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Epidemiological studies of the bacterial fish pathogen Yersinia ruckeri

Rodgers, Christopher John

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C. J. RODGERS

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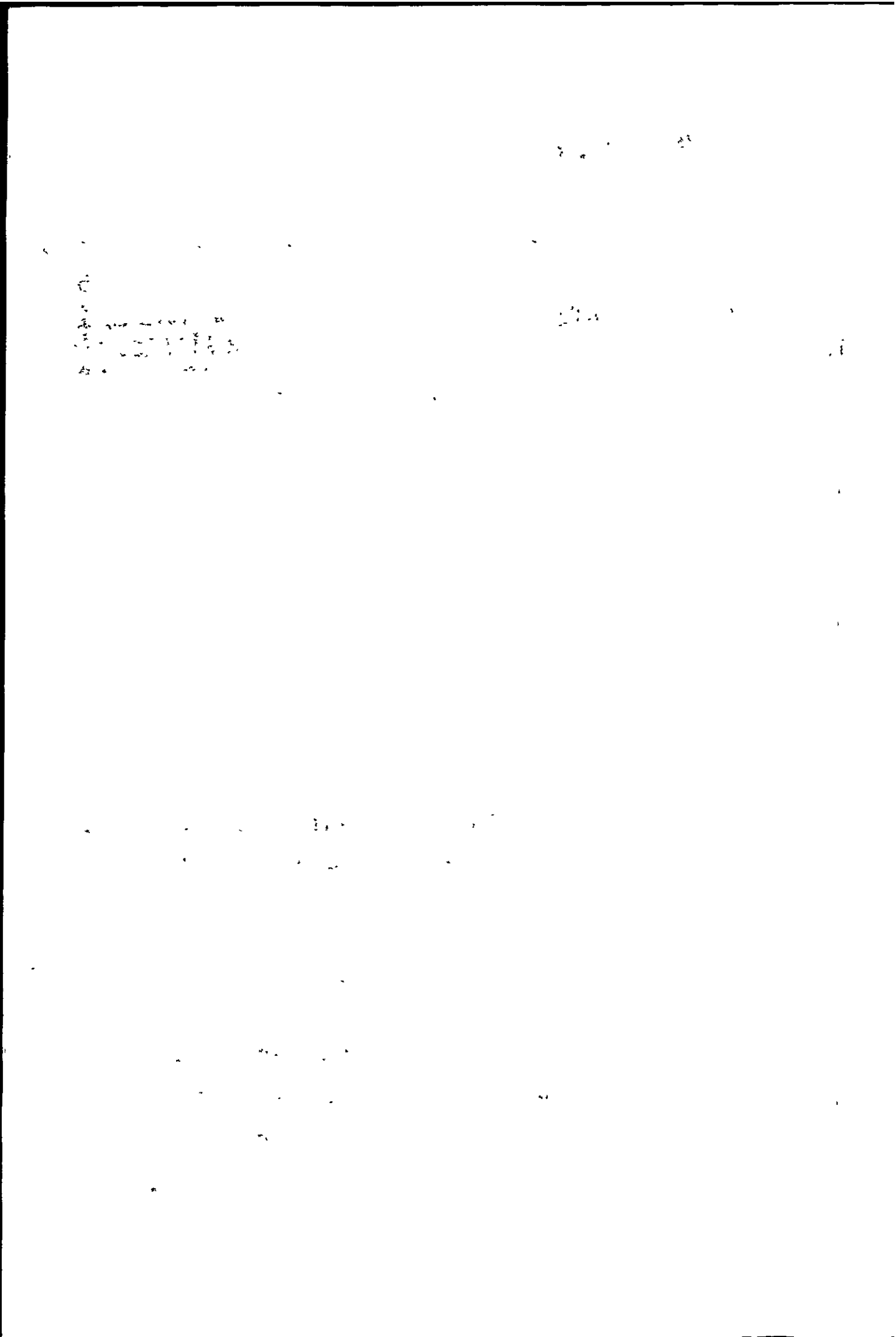

Epidemiological studies of the bacterial fish pathogen *Yersinia ruckeri*

by

Christopher John Rodgers

A thesis submitted to the Council for National Academic Awards in partial fulfilment for the degree of DOCTOR OF PHILOSOPHY.

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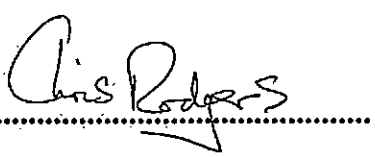
Research was conducted at the Department of Biological Sciences, Polytechnic South West, Plymouth, Devon in collaboration with the Ministry of Agriculture, Fisheries and Food, Fish Diseases Laboratory, Weymouth, Dorset.

DECLARATION

I hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work has been performed by myself, and that all sources of information have been specifically acknowledged.



Christopher J. Rodgers

Colin B. Munn

(Director of Studies)

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ABSTRACT

A new medium, designated Ribose Ornithine Deoxycholate agar (ROD), was developed and used in field trials at two fish farms where fish were known to have ERM. The medium indicated that *Yersinia ruckeri* could occur in faeces four to six weeks before appearing in the kidney. Further epidemiological studies dealing with fish condition, performance, disease signs and water isolation are presented. These factors are discussed in relation to husbandry and management practices.

The minimum inhibitory concentration (MIC) values for 124 strains of *Y. ruckeri* were determined. The effect of oxolinic acid, oxytetracycline and a potentiated sulphonamide, on growth patterns over a 72 h period, was also determined. Results showed the bactericidal or bacteriostatic nature of each antimicrobial agent. It was possible to increase the MIC for oxolinic acid, oxytetracycline and a potentiated sulphonamide using an *in vitro* technique. Attempts to decrease resistance to oxolinic acid were unsuccessful. However, it was possible to reduce the MIC's for oxytetracycline and a potentiated sulphonamide.

The recovery of *Y. ruckeri*, after artificial challenge, was less from the faecal and kidney material of a vaccinated group of fish compared with a non-vaccinated group. An ELISA technique indicated that although there was no detectable serum antibody there was a local mucosal response in vaccinated fish. Uptake of *Y. ruckeri* antigen was demonstrated in vaccinated and non-vaccinated fish using an immunoperoxidase technique. However, only vaccinated fish appeared to take up the antigen by an active process.

