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Research Completion Report*

CULTURE AND DETECTION OF THE BACTERIAL KIDNEY DISEASE AGENT

A RESEARCH REPORT AND REVIEW

by

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prepared for

Great Lakes Fishery Commission

August, 1988

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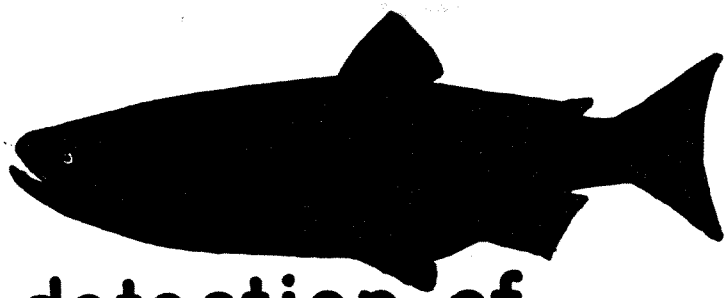
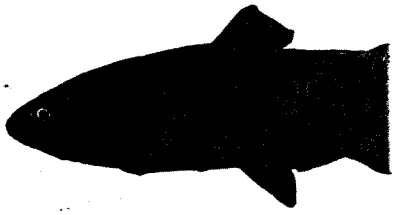


Culture and detection of the Bacterial Kidney Disease agent.



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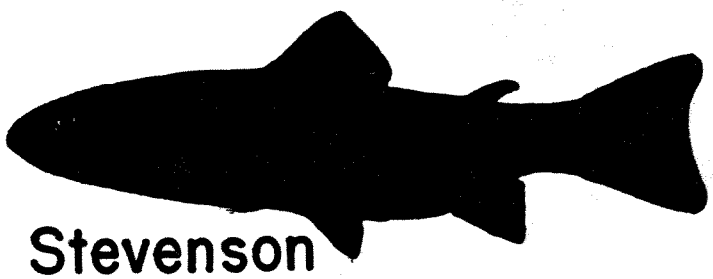

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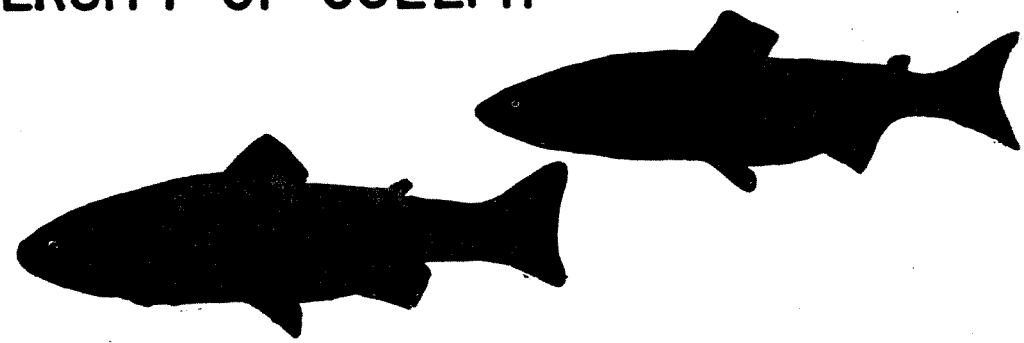

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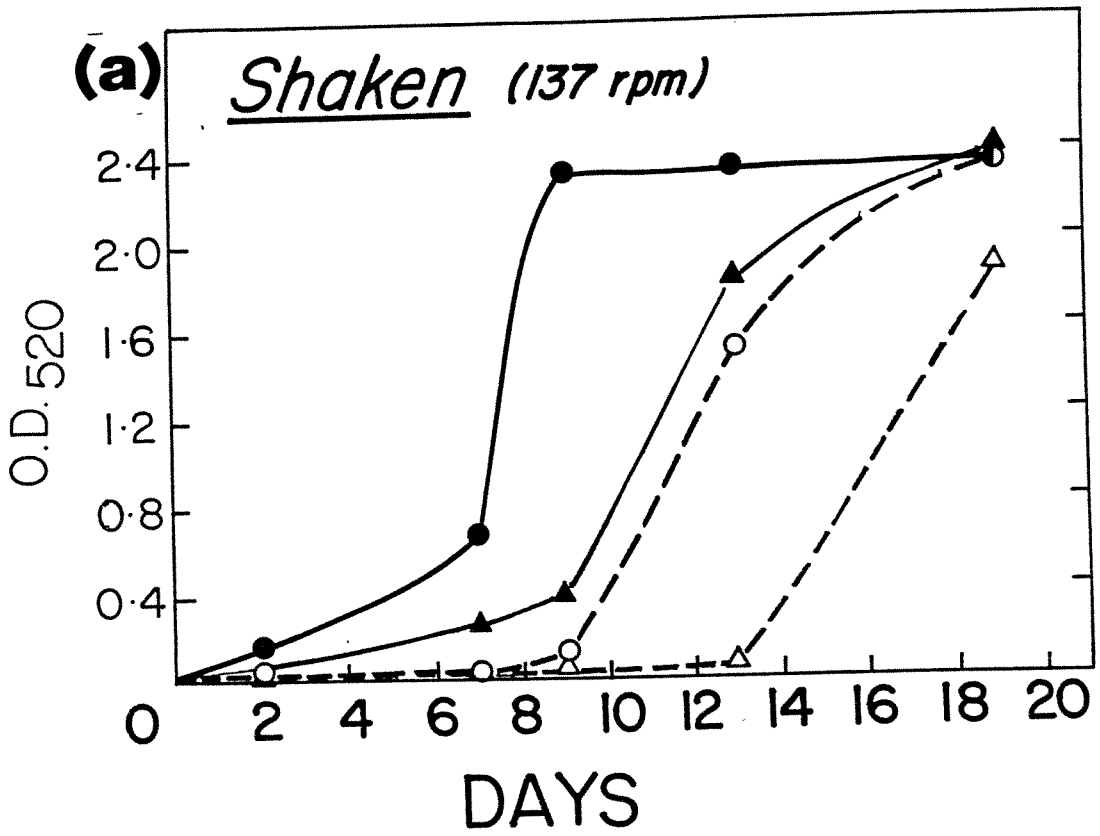
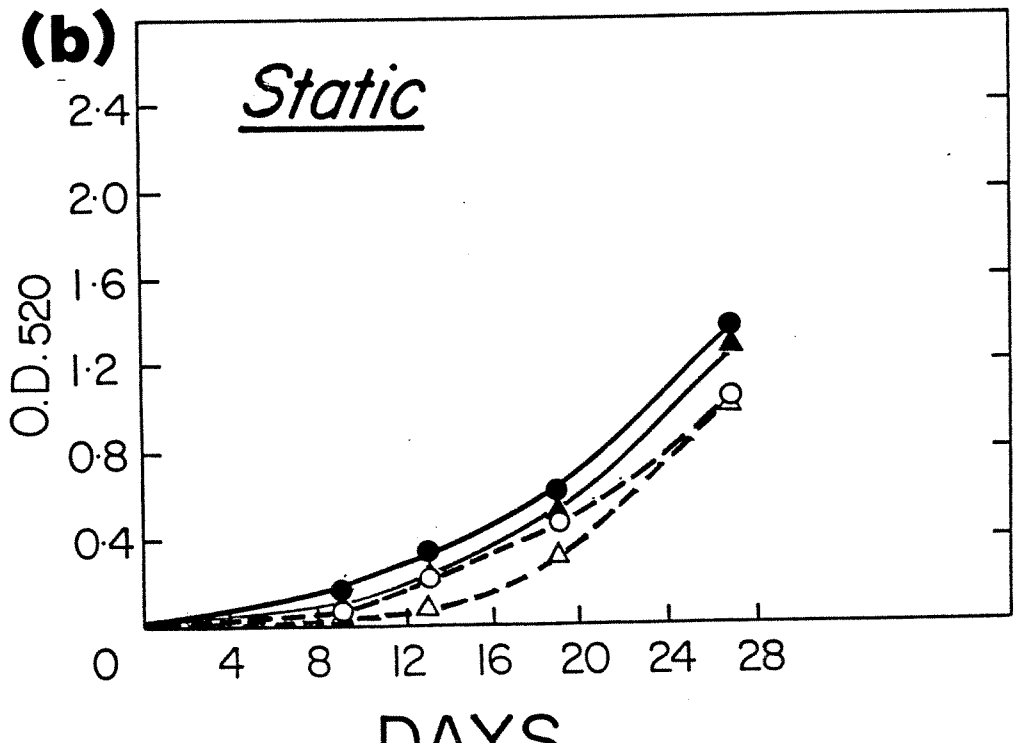
FIGURE 1. Growth of R. salmoninarum in broth culture, with and without added charcoal. (Black symbols indicate cultures with charcoal added to the medium.) Flasks were incubated with shaking (a) or as static cultures (b). Circles indicate tests with R. salmoninarum ATCC 33209, a high-passage strain; triangles indicate tests with Coho, a passage-2 strain from diseased fish. (Results shown are from a single, representative trial.)

