undiluted anti-KD serum was added to the bacterial suspension, mixed using the loop, and examined after a few minutes. Agglutination was rapid and clumps could be easily seen with the naked eye.

d) Indirect Fluorescent Antibody Technique

Impression smears, tissue sections and culture smears or bacterial suspensions on microscope slides were allowed to air dry and then fixed in

cold anhydrous acetone for 10 minutes. Slides were flooded with anti-KD serum and incubated in a moist chamber for 30 minutes at room temperature. After rinsing and washing for 10 minutes in phosphate buffered saline pH 7.2 the slides were air dried and then flooded with appropriate labelled antiserum and incubated as above for 30 minutes at room temperature. The slides were then washed for 10 minutes in carbonate-bicarbonate buffer pH 9.4, air dried, mounted in glycerol buffer to pH 9.0 and examined immediately by fluorescence microscopy. As controls the following combinations of reagents were employed:

Positive antigen + anti-KD serum + fluorescent labelled antiserum.

Negative antigen (Corynebacterium sp.) + anti-KD serum + fluorescent labelled antiserum.

Positive antigen + fluorescent labelled antiserum.

Positive and negative antigens + normal rabbit serum

Standard rabbit and goat anti-KD sera were obtained from Bullock (U.S. Eastern Fish Disease Laboratory, Kearneysville, W.Virginia). Optimum working dilutions for the standard antisera were 1:40 and 1:160 respectively.

Optimum working dilutions for the labelled sheep anti-rabbit and rabbit anti-sheep (Wellcome Reagents) were 1:50 and 1:75 respectively.

Counterstain

0.1ml of appropriate labelled conjugate (anti-rabbit at 1:5 and anti-sheep at 1:7.5) was added to 0.9ml of 0.2% Evans blue and applied at the second stage of the test.

7. Biochemical Tests

Bacterial inoculum

Cultures were harvested from KDM_2 by scraping, and suspended in sterile distilled water, washed three times and adjusted to an approximate OD 0.4 @ 650nm on the SPL600.

Carbohydrate Fermentation

All media were supplemented with essential nutritional requirements: 0.1% cysteine hydrochloride and 20% foetal calf serum.

Basic peptone water sugars were prepared by the Wellcome Media Production Unit (M.P.U.).

The following tests were performed:-

1) Monosaccharides - Arabinose, Xylose, Rhamnose, Glucose, Galactose.
2) Disaccharides - Sucrose, Maltose, Lactose, Trehalose.
3) Trisaccharides - Raffinose.
4) Polysaccharides - Starch, Inulin.
5) Sugar alcohols - Glycerol, Aldonitol, Mannitol, Dulcitol, Sorbitol, Inositol.
6) Glycosides-Salicin, Aesculin.

(Protein hydrolysate 1.5g/litre, Sodium chloride 0.5%, Sugar 1.0% (Aesculin - 0.5%, Starch-soluble 1.0%), 0.6% Bromo-cresol purple in 50% meth. 0.2%).

A positive reaction was denoted by the presence of acid accompanied by a colour change from violet purple at pH 6.8 to yellow at pH 5.2.

Tests for Proteinases and Amino-Acid Breakdown

Gelatin Liquefaction

Nutrient Gelatin; Wellcome Medium Production Unit (M.P.U.) (Gelatin (Croda) 200g, Nutrient Broth to 1.0 litre).

A loopful of culture was inoculated into the medium by stabbing. After 28 days incubation the medium was examined for liquefaction.

Amino-Acid Decarboxylase Tests

Ornithine, Lysine and Arginine (Oxoid Laboratories)

Sterile mineral oil was added to the medium to form a surface layer. A positive reaction was indicated by a violet colour.

Indole Test

Growth medium; Wellcome M.P.U. (Peptone 20g, Sodium chloride 5g, Distilled water 1 litre).

After the 28 day incubation period, 0.5ml of Kovaks Reagent was added. A red colour indicated a positive reaction.

Kovacs Reagent; Wellcome M.P.U. (Amyl alcohol 150ml, p-Dimethyl-aminobenzaldehyde 10g, Conc. hydrochloric acid 50ml).

Miscellaneous Tests

Urease Test

Wellcome M.P.U. (Peptone 1g, Sodium chloride 5g, Monopotassium dihydrogen phosphate 2g, Phenol red 6ml, Agar 20g, Distilled water 1.0 litre).

Slopes were inoculated from plate cultures and incubated for 28 days. Cultures were examined for a pink colour indicating a positive reaction.

Catalase Test

A small amount of culture using a sealed capillary tube was placed in a drop of 30% hydrogen peroxide on a glass slide. A positive reaction was indicated by the production of gas bubbles.

Oxidase Test

A piece of Whatman's No.1 filter paper was saturated with 1% tetramethyl-p-phenylenediamine HCl, and using a sealed capillary tube, a small amount of culture was rubbed on the filter paper.

A positive reaction was indicated by the appearance of a dark purple colour within 10 seconds.

Citrate Utilization Test

Koser's Citrate Medium (modified); Wellcome M.P.U. (Sodium chloride 5.0g, Magnesium sulphate 0.2g, Ammonium dihydrogen phosphate 1.0g, Potassium dihydrogen phosphate 1.0g, Sodium citrate 5.0g, Distilled water 1 litre).

The medium was examined for growth after 28 days incubation.

Malonate Utilization and Phenylalanine Deaminase Test

Wellcome M.P.U. (Method described by Shaw and Clarke, 1955).

The medium was observed for a colour change from green to blue which indicated malonate utilization. A few drops of 0.1N HCl followed by a few drops of aqueous ferric chloride were added to the medium. A colour change from yellow to dark green indicated a positive deamination.

Bromo-Cresol Purple Milk

Wellcome M.P.U. (0.6% Bromo-Cresol Purple in 20% Alcohol 3.0ml, Dried skimmed milk 100g, Deionised water to 1 litre).

Fermentation of lactose was indicated by a colour change from blue to yellow. Clotting and peptonization was recorded.

8) Electron Microscope Preparations

Bacterial inoculum

Suspensions of bacteria were prepared as on Page 22.

A) Negative stain

Using a micropipette, two drops of the suspension and one drop of 4% Phospho tungstic acid were mixed on a glass microscope slide. Fine forceps were used to hold the 400 mesh grid and the "dull side" was placed in the mixture. Excess fluid was carefully removed with blotting paper, and the grid placed in a labelled petri-dish lined with filter paper. The grid was allowed to dry and then examined in a Phillips 300 Transmission Electron Microscope.

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Wellcome M.P.U. (Method described by Shaw and Clarke, 1955).

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8) Preparation of sectioned material

Bacteria were emulsified in 2.5% Gluteraldehyde in Cacodylate buffer and stored at +4°C until processed. They were removed from the fixative by haematocrit centrifugation and the resultant pellet placed in a solution of 1% osmium tetroxide for 1 hour. The pellet was then treated as follows: aqueous uranyl acetate 30 minutes, 70% alcohol 10 minutes, 90% alcohol 10 minutes, 25 minutes in 100% alcohol, 30 minutes in 50/50 propylene oxide and araldite, and then finally 100% araldite for 1 hour. After embedding in fresh araldite at 60°C overnight, 90nm sections were cut, stained in an LKB ultrastainer and examined as above.

9) Cell Wall Preparations and Analysis

Organisms from 30 KDM_2 plate cultures were harvested in 0.85% normal saline and washed three times. The organisms were then ruptured with glass ballotini No.14 in a rotary disintegrator. After the removal of the ballotini by filtration through a sintered glass filter funnel, the suspension was subjected to a low speed centrifugation (300g for 5 minutes) to remove intact cells. The supernatant containing the broken cells was centrifuged at 12,000g for 20 minutes on an HS25 centrifuge. The deposit was re-suspended in distilled water and the supernatant was stored at -20°C (S/N 1). A small sample of the re-suspended deposit was negatively stained and examined under the electron microscope (as in 8(A) above, also see Figure 1a). The suspension was then washed three times and the supernatant was retained and stored at -20°C after each washing (S/N (2), (3), (4)).

Digestion with Pronase (Sigma)

The membrane deposit was re-suspended to 10% in 0.05m phosphate buffer pH 7.4 containing 0.22µm filtered pronase at 1mg/ml and sodium azide 0.02% as preservative. Digestion was carried out with agitation at 37°C for 2 hours, after which time the suspension was centrifuged at 12,000g for 20 minutes and the supernatant taken off, labelled Fraction (1), the OD measured at 650nm and stored at -20°C. This procedure was repeated until no further release of free amino groups was detected.

Digestion with Ribonuclease (Sigma, Type IIIB)

The resultant material was washed and re-suspended as a 10% suspension in 0.05m phosphate buffer at pH 7.2 containing 1mg/ml of 0.22µm filtered ribonuclease and sodium azide and digested at 37°C until release of 260nm absorbing nucleotides was complete.

Digestion with D'Nase (Sigma, 0.25mg/ml)

The material was then similarly treated with 0.22µm filtered D'Nase.

The final product was then washed three times in distilled water and a sample examined under the electron microscope (Figure 1b). The pellet of membranes was then freeze dried.

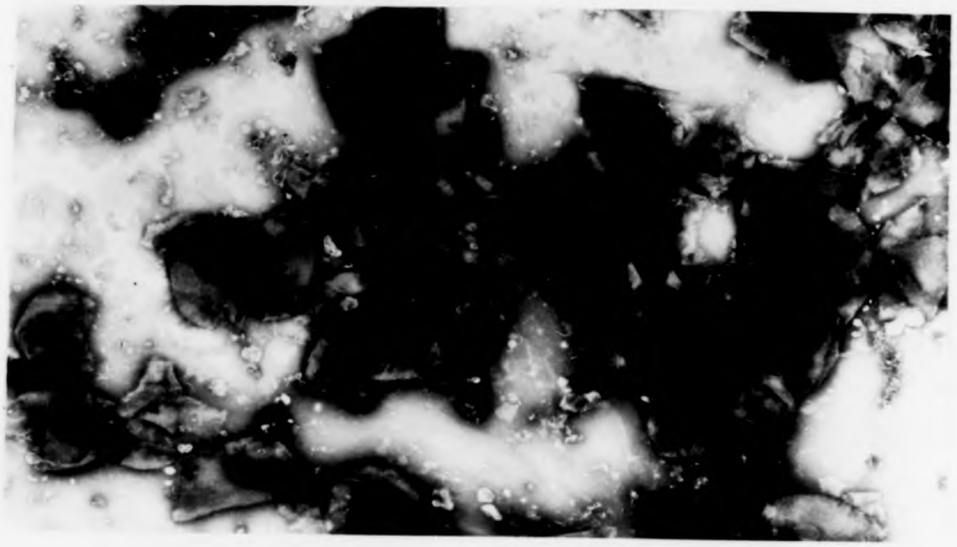
Amino-acid Analysis

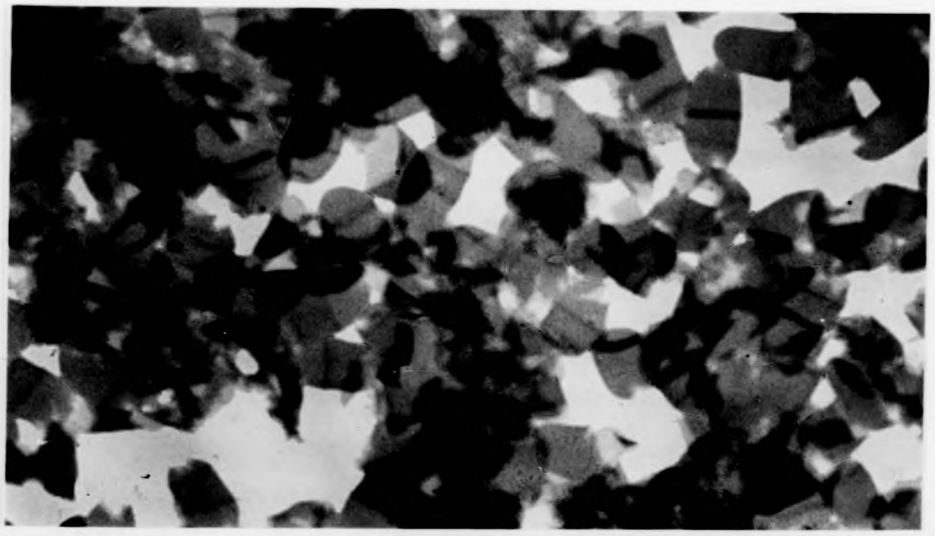
Hydrolysis of the sample and analysis on a Rank Hilger Chromaspek automatic amino-acid analyser was performed by workers in the Physical Chemistry Department, Wellcome Research Laboratories.

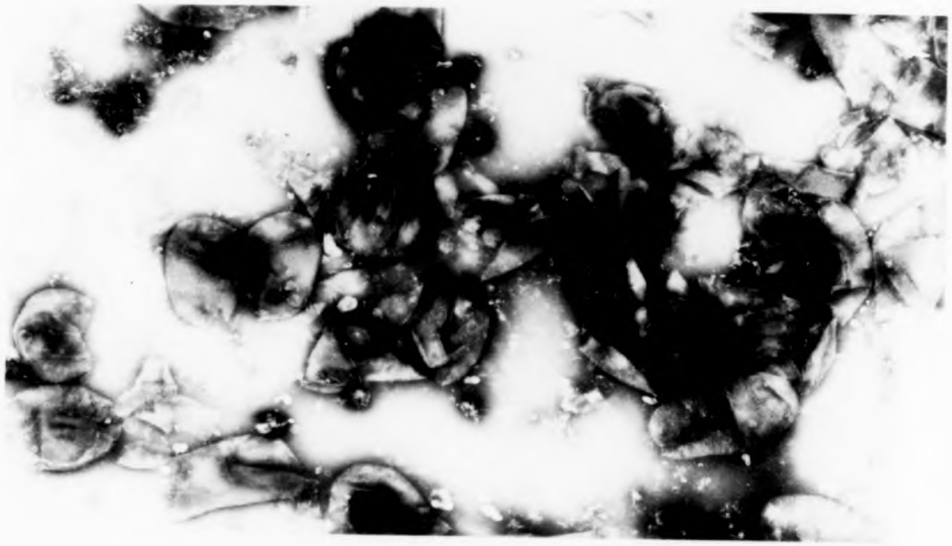
Figure 1

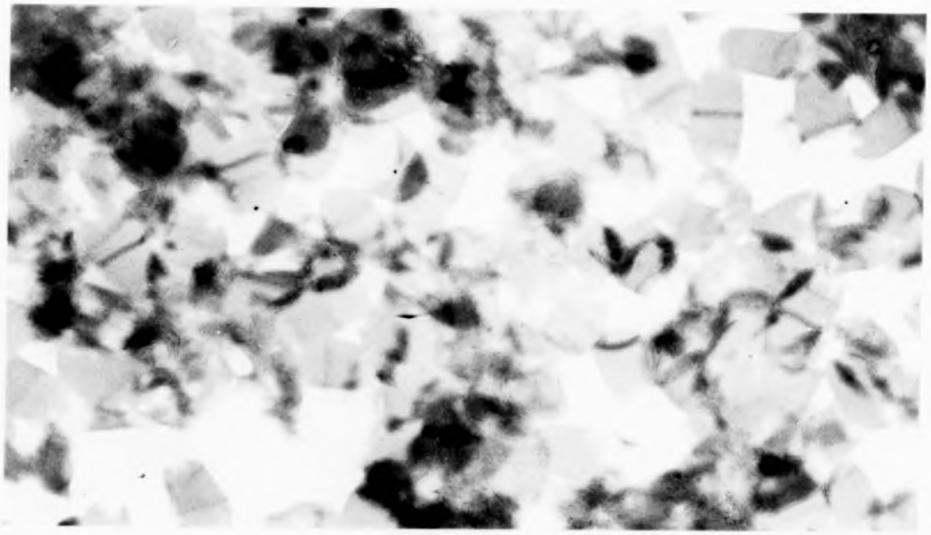
a) Residue of R.salmoninarum after homogenisation of culture suspension x 18,800.

b) Final product after full extraction procedure consisting of the remains of cells from which all the contents have been released x 15,200.









10) Amino-Acid Chromatography (Haworth Heathcote, 1969)

The same hydrolysed sample was used for thin layer chromatography. The dimensional thin layer chromatography was performed on cellulose F Plates (Marck and Darnstadt, West Germany) by the method of Haworth and Heathcote (1969). Amino acid stock solutions (0.025m) were made in aqueous N-propanol (10% v/v). Samples were applied at a position 1.5cm up from the lower edge and 1.5cm inside from the left hand edge of the plate using 1µl capillary pipettes (Microcaps). The spots were dried in a stream of warm air.

The composition of the solvent system for development in the first dimension was 2 propanol:butanone:1N hydrochloric acid (60:15:25 v/v). The plates were removed from the tank when the solvent front had ascended to within 2-3cm of the upper edge of the plate and were dried in a stream of cold air for 15 mins and heated in an oven at 60^{0}C for a further 15 mins.

The solvent for the second dimension consisted of 2-methylpropanol:2 butanone:acetone:methanol:water:0.880 ammonia (40:20:20:1:14:5 v/v). The plate was developed at right angles to the first dimension by the ascending technique and dried as described.

Spots were visualised with ninhydrin. This reagent was prepared by dissolving 0.5g of calcium acetate in 50mls of water to which 10ml of glacial acetic acid had been added. Propanone (Acetone) was then added until the total volume was 500ml. To required volumes of this solution solid ninhydrin was added until the final concentration was 0.2% (w/v).

Section I

RESULTS

Isolation and Characterisation of the Organism

A) 1. Isolation and identification

Impression smears of kidney, heart, liver and spleen were prepared from fish suspected of carrying the BKD organism. These were used for Gram staining and in the indirect fluorescent antibody test (IFAT).

Two patterns were noted on surveying the Gram stained smears. The first, seen in the majority of fish, showed moderate to large numbers of Gram positive coccobacilli in all organs examined, while the second, seen in a minority of the fish, showed large numbers of organisms in the kidney and small numbers in other tissues (Figure 2a, b). No correlation between the number of organisms and overt signs could be established.

The IFAT was not satisfactory without a counterstain since the distinction between a high background fluorescence and specific fluorescence was not adequate (Bullock and Stuckey, 1975). Better results were obtained using 0.1% Evans blue as a counterstain, as this more clearly defined the bacteria (Figure 3).

Good correlation between the findings from the smear and the validity of using these tests as presumptive diagnostic tests was confirmed by successful culture of the BKD organisms only from those samples showing large numbers of organisms in both tests (Table 2).

Table 2

Correlation between results obtained from impression smears and isolation from 10 representative kidney specimens

	Impression Smears		Culture on KDM_2
Kidney	Grams stain	IFAT	Isolation
1	-	-	-
2	++	++	+
3	+	++	+
4	+++	+++	+
5	-	+	-
6	+++	+++	+
7	-	-	-
8	+	++	+
9	+	+	-
10	-	-	-

Key

\+ small numbers of organisms (5-10/fld)

++ moderate numbers of organisms (10-50/fld)

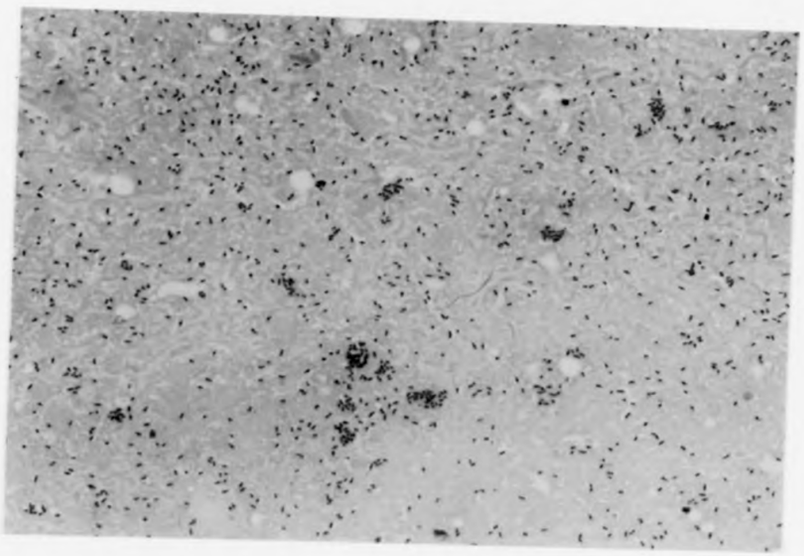
+++ large numbers of organisms (>50/fld)

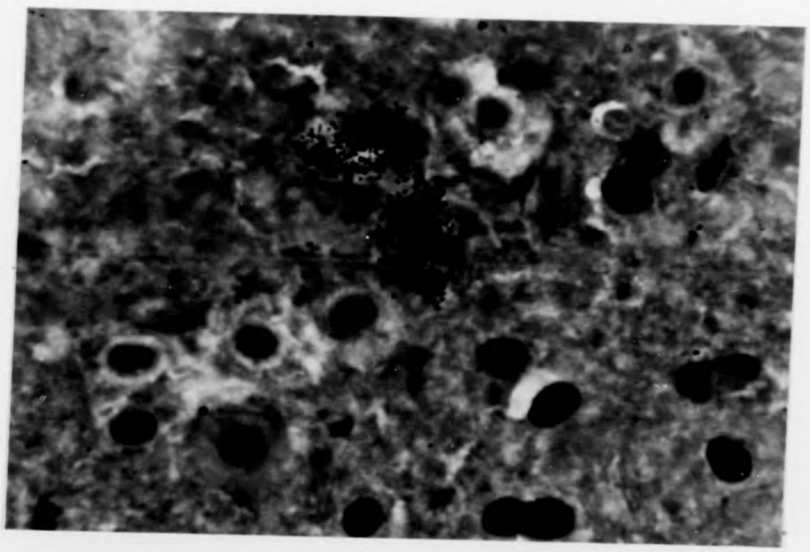
\- no organisms detected

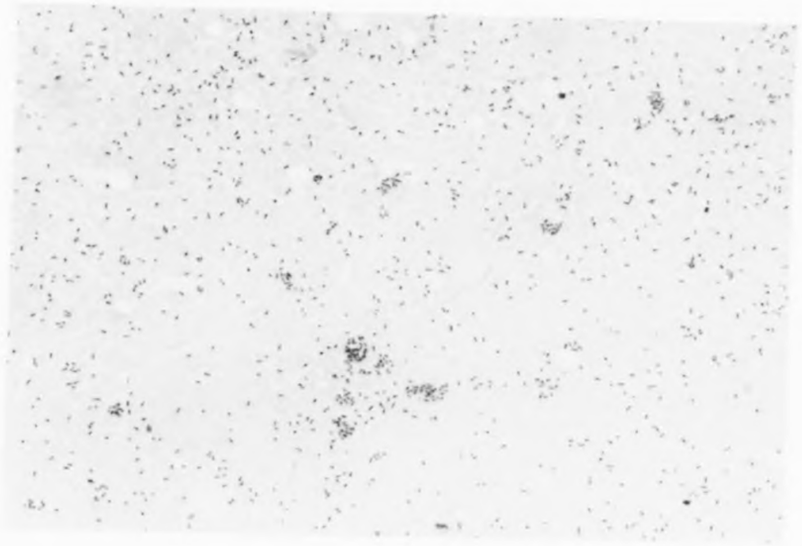
Figure 2 Gram stained impression smears demonstrating Gram positive coccobacili in:

a) kidney x 1400

b) heart x 1400







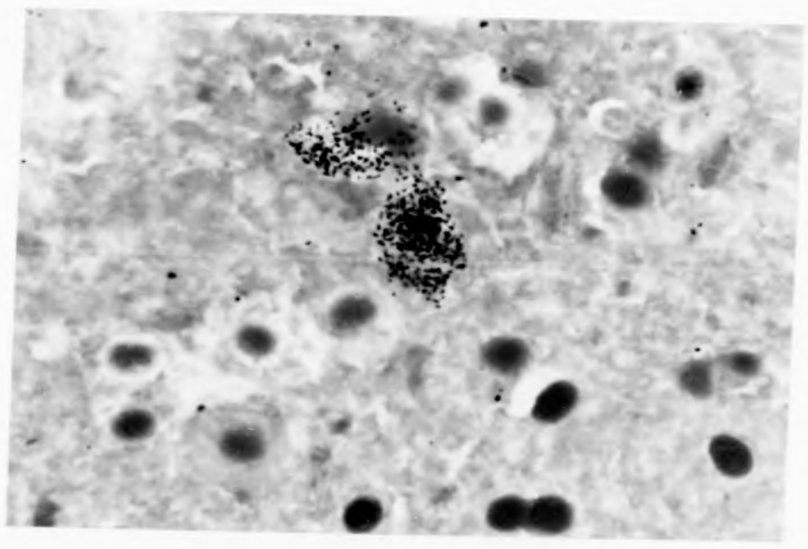
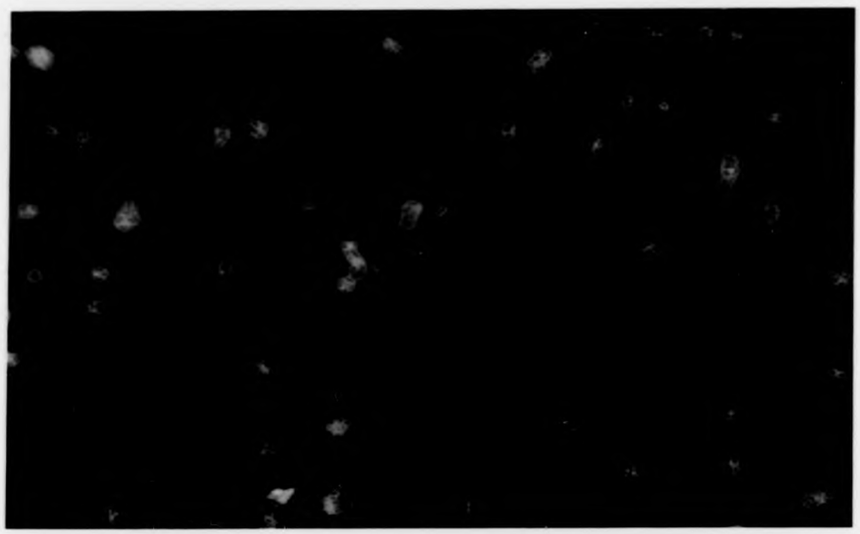


Figure 3 Fluorescein labelled R.salmoninarum in kidney smear taken from a naturally infected rainbow trout x 1400.



2. Cultural characteristics and microscopic examination

Successful isolation of organisms from a large number of homogenised tissues from infected fish was achieved on Mueller Hinton agar, Loeffler's serum slopes and KDM_2 at $15^{o}C$. Repeated attempts to grow the organism on Dorset's egg medium failed.

KDM_2 however, was found to be far superior to the other media in terms of success rate and yield.

On KDM_2, a fine transparent film of organisms appeared after 5-8 days, and progressively thickened over the next 4 to 7 days and became opaque.

After about 15 days the plate bore a profuse growth, which was white to cream in colour with a smooth shiny surface. Colonies visible at the edge of the smears were white, smooth convex discs with regular borders showing considerable variation in size (Figure 4a, b & c).

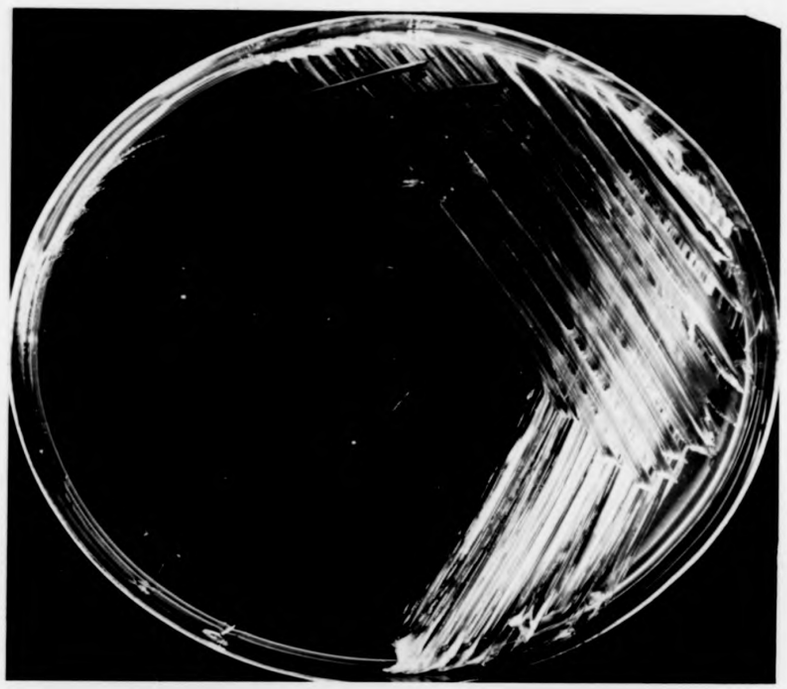
Smears prepared from cultures and stained by the Gram's method showed small Gram positive coccobacilli, morphologically uniform, but with some pleomorphism and a tendency to lie at acute angles to one another (Figure 5).

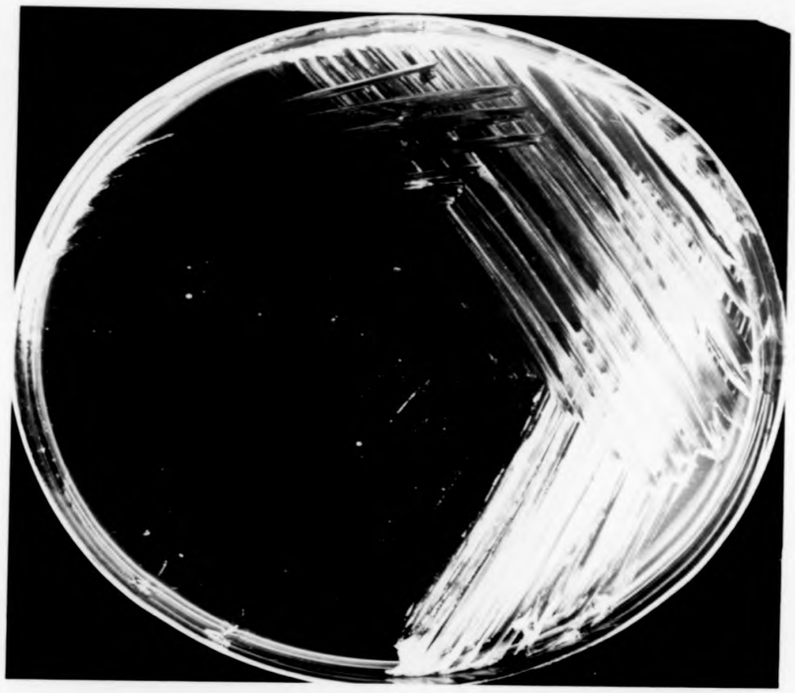
3. Confirmatory identification

Isolates grown on KDM_2 were identified as KD organisms using the IFAT and by immuno-gel diffusion.

Figure 4a 15 day culture of R.salmoninarum on KDM_2 x 1.4.

Figure 4a 15 day culture of R.salmoninarum on KDM_2 x 1.4.





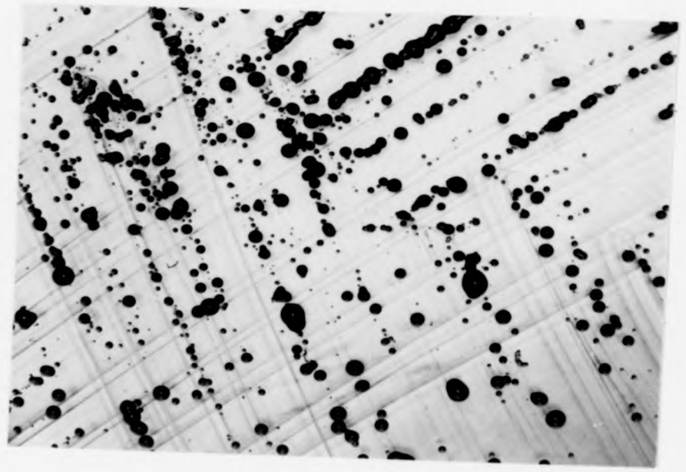


Figure 4b x 4

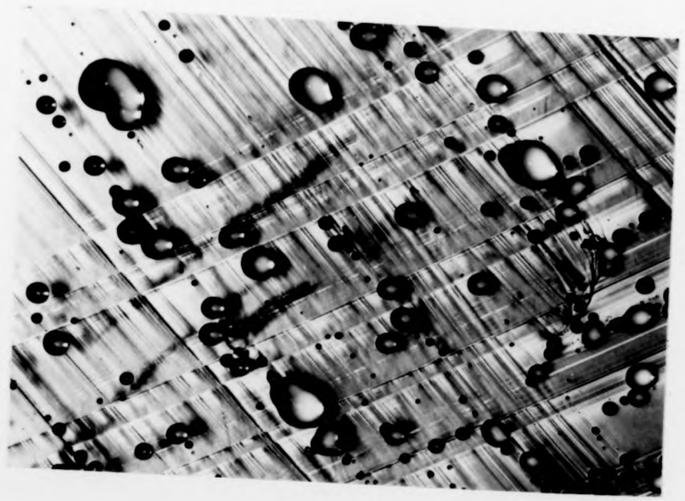


Figure 4c x 8.7

Demonstration of the variation in colony size from 15 day culture of R.salmoninarum on KDM_2.

Figure 4b x 4

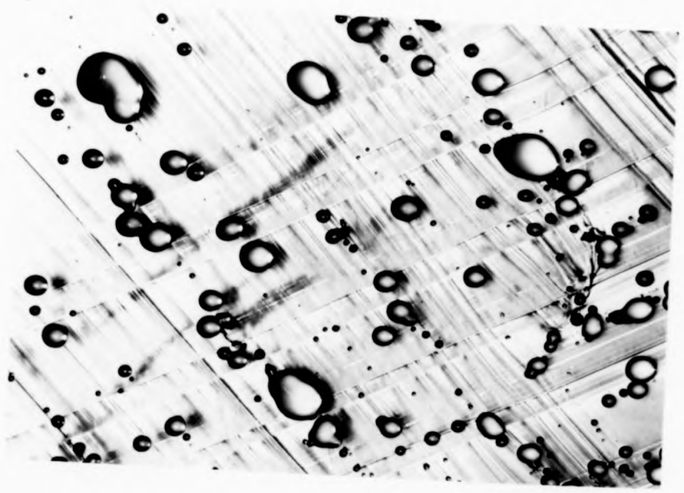


Fig. 4c x 8.7

Demonstration of the variation in colony size from 15 day culture of R. tenuoninarum on KDM_2.

Figure 5 A Gram stained smear of R.salmoninarum from 15 day plate culture on KDM_2 x 1400.

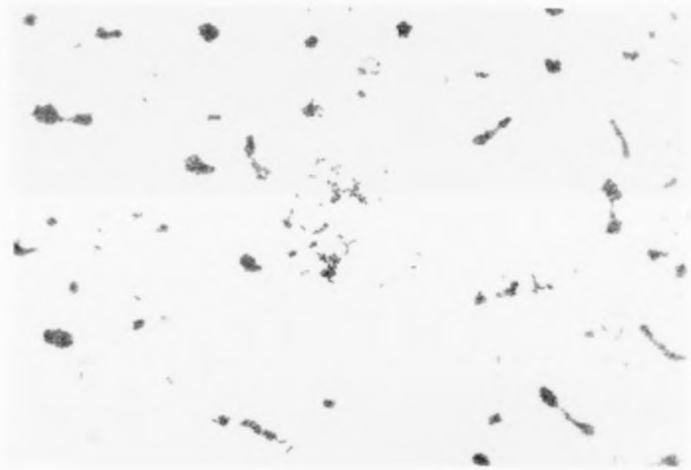
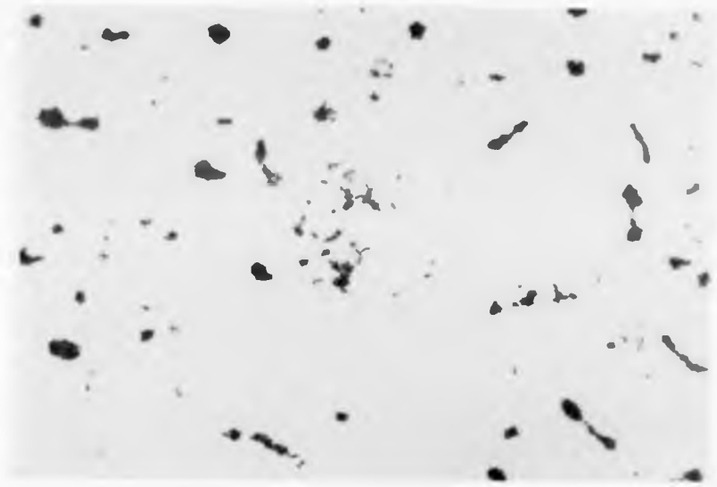


Figure 5 A Gram stained smear of R.salmoninarum from 15 day plate culture on KDM_2 x 1400.



In the immuno-fluorescence test, good fluorescence was obtained with all presumptive KD cultures. Control cultures were negative, as were all cultures when normal rabbit serum was substituted for the anti-KD serum.

The antigen used in immunodiffusion was prepared by saline extraction of harvested whole cells. Antigens from a number of isolates were allowed to diffuse against both goat and rabbit anti-KD sera. Continuous precipitin lines (Figure 6a, b) demonstrated antigenic homogeneity between the antigens tested suggesting that the strains used to raise the antisera and the fresh isolates shared similar extractable antigens.

4. Infectivity

To confirm Koch's postulates for some of these isolates infectivity experiments were performed and will be described in Section II.

B. Production of Antisera

Antisera with high agglutinating titres were obtained in rabbits using vaccines containing strains WSO3, WS04, WS05, WS07 and WS08 emulsified in Incomplete Freunds Adjuvant. Rabbits immunised in the same way with strains WS06, WS09, WS10 and WS11 gave poor antisera and little improvement was obtained by repeated intramuscular injections over a period of a further four weeks. Homologous titres ranged between 1:32 and 1:4096 (Table 3).

Table 3

Agglutinating antibody response of rabbits to intramuscular injections of killed R.salmoninarum emulsified in Freund's incomplete adjuvant

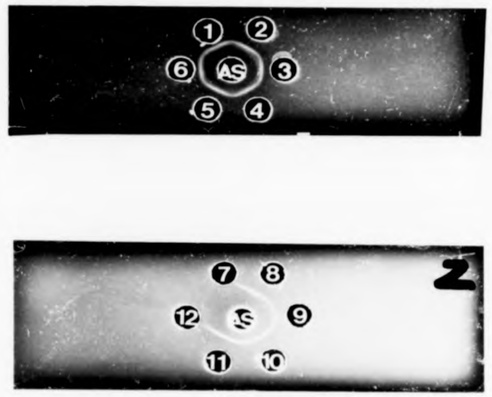
Strain	Antibody Titre
WS03	> 1:4096
WS04	1:1024
WS05	1:1024
WS06	1:32
WS07	> 1:4096
WS08	1:2048
WS09	1:128
WS10	1:32
WS11	1:64
Positive Control	> 1:4096
Negative Control (Pre-immune)	< 1:4
Saline	< 1:4

Figure 6a Immunogel diffustion tests : a continuous precipitin band formed by eleven isolates and rabbit anti-KD serum. The non-reacting wells contain 2 strains of Corynebacterium sp. Actual size.