A survey of salmonid farms showed that there was a tendency for those sites where ERM had been diagnosed, irrespective of vaccination, to be larger table farms with production ranging up to over 200 tonnes p.a. Certain environmental and husbandry factors were reported as coinciding with the appearance of *Y. ruckeri*. A slight tendency to consider that vaccines had failed in some way was also indicated. This and the use of antimicrobial agents are discussed.

Parts of this work have been published or presented at conferences:

1. Rodgers, C.J. (1991) The usage of vaccination and antimicrobial agents for control of *Yersinia ruckeri*. *Journal of Fish Diseases* **14**, 291-301.
2. Rodgers, C.J. (1991) The control of enteric redmouth disease in fish. *Trout News* **12**, 27-30.
3. Rodgers, C.J. Development of a selective-differential medium for the isolation of *Yersinia ruckeri* and its application in epidemiological studies. *Journal of Fish Diseases* (in press).
4. Rodgers, C.J. (1991) In vitro resistance of *Yersinia ruckeri* to three fisheries antimicrobial agents. E.A.F.P. 5th International Conference, Budapest, Hungary.
5. Rodgers, C.J. (1990) The usage of vaccination and antimicrobial agents for control of *Yersinia ruckeri*. Bacterial Diseases of Fish Conference, Stirling, Scotland.
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I would like to dedicate this thesis to my family, but particularly my parents Betty and John Rodgers for their support and understanding.

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CONTENTS

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

ABBREVIATIONS

A_x	absorbance at a specific wavelength in nanometres
AB	alcian blue
ANOVA	analysis of variance
BDH	British Drug Houses
BG	Brilliant Green agar
BHI	brain heart infusion
BSA	bovine serum albumin
c.f.u.	colony forming units
cm	centimetre
°C	degree centigrade
DAB	3,3'-diaminobenzidine tetrahydrochloride
DCLS	Desoxycholate Citrate Lactose Sucrose agar
DESOX	Desoxycholate agar
DNA	deoxyribonucleic acid
EDTA	ethylene diamine-tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
EMB	Eosin Methylene Blue agar
ERM	enteric redmouth disease
FCA	Freund's complete adjuvant
FDBG	freeze-drying broth with the addition of 0.1% glycerol
FDL	Fish Diseases Laboratory, Weymouth
FIA	Freund's incomplete adjuvant
g	gram
x g	acceleration due to gravity
HE	Hektoen Enteric agar
HSF	heat sensitive factor
h	hour
HCl	hydrochloric acid
im	intramuscular
io	intraoesophageal
ip	intraperitoneal
kg	kilogram
l	litre
lb	pound (weight)
LD_{50}	lethal dose 50 (i.e. 50% mortality)
M	molar
McC	McConkey agar
µg	microgram
µl	microlitre
µm	micrometre
mg	milligram
ml	millilitre
mm	millimetre
MIC	minimum inhibitory concentration
min	minute
MS222	3-aminobenzoic acid ethyl ester
NA	nutrient agar
nm	nanometre

OA	oxolinic acid
O/F	oxidative/fermentative reaction (Hugh and Leifson)
OGC	optimum growth concentration
OPD	o-phenylenediamine
OT	oxytetracycline
%	percent
p	statistical probability
PAS	periodic acid Schiff
pers. com.	personal communication
PBSa	phosphate buffered saline
PBST	phosphate buffered saline + 0.5% tween 20
PCB	plate count broth
PMSF	phenylmethylsulphonylfluoride
pnr	positive-negative ratio
PSu	potentiated sulphonamide
ROD	ribose ornithine deoxycholate
RPS	relative percentage survival
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	seconds
TM	trimethoprim
Tris	Tris (hydroxymethyl) amino methane
TBS	Tris buffered saline
TSA	tryptone soya agar
TSB	tryptone soya broth
TVC	total viable count
VC	viable count
VRB	Violet Red Bile agar
v/v	volume/volume
w/v	weight/volume
XLD	Xylose Lysine Deoxycholate agar

CHAPTER 1

INTRODUCTION

Fish farming is not a new phenomenon, in fact this type of culture can be traced back over many centuries. It is widely believed that the origins of rearing fish for food lie with the early attempts at carp production in China (Shepherd, 1988a). However, the increase in aquaculture, particularly the rearing of salmonids, has been most marked in the last four or five decades. Technical progress has led to a fish farmer now being able to produce marketable fish all year round. Unfortunately the pressure to produce more fish has meant that intensification can lead, in some cases, to poor husbandry and management practices. These less than adequate measures often mean that water quality suffers a decline. Consequently, the optimum requirements for growth, such as low stocking rates, sufficient dissolved oxygen, efficient nutrition and low levels of excretory products, become unbalanced (Shepherd, 1988b). These in turn promote unacceptable levels of stress in fish being increasing reared under unnatural environmental conditions. Continual stress does not allow fish to adapt to their environment and the resultant impairment in the immune response gives rise to lowered disease resistance.

Fish diseases continue to threaten the commercial viability of many intensive systems (Shepherd, 1988b) and bacterial infections in particular are capable of significant economic implications for fish farmers.

Enteric redmouth disease (ERM) is an acute-to-chronic bacterial disease of salmonid fish. The causal agent, *Yersinia ruckeri*, has become increasingly widespread in farmed stocks of rainbow trout (*Oncorhynchus mykiss*) in the USA since it was first recognised as a serious infection of salmonid fish in the late 1950's on farms in the Hagerman Valley, Idaho (Rucker, 1966). *Y. ruckeri* was classified as a member of the Enterobacteriaceae by Ewing *et al.* in 1978 and in the same year the first isolation occurred in the UK (Sweeting, pers. com.). More recently, however,

the disease has been confirmed in central Europe with outbreaks reported within the last 10 years from Denmark (Dalsgaard *et al.*, 1984), England (Roberts, 1983), Finland (Rintamäki *et al.*, 1986), France (Lesel *et al.*, 1983a, 1983b), Ireland (McArdle and Dooley-Martyn, 1985), Italy (Giorgetti *et al.*, 1985), Norway, (Sparboe *et al.*, 1986), Scotland (Frerichs and Collins, 1984), Spain (De La Cruz *et al.*, 1986), Switzerland (Meier, 1986), West Germany (Fuhrmann *et al.*, 1983) and Yugoslavia (Ocvirk *et al.*, 1988).