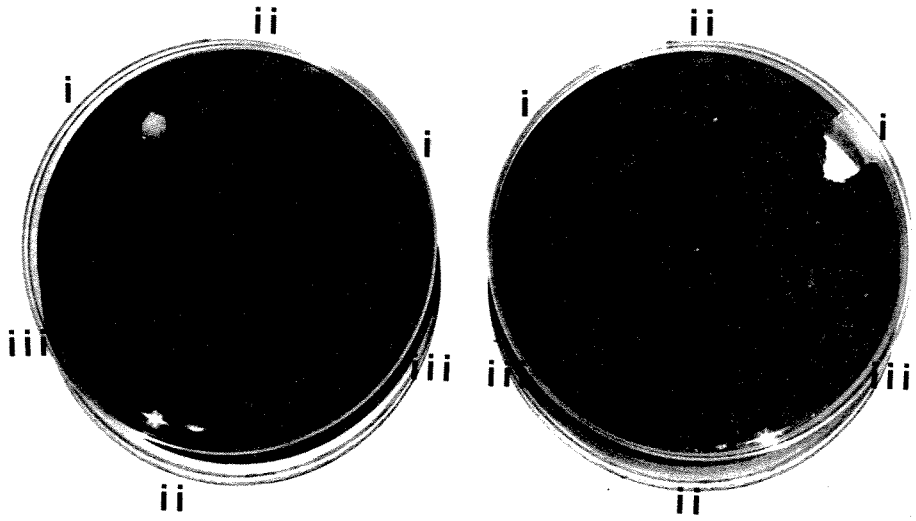




3.2.4 Detoxification by spreading dilution: When rainbow trout tissues were examined on the agar plates, it appeared that the toxicity was reduced when the spot inocula were more spread out. The bacteria appeared to grow well at the outer edge of the spot-inoculum. This suggested that the toxicity might be reduced by actively streaking out the inocula, in effect, doing an in situ dilution of the tissue.

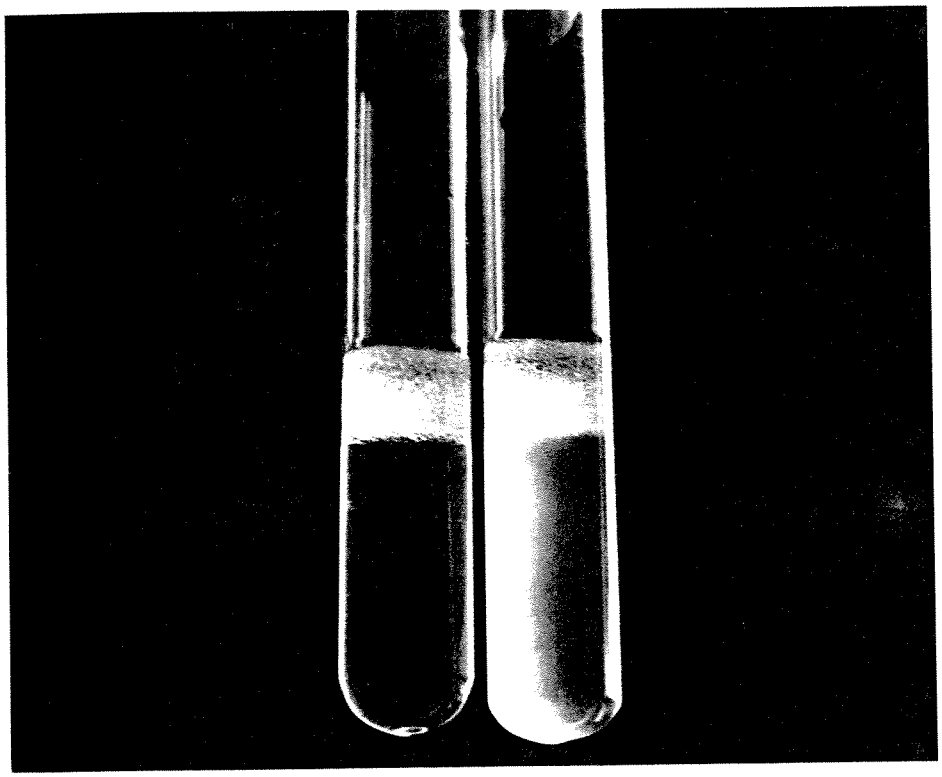
Procedure and results: The 25 µl spot inocula were spread with an inoculating loop, so that approximately 1/6 of the plate was covered per spot. With both liver and kidney tissue, this procedure did allow growth of the bacteria at the 50% dilution, where it would otherwise be inhibited (Fig. 2).

FIGURE 2. **Growth on KDM-C agar from dilutions of rainbow trout kidney homogenate seeded with Renibacterium salmoninarum.** For the plate on the left, drops were allowed to absorb to the agar directly where placed; for the plate on the right, duplicate drops were spread out with a sterile loop. The dilutions, in duplicate, are: (i) 1/2, (ii) 1/4, and (iii) 1/8. (A contaminant appears at the top of one plate; a similar one has been excised from the second plate to prevent overgrowth during prolonged incubation.)



Results: The attempt to selectively extract and concentrate bacterial cells from tissue homogenates failed, largely because of the excessive amount of tissue fats and debris present. Additional extraction steps to improve separation do not appear to be feasible as the cells may loose viability and/or antigenicity from the hexadecane treatments or from extra washing. This approach to detection was abandoned.

FIGURE 3. Extraction of cells of R. salmoninarum into hexadecane overlay. In the left tube, the hydrophobic cells have been concentrated in the hexadecane phase; in the right tube, the bacterium cells had previously been stripped of the haemagglutinin by boiling, and remained in the aqueous phase.



CULTURE AND DETECTION OF THE BACTERIAL KIDNEY DISEASE AGENT

A research report and review

Prepared for the **Great Lakes Fishery Commission**

August 1988

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ABSTRACT

Renibacterium salmoninarum is the agent responsible for bacterial kidney disease of salmonid fish. This research project examined potential improvements that could be made in the methods for growth and detection of the bacterium, and investigated aspects of pathogenesis and therapy.

Charcoal has been found to be a suitable replacement for serum in the medium used to grow R. salmoninarum. This medium was used to demonstrate that the organism has a stereospecific requirement for L-cysteine. Precursors such as methionine could also support growth, but to a lesser degree. Charcoal (or serum) is required for adequate growth on agar, but it can be omitted from broth medium if the culture receives adequate agitation. This would allow R. salmoninarum to be grown in fermenter culture for vaccine studies.

If culture is used as a detection method, it is necessary to wash and dilute the fish organ samples to reduce toxicity effects of the tissues. We were not able to replace this step by direct addition of charcoal to the tissue homogenates. With rainbow trout samples, toxicity of unwashed homogenates could be reduced if drop inocula were spread over a larger surface area with a sterile loop. Attempts to concentrate bacterial cells from samples by hydrophobic phase-separation with hexadecane were hampered by the large amounts of fatty material and tissue debris released by homogenization.

Macrophages could be separated from kidney homogenates of naturally-infected coho salmon by Percoll gradient centrifugation, and R. salmoninarum could be cultured from these preparations. This provides a potentially useful procedure for enriching samples for BKD detection. R. salmoninarum cells can be seen within macrophages maintained as in vitro cultures. A critical observation with respect to detection methodology is that R. salmoninarum cells are not readily visible by Gram-stain or FAT stains if the slides have been heat-fixed. Methanol or acetone fixation should be used in order to de-fat the membranes of cells containing the bacteria. Macrophages loaded with R. salmoninarum may be useful for testing efficacy of antimicrobial agents. For this intracellular pathogen, these results could be more informative than those from in vitro assays by disc or agar dilution methods. These loaded macrophages can be readily prepared by intraperitoneal injection of fish. It was also possible to infect fish per os. This route may resemble natural infections more closely than injected challenges, but the response is much slower to become apparent.

Immunofluorescent antibody techniques, IFAT and DFAT, appear to remain the most convenient, rapid and reliable detection methods for BKD. ELISA procedures are sensitive and promising but still require evaluation of their use with fish samples. Information about the nature of the major surface antigen(s) of R. salmoninarum will be critical for preparing specific diagnostic serum and designing methods for processing samples before examination.

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1.0 INTRODUCTION

1.1 Background: Bacterial kidney disease is a significant problem in salmonid aquaculture, and is a matter of concern in fish health control. The etiological agent of BKD is Renibacterium salmoninarum, a slow-growing Gram-positive bacterium. The slow growth presents special problems for detection methodology and, in general, for studies of the disease and its control.

The ability to grow R. salmoninarum effectively in laboratory culture is pivotal to many approaches to controlling bacterial kidney disease. First, cultural methods have been shown to be very sensitive means of BKD detection, although tedious and slow. Second, development of useful BKD vaccines will depend on ready availability of antigen for immunization and challenges. Finally, at a very basic level, growth and study of the organism in the laboratory is necessary to design of refine approaches to therapy and prevention of disease.

1.2 Objectives: This report considers research work carried out at the University of Guelph, between November 1985 and November 1987, supported by a grant from the Great Lakes Fishery Commission. The results of specific studies are presented, and put into perspective by the accompanying review of the relevant literature.

The stated objectives of the work, initially proposed in the summer of 1984, were as follows:

(1) To analyze the factors which enhance or inhibit the growth of R. salmoninarum in culture;

(2 To compare and improve upon the present methods for detection of R. salmoninarum in fish by culture technique;

(3) To assess, in vivo as well as in vitro, the most effective antimicrobial therapy for bacterial kidney disease.

Changes were made from the initially stated objectives in that funding for the project was not confirmed until late in 1985. During that time, some further information about aspects of the projected work became available in the published literature, making duplication unneccessary. Thus, for example, we did not carry out extensive in vitro tests of antibiotic susceptibility as Austin (1985) published an exhaustive summary of his antibiotic test results. For the purposes of this report, we have included brief reviews of the current status of some aspects of BKD research as well as reports of our own findings in studies sponsored by the Great Lakes Commission. These sections should provide an overview of some recent progress in BKD research.

1.3 An update on Renibacterium salmoninarum.

1.3.1 Taxonomic position: The name Renibacterium salmoninarum was applied to the bacterial kidney disease bacterium by Sanders and Fryer (1980), following a brief taxonomic appearance as "Corynebacterium salmoninus". Recent analysis of the phylogeny of R. salmoninarum by 16s ribosomal RNA cataloging suggests that it is most closely related to members of Arthrobacter and Micrococcus (Stackebrandt et al. 1988).

1.3.2 Uniformity of isolates: Strains of R. salmoninarum gave very similar reactions in biochemical tests, regardless of the geographic location or host species from which they were isolated (Bruno & Munro, 1986). The chemical composition of the cell envelope also appeared to be consistent for different isolates (Fiedler & Draxl, 1986).