1 - 10 : Saline extracts (SE) of isolates
11 & 12 : SE of Corynebacterium spp.
AS : Rabbit anti-KD serum

Figure 6b A continuous precipitin band formed by 3 smooth isolates and 1 rough culture and 2 strains of Corynebacterium spp. Actual size.

1 - 3 : SE of smooth cultures
4 : SE of rough culture
5 & 6 : SE of Corynebacterium spp.



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4 : SE of rough culture
5 & 6 : SE of Corynebacterium sop.

The response was measured by a microtitre agglutination test, and immuno-gel diffusion was used to assess the homogeneity of the sera. The specificity of these antisera was checked using authentic KD organisms and controls. Specific antisera were selected for future use in IFAT and for diagnostic work.

Gel diffusion results showed precipitin reactions demonstrating the homogeneity between the soluble antigens of the KD strains used for antiserum production.

Good fluorescence of all R.salmoninarum strains was achieved with WS03, WS07 and WS04 anti-KD sera. Poor fluorescence was obtained with the other sera. Final bleedings were taken seven days after last injection, distributed into 2ml aliquots and stored at -20^{o}C to provide a supply of diagnostic sera.

C. Examination of the Diversity of Colonial Morphology

1) Surface growth

Most of the strains did not present any problem in culture and grew well on KDM_2 as long as freshly prepared medium was used. Colonies were discernible at about Day 12, and a variation in size was noted which was recognised as being characteristic of R.salmoninarum. The larger colonies were creamy-white in colour, convex, circular, smooth edged, shiny and about 1.5mm in diameter. Smaller colonies with the same characteristics were about 0.5 - 1.0mm in diameter, and transparent discs about 0.2 - 0.5mm in diameter were just visible to the naked eye. Sub-culture of each colony type produced a growth containing the three colony variants. It was found that the incubation time was reduced considerably for growth from sub-culture, as compared to that for direct isolation (e.g. from 12 to 5 days).

The most readily noticeable feature was produced by some strains after repeated subculture. A spontaneous transition occurred from a smooth culture to a rough crystalline form consisting of dull and dry colonies of similar size to those found in smooth cultures. The colonies were white in colour and did not emulsify in saline, distilled water or serum. A reversion to the smooth form was not achieved on further subculture and liquid cultures produced a granular growth with the development of a pellicle after 14 days incubation.

2) Immunogel diffusion

Whole cell formalized suspensions adjusted to OD 0.4 at 650nm on an SPL600 were prepared from smooth and rough cultures and compared by immuno-gel diffusion using sera raised against different strains of R.salmoninarum. The antigen preparations of both produced single precipitin bands against all the anti-KD sera tested (Figure 6b).

3) Electron microscope examination

Electron microscope examination of negatively stained and sectioned preparations of bacteria from rough and smooth cultures revealed no significant morphological differences between the bacteria, although a high degree of clumping was observed by organisms from rough colonies.

A marked variability in the layering of the cell wall and a very irregular exterior in the form of surface projections was noted in organisms from each colonial type (Figure 7, 8). Further investigations using an immunocytochemical technique were performed by the Electron Microscope Department (Wellcome) using the method of Beesley, Orpin & Adlam (1984).

A colloidal gold marker was labelled using the WS03 antiserum and examined by transmission electron microscopy. An affinity for the surface projections was demonstrated by the marker suggesting that with the serum used there are specific antigenic binding sites for R.salmoninarum antibody (Figure 9).

In contrast, control samples using unlabelled colloidal gold were negative.

4) Challenge studies

Challenge experiments in rainbow trout showed a slightly decreased virulence with the rough strain compared with the smooth strain in terms of numbers of deaths. However, similar macroscopic lesions were observed regardless of the organism used to infect.

All ten fish challenged intraperitoneally with a suspension of smooth bacteria adjusted to an optical density of 0.2 at 650nm on an SPL600, had died by Day 25.

Seven out of the ten fish challenged with a similar suspension of rough bacteria had died by Day 25. No further deaths were recorded for the duration of the experiment.

All ten control fish injected with 0.85% saline survived the experiment.

The experiment was terminated at Day 25.

Figure 7 Electron Microscopy
The ultrastructure of bacteria from rough and smooth cultures.
The cell wall layers and the irregularity of the surface is demonstrated.

a) Negatively stained bacteria from smooth culture x 73,200.

b) Negatively stained bacteria from rough culture x 73,200.

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The ultrastructure of bacteria from rough and smooth cultures.
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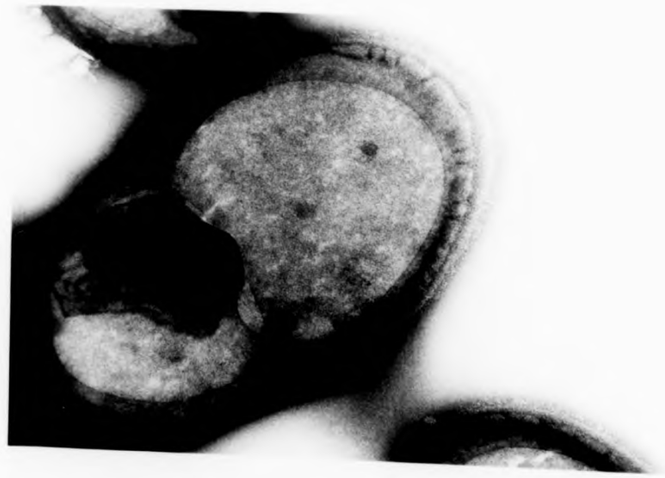
a) Negatively stained bacteria from smooth culture x 73,200.



b) Negatively stained bacteria from rough culture x 73,200.

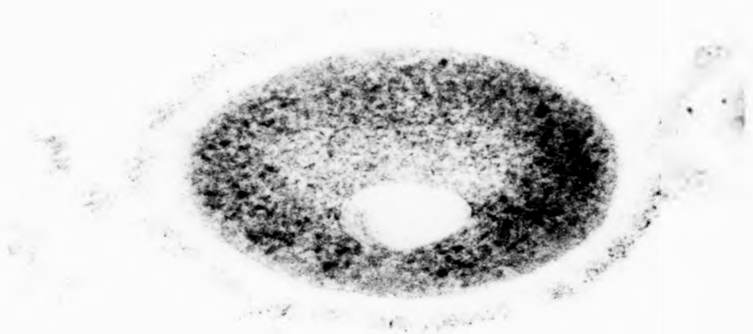


Figure 8 Thin sections of bacteria prepared from:

a) Smooth culture on KDM_2 x 144,800.

b) Rough culture on KDM_2 x 144,800.

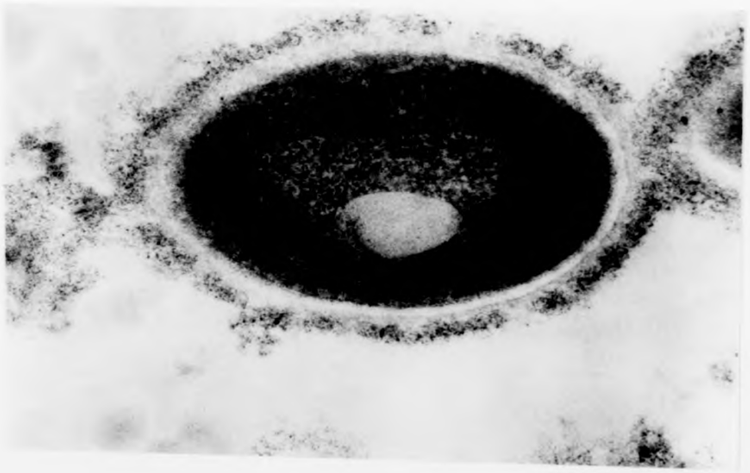


Figure 8 Thin sections of bacteria prepared from:

a) Smooth culture on KDM_2 x 144,800.

b) Rough culture on KDM_2 x 144,800.

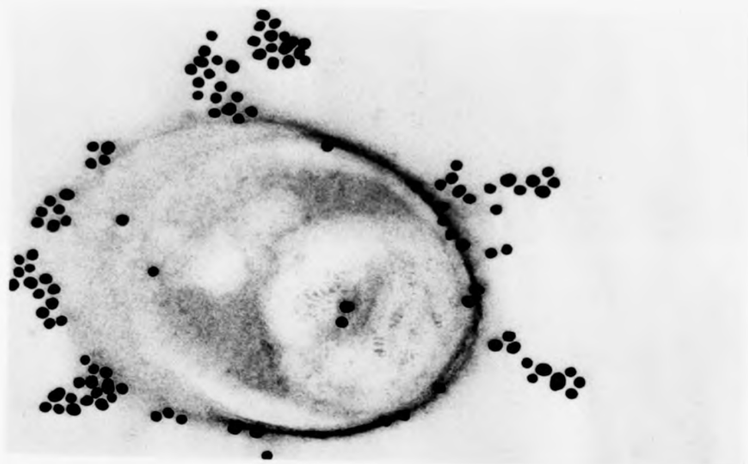


Figure 9 Thin section of osmium fixed R.salmoninarum x 105,000.

Demonstrates the affinity of colloidal gold particles labelled with specific anti-KD serum to the cell wall surface projections.

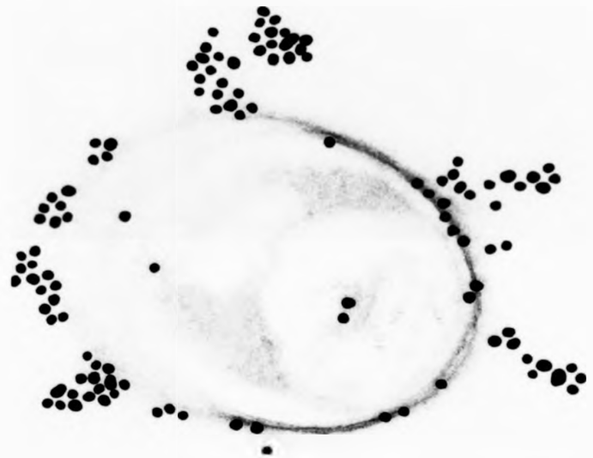


Figure 9 Thin section of osmium fixed R.salmoninarum x 105,000.

Demonstrates the affinity of colloidal gold particles labelled with specific anti-FD serum to the cell wall surface projections.

D. Comparison of BKD strains with C.pyogenes

1) Culture

A comparative study between BKD strains and C.pyogenes included an investigation of the cultural and biochemical characteristics. Marked differences were observed in the cultural requirements and the biochemical reactions. The most obvious cultural differences were the inability of the BKD strains to grow on blood agar medium, and its failure to grow at 37°C on blood agar or KDM_2. C.pyogenes did not grow at 15°C but grew well on blood agar at 37°C producing tiny ß-haemolytic colonies after 24 hours. C.pyogenes also grew on KDM_2 at 37°C producing cultures of similar appearance to those of the BKD strains.

Morphologically, the organisms from all cultures were very similar; Gram positive coccobacilli with a tendency to lie at acute angles to one another. C.pyogenes exhibited a greater degree of pleomorphism and coccoid forms were seen occasionally to form chains.

2) Biochemical Results

The results obtained demonstrated the non-reactive nature of the BKD isolates. Very weak fermentation reactions were indicated by a slow but not complete acid production and a colour change from purple to blue-green in a small number of sugar media.

Gelatin or serum were not liquefied, but a slight softening of the medium was noted.

In contrast, C.pyogenes were fermentative, liquefied gelatin and serum, and produced acid and a clot in the BCP milk (Table 4).

Table 4 Differential biochemical characters of 11 strains of R.salmoninarum and 3 strains of C.pyogenes

	STRAIN WS											C.PYOGENES		
	01	02	03	04	05	06	07	08	09	10	11	1	2	3
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Lactose	±	±	±	±	±	±	±	±	-	±	±	-	-	+
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Inulin	-	-	-	±	±	-	±	±	-	±	±	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	±	±	±	±	-	±	±	-	-	±	±	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	±	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	±	±	±	±	±	-	-	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Serum slope	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malonate	+A	+A	±	±	±	±	±	±	±	±	-	-	-	
Bromo-cresol purple milk (BCP)	-	-	-	-	-	-	-	-	-	-	-	+A+C	+A+C	+P
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+

A = Acid; C = Clot; P = Pepronisation

E. Amino Acid Composition of the Peptido Glycan Cell Wall Layer

A) Amino acid analysis of the purified cell walls indicated that the major amino acids contained in the peptido glycan cell wall layer were alanine, glutamic acid, lysine and glycine. A small broad double peak was observed which was considered to be muramic acid.

The molar ratios of the "major" amino acids were determined as values relative to lysine.

Lysine, glutamic acid, glycine and alanine were calculated in molar ratio of approximately 1:1:1:3.5 (Table 5, Figure 10).

B. Two dimensional chromatography

A failure of the two dimensional chromatographic test to separate and detect the amino-acids contained in the cell wall preparations was attributed to insufficient concentrated material available.

Table 5

Amino-acid analysis of cell walls prepared from R.salmoninarum

Amino acid	Molar Ratios	
	Run 1	Run 2
Aspartic acid	0	0
Threonine	0	0
Serine	0	0
Glutamic acid	1.16	1.24
Glycine	0.91	0.94
Alanine	3.37	3.45
Leucine	0	0
Hexosamines	Not calculated	Not calculated
Lysine	1.0	1.0
Ammonia	Not calculated	Not calculated

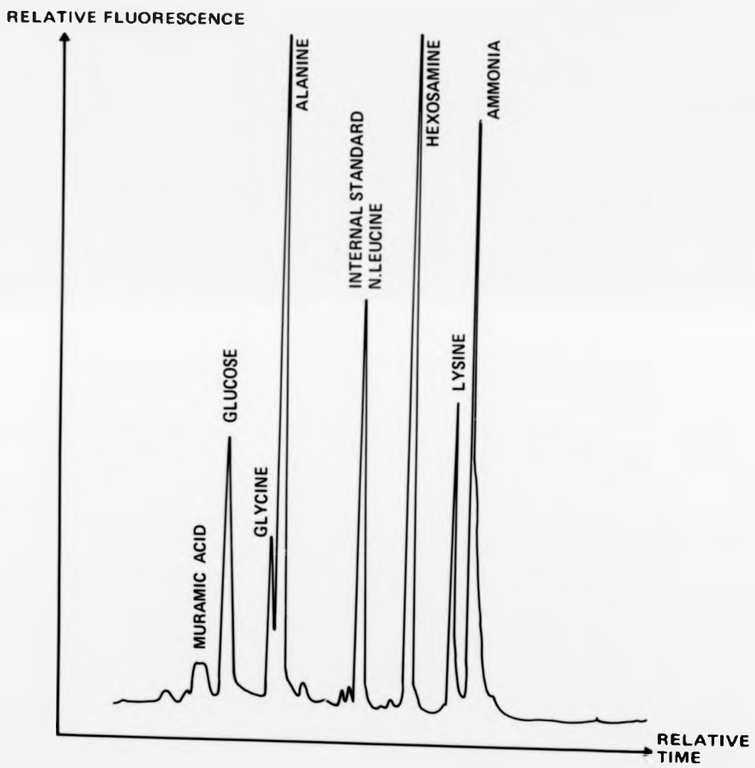


Figure 10 Amino-acid profile of cell walls of R.salmoninarum

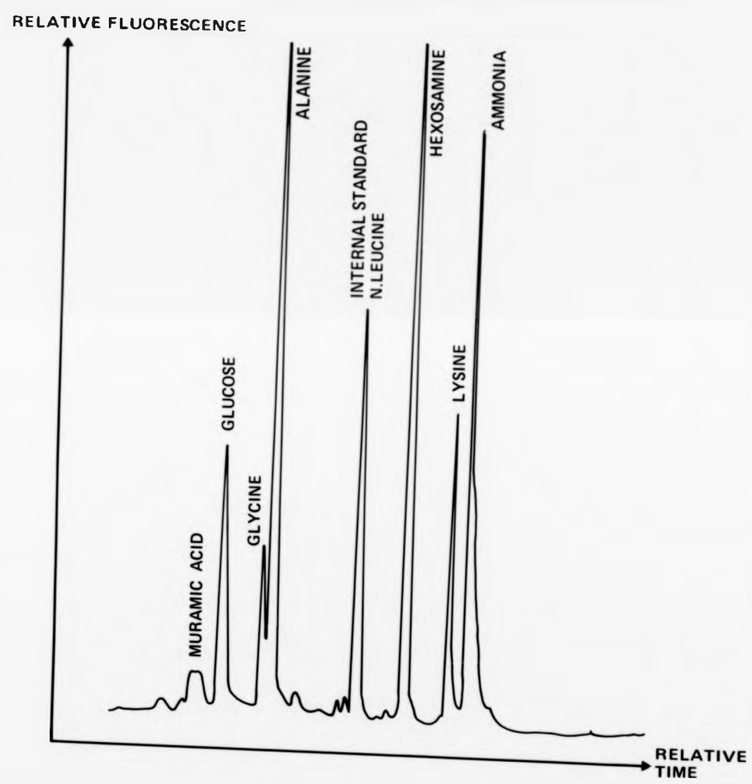


Figure 10 Amino-acid profile of cell walls of R.salmoninarum

DISCUSSION

Isolation and Characterisation of R.salmoninarum

Direct isolation of Renibacterium salmoninarum from infected specimens presented many problems. Difficulties arose with contaminants, either post mortem invaders or airborne, and commensals. The degree of contamination varied between organs examined; for example, commensals from the gut became a major problem. The condition of the fish on receipt was also an important factor. The organism was isolated most successfully from moribund fish and if these were not available it was found that the time between death and examination had a great bearing on whether isolation was achieved. The addition of fungizone to the culture medium, the excision of contaminants at regular 2 day intervals, and the subculture of suspected growth at the earliest possible stage helped to overcome this problem. Incubation time was greatly reduced to 5-10 days for subculture. Although several culture media were used for direct isolation work including Mueller Hinton, Dorset egg and Loeffler, KDM_2 with the addition of fungizone proved to be the medium of choice. The first sign of growth on KDM_2 invariably appeared as a fine transparent film at about day 12-15. Subculture to obtain pure cultures was necessary for confirmatory investigations. Temperatures between 15-18^{o}C were found to be optimum for isolation. Temperatures outside this range, up to 22^{o}C and down to 9^{o}C allowed a slow growth, but this was unsatisfactory for direct isolation. It was important to maintain a moisture free atmosphere throughout incubation. Freshly prepared KDM_2 plates were thoroughly dried to avoid surface moisture, and inoculated plates were placed in plastic bags, which were evacuated and tightly sealed after each examination.

Evelyn (1977) compared KDM_2 medium with KDM_1 supplemented with serum instead of blood (Ordal and Earp, 1956), and cysteine supplemented Mueller Hinton (MH) (Wolf and Dunbar, 1959). For primary isolation of the KD organism serum was found to be an essential constituent of the medium. although KDM_1 and KDM_2 gave comparable estimates of the number of bacteria present, and MH gave low estimates in terms of growth yields depending on the strain involved. KDM_2 was 8.36 times better than MH and 3.5 - 10 times better than those on KDM_1. The cultural technique involved the preparation of 10 fold serial dilutions of homogenised tissues and "drop plating" 0.025ml drops of each dilution onto thoroughly dried agar. Although laborious, this method has proved to be highly sensitive for detecting BKD infections. He found that growth on KDM_2 from overtly infected specimens occurred within 4 - 10 days, but incubation periods of 3 -5 weeks were sometimes required for highly diluted inocula.

Suspect cultures were examined by a battery of tests, including Gram stain, IFAT and immuno-gel diffusion. Results from these tests confirmed the identity of isolates. Microbiological examinations for other known fish pathogens were negative.

Colonies developed in the streaked portion of the plate. A wide diversity of colony size was noted even in subcultures from a single colony. Immuno-gel diffusion showed no antigenic difference between them. Repeated subculture on KDM_2 of certain isolates produced a marked change in the colonial appearance. The colour changed from cream to white and the texture from smooth and translucent to a rough dry growth. Investigations were carried out to determine whether a possible mutation

had occurred which had also altered the virulence of the organisms and their ability to produce precipitins and agglutinins. This apparent smooth-rough transition was irreversible on subsequent subcultures which autoagglutinated in saline.

Smooth-rough mutation commonly occurs in the Gram negative bacteria of the genus Enterobacteriaceae. The outer surfaces of these bacteria consist of a variety of components:- cell wall antigens, including lipopolysaccharide (LPS) protein, and lipid or lipoprotein, extracellular capsular or slime substances, and appendages such as flagellae and pili. The LPS molecule consists of three main parts i) a polysaccharide core, ii) a lipid portion (lipid A); and iii) the O-specific antigenic polysaccharide side chain (Luderitz, Westphal, Staub & Nikaido, 1971).

It is thought that the 'O' specific side chains of the LPS complex are orientated towards the outer cell surface, and are thus more accessible than the rough core polysaccharide. During rough-smooth dissociation, the smooth 'O' side chain is lost, leaving the core region accessible (Freer & Salton 1971). According to Rowley (1971), regions of the bacterial surface in which rough core polysaccharide is accessible to antibody are particularly susceptible to attack by the bacterial complement system.

In many species of organisms a loss of virulence is correlated with a change of colony texture from smooth to rough and a corresponding loss of o-somatic antigens. This is called the smooth-rough mutation.

Artificial attenuation is often achieved by repeated subculture of laboratory stock cultures. The resultant R type tends to autoagglutinate though it may remain stable in weak saline solutions (0.2%). These types are antigenically different, thus an S.antiserum may not agglutinate an R mutant. Analogous changes in cell surface components occur in bacteria other than members of the Enterobacteriaceae including Gram positive species. Owing to the wide diversity in the composition of cell surface components S (virulent) and R (avirulent or less virulent) strains do not always correspond to 'smooth' and 'rough' colonies respectively. For example, strains of Streptococcus may form mucoid colonies, while less virulent strains form 'shiny' or glossy colonies. The change from the mucoid to glossy colony would in this case be an instance of smooth-to-rough variation. In Streptococcus pneumoniae S-R variation is associated with the loss of capsule.

Brodie and Shepherd (1950) found that batches of the same brand of agar used at a concentration between 1-2% w/v differed in gelling power, and some affected the colonial morphology, notably matching genotypically smooth Enterobacteriaceae form phenotypically rough looking colonies.

In view of what is generally understood to be the cause of smooth-rough mutations, it is difficult to explain the colonial variation - smooth-rough transition exhibited by Renibacterium salmoninarum. As a Gram positive bacterium, the cell wall consists of peptidoglycan intimately associated with techoic acids and or, proteins and techuronic acids. It lacks LPS which confers the smooth rough characteristics to Gram negative bacteria. Electron microscopy examination using a labelled

antibody has shown that the organism does not have a capsule. The same batch of medium was used for all cultures at each subculture. A small number of isolates only showed a smooth-rough transition which tends to rule out the possibility of the medium being the cause.

There is a possibility that under certain conditions e.g., after repeated subculture, a component of the cell wall cannot be synthesised and the defect produces rough outgrowths from the edge of the colony. Unfortunately, this speculation has not been confirmed by EM examination carried out so far.

Ultrastructural examination failed to show any pronounced morphological difference between rough and smooth organisms, but a marked clumping of the organisms from the rough colonies was noted.

Injection into fish of suspensions of rough and smooth organisms of the same opacity revealed a reduction in mortalities in the group injected with the rough suspension. Similar macroscopic lesions, however, were observed in fish from both groups. If further challenge work revealed an obvious reduced virulence of the rough bacteria, the significance of this finding could have far reaching applications in vaccine preparation.

Young and Chapman (1978) examined the ultrastructural morphology of two strains of R.salmoninarum. One, the Lamar strain was examined within the host tissues of naturally infected brook trout and the other the R11 strain as harvested from culture media and as it appeared in experimentally infected tissue. They found that cells maintained in culture

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showed a consistent bacillary form whereas cells growing within host cells were irregular in shape. They also found that the cell wall layering was generally variable and its exterior surface frequently irregular. There was no evidence of a "part fusion" snapping process which is characteristic of coryneform bacteria (Barksdale 1970).

The features described by Young and Chapman agree with those of the WS03 isolates, in particular the variability of the cell wall layers and the irregularity of the cell surface. They did not describe the surface projections reported in the present study.

The immunocytochemical method employed to examine the surface projections, common to both rough and smooth organisms, resulted in their identification as antigenic binding sites of R.salmoninarum antibody labelled gold particles by acquisition of the specific activity of the adsorbed macromolecule.

This raises the question of whether such binding sites are immunogenic and capable of eliciting an immune response which provides protective immunity.

Surface antigens which induce virulence in bacteria have been identified for certain bacteria of veterinary importance, for example the K88 pillus antigen found on some E.coli pig pathogens.

Aeromonas salmonicida the causative organism of the fish disease furunculosis and probably the most important pathogen of salmonid species (Herman, 1968) possesses external to the cell wall an extra surface layer of protein known as the 'A layer' which has been shown to be responsible for the virulence of the organism.

However, it must be noted that there are many technical problems and complexities associated with adsorption of macromolecules to gold particles and further work is necessary to corroborate these results.

Culture in broth was most successful using shake flasks and maximum growth was achieved after approximately 3 weeks incubation of 10^9 orgs/ml maintaining a high rate of aeration. The starting culture had a pH of about 6.5, a period of autoagglutination with the formation of a sediment then occurred about the mid-growth cycle, and the growth cycle ended when a pH of between 8.0 and 8.5 was reached. A soluble antigen was detected in the broth supernatants which precipitated in immunodiffusion experiments using homologous WSO3 anti-R salmoninarum serum.

To test the homogeneity of strains isolated in this study with those involved in BKD outbreaks, production of suitable antisera was necessary. No difficulty was experienced in raising suitable precipitating and agglutinin antisera in rabbits according to the method of Bullock and Stuckey (1974). The responses of the rabbits to the adjuvanted type strains were good, and sera with high titres were produced. A comparative study showed that there was little antigenic diversity among isolates of

R.salmoninarum using gel diffusion and agglutination titres diminished at the same rate on adsorption with homologous and heterologous antigens. Biochemical tests also showed identical fermentation and biochemical patterns between strains. The widespread use of the term "coryneform" to describe any non sporing Gram positive rod of irregular outline gave rise to the inclusion of the causative organism of BKD into this genus, and it was called a Corynebacterium spp. Further suggestions were made that the organism may have been closely related to Corynebacterium pyogenes (B.Austin, 1980). It was this proposal which prompted the comparative study between strains of C.pyogenes and Type strains of the causative organism of BKD.

C.pyogenes is an aerobic and facultative organism. It is fermentative and produces acid from glucose, fructose, galactose, xylose, maltose, sorbitol, dulcitol, glycerol, inositol, arabinose, rhamnose, raffinose, sucrose and trehalose. All the kidney disease strains tested however, proved to be totally non fermentative. C.pyogenes is catalase negative, although some strains have shown a weak catalase reaction, indole is not produced, nitrate not reduced and litmus milk is digested and produced an acid clot. The type strains of R.salmoninarum were biochemically non reactive.

Cultural characteristics at 15°C on KDM were found to be similar in colonial morphology but incubation time varied considerably between BKD strains and C.pyogenes strain. On blood agar C.pyogenes produced a zone of β haemolysis around its tiny colonies after 24 hours. The KD strains did not grow on BA. The optimum temperature for C.pyogenes was 37°C with a range of between 15°C to 40°C.

As with the KD strains, colonial variants are found to occur in the C.pyogenes group. A spontaneous conversion of a small colony type (C.pyogenes S to the large colony type C.pyogenes L) was observed in culture studies. (Barksdale, Cummins & Harris, 1957).

Sera raised in rabbits against C.pyogenes strains did not produce precipitin lines in gel diffusion or agglutinate the WSO3 or type strains. Immunologically therefore, they appeared to be unrelated.

Fish inoculated intraperitoneally with 0.1 ml suspension containing 5 x 10^8 cells per ml prepared from a C.pyogenes culture did not show any signs of BKD and remained healthy for the duration of the experiment. Histological examination of kidney, spleen, heart, liver and gut revealed no pathological changes which resembled those found in BKD.

To prove conclusively that C.pyogenes is not related to the causative organism of BKD it was necessary to study the cell wall properties of the isolate. The composition of the peptidoglycan and the principal sugars were therefore examined in the WSO3 strain and compared with C.pyogenes.

C.pyogenes bears little similarity to other animal pathogen corynebacteria. It has a different cell wall composition and shares a cell wall polysaccharide antigen with Streptococci of the Lancefield Group G.

The reported Guanine and Cytosine (G & C) contents of the DNA of corynebacteria cover a wide range between 48 to about 70%. C.pyogenes have G & C contents of 58-59%. It would appear, therefore, that determinations of DNA base ratio are of limited value in the taxonomy of coryneform organisms.

The features most readily used in the taxonomy of the coryneform organisms are the principal sugars identified in cell wall hydrolysates and for the diamino acid of the peptidoglycan. The presence of mesodiaminopimelic acid as the diamino acid of the peptidoglycan and a polysaccharide containing arabinose, galactose and often mannose are characteristic. The cell wall sugar components of C.pyogenes are rhamnose and glucose and most importantly the diamino acid of the peptido glycan is lysine instead of mesodiaminopimelic acid and also contains alanine and glutamic acid. They do not possess mycolic acids. Because of this, and that the polymer containing arabinose is not present but in its place is a rhamnoyl polymer which cross reacts with Group G Streptococci antisera, it was proposed by Barksdale (1970) that C.pyogenes be removed from the family Corynebacteriaceae on the grounds that it has too little in common with the type species.

The WSO3 strain was found to share similar cell wall characteristics to C.pyogenes. Four major amino acids were determined:- Lysine, Glutamic acid, Glycine and large amounts of Alanine. The molar ratios were 1:1:1:3.5 respectively and the diamino acid was found to be lysine. Mycolic acids and galactose were not present. These results correlated well with those of Sanders and Fryer (1980) who also examined the DNA

base composition and the neutral and amino sugar composition. The average % G & C contents of the DNA's for these BKD isolates was 53 ± 0.46 mol %. This differs markedly from the G & C value for C.pyogenes. Glucose was the prinicipal sugar detected. Others were rhamnose, mannose, arabinose and the amino sugars, glucosamine and muramic acid.

On the basis of these findings they proposed that a new genus be created for the kidney disease organism, Renibacterium and that members of the same species belonging to this genus be called R.salmoninarum. It may, therefore, be assumed that the WSO3 strain which conforms closely to the characteristics outlined for this new genus, also belongs to the genus Renibacterium and shall be called R.salmoninarum.

The question of classification of KD organisms has provoked a variety of suggestions. Bullock Stuckey and Wolf (1975) suggested a resemblance between the so-called KDB and the genus Listeria. Sanders and Fryer (1980) however, have shown that the G & C content of the DNA of R.salmoninarum is 53.00 ± 0.46 mol %, whereas that of the family Listeriacae is 38-42%, and that the peptidoglycan of Listeria sp contains mesodiaminopimelic acid and not lysine as in R.salmoninarum.

Austin & Rodgers (1980) proposed a relationship between KDB and Lactobacillus on the basis of general biochemical preperties. This suggestion may also now be discounted in the light of the BKD cell wall biochemistry.

A pseudo kidney disease was described by Ross & Toth (1974) from which a Lactobacillus spp was identified on the basis of its biochemical characteristic as the causative organism, and conclusively distinguished it from the causative organism of BKD.

It has since been suggested that organisms belonging to the Lactobacillus genus are relatively common contaminants in cases of BKD, but are easily differentiateed from R.salmoninarum by their larger size, tendency to form chains, cultural and biochemical chacteristics.

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Section II DEVELOPMENT OF A CHALLENGE SYSTEM

Introduction and Literature Review

The mode of transmission of the causative organism of BKD has not been fully determined. Experimental work has shown that the disease can, with difficulty, be contracted by in-contact fish but in field outbreaks vertical transmission is commonly involved (Klontz, 1982).

The first reported success in transmitting BKD experimentally was recorded by Earp et al, (1953), who succeeded in infecting fingerling sockeye salmon by feeding infected flesh and viscera from adult chinook salmon. Although Snieszko and Griffin (1955) did not achieve the same success using fingerling brook trout, Wood and Wallis (1955) suggested that the natural disease might be transmitted to fish feeding on infected tissues.

Wolf and Dunbar (1959) investigated the portal of entry of infection by artificial challenge under hatchery conditions. They attempted to infect large numbers of trout with isolates from either Scotland or the U.S.A. The organisms were incorporated into the food, an abrasive was added, and the suspension introduced into troughs containing fish with natural or artificial skin scarifications. These attempts failed. In contrast, the eastern U.S.A. strain proved to be more virulent and was readily transmitted by direct application to abraded skin. They concluded from this work that the organism was probably transmitted through the skin, especially under crowded hatchery conditions.

Experimental transmission of BKD has been most successful by intraperitoneal injection of infected tissue homogenates or of pure culture suspensions. Thus Ordal and Earp (1956) produced 100% mortality by IP inoculation of a culture suspension in saline and of homogenised frozen infected tissue. Characteristic coccobacilli were found in large numbers in smears taken from each test fish, and the organisms were isolated from a number of fish examined shortly after death.

Smith (1964) also used the IP route of inoculation to test the virulence of 19 strains of the organism isolated from fish with Dee disease, in rainbow trout and Atlantic salmon parr. Disease developed in 80% of the experimental fish and the organisms were demonstrated in smears from livers and kidneys. Reisolation of 15 of the 19 strains used in the experiment was achieved.

Evidence produced by Bullock, Stuckey & Mulcahy (1978) and Klontz (1978) suggested that BKD is transmitted in the egg. Bullock and Stuckey obtained eggs from two sources, chinook salmon with clinical BKD and rainbow trout that had a history of the disease. Clinical signs of the disease were absent at the time of spawning, but occurred in all groups at fry and fingerling stages. Klontz obtained similar results and further showed that disinfection with organic iodine at 100 p.p.m. reduced the severity of the clinical signs but not the incidence of infection, suggesting transmission of the organisms within the egg.

The prevalence of kidney disease has been found to be associated with certain predisposing environmental factors. Conditions which can produce stress increase both the incidence and the severity of the disease. Earp et al, (1953), Wolf & Dunbar (1959) and Smith (1964) reported that losses were higher at water temperatures lower than 13°C. Sanders, Pilcher and Fryer (1978) confirmed these findings experimentally and also showed that BKD was suppressed by water temperatures between 17.8 - 20.5°C.

It had been noted that BKD was more severe in "soft water" stations than in those with "hard water". Warren (1963) analysed the water from various hatcheries and found that as the mineral content of the water decreased, the severity of BKD increased, but he was unable to determine the constituent involved.

Bullock & Stuckey (1978), however, found no apparent effect on the incidence and severity of BKD in soft water as opposed to hard water areas.

An increased mortality has been shown when overtly and covertly infected fresh water fish enter sea water during their rearing phase (Frantsi, Ritter & Elson 1977). An alteration in the intrarenal environment which favours growth of the organism may be the reason for this (Klontz, 1982).

Diet may also be a predisposing factor. Wedemeyer & Ross (1973) fed dry diets containing corn gluten to Pacific salmon and increased the mortality from BKD. He concluded that this was because cases of the disease were more severe rather than that their incidence was increased.

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The occurrence of clinical BKD has been linked with the dietary influence of high levels of trace elements and low levels of calcium. Fish fed increased levels of iron, copper, manganese, cobalt, iodine and fluorine experienced lower BKD mortality rates than control fish fed recommended levels. A therapeutic reduction of BKD infections in chinook salmon fed high levels of iodine was demonstrated. Although iodine and fluorine showed the greatest effects on reducing BKD mortalities, when combined with zinc and/or magnesium the same response was not found (Woodall & Laroche, 1964). It has since been reported by Paterson, Lall and Desautels (1978) that minimal levels of BKD occurred in fish fed high trace element or low calcium diets. They attributed this therapeutic effect to the increased availability of trace elements for dietry purposes. Their results lent further support to the view that BKD infections influence trace element utilization.

It has also been shown that fish of different transferrin genotypes differ in their resistance to BKD (Suzumoto, Schreck & McIntyre 1977).

This section investigates artificial transmission of R.salmoninarum to rainbow trout. The primary objective was to establish a reliable, reproducible challenge system, and to compare the histopathology of the artificially infected fish with that of the natural disease. Variations in virulence between different strains of the organism were determined and diagnostic procedures evaluated with particular reference to their ability to detect varying degrees of infection.

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Section II

MATERIALS AND METHODS

1. Maintenance of Fish

Rainbow trout which had previously been screened and found free of BKD, were obtained from commercial trout farms and transported in heavy duty plastic bags in ice cold and well oxygenated water. Fish were held in glass fibre tanks of 2' diameter in water maintained at 15°C and oxygenated by passage through fine sprays, exchanging at 2 tank volumes/hour. Fish were fed on a commercial trout pellet diet (Omega) during the course of the experiments. Observation of fish for 10 days prior to challenge was routinely carried out.

2. Inocula

Cultures of R.salmoninarum were grown on KDM_2, harvested by scraping, washed twice in 0.85% saline, and the opacity of the suspensions adjusted using standard optical density methods (SPL600). Challenge was administered immediately at varying required dose levels through a 25 gauge needle. Control fish each received a similar injection of saline.

3. Examination and Investigations

Observations of the fish for clinical signs of BKD were recorded daily. Fish were killed by immersion in MS222 (Tricaine methane-sulfonate-Sigma) solution and requisite organs were dissected out. Visible signs of the disease were noted.

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a) Bacteriology and serology

Homogenised tissues from putatively infected fish were plated onto KDM_2 containing fungizone (Gibco) as in Section I. Organisms suspected of being R.salmoninarum were examined both by Gram stain and by the indirect FAT, using serum prepared as described in Section I. The relative numbers of organisms in various organs were estimated using the Gram stain and IFAT on impression smears and organ sections.

The supernatants from approximately 50% organ homogenates in P.B.S. pH 7.2 were examined for bacterial antigens by immunogel diffusion against the anti-KD serum as described in Section I.

b) Histology

Tissue samples were fixed immediately in 10%. neutral buffered formalin, dehydrated, embedded in paraffin wax, sectioned at 5μ on a Leitz Base sledge, Model 1300 and stained with Haematoxylin and Eosin (Culling, 1974) and Gram-Twort (Wellcome modification TOX/SOP/7, Appendix D/45).

Gram-Twort Stain

The sections were stained with Aniline crystal violet for 3-5 minutes and then in filtered Ammonium oxalate crystals. After rinsing in distilled water Grams iodine was applied for 3 minutes, immediately rinsed in distilled water and blotted dry. Acetone was used to decolourise until the sections were a deep straw colour. After rinsing in distilled water the sections were stained in a modified Twort's stain for 5 minutes, rinsed again in distilled water and decolourised in acetone until the red colour was lost from the sections. Once cleaned in xylene the sections were mounted in DPX.