Clinical cases of ERM have been described in salmon, trout and white fish (Rintamäki *et al.*, 1986) but *Y. ruckeri* has also been isolated from cyprinid fish in freshwater (Roberts, pers. com.), freshwater invertebrates (Dulin *et al.*, 1976) and the muskrat (Stevenson and Daly, 1982).

Signs of ERM may include haemorrhages in various tissues and organs, particularly around the mouth, in musculature, body fat, viscera and lower intestine. In addition, clinical infection can be characterised by a yellow, often mucoid, discharge from the vent.

Asymptomatic carrier fish can excrete large numbers of *Y. ruckeri* into the water via the faeces (Busch, 1978, 1983; Busch and Lingg, 1975; Rucker, 1966) and approximately 25% of rainbow trout can carry *Y. ruckeri* in the lower intestine (Busch and Lingg, 1975). Cyclical shedding can also precede systemic infection and resultant mortalities. However, this can lead to localisation or colonisation of *Y. ruckeri* in the lower intestine of surviving fish. In addition, even immunised fish can become temporary carriers of *Y. ruckeri* in the intestine but only 25-50% of such carriers can be identified by using classical methods of isolation from kidney and spleen material. These cyclical patterns of infection are not only affected by seasonal variations in water temperature but also by stocking densities and grading.

Although *Y. ruckeri* grows well on general purpose bacteriological media, there are problems when isolation from faeces is attempted. A selective isolation medium has been developed for *Y. ruckeri* (Waltman and Shotts, 1984) but some

published reports have indicated that there are limitations in its use (Hastings and Bruno, 1985) and it can be less satisfactory than tryptone soya agar (TSA) for recovery from both kidney and faecal samples when used for field studies (Rodgers and Hudson, 1985).

For this reason, the development of a selective-differential medium for the isolation of *Y. ruckeri* from carrier and clinically infected rainbow trout was one of the principal aims of this project. This medium, designated ribose ornithine deoxycholate agar (ROD), subsequently aided the detection of *Y. ruckeri* in faecal material and allowed the epidemiology of enteric redmouth disease to be studied in greater detail. Following laboratory trials, the medium was used regularly at two fish farms in southern England to study the carrier status of both vaccinated and non-vaccinated fish. In addition, this medium proved useful for isolating *Y. ruckeri* from river water samples.

Prolonged use of antimicrobial agents leading to potential misuse, can cause the appearance of drug resistance in strains of *Y. ruckeri* and this has severe implications for future treatment programmes on fish farms. Multiple resistance to tetracyclines and sulphonamides has been demonstrated (De Grandis and Stevenson, 1985; Post, 1987). Although the treatment of clinical outbreaks of ERM should follow laboratory antimicrobial sensitivity testing, the dosages used for salmonid fish have varied widely. As a result, a problem may occur in the weeks or months following cessation of antimicrobial treatment when ERM can recur. Consequently, a further aim of this project was to investigate the potential of *Y. ruckeri* to develop *in vitro* resistance to three fisheries antimicrobial compounds. In addition, subsequent loss of this resistance was studied and the bactericidal/bacteriostatic nature of the minimum inhibitory concentration of each compound was determined.

The general immune response of fish to the antigenic components of disease agents is already well documented (Corbel, 1975; Dorson, 1981). Some reports indicate that rainbow trout can develop agglutinating antibodies against soluble or particulate antigens (Busch, 1978) but others show a highly variable response which has led to the conclusion that protection may be independent of agglutinating antibody and possibly due to cell-mediated immunity instead (Cossarini-Dunier, 1986b). The early results from the field epidemiological studies in this project indicated that faecal carriage and subsequent clinical infection in vaccinated fish were inconsistent and suppressed when compared to non-vaccinated fish. Consequently the role of the intestinal immune response, particularly at the mucosal level, was further studied under laboratory conditions using histochemical techniques and cytochemical staining. The localisation of *Y. ruckeri* antigen after artificial anal infection was followed over a ten day period in vaccinated and non-vaccinated fish.

Since ERM is not a notifiable disease in England and Wales, background details are scarce. Consequently, further information on the potential of *Y. ruckeri* as a serious economic bacterial pathogen affecting salmonid culture would be a valuable aid to the understanding of the disease process. For this reason, an epidemiological study was conducted and showed that vaccination has a beneficial influence on the level of infection in farmed rainbow trout. Certain factors which seemed to reduce the efficiency of vaccination were investigated. Fish condition, particularly at the time of vaccination, had an important bearing on subsequent survival and performance. In addition, the early field studies of this project indicated that the design, husbandry practices and management of a fish farm may also affect the performance of fish exposed to repeated natural infections of *Y. ruckeri.* Consequently, despite vaccination, there is still a need to use expensive antimicrobial therapy in certain circumstances. Therefore, a survey was designed in order to understand more fully these potential problems in the fish farming industry. Information about the current usage of vaccines and antimicrobial agents for ERM

was collected by designing a confidential questionnaire which was sent to selected farms. The findings were used to give an insight into efficacy, cost and the incidence of ERM outbreaks on salmonid farms of all sizes with a variety of water sources.

. The initial aims of this project concerned certain epidemiological aspects of *Y. ruckeri*. However, the results of field trials at two fish farms, intensively rearing rainbow trout, dictated the progression of the experimental work and resulted in the following objectives:

a) the development of a selective-differential medium for the isolation of *Y. ruckeri* from carrier and clinically infected rainbow trout, to allow the epidemiology of enteric redmouth disease to be studied in greater detail.

b) an investigation into the potential of *Y. ruckeri* to develop *in vitro* resistance to three fisheries antimicrobial compounds (oxolinic acid, oxytetracycline and a potentiated sulphonamide).

c) the role of the intestinal immune response, particularly at the mucosal level, and the localisation of *Y. ruckeri* antigen was followed in vaccinated and non-vaccinated fish.

d) information on the potential of *Y. ruckeri* as a serious economic bacterial pathogen and information about the current usage of vaccines and antimicrobial agents for ERM was gathered by using a confidential questionnaire which was sent to selected salmonid fish farms.