Table 1. Media used for growth of Renibacterium salmoninarum.

Medium	Comments	Reference
KDM-2	serum in a peptone-yeast extract base, with cysteine	Evelyn, 1977
MH with cysteine	base Mueller-Hinton agar contains starch; cysteine added	
KDM-C	charcoal replaces serum in KDM-2	Daly & Stevenson, 1985
SKDM	4 antibiotics added to KDM-2 as a selective medium for isolation (cycoheximide, D-cycloserine, oxolinic acid, polymyxin B)	Austin, 1983
Semi-defined	tryptone (10g/L) added to mineral salts, vitamins, nucleic acid bases, cysteine and glucose.	Embley et al. 1982

2.0 CULTURE MEDIA FOR RENIBACTERIUM SALMONINARUM

2.1 Culture media: Generally, R. salmoninarum has been grown on a variety of complex media, such as cysteine-blood agar, or Evelyn's serum-containing KDM-2 (Table 1). It is possible to replace the fetal calf serum used by Evelyn with normal cow, horse or rabbit sera. However, even on these relatively rich media, growth is slow, and plates are often over-grown by contaminants or other organisms from fish tissues. A selective medium was described by Austin et al. (1983), containing serum and four antimicrobial compounds. Other variations on KDM-2 involving addition of antibiotics have also been used (Bell, 1984).

Embley et al. (1983) were not able to formulate a completely defined medium suitable for physiological studies. The starch-containing Mueller-Hinton agar formulation could support growth of the organism when supplemented with cysteine, suggesting that the major function of serum in common media was not as a nutrient. All of the media used successfully for growing the kidney disease bacterium appeared to have in common some component that could act to bind toxic substances. This was the basis for the development of charcoal agar for the culture of R. salmoninarum.

2.2 KDM-C, Charcoal medium: The serum in KDM-2 was replaced with, in turn, starch, skim milk powder, and charcoal, and the ability of these media to support the growth of eight isolates of R. salmoninarum were compared by end-point dilution of growth (Daly & Stevenson 1985). The results of these tests led to the use of KDM-C, a medium in which serum is replaced with 0.1% (w/v) charcoal, with no reduction in its ablility to support growth. It is important to note that KDM-2 and KDM-C will give the same recovery rates from dilute suspensions; other media give lower recovery rates (Evelyn 1977; Daly & Stevenson 1985), and less sensitive detection. The medium is both cheap and convenient, and has the significant advantage of avoiding the presence of extraneous serum antigens in bacterial cell preparations used for preparation of diagnostic antisera.

KDM-C Components (per litre):

10	g	peptone
0.5	g	yeast extract
1	g	L-cysteine- hydrochloride
1	g	activated charcoal
15	g	agar

... adjusted to pH 6.8 with NaOH before autoclaving.

Comments on the use of KDM-C: The charcoal agar has obtained a fairly widespread acceptance for research and screening purposes. Initial problems experienced by others using the formulation included some due to the coarseness of charcoal used. For the best results a finely powdered charcoal is required. The SIGMA Chemical Co. product #C 5260 has been satisfactory in our hands. When pouring plates, occasional gentle swirls of the flask helps to maintain the charcoal in suspension.

As with the serum-containing KDM-2, this medium is best used fresh, as the cysteine becomes oxidized. (The oxidation of cysteine on KDM-C plates can result, disconcertingly, in tiny white crystalline "colonies" when old plates are streaked.) A shelf life of 2 weeks is appropriate for both KDM-2 and KDM-C.

2.3 EXPERIMENTAL WORK: Culture media and growth conditions.

2.3.1 Components: KDM-C permitted further studies into the role of the various components of the culture medium. In particular, we were interested in establishing whether L-cysteine served as a reducing agent, or as an essential nutrient. Also, we wanted to determine whether cysteine was an absolute requirement for R. salmoninarum and whether it was required under all conditions of growth.

Methods: KDM-C was prepared according to the recipe above except that the 0.1% L-cysteine hydrochloride was replaced with other amino acids, or with lower amounts of L-cysteine. Serial 10-fold dilutions of R. salmoninarum ATCC 33209^T or a low-passage coho isolate were prepared in peptone-saline (0.85% NaCl with 0.1% peptone, wt/vol). Drops (25 uL) of the dilutions were spotted onto the agar plates. The plates were then placed in plastic bags, incubated at 15°C, and periodically examined. Growth was recorded as the endpoint dilution, the last dilution of culture at which growth was observed on spot plates. (Note: each table records the results of a separate experiment. The endpoint dilution values for the standard medium vary between experiments due to variations in the viable cells in the inoculum used.)

Results: Optimal growth occured when the L-cysteine concentration was either 0.05% or 0.1% (Table 2.1). The requirement for cysteine is stereospecific, as D-cysteine could not replace L-cysteine in KDM-C (Table 2.2).

Table 2.1 Growth of R. salmoninarum ATCC 33209 on KDM-C with varied concentrations of L-cysteine.

L-cysteine concentration	growth endpoint dilution
0.1 %	10^{-5}
0.05 %	10^{-5}
0.02 %	10^{-4}
0.01 %	10^{-2}
0 %	no growth

Table 2.2 D-cysteine does not replace L-cysteine in KDM-C

Isomer and concentration	growth endpoint dilution
0.1 % D-cysteine	10^{-3}
0.1 % L-cysteine	10^{-6}

Cystine was not utilized as a replacement for L-cysteine (Table 2.3). Methionine, glutathione and homocysteine, which are precursors of cysteine biosynthesis, allowed growth at a reduced rate, while homocysteine thiolactone gave the same growth rate as cysteine (Table 2.3). With some of the tested compounds, particularly methionine, it was not neccessary to adjust the pH of the medium with NaOH, and we considered whether an increased NaCl level in the medium might be involved in the better growth with L-cysteine. However, 0.5% added NaCl did not affect the growth endpoint dilutions shown in Table 2.3. Further increases in the NaCl concentration of the methionine-containing medium also did not affect growth until 1.5%, when it became inhibitory (Table 2.4). When passed on media containing methionine or glutathione, the bacteria did not appear to adapt to these components, as the growth rate never attained that seen on cysteine-containing medium.

Table 2.3 Growth on charcoal agar with L-cysteine replaced by other amino acids, with or without 0.5% NaCl.

amino acids (6.3 mM)	Growth endpoint dilution for: ATCC 33209	coho strain
cystine	no growth	no growth
cystine + NaCl	no growth	no growth
methionine	10^{-3}	10^{-2}
methionine + NaCl	10^{-4}	10^{-3}
glutathione (reduced)	10^{-1}	10^{-1}
glutathione + NaCl	10^{-1}	10^{-1}
homocysteine	10^{-3}	10^{-3}
homocysteine + NaCl	10^{-3}	10^{-3}
homocysteine thiolactone	10^{-6}	10^{-5}
homocysteine thiolactone + NaCl	10^{-6}	10^{-5}
cysteine	10^{-6}	10^{-5}
cysteine + NaCl	10^{-5}	10^{-4}

Table 2.4 Growth of R. salmoninarum ATCC 33209 on KDM-C with cysteine replaced with 6.3 mM methionine and various concentrations of NaCl.

NaCl concentration	Growth endpoint dilution
0 %	10^{-8}
0.5 %	10^{-9}
1.0 %	10^{-8}
1.5 %	10^{-5}

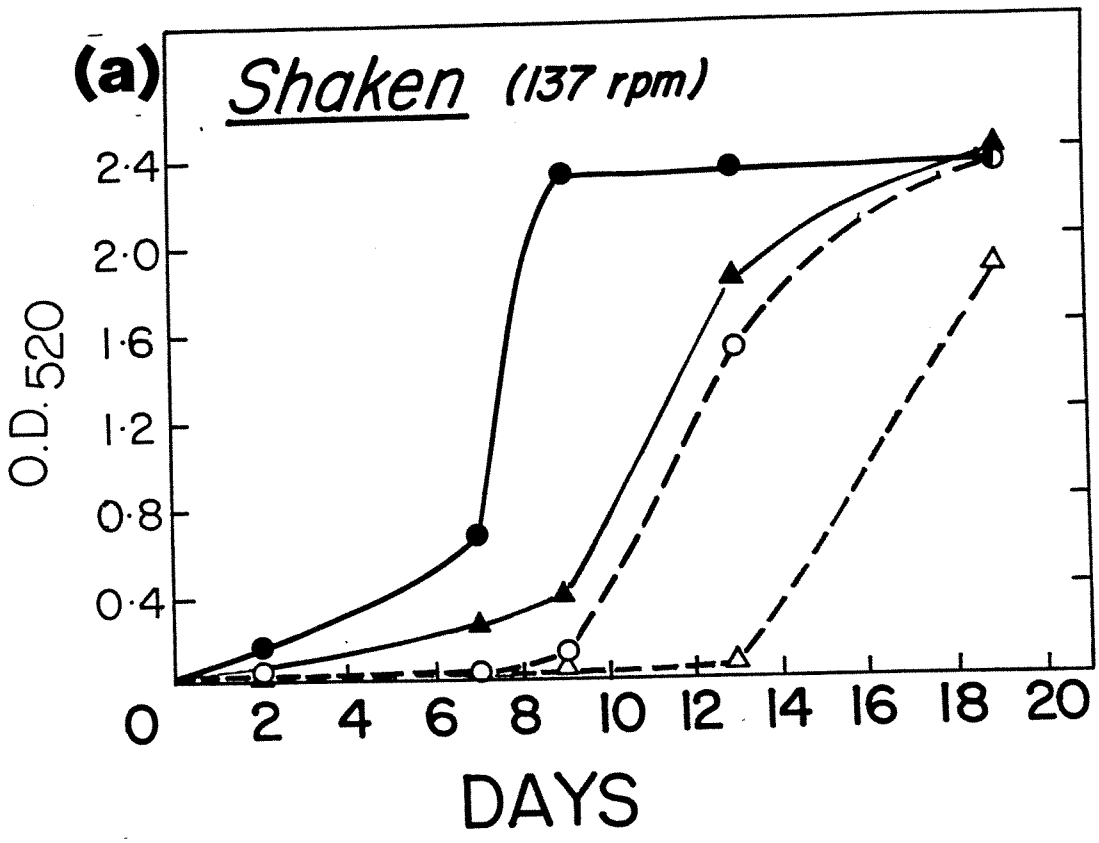
2.3.2 Growth conditions: KDM-C is more flexible than serum-containing media for investigations of the effects of conditions of growth. We previously described the use of a dialysis sac containing charcoal as a means of permitting growth in a liquid medium, which suggested possible methods of direct detoxification of tissue samples (see 3.2). Biphasic growth, in which a small volume liquid culture is in shifting contact with a large area of solid medium has significant advantages in increasing the density of cell suspensions collected from agar slants. This procedure can be scaled-up using Roux culture bottles with 90 mL KDM-C agar to give a larger surface area. Charcoal (or serum) was required for growth on agar, as colonies or as biphasic cultures. However, we found that in broth neither was an absolute requirement provided there was sufficient aeration.