Gram positive organisms were indicated by a dark blue colour, Gram negative - pink, nuclei - red, cytoplasm - light green and RBC - green.

Primary Transmission Experiment

Fish approximately 100g in weight were used for this experiment.

The WS03 isolate was used to meet the requirements of Koch's postulates and a suspension in 0.85% N saline was prepared from a KDM_2 culture, washed twice and adjusted to an opacity of 0.2 OD at 650nm on an SPL600.

Two groups each of 10 fish were used. Group 1 was inoculated intraperitoneally with 0.1ml of bacterial suspension and Group 2 with saline.

Development of a Standard Challenge System

Preliminary experiments were carried out to determine the essential criteria required to obtain optimum results. Attempts were made to determine:

a) the optimum dose of organisms required to produce disease and death using bacterial suspensions at predetermined optical densities.
b) the most successful route of infection; intramuscular, intraperitoneal or bath.
c) the ideal age/size of fish to use as regards susceptibility to disease, facility in handling and for tissue sampling.

All experiments were continued for 60 days when all remaining fish were sacrificed.

a) Dose response experiment

Five groups, each of 10 fish 50-100g in weight were used. The bacterial suspension was prepared as above, and 0.1ml injected intraperitoneally at approximate 4 dose levels: 10^9, 5×10^8, 10^8 and 5×10^7/ml.

Control fish were inoculated with 0.1ml sterile normal sterile.

b) Experiment to determine the ideal route for challenge

Three groups each of 10 fish approximately 50g in weight were injected with 0.1ml of a bacterial suspension containing approximately 5×10^8 organisms per ml. Group 1 were inoculated by the intraperitoneal route and Group 2 by the intramuscular route. Group 3 were immersed in a bath containing approximately 5×10^8 organisms per ml for 10 minutes.

Three control groups each of 5 fish were used.

c) Experiment to determine the ideal size of fish for challenge

Three groups, each of 10 fish were used. Group 1, of 200-250g in weight, were inoculated with 0.4ml of bacterial suspension containing approximately 5×10^8 organisms per ml. Group 2, 100-120g in weight with 0.2ml and Group 3, 50-60g in weight, with 0.1ml.

Five control fish of each size were used.

Using results obtained from the preliminary challenge experiments a final protocol for a standard challenge system was drawn up.

Challenge Experiment

Groups of 25 fish approximately 50g in weight were used. Sample fish were sacrificed before challenge for bacteriological and histological examination.

Inoculum:

The inoculum was prepared as above to contain 5×10^8 organisms per ml. Fish were immediately challenged with a dose of 0.1ml administered intraperitoneally through a 25 gauge needle.

The fish were observed daily and moribund or dead fish examined for clinically detectable signs of disease, and kidney, heart, spleen, liver, muscle and swimbladder were investigated as described above.

The challenge experiment was used to compare the virulence of different strains of R.salmoninarum with the WS03 isolate.

Section II

RESULTS

Primary Transmission Experiment using Laboratory Isolate

The first deaths occurred on the eighth day after challenge and by Day 22 all the fish had died.

All control fish had survived at Day 60.

Clinical Signs

Affected fish were lethargic, unresponsive to food and tended to swim to the sides of the tank. Skin was darker than normal in colour and external lesions were apparent on some fish, including exophthalmos and small "blister-like" lesions.

Post Mortem Findings

The internal organs of challenged fish showed gross signs of the disease and some of the fish had enlarged spleens. Most of the fish were found to have characteristic small white lesions on the spleen, liver or kidney. The heart appeared unaffected.

Bacteriology

Impression smears, prepared from internal organs and stained by Gram's method showed the presence of a large number of Gram +ve coccobacilli. The identification of the organism was confirmed by applying the IFAT to smears of organ sections. Moderate to large numbers of the organisms were observed in all kidney smears. Five fish showed large numbers of organisms in the heart, seven in the spleen and one in the liver.

Isolation was achieved after 13 days incubation at 15°C on KDM_2 from eight of the kidney and two of the spleen homogenates. A transparent, smooth film was first observed, which after 18 days incubation became a creamy white culture.

Whole cell saline extracts prepared from the isolations produced strong, precipitin lines in immuno-gel diffusion against standard anti-KD rabbit sera.

Development of a Standard Challenge System

a) Dose Response Experiment

Mortality

The first mortalities occurred in Group 1 challenged with 5×10^9 orgs/ml, and Group 2 with 5×10^8 orgs/ml at Day 8. By Day 28 all fish in Group 1 had died and nine in Group 2. At Day 60 the remaining fish from Group 2 and all fish from Groups 3 (10^8 orgs/ml), 4 (5×10^7 orgs/ml) and 5 (Control) survived. Three further experiments showed a similar pattern of results.

Clinical signs

Fish from Groups 1 and 2 exhibited similar clinical signs to those seen in Experiment A. Fish from Groups 3, 4 and 5, however, remained apparently normal.

Post Mortem Findings

Typical lesions of the liver, spleen and kidney were observed only in fish which died in Groups 1 and 2.

Bacteriology

Microscopic examination of organ smears using Gram stain and IFAT revealed large numbers of Gram positive, fluorescent coccobacilli in fish from Groups 1 and 2. Three fish from Group 3 showed moderate numbers of Gram positive, fluorescent coccobacilli in heart and spleen smears.

Reisolation of R.salmoninarum was achieved on KDM_2 from organ homogenates from seven fish in Group 1 and five fish in Group 2 and one fish in Group 3.

b) Experiment to determine the ideal route for challenge

Mortality

Fish challenged intraperitoneally with approximately 5×10^8 orgs/ml began to die eight days after inoculation, and by Day 30 all fish were dead. Fish challenged intramuscularly did not die as quickly as those in Group 1, but the first deaths occurred at Day 13 and at Day 60 one fish survived. In contrast, all control fish survived the experiment.

Clinical signs

In Group 1 and 2 as above. External lesions were clearly apparent at the site of inoculation in fish injected intramuscularly.

Post Mortem Findings

As above of Groups 1 and 2.

Bacteriology

Varying numbers of organisms were demonstrated in the kidney, liver, spleen and heart using the Gram stain and IFAT. Reisolation was achieved from a considerable number of organ homogenates from fish in Groups 1 and 2.

c) Experiment to determine the ideal size for challenge

Fish in Groups 2 and 3 began to die 7 days after incoulation and by Day 12, 50% were dead. At Day 60 all the fish were dead in Group 2 and 3 survived in Group 3. The clinical, post mortem and bacteriological findings were comparable to those found in previous experiments.

It was noted with some interest that nine out of ten fish in Group 1 had survived the experiment and were apparently normal. These fish were not sacrificed at this stage, but bled and rechallenged with a further 0.4ml. At the same time, ten fish of similar size were also inoculated with the same challange. All the fish were bled and rechallenged at 30 day intervals up to Day 180 when they were sacrificed.

Throughout the experiment the fish remained clinically normal, except for lesions at the site of inoculation which were apparent on some fish. Few visible post mortem abnormalities were found, but the most common were enlarged spleens and haemorrhagic and inflamed intestines. Some white lesions were observed on the kidney, heart and spleen in three fish. R.salmoninarum was detected by Gram stain and IFAT in organ smears and isolated from liver, kidney and heart and sites of inoculation from five fish.

Table 6

Agglutinating Antibody Response of Rainbow Trout to Sequential Intraperitoneal Injection of Live R.Salmoninarum

Days/ Injection	Number of Fish (A)									Number of Fish (B)									
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	10
30	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	4	<4	<4	8	<4	<4	<4
60	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	D	<4	8	<4	<4	<4
90	32	64	64	128	8	32	32	128	64	32	16	16	32	-	16	64	32	64	64
120	128	128	256	128	64	64	64	256	64	128	64	64	32	-	64	128	128	64	128
150	128	512	256	128	64	128	64	256	128	128	64	64	64	-	128	128	64	128	64
180	128	512	512	128	64	128	64	128	128	128	64	128	128	-	D	256	128	128	128

(A) = Fish survived original Experiment
(B) = Fish included in Experiment
D = Died

Control sera titres
WS03 - 1:4096
Goat anti kD - 1:2048
Pre-exposure - <4

Histological examination revealed small necrotic lesions containing Gram positive coccobacilli in liver and kidney from a number of fish and small necrotic lesions were found in the heart from 3 fish.

The sera were tested in parallel with standard rabbit and goat anti-KD sera and pre-exposure serum using the microtitre agglutination test described in Materials and Methods, Section I (Table 6).

No, or low, humoral agglutinin response was observed in fish at 30 and 60 days after challenge. Agglutinating titres were demonstrated at 90 days and persisted for the duration of the experiment. Repeated challenge did not significantly elevate the antibody response, and high titres were not produced.

Standard Challenge Experiment

1. Comparison of virulence between WS03 and WS07 strains and an evaluation of diagnostic procedures

Mortality

The cumulative mortalities are shown in Figure 11. Fish challenged with strain WS03 began to die seven days after inoculation, by 13 days 50% were dead. At Day 60 only one fish survived.

In contrast, fish challenged with strain WS07 did not begin to die until the 21st day, but by Day 34 50% were dead and only four fish survived at Day 60.

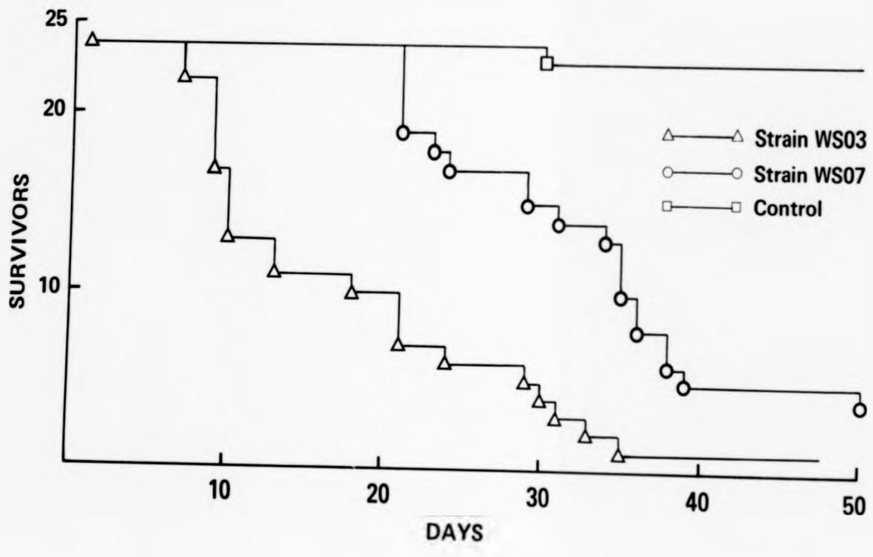


Figure 11 Cumulative mortalities of fish receiving KD strains WS03, WS07

Clinical signs

Affected fish were lethargic, swam at the sides of the tank and were inappetant. Their skin colour was darker than normal and other signs included exophthalmos, small "blister-like" lesions containing clear fluid and larger raised swellings at the site of injection (Table 7a, b).

Post mortem signs

No gross abnormalities were visible in fish inoculated with WS03 which died before Day 13 p.i. Fish which survived longer showed inflamed injection sites, enlarged spleens and a granular appearance of the kidney More severe changes became evident as the experiment progressed.

Increasing numbers of white lesions of varying size appeared throughout the substance of the liver, spleen and kidney, and pericarditis was also commonly visible. In the latter stages of the disease, nodule formation became more extensive, and the markedly milky appearance of the swim bladder was noted (Table 7a).

In fish inoculated with WS07 gross changes were generally not present (Table 7b).

Control fish showed no macroscopic abnormalities.

Bacteriological Examination

The results of examination using the four techniques described revealed marked differences between the two strains (Table 8a, b).

Table 7a Clinical Signs and Gross Pathology in Fish Challenged with R.salmoninarum Strain WS03

Day post-challenge	Fish No.	External Lesions: Darkened skin-colour	External Lesions: Site of inj.	External Lesions: Exoph-thalmos	"Blister-like" lesions	Internal Lesions: White nodules: Kidney	Spleen	Liver	Heart	Swim bladder
13	13	-	-	-	-	-(G)	-(E)	-	-	-
	14	+	+	-	-	-	-	-	-	-
18	15	+	+	-	+	-(G)	-	-	-	-
21	16	+	+	+	-	+(G)(E)	-	-(M)	-	-
	17	+	+	-	-	-	-	-	-	-
	18	+	-	-	-	-	-	-	-	-
24	19	+	-	+	-	+	-	-(M)	-	-
29	20	-	-	+	-	-	-(E)	+	-	-
30	21	+	+	-	++	+(E)	+	+	+	O
31	22	+	+	+	+	+(E)	+	-	-	-
33	23	+	-	+	+	+	+	+	-	O
35	24	+	-	+	+	+	-	-	-	O
60	25	+	-	-	-	-	-	-	-	-

Key:
E = Enlarged, G = Granular, M = Mottled, O = Opaque - Milky

Table 7b Clinical Signs and Gross Pathology in Fish Challenged with R.salmoninarum Strain WS07

Day post-challenge	Fish No.	Darkened skin-colour	External Lesions			Internal Lesions				
			Site of inj.	Exoph-thalmos	"Blister-like" lesions	White nodules				
						Kidney	Spleen	Liver	Heart	Swim bladder
21	1	+	-	-	++	-	-	-	-	-
	2	+	-	-	+	-	-	+	-	-
	3	-	-	-	-	-	E	-	-	-
	4	+	+	-	-	-	-	-	-	-
	5	+	-	-	-	-	-	-	-	-
23	6	+	-	-	-	-	-	-	-	-
24	7	+	-	-	-	-	-	-	-	-
29	8	+	-	-	-	-	-	-	-	-
30	9	+	-	+	+	-	-	M	-	-
	10	+	-	-	-	-	-	-	-	-
31	11	+	-	-	+	-	-	-	-	-
34	12	+	-	-	-	+	-	-	-	-
35	13	+	-	-	-	-	-	-	-	-
	14	+	+	+	-	-	-	-	-	-
	15	+	-	-	-	-	-	-	-	-
36	16	+	-	-	-	-	-	-	-	-
	17	+	-	-	-	-	-	-	-	-
38	18	+	-	-	+	-	-	-	-	-
	19	+	+	+	+	-	-	-	-	-
	20	+	-	+	+	-	-	-	-	-
39	21	+	-	+	-	-	-	-	-	-
60	21	+	-	+	-	+	-	+	-	-
	23	+	-	+	-	+	-	-	-	-
	24	+	-	+	-	+	-	-	-	-
	25	+	-	+	-	+	-	-	-	-

Key:
E = Enlarged, M = Mottled

Table 8a The relative sensitivities of the Gram, IFAT, Gel diffusion and culture methods at detecting R.Salmoninarum in specimens from fish challenged with strain WS03

CULT			0 0 0 0										
GD			0 0 0 0										
IFAT			2 2 2 0										
GRAM			2 0 0 0										
CULT			0 0 0 0	4 4 0 0									
GD			0 0 0 0	4 4 4 4									
IFAT			0 0 0 0	3 4 4 4									
GRAM			0 0 0 0	3 4 4 3									
CULT			0 0 0 0	4 0 0 0			0 0 0 0						
GD			0 0 0 0	2 2 0 0			0 0 0 0						
IFAT			0 0 2 0	4 4 0 0			2 2 0 2						
GRAM			0 0 2 0	4 3 0 0			2 2 0 2						
CULT		0 0 0 0	4 4 4 4	0 0 0 0	0 4 0 0		0 0 0 0						
GD		0 0 0 0	4 4 4 4	2 0 2 2	4 4 4 4		0 0 0 0						
IFAT		0 0 0 0	4 4 4 4	2 0 3 3	4 4 4 4		2 1 0 0						
GRAM		0 0 0 0	4 4 4 4	2 0 3 3	4 4 4 4		1 0 0 0						
CULT	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	4 4 0 0	0 4 0 0	3 0 2 0	0 0 0 0	0 0 0 0	3 2 0 0	4 4 4 4	4 0 0 0	0 0 0 0
GD	0 0 0 0	0 0 0 0	0 0 0 0	0 1 0 1	2 2 2 2	4 4 4 4	3 3 3 3	1 0 0 0	0 2 1 2	2 3 0 3	4 4 4 4	4 4 4 4	0 0 1 0
IFAT	0 0 0 0	2 2 2 2	0 0 0 0	2 3 0 2	2 4 3 3	4 4 4 4	4 4 4 4	2 2 2 0	1 4 3 4	3 4 4 4	4 4 4 4	4 4 4 4	0 0 3 0
GRAM	0 0 0 0	2 1 3 3	0 0 0 0	0 3 0 2	2 4 3 3	4 4 4 4	4 4 4 4	1 0 2 0	0 4 0 4	0 4 4 4	4 4 4 4	4 4 4 4	0 0 2 0
	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H
NO. OF MORTALITIES	1	2	3	4	2	1	3	1	1	1	1	1	1
DAYS POST-CHALLENGE	3	7	9	10	13	18	21	24	29	30	31	33	35

K = Kidney
L = Liver
S = Spleen
H = Heart

GRAM, IFAT
0 = Negative
1 = C<1 orgs/field
2 = 1-10/field
3 = Moderate numbers
4 = Large numbers

GEL DIFFUSION (GD)
0 = Precipitin line NOT detected
2 = Faint precipitin band
3 = Moderate precipitin band
4 = Heavy precipitin band

CULTURE (CULT)
0 = No growth
2 = Light growth
3 = Moderate growth
4 = Profuse growth

Table 8b The relative sensitivies of the Gram, IFAT, Gel diffusion and culture methods at detecting R.Salmoninarum in specimens from fish challenged with strain WS07

CULT	4 4 0 0											
GD	4 4 4 4											
IFAT	4 4 4 4											
GRAM	4 4 4 4											
CULT	4 0 4 0											
GD	4 4 4 4											
IFAT	4 4 4 4											
GRAM	4 4 4 4											
CULT	4 0 0 0								0 0 0 0		0 0 0 0	
GD	4 4 4 4								0 0 0 0		0 0 0 0	
IFAT	4 4 3 4								0 0 0 0		0 0 0 0	
GRAM	4 4 2 4								0 0 0 0		0 0 0 0	
CULT	0 0 0 0				2 0 0 0				0 0 0 0	0 0 0 0	0 0 0 0	
GD	2 2 2 3				0 0 0 0				0 0 0 0	0 0 0 0	0 0 0 0	
IFAT	4 4 4 4				2 2 2 0				2 0 2 0	2 2 0 0	0 0 0 0	
GRAM	4 4 4 4				2 2 2 0				2 0 2 0	2 2 0 0	0 0 0 0	
CULT	4 0 0 2	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
GD	2 2 2 2	4 4 4 4	0 0 0 0	1 1 0 1	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
IFAT	4 3 0 4	4 4 4 4	2 2 2 2	3 3 2 3	0 0 0 0	0 0 0 0	2 0 2 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
GRAM	4 2 0 4	4 4 4 4	2 2 2 0	0 2 0 2	0 0 0 0	0 0 0 0	2 0 2 0	0 0 0 0	0 0 0 0	0 0 0 0	2 0 0 0	0 0 0 0
	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H	K L S H
NO. OF MORTALITIES	5	1	1	1	2	1	1	3	2	3	1	
DAYS POST-CHALLENGE	21	23	24	29	30	31	34	35	36	38	39	

K = Kidney
L = Liver
S = Spleen
H = Heart

GRAM, IFAT
0 = Negative
1 = C<1 org/field
2 = 1-10/field
3 = Moderate numbers
4 = Large numbers

GEL DIFFUSION (GD)
0 = Precipitin line NOT detected
2 = Faint precipitin band
3 = Moderate precipitin band
4 = Heavy precipitin band

CULTURE (CULT)
0 = No growth
2 = Light growth
3 = Moderate growth
4 = Profuse growth

Impression smears from the kidney, liver, spleen and heart of the first five fish which died in each group were stained by Gram and by IFAT and revealed R.salmoninarum in very large numbers from all the fish in Group 2 (WS07) but in only one fish in Group 1 (WS03).

Subsequently, the majority of the smears examined from fish in Group 1 showed large numbers of organisms, but only small numbers were detected in smears from Group 2. In seven fish from this group, no bacteria were detected.

The success rate achieved by culture varied in proportion to the number of organisms demonstrable by staining. Fast growing contaminants were a particular problem in light infections.

Immuno-gel diffusion

The results of immuno-gel diffusion tests were consistent with the bacteriological findings. Marked precipitin reactions were obtained against homogenates diluted 1:2 in PBS pH 7.2 prepared from the tissues which had shown large numbers of organisms by Gram stain and IFAT. The intensity of the precipitin bands varied in proportion to the numbers of the organisms observed in smears, although the Gram and the IFAT in particular were more sensitive than the immunodiffusion test (Figure 12).

Histological Examination

The pathological and histological changes produced by the artificially induced disease were comparable to those found in naturally infected fish (Tables 9 and 10).

Figure 12 Gel diffusion pattern obtained with homogenates prepared from infected tissues vs anti-KD serum. Actual size.

1 - 5 : Supernatants from 50% organ homogenates
6 : SE of Corynebacterium spp.
AS : Rabbit anti-KD serum

Figure 12 Gel diffusion pattern obtained with homogenates prepared from infected tissues vs anti-KD serum. Actual size.

1 - 5 : Supernatants from 50% organ homogenates
6 : SE of Corynebacterium spp.
AS : Rabbit anti-KD serum

Table 9

Pathology of BKD

External Signs

	Belding and Merrill 1935	Snieszko and Griffin 1955	Smith 1964
Exophthalmos	One or both eyes	Present	-
'Blebs' blisters	Trunk of fish	Sides of fish filled with clear or turbid fluid	-
Abscesses	Muscle - kidney region	Muscle	-
Haemorrhage	Between pectoral fins	-	Vents and areas on muscle
Congestion of fins	-	-	Injection of fins
Distended abdomen	-	Distended abdomen	-

Internal Signs

Kidney	Abscesses, grey-white areas of varying size. Capsule distended by haemorrhagic exudate.	Swollen - small-large grey white necrotic areas (abscesses)	Advanced cyst-like appearance Grey-brown lesions. Swimbladder - occasional petechial haemorrhage white membrane.
Peritoneum	Excess of fluid, sometimes haemorrhagic	Petechiae appear in peritoneum and skin. Fluid accumulation. Semi-transparent opaque red.	Petechial haemorrhage. Peritoneum lining.
Liver	Abscesses of varying size discolouration, mottled	Abscesses	Abscesses. White membrane.
Spleen	Abscesses (infrequently seen) increased size (enlarged). Stippled appearance.	Abscesses. False membrane (white)	Abscesses. White membrane. Peppered appearance.

Table 9 (continued)

Pathology of BKD (continued)

Internal Signs (continued)

	Belding and Merrill 1935	Snieszko and Griffin 1955	Smith 1964
Intestine	Congestion of hind gut. Distended blood vessels.	Swollen anal region. Distended with yellow purulent fluid. Lesions stomach oesophagus increase in size. Intestine and pyloric caecae.	-
Pericardium	Fibrinous, purulent effusion. Adhesions, pale appearance.	Heart. False membrane.	-
Reproductive Organs	Haemorrhagic, dilated blood vessels, congestion. Small abscesses.	-	Gonads. White membrane
Musculature	-	Abscess filled with colourless or turbid fluid	Petechial haemorrhage (muscle lining).

Table 10

Histopathology of BKD

	Wood and Yasutake 1956	Snieszko and Griffin 1955	Smith 1964
Granulomatous Lesions	Systemic - kidney, intestine, pancreas, liver, spleen, gills, cerebral meninges, gall bladder swimbladder, eyeball, muscle etc.	Described as a widespread tubercle. Appearance small tubercles in gills, liver, spleen, kidney, eyes musculature and anterior intestinal tract.	Described as small areas of focal necrosis in spleen, liver and kidney - few necrotic areas.
Fibrotic Lesions	Every organ	-	Few found.
Giant Cells	Infrequent	Common	Not seen
Eosinophilic Inflammation	Muscular wall of oesophagus - SI	-	-
Peritonitis	Gastric caecae	-	-
Cytoplasmic Inclusions	Liver parenchymal cells and pancreatic acinar cells	-	-
Myocarditis	Frequently seen. Heart - massive destruction of heart muscle.	-	-
'False membrane'	-	-	Liver and spleen - thin membrane of fibrin and leucocytes - 3 layered structure.

The earliest lesions detected were focal infiltrations of mononuclear cells, the majority of which were recognisable as macrophages. Such lesions were most commonly seen in kidney and liver (Figure 13).

On some occasions foci of pyknotic cells were seen with little inflammatory response (Figure 14). Bacteria were readily demonstrated in such lesions using Gram stain.

Lesions then increased in number and size and were found in all body organs examined. In affected kidneys the lesions replaced large areas of haemopoietic tissue, but tubular elements often remained intact (Figure 15). In affected livers lesions were most commonly found around the bile ducts. (Figure 16).

As the disease progressed lesions enlarged further and in many areas almost completely replaced normal tissue structure. Such lesions in the heart contained large numbers of macrophages, necrotic cells and fibrin deposits (Figure 17).

Extensive kidney lesions showed complete replacement of normal tissues and were somewhat lobular in nature, with an outer zone of fibrous tissue and an inner zone of macrophage cells and many pyknotic cells (Figure 18). At this stage also muscle lesions became prominent and consisted of extensive infiltration of macrophages with associated myophagia (Figure 19a & b). Such lesions also spread to involve bone and nerve (Figure 20). At the same time extensive peritonitis was commonly found involving all internal organs. A wide zone of inflammatory cells and necrotic tissue debris containing masses of bacteria was found on the peritoneal surface of visceral organs, and immediately adjacent to the

organ capsule was a narrow band of pyknotic nuclei (Figure 21). It was common to find such extensive peritonitis in the absence of major parenchymatous lesions.

When the skin was affected from deeper lesions in the muscle the stratum compactum of the dermis appeared to act as a natural barrier to the spread of infection, so that lesions in the underlying muscle spread along the hypodermis and fascial planes and only penetrated the stratum compactum in severe cases (Figure 22).

Gram-Twort stained sections are shown in Figures 23-25 to demonstrate bacteria not readily visible in black and white photography.

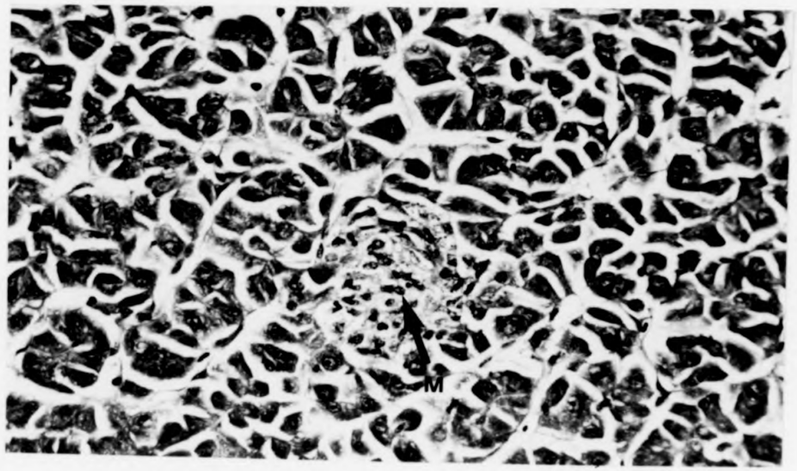


Figure 13 Liver
An early change showing a focal infiltration of predominantly mononuclear cells. Haematoxylin and Eosin x 356.

M = Mononuclear cell

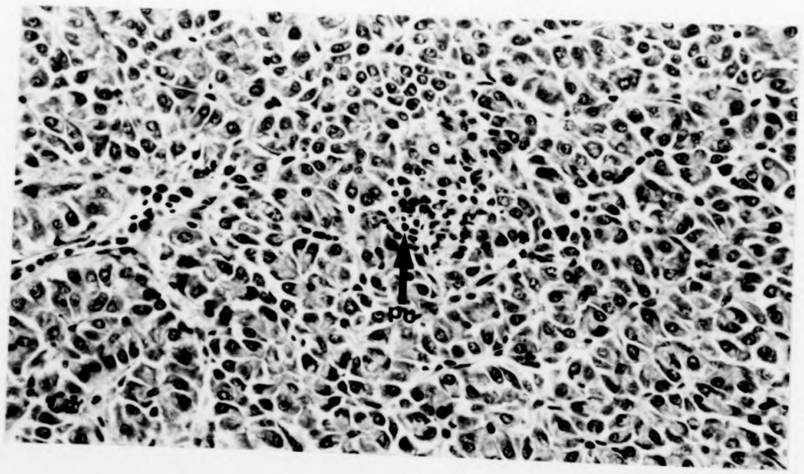
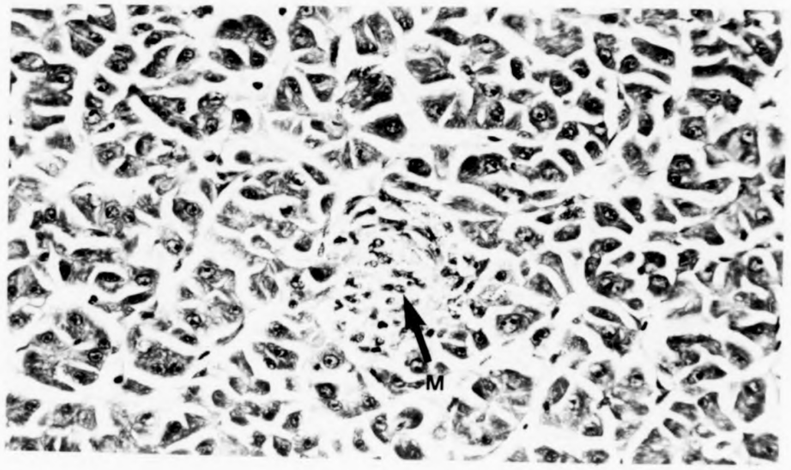


Figure 14 Liver
Early necrotic lesion, demonstrating pyknotic nuclei. Haematoxylin and Eosin x 218.

PC = Pyknotic cell



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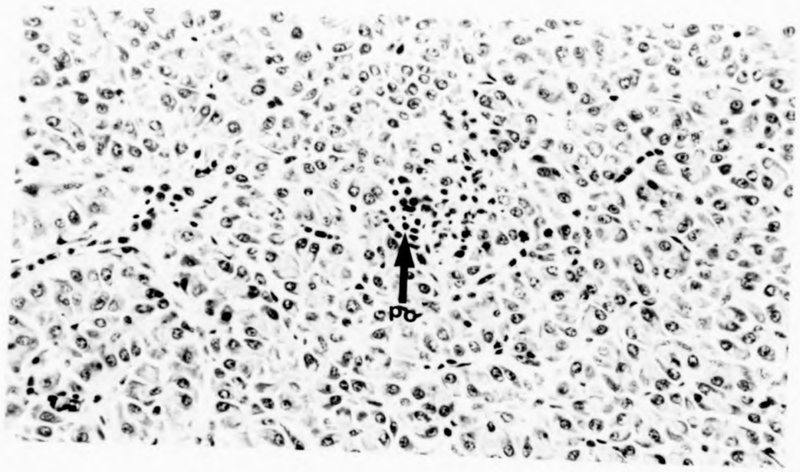

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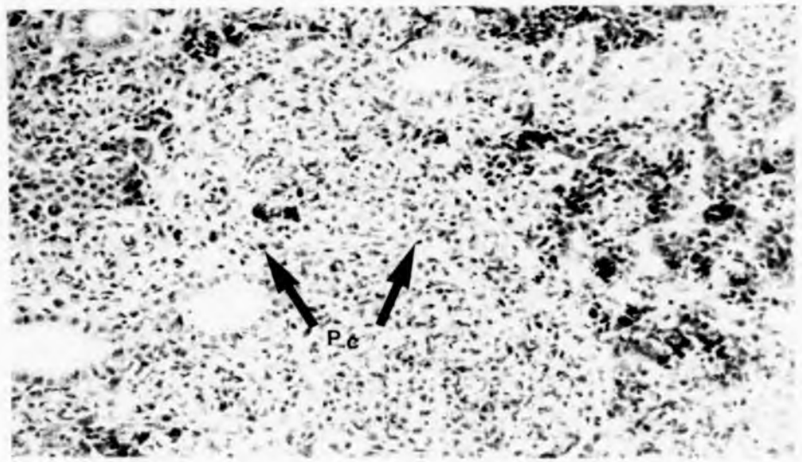


Figure 15 Kidney
Haemopoietic tissue infiltrated with large numbers of macrophages and fibrocytes. A marked necrotic change is not evident. The presence of pyknotic cells is, however, noted. Haematoxylin and Eosin x 85.

PC = Pyknotic cells

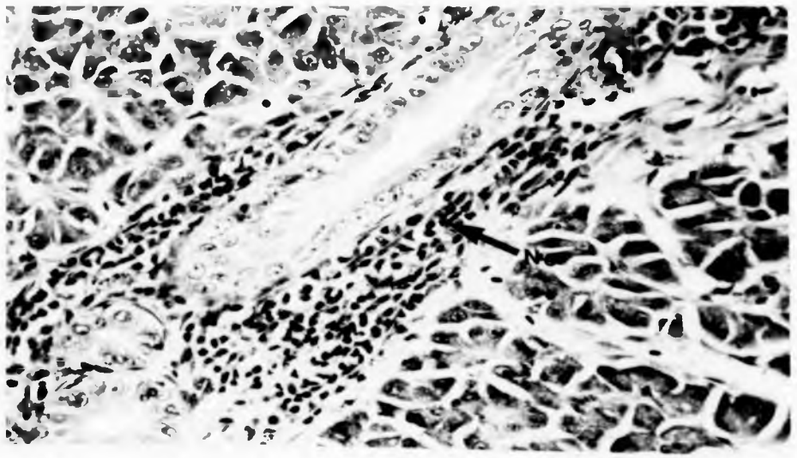
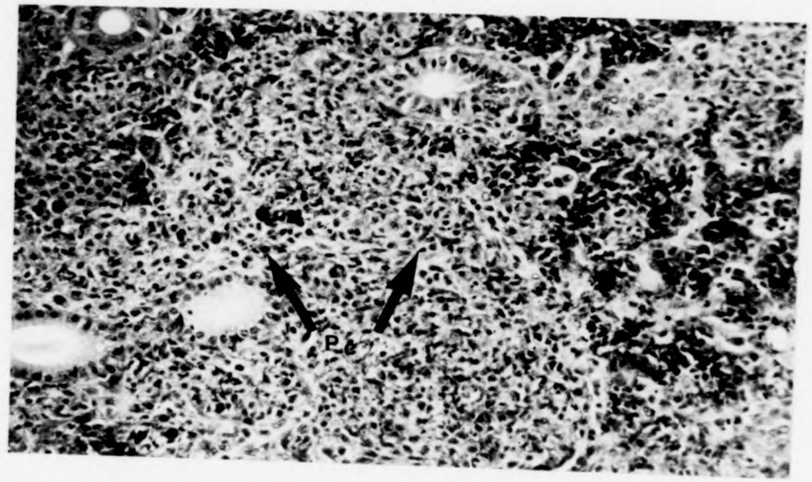


Figure 16 Liver
An infiltration of macrophages and a small number of necrotic cells around the bile ducts. Haematoxylin and Eosin x 356.

NC = Necrotic cell



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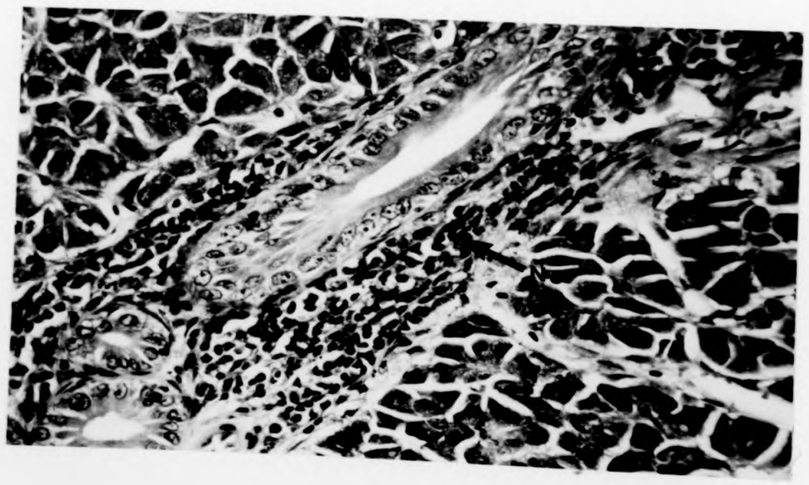

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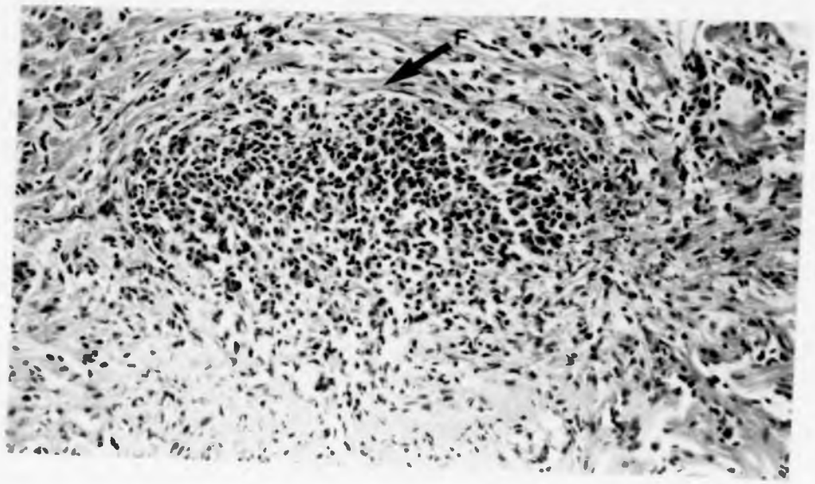


Figure 17 Heart
An advanced lesion demonstrating extensive infiltration of macrophages, necrotic cells and fibrin deposits. Haematoxylin and Eosin x 85.