CHAPTER 2 - LITERATURE REVIEW

YERSINIA RUCKERI, THE CAUSAL AGENT OF ENTERIC REDMOUTH DISEASE (ERM) IN FISH

2.1 AETIOLOGY AND CLASSIFICATION

2.1.1 Taxonomic position

Ross *et al.* (1966) first described a Gram negative bacterium that had been isolated frequently from kidney material of rainbow trout (*Oncorhynchus mykiss*) infected with "redmouth" disease. This rod-shaped, oxidase-negative, catalase positive, fermentative isolate was thought to be a member of the Enterobacteriaceae and was termed the redmouth or RM bacterium. However, it was not until 1978 that Ewing *et al.* further characterised the organism and proposed the name *Yersinia ruckeri* as the causal agent of enteric redmouth disease (ERM). This study used the deoxyribonucleic acid (DNA) hybridization technique to confirm that the RM bacteria were members of the Enterobacteriaceae and comprised a single species. In addition, representative strains appeared to be 30% related to *Serratia* as well as to *Yersinia*. The biochemical reactions and a guanine-cytosine (G+C%) ratio between 47.5 and 48.5%, however, indicated that they were most closely related to the yersiniae. These values were confirmed in a study by Stevenson and Airdrie (1984) when all but one of their strains fell within the range reported by Ewing *et al.* (1978). A further characterisation of *Y. ruckeri* was made by O'Leary *et al.* (1979); their reported G+C% content, biochemical and physical characteristics supported *Y. ruckeri* as the genus and species designation for the ERM bacterium. Nevertheless, Bercovier and Mollaret (1984) consider that the bacterium may be a genus by itself whereas De Grandis et al. (1988) indicate that its position should be re-evaluated.

In contrast to the DNA hybridization work of Ewing *et al.* (1978), Green and Austin (1983) concluded, after a numerical phenetic study, that *Y. ruckeri* was more closely related to *Salmonella arizonae*. The results of this study indicated that there

were comparatively few phenotypic traits to distinguish *Y. ruckeri* from *S. arizonae.* These included degradation of gelatin and Tween 20, fermentation of arabinose and rhamnose, and the production of arginine dihydrolase and hydrogen sulphide. In addition, there was some evidence for a low level antigenic relationship between the taxa. Earlier work had even indicated that *Y. ruckeri* may be related to the *Erwinia* group (Ross *et al.*, 1966; Busch 1978). In light of these apparent contradictions it would seem that further work is required in order to confirm the taxonomic position of *Y. ruckeri.* There seems to be no doubt that known strains are members of the Enterobacteriaceae. However, additional experimental evidence should show whether representative isolates either belong in another genus or are perhaps distinct enough to form a new genus.

Y. ruckeri is generally considered to be a short (0.5 x 1.5-2.0 μm) Gram-negative peritrichously flagellated bacillus. Variations in size have been recorded between strains and with the age of the culture (Bullock *et al.*, 1978). Davies and Frerichs (1989) found that the majority of *Y. ruckeri* strains could be divided into two morphological groups, namely, very short, coccobacillary forms (0.5 x 0.5-1.0 μm) or medium sized rods (0.5 x 1.0-2.0 μm). The remaining strains in their study differed in that the cells were longer (0.5 x 1.0-3.0 μm) and also occurred with a high frequency of chain formation. This difference in size was also noted by Austin *et al.* (1982) but, in addition, these workers reported filamentous cells and long, thin rods (0.5 x 4.0 μm). This cell size difference was less marked on nutrient rich media and more apparent on media containing high (3%) salt levels.

Cultures of *Y. ruckeri* in the log phase of growth are motile between 18-27°C, although the optimum temperature for growth is 22-25°C. However, at the lower temperature of 9°C flagella can be present but non-functional, whilst at the higher temperature of 35°C they are absent (O'Leary, 1977). In a study by Davies and Frerichs (1989), 82% of strains were motile at 22°C. However, this investigation was heavily biased geographically in that 90% of the non-motile strains were isolated in the UK. This type of finding should be verified by other workers since the strains

examined in the study by Davies and Frerichs (1989) were collected largely from other culture collections. Consequently, as the authors admit, the strains could have been subjected to an element of selection by the contributors. Nevertheless, Austin *et al.* (1982) found that there were discrete differences in the number of flagella around cells also at 22°C, with longer rods having twelve peritrichous flagella in contrast to the three or four found around smaller cells.

2.1.2 Growth media

Y. ruckeri grows well on general purpose bacteriological media such as tryptone soya agar (TSA). Normally, colonies are 1-2 mm in diameter, smooth, round, raised and with entire edges. Colonies are non-fluorescent under ultra-violet light but are slightly iridescent under reflected light. In addition, they are white-cream in colour, translucent and exhibit a butyrous type of growth. There is a lack of pigment and colour does not diffuse into the surrounding media (Ross *et al.*, 1966; Busch, 1978; 1983). Exceptions to these characteristics have been noted by Bullock *et al.* (1978) who reported that 15 strains formed colonies 2-3 mm in diameter with entire edges but three strains produced 3-4 mm colonies with irregular edges.

Blood enriched agar has also been used for primary isolation of *Y.ruckeri* from kidney, spleen or liver material (Fuhrmann *et al.*, 1983; Dalsgaard *et al.*, 1984; Rübsamen and Weis, 1985; Bragg and Henton, 1986; Meier, 1986; Rintamäki *et al.*, 1986; Sparboe *et al.*, 1986; Ocvirk *et al.*, 1988; Willumsen, 1989).

Y. ruckeri is reported to give typical red to magenta colonies on deoxycholate-citrate-mannitol agar (Y-M) (Hunter *et al.*, 1980). The use of Y-M medium was also mentioned by Stevenson and Daly (1982), especially in conjunction with primary enrichment in tryptic soy broth (TSB). This type of enrichment for kidney, heart, spleen or liver material, prior to streaking onto routine solid agars, has also been used by Daly *et al.* (1986). Rimler-Shotts agar (Shotts and Rimler, 1973) has been proposed as a primary differential medium (Shotts and Bullock, 1975) for isolation of *Y. ruckeri.* In addition, an alkaline over acid reaction is produced on triple sugar iron (TSI) agar (O'Leary *et al.*, 1979; Cipriano *et al.*, 1986; De La Cruz *et al.*, 1986).