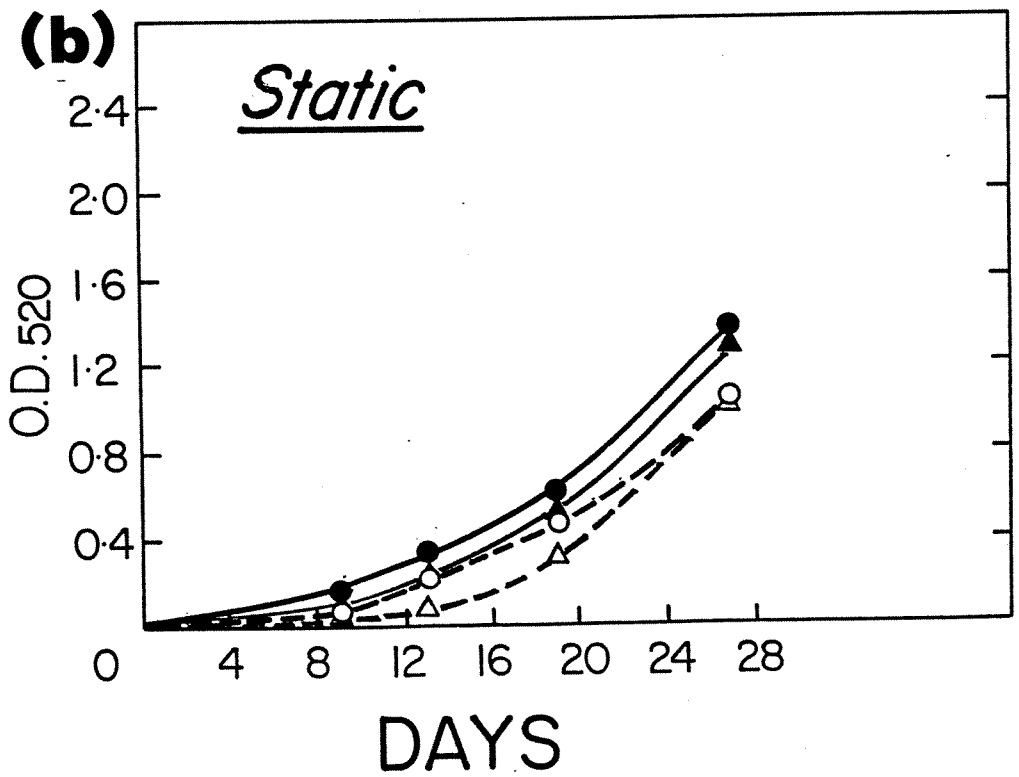
Methods: The strains tested were the high passage R. salmoninarum ATCC 33209 type strain and the passage 2 coho isolate, which were grown in broth at 15°C for 2 weeks, with agitation on a shaker. Inocula of 2% v/v from these were added to 100 mL of media in 250 mL flasks. Charcoal, when present, was in a dialysis sac. The flasks were incubated statically or with shaking at 137 rpm.

Results and Discussion: Charcoal was not required for growth in broth if the cultures were agitated, but addition of charcoal did enhance the rate of growth (Figure 1a). This response was seen with both the highly passaged type-strain and with a low-passage, recent isolate.

The observation that sufficient aeration reduced the requirement for charcoal (and presumably serum also) is significant in considering how large amounts of cells could be grown for potential vaccine development studies. Serum would be an expensive ingredient for scaled-up growth, and would cause foaming problems in fermenters. Charcoal may also pose some problems, so it is useful to know that essentially the same levels of growth can be attained in the basic broth if aeration is sufficient, albeit over a longer period (Figure 1b). Adequate inocula, agitation, and the presence of charcoal in the medium combine to produce optimal cell yields. It has been possible to scale up growth to 2 L and 10 L in fermenters.

FIGURE 1. Growth of R. salmoninarum in broth culture, with and without added charcoal. (Black symbols indicate cultures with charcoal added to the medium.) Flasks were incubated with shaking (a) or as static cultures (b). Circles indicate tests with R. salmoninarum ATCC 33209, a high-passage strain; triangles indicate tests with Coho, a passage-2 strain from diseased fish. (Results shown are from a single, representative trial.)





3.0 DETECTION BY CULTURE METHODS:

3.1 Inhibition by fish tissues: R. salmoninarum has fastidious growth requirements and has been generally cultured on such media as KDM-2, containing serum and cysteine (Evelyn, 1977). Under ideal conditions, and with a heavy inoculum, growth appears on agar after approximately 10 days. With low inocula, growth may not be evident before six weeks (Fryer and Sanders, 1981). Isolation of the bacterium from infected fish is further complicated by toxic effects of kidney tissue from coho salmon (Oncorhynchus kisutch) and sockeye salmon (O. nerka) towards R. salmoninarum (Evelyn et al. 1981). Growth was inhibited unless tissue samples were washed and/or diluted to 5% of their original volume.

The inhibitory material was found in the supernatant fraction when kidneys were homogenized in peptone-saline and then centrifuged. A sample of kidney tissue which contained 4×10^6 bacteria and had been diluted 50% with peptone saline showed no growth. After washing the kidney tissue three times with peptone saline, a 50% homogenate did show growth. Evelyn (1977) had recommended that tissue homogenates be diluted in a 10-fold series dilution, and then drop-plated onto KDM-2 plates. The inhibitory effect of the tissue material may be evident with the 50% tissue homogenate, but reduced at 5% or 0.5%. Fatty acids are known to have an inhibitory effect on the growth of a variety of bacteria, and it has been postulated that fatty acids from the tissue homogenate may be the substance retarding growth of R. salmoninarum from homogenates.

3.2 EXPERIMENTAL WORK

3.2.1 Processing tissues: The process of washing tissue homogenates and then plating dilutions has two advantages over fluorescent-antibody stains in that a larger sample of tissue is taken initially, and a live culture is obtained for examination. However, this is a very labour-intensive screening process. Our major objective was to try to improve the ease with which tissue could be processed and detoxified.

Preliminary to this, we asked whether the tissue toxicity was in fact still in evidence when samples were plated on a serum-free charcoal agar (KDM-C). Charcoal could be replacing serum in the medium by acting as a detoxifying agent, and this might relieve the observed tissue toxicity phenomena. As another aspect of this study, we also wanted to know if tissue toxicity occurred with other salmonid genera, particularly the widely cultured rainbow trout. Then, in an attempt to eliminate the necessity of laborious washings of tissue, we examined the effect of charcoal on tissue toxicity when it was added directly as a pre-treatment of homogenized tissues.

Methods: Liver and kidney tissues from rainbow trout (Salmo gairdneri) and brook trout (Salvelinus fontinalis) were obtained from BKD-free stocks maintained for experimental use in this laboratory. Coho salmon were obtained from a provincial hatchery that was experiencing a chronic problem with BKD. All fish were held in running well-water at approximately 10° C. Fish were killed by an overdose of MS-222 in water, and liver and kidney tissues were

aseptically removed, placed in sterile plastic bags (Whirl-Pac), and weighed.

For seeding the tissues, a culture of R. salmoninarum ATCC 33209 was grown biphasically on KDM-C for 10-14 days, as described previously (Daly and Stevenson, 1985). A bacterial suspension was made by adding 1 ml of the liquid overlay phase from a culture to 100 ml of peptone saline (0.1% peptone in 0.85% NaCl). Rainbow and brook trout tissues were seeded by adding the bacterial suspension to the plastic bags at ratio of 0.1 ml to 0.1 g of tissue. The mixtures were homogenized for approximately 2 min with a mechanical stomacher (Seward Laboratory, London, England). The coho salmon were naturally infected with BKD and tissues did not require artificial seeding with the bacterium. They were treated in the same manner except that sterile peptone saline was used rather than the bacterial suspension.

In the experiments to determine if tissues could be directly treated with charcoal to remove toxicity, 0.1 g, 1 g or 2 g of activated charcoal was added to 100 mL of peptone-saline. To this suspension, 1 mL of biphasically-grown bacterial mixture was immediately added to the tissue at a ratio of 0.1 mL to 0.1 g of tissue, providing a 1/2 tissue homogenate. The tissue homogenates were diluted further by making serial doubling dilutions to 1/64 in peptone saline. Two 25 µl samples of each dilution were then spot-inoculated in duplicate onto KDM-C agar plates, three dilutions per plate. Inoculated plates were allowed to dry overnight in a 15°C incubator, then placed in bags to prevent dehydration during further incubation at 15°C for 1 month.