F = Fibrin

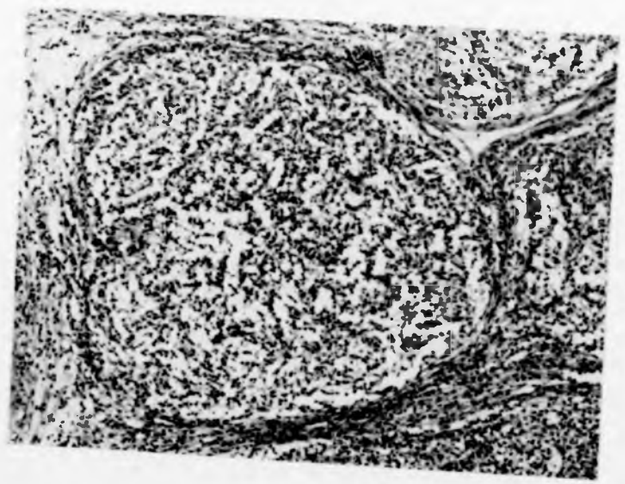
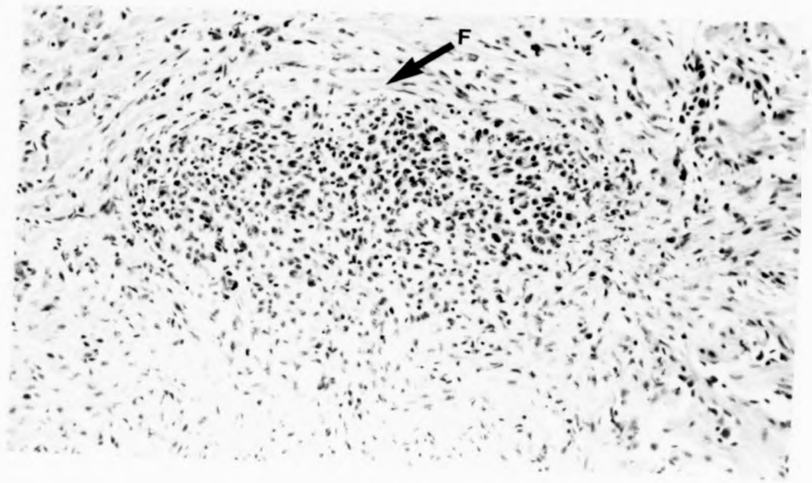
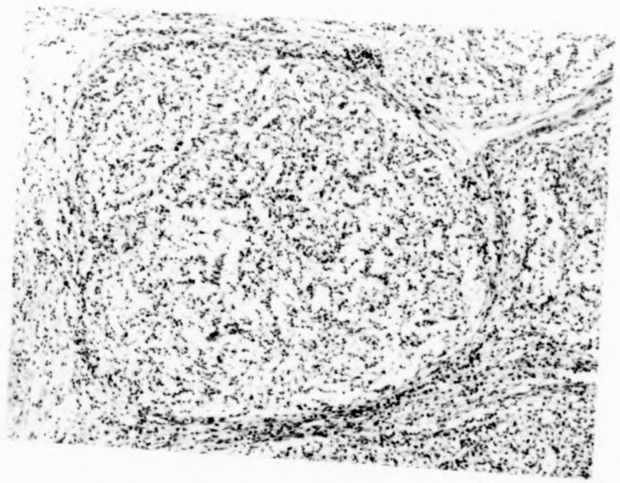


Figure 18 Kidney
Lobular areas of macrophages and pyknotic cells bounded by fibrin tissue. Haematoxylin and Eosin x 85.



Figu 17 Heart
An advanced lesion demonstrating extensive infiltration of macrophages, necrotic cells and fibrin deposits. Haematoxylin and Eosin x 85.

F = Fibrin



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Lobular areas of macrophages and pyknotic cells bounded by fibrin tissue. Haematoxylin and Eosin x 85.

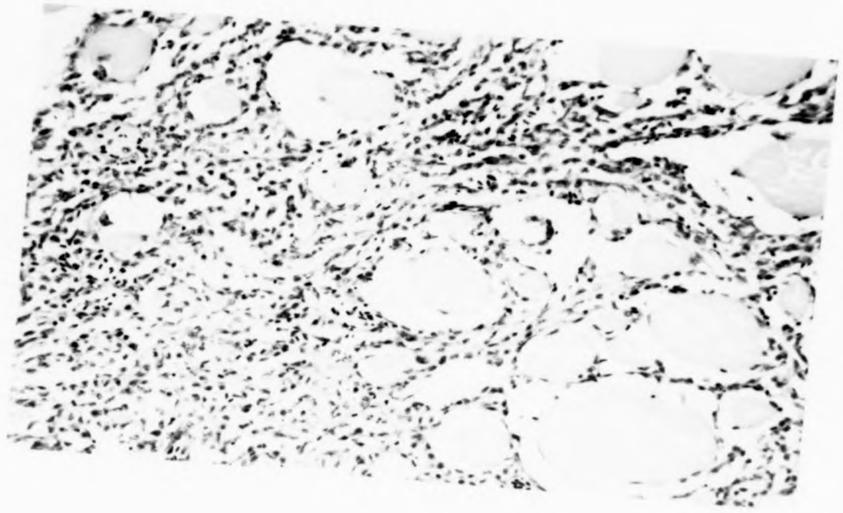
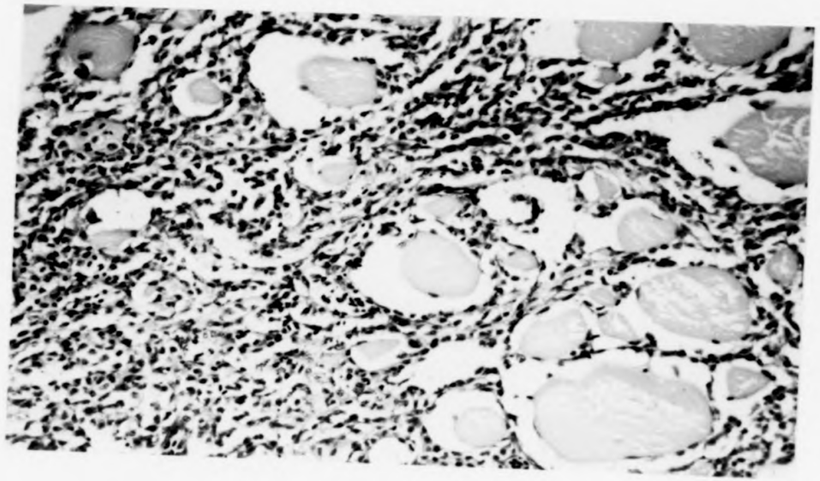


Figure 19a Muscle
Massive infiltration of inflammatory tissue predominantly macrophages, causing gross degeneration of muscle fibres. Haematoxylin and Eosin x 85.

Figure 19b Muscle
Demonstration of probable active myophagia. Haematoxylin and Eosin x 830.



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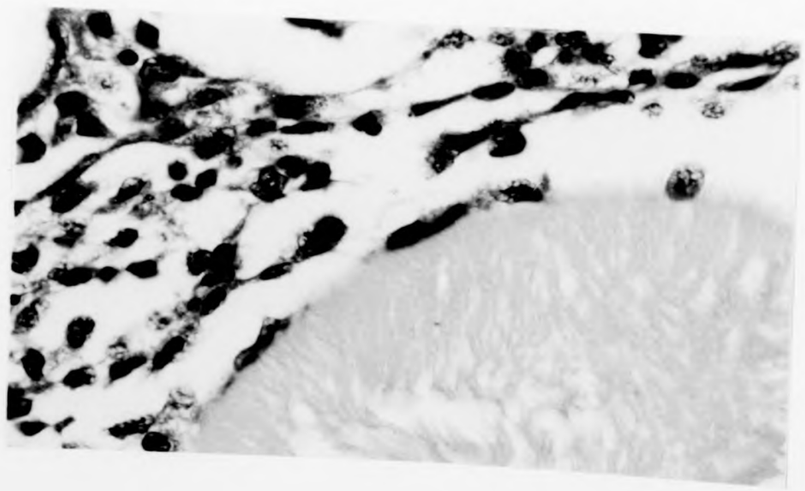


Figure 19b Muscle
Demonstration of probable active myophagia. Haematoxylin and Eosin x 830.

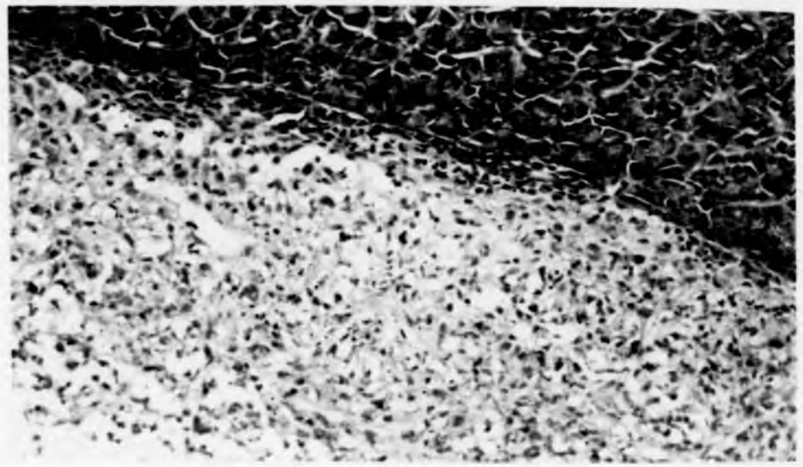


Figure 20 Muscle
Infiltration of inflammatory tissue with extensive involvement of bone tissue. Haematoxylin and Eosin x 85.

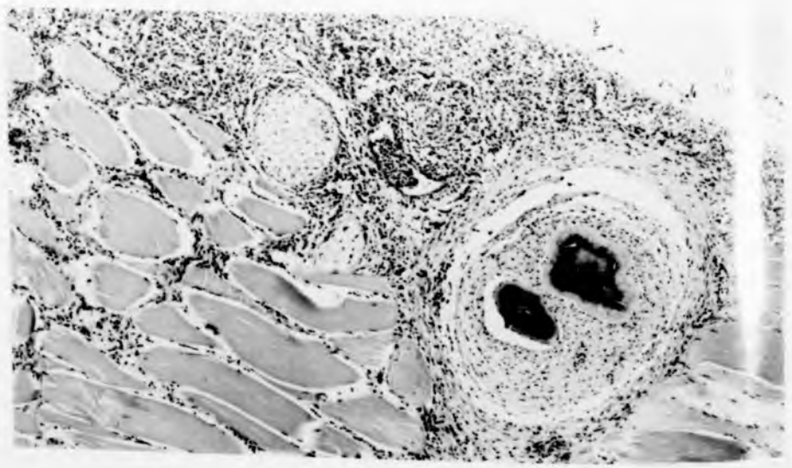
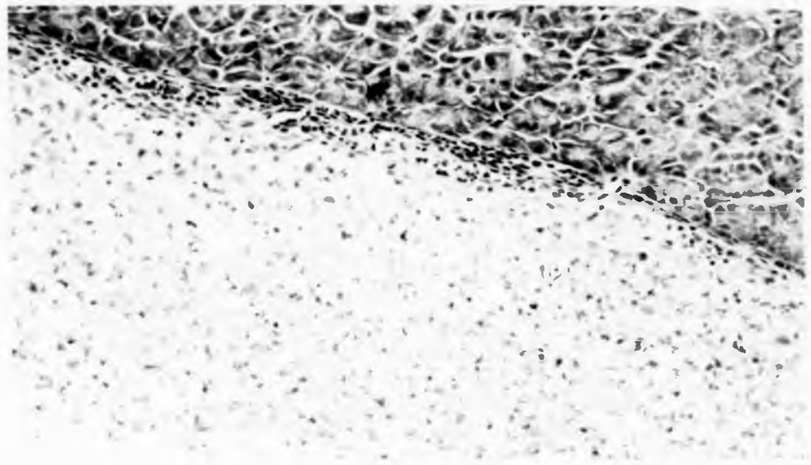


Figure 21 Liver
Peritonitis of the liver capsule. The capsule area adjacent to the organ shows an increased number of darkly stained nuclei. The outer zone is composed of unidentifiable degenerate cells with pale staining nuclei. Haematoxylin and Eosin x 218.



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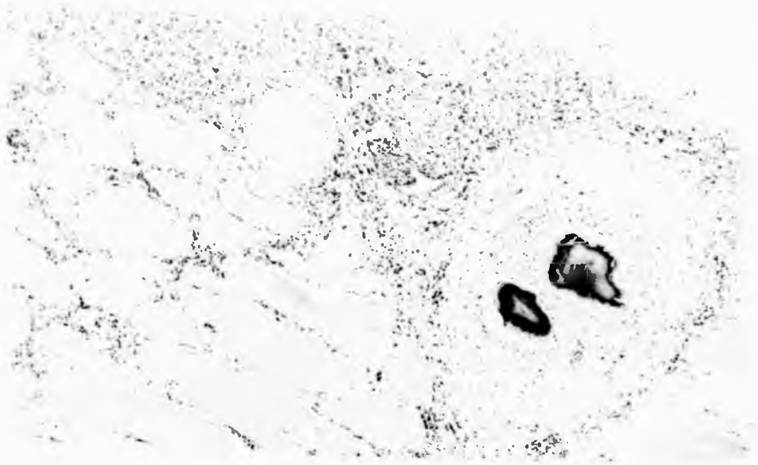

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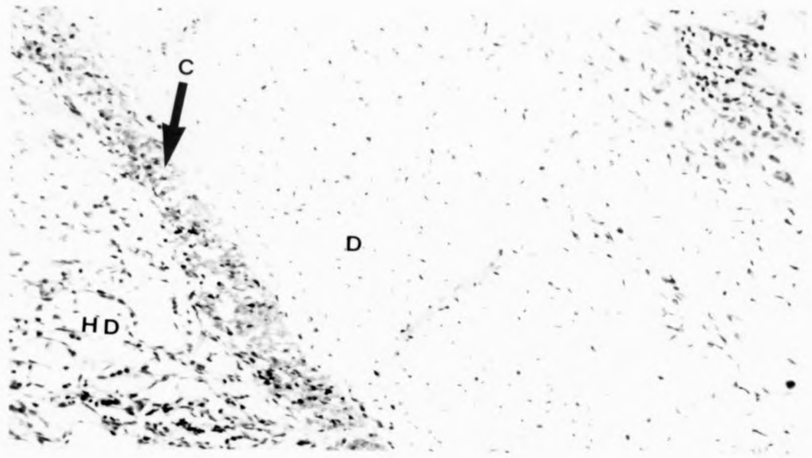
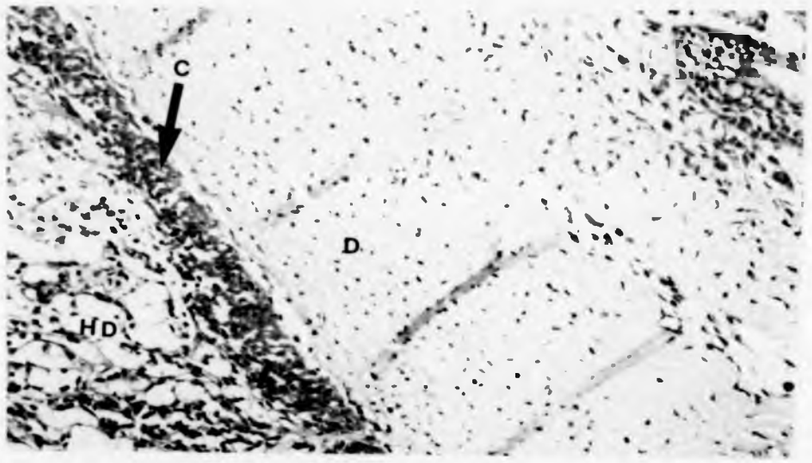


Figure 22 Muscle/Skin
The collagen layer (C) of the stratum compactum of the dermis acts as a natural barrier to infection.

Note the apparently normal dermis (D) with the underlying degenerating hypodermis (HD) containing many bacteria. Haematoxylin & Eosin x 218.



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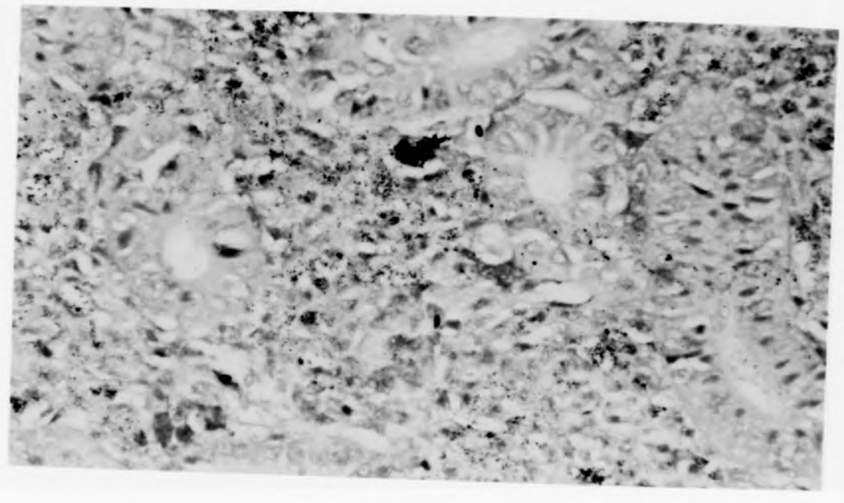


Figure 23 Liver
Demonstration of intracellular bacteria within capsule area. Modified Gram Twort x 450.

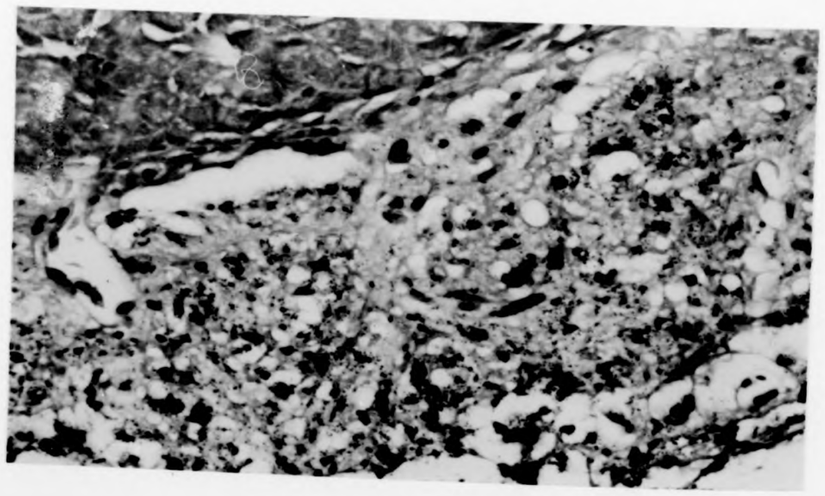
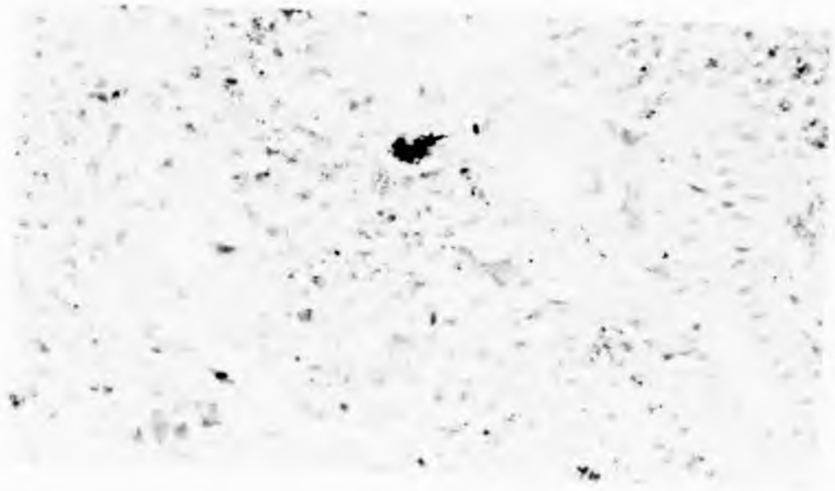
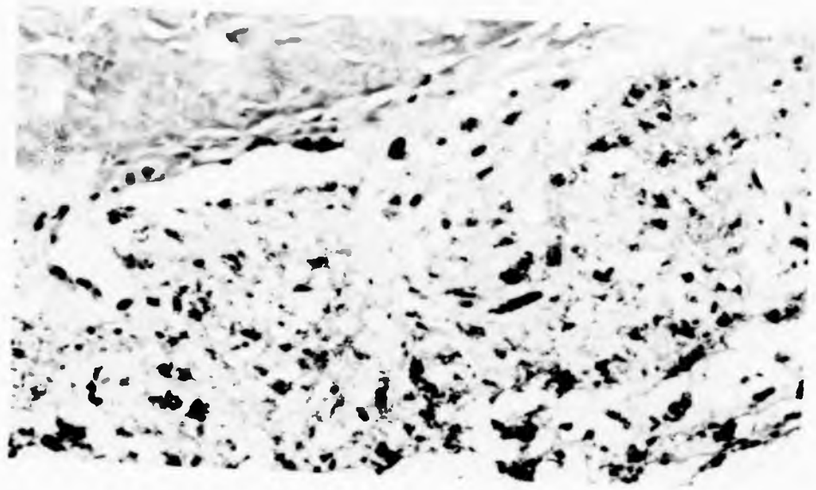


Figure 23 Kidney
Demonstration of intracellular bacteria in large numbers of macrophages in inflammatory tissue. Modified Gram Twort x 450.



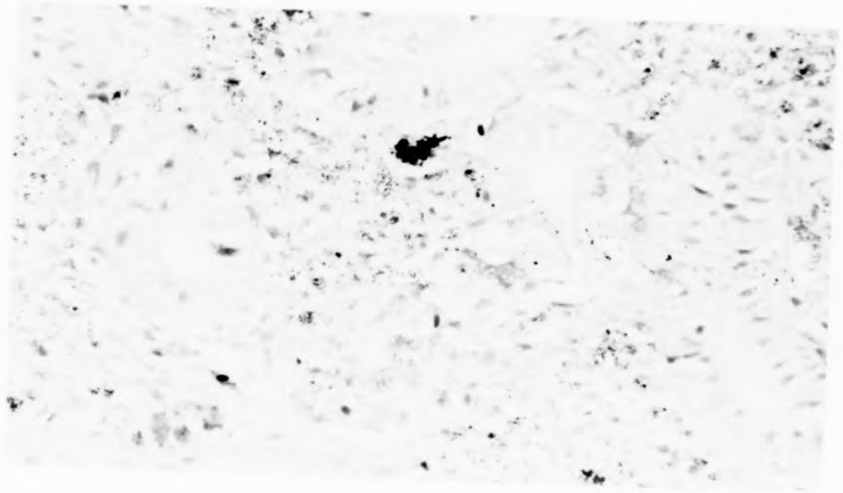
Figur

Liver
Demonstration of intracellular bacteria within capsule area. Modified Gram Twort x 450.

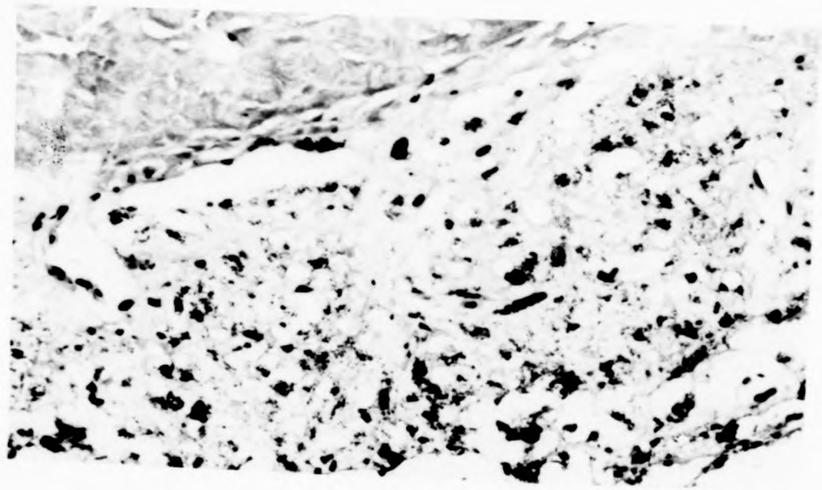


Figu

Kidney
Demonstration of intracellular bacteria in large numbers of macrophages in inflammatory tissue. Modified Gram Twort x 450.



igure 23 Liver
Demonstration of intracellular bacteria within capsule area. Modified Gram Twort x 450.



igure 23 Kidney
Demonstration of intracellular bacteria in large numbers of macrophages in inflammatory tissue. Modified Gram Twort x 450.

Figure 25 Muscle
Demonstration of intracellular and extracellular bacteria in inflammatory tissue. Modified Gram Twort x 1750.

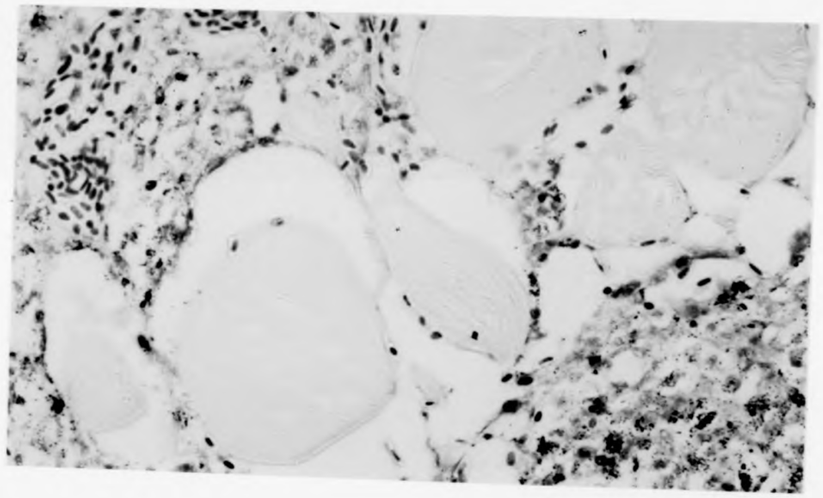


Figure 25 Muscle
Demonstration of intracellular and extracellular bacteria in inflammatory tissue. Modified Gram Twort x 1750.

2. Comparison of the virulence between strains WS04 and WS06

Mortality

The cumulative mortalities are shown in Figure 26. Fish challenged with strain WS04 began to die 13 days after inoculation. By Day 26, 12 fish had died and only one more death occurred on Day 45. Fish challenged with strain WS06 did not start to die until Day 39. Five fish died on Day 45 and no further deaths were recorded.

No deaths occurred in the control group.

Clinical signs

The only obvious clinical signs were a darkening of the skin colourisation and a very pronounced bilateral exophthalmos.

Post mortem findings

Gross signs of the disease included small white lesions on the kidneys in only 2 fish with haemorrhagic gut and milky swim bladder in 2 fish only.

Bacteriological Examination

Examination of impression smears from kidney, liver, heart, spleen and gut using Gram stain and IFAT revealed small numbers of Gram positive coccobacilli in kidney smears, in six out of the 13 fish which died in fish challenged with WS04 and very small numbers in the heart of 2 of these fish detectable only by the IFAT method.

R.salmoninarum was not detected in smears prepared from fish challenged with WS06.

Reisolation was achieved from four kidney homogenates.

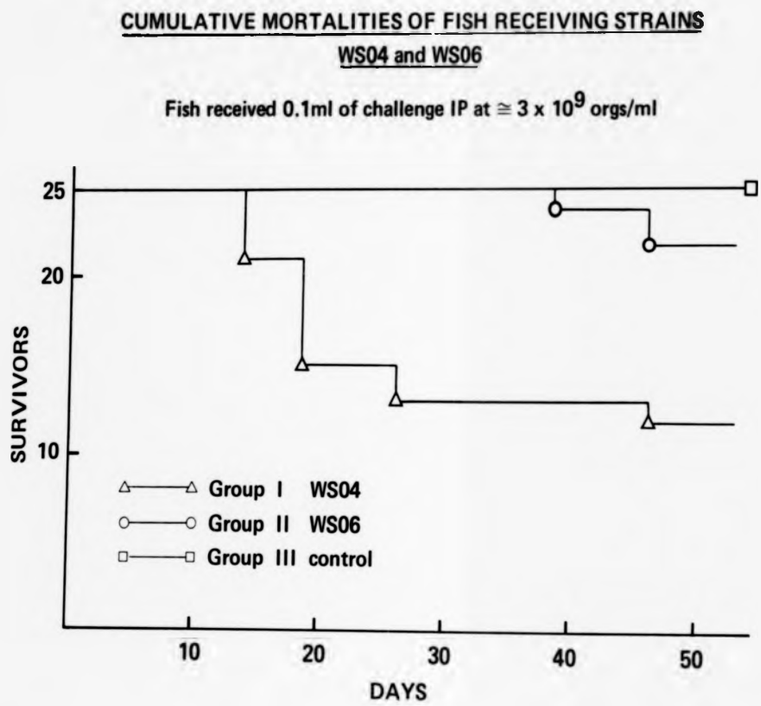


Figure 26 Cumulative mortalities of fish receiving strains WS04 and WS06

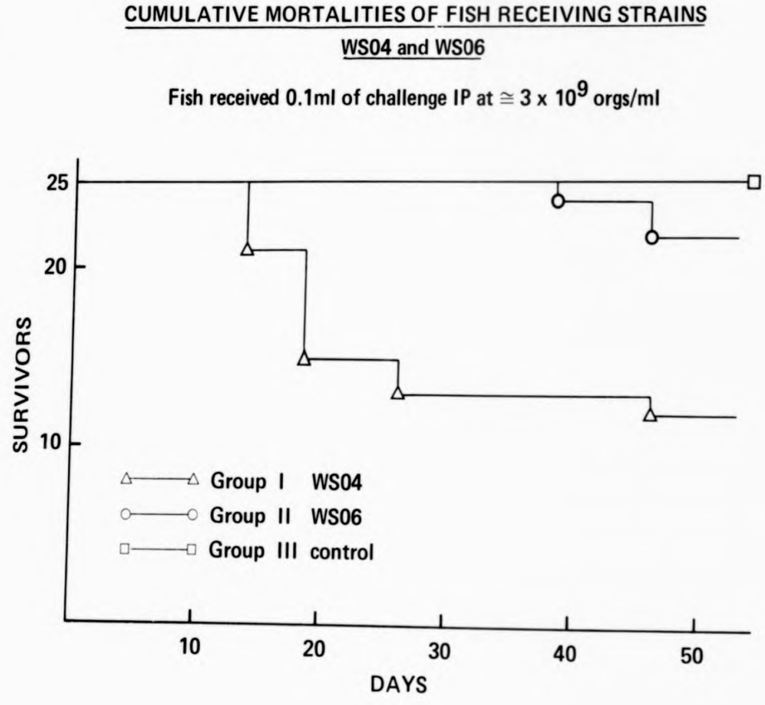


Figure 26 Cumulative mortalities of fish receiving strains WS04 and WS06

Histological Examination

Kidney sections from fish challenged with WS04 showed a mononuclear infiltration of the interstitial tissue. Variation in the numbers of mononuclear cells was found but there was no evidence of tubular or glomerular damage. Intra- and extracellular Gram positive coccobacilli were detected. The liver, spleen and heart showed no evidence of inflammation or necrosis.

Section II

DISCUSSION

Preliminary experiments using the WS03 isolate fulfilled the requirements of Koch's Postulates, and allowed the development of a reproducible challenge system. Results showed that large numbers of organisms were required to produce disease and death, that fish of 50-100g in weight were most susceptible to challenge, and that the intraperitoneal route of challenge was the most successful in terms of speed of manifestation of clinical signs and death.

It is important to note here that in the large fish used in the experiment to determine the most susceptible size fish to challenge, a carrier state was created. Although a severe challenge was administered to these fish and then repeated at 30 day intervals, they suffered no obvious adverse effects, and a slow moderate antibody response was recorded. Isolation of R.salmoninarum from various tissues revealed that a sub-clinical infection had developed.

These results indicated that small residual populations of viable organisms were able to establish themselves within the protective environments and to maintain a state of premunity within the host so that these carriers were highly resistant to re-infection by the same organism.

In nature, few outbreaks are recognised, except in massive epizootics. Sub-clinical disease in wild fish therefore is very significant, since some farmers still use wild salmon as replacement brood stock. Such carrier fish liberate the organism in the urine and defeat any attempt to eradicate the problem on commercial farms.

With the data obtained a standard challenge system was established, and this demonstrated that infection could consistently be produced by intraperitoneal injection of R.salmoninarum into rainbow trout, and that artificially provoked disease paralleled the natural disease in both gross pathology and histopathology when recent isolates were used.

The gross pathology of the natural disease has been described by Belding and Merrill (1935), Snieszko & Griffin (1955) and Smith (1964). In their description of the disease in brook (Salvelinus fontinalis) and brown trout (Salmo trutta), Belding and Merrill divided the lesions into external and internal groups. The external signs included exophthalmos or "popeye" due to oedema and serous effusion into the periorbital area; superficial blebs or blisters restricted to the trunk of the fish; abscesses filled with pus or fluid with varying degrees of haemorrhagic contamination in the musculature of the kidney region and between the pectoral fins, and occasional haemorrhages at the base of the pectoral fins and on the outer surface of the operculum.

The presence of exophthalmos and superficial blisters was confirmed in the brook, brown and rainbow trout by Snieszko & Griffin who also described a distended abdomen in many fish. Smith, on the other hand, found little evidence of diagnostic external signs in salmon, except for occasional congestion of the fins and haemorrhage at the vent and in the muscle.

In the artificially injected fish in this study external signs with the exception of exophthalmos were rare. A direct eye infection was not seen suggesting a dysfunction in the maintainance of fluid balance. As the experiment progressed exophthalmos became more common, and was seen in all infected fish; superficial haemorrhagic and blister-like lesions sometimes appeared in fish which died late in the experiment. This may indicate that bacterial multiplication was necessary for these lesions to appear, since large numbers of bacteria were seen in impression smears of the blisters.

Belding and Merrill found involvement of nearly all internal organs, in particular the kidney, liver, spleen and serous cavities. Abscesses of varying sizes were frequently seen in the kidney, liver and spleen and excess fluid in the peritoneal cavity and the pericardial sac was characteristic. The reproductive organs were often haemorrhagic and congestion of the hind gut common.

Snieszko & Griffin found similar lesions in the kidney, liver and spleen. They also described swollen anal regions and an accumulation of semi-transparent or opaque red fluid in the peritoneal cavity. They were the first to describe an opaque false membrane which covered the heart and the spleen. Smith also noted the false membrane in salmon. She found that below 9.5^{o}C, the spleen, liver, gonads, swimbladder and peritoneal muscle were totally or partially enveloped. At higher temperatures it was not seen and the clinical picture was similar to that described by Belding and Merrill.

In the present studies, only fish infected with strain WSO3 consequently developed gross internal lesions, and these tended to be severe and extensive. The kidney was enlarged as described by Snieszko and appeared granular; numerous white nodular lesions were visible along its length and the swimbladder frequently appeared markedly thickened and milky white. The latter observation may correspond to the false membrane described by Smith.

Focal lesions were also seen in the liver, which sometimes presented a mottled appearance, and in the spleen which was often enlarged. Some of the fish in this group also showed a pericarditis which appeared as a patchy grey discolouration of the heart. This may be a partial pseudo-membrane as described by Smith, Snieszko & Griffin, although it was firmly adherent to the heart. The intestine remained unaffected throughout the disease but excess semitransparent peritoneal fluid was noted in the late stages.

Histologically the picture described by Snieszko and Griffin (1955), Wood and Yasutake (1956), and Smith (1964) was similar although the terminology differed. Wood and Yasutake considered the disease to be systemic chronic infection which histologically was characterised by granulomatous lesions with proliferating fibroblasts forming distinct nodules. Giant cells were found but infrequently, particularly in the muscle. Snieszko, on the other hand, likened the histology of the disease to the granulomas associated with mycoses and tuberculosis of mammals. He described the appearance of typical tubercles with which a fungus-like organism was always associated. However, isolation of a small Gram positive organism was achieved from these granulomatous lesions. Giant cells were commonly found.

Smith studied histological sections of the spleen, liver and kidney and found small areas of focal necrosis composed of tissue debris in both the spleen and the liver. Few necrotic areas were found in the kidney. Gram positive organisms were found around the edge of the necrotic areas in the liver and spleen and in large numbers in the parenchyma surrounding the renal tubules. Giant cells were not found and few fibrotic lesions were seen.

In the artificially provoked disease, no histological changes were seen until 18 days post-injection, although organisms were recovered from many of the tissues. It was therefore difficult to ascertain histologically which organ was the first to be affected.

In the challenged fish which showed progressive disease, the first pathological changes detected were in kidney and liver and normal tissue was gradually replaced by macrophage accumulation, necrosis, peritonitis and occasionally fibrosis. Large numbers of small Gram positive coccobacilli were seen both intra-and extracellularly in most organs. The majority were concentrated in lesion areas whereas relatively few were seen in the unaffected parenchymal tissues. This latter finding agrees with the report of Wood and Yasutake who, unlike Smith, found that in the natural disease organisms were rarely seen in large numbers in the parenchyma of any organ. The overall histological picture of the later stages of the artificially provoked disease is very similar to that which they described in the natural disease. The lesions, however, are best described as inflammatory rather than granulomatous. The smaller lesions consisted for the most part of mononuclear cells often discernible as

macrophages, while the more advanced lesions contained large numbers of macrophages, necrotic cells, fibrin and fibrocyte deposits. Giant cells were not found in any organ examined.

In natural outbreaks in Britain a histological picture of pronounced macrophage infiltration has generally been found. This is often virtually the sole cell type present. In long standing infections seen less frequently, nodule formation with a distinct rim of fibrous tissue is also seen (Personal communication R.Richards).

Wood and Yasutake's description certainly appears to be consistent with that of chronic infection, whilst the Snieszko and Griffin infection in which he describes a fungal-like organism is more typically that of chronic fungal infections, such as that caused by Exophiala (Richards, Holliman and Helgason 1978).

Giant cells have not been observed in any natural or artificial induced infection.

Smith was the first to describe the histology of the so called false membrane found on the surface of the liver and spleen. It varied from a very thin layer of fibrin and leucocytes to a three layered structure consisting of fibrin and nucleated cells, leucocytes being phagocytosed by histiocytes, and an innermost layer of fibroblasts and histiocytes. The membrane was originally likened to the diphtheritic membrane which characterises diphtheria. In this disease, bacteria kill the superficial cells of the mucous membrane on the human tonsil or throat, multiply in the necrotic layer and produce toxin which extends the superficial necrosis.

The products of the necrotic cells stimulate an inflammatory reaction in the underlying tissues and the fibrin produced binds the necrotic layer into a firm, pale membrane. In salmon however, the membrane did not penetrate the parenchyma of the liver and spleen and, in addition, included organised deeper layers.

The change in the peritoneal surface of the internal organs observed in experimentally infected fish were those of acute peritonitis, fibrin deposition not being a regular feature of the reaction. The production of a "pseudo-diphtheritic" membrane may be the result of more chronic inflammatory change and be more likely to be produced at the lower temperatures noted by Smith.

It is interesting to note that where "blebs and blisters" occurred, the stratum compactum of the skin was not penetrated. Inflammation occurred within the subcutaneous muscle, but did not spread outwards. This was also found in the experimental infections and would explain the regular occurrence of raised blister-like lesions and the absence of ulceration found in this and other studies.

Detection and reisolation of R.salmoninarum were also necessary to satisfy Koch's Postulates. Four methods of detection were used:- culture, indirect fluorescent antibody test, Gram stain and gel diffusion. The cultural method had limitations because it frequently failed to detect R.salmoninarum even at high levels of infection, most commonly due to complete overgrowth of plates by contaminants.

It should be pointed out here, and will be discussed later, that more sensitive culture methods are now available and indicate the use of a variety of antibacterial substances often with dilution of the initial inoculum to remove the so-called anti-BKD factor present (Evelyn, Ketcheson & Prosperi-Porta 1981, Laidler, 1983).