A selective isolation medium containing Tween 80, sucrose and bromothymol blue was developed by Waltman and Shotts (1984) for the isolation and differentiation of *Y.ruckeri.* Colonies of *Y. ruckeri* appear green with a zone of Tween 80 hydrolysis. Unfortunately, experience with this medium in other countries has suggested that there are limitations when it is used for detecting *Y. ruckeri.* A study by Hastings and Bruno (1985) indicated that UK strains of *Y. ruckeri* promoted weak colour changes in the medium. In addition, the strains failed to hydrolyse Tween 80. This finding was also reported by Rodgers and Hudson (1985) who also showed that the medium was less satisfactory than TSA for recovery from both kidney and faeces samples when used for field studies. A more recent publication (Shotts, 1991) has revealed an unfortunate printing error in the original Waltman and Shotts (1984) paper that lead to a bromothymol blue concentration of 0.003 g l^{-1} instead of 0.03 g l^{-1}. In addition, pH stabilization was made more difficult by the use of rancid Tween 80. Nevertheless, the use of this medium, in the UK at least, would still be very limited since the majority of UK isolates of *Y. ruckeri* are Tween-negative and non-motile (Davies and Frerichs, 1989). This is in contrast to strains isolated in USA (Waltman and Shotts, 1984; Shotts, 1991).

2.2 NATURE OF ERM

Salmonid fish with ERM present disease signs which are easily confused with other bacterial haemorrhagic septicaemias. Such non-specific signs, usually occurring during early or sub-acute infections, are for example also seen where pseudomonads, motile aeromonads or members of the Vibrionaceae are implicated (Ross *et al.*, 1966; Rucker, 1966; Busch, 1983).

2.2.1 External disease signs

In the early stages of ERM, fish appear lethargic, dark in colour and often exhibit a wasting condition due to anorexia. There may also be a characteristic reddening of the mouth, tongue, jaw area and operculum (Rucker, 1966; Busch, 1983; Warren, 1983). However, during UK outbreaks of the disease the classic "red

mouth" sign is not common (Roberts, 1983; Frerichs *et al.*, 1985) but is present occasionally in late stages of chronic infection (Rodgers, unpublished observation). In addition, further subcutaneous haemorrhages, caused by a loss of capillary integrity, can be seen at the base of the fins, between the rays, on the ventral surfaces and around the anus. The gills may also appear haemorrhagic towards the tips of the filaments (Rucker, 1966; Busch, 1983; Warren, 1983).

Unilateral or bilateral exophthalmia and haemorrhage in the iris, as a result of tissue oedema, occurs in later stages of infection. Subsequent increased fluid pressure in the eye can lead to rupture, lens opacity and blindness (Rucker, 1966; Busch, 1983; Warren 1983). Fish in this advanced stage of infection are often seen around the periphery of tanks and tend to collect towards the outlet screens (Busch, 1983).

2.2.2 Internal disease signs

Internal signs can be characterised by petaechial haemorrhages on the liver, pyloric caecae, adipose tissue, pancreas, swim bladder and body musculature. In addition, the spleen is enlarged and haemorrhages can occur in gonad tissue (Rucker, 1966; Busch, 1983; Warren, 1983; Sparboe *et al.*, 1986). As well as "red mouth", the appearance of the lower intestine is a distinctive feature in some cases of ERM. The intestine becomes filled with an opaque, often yellow, mucoid or watery material and the intestinal structure itself becomes flaccid, translucent, haemorrhaged and devoid of food (Busch, 1983; Warren, 1983). Accumulation of blood tinged serosanguinous fluid often results in the abdomen becoming distended (Rucker, 1966; Busch, 1978). Slow, chronic forms of ERM result in a progressive necrosis and sloughing of the intestinal mucosa (Busch, 1978, 1983). As a result, poor nutritional condition and secondary infection can develop.

The various signs associated with ERM occur at different stages of the disease and a wide variety can be seen in a population of infected fish during a single epizootic (Rucker, 1966; Wobeser, 1973).

2.2.3 Histopathological signs

In some acute forms of the disease there may even be no outward gross signs apparent, although the bacterium can usually be found in the blood (Busch, 1978). Histopathological examination indicates that ERM is similar to other bacterial haemorrhagic septicaemia conditions. Disease progression is characterised by bacterial colonisation of the capillaries of the highly vascularised tissues such as the gills, muscle, kidney, liver, spleen and heart (Busch, 1978; 1983). This results in haemorrhages, systemic tissue oedema caused by disruption of the ionic balance. Necrosis of haematopoietic tissue in the spleen and kidney leads to anaemia with consequent lowered haematocrit values (Busch, 1983). Wobeser (1973), in a detailed study also observed a severe loss of haematopoietic tissue in the anterior and posterior kidney. In addition, there was dilation of the sinusoids, a decrease in the size and numbers of pigment cells, and swelling and vacuolation of reticuloendothelial cells. In the spleen there was total loss of the normal lymphoid follicular structure. Pathological studies of liver demonstrated that there were few vacuoles in the hepatocytes of diseased fish which contrasted with the prominent vacuolation in apparently healthy fish. Mononuclear cells, resembling lymphocytes, were present in the periportal areas of diseased fish livers.

2.2.4 Effect of fish size

Although all sizes of fish may be affected by ERM, Rucker (1966) reported that fingerling rainbow trout of approximately 7.5 cm (3 inches) in length are most commonly affected. Larger fish of 12.5 cm (5 inches) have a more chronic but less severe form of the disease. In addition, Busch (1978) states that peracute to acute infection usually occurs in the spring and early summer in curiously named "young-of-the-year fish", whereas acute to subacute infections usually occur in yearling fish in the autumn and early winter. Mortalities in 25-30 cm fish in Europe have been reported by Bentley (1982), Fuhrmann *et al.* (1983) and Meier (1986). Outbreaks of ERM in smaller fish between 5-20 cm were noted by Rübsamen and Weis (1985), De La Cruz *et al.* (1986) and Meier (1986).