3.2.2 Specificity of toxicity: Evelyn et al. (1981) described tissue toxicity for kidney tissue from coho salmon and sockeye salmon. Liver and kidney tissue from rainbow trout, brook trout and coho salmon were all toxic towards R. salmoninarum when tested on charcoal agar. As with the previous studies by Evelyn et al. (1981) on KDM-2, toxicity disappeared when the tissues were diluted to 1/16 of their original volumes. These findings demonstrate that tissue toxicity affecting R. salmoninarum occurs not only in isolations from O. nerka and O. kisutch, as described by Evelyn et al (1981), but in those from other salmonids as well. The toxicity is not restricted to kidney tissue but was also found in liver tissue, and is potentially present in all organ tissues.

The charcoal agar, KDM-C, does not appear to be any better than KDM-2 in its ability to reduce tissue toxicity. The amount of charcoal in the agar could not be increased significantly in the hope of binding more toxic material as the surface tension increased to a point where drop inocula did not spread on the agar surface.

3.2.3 Direct charcoal detoxification: Plastic bags were used in the stomacher in order to homogenize the tissue, with charcoal added to remove the inhibitory factors in situ. However, these attempts to remove the toxicity by mixing charcoal directly with the tissues during homogenization in peptone-saline were unsuccessful with concentrations of charcoal up to and including 2%. Higher concentrations of charcoal were not tried because of the danger of puncturing the bags containing the tissue.

3.2.4 Detoxification by spreading dilution: When rainbow trout tissues were examined on the agar plates, it appeared that the toxicity was reduced when the spot inocula were more spread out. The bacteria appeared to grow well at the outer edge of the spot-inoculum. This suggested that the toxicity might be reduced by actively streaking out the inocula, in effect, doing an *in situ* dilution of the tissue.

Procedure and results: The 25 µl spot inocula were spread with an inoculating loop, so that approximately 1/6 of the plate was covered per spot. With both liver and kidney tissue, this procedure did allow growth of the bacteria at the 50% dilution, where it would otherwise be inhibited (Fig. 2).

FIGURE 2. **Growth on KDM-C agar from dilutions of rainbow trout kidney homogenate seeded with Renibacterium salmoninarum.** For the plate on the left, drops were allowed to absorb to the agar directly where placed; for the plate on the right, duplicate drops were spread out with a sterile loop. The dilutions, in duplicate, are: (i) 1/2, (ii) 1/4, and (iii) 1/8. (A contaminant appears at the top of one plate; a similar one has been excised from the second plate to prevent overgrowth during prolonged incubation.)

This technique was, however, not very effective in reducing the toxicity of coho salmon and brook trout tissues. Sometimes it allowed the growth at the 1/8 dilution but not consistently. It is not evident why rainbow trout appear to be different, nor whether in situ dilution of tissues would reduce toxicity for all rainbow trout strains. However, in attempts to culture R. salmoninarum from tissues, it would still seem to be a useful procedure to spread out spot inocula on agar plates in order to separate bacteria from tissue.

Discussion: R. salmoninarum has been found to possess a hydrophobic cell surface (Daly and Stevenson, 1987). This would facilitate the binding of fatty acids liberated from the tissues due to homogenization, and account for the toxicity effects observed by Evelyn et al. (1981). In the case of a number of other bacteria, fatty acids of various chain-lengths have been found to have a toxic effect on the growth of the organisms.

An additional or alternate explanation for the reduction in growth of R. salmoninarum when mixed with fish tissue may be binding of the bacteria to the tissue debris. A cell-associated rabbit erythrocyte agglutinin (haemagglutinin) has also been described (Daly and Stevenson, 1987) but the ligand to which it naturally binds has not yet been found. Perhaps the bacteria are binding to brook trout and coho salmon tissue but not as effectively to rainbow trout. This may explain why in situ dilution only worked for the latter species.

One fish tissue that does respond to haemagglutinin is spermatozoa. Recently, Daly & Stevenson (manuscript submitted) demonstrated that both cells of R. salmoninarum and extracted haemagglutinin agglutinated spermatozoa from brook trout, rainbow trout, chinook salmon, common white sucker, and goldfish. Spermatozoa from walleye and from bulls were not agglutinated. When examined microscopically, bacterial cells were seen to bind to the tails but not the heads of these cells. It is not clear if this reaction has significance in connection with vertical transmission of BKD, but it is of interest in analyzing specific interactions between this bacterium and its host.

4.0 ENRICHMENT STEPS FOR RECOVERY AND CULTURE:

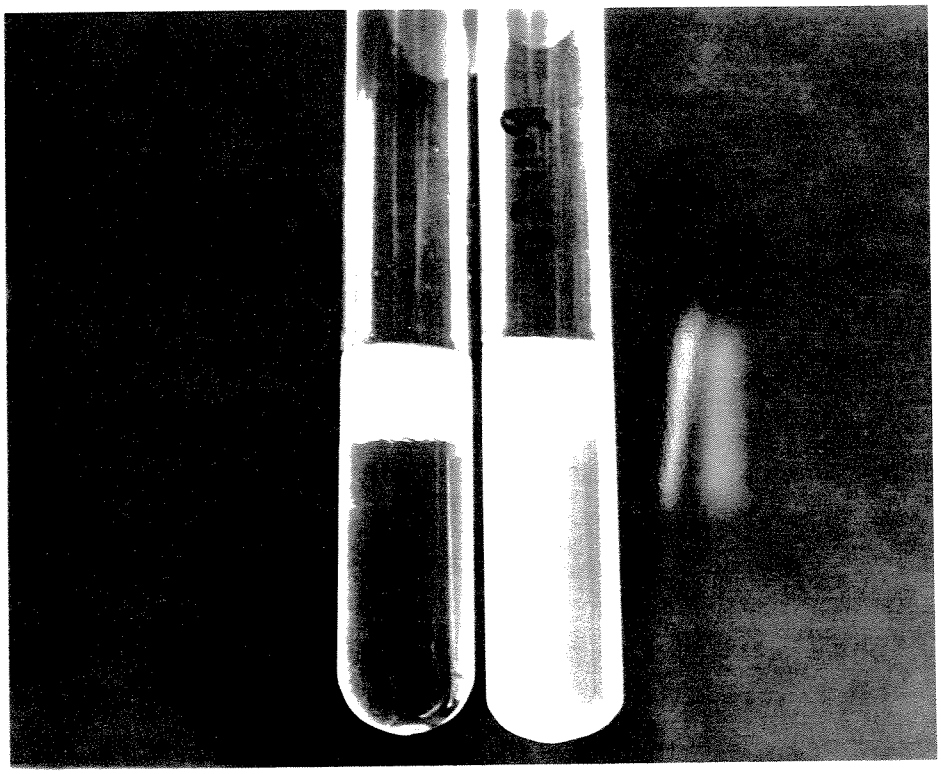
4.1 EXPERIMENTAL WORK - Hydrophobicity: The hydrophobic cell suface of R. salmoninarum (Daly & Stevenson, 1987) suggested a possible enrichment procedure based on partitioning of the bacterial cells.

Methods:

Adherence to hydrocarbons: The general procedures are as described by Daly & Stevenson (1987). Generally, bacterial cells in a potassium phosphate-urea buffer were overlaid with small (50-400 µL) quantities of hexadecane. The tubes were agitated on a vortex mixer, each for a standard amount of time, then allowed to sit for phase-separation (Figure 3). This general procedure was followed with samples consisting of tissue homogenates seeded with cells of R. salmoninarum.

Results: The attempt to selectively extract and concentrate bacterial cells from tissue homogenates failed, largely because of the excessive amount of tissue fats and debris present. Additional extraction steps to improve separation do not appear to be feasible as the cells may loose viability and/or antigenicity from the hexadecane treatments or from extra washing. This approach to detection was abandoned.

FIGURE 3. Extraction of cells of R. salmoninarum into hexadecane overlay. In the left tube, the hydrophobic cells have been concentrated in the hexadecane phase; in the right tube, the bacterium cells had previously been stripped of the haemagglutinin by boiling, and remained in the aqueous phase.



4.2 Macrophage location of the bacterium: One possible effect of hydrophobicity noted is association with macrophages. Hydrophobicity is postulated to enhance the uptake of BKD by phagocytic cells, in which the bacteria may be able to survive and grow (Daly & Stevenson 1987).

Symptomatically, bacterial kidney disease involves production of granulomatous tissue in many of the internal organs. These clinical findings indicate that the bacterium is capable of surviving and possibly growing within macrophages and other phagocytic cells. Electron microscopy studies by Young and Chapman, (1978) also suggested intracellular survival of Renibacterium, as did the comments by Bruno (1986).

4.3 EXPERIMENTAL WORK

4.3.1 Macrophage separations from tissues: Isolation of macrophages from fish kidney tissue may provide a means of enriching tissue samples being tested to detect presence of the bacterium in lightly-infected fish or in carrier fish among broodstock.

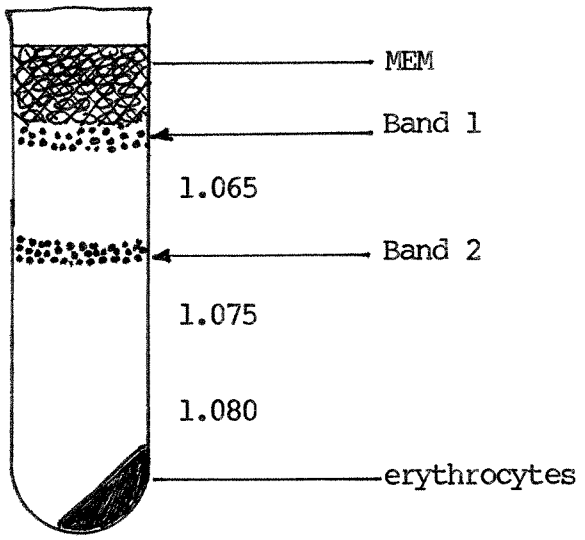
Methods:

Collection of peritoneal macrophages: In the first studies, macrophages were collected from adult brook trout after intraperitoneal injection with 1 ml of 2.5% glycogen in saline (w/v). After 6 days, 1 ml of a culture of R. salmoninarum ($OD_{600\ nm}$ = 0.75) was injected intraperitoneally. On day 11, the fish were sacrificed, and the peritoneum was filled with 10 ml of MEM (Minimal Essential Medium) containing 20 IU heparin/ml, and gently massaged. The peritoneal wash was removed with a syringe, and layered onto a discontinuous Percoll gradient. The gradient was prepared using Percoll densities of 1.065, 1.075 and 1.080. After centrifugation at 400 xg for 40 min, the macrophages typically banded on the 1.075 band (Figure 4).