The other three methods all gave statistically similar results, mainly because of the low numbers of fish involved. In general the IFAT and Gram stain gave comparable results although IFAT appeared preferable when small numbers of organisms were present. Gel diffusion did not appear to be as sensitive as the other tests and therefore would not provide any advantage. However more data are needed to confirm this. The method of choice would thus seem to be IFAT, not only because of its sensitivity, but also its specificity, ease and speed.

The cumulative mortalities and the histopathology results show that the WSO3 strain was notably more virulent than WSO7, WS04 and WS06. The WSO3 strain was a fresh isolate from an outbreak of BKD in a stock of rainbow trout causing very high mortalities. The WSO7 strain was obtained from the NCMB and was originally isolated from a case of BKD in Atlantic Salmon (Salmo salar), WSO4 was isolated from a Salmo species and WSO6 from rainbow trout (Salmo-gairdneri). The repeated subculture since isolation and the change in host could account for the reduced virulence and relative lack of clinical and histological signs of disease.

The results clearly indicate that for accurate diagnosis it is necessary to examine more than one organ. Although the kidney gave consistently good results from fish infected with WSO7 and WSO3 and routinely provided the highest number of positive results, some infections were only detected in the liver, spleen and heart.

Examination of heart, spleen and liver as well as the kidney would reduce false negatives to very low numbers.

These findings corroborate those in fresh water cage farming systems in Scotland, where in several clinical cases in rainbow trout the heart is commonly involved in disease, and is sometimes the only organ grossly affected (Personal communication R.Richards).

It should also be pointed out that histological examination often reveals early cases of BKD in the absence of convincing clinical signs and is an extremely useful additional diagnostic tool.

Due to the rapidly escalating requirement for diagnosis of BKD, work has proceeded to find new diagnostic techniques and improvements on old techniques which may ultimately increase accuracy and allow a more rapid identification of the disease.

Many authors have compared the sensitivities of the available diagnostic techniques. Chen, Bullock and Stuckey, (1974) used an immuno-diffusion test to detect and identify the soluble KD antigen in infected tissues within twenty four hours. Although this eliminated the considerable time needed for growing the organism on a medium, doubt was expressed on the possibility of diagnosing covert cases of BKD.

The development of the indirect fluorescent antibody technique (IFAT) allowed the detection of low numbers of R.salmoninarum in fresh, frozen and formalin fixed tissue within 2 hours. Bacteria which occurred intracellularly however were not detected by fluorescence microscopy.

Paterson, Gallant & Desautels (1979) used the IFAT technique to detect BKD in wild salmon and by direct comparison with the cultural method on supplemented Mueller Hinton medium, Gram stain and immuno-gel diffusion technique found it to be twice as sensitive as any other technique employed.

Bullock, Griffin & Stuckey (1980) described a direct FAT which required only seven minutes to produce results. In a comparative study it was found to be clearly more sensitive than the Gram stain. Apart from being a superior method over Gram stain it was as effective as the IFAT and therefore more practical for screening large numbers of samples and detecting subclinical infections. It is interesting to note here that Bullock et al. have suggested examination of faecal samples in preference to kidney samples for detecting subclinical infection, and that concentration of faecal samples might be employed as a follow up procedure.

These findings are in accordance with Lobb (1976) who suggested that in covertly affected fish the best organ for detection of low level bacterial infection was the intestine. The pyloric caecae and liver were also shown to be significantly better than the kidney. The presence of large numbers of Gram +ve bacilli in the intestinal contents greatly increases the risk of losing the specifity for the anti-KD serum for R.salmoninarum.

Contradictory results were obtained by Evelyn et al. (1981) when he failed to isolate R.salmoninarum using a highly sensitive culture method from kidney and faecal samples from covertly infected sockeye salmon which had shown large numbers of FA positive organisms in smears. Attempts to unmask the infection by stressing the fish with an increase in water temperature from 9 - 15°C and by using an anti-inflammatory drug also failed. He concluded that the organisms were either variant forms of R.salmoninarum, Gram variable bacilli sharing one or more antigens in common with R.salmoninarum, non-viable organisms or organisms persisting in the host in a non-culturable form. He suggested that on the basis of these findings, FA based diagnosis of the R.salmoninarum carrier is open to interpretation.

Two new diagnostic techniques were described by Kimura et al. (1978). One was an immunodiffusion test using a heat stable antigen extracted from infected kidney, and the other a co-agglutination test performed by the agglutination of antibody sensitized Protein A containing staphylococci. Each had its advantages; the heat treated samples for the precipitin test were less hazardous for transportation to the laboratory, and the co-agglutination test, as a simple, rapid and reliable test, was suitable to be used in the field or laboratory. It was later reported that the co-agglutination test was highly specific and more sensitive than the immunodiffusion test (Kimura & Yoshimizo 1981).

The culture method was shown by Evelyn et al. (1981) to be more sensitive than the IFAT, Gram stain and immunodiffusion. The success of the cultural technique was achieved by serially diluting each sample, and removing the anti-R.salmoninarum activity in the supernatants of 50% kidney homogenates by washing once in 0.1% peptone in 0.85% saline.

Paterson et al. (1981) examined various organs from two year old smoltifying Atlantic salmon using the IFAT. R.salmoninarum was present in all organs and tissues monitored including, blood, faeces, kidney, liver, spleen, stomach, heart and intestine. Larger numbers of organisms were detected in the kidneys of infected animals and the gross pathology was more common in the kidney than in any other organ. The IFAT method was also used for detection of R.salmoninarum in 20 year old formalin preserved samples, and in covert infection in wild salmon, and provided useful data on the enzootic nature of the disease.

Despite the recent developments in the culture technique, for the detection of R.salmoninarum in overt and covert infection of BKD, the IFAT or the direct FAT must be the method of choice for routine screening of large numbers of samples. However, for field surveys requiring extreme detection sensitivity the culture method has proved to be of great value (Evelyn et al. 1981).

In the present study approximately 50% homogenates were prepared from kidney, liver and spleen in PBS. Heart and muscle were plated directly onto the culture medium. In the light of the findings of Evelyn et al. (1981) the lack of detection by the culture method of low level infections from kidney may be attributed to the anti-R.salmoninarum activity in these samples, but this does not account for the unsuccessful attempts at isolation from the other organs. It would therefore seem appropriate when examining a wide range of organs and tissues to use the IFAT with its increased diagnostic sensitivity.

Section III

Investigation of the Initial Stages of BKD Following Artificial Infection

Introduction and Literature Review

There is little published data to be found on the initial stages of Bacterial Kidney Disease. It is believed that the disease is transmitted to in-contacts by ingestion of infected tissues. It is possible that the macrophages in the gut lumen engulf the bacteria and transport them to the lamina propria where multiplication may lead to progression of disease. Alternatively, the organisms may gain access to the lamina propria through damaged intestinal mucosa. Subsequently, the bacteria may be distributed via the lymphatic or circulatory systems to target organs where colonisation takes place. Under conditions of stress, acute disease then develops. Otherwise, chronic carriers are produced.

Coulson (1977) attempted to infect healthy S.fontinalis by exposure to the KD organism under natural conditions. He produced stress by stopping exchange of water in the holding tanks which increased the concentration of waste products, raised the water temperature and decreased the oxygen supply. A suspension of bacteria was then added. Macroscopic and microscopic investigation for up to 9 months after challenge did not reveal any evidence that infection had been established.

These findings agree with those found in the bath experiments described in Section II.

Artificial challenge resembling natural transmission of the disease would be desirable for the present studies. Unfortunately, the experiments previously described in Section II indicated that the artificial disease most similar to the natural infection was only achieved by intraperitoneal inoculation.

The WSO3 strain, shown to be the most virulent available (Section II) was used to examine the initial distribution of organisms, the onset and establishment of infection and the development of the acute and chronic forms of the disease.

Evidence of early infection might be useful to produce criteria for early diagnosis.

Diagnosis of BKD is usually made when characteristic clinical signs are already obvious. For example, the diseased fish may exhibit exophthalmos, enlarged abdomen, small skin ulcers and irregular reddened areas in the skin and fin bases. These gross findings are usually in moribund fish. Detection at this stage may be too late to save a stock of cultured fish. It is, therefore, important that the disease should be detected at the earliest possible stage.

Such information would also be essential to determine effective vaccine formulations and routes of administration. Once the sequence of target organs has been established, the route of immunisation directed at the primary site could be investigated. Thus, the route of migration of the organisms could determine whether vaccine should be utilised by ingestion, or by parenteral or external application. To this end the following features were investigated.

(i) The distribution of bacteria following i.p. inoculation using classical bacteriological and immunological techniques.

(ii) The distribution of radioactive labelled bacteria, administered by i.p. inoculation using a whole body autoradiography technique and radioactivity measurement in a scintillation counter.

(iii) The histopathology of target organs for early diagnostic changes.

Experiments were then extended to examine the role of the immune system in the distribution of the organisms and to investigate the local cellular response to intramuscular presentation of live organisms.

Section III

MATERIALS AND METHODS

Fish — Rainbow trout weighing between 60-100g were used in all experiments and maintained as described in Section II.

Strain — The WS03 strain was used throughout in view of the results previously obtained.

i) Investigations to determine the distribution of R.salmoninarum following i.p. inoculation.

These experiments were designed to enable close monitoring of the early course of the infection by sequential samples of blood, tissues and peritoneal fluid over a period of 48 hours.

To substantiate these findings and obviate individual variation the experiments were repeated three times.

Administration

Fish were anaesthetised in a 1:10;00 dilution of (MS222) Tricaine methane sulphonate and an incision of about 2mm made with a scalpel blade through the hypaxial muscle wall. An irrigating needle (Arnolds Veterinary Products Ltd) was inserted into the peritoneal cavity and 0.1ml bacterial suspension inoculated. Ten fish were used and five control fish were similarly injected but with 0.1ml of saline.

Sampling

Two challenged fish and one control were sacrificed at 2, 4, 6, 24 and 48 hours after challenge. From one of the test fish, tissue samples or whole organs, from the spleen, kidney, pronephros, swim bladder, thymus, heart, liver, muscle, pyloric cacae, mid gut and large intestine were dissected out and immediately immersed in 10% phosphate buffered formal saline for histological examination.

Prior to post mortem examination of the other test fish a peritoneal washing was obtained by irrigating needle and syringe using 0.85% saline. Similar tissue samples were collected aseptically, placed in a sterile container and stored at -70°C for further examination.

The control fish were examined as for the second test fish.

Bacterial techniques

Impression smears prepared from tissue samples and smears from the peritoneal washings were examined by Gram stain and the indirect FAT. Tissue homogenates (approximately 5% in 0.85% saline) were inoculated onto KDM_2 and incubated at 15°C.

Blood sampling

Blood samples were withdrawn from the caudal vein using a 2ml syringe and a 25 gauge needle, placed immediately into a bottle containing sequestrene and mixed thoroughly. Films were prepared by placing a small drop of blood close to one end of a glass microscope slide and on it placing the narrow edge of a second slide kept at an angle of 45°. The blood was allowed to spread out across the slide before drawing out the film.

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Films were air dried and fixed in methanol for 20 minutes. They were flooded with Leishman's stain (Gurr) for 2 minutes and then with phosphate buffered saline pH 7.0 the stain was diluted 1:3 and left for 10 minutes. The slides were washed for a further 5 minutes in PBS pH 7.0 and then air dried.

ii) Distribution of radioactive labelled R.salmoninarum following i.p. inoculation

Shake flask cultures

Baffled shake flasks containing 50ml of KDM_2 were prepared with the cysteine hydrochloride component omitted. After autoclaving for 15' at 121°C, 250µCi ^{35}S labelled cysteine hydrochloride was added to give a final concentration of 5µCi/ml. The flasks were then inoculated with 0.5ml of a bacterial suspension in 0.85% saline prepared from an 8 day plate culture. After incubation at 15°C on a shaker (100rpm), 2ml samples were taken at 2 day intervals and the optical density measured in an SPL600 at 650nm. When the maximum growth had been achieved the organisms were harvested by centrifugation, washed three times in saline, adjusted to an OD of 0.2 on an SPL600 at 650nm, and 15µl aliquot samples examined for activity in a liquid scintillator. The results were expressed as mean disintegrations per minute (dpm) (Dent & Johnson, 1973).

0.5ml of this suspension of labelled bacteria was injected intra-peritoneally as previously described into each of ten fish. Two fish were anaesthetised in MS222 solution at 2, 4, 6, 24 and 48 hours.

One fish from each pair was labelled and immersed in isopentane for whole body autoradiography. The second fish was examined post mortem and the following organs were dissected out for quantitative analysis in a liquid scintillator using the method of Dent & Johnson (1973); liver, spleen, kidney, swim bladder, heart, pyloric caecae, large intestine, carcase.

Whole Body Autoradiographic (WBA) Technique

Whole body sections were prepared by Ullberg's technique (1954) on 3M Type 810 tape using a Jung Tetrander Microtome in a Bright freezing cabinet. Longitudinal sections through the mid-line were cut at 15µm thickness. The sections were then transferred to a -20°C chest freezer to dehydrate for 24 hours.

Section Identification

Before exposure, the dried sections were identified using radioactive indian ink (^{14}C 0.3µCi/ml). The sections were labelled with the WBA number, the isotope, the section thickness and the exposure time. The tapes surrounding the sections were dusted with french chalk to remove stickiness and to eliminate static electricity which would fog the film.

Mounting

The dried sections were pressed directly against photographic film with a standard radioactive ladder for calibration (prepared by serial dilutions of ^{14}C labelled polymethyl methacolate in unlabelled polymethyl merthacolate ranging between 7.0nCi to 0.27nCi/ml activity).

The mounted film was then placed on a piece of cardboard between pieces of black paper and tin foil to avoid cross contamination during exposure, replaced in the original envelope with the film and stored at -20°C during exposure.

Two types of autoradiographic film were used in this study. Agfa Osray M3, a so-called "fast film" was chosen for the shorter exposure times of 4 and 8 weeks, and Kodak Industrex C "slow film" for the 12-16 and 20 week exposures.

Photographic Development

After exposure the films and sections were separated. The films were developed in DX80 Kodak diluted 1:3 for 4 minutes, immersed in an acid stop of 2% acetic acid for 30 seconds, then Kodak fixing fluid diluted 1:4 for 8 minutes, rinsed in running tap water for 30 minutes and allowed to dry.

Preparation of stained whole sections

Separate sections were thawed and partially dried in front of a fan at room temperature for approximately 20-30 seconds. The tissue was fixed by floating face downwards on 10% buffered formal saline for 2-3 minutes so that the tape kept dry and therefore remained quite flat. Sections were washed in running tap water for 30 seconds and stained by staining techniques involving minimal exposure to alcohol.

Alcian blue

The sections were floated on Alcian blue (0.5% Alcian blue in 0.5% acetic acid) for 5-10 minutes. After washing in tap water they were counterstained with Harris's Haematoxylin for 2 minutes, then "blued" in a running water bath for 2 minutes and differentiated in 1% HCl if necessary. Glycerol was used to dehydrate and clean the sections for 2-5 minutes. Excess glycerol was drained off, the tapes laid face down on blotting paper and blotted until dry.

A light coat of 'Trycolac' (Aerosol Marketing Co.Ltd) was sprayed over the sections on the tapes which were then laid flat and secured for several hours to dry (Farebrother & Woods, 1973). This clear antifading varnish protected the sections by forming a plastic film so that they could be safety stored in polythene envelopes.

Haematoxylin and Eosin

Sections were floated on Harris's Haematoxylin for 2 minutes, "blued" in running tap water and counterstained with 0.4% aqueous Eosin for 20 seconds. They were then rinsed in tap water, dehydrated, dried and sprayed as above.

The autoradiograms were superimposed on these stained sections to facilitate interpretation and were prepared as positive and negative prints for comparison of respective advantages.

iii) The cellular responses to intramuscular injection with R.salmoninarum

Preliminary histological examinations of developing lesions in artificial and natural infections revealed extensive infiltration by macrophages. Relatively few polymorphonuclear cells (PMN) were present.

The primary local cellular responses to infection were therefore studied in greater detail.

Inocula

R.salmoninarum was grown on KDM_2 medium and the challenge prepared as described in Section II and standardised by OD measurements at 650nm.

Two experiments were performed:

a) 0.1ml suspension of viable organisms containing approximately 5×10^8 orgs/ml was injected via a 25 gauge needle into the apaxial muscles of anaesthetised fish.

b) A dose reponse was investigated by injection of 0.1ml suspensions at three dose levels (5×10^8, 1×10^9 or 1×10^{10} orgs/ml).

Investigations

Injection sites were examined 2, 4, 6, 24 and 48 hours after inoculation. Sections were stained by H & E, Gram Twort (Section I) and by IFAT.

Section III

RESULTS

i) Distribution of R.salmoninarum following i.p. inoculation

a) Impression smears and peritoneal washings

The dissemination of organisms from the peritoneal cavity, and the subsequent tissue localisation is demonstrated in Table 11. All smears showing Gram positive organisms were confirmed to be R.salmoninarum by the IFAT (Figure 27-30).

At 2 hours p.i. organisms were consistently located in the peritoneal cavity only. At 4 and 6 hours p.i. a marked individual variability between fish in their distribution of organisms was observed. In one fish at 4 hours and two fish at 6 hours organisms were not detected at all. In other fish at 4 and 6 hours small numbers of organisms were found to be localised primarily in the spleen and liver, although in some fish organisms were detected in smears from gut and swim bladder.

At 24 hours large numbers were found in the spleen and liver, and most smears from the swimbladder and crude transverse sections of gut were positive. Small numbers were also detected in the kidney. No organisms in the peritoneal washings were noted at this stage, indicating a complete dispersion from the peritoneal cavity, or a firm adherence to the visceral organs.

At 48 hours a similar pattern of localisation was observed.

Table 11 Detection of R.salmoninarum in smears by Gram stain and IFAT demonstrating their in vivo distribution following i.p. inoculation

	2 hours				4 hours				6 hours				24 hours				48 hours			
Experiment No:	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Sample																				
Spleen	-	-	-	-	+	+	-	-	-	+	+	-	+++	+++	++	+++	+++	+++	+++	+++
Liver	-	-	-	-	+	+	-	+	-	+	+	-	+++	+	+	+++	+	+	+	-
Kidney	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	++	+	+	+
Heart	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyloric cacae	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-
Mid gut	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-
Hind gut	-	-	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+	+	+	-
Swimbladder	-	-	-	-	++	+	-	-	-	+	+	-	+	+	+	+	+	+	+	-
Thymus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pro-nephros	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Peritoneal wash	+++	+++	+++	+++	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-

Key
+ - small number, 1-10 orgs/fld
++ - moderate number, 10-50 orgs/fld
+++ - large number >50 orgs/fld

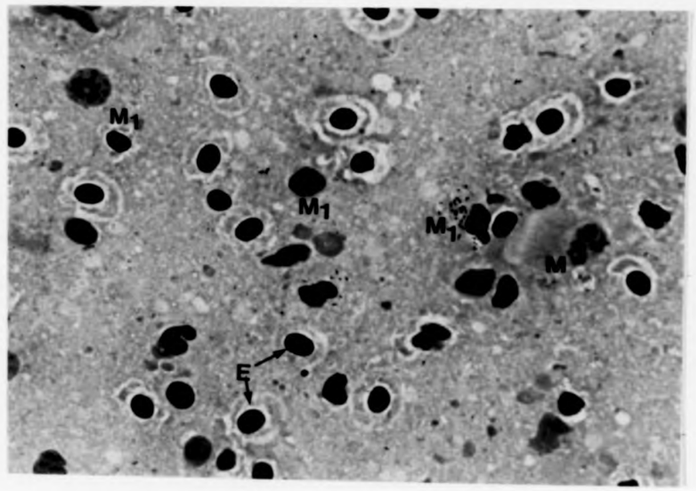


Figure 27 Impression smear of spleen. Gram stain x 840.

M - Macrophage
M1 - Macrophage containing bacteria within its cytoplasm
E - Erythrocyte

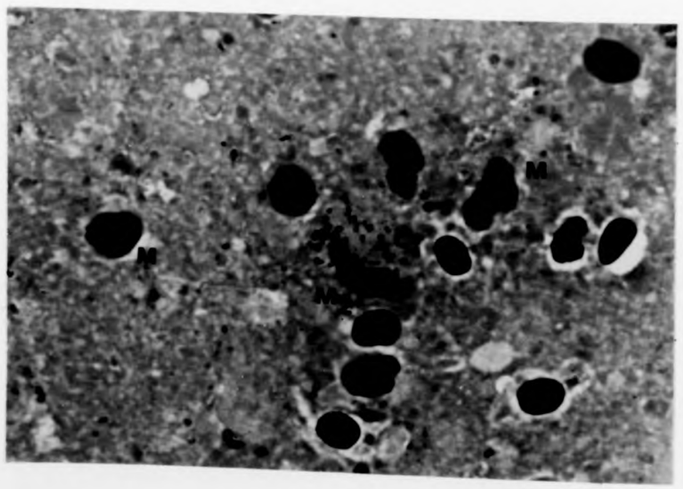
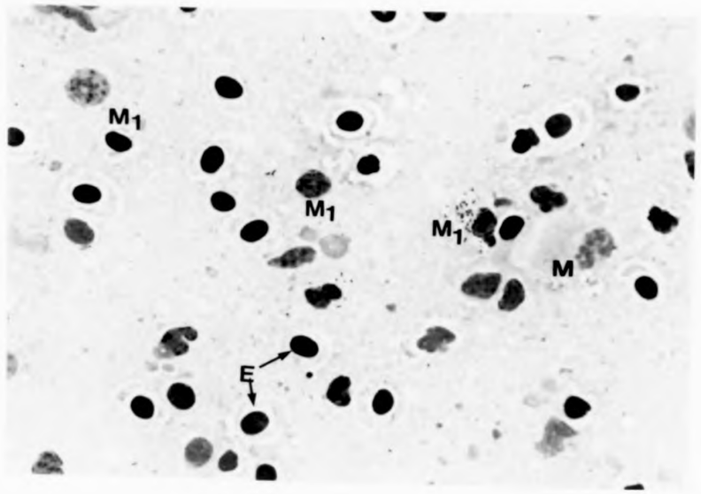


Figure 28 Impression smear of kidney. Gram stain x 1400

M - Macrophage
M2 - Macrophage actively phagocytosing bacteria



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M1 - Macrophage containing bacteria within its cytoplasm
E - Erythrocyte

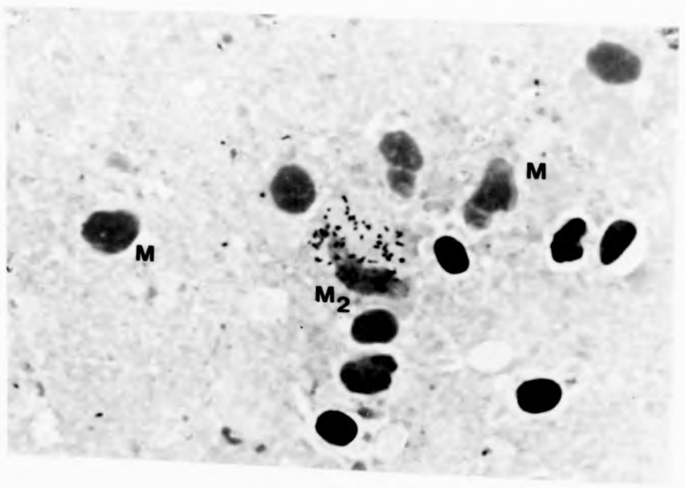
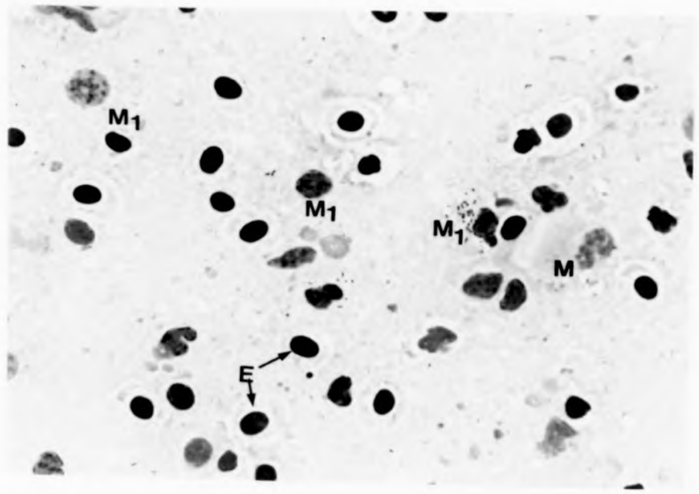


Figure 28 Impression smear of kidney. Gram stain x 1400

M - Macrophage
M2 - Macrophage actively phagocytosing bacteria



gure 27 Impression smear of spleen. Gram stain x 840.

M - Macrophage
M1 - Macrophage containing bacteria within its cytoplasm
E - Erythrocyte

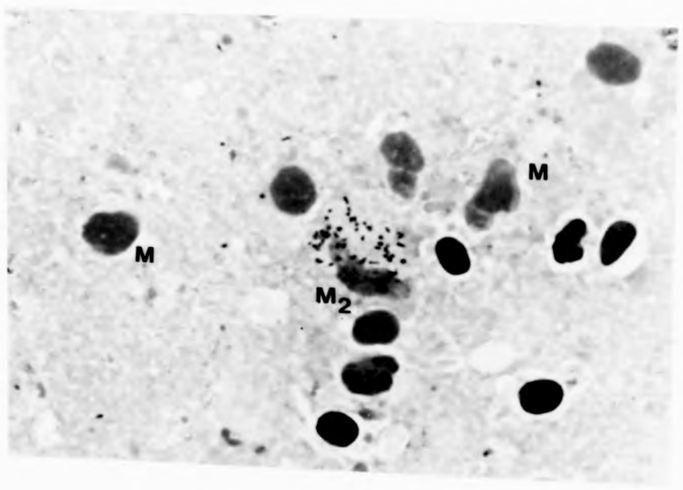


Figure 28 Impression smear of kidney. Gram stain x 1400

M - Macrophage
M2 - Macrophage actively phagocytosing bacteria

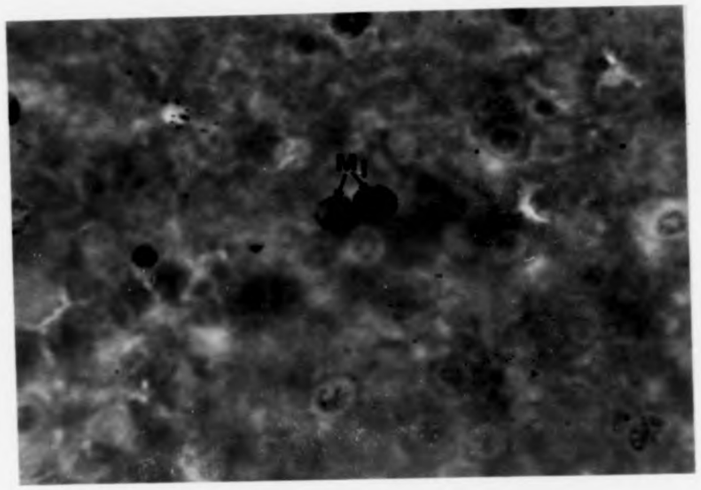


Figure 29 Peritoneal wash smear. Gram stain x 1400

M1 - Macrophage containing bacteria within its cytoplasm

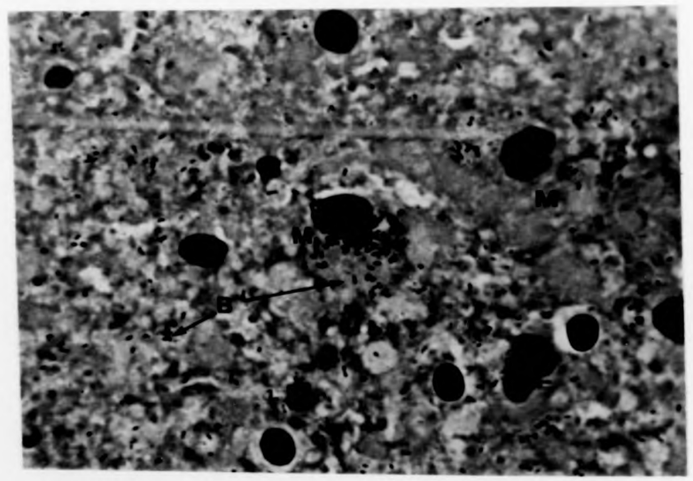


Figure 30 Impression smear of liver. Gram stain x 1400.

M - Macrophages
M1 - Macrophage containing bacteria within its cytoplasm
B - Free bacteria

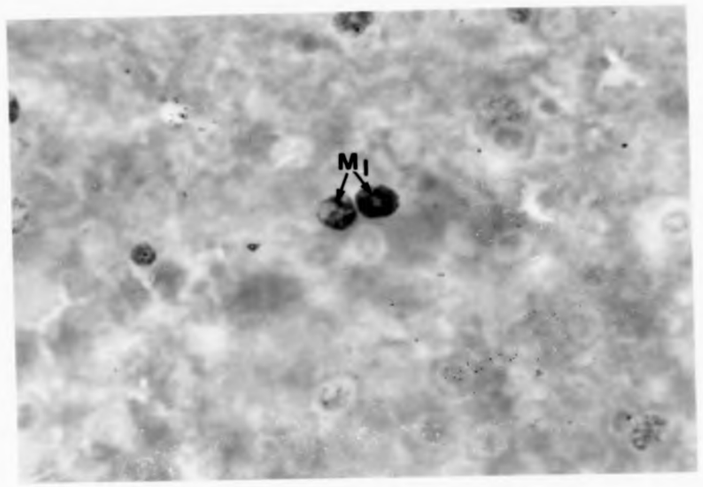


Figure 29 Peritoneal wash smear. Gram stain x 1400

M1 - Macrophage containing bacteria within its cytoplasm

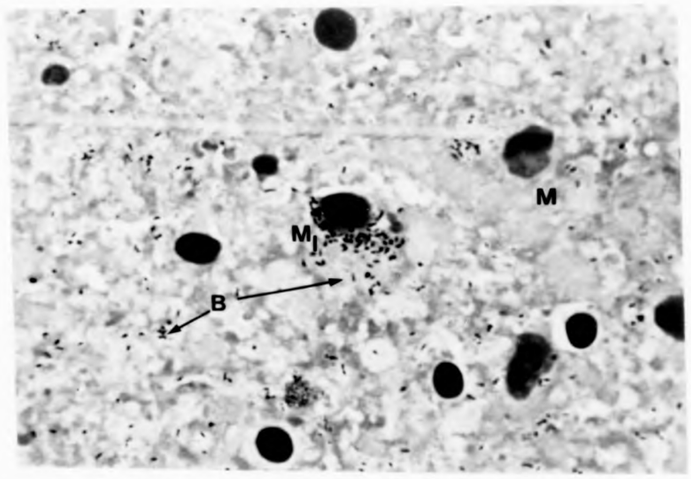


Figure 30 Impression smear of liver. Gram stain x 1400.

M - Macrophages
M1 - Macrophage containing bacteria within its cytoplasm
B - Free bacteria

b) Culture

Isolation was achieved from all 2 hour peritoneal washings, and liver, spleen, kidney and swimbladder samples at 24 and 48 hours p.i.

c) Histological examination

Histopathological changes were not found in sections of specimens taken at 2, 4 and 6 hours p.i. The first histological changes were seen in the liver at 24 hours p.i. and were small focal infiltrations of mononuclear cells. Organisms were not detected in the Gram/Twort stained sections, or by IFAT. Similar lesions were also found in the liver at 48 hours p.i., but no other changes were observed. These lesions closely resembled the early changes found in the challenge experiment described in Section II.

d) Differential white cell count

A high percentage of smear cells (Figure 31a, b) encountered in all blood films made interpretation of differntial white cell counts very difficult.

ii). Distribution of radioactive labelled R.salmoninarum following i.p. inoculation

a) Liquid scintillation count on inoculum

Activity of inoculum was calculated from an average of six 15µl homogenised samples.

Samples 1 - 6 = 5.306422 dpm/ml = 884408q/ml = 2.388µCi/ml

b) Culture

Isolation was achieved from all 2 hour peritoneal washings, and liver, spleen, kidney and swimbladder samples at 24 and 48 hours p.i.

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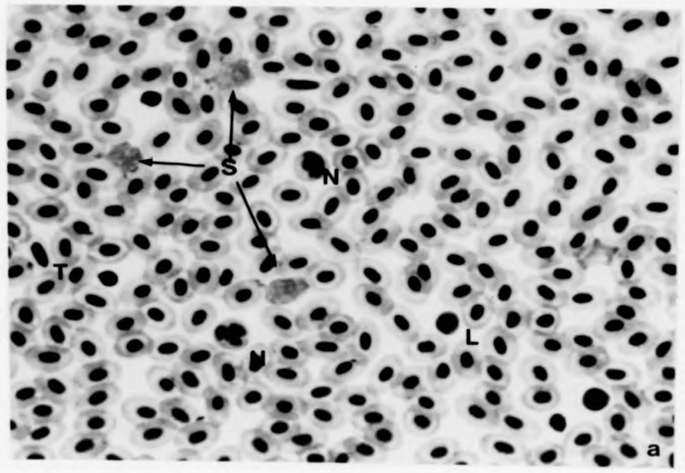


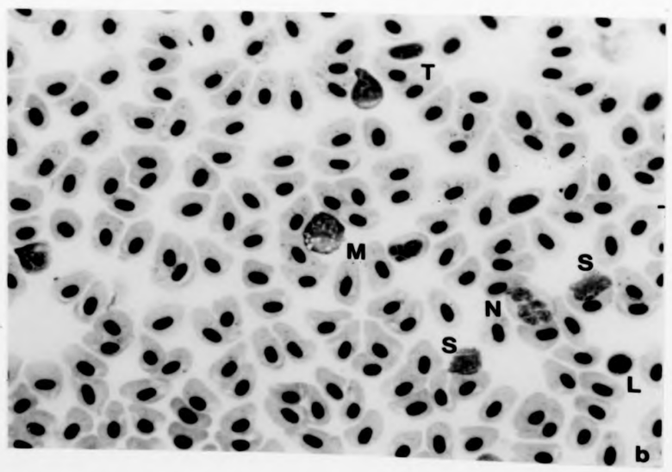


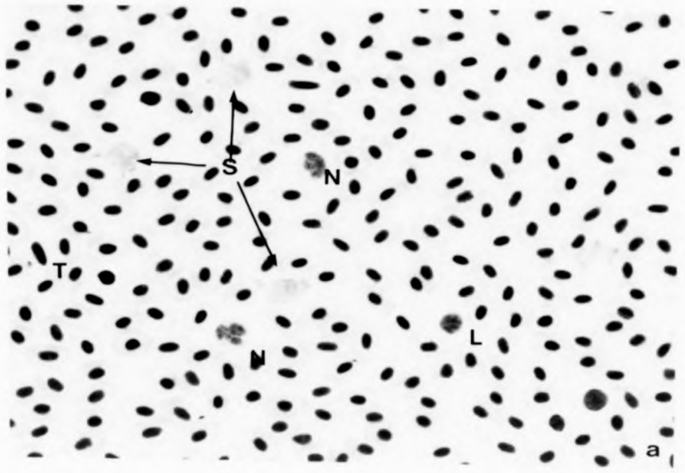
Figure 31 Peripheral blood film. Leishman stain x 450

a) 2 hours p.i.

N - Neutrophil
L - Lymphocyte
T - Thrombocyte
S - Smear cell

b) 48 hours p.i.

M - Vacuolated monocyte
N - Neutrophils
L - Lymphocytes
T - Thrombocytes
S - Smear cells



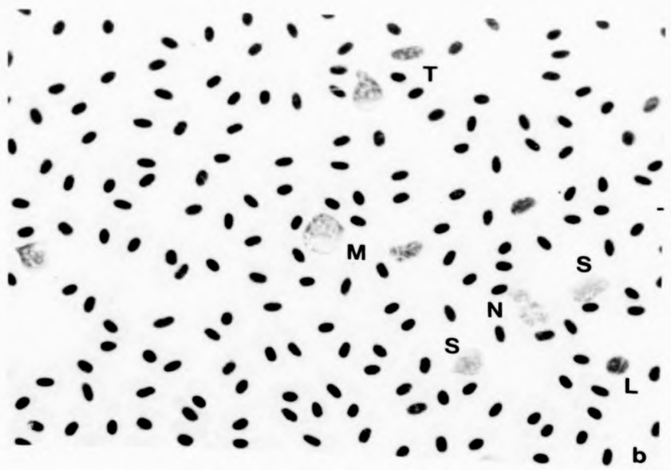


Figure 31 Peripheral blood film. Leishman stain x 450

a) 2 hours p.i.

N - Neutrophil
L - Lymphocyte
T - Thrombocyte
S - Smear cell

b) 48 hours p.i.

M - Vacuolated monocyte
N - Neutrophils
L - Lymphocytes
T - Thrombocytes
S - Smear cells

b) Liquid scintillation counts on organ and tissue samples

The results presented in Table 12 demonstrate detection of the 35S labelled organisms in all tissues examined.

At 2 hours, high concentrations of activity were found in the liver, spleen, heart, gut and swimbladder. This trend persisted until 24 hours when localisation in the kidney was detected and increased significantly at 48 hours.

After 2 hours, the initial high activity in the heart decreased and remained low for the remainder of the experiment.

Low levels were also detected in the muscle and carcase throughout the experiment.

c) Whole Body Autoradiography

The results were presented as a sequence of autoradiograms obtained from fish sacrificed at 2, 4, 6, 24 and 48 hours following intraperitoneal injection of ^{35}S labelled R.salmoninarum.

Autoradiograms were interpreted by comparison with their corresponding stained sections (Figure 32a, b).

By 2 hours p.i. there had been rapid uptake into the blood stream and/or the lymphatic system as shown by radioactivity in the main vessels of the head and gills and in the skin. Accumulations of bacteria bound to the liver capsule, intestinal surfaces and swim bladder were revealed as dark areas or "hot spots". Diffuse areas of moderate activity were seen in areas of high blood flow such as the

Table 12 ^{35}S content ($dpmg^{-1}$) in tissue samples following i.p. inoculation

Organ	Time				
	2 hours	4 hours	6 hours	24 hours	48 hours
Liver	39446.1	21196.7	28264.6	4534.5	150591.04
Spleen	33777.8	13341.2	480771.9	5794.4	20960.5
Kidney	4242.4	4740.6	4319.72	15445.845	252050.57
Heart	32356.1	5830.1	8030.4	2644.5	8880.9
Swim bladder	63988.7	15826.2	60786.6	9080.73	140919.1
Pyloric cacae	32105.3	24959.5	50537.7	13109.5	27488.7
Large intestine	24395.1	10711.6	20871.2	5453.0	15598.5
Muscle	10046.96	3256.2	2189.3	2251.0	5401.0
Carcase	4782.5	2629.2	4448.9	1133.2	1830.86

liver and stomach lining. No activity was detected in the muscle (Figure 33a, b, c).