2.2.5 Effect of temperature

The disease is most severe at a water temperature of 15-18°C and this is confirmed in outbreaks recorded by Dalsgaard *et al.* (1984) and Ocvirk (1988) with Rübsamen and Weis (1985) reporting rising mortalities at 20°C. Rucker (1966) indicated that ERM is less severe at 10°C or below. Handling stress can trigger outbreaks of ERM (Sparboe *et al.*, 1986), especially when water temperatures exceed 13°C (Busch and Lingg, 1975; Dulin *et al.*, 1976). Hunter *et al.* (1980) showed in a laboratory study that heat stress can play an important part in triggering the release of *Y. ruckeri* from carrier fish. Their study indicated that only carriers stressed by being held in water with the temperature raised to 25°C transmitted *Y. ruckeri* to healthy recipient fish. Unstressed carrier fish did not transmit the pathogen to healthy fish. Such a high temperature would have reflected an artificial situation and data comparing more natural elevated temperatures of 15°C to 20°C would have been useful. Heat-stressed rainbow trout exposed to *Y. ruckeri* in water at 20°C for 1 hour were shown by Busch and Lingg (1975) to produce an LD_2 rate of total mortality after 7 days. This method of expressing mortalities is normally reserved for LD_{50} experiments. Consequently, the use of lethal dose (LD) to denote such a low mortality (2%) could be confusing.

2.2.6 Disease course

The incubation time for the development of ERM infection to the point of first mortality is approximately 5-7 days at 15°C in newly exposed fish as reported in the review by Busch (1983). However, this time period can be affected by the stress factors already mentioned. Consequently, fish with prior exposure to *Y. ruckeri* may start dying within 3-5 days given unfavourable environmental conditions.

The acute form of the disease can result in a rapid course of infection lasting 4-10 days (Busch, 1978). These initial acute epizootics can lead to losses of 30-70%, depending on the size of fish, water temperature, stress level and the relative susceptibility (McDaniel, 1971; Wobeser, 1973; Mitchum, 1981). Mortality in acute to subacute infections can be 10-50% but over a 2-6 month period.

In addition to the more rapid disease form, ERM can cause sustained, low-level mortalities lasting several months (Bullock, 1984; Rodgers, unpublished observation) that have the potential to cause heavy losses given unfavourable on-farm conditions. This chronic form of the disease may result in an acute epizootic following stress arising from handling, low dissolved oxygen, over-crowding, fluctuating water temperatures, increased suspended solids, elevated levels of ammonia or other waste products (Busch, 1978, 1983; Austin, 1982; Bullock, 1984; Frerichs *et al.*, 1985). However, very low level losses of approximately 10% can also occur (Busch, 1978). Fish that recover show a recurrent, low level mortality and a poor conversion rate (Horne *et al.*, 1984). In addition, Rucker (1966) mentioned that the nutritional state of affected fish was important and fat or debilitated fish were more susceptible to severe epizootics. Dear (1988) reported that mortalities of Atlantic salmon (*Salmo salar*) at a fresh water Scottish loch site were limited to cages where fish had suffered environmental stress caused by fouled nets.

2.2.7 Carrier status

Reservoirs of infection leading to recurrent ERM problems and new outbreaks of disease are primarily attributable to the presence of asymptomatic carrier fish (Bullock, 1984; Busch, 1978, 1983; Busch and Lingg, 1975; Rucker, 1966; Warren, 1983). These fish have the ability to excrete large numbers of *Y. ruckeri* into the water via the faeces (Busch, 1978, 1983; Busch and Lingg, 1975; Rucker, 1966). The existence of a carrier state was demonstrated by Busch and Lingg (1975) in a key experiment who showed that approximately 25% of rainbow trout carried *Y. ruckeri* in the lower intestine 45 days after surviving an artificial infection. In addition their work also indicated subsequent cyclical shedding of 36-40 day intervals at 14.5°C, that could precede systemic infection and mortality by 3-5 days. This led to localisation of *Y. ruckeri* in the lower intestine of 50-75% of survivors, 60-65 days following infection. Hunter *et al.* (1980) found that colonisation of the lower intestine of experimentally infected fish was detectable among survivors 20 days after

infection. They also showed that 20% of the remaining fish were positive for *Y. ruckeri*. However, this increased to 36% after 25 days and remained at this level until 60 days post infection. The percentage of carriers then declined, eventually reaching an undetectable level 80 days after the initial infection. In addition, Hunter *et al.* (1980) reported that when immunised fish were challenged with *Y. ruckeri* they became temporary carriers of the bacterium for up to 3 days but were unable to transmit the infection to healthy recipient fish. Busch and Lingg (1975) concluded that the cyclical presence of *Y. ruckeri* in the intestine would lead to only 25-50% of carriers being identified using classical methods of isolation from kidney and spleen. McDaniel (1971) showed yearly cyclical mortality patterns in a hatchery population chronically infected with ERM. This phenomenon has also been reported by Lesel *et al.* (1983a) and Rübsamen and Weis (1985), even after antibiotic treatment. These cyclical patterns are affected by seasonal variations in water temperature, stock densities and grading, as well as natural resistance and immunity. Rübsamen and Weis (1985) reported that losses in treated fish declined only gradually after the use of antibiotics. It was only when the fish were transferred to a larger pond with consequent reduced stocking density that further mortalities finally ceased. Ross *et al.* (1966) showed that *Y. ruckeri* could be recovered from kidney tissue of rainbow trout 30 and 60 days after a water-borne challenge. Willumsen (1989) reported a latent carrier status of 2-10% in Atlantic salmon following treatment with oxytetracycline.

2.2.8 Transmission

Transmission of *Y. ruckeri* is primarily horizontal (from fish to fish) through the water (Busch, 1983) and several workers have reported such a spread in both hatchery and laboratory fish populations (Rucker, 1966; Dulin *et al.*, 1976-cited by Bullock, 1984; Hunter *et al.*, 1980). Water which has been inhabited by other fish with ERM can also infect other susceptible fish (Ross *et al.*, 1966; Rucker, 1966; Bullock *et al.*, 1976). Dulin *et al.* (1976) reported that infected male broodstock used to fertilize ova did not lead to transmission after disinfection of the egg with an

unnamed iodophor. In fact, vertical transmission via the egg had not, until recently, been shown by any study. However, the first reported outbreak of ERM from Venezuela indicated that the importation of infected eggs may have been the source of the disease (Anon, 1990). The role of invertebrates in transmission of *Y. ruckeri* is unclear, but crayfish inhabiting hatchery water supplies have been found to be carrying the pathogen (Dulin *et al.*, 1976-cited by Post, 1987). Apparently healthy coarse fish (cyprinids) can also carry the organism and may excrete it into the surrounding water (Roberts, pers. com.). Under these conditions, it is a distinct possibility that infected fish farms which are river fed could recycle the disease (MAFF, 1984).