Culture of macrophages: Macrophages in Bands 1 and 2 were allowed to surface-attach in separate Leighton tubes overnight and cultured in CMEM (complete MEM) with the addition of 2.5 µg/ml gentamycin to prevent extracellular growth of the bacteria.

Macrophage enrichment from coho salmon kidney: Kidneys were removed from naturally-infected coho salmon smolts and placed in a stomacher bag containing MEM with 20 IU/ml heparin. The bag of tissue was processed in the stomacher for 1 min, and samples were removed for serial 10-fold dilution and spot-inoculation of 25 ul samples on KDM-C plates, as in the usual procedure for determining viable numbers. A 2 ml sample of the minced tissue was then layered onto a Percoll gradient and centrifuged at 400 xg for 40 min. Band 2 (Fig. 4) was removed and the cells washed in peptone-saline to remove Percoll. Serial 10-fold dilutions were made, and 25 µl samples again plated on KDM-C agar.

FIGURE 4: Location of macrophages on Percoll gradient



Results:

Macrophage collection: Band 1 from the Percoll gradient typically contained single cells and cellular debris. Band 2 contained cells that were clumped together. After 1 day of in vitro growth, macrophages could be seen spreading out and elongating.

No tissue toxicity was observed in the Percoll samples from coho salmon kidney. Of 11 coho salmon with natural infections with R. salmoninarum, 3 appeared to show more growth from the Band 2 Percoll sample than on direct kidney isolated, judged by relative end point of growth on dilution. Accurate quantitative comparisons of the two treatments are not possible from this experiment as dilution factors were not recorded for comparison. However, the Percoll enrichment of macrophages does appear to have been successful. As bacteria are found in both Bands 1 and 2, a repeat of this study should involve pooling both bands to further concentrate the bacteria present.

Intracellular location of Renibacterium salmoninarum: When these macrophages were recovered and purified on discontinuous Percoll gradients, they appeared to contain numerous R. salmoninarum within their cytoplasm, based on bacterial morphology by Gram-stain, and by IFAT staining. When examined after 5 days in culture, the Gram-stained bacteria appeared to occur within the macrophages. Bacteria did not appear in the culture supernatant, nor did the distribution suggest attachment to the external surface of the cells. Some macrophages appeared to have fused, possibly forming giant cells.

Discussion:

The collection of macrophages using Percoll gradients provides a method for concentrating R. salmoninarum from infected kidney that has potential applications in diagnostics and other studies. Since R. salmoninarum is a facultative, intracellular pathogen, vaccination schemes aimed at preventing BKD must activate the cellular immune system. Our in vitro assay system has the potential to compare the effects of unactivated and activated macrophage on the intracellular survival of this important fish pathogen.

After the first collection of macrophages from the peritoneal cavity of brook trout, we found that the initial glycogen injection was not necessary for the recovery of these cells. The purpose of the glycogen injection is to stimulate macrophage migration into the peritoneal cavity, where they would phagocytose the bacterial cells. However, it appears that injection of R. salmoninarum provides the same stimulation to macrophage migration as does glycogen. This has potential implications in considering how the bacterium interacts with the host immune system.

4.3.2 Staining techniques for cells in macrophages: As noted above, cells of R. salmoninarum appear to locate preferentially within macrophages. In this location, they were not evident when heat-fixed tissue smears were examined after Gram-stain or IFAT staining procedures. When methanol or acetone was used to fix the smears, a process which extracts the lipids of the cell membranes, the bacterial cells became clearly evident.

This observation is of interest as an additional demonstration of the intracellular location of R. salmoninarum. It is of much more immediate interest because of the implications for diagnostic methodology. While Canadian Fish Health Protection procedures call for acetone-fixing kidney smear slides (page 22, IX.C.2.g.2), the American Fisheries Society "Blue Book" (3rd edition) specifies a 60°C heat-treatment for all fluorescent antibody staining (p.71, XVI.A.1.a and 2.a). Use of heat-fixed smears for BKD detection is likely to result in false-negative reactions because the intracellular location of the bacterium masks staining or antibody reactions.

RECOMMENDATION: Tissue smears being examined for the presence of Renibacterium salmoninarum should be fixed by methanol or acetone, not by heating.

5.0 IN VIVO FISH CHALLENGE PROCEDURES:

5.1 Artificial challenges: Only a single trial of macrophage-enrichment as a means of improving recovery of R. salmoninarum from tissue was attempted. The results appear promising, but additional trials were required. Coho salmon smolts from a source with a history of BKD had been used, but seasonal availability limited carrying out further trials. Tissue seeded with bacterial cells would not be a useful approximation, as the bacteria would not be localized as in the infected animals. A reliable method for in vivo infection of fish would be useful for further work.

Most studies requiring an artificial challenge for disease or vaccination studies have used an intraperitoneal (IP) injected challenge of between 10^3 to 10^8 cells, although other methods also have been employed (Table 3). Some work has relied upon natural challenges (Paterson et al. 1981). Vigneulle (1981) and Bell et al. (1984) used waterborne challenges, placing uninfected fish in the same tank as infected animals. The immersion challenge is effective, and it has advantages over injection as far as representing a natural challenge. A natural route of pathogen entry is important in studies of pathogenic mechanisms and for considering interactions with the host immune system. Some comparisons of naturally and artificially infected fish suggest that the slower development of natural infections may result in more chronic inflammatory responses (Bruno, 1986). It may be that some disease conditions, such as skin blistering, may only be seen when pathogen entry has been by a specific route.

For immersion challenges, relatively large quantities of R. salmoninarum cells would be required, especially if it is desirable to expose fish to large doses of the bacterium. An oral infection would be a useful alternative if small volumes of cells could be administered to the animals individually, to ensure each got a specific dose. Fish, in a natural situation, might become infected very efficiently through the intestine, by eating infected fish or fecal material in cage-culture situations. In this study, we infected fish via the oral route, per os, and followed the course of the subsequent infection.

Table 3. ARTIFICIAL TRANSMISSION OF BKD - SUMMARY OF STUDIES

Route and dose	Species	Result	Reference (see below)
INJECTION			
injected (IM or SC) tissue emulsions from Atlantic salmon	brown trout	mortalities after 3-5 weeks with lesions	Mackie et al. (1933)
injected (IM) with kidney abscesses	brook trout	mortalities in 18-35 days	Belding & Merrill (1935)
injected (IP) with bacteria grown for 3 passages on agar	sockeye salmon	mortalities after 12-23 days; re-isolated bacteria	Ordal and Earp (1956)
injected (IP) with bacterial suspensions	sockeye salmon	mortalities in 17 to 31 days	Evelyn et al. (1973)
injected (IP) with 10^8 viable cells	coho salmon, sockeye, steelhead trout	mortalities, with temperature effect (some fish were pre-infected)	Sanders et al. (1978)
injected (IP), varied concentrations (10^3-10^7)	sockeye salmon	fish died from BKD, including controls in same tank	Bell et al. (1984)
injected IP with 2×10^7 cells	rainbow trout, Atlantic salmon	systemic infections after 40 days; 75-85% mortalities in 35 days	Bruno (1986)
injection IP of ATCC 33209	rainbow trout	signs of disease in 10-12 days; mortalities up to 28 days	Austin (1985)
injection IP of 3×10^7 cfu/fish	rainbow trout, 5 & 15 g	75% mortalities with small fish; infection (87%) only with 15 g fish	McCarthy et al. (1984)

CONTACT			
water contact with infected coho in same tanks	coho salmon, rainbow trout	transmission with mortalities in coho but very low in rainbow trout	Vigneulle (1981)
self-abrasion with bacterial exposure	brook trout	infected, with mortalities between 48- 102 days.	Wolf & Dunbar (1959a)
immersion, dose not noted	rainbow trout	14% mortality after 40 days, begining day 39	McCarthy et al. (1984)
ORAL			
exposure to infected fish, then fed viscera	brook trout	no transmission	Sniesko & Griffin (1955)
feeding infected viscera and flesh for 52 days	chinook salmon	mortalities at 32 days; 95% at 12 weeks	Wood & Wallis (1955)
fed bacteria, plus bile salts, glass shards	brook trout	no transmission	Wolf & Dunbar (1959)
intubation with cells, 4.6×10^{11}	brook trout	infection and low mortalities	(this report)

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Vigneulle (1981) Thesis, Doctorat Veterinaire, Ecole Nat. Vet. D'Alfort.
Bell et al. (1984) AFS Fish Health Newslet. 12(1):8.
Bell et al. (1984) Aquaculture 36: 292-311
Bruno (1986) J.Fish Dis. 9: 523-537.
Austin (1985) J.Fish Dis. 8: 209-220.
McCarthy et al. (1984) J.Fish Dis. 7: 65-71.

5.2 EXPERIMENTAL WORK - per os infection:

Methods: The bacterium was a strain of R. salmoninarum isolated from diseased Atlantic salmon. The bacteria were passaged in brook trout, using the per os route of infection described below, and reisolated from diseased fish. The bacteria were isolated on charoal agar (Daly and Stevenson, 1985), passaged once and then administered per os to brook trout. Thirty 1+ year old fish were given 1 ml of a R. salmoninarum suspension from overlay fluid, containin 4.6×10^{11} cells per mL. The bacteria were not washed before being given to the animals.