At 6 hours p.i. "hot spots" of radioactivity were still localised around the gut. Low grade radioactivity demonstrable in the skin and gills suggested the presence of organisms still in the blood stream, or lymphatic vessels (Figure 34a, b, c) but preparation problems precluded further detailed examinations.

At 24 hours p.i. a pronounced accumulation of radioactivity was seen in the head, trunk and kidney. A low grade radioactivity was present in the liver and gills and "hot spots" were located around the peritoneal surface of the pyloric caecae (Figure 35a, b, c).

High concentrations of radioactivity were seen in the stomach mucosa which may represent an accumulation of free sulphur.

At 48 hours p.i. radioactivity was more generalised which may indicate widespread distribution of organisms in capillaries. Radioactivity was present in the kidney and there were signs of accumulation in the walls of the gut. There were particular areas of concentrated radioactivity distributed along the peritoneal surface of the pyloric caecae, mid gut and hind gut. Low grade radioactivity was seen in the liver, gills and skin (Figure 36a, b, c).

A marked accumulation of radioactivity in the gall bladder was noted, which probably represented excretion of soluble products liberated by the destruction of the bacteria.

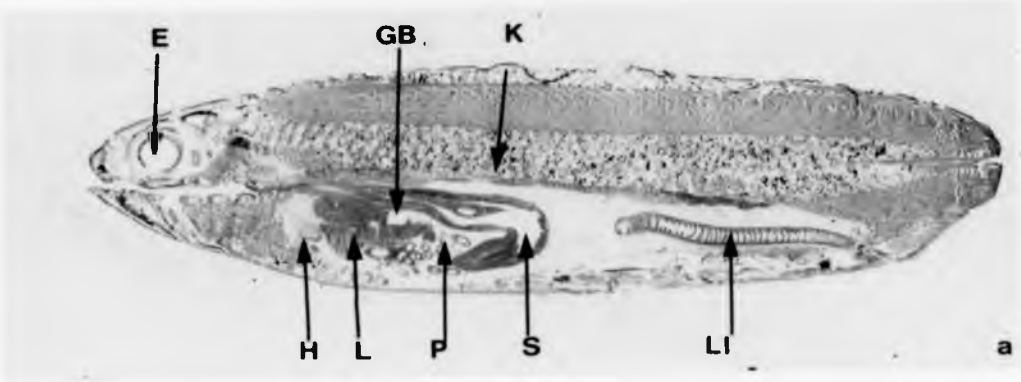
Figure 32 Stained whole body sections of rainbow trout

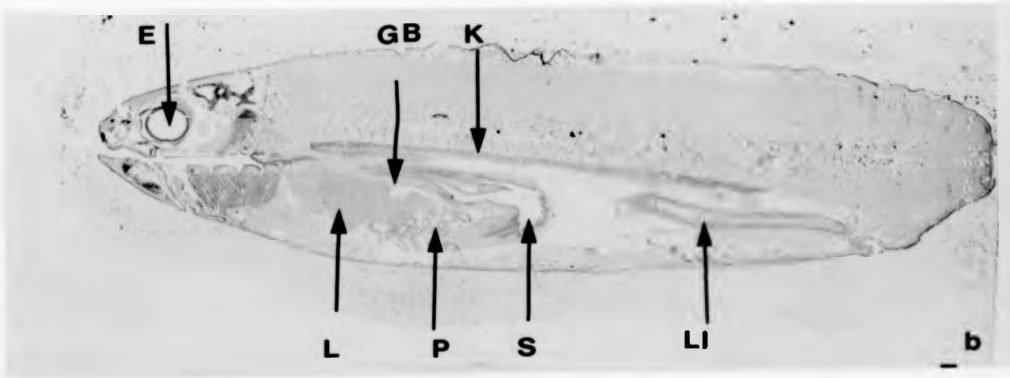
a) Haematoxylin and Eosin stain

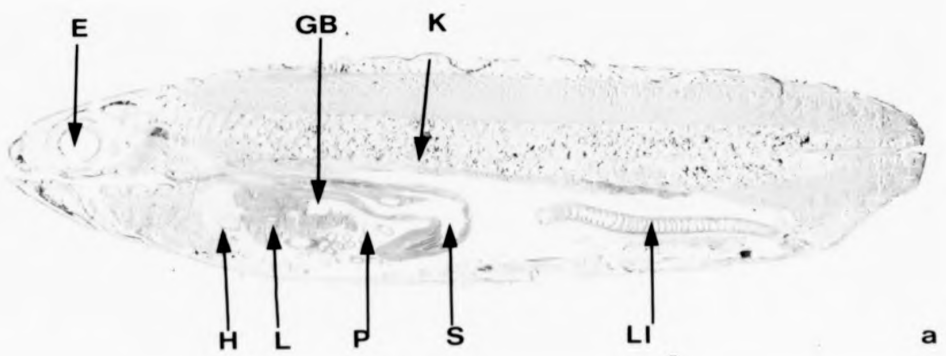
E - Eye
GB - Gall bladder
LI - Large intestine
S - Stomach
P - Pyloric caecae
L - Liver
H - Heart

b) Alcian blue stain (actual size)

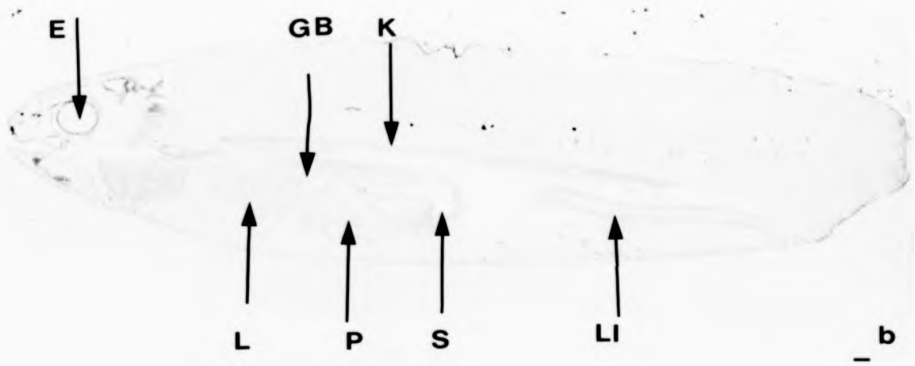
E - Eye
GB - Gall bladder
K - Kidney
LI - Large intestine
S - Stomach
P - Pyloric caecae
L - Liver


E
GB
K
H
L
P
S
LI
a


E
GB
K
L
P
S
LI
b



a



b

Figure 33 Distribution of ^{35}S labelled R.salmoninarum 2 hours post-injection

a) Negative print : black areas correspond to high activity

L - Liver
S - Stomach
P - Pyloric caecae
Lc - Liver capsule
G - Gill

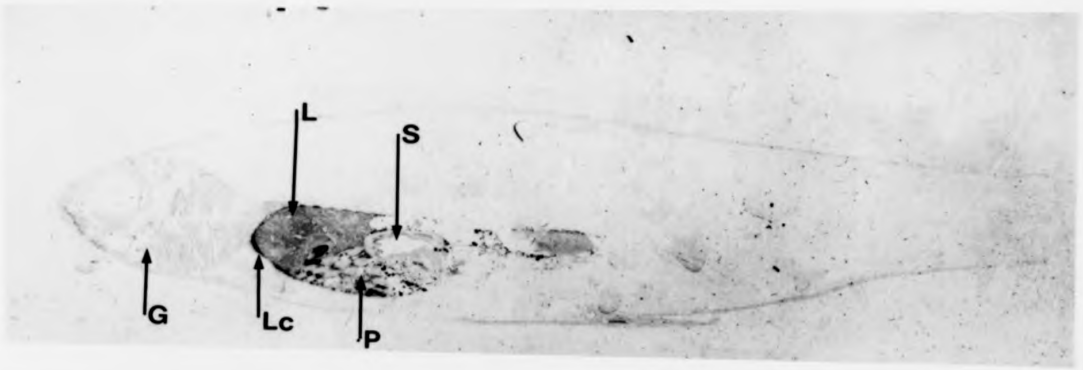
b) Positive print : white areas correspond to high activity

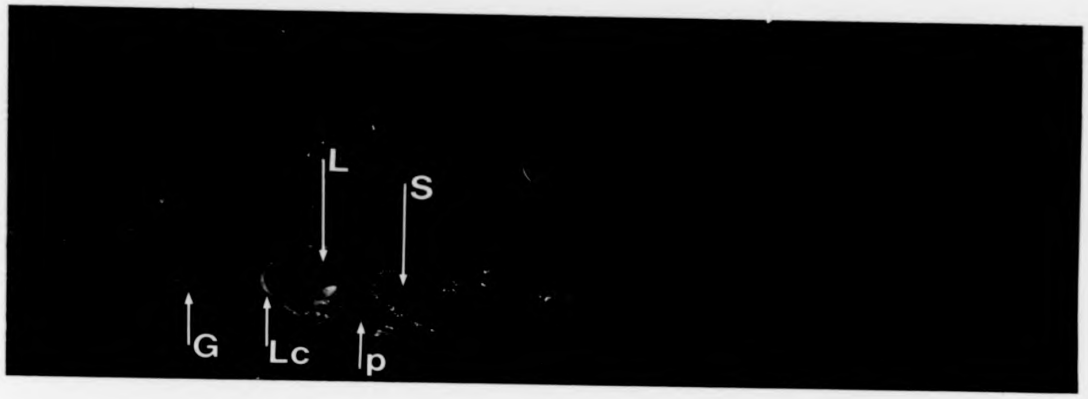
L - Liver
S - Stomach
P - Pyloric caecae
Lc - Liver capsule
G - Gill

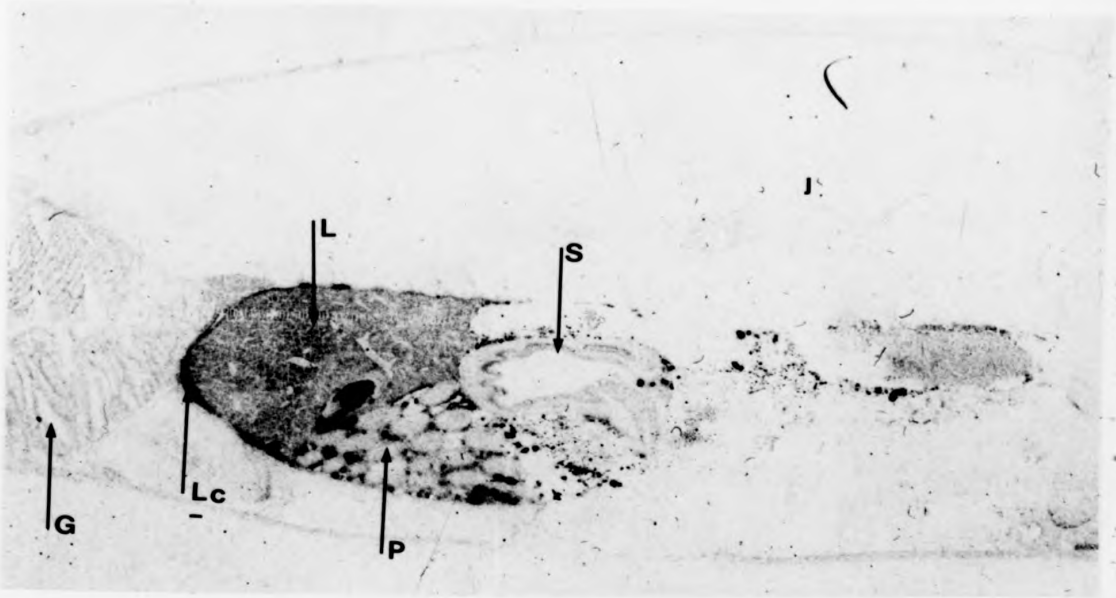
c) Negative print x 2.5

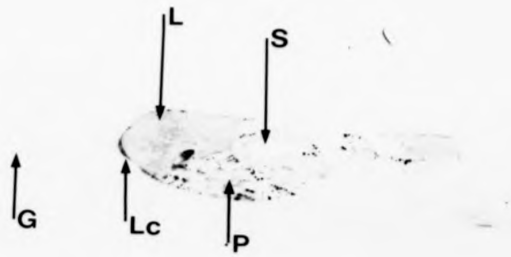
L - Liver
S - Stomach
P - Pyloric caecae
Lc - Liver capsule
G - Gill

Note: "hot spots" of activity adherent to the outer surfaces of the gut and liver capsule.


L
S
G
Lc
P


L
S
G
Lc
p


L
S
G
Lc
P


L
S
G
Lc
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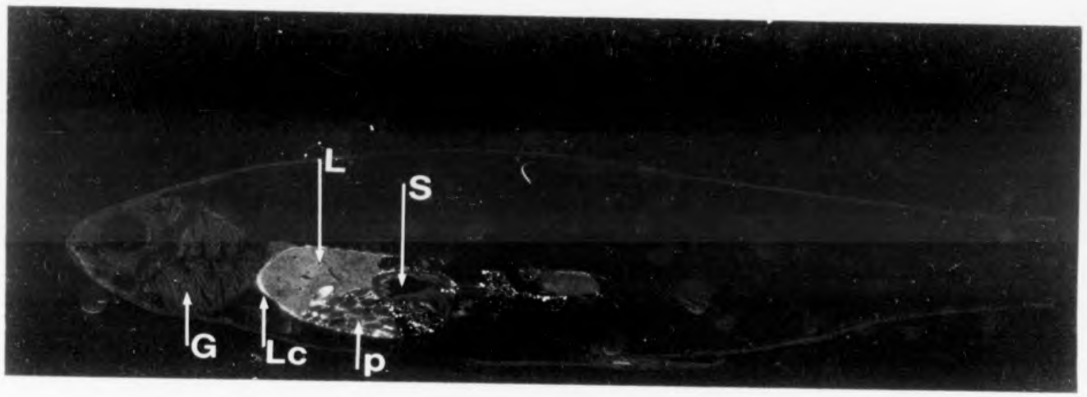
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S
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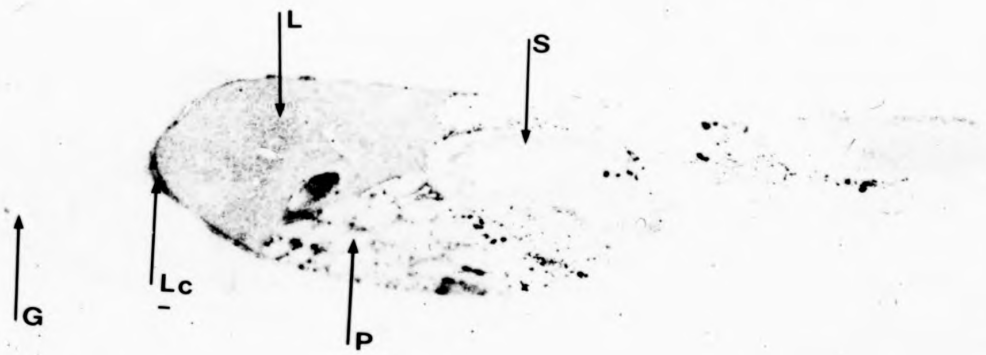
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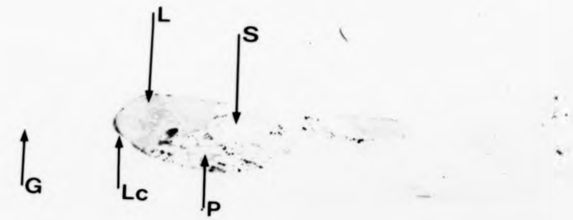
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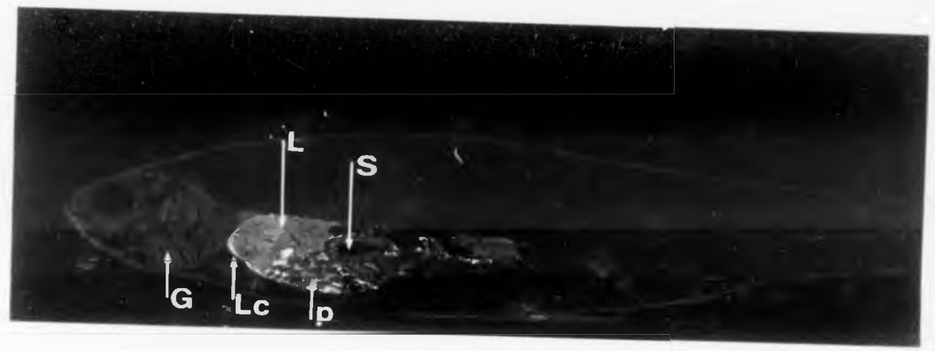
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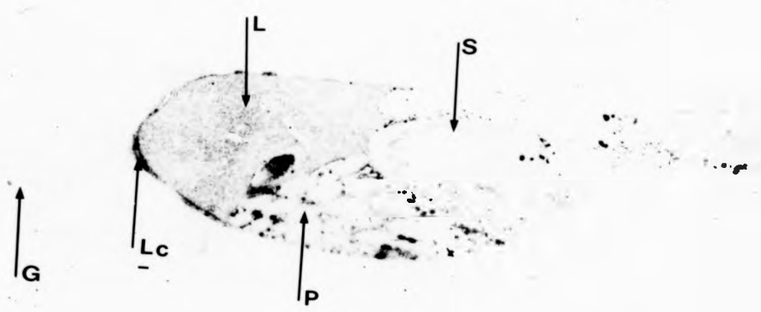
L
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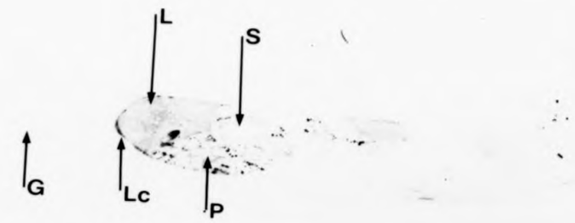
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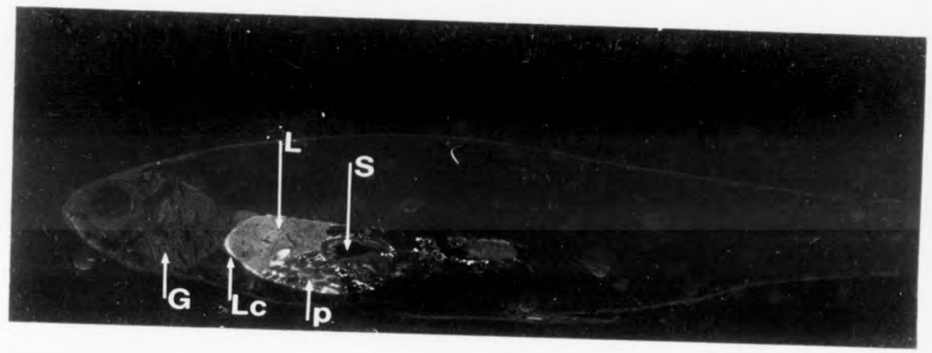
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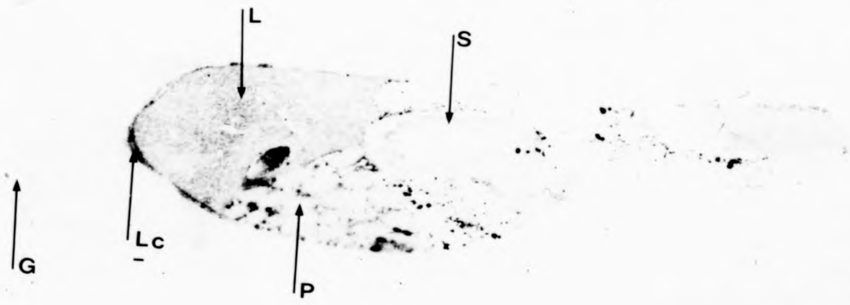
S
G
Lc
p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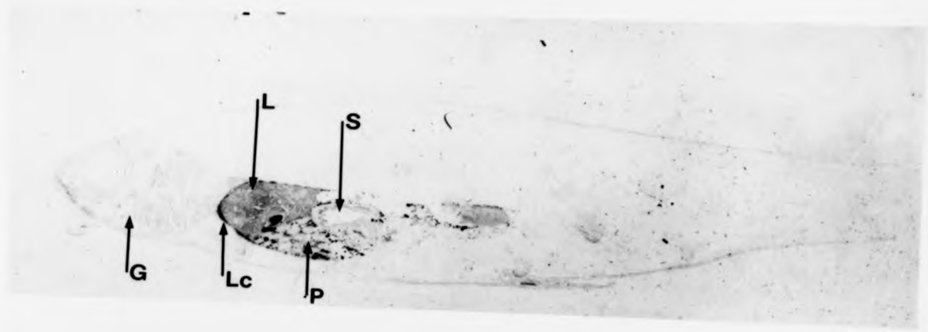

L
S
G
Lc
P


L
S
G
Lc
p


L
S
G
Lc
P

igure 34 Distribution of ^{35}S labelled R.salmoninarum 6 hours post-injection

a) Negative print

S - Stomach
G - Gut

b) Postive print

S - Stomach
G - Gut

c) Negative print x 2.5

S - Stomach
G - Gut

Figure 34 Distribution of ^{35}S labelled R.salmoninarum 6 hours post-injection

a) Negative print

S - Stomach
G - Gut

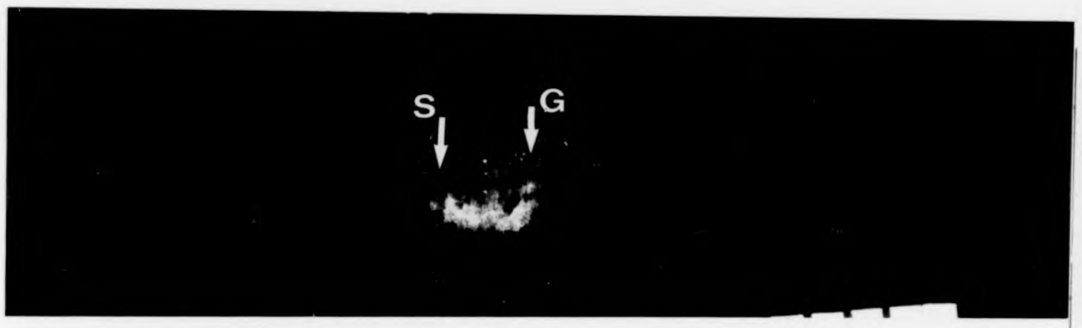
b) Postive print

S - Stomach
G - Gut

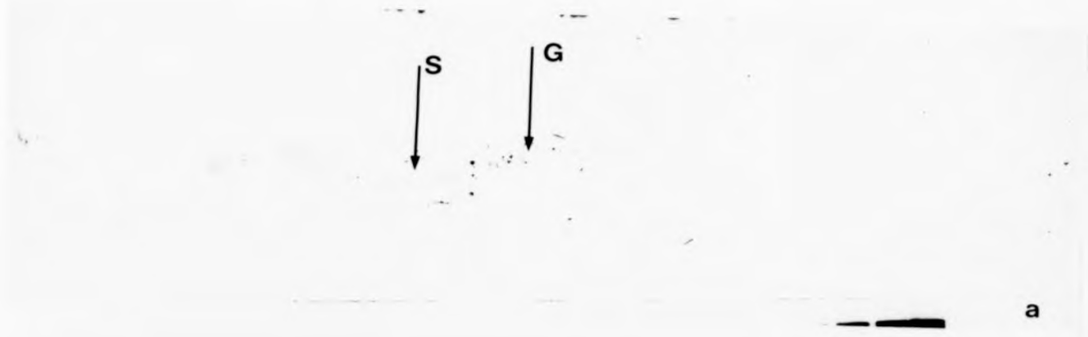
c) Negative print x 2.5

S - Stomach
G - Gut


S
G
a

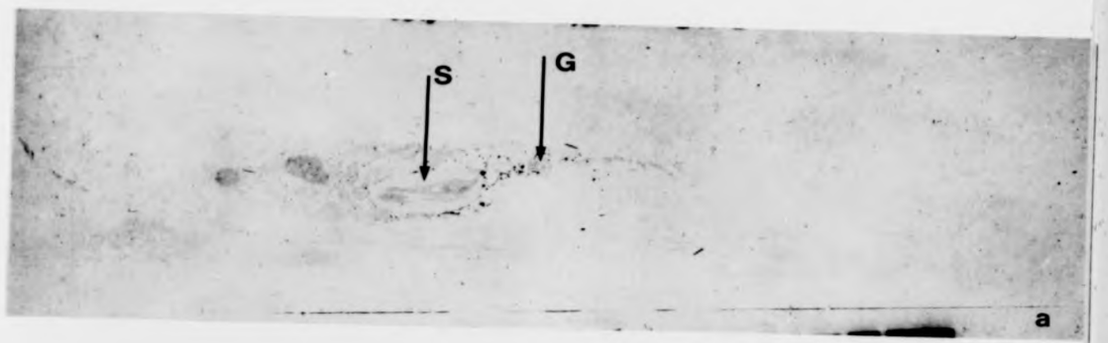

S
G

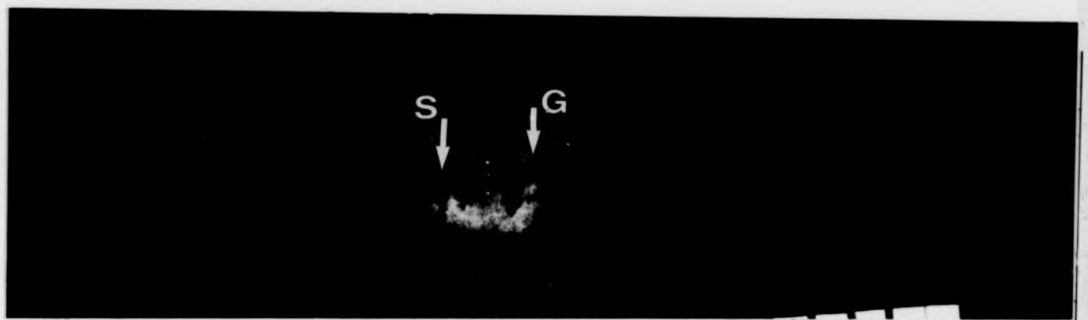

S
G
c



a

c


S
G
a


S
G

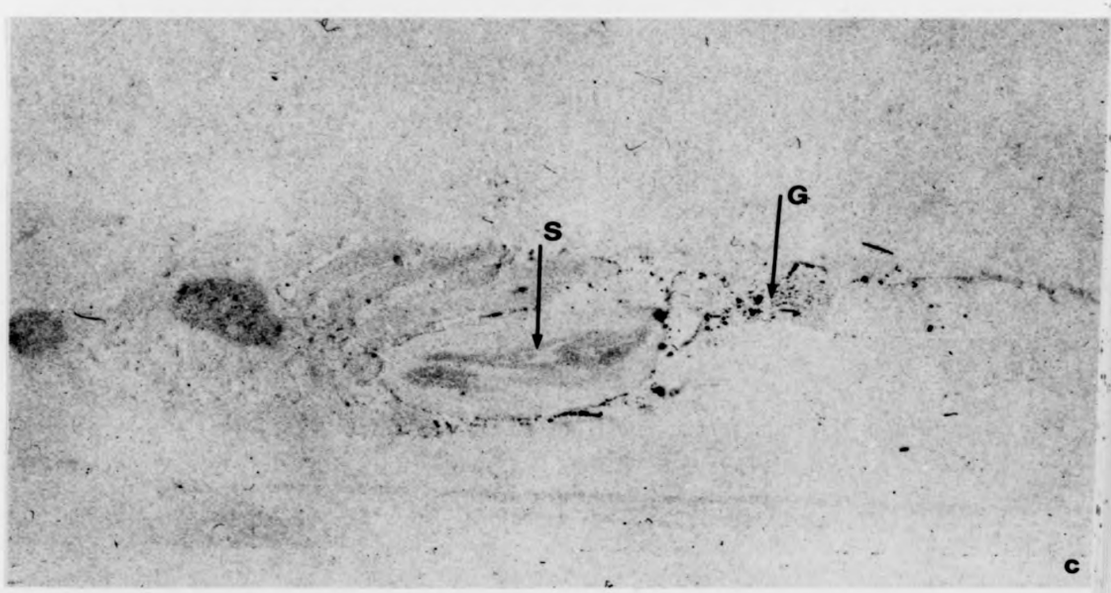

S
G
c

Figure 35 Distribution of ^{35}S labelled R.salmoninarum 24 hours post-injection

a) Negative print

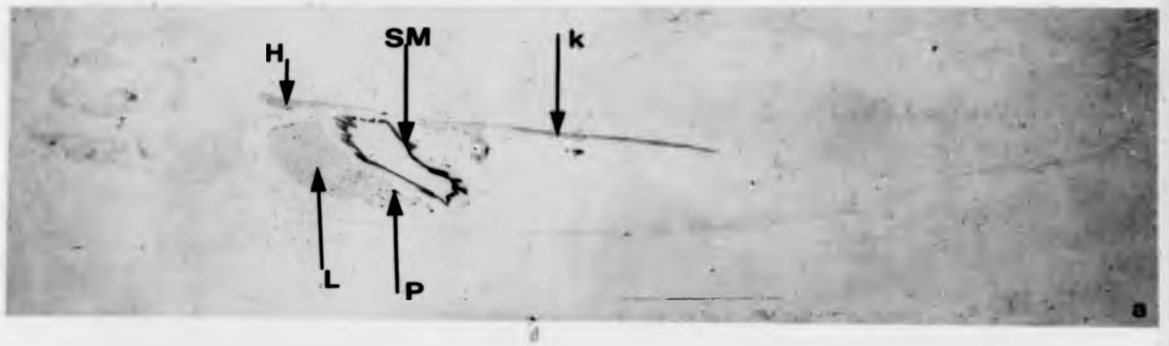
HK - Head kidney
SM - Stomach mucosa
K - Kidney
P - Pyloric caecae
L - Liver

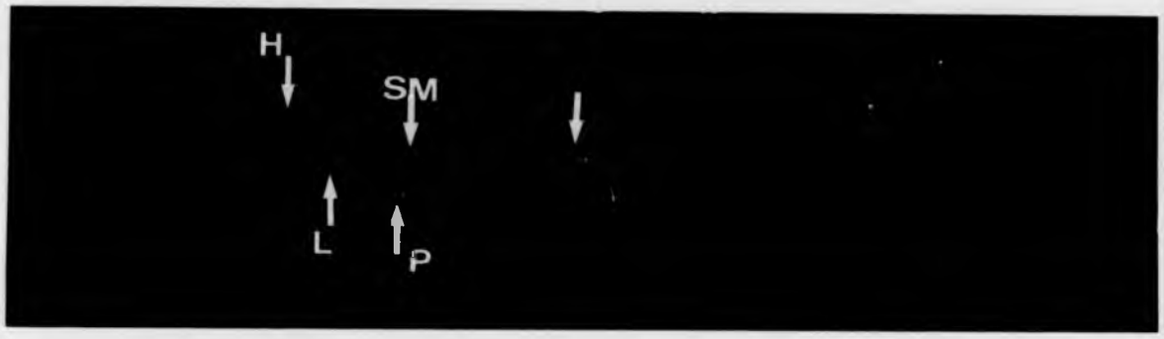
b) Positive print

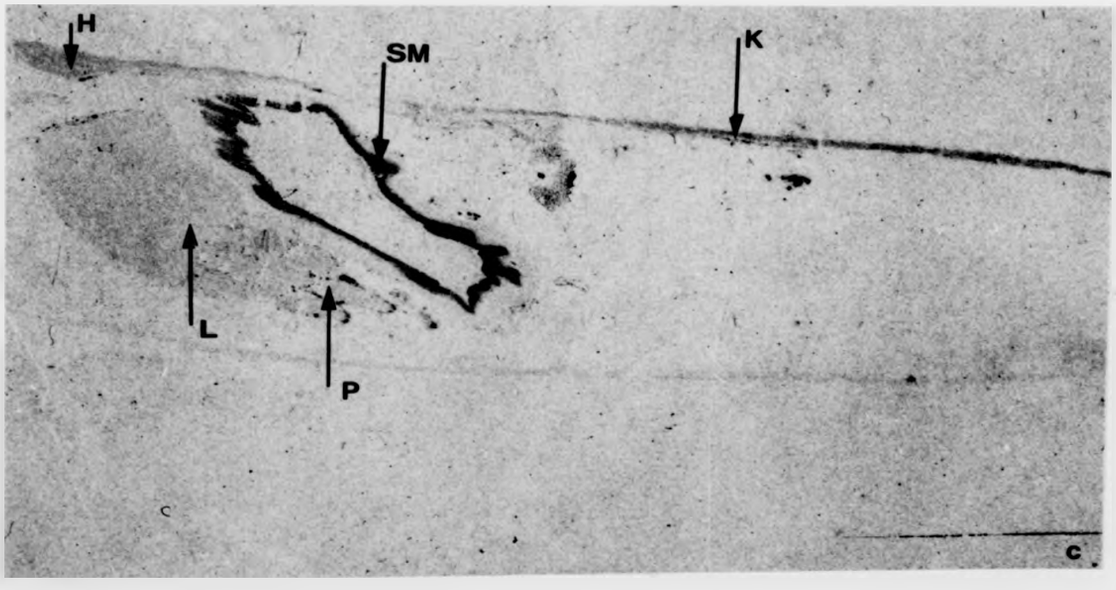
HK - Head kidney
SM - Stomach mucosa
K - Kidney
P - Pyloric caecae
L - Liver

c) Negative print x 2.5

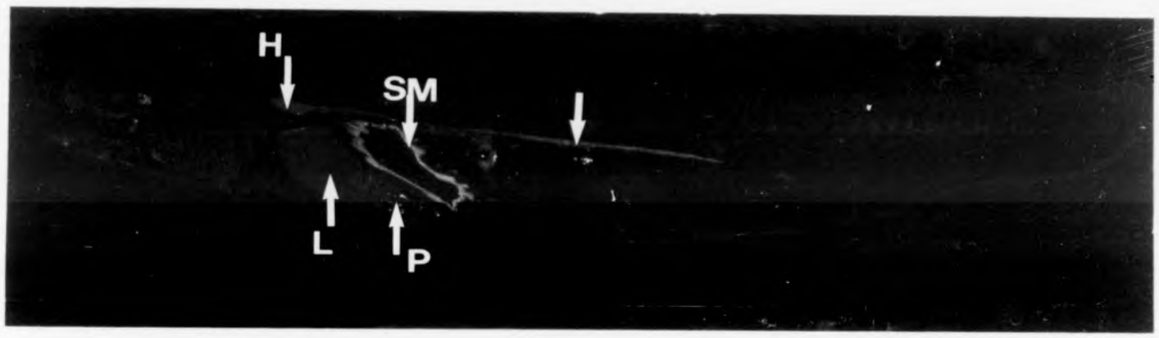
HK - Head kidney
SM - Stomach mucosa
K - Kidney
P - Pyloric caecae
L - Liver


H
SM
k
L
P


H
SM
L
P


H
SM
K
L
P


H
SM
k
L
P
a


H
SM
L
P

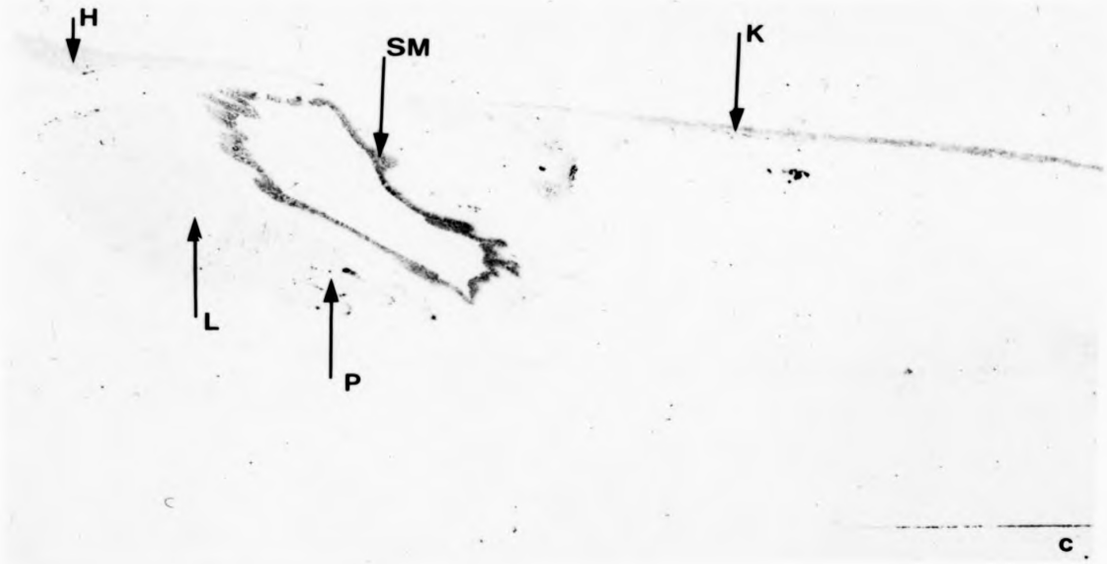

H
SM
K
L
P
c

Figure 36 Distribution of ^{35}S labelled R.salmoninarum 48 hours post-injection

a) Negative print

HK - Head kidney
K - Kidney
LI - Large intestine
G - Gut
P - Pyloric caecae
L - Liver
GB - Gall bladder

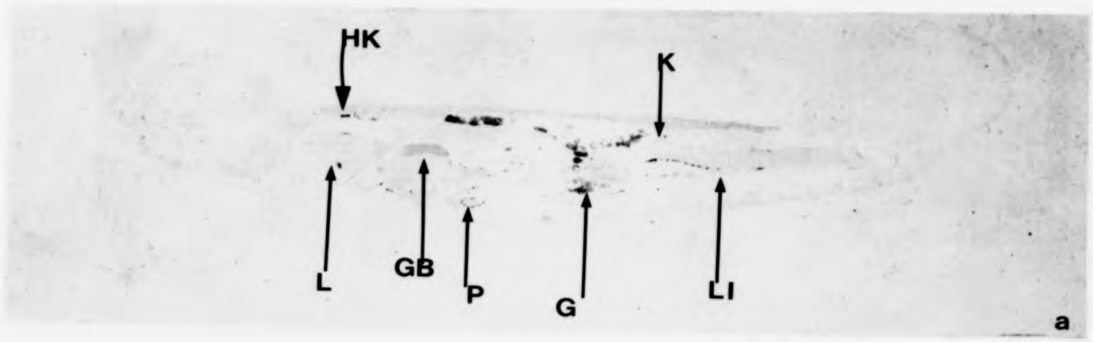
b) Positive print

HK - Head kidney
K - Kidney
LI - Large intestine
G - Gut
P - Pyloric caecae
L - Liver
GB - Gall bladder

c) Negative print x 2.5

HK - Head kidney
K - Kidney
LI - Large intestine
G - Gut
P - Pyloric caecae
L - Liver
GB - Gall bladder

Note: "hot spots" of radioactivity distributed along the peritoneal surface of the gut.