Potential inter-continental spread of ERM has been inferred by Michel *et al.* (1986) with the important finding of *Y. ruckeri* being isolated from bait fish (minnows) imported into France from USA.

2.3 HOST AND GEOGRAPHIC RANGES

ERM was first recognised as a serious infection of salmonid fish in the 1950's at a hatchery in Idaho, USA (Rucker, 1966). *Y. ruckeri*, the causal agent of ERM, is now known to be widespread in farmed populations of salmonids in the USA and Canada (Bullock *et al.*, 1978). The disease is most prevalent in rainbow trout. It has also been reported from cut-throat trout (*Salmo clarki*), coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), Atlantic salmon (Bullock and Snieszko, 1979), brook trout (*Salvelinus fontinalis*) (Cipriano *et al.*, 1987), brown trout (*Salmo trutta*) and sockeye salmon (*Oncorhynchus nerka*) (McDaniel, 1979). Non-salmonids such as emerald shiners (*Notropis atherinoides*) have been reported as undergoing a natural epizootic of ERM (Mitchum, 1981). In addition, Canadian studies have demonstrated isolation of *Y. ruckeri* from moribund cisco (*Coregonus artedii*) and the intestine of a muskrat (*Ondatra zibethicus*) (Stevenson and Daly, 1982). Dwilow *et al.* (1987) reported isolation of *Y. ruckeri* from burbot (*Lota lota*) during routine bacteriological screening of fish in the

Mackenzie River, North West Territories. There has even been a disturbing report of a strain of *Y. ruckeri* being isolated from the bile of a human clinical patient (Farmer *et al.*, 1985). However, no further published information was presented and consequently the potential clinical significance is not known.

Reports of *Y. ruckeri* in countries outside North America and Canada started in 1978 with the first isolation occurring in the UK (Sweeting, pers. com.), from brown trout on a fish farm in southern England. Since that time outbreaks have been reported in Australia (Bullock *et al.*, 1978; Llewellyn, 1980), Bulgaria (Gelev *et al.*, 1984), Chile (Enriquez and Zamora, 1987), Denmark (Dalsgaard *et al.*, 1984), England (Roberts, 1983), Finland (Rintamäki *et al.*, 1986), France (Lesel *et al.*, 1983a, 1983b), Ireland (McArdle and Dooley-Martyn, 1985), Italy (Giorgetti *et al.*, 1985), Norway (Sparboe *et al.*, 1986), Scotland (Frerichs and Collins, 1984), South Africa (Bragg and Henton, 1986), Spain (De La Cruz *et al.*, 1986), Switzerland (Meier, 1986), Venezuela (Anon, 1990); West Germany (Fuhrmann *et al.*, 1983) and Yugoslavia (Ocvirk *et al.*, 1988).

Clinical cases of ERM in fish species outside North America and Canada have been described in salmon, trout and white fish by Rintamäki *et al.* (1986) and Dear (1988). In addition, *Y. ruckeri* has also been isolated from other fish in freshwater, such as carp, *Cyprinus carpio* (Fuhrmann *et al.*, 1984; Enriquez and Zamora, 1987), eels, *Anguilla anguilla* (Fuhrmann *et al.*, 1984; Lehmann *et al.*, 1989), goldfish, *Carassius auratus* (McArdle and Dooley-Martyn, 1985), grayling, *Thymallus thymallus* (Ceschia *et al.*, 1984), bait fish (minnows), *Pimephales promelas* (Michel *et al.*, 1986), turbot, *Scopthalamus maximus* (Baudin-Laurencin and Tixerant, 1985), and sturgeon, *Acipenser baeri* (Vuillaume *et al.*, 1987). In addition, Willumsen (1989) reported isolating *Y. ruckeri* from wild populations of arctic char (*Salvelinus alpinus),* coal fish (*Pollachius virens*) and even seagulls (*Larus* sp.) caught in the vicinity of fish farms rearing Atlantic salmon (*Salmo salar*) infected with *Y. ruckeri.* This type of local vector was suggested as an explanation for the rapid spread of

ERM, particularly in Norway. Lewis (1981) described an artificial infection of 25 cm laboratory-reared channel catfish (*Ictalurus punctatus*) with *Y. ruckeri*.

Bangert *et al.* (1988) reported isolating *Y. ruckeri* from the faeces of a kestrel (*Falco sparverius*) and a turkey vulture (*Cathartes aura*). However, although the diet of a turkey vulture occasionally consists of live fish, kestrels are not known to eat fish at all. The significance of this finding was not discussed but it has obvious implications for transmission.

2.4 VIRULENCE AND PATHOGENICITY

The early work by Ross *et al.* (1966) and Rucker (1966) first showed that an isolate could be a primary pathogen of rainbow trout when the bacterium was successfully transmitted by a water-borne challenge. Bullock *et al.* (1976) found that *Y. ruckeri* and *Aeromonas salmonicida* were equally pathogenic for fingerling Atlantic salmon. The study showed that intraperitoneal (ip) injection of 5×10^5 cells of either pathogen killed all salmon within 96 h, but within 72 h at the higher concentration of 5×10^7 cells. However, a water-borne challenge was less effective. Parenteral infection of rainbow trout by ip injection was also used by Busch and Lingg (1975) to show that a surprisingly low average of 30 bacteria constituted an LD_{50}. In fact, only 18 bacteria, similarly administered, resulted in an unconventially expressed LD_{26}. The infectivity of the pathogen was indicated when it was possible to recover *Y. ruckeri* from all fish sacrificed after 21 days.