The bacteria were put into a syringe fitted with a 22 G needle. To the needle was attached 10 cm of polyethylene tubing (external diameter 1 mm and internal diameter of 0.56 mm). The fish were anaesthetised and the tubing was inserted down the throat of the animal. To do this, the tubing was placed at the back of the mouth and inserted down the throat after the animal exhibited the gag reflex. Fish were held in flowing water with effluent disinfection by chlorination. They were monitored daily and killed with an overdose of MS 222 when they became obviously sick and distressed. After 154 days the experiment was terminated and all remaining animals sacrificed.

Fish examination: Heart, liver, spleen, and kidney were removed from the animal and placed in sufficient peptone saline to give a final tissue concentration of 5% (w/v). For culture, 25 uL aliquots were placed on charcoal agar plates and incubated for 1 month at 15°C. Slides were smeared with the peptone saline suspension for IFAT staining. For this, 50 fields were examined at 1000 x magnification.

Results: The results of the 128 day per os trial are shown in Table 4. Only 5/13 infected fish were thought to have died of BKD. However the fish were not very stressed, which might have increased the mortality rate. Of the 30 fish, 13 were shown to be infected based on culture of the organs, while 8 showed a positive IFAT test. The only organ from which the bacterium was always isolated was the kidney, although in one fish the number of bacteria per gram of tissue was 100 times greater in the heart than the kidney. The IFAT detection method appeared relatively insensitive as, in some cases, 10^4 bacteria per gram of tissue, based on culture, were required before a positive IFAT test could be seen.

Discussion: The per os method of infecting the fish has an advantage over IP or IM injections since the animal's natural mucosal barriers have not been breached. Indeed, the oral route may be one means by which R. salmoninarum is transmitted horizontally among fish. Immersion could provide a similar natural challenge, but the per os route has the advantage of requiring fewer cells, which with BKD may be a limiting factor for experiments. Challenging by natural routes may not produce effects as strongly and as rapidly as with injected challenges, but they are important as the route may have an influence on the course of the disease. Bruno (1986), for example noted the more chronic inflammation in natuarally infected fish and the development of skin lesions only in these fish.

TABLE 4: Mortalities and infections of per os infected brook trout:

Time of Death (days post-infection)	Number dead	Reason	Number infected
39	4	water shut off	0/4
48	1	discoloured skin	0/1
80	3	sacrificed	2/3
88	1	died	1/1
90	1	died	1/1
118	1	died	1/1
139	1	died	1/1
146	2	died	1/2
150	1	died	0/1
154 (experiment terminated)	15	sacrificed	6/15
TOTAL	(30)		13

6.0 ANTIMICROBIAL THERAPY

6.1 In vitro tests of antimicrobial activity: Testing the effect of antibiotics on bacteria in culture serves to identify the reagents that have potential for in vivo application. Wolf and Dunbar (1959) used the disc procedure, placing filter discs impregnated with the antibiotics on freshly-inoculated slants of Mueller-Hinton agar plus 0.1% cysteine. The broth or agar dilution procedures used by Austin et al. (1983) and Austin (1985) allow a more quantitative assessment of the minimum inhibitory concentration. A summary of the published studies on in vitro antibiotic sensitivity of R. salmoninarum and the antibiotics reported to have inhibitory effect is given in Table 5.

6.2 Use of antibiotics as the basis of selective media for culture: Antibiotics can be useful as a selective agent when incorporated into agar or broth media for growth or isolation by culture, as in SKDM (Austin et al. 1983), which contains cycloheximide, D-cycloserine, oxolinic acid and polymyxin B. This formulation was the most effective agar for primary isolation of R. salmoninarum from fish tissues in an extensive study involving washed and diluted tissue samples from 517 fish in Iceland (Guomundsdottir et al. 1988). Of all positive samples, 91% were cultured on SKDM, 60% on KDM-C supplemented with the same antibiotics (SKDM-C), and 35% on KDM-2 alone. SKDM and SKDM-C both contain antibiotics and an adsorbing compound - serum or charcoal. The extent to which these substances bind the antibiotics is a concern in assessing the effective level of antimicrobials available in the medium.

6.3 In vivo effects of antibiotics: Antimicrobial agents that are effective in vitro may not be useful as therapeutics if there are problems with their penetration to the bacterium in the fish. Specifically, for BKD, antibiotics must be able to penetrate to R. salmoninarum within macrophages. Potentially, a test system involving macrophage-engulfed bacteria (as in section 4.3) could be used to predict efficacy, particularly if we could improve techniques for assessing changes in the viability of the intracellular bacteria.

Currently, evaluation of therapeutic effects depend on trials with infected fish. As a follow-up to their in vitro antibiotic tests, Wolf and Dunbar (1959) demonstrated that feeding erythromycin to brook trout for 21 days was helpful to the survival of fish injected with the BKD organism. Five of the eleven antibiotics effective in vitro in the tests by Austin (1985) were also able to reduce mortalities when administered in feed for 10 days, beginning 10 days after challenge with injected Renibacterium. The effective compounds included erythromycin and three other macrolides (spiramycin, clindamycin, and kitasamycin). Two other macrolides, oleandomycin and lincomycin were effective in vitro but not in therapeutic tests. Penicillin G was also effective, but it can present specific problems for humans if used in food fish.

Groman and Klontz (1983) have reviewed work in which erythromycin feeding and injection have been used as therapy for BKD. Emphasis has recently been onits use as a preventative rather than a treatment, particularly when applied

to pre-spawning fish to combat vertical transmission (Groman & Klontz 1983, Evelyn et al. 1986, Bullock & Leek 1986). Another approach has been water-hardening of eggs in erythromycin, although subsequent studies have shown that R. salmoninarum can still be cultured from eggs treated in this manner (Evelyn et al. 1986; Bruno & Munro 1986).

Fairly extensive use has been made of erythromycin in field treatments of salmonids, with still varied verdicts about optimal timing and conditions. One matter of interest will be the effects of erythromycin regimes on fish that are exposed to BKD by a natural route rather than experimental injections. In such situations, the interaction of the antibiotic with the fish immune system may be very significant. An additional concern is the possibility of developing resistant strains of R. salmoninarum, such as the isolate described by Bell et al. (1988).

Table 5: Antimicrobial sensitivity of Renibacterium salmoninarum

Method used	No. of strains	No. of drugs	Inhibitory antibiotics	Reference
discs on MH-cys	16	34	bacitracin, candicidin, carbomycin, chloramphenicol erythromycin, fungichromin, novobiocin, penicillin, streptomycins, tetraccylines, thiomycetin.	Wolf & Dunbar (1959)
broth dilution	8	11	ampicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, penicillin G, tetracycline, vancomycin	Getchell (1984)
agar dilution in KDM-2	44	22	cephalothin, Dynamutalin, gentamycin, lincomycin, novobiocin, oxytetracycline, rifampicin	Austin et al. (1983)
agar dilution in KDM-2	3	1	erythromycin, MIC between 0.1 µg/mL and 0.15 µg/mL	Groman & Klontz (1983)
agar dilution in KDM-2	40	79*	cephalosporins, chloramphenicol, gentamycin, macrolides including erythromycins, novobiocin, penicillins, rifampicin, tetra-cyclines, vancomycin.	Austin (1985)

* Includes some stains, as crystal violet and malachite green.

7.0 IMMUNOLOGICAL DETECTION METHODS:

7.1 Update and comparison of methods

7.1.1 Gram-stain and culture: Initial detection methods for BKD involved observations of gross pathology and examination of Gram-stained tissue smears for cells of R. salmoninarum. These methods are relatively insensitive, and positive tests require high levels of the bacterium and/or well-established disease. Culture methods are very much more sensitive, and have the advantage that they detect viable cells. However, growth of R. salmoninarum from infected tissues may take 4 to 10 days from overtly infected fish and 6 weeks from highly dilute inocula (Evelyn 1977; Fryer and Sanders 1981). (Recently it has been suggested that detection is increased when culture plates are reexamined after 3 months incubation.)

The time required for the culture technique to yield results, the loss of samples due to contamination, and the labour involved in preparing washed tissues for culture (see section 3.0) make this impractical for routine screening of fish. The prefered methods for rapid routine testing are serological tests, notably the direct and indirect immunofluorescent antibody staining techniques, DFAT and IFAT.

7.1.2 Fluorescent antibody staining: In fluorescent antibody staining techniques, the surface antigens of whole bacterial cells are detected by antibody-staining of tissues carrying the organism. The advantage of this is that cross-reacting organisms may be distinguished from R. salmoninarum on the basis of their microscopic morphology (Austin et al. 1985), while with other immunological methods, this is not possible. (For immunodiffusion, coagglutination, or ELISA, the antigen is extracted from tissues, generally by a heat treatment (Kimura et al. 1978, Kimura and Yoshimizu 1981).) A variation of the FAT staining procedure involving concentration of coelomic fluid by membrane filtration was developed by Elliot and Barila (1987) in order to make the test applicable to spawning fish. Similarly, Lee and Gordon (1987) applied a combination of IFATs on kidney and ovarian fluid and culture of egg samples as a means of screening broodstock chinook salmon.

7.1.3 ELISA systems: Pascho and Mulcahy (1987) developed an ELISA procedure for detecting a soluble antigen from R. salmoninarum, prepared by ammonium sulfate precipitation from KDM-2 culture supernate. Antiserum was prepared against this antigen preparation, and the intended test samples are extracts of tissue or ovarian fluid are the intended antigens. In the ELISA procedure described by Dixon (1987), tests were carried out on dilutions of kidney homogenates. The antiserum used had been raised against whole bacterial cells, and it was necessary to increase its specificity for R. salmoninarum by absorbing with Rothia dentocariosa, a cross-reacting bacterium. A commercially-available antibody to R. salmoninarum (Kirkegaard and Perry Laboratories, Gaithersburg, MD) has been shown to detect both bacterial cells and the soluble antigen prepared by Pascho (Wetherell and Ruppenthal, 1988). Variations on the ELISA procedure include the dot-blot method used by Sakai et

al. (1987), who assessed the procedure using the supernate from centrifuging heat-treated (100°C) samples of kidney homogenates seeded with bacterial cells.