HK
K
L
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P
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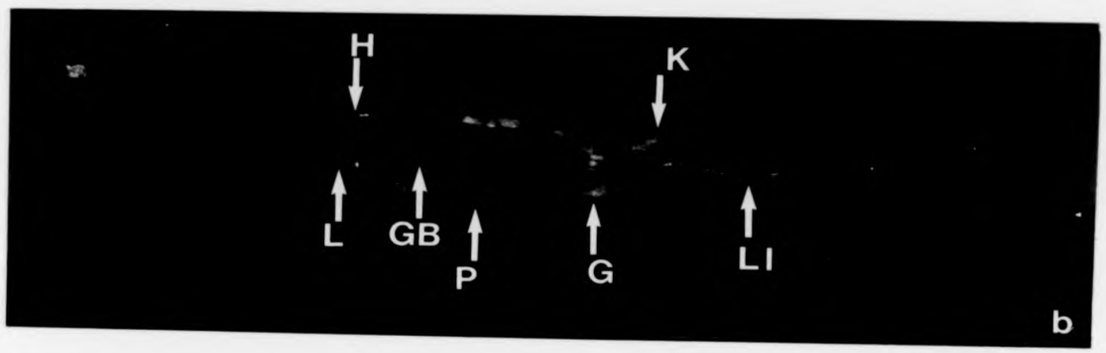
LI
a


H
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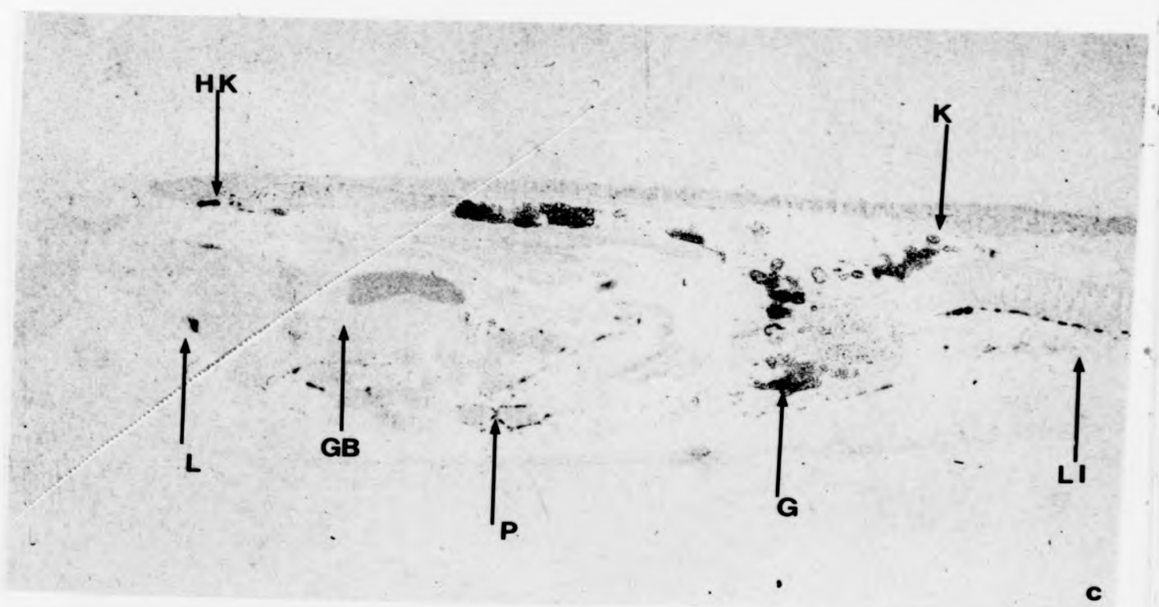
HK
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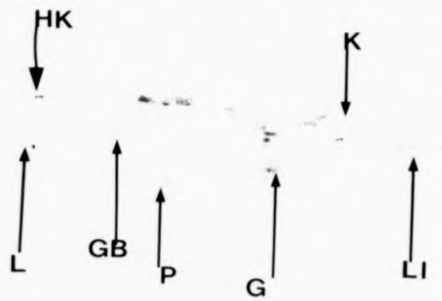
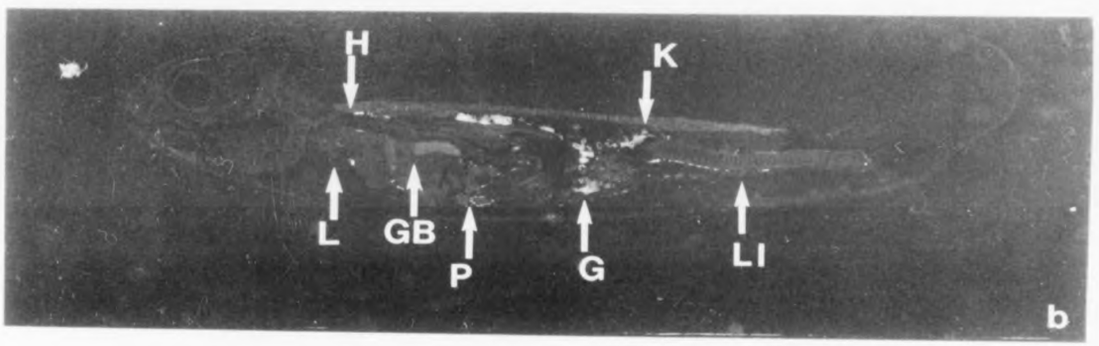
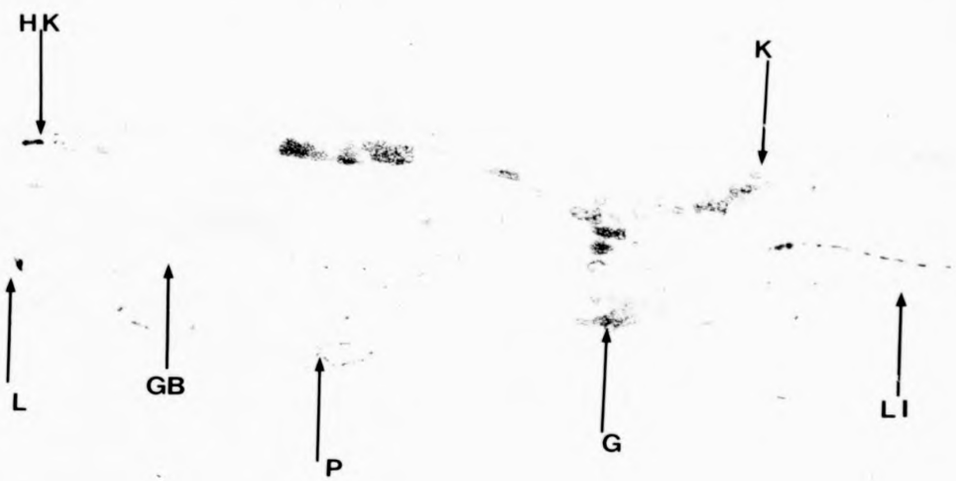


a



b



c

iii) The cellular responses to intramuscular injection with R.salmoninarum

Experiment a) and Experiment b), Group I - Fish inoculated with 0.1 x 5×10^8 orgs/ml

Histological examination of lesions after 2 hours revealed the onset of a cellular response consisting of small accumulations of inflammatory cells. Muscle necrosis and rupture of the cell membranes or sarcolemmae was evident.

A progressive infiltration of inflammatory cells, predominantly macrophages, was noted at 4, 6, 24 and 48 hours with an increased muscle necrosis and complete loss of striations in affected muscle fibres. Invasion of muscle fibres by macrophages was evident, (Figure 37a, b, c, d and 39a, b). Phagocytosis was demonstrated in Experiment a and Experiment b at 24 and 48 hours (Figures 38 and 40).

Group II - Fish inoculated with 0.1×10^9 orgs/ml

A slower response was found in this group and the first sign of a cellular response was seen at 6 hours post injection. A reduced chemotaxis with fewer infiltrating leukocytes was noted at 24 and 48 hours as compared with that seen in Group I. Muscle necrosis and disorganisation was noted as above (Figure 41a, b).

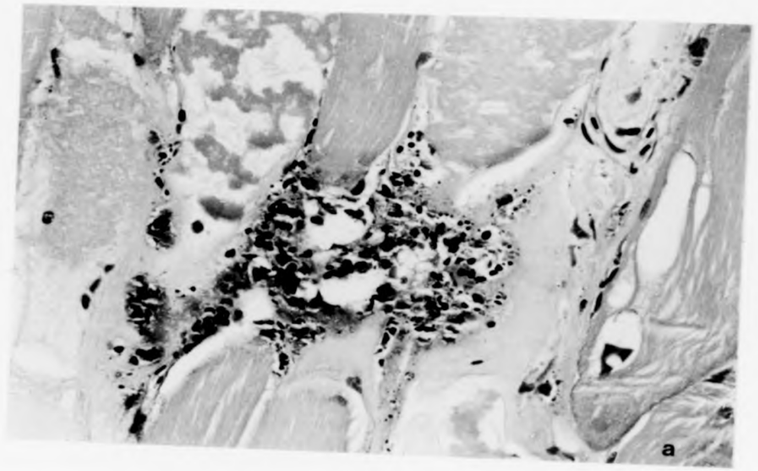
Group III - Fish inoculated with 0.1×10^{10} orgs/ml

Haemorrhages were seen between the muscle fibres at 2, 4, 6 and 24 hours post injection (Figure 42a, b). There was no evidence of chemotaxis before 48 hours when phagocytosis by macrophages was detected (Figure 43).

The bacteria appeared to be attracted to the periphery of the muscle fibres.

Group IV - Control Group - Fish inoculated with saline

No cellular response was detected although there was a little muscle necrosis (Figure 44a, b).



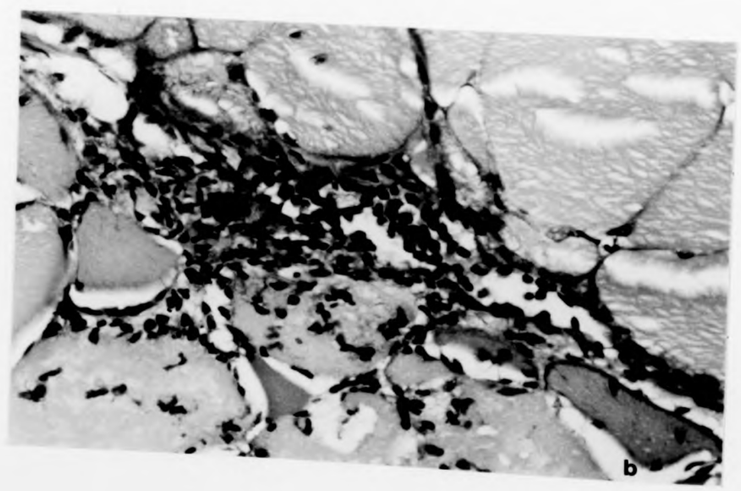


Figure 37 Experiment (a). Fish inoculated with 0.1 x 5 x 10^8orgs/ml

a) 2 hours p.i.

Small accumulations of inflammatory cells and muscle necrosis

Haematoxylin and Eosin x 406

b) 6 hours p.i.

Inflammatory lesion containing predominantly macrophages

Haematoxylin and Eosin x 406

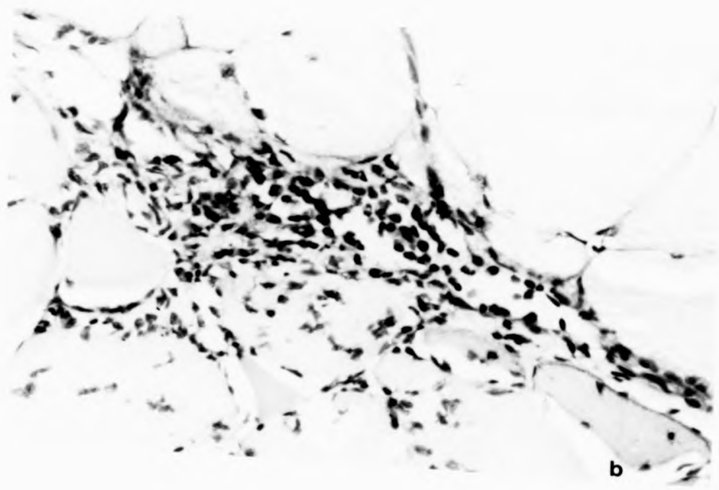


Figure 37 Experiment (a). Fish inoculated with $0.1 \times 5 \times 10^8$ orgs/ml

a) 2 hours p.i.

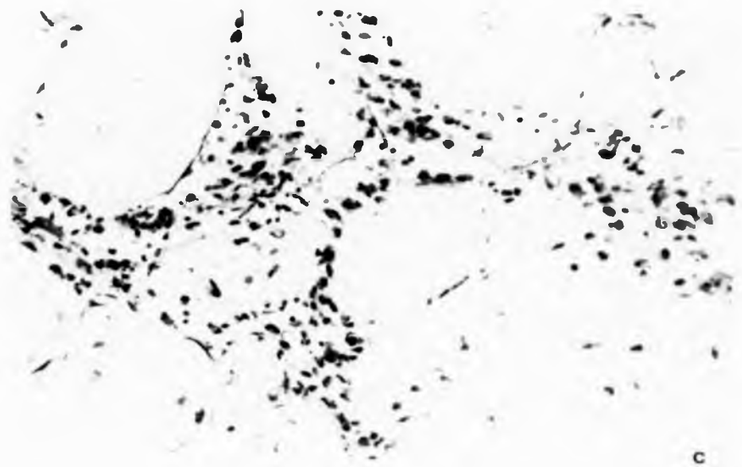
Small accumulations of inflammatory cells and muscle necrosis

Haematoxylin and Eosin x 406

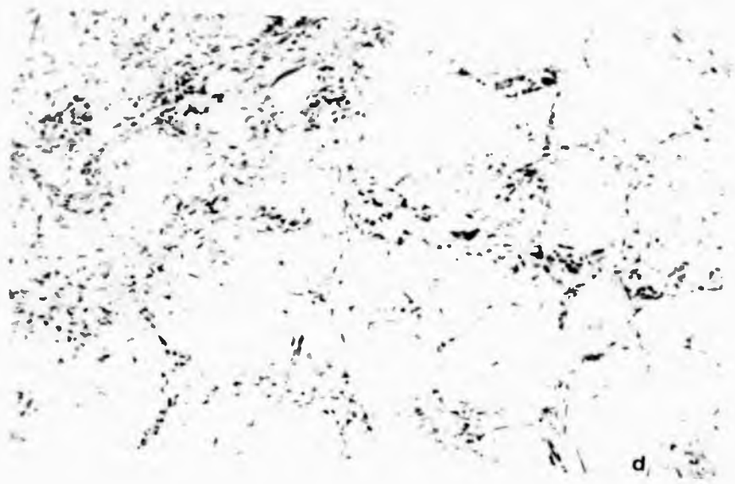
b) 6 hours p.i.

Inflammatory lesion containing predominantly macrophages

Haematoxylin and Eosin x 406



c



d

ure 37 Experiment (a). Fish inoculated with 0.1 x 5 x 10^8 orgs/ml.

c) 24 hours p.i.

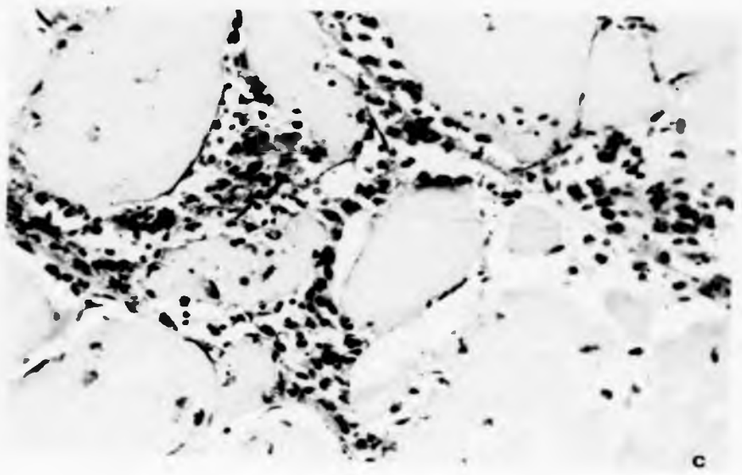
Muscle necrosis and loss of striations.
Note: bacterial phagocytosis by macrophages

Haematoxylin and Eosin x 406

d) 48 hours p.i.

Invasion of muscle fibres by macrophages. Widespread muscle necrosis

Haematoxylin and Eosin x 248



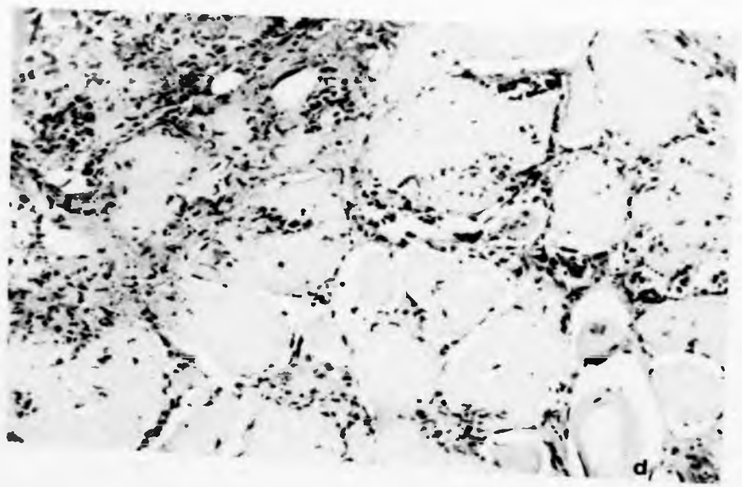


Figure 37 Experiment (a). Fish inoculated with $0.1 \times 5 \times 10^8$orgs/ml.

c) 24 hours p.i.

Muscle necrosis and loss of striations.
Note: bacterial phagocytosis by macrophages

Haematoxylin and Eosin x 406

d) 48 hours p.i.

Invasion of muscle fibres by macrophages. Widespread muscle necrosis

Haematoxylin and Eosin x 248

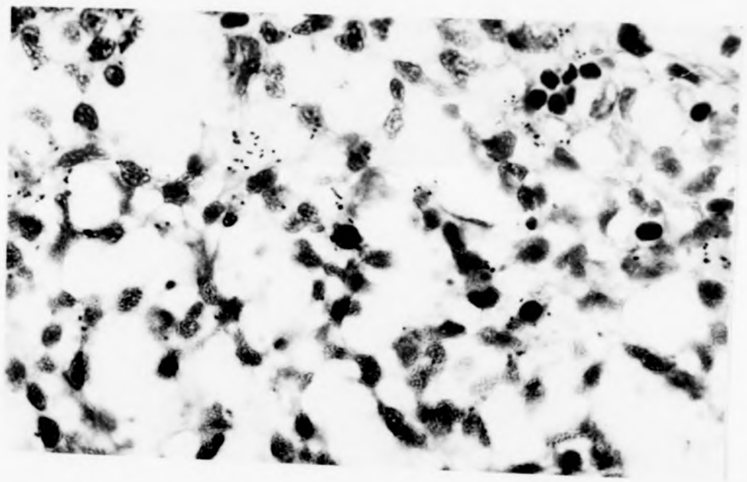
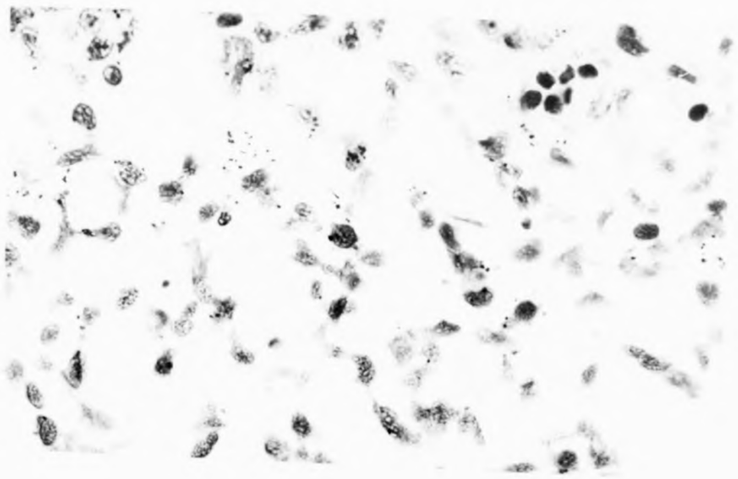


Figure 38 Experiment (a). Fish inoculated with $0.1 \times 5 \times 10^8$ orgs/ml.

Bacterial phagocytosis by macrophages demonstrated in inflammatory lesion

Haematoxylin and Eosin x 1578



ure 38 Experiment (a). Fish inoculated with $0.1 \times 5 \times 10^8$ orgs/ml.

Bacterial phagocytosis by macrophages demonstrated in inflammatory lesion

Haematoxylin and Eosin x 1578

a

b

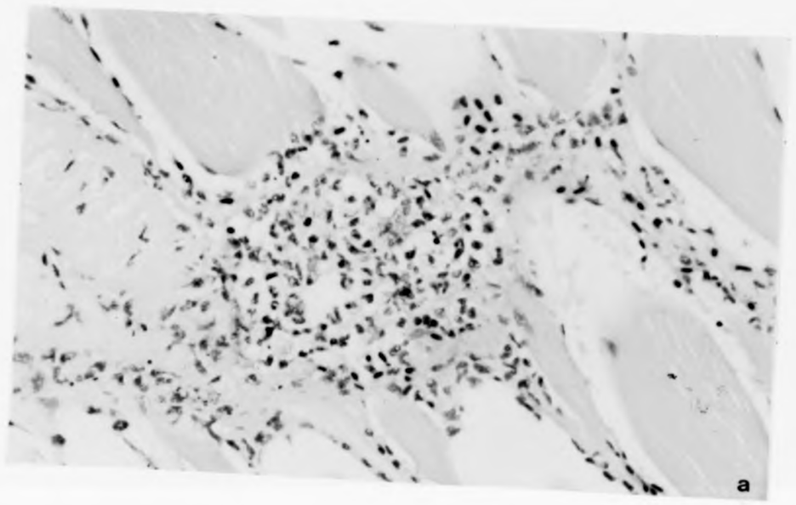
Figure 39 Experiment (b). Group 1 - fish inoculated with $0.1 \times 5 \times 10^8$orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

Inflammatory lesions containing predominantly macrophages. Few infiltrating polymorphonuclear cells.

Haematoxylin and Eosin x 360.



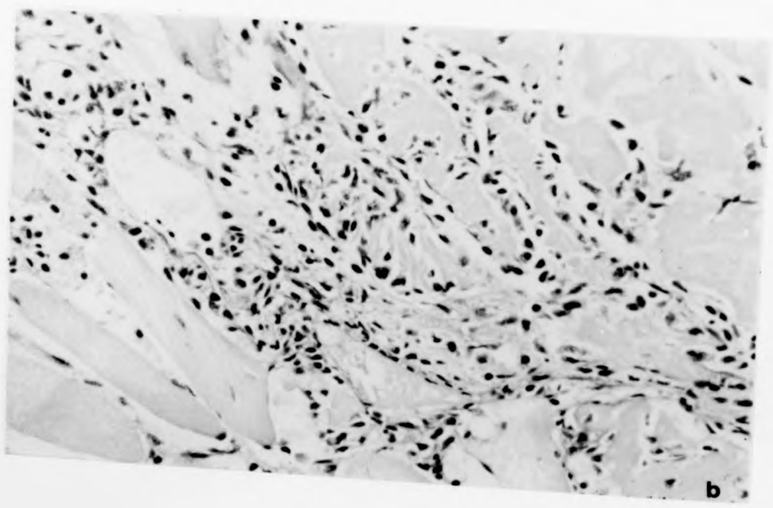


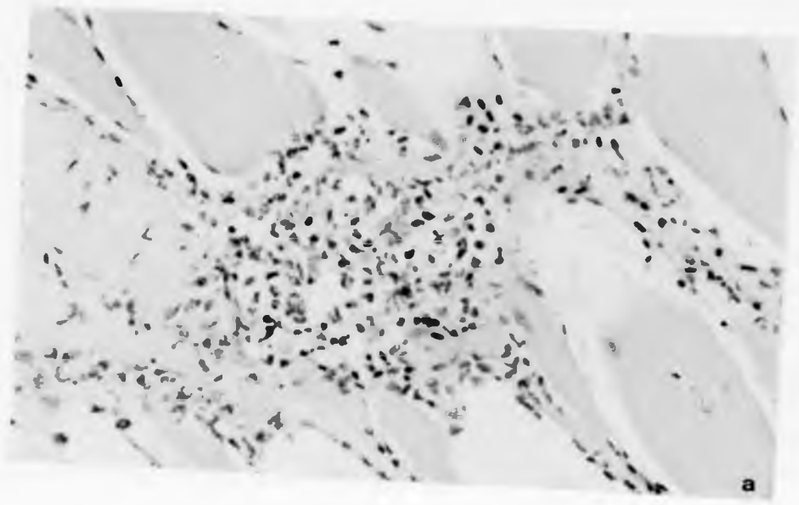
Figure 39 Experiment (b). Group 1 - fish inoculated with 0.1 x 5 x 10^8orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

Inflammatory lesions containing predominantly macrophages. Few infiltrating polymorphonuclear cells.

Haematoxylin and Eosin x 360.



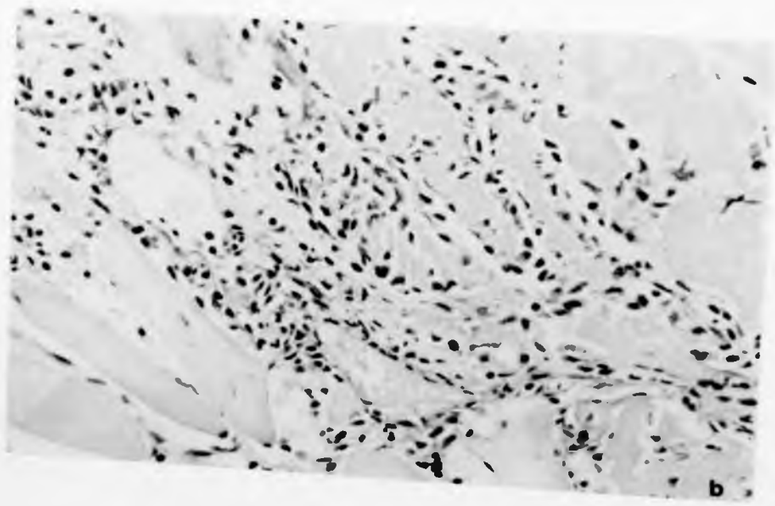


Figure 39 Experiment (b). Group 1 - fish inoculated with 0.1 x 5 x 10^8orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

Inflammatory lesions containing predominantly macrophages. Few infiltrating polymorphonuclear cells.

Haematoxylin and Eosin x 360.

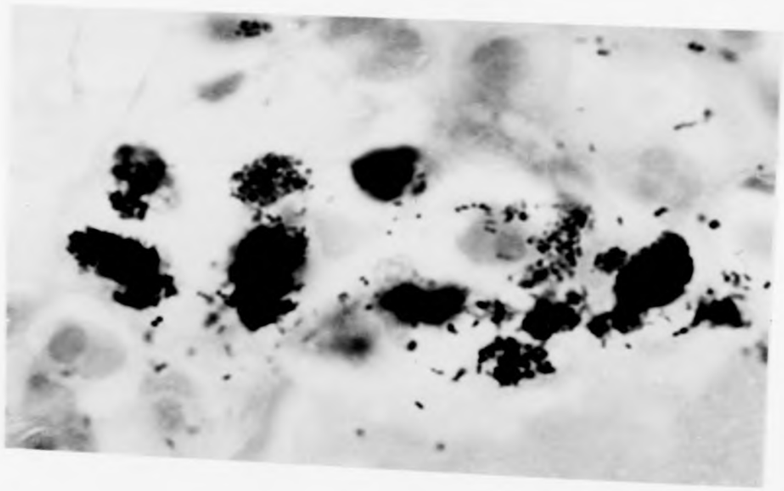


Figure 40 Experiment (b) Group I - fish inoculated with $0.1 \times 5 \times 10^8$ orgs/ml

Phagocytosis of R.salmoninarum by macrophage demonstrated in inflammatory lesion 48 hours post inoculation.

Haematoxylin and Eosin x 2158

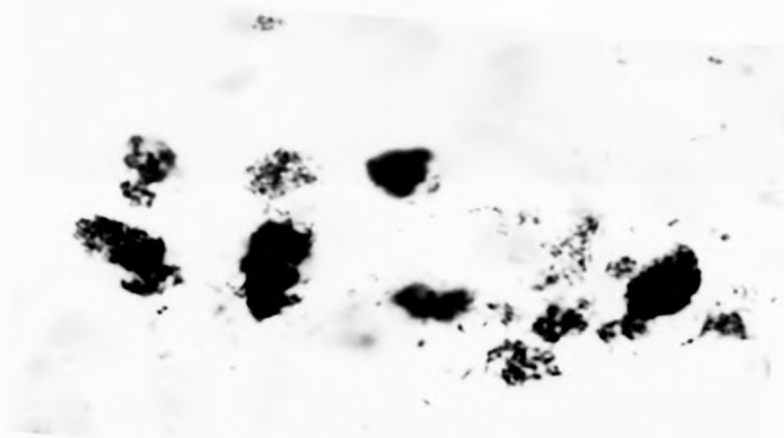


Figure 40 Experiment (b) Group I - fish inoculated with $0.1 \times 5 \times 10^8$ orgs/ml

Phagocytosis of R.salmoninarum by macrophage demonstrated in inflammatory lesion 48 hours post inoculation.

Haematoxylin and Eosin x 2158

a

b

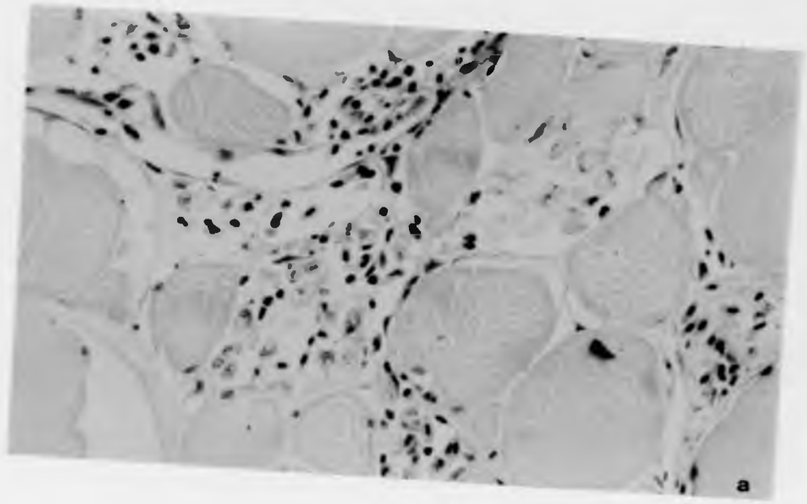
ıre 41 Experiment (b). Group II - fish inoculated with 0.1 x 10^9 orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

ow cellular response and reduced chemotaxis. Inflammatory lesion ıntaining predominantly macrophages.

aematoxylin and Eosin x 360.



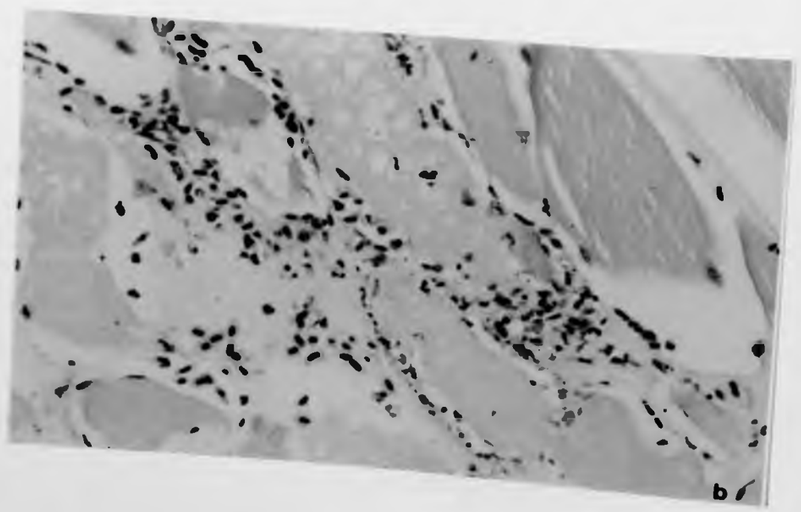


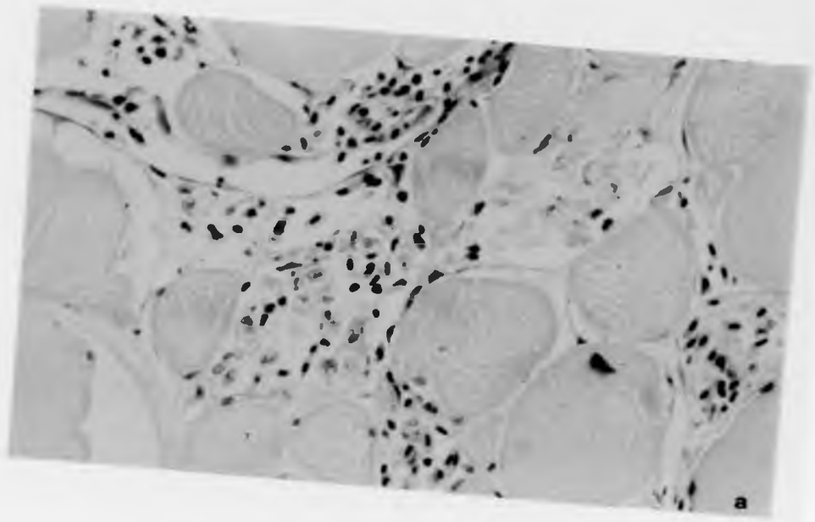
Figure 41 Experiment (b). Group II - fish inoculated with 0.1 x 10^9 orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

Slow cellular response and reduced chemotaxis. Inflammatory lesion containing predominantly macrophages.

Haematoxylin and Eosin x 360.



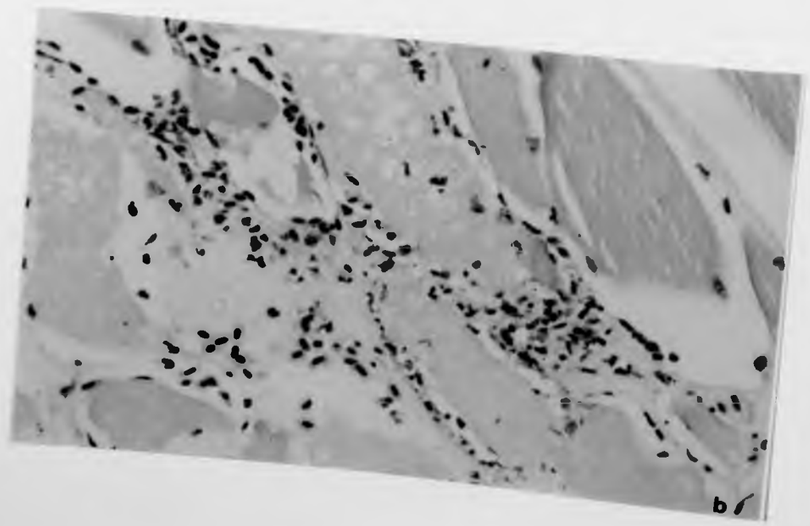


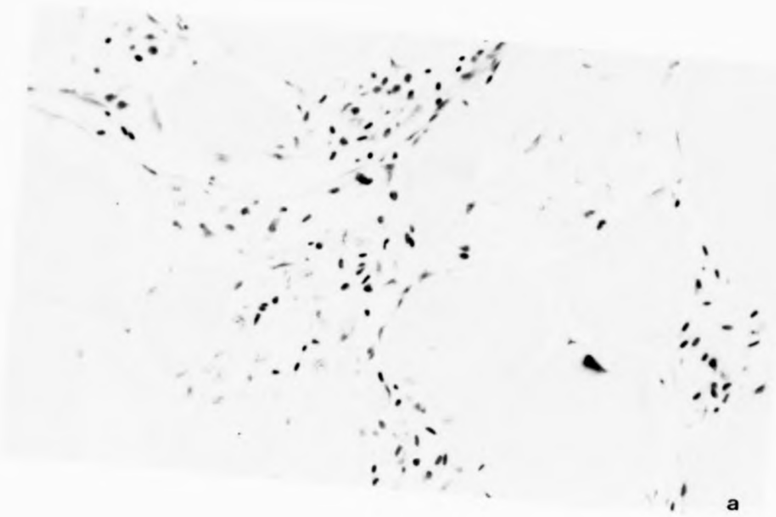
Figure 41 Experiment (b). Group II - fish inoculated with 0.1 x 10^9 orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

Slow cellular response and reduced chemotaxis. Inflammatory lesion containing predominantly macrophages.

Haematoxylin and Eosin x 360.



a

b

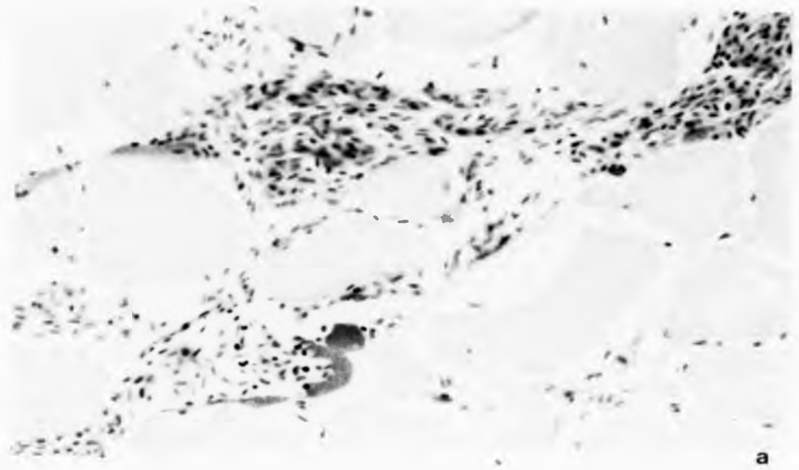
Figure 41 Experiment (b). Group II - fish inoculated with 0.1 x 10^9 orgs/ml.

a) 24 hours p.i.

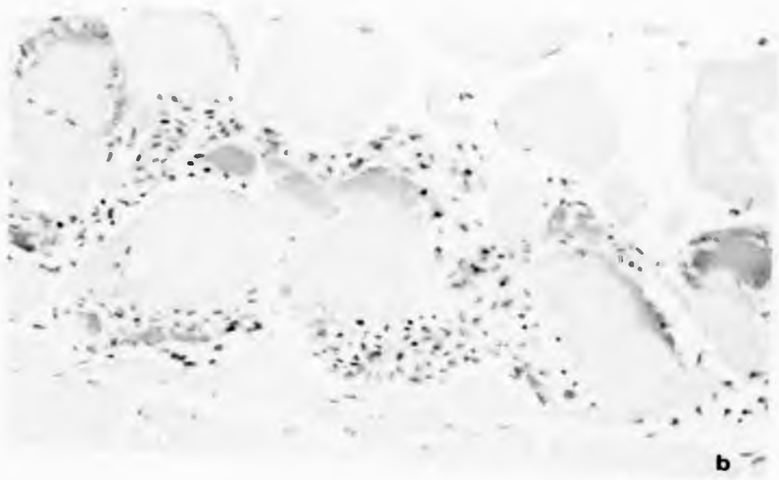
b) 48 hours p.i.

Slow cellular response and reduced chemotaxis. Inflammatory lesion containing predominantly macrophages.

Haematoxylin and Eosin x 360.



a



b

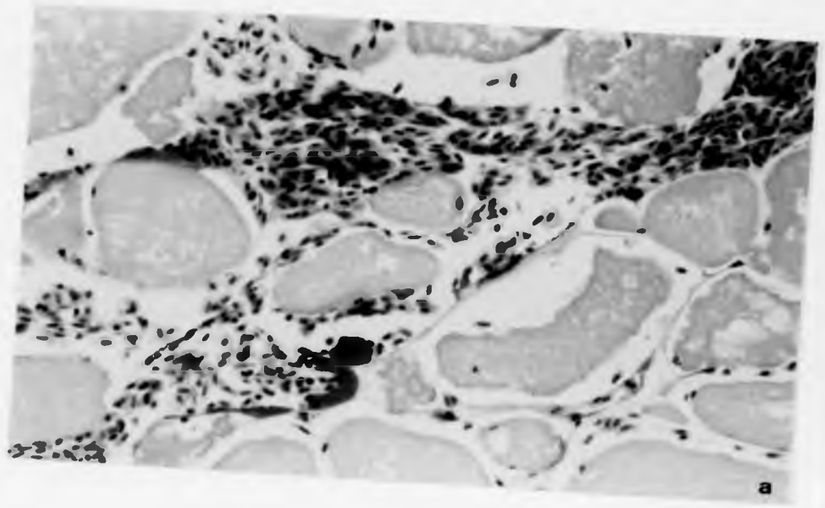
Figure 42 Experiment (b). Group III - fish inoculated with 0.1 x 10^{10} orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

Haemorrhages between muscle fibres and muscle necrosis. No evidence of chemotaxis before 48 hours when small numbers of macrophages and active phagocytosis seen.

Haematoxylin and Eosin x 360.



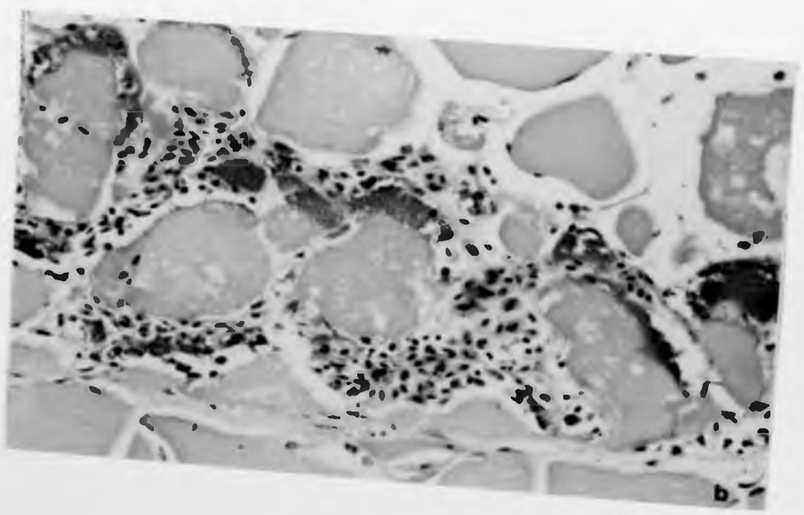


Figure 42 Experiment (b). Group III - fish inoculated with 0.1 x 10^{10}orgs/ml.

a) 24 hours p.i.

b) 48 hours p.i.

Haemorrhages between muscle fibres and muscle necrosis. No evidence of chemotaxis before 48 hours when small numbers of macrophages and active phagocytosis seen.

Haematoxylin and Eosin x 360.

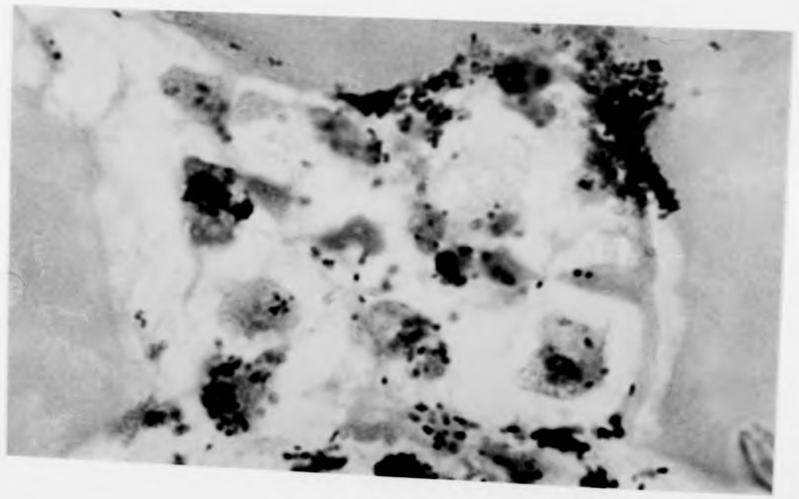
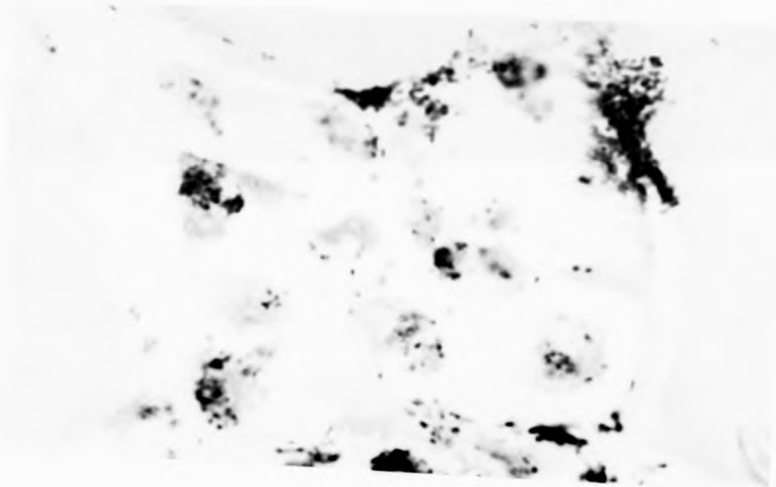


Figure 43 Experiment (b). Group III - fish inoculated with 0.1×10^{10} orgs/ml

Phagocytosis of R.salmoninarum by macrophages is clearly demonstrated at 48 hours p.i.

Haematoxylin and Eosin x 2158



Fic re 43 Experiment (b). Group III - fish inoculated with 0.1 x 10^{10} orgs/ml

Phagocytosis of R.salmoninarum by macrophages is clearly demonstrated at 48 hours p.i.

Haematoxylin and Eosin x 2158

a

b

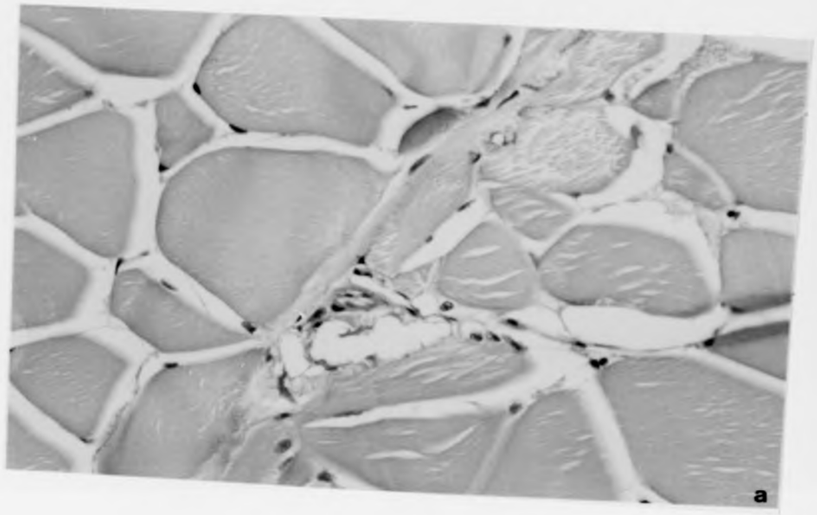
Figure 44 Experiment (b). Group IV - fish inoculated with saline

a) 24 hours p.i.

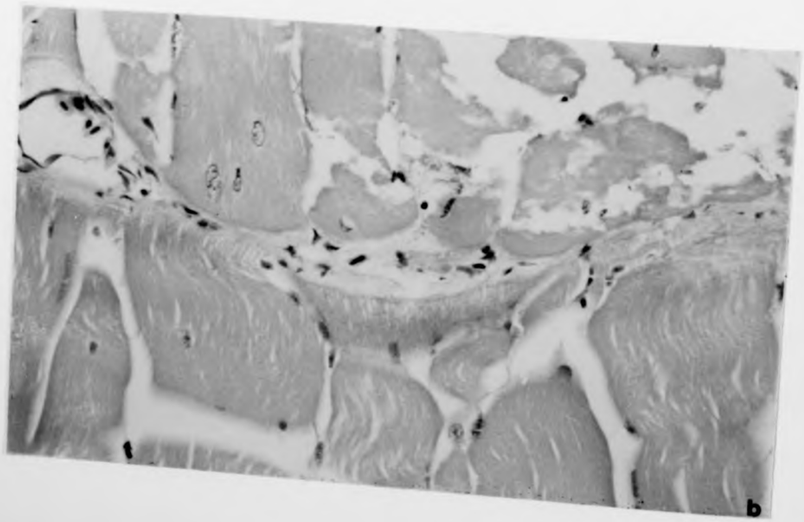
b) 48 hours p.i.

Loss of striations and muscle necrosis caused by trauma of injection.

Haematoxylin and Eosin x 360.



a



b

Figure 44 Experiment (b). Group IV - fish inoculated with saline

a) 24 hours p.i.

b) 48 hours p.i.

Loss of striations and muscle necrosis caused by trauma of injection.

Haematoxylin and Eosin x 360.

Section III

DISCUSSION

Although the pathogenesis of BKD is not fully understood, much work on various aspects of the disease have led to a greater understanding of the clinical, physiological and histopathological picture.

In the vertically transmitted infection the course of the disease has been described by Bullock et al. (1978) and Klontz (1978). It is known that organisms are found within and on the surface of the egg, and have a deleterious effect on the embryo and on subsequent hatching. An appreciable difference in growth rates between infected and non-infected groups of fish was noted by Bullock et al. (1978). Organisms were located in the posterior kidney as the fish grew, and Hendricks & Leek (1975) suggested that the post orbital tissues may also be predilection sites. Organisms have also been detected in the folds of the intestinal lumen. Because of the lack of any histopathological changes Klontz (1982) suggested that a type of immunotolerance may have developed as a result of infection during the embryonic stage.

Horizontal transmission has been demonstrated by many authors and the most common route of infection was by ingestion (Earp et al. 1953). The mechanisms whereby the organisms gain access through the intestinal tract and the course of the disease are unknown. Many bacterial infections are confined to the epithelial surfaces mainly because the host antibacterial defences do not permit further penetration, or because the organism does not possess an invasive mechanism. If these defences are impaired, as in debilitated, malnourished or immunosuppressed hosts, penetration of the intestinal epithelium, entry into the lymphatic system and systemic spread throughout the body will cause serious generalised infection.

Entry into the peritoneal cavity from the intestinal tract may take place as a result of injury, mechanical or toxin induced, or by focal infection causing epithelial degeneration. Mobile macrophages in the intestinal lumen may engulf pathogens, return to subepithelial lymphatic vessels and carry the organisms to other parts of the body. This phagocytic ability has been described in fish by several authors (Jakowska & Nigrelli 1953, Tetz, 1969, Stossel, 1975, Ellis, Monro & Roberts 1976, McKinney, Smith, Haines and Sigel, 1977 etc). Ellis et al. (1976) studied the phagocytic system and fate of intraperitoneally injected colloidal carbon in plaice (Pleuronectis platessa). They found that although some phagocytosis by peritoneal macrophages occurred, most of the carbon had apparently gained access to the circulation as free particles, and phagocytosis was performed by the ellipsoids of the spleen, the reticulo-endothelial (RE) cells throughout the haemopoietic tissue of the kidney and the RE cells in the intramuscular spaces of the heart. In view of the inert nature of carbon particles, the means by which they gained access to the circulation if not within phagocytic cells is questionable.

The results of this work, however, demonstrated the presence of scattered carbon particles within the ellipsoid walls of the spleen after one hour, and by the tenth hour aggregates of carbon were seen in macrophages of the ellipsoid walls and in free phagocytes in the pulp adjacent to the ellipsoids. Because of the absence of haematogenous phagocytes in the blood vessel lumens it was therefore assumed that the carbon particles were carried free in the blood and extracted by phagocytes resident in the ellipdoid wall.

McKinney et al. (1977) showed that in contrast to the mammalian phagocytic system, where the granulocytes play a predominant role, the phagocytic function in the gar Lepisosteus platyrhincus was performed by the macrophage-monocytic cell.

An important factor in the spread of R.salmoninarum is that, as a facultative intracellular bacterium it is able to resist destruction by phagocytes, and as such constitutes a member of an important group of pathogens. In mammals Salmonella typhimurium is also a successful facultative intracellular organism and this was demonstrated by Carrol, Jackett, Aber and Lowrie (1979), within mouse peritoneal macrophages.

Stressor agents, such as described in Section II, predispose to a clinically acute systemic disease which results in death of the host, or progresses to a chronic carrier state with infection contained within focal abscesses.

Information referring to the initial stages and course of BKD would be of great value for early diagnosis and for prophylaxis. Bacterial investigations directed towards target organs, sites of adhesions etc, would be particularly useful in the examination of clinically healthy fish which may be carriers.

Knowledge of the stages involved in the development of many infectious diseases had contributed to the formulation of successful vaccines and to their appropriate administration. Thus E.coli vaccines are now prepared to include the antigens which are known to be responsible for the adhesion of E.coli to the intestinal mucosa of piglets and calves. The K88 and K99 preparations are administered during pregnancy so that the

antibodies produced will protect the new born animal against E.coli diarrhoea before weaning. Nasalgen, a herpes virus vaccine is designed for use against infectious bovine rhinotracheitis (IBR) by topical application as a nasal spray to give effective protection against infection of this target organ.

In view of the complexity of host-parasite relationships, methods to study the distribution and fate of the organisms involved are essential to elucidate the progress of infectious disease processes. The first method used in this study consisted of conventional bacteriological and histological procedures to investigate the distribution of the organisms.

Distribution of R.salmoninarum following intraperitoneal injection

The i.p. route of transmission was chosen to follow the early course of the disease in view of the successful challenge experiments undertaken in Section II. Although these experiments do not represent the naturally aquired infection, the results demonstrated the distribution of organisms once they have entered the body.

a) Bacteriological Investigation

Using bacteriological techniques, organisms were found to localise in the liver and spleen at 4 hours post exposure. Examination of stained smears revealed that their numbers had markedly increased by 24 and 48 hours p.i. and many were contained within phagocytes. Isolation confirmed the viability of these organisms. Detection in smears of gut and swimbladder may be due to adhesion rather than localisation.

The lack of early involvement of the kidney is unexpected in view of the localisation in liver and spleen which most certainly occurred by means of the blood stream or lymphatic system.

The clearance of organisms from the peritoneal cavity by 24 hours p.i. suggests that the R.salmoninarum challenge is rapidly dispersed either directly into the lymphatic system, or indirectly after ingestion by macrophages. These results are not in agreement with the work by George, Ellis and Bruno (1982) who inoculated R.salmoninarum intraperitoneally into rainbow trout and found them to be extruded either free or within macrophages through the abdominal pores in the fish. Of other organs examined in their study, only the liver showed small numbers of Gram positive coccobacilli.

The first histological changes were seen in the liver at 24 hours p.i. These were small discrete focal infiltrations of mononuclear cells. Organisms were not detected by the IFAT, but control fish did not show these changes which are commonly found in the natural disease.

b) Whole Body Autoradiography

Whole body autoradiography was then used to examine the distribution of radioactive labelled bacteria throughout the body.

Whole body autoradiography was first introduced in 1954 by Ullberg. Since then the technique has been extensively modified and improved, and elaborate equipment developed for cryosectioning. The distribution pattern of various labelled compounds in the animal body was also studied. Interest in whole body autoradiography has rapidly been increasing, and the technique is much used in basic research and in the pharmaceutical

industry to study the detailed distribution of putative therapeutic compounds.

One of the advantages of the technique is the comprehensive information it provides. A general view of the distribution pattern throughout practically all the body can be determined. In sections taken at different levels of the body, most tissues and fluids can be studied. The labelled material may thus be studied in situ and not only in certain pre-selected tissue. This may reveal unexpected localizations and new perspectives regarding its mode of action.

The technique was applied in this particular study to determine the affinity of labelled R.salmoninarum for various tissues in experimentally infected fish and to reveal organism localisation. Whole body autoradiography samples were also taken for quantitative analysis in a liquid scintillator.

The results obtained by autoradiography did not fully agree with those using conventional microbiological methods. Labelled organisms rapidly reached the blood stream and/or the lymphatic system and were demonstrated in the autoradiograms. Concentrations of bacteria were visualised as "hot spots" adherent to the surface of visceral organs. Bacteriological examination of peritoneal washings confirmed the presence of large numbers of organisms in the peritoneal cavity at this stage and up to 24 hours p.i. The accumulation of activity seen in the liver, however, was not confirmed bacteriologically until 4 and 6 hours p.i. At 24 hours p.i. involvement of the kidney was seen and at 48 hours increased radioactivity in the gastro-intestinal mucosa was noted.

Unfortunately, the method employed made interpretation of the results difficult because of the inherent nature of the technique. The main problem encountered was to distinguish between the labelled bacteria under investigation and their metabolic products and the destruction of the organisms by defence mechanisms within the host which releases catabolic products. The lack of ^{35}S uptake in areas of high cartilage content as seen in the dark blue regions of the Alcian blue stained sections, suggested that a high percentage of the radioactivity of label attached to whole undegraded bacteria was a genuine measure.

The low uptake of radioactive label by organisms during culture also caused difficulty in assessing autoradiograms, because of low contrast. Bonventre, Nordberg & Schmiteslow (1960) found that high concentrations of organic compounds in growth media inhibited the incorporation of inorganic ^{35}S into Bacillus cereus. Further work to modify the medium employed for incorporation may resolve this problem.

Another problem encountered was the difficulty in cutting fish tissue which resulted in areas of section not being mounted onto tape and consequently not represented in the autoradiogram.

The variation between fish also presented difficulties for analysis of quantitative determinations and comparison with WBA results.

Consistently accurate results may only be obtained by using the same specimen for WBA and analysis by sampling pieces of tissue from the whole body sections. If larger tissue samples are required, they may be taken from the surface of the sectioned specimen. Compared with sampling during dissection at room temperature, specimens from cryosections could

have several advantages for quantitative analysis. The samples could be cut with greater precision since the specimen would be frozen and firm, and post-mortem changes and contamination of tissues would be avoided.

The route of administration had little influence on the qualitative distribution of radioactivity. However Ullberg (1977) suggested that in mammals, the intravenous route had advantages, because short time studies could be made and quantitative estimations of the autoradiogram would not be disturbed by variations in the route of absorption from the site of infection.

Despite the obvious shortcomings of these preliminary experiments, whole body autoradiography should not be dismissed. It is a very useful technique for studying the distribution of bacteria, and the initial stages and course of disease following artificial infection. Visual examination and tissue analysis provides a more accurate picture of the disease and important information regarding primary target organs. However, the problems of interpretation, outlined above, must be recognised when making a final assessment.

A combined assessment of the investigations described indicate a rapid dissemination of bacteria from the peritoneum followed by accumulation in the primary target organs, the liver and spleen. The spleen was found to possess intense phagocytic activity.

The kidney was another site of localisation but relatively small numbers were detected at 24 and 48 hours p.i.

It is interesting to note the involvement of the gut. Localisation was uncertain, but adherence of large numbers of bacteria were demonstrated in autoradiograms. Histological investigation involving a more comprehensive examination of serial sections at predetermined sites along the length of the gut might provide evidence of adherence or localisation.

Horne and Baxendale (1983) demonstrated adherence of Vibrio anguillarum in excised pieces of gut from sacrificed rainbow trout during the course of experimental vibrisis. They suggested that the gut may be an important site of bacterial multiplication in slowly developing infections of mature fish.

Tatner, Johnson & Horne (1984) recovered Aeromonas salmonicida from fore, mid and hind gut of rainbow trout following intraperitoneal injection. She suggested that the bacteria were probably adhering to the outer wall following flooding of the peritoneal cavity.

It may be speculated that sites showing the most active uptake of radioactivity are associated with involvement of the lymphatic system. The visceral lymphatic vessels surround all of the abdominal organs, the liver and spleen at the point of entry of the blood vessels, the gall baldder, swimbladder and the ventral surface of the kidney. Generally, all parts of the intestine are covered by a network of lymph vessels. Further studies are required to investigate this.

The cellular response to intramuscular injection

Because of the widespread nature of BKD, the apparently rapid spread of organisms and the histologically demonstrable areas of macrophage infiltration in many organs, the investigations were extended to include the role of the immune response and the cellular response to intramuscular infection.

The results obtained in experiments were in accordance with the results obtained by WBA and conventional bacteriology in terms of the speed of the cellular response and the rapid uptake of bacteria by macrophages.

A rapid progressive infiltration of inflammatory cells was observed. Macrophages predominanted and few polymorphs were seen during the first 48 hours.

A number of authors have studied inflammatory responses in fish to various irritants and bacteria. Mesnil (1895) reported that various types of mononuclear leukocytes in fish could phagocytose anthrax bacilli. Jakowska & Nigrelli (1953) observed initial infiltration of eosinophils around wounds in guppies injected with a Mycobacterium spp. They ingested these bacteria and were replaced after 2 days by macrophages.

The inflammatory response of fish skeletal muscle to heat killed Staphylococcus aureus at 10^{10}orgs/ml was studied by Finn & Nielson (1971a) in rainbow trout. They found that by 12 hours p.i. extravascular polymorphonuclear cells (PMN) were present at the periphery of the lesion, and after 24 hours PMN and monocytes (macrophages) were present in approximately equal proportions. The majority of bacteria had been

phagocytosed by 48 hours p.i. By 4 days the lesions were clearly circumscribed by fibrous perimysium; the PMN's exceeded the macrophages in number. Petechial haemorrhages were present between the muscle fibres. Subsequent resolution of the lesion by fibrous granulation followed. If the fish were maintained at 5°C, rather than 15°C there was delay in macrophage response, clearance of bacteria, appearance of necrotic tissue and fibroplasia (Finn & Neilson, 1971b).

The results of these studies indicated that while the inflammatory responses in fish compare closely with those in mammals, the speed and degree of cellular responses were considerably less.

The inflammatory responses to bacteria have been studied intensively in mammals. Once bacteria have invaded the body they usually produce and provoke powerful chemotactic stimuli which attract large numbers of phagocytic cells. The predominant feature in the early stages of acute pyogenic infections is oedema and a local polymorphonuclear influx followed by a rise in the blood neutrophil count. In more chronic infections and during resolution, mononuclear phagocytes and macrophages predominate. Both these cell types are derived from corresponding blood cells which adhere to the endothelium, emigrate across the vessel wall, and once in the tissue become mobile and phagocytose bacteria and cellular debris. Both respond by chemotaxis to invading and multiplying bacteria, but there may be certain differences in chemotactic responsiveness between them (Ryan & Majno 1977). For instance monocytes respond significantly to a neutrophil derived lysosomal cationic protein (Ward & Becker 1968).

Some viruses produce inflammation by the formation of antigen-antibody complexes. These are usually less potent than many bacterial metabolic products so that the initial inflammatory response is of a shorter duration and characterised by mononuclear cells.

The inflammatory responses are part of the defence mechanisms evolved to protect the host. Suppression of early related oedema and a related chemotaxis resulting in inhibition of polymorphs may promote the spread of infection. For example, virulent strains of Staphylococcus aureus produce a mucopeptide which suppresses early inflammatory oedema. A related staphylococcal factor inhibits the locomotion of polymorphs and macrophages (Easmon & Glynn 1980). Streptococcal streptolysins kill phagocytes and suppress polymorph chemotaxis at very low concentrations (Hirsch, Bernheimer & Weissman 1963).

The lack of polymorphs noted in the present study at the site of proliferation of R.salmoninarum indicates a lack of positive polymorphonuclear leucocyte chemotaxis or possibly destruction of these cells by toxin. In contrast, a fast and extensive macrophage response was demonstrated. In mammals the chemotactic mediators for macrophages differ from those which attract polymorphs. The experimental results indicate that in BKD the same principle applies and that a strong macrophage chemotactic substance is produced by R.salmoninarum which results in massive macrophage infiltrations in most organs. The dose response experiment revealed that an increased challenge produced a marked decrease in the cellular response. This indicated that R.salmoninarum produced negative chemotaxis at high dose levels which may inhibit or reduce an immunological respnse.

In many infections stimulation or suppression may occur at any one stage of the immune process. It would seem that severe challenge with R.salmoninarum may result in an immunosuppression.

There are a number of mechanisms which are recognised to be responsible for such phenomena. For example, some bacteria produce a substance which can desensitize or inactivate T and B lymphocytes. B (bursa of fabricius in birds or its mammalian equivalent) and T (thymus) dependent cells are both immunologically reactive. B cells are concerned with antibody responses and T cells largely with cell mediated immune (CMI) responses. Alternatively, some bacteria contain surface antigens which are so similar to those of the host cells that the immune responses are weak and a degree of tolerance results. This is known as molecular mimicry. The hyaluronic acid capsule of streptococci, for example, appears to be identical to a major component of mammalian connective tissue. R.salmoninarum may produce a surface component similar to fish cell antigens which allows it to reside within host tissues and evade the host defences. This possibility remains to be explored and could explain the chronic nature of BKD, particularly in wild fish. In nature, few outbreaks are recognised except in massive epizootics. The stress of intensification in cultured fish produces an acute, highly contagious form of the disease.

Facultative Intracellular Organisms

The circulating precursors of macrophages are monocytes which as soon as they migrate into the tissues become potential phagocytes. They are strategically placed throughout the body in the liver, spleen, lymph nodes, intestinal submucosa, peritoneal cavities etc. (Nelson, 1972) where their primary function in the mammal is to remove foreign particles and

destroy any invading bacteria, mainly in situ but sometimes after migration to the lymphatic nodes.

Within the phagocyte, the organisms are retained within a vacuole or phagosome and exposed to a battery of lysosomal enzymes which quickly kill and digest them. In most cases, free or "processed" antigens will then be released onto the cell surfaces and stimulate a series of secondary immune responses (Rosenthal & Shevach 1976).

The bacterial capacity of the macrophage is determined by the nature of the bacterial cell wall antigens; the presence of specific opsonins; the ratio of bacteria to phagocytes and their previous level of immunological activation (Ginsberg & Sela, 1976; Spitalny & North, 1981).

As a facultative intracellular bacterium, R.salmoninarum has the ability to multiply within host phagocytes, in vivo or possibly in vitro. The phagocyte may then transport the organisms throughout the body and even provide an environment essential for continual multiplication. A carrier state may thus be developed and localized foci become potential sources of active proliferation if environmental or other stress factors temporarily produce immunosuppression. However, as a stable carrier state persists, a state of premmunity will maintain resistance against further homologous challenge (Collins, 1971).

Facultative intracellular organisms of veterinary importance such as Mycobacterium tuberculosis, Brucella abortus and Listeria monocytogenes (Mackaness, 1964) when introduced into tissues by the intravenous, subcutaneous, intradermal, or intranasal routes are exposed to large numbers of phagocytes. In many cases the organisms survive and spread

via the lymphatic system to the blood stream and eventually colonise target organs such as the liver and spleen (Collins, Auclair and Mackaness, 1977) to induce a carrier state.

The composition of the bacterial cell wall is important in conferring resistance to intracellular digestion. Thus, cell walls of virulent mycobacteria and pathogenic corynebacteria contain high concentrations of mycolic acids (Barksdale & Kim, 1977). These large amounts of lipid and lipopolysaccharide within the cell wall protect against lysis by the lysosomal enzymes within the phagocyte. Carrol et al. (1979) believed that the success of S.typhimurium as an intracellular parasite was due to the LPS content in its cell wall.

The striking reduction in virulence in rough mutants of Salmonella, as discussed in Section I is also strong evdience of the importance of cell wall components.

R.salmoninarum does not contain mycolic acid, and yet still survives phagocytic destruction. The ability of some organisms to prevent the fusion of lysosomes to the phagosome is another mechanism by which virulent intracellular organisms, e.g. M.tuberculosis survives destruction (Hart, Armstrong, Brown and Draper, 1972). This may be one of the possible reasons for the intracellular survival of R.salmoninarum.

Many other factors may be involved in resistance to destruction by phagocytes, but they have not yet been adequately studied.

Further research on the precise phagocytic and bactericidal capacity of macrophages and the mechanisms of resistance by important veterinary pathogens including R.salmoninarum is required.

GENERAL DISCUSSION

The initial investigations of naturally infected rainbow trout by R.salmoninarum showed that culture were less reliable for diagnosis than the examination of smears especially by the IFAT. Since then however, growth inhibitory factors produced in kidney tissue have been recognised (Evelyn et al. 1981). These may be removed by dilution techniques to improve the sensitivity of examinations by culture.

Repeated in vitro subculture of some strains revealed an apparent transition from a smooth translucent colony type to a rough dry form. Organisms from rough and smooth colonies were examined to determine whether any other significant differences could be detected. By electron microscopy examination and in immunodiffusion tests no changes in morphology or immunology were found, although a slightly decreased virulence was shown by the rough strain. However, this may have been due to individual variation between fish as similar macroscopic lesions were observed regardless of which strain had been used.

Further work is required to examine this so-called smooth-rough transition in particular with reference to possible loss of virulence. The development of an avirulent or less virulent strain could be particularly important for control by vaccination in view of the problems in therapy of BKD.

The comparison of the R.salmoninarum strains and with C.pyogenes, as regards cultural, biochemical, precipitin and cell wall properties showed that KD strains form a closely homogeneous group. The amino-acids of the peptido-glycan cell wall layer correlated with those first described by Sanders and Fryer (1980) and suggestions of a possible relationship between R.salmoninarum and C.pyogenes can be disregarded.

A reliable standard challenge system has been established by intraperitoneal injection of R.salmoninarum into rainbow trout. The macroscopic and histological changes produced when recent isolates were used were identical with those of the naturally occurring BKD.

It will now be possible to test the efficacy of vaccination, and to examine protection against R.salmoninarum.

Detection techniques used to monitor disease in the experimentally infected fish were evaluated. Culture methods frequently failed to detect R.salmoninarum in seriously diseased fish, most commonly because the plates were overgrown by contaminants. The use of selective media as described by Laidler (1983) should prove valuable for screening large numbers of fish despite the inconvenience of the time involved for preparation of homogenates.

IFAT gave consistently better results than the other techniques employed in these studies. But, difficulties in interpretating the findings have been recognised by Evelyn et al. (1981) especially in samples from covertly infected fish. A combination of IFAT and culture is recommended to resolve such problems.

It must also be emphasised that, particularly to detect R.salmoninarum carriers, more than one organ should be examined, and that histological examinations often reveal early pathological changes before clinical signs of disease can be detected.

To provide greater understanding of the progress of development of the disease the initial distribution and fate of challenge organisms were studied in greater detail. It was found that viable free and intracellular organisms were rapidly cleared from the peritoneal cavity into the liver and spleen as primary target organs, and subsequently localised in the kidney.

These results highlighted one of the significant problems associated with this disease, namely that of intracellular survival. The histological characteristics of both the natural and artificial disease were of major macrophage involvement in most organs examined. Following intramuscular injection of R.salmoninarum there was rapid infiltration by predominantly mononuclear cells which subsequently phagocytosed many of the organisms.

The important role of macrophages in the initial and later stages of BKD has clearly been shown. Whilst growing within phagocytes, the R.salmoninarum are protected from the activity of extracellular factors such as complement, specific antibody and antibiotics, so that balanced infections develop in which the host parasite eventually co-exist. A small residual infection may persist within parenchymal cells and develop the carrier state so common in wild salmon. Being impossible to detect clinically it is therefore difficult to control.

Eradication of BKD from salmonids would appear to be unlikely because of the problems outlined above. Control by vaccination or chemotherapy has been investigated by many workers with only partial success.

Treatment of BKD with erythromycin chemotherapy has been the most successful to date. Administration by feeding was introduced by Wolf and Dunbar (1959) and is still practised by many hatcheries in the USA, but is generally restricted to presmolting salmon and trout populations that have a history of clinical BKD.

Major outbreaks of severe disease in pacific salmon broodstock led to the use of erythromycin phosphate administered subcutaneously at 11mg per kg body weight (Holt, Smith, Zakel and Maher, 1979). A condition called "bronzing" considered to be a liver abnormality, which may reduce its capacity to metabolise erythromycin, was noted in 4% of infected fish.

Treatments of vertically transmitted disease and of carriers of R.salmoninarum are still being explored. Bullock et al. (1978) showed that BKD is egg transmitted and that disinfection with organic iodine will reduce severity, but does not eliminate transmission. Groman (1979) suggested that iodophors added as water hardening agents to replace erythromycin phosphate therapy or a combination of multiple pre-spawning injections with erythromycin phosphate, iodophor water hardening, as well as prophylactic feeding and/or bath treatments of suspect pre-smolting populations should be investigated.

A useful vaccine to prevent widespread losses of economic importance has yet to be developed.

As R.salmoninarum is a facultative intracellular oganism protection against infection would require a cell-mediated immune response involving T-cells and immunologically-activated macrophages. Killed vaccines generally are less effective against such organisms than living attenuated vaccines, although killed vaccines may induce specific opsonin production and some non-specific stimulation of the resident macrophage population. When these responses are combined, some protection may be achieved. Examples of this have been reported by Jenkin and Rowley (1963) against intraperitoneal challenge with Salmonella and Brucella sp.

Research into killed R.salmoninarum vaccine has had only limited success. Paterson et al, (1981) showed that a single intraperitoneal injection of killed R.salmoninarum emulsified in Freund's complete adjuvant was not sufficient to protect in underyearling parr after two years when the fish had become smolts, despite high humoral agglutinating responses. Overt infection was, however, reduced considerably by inoculating post yearling parr Atlantic salmon with one dose of this vaccine.

Greater success was reported by McCarthy, Croy & Amend (1984) using fermenter grown formalin-inactivated R.salmoninarum without adjuvant under laboratory conditions. They found that single intraperitoneal injections of bacteria, lysed by adjusting the pH from 6.0 to 9.5 with 10N NaOH, then readjusting back to 7.2 with 10 NHCl, induced most protection. Typically 80% or more of unvaccinated controls became infected, 10% or less of the vaccinated fish were affected.

This work is encouraging, and suggests that lysing the cells released otherwise masked protective antigens and induced a protective response. Further work to obtain efficacy data against the natural disease is required.

Cell fraction vaccines have been employed experimentally against several facultative intracellular bacteria. Most of these have consisted of cell wall components, but some consist of ribosomal fractions and have been used effectively against experimental tuberculosis (Youmans and Youmans, 1972) and salmonellosis (Vennemah, Bigley, Klun, Zackery & Dodd 1968).

Clearly, many factors influence the incidence of this disease and although effective control by chemotherapy and vaccination is desirable improved management and feeding practises would do much to control the disease on many fish farms.

Future Developments in Control

Variations in the expression of this disease are governed by complex inter-relationships between the host, the parasite and their external and internal environments. The host is predisposed to disease by inadequate control of its environment especially under intensive conditions if the fish are improperly handled and exposed to unfavourable water conditions so far as temperature, pH, oxygenation etc. are concerned.

If the fish are subjected to such stress over prolonged periods in the presence of R.salmoninarium BKD will follow. If the predisposing factors can be avoided outbreaks of the disease will be controlled. If this is

practical, it is clearly the method of choice. But the inherent difficulties in control by management, and if this fails the inadequacy of current antibacterial therapy, emphasise the need for immunological control.

However, the intracellular distribution of R.salmoninarium and their ability to survive even within phagocytic cells enables them to elude normal immunological defence mechanisms. The mechanisms by which organisms survive intracellularly are worthy of future research, and examination of cell wall components might elucidate the role of various virulence factors.

Most of the immune responses recognised in mammals are also shown by fish. There is however evidence of quantitative rather than qualitative deficiencies which are probably due to lower body temperature and optimum response is determined by the optimum temperature for the species. Studies on fish immunity have largely been concerned with humoral mechanisms, but cell mediated responses are now recognised to have an important if not primary role in fish defences. Cell mediated responses to live vaccines, mediated by T-lymphocytes and activated macrophages are more likely to protect against R.salmoninarium than those produced by killed vaccines.

The present study suggests vaccine development may require particular attention to the following aspects.

1. Further examination of the smooth-rough transition after repeated in vitro subculture. To achieve progressive reduction of virulence without loss of immunogenicity and provide the basis for an affective live attenuated vaccine.

Problems associated with such a product could be in maintaining adequate stability and in distinguishing BKD carriers from vaccinated fish by the IFAT.

2. Investigation of a cell fraction vaccine.

The results of the immunocytochemical tests support attempts to extract and examine cell wall components, cell fractions or purified cell walls as potential cell fraction vaccines. In mammals, some such vaccines are associated with the development of delayed type hypersensitivity reactions.

3. Investigation of antibody dependent cell mediated cytoxicity reactions.

Macrophages are involved in delayed hypersensitivity reactions in mammals and higher vertebrates. A specific inflammatory reaction occurs on secondary exposure to an antigen after primary sensitization. Macrophages are prevented from leaving the site of antigen challenge by lymphokines released by T cells : Macrophage Migration Inhibition Factor.

Sufficient evidence to show that delayed hypersensitivity occurs in at least some fish species has been reported by several authors.

The present study has demonstrated that rainbow trout show a strong inflammatory reaction following exposure to R.salmoninarium. Evidence for a more specific delayed hypersensitivity reaction of the kind seen in mammals may be more difficult to obtain. The Migration Inhibition Factor test (M.I.F) is an in vitro test of delayed hypersensitivity and might be employed. It has been used to screen

rainbow trout for resistance to furunculosis after immunization by various methods (Smith, McCarthy and Paterson, 1980). The percentage of fish producing a positive cellular response against the natural challenge strain of Aeromonas salmonicida corresponded very closely with the percentage found to be resistant to furunculosis in vaccine field trials.

4. Genetic engineering methods could make possible the stable deletion or insertion of mutations (such as virulence factors) at suitable sites to confer irreversible attentuation on bacterial, viral, protozoan and metazoan parasites. Application of such techniques to fish pathogens might be a major leap forward in the development of new vaccines.

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