7.1.4 Monoclonal antibodies: Monoclonal antibodies are becoming widely used in microbiological detection systems because they can be used to detect very specific antigenic determinants in a consistent fashion. Arakawa et al. (1987) described hybridoma clones that reacted with R. salmoninarum, but these either cross-reacted with other Gram-positive bacteria or failed to react with all strains of BKD. More recently, Weins and Kaattari (1988) were able to develop two monoclonal antibodies that appeared to recognize different epitopes of the major surface antigen of Renibacterium (see 7.2 below). Such antibodies have potential for improving diagnostic methods by more precise recognition of BKD antigens.

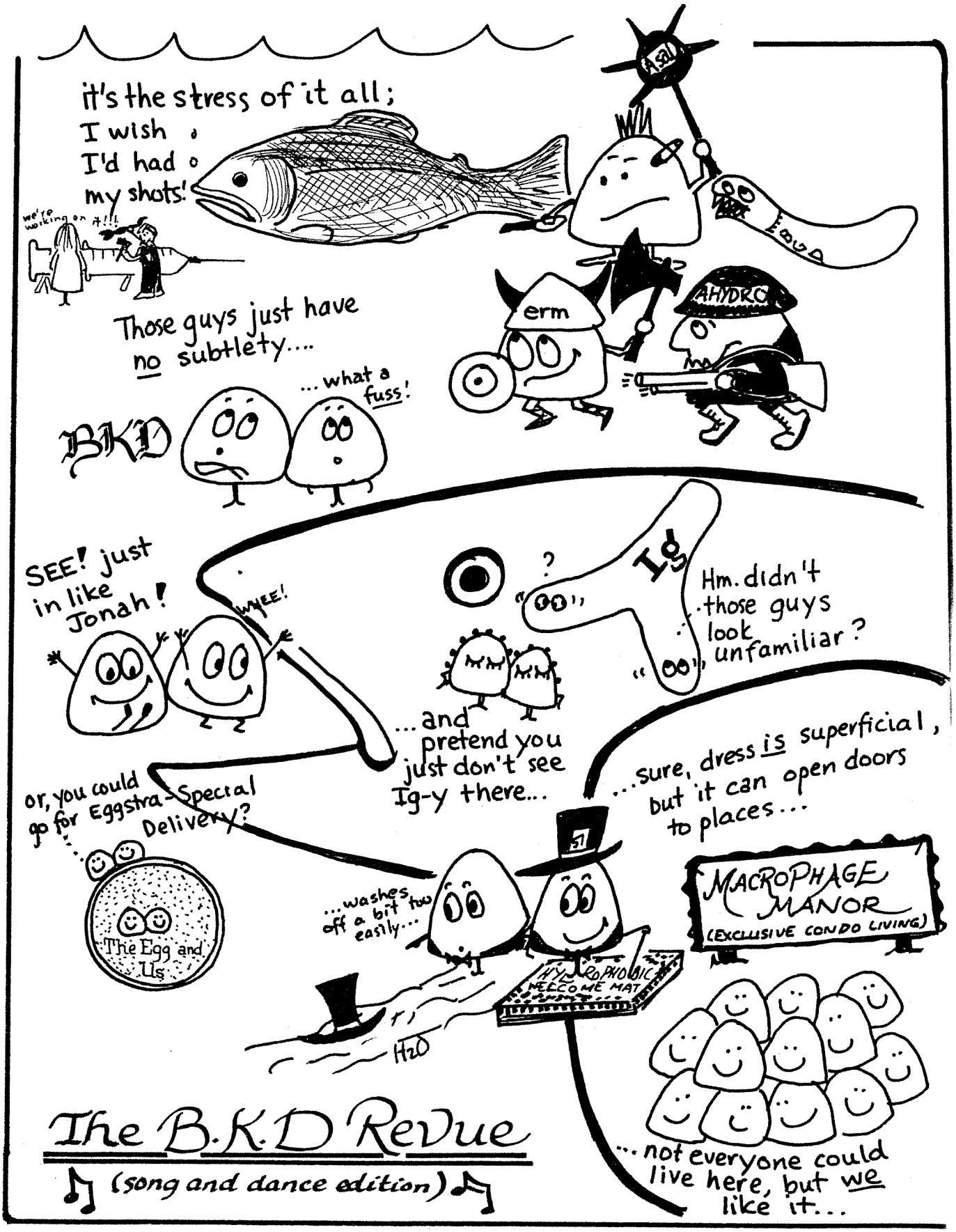
7.1.5 Comparison of methods: Evelyn et al. (1981) found culture to be 100-times more sensitive than IFAT for detecting R. salmoninarum. Lallier et al. (1981) indicated that IFAT was more sensitive than culture, even when tissues were washed prior to plating. However, these results were unusual in that R. salmoninarum could not be cultured from some fish with macroscopic kidney lesions, suggesting that the cells could have been non-viable. Cipriano et al. (1985) reported that the highest detection, 73%, of the organism in coho salmon was with counterimmunoelectrophoresis (CIE), followed by 48% detection by culture, 10% by DFAT, 5% by Gram-stain, and 0% by immunodiffusion. In the report of this comparison, it is not clear whether the plates of KDM-2 were inoculated with a direct streak or with washed tissues. The small amount of sample in a direct streak, or the toxicity effect with unwashed tissues would reduce detection, as would the fact that plates were incubated for only 21 days. Sakai et al. (1987) compared FAT and CIE with various versions of the dot-blot ELISA procedure, expressing the results in terms of the number of organisms required for a positive result. The value of 10^3 to 10^4 bacteria required for an IFAT result is consistent with the results for IFAT detection of other bacteria, such as Legionella. The rapidity and convenience of testing by some methods, notably ELISA-"sticks" and latex agglutination beads, are a consideration in assessing methods. Dixon (1987a) points out that the higher sensitivity of the ELISA may be balanced by the ease and cost of performing tests by other methods. To some extent, the sensitivity of the detection method chosen will reflect the level of detection desired in the diagnostic process.

7.2 Major surface antigen: Success with the ELISA procedure and with the use of monoclonal antibodies in detection work requires a clear understanding of the antigen(s) being used in the diagnostic procedures. The use of serological methods in identification has been feasible because isolates of R. salmoninarum have a shared major antigen, based on the results of precipitin tests (Bullock et al. 1974). Banowetz (1974) reported finding two soluble antigens, with molecular weights of more than 200,000, which were heat-stable at 60°C for 1 hour. From the results of various immunoelectrophoretic tests, Getchell et al. (1985) claimed that isolates shared seven antigens, including a 57,000 molecular weight surface component.

The "soluble antigen" mixture of Getchell et al. (1985) is prepared from the culture supernate of R. salmoninarum grown as shake-cultures in KDM-2

broth without the serum supplement. This preparation supresses *in vitro* antibody production and affects haematocrit values in coho salmon (Turaga et al. 1987a). An ELISA assay was used to demonstrate an increase in the levels of SA (soluble antigen(s)) in sera of fish infected with BKD (Turaga et al. 1987b). These would seem to be the antigens detected in tissue samples in the techniques described by Sakai et al. (1987), Kimura and Yoshimizu (1981) and Cipriano et al. 1985).

The haemagglutinin on the surface of *R salmoninarum* (Daly & Stevenson 1987) has a molecular weight of 57K, and can be removed from the surface of the cell along with other components when the bacteria are washed with distilled water. Antibody reactions of the separated haemagglutinin suggest this activity is associated with the major surface antigen of the cell (Daly & Stevenson 1988). This component is heat-stable and extracted from the cells by heating (Daly & Stevenson 1987). However, extensive heating of tissue samples in preparing them for diagnostic tests may present problems of antigen denaturation, as suggested by Dixon (1987b).


it's the stress of it all;
I wish
I'd had
my shots!
we're working on it!!!
ASD
EDVD
AHYDRO
erm
Those guys just have no subtlety....
... what a fuss!
BKD
SEE! just in like Jonah!
WHEE!
Hm. didn't those guys look unfamiliar?
Ig
... and pretend you just don't see Ig-y there...
or, you could go for Eggstra-Special Delivery?
The Egg and Us
...sure, dress is superficial, but it can open doors to places...
MACROPHAGE MANOR
(EXCLUSIVE CONDO LIVING)
...washes off a bit too easily...
H2O
HYDROPHOBIC WELCOME MAT
The B.K.D Revue
(song and dance edition)
... not everyone could live here, but we like it...

8.0 SUMMARY OF FUNDED STUDIES

The practical research and the literature review comprising this report were financially supported by a grant of $10,000 from the Great Lakes Fishery Commission. These funds supported a summer research assistant to help with the toxicity studies, and provided supplies and small equipment for some other aspects of the work.

The key acheivements relating to the Great Lakes sponsored work include:

1. Analysis of cysteine and charcoal/serum requirement for growth of R. salmoninarum.
2. Demonstration of conditions for growth in fermenter culture.
3. Improved dilution-spreading technique for culture of organs.
4. Macrophage-enrichment as a potential detection aid.
5. Observation of the significance of fixing methods in FAT and Gram-stain tests.
6. Method established for per os challenge with BKD.

PUBLICATION OF RESULTS:

Daly, J.G. and R.M.W. Stevenson. 1988. Inhibitory effects of salmonid tissue on the growth of Renibacterium salmoninarum. Diseases of Aquatic Organisms 4: 169-171.

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