The LD_{50} dose achieved by immersion varies with the size of fish and the duration of exposure (Busch, 1983). Nevertheless, this author reported that a 1 hour exposure to a diluted 24 h broth grown culture could result in not only a 100% infection but also a 30-70% mortality within 28 days at 15°C. The author does not make clear whether this implies a 100% carrier state followed by a variable mortality of 30-70% in repeated experiments. This type of detail is important since reliable challenges leading to reproducible LD_{50} rates are difficult to achieve, without careful

experimentation designed to standardize the many variables involved (Furones, pers. com.).

2.4.1 Virulence of serotypes

O'Leary *et al.* (1979) showed that division of strains into the two main serotypes (I and II) of *Y. ruckeri* correlated biochemically with the ability of some strains to ferment sorbitol. Other studies found that strains that did not ferment sorbitol were virulent for fish (Bullock *et al.*, 1978; McCarthy and Johnson, 1982) and that serotype II was avirulent (McCarthy and Johnson, 1982). It has also been reported that serotype I can be more virulent than either serotype II or serotype III strains for rainbow trout, following water-borne exposure (Bullock and Anderson, 1984). Serotype I sorbitol-negative isolates cause most epizootics of enteric redmouth disease (Bullock *et al.*, 1978). However, Cipriano *et al.* (1986) reported an epizootic in chinook salmon caused by a sorbitol positive serotype II strain. Laboratory virulence studies also showed that the isolate was pathogenic, after an immersion challenge, for brook trout and Atlantic salmon. This work was extended by Cipriano *et al.* (1987) who reported serotype II, sorbitol-fermenting isolates of *Y. ruckeri* as pathogenic for brook trout. The study indicated that serotype I (sorbitol-negative) isolates induced a mean mortality of 65% following an immersion challenge, whereas serotype II (sorbitol-positive) strains gave a mean of 59%. The authors concluded that sorbitol-fermenting isolates of *Y. ruckeri* were potentially as virulent as their sorbitol-negative counterparts. However, they advocated caution where experimental challenges are used for measuring the relative susceptibility of fish to disease since the procedures do not necessarily mimic the natural route of infection.

More recent work has shown that virulence in serotype I strains of *Y. ruckeri* could be closely correlated with the possession of a heat-sensitive factor (HSF) in cell extracts (Furones *et al.*, 1990). It was suggested that the factor could have a lipid component and might be associated with the cell envelope. Pathogenicity studies indicated that strains possessing HSF caused high mortalities in rainbow trout

challenged by ip injection, whereas isolates lacking the factor did not kill any fish. Results with bath challenge, however, seemed less clear cut and led the authors to conclude that other bacterial components, such as adhesin, are also necessary for virulence.

Davies (1991) showed the presence of six distinct serotype I clonal groups, differing in geographic distribution, virulence characteristics and serum-sensitivity. The author concluded that this finding should help the understanding of *Y. ruckeri* virulence mechanisms.

2.4.2 Experimental infection

The potential virulence of *Y. ruckeri*, determined by artificial challenge, has been used in many reported studies. However, these have largely been to determine the relative percentage survival of vaccine preparations or for efficacy testing of antimicrobial agents.

2.4.2.1 Immersion challenge

The duration of immunity in rainbow trout vaccinated with *Y. ruckeri* was determined by Johnson *et al.* (1982b) by using a 60-min bath challenge. This experimental work with vaccine development was continued by Amend *et al.* (1983) in a study investigating some factors affecting the potency of *Y. ruckeri* bacterins. Thirty days after immunisation, rainbow trout were challenged by adding a 24 to 48 h TSB culture of virulent *Y. ruckeri* directly to water after the temperature had been raised from 10°C to 17°C. The fish were challenged for 1 h at a concentration predetermined to kill about 70% of the unimmunized control fish (approximately 1 x 10^8 c.f.u. ml^{-1}). In addition, the same authors challenged fish by administering 0.05 ml per fish by ip injection (approximately 2.6 x 10^4 c.f.u. ml^{-1}). Cipriano and Ruppenthal (1987) investigated the significance of humoral immunity and cross protection between serotypes following the immunisation of brook trout against *Y. ruckeri*. Groups of fish were challenged with either serotype I or II isolates by exposing them for 1 min in 1 l of BHI containing about 1 x 10^8 c.f.u. ml^{-1}. The susceptibility of this fish species to experimental infections with different serotypes

of *Y. ruckeri* has also been determined by using a 1 min immersion in a 48 h TSB culture containing about 10^9 c.f.u. ml^{-1} (Cipriano *et al.*, 1987). A study by Bruno and Munro (1989) investigated immunity in Atlantic salmon fry following vaccination against *Y. ruckeri*. An isolate obtained during an outbreak of ERM among farmed rainbow trout in Scotland was used to bath-challenge groups of fish with 2×10^5 c.f.u. ml^{-1} for 30 min in 15 l water. Hunter *et al.* (1980) investigated the stress-induced transmission of *Y. ruckeri* from carriers to recipient steelhead trout (*Salmo gairdneri*). The fish were infected by adding 200 ml of a TSB culture to aquarium water which had the water flow stopped and the volume reduced to 10 l. Then the culture was allowed to remain in contact with the fish for 1 h and the water flow was resumed. This method was also used by Knittel (1981); however, the TSB culture was incubated at 25°C for 24 h and subsequently diluted to an optical density of 0.6 at 660 nm. The addition of the culture to the aquarium water then resulted in an inoculum of 1×10^3 c.f.u. ml^{-1}. Busch and Lingg (1975) created a carrier status in trout by using a bath challenge method which gave a final water concentration of 2.75×10^6 c.f.u. ml^{-1} with a 1 h exposure at 20°C. Artificial infection of rainbow trout by water-borne challenge was used by Bullock *et al.* (1983) to determine whether a potentiated sulphonamide (sulphadimethoxine/ormetoprim) could effectively control ERM. Trout were challenged by first draining down the water then exposing them for 90 s to 1 l of a 24 h BHI culture of *Y. ruckeri* containing 1×10^9 c.f.u. ml^{-1} before the water was turned on again. Bullock *et al.* (1976) attempted horizontal transmission by holding salmon in aquaria receiving effluent from rainbow trout infected with ERM. Anderson and Nelson (1974) used a simple water-borne challenge when heavily inoculated fish were placed into a tank along with previously immunised fingerling rainbow trout. Bosse and Post (1983) used the immersion method as the basis for determining the effectiveness of prospective drugs against a spontaneous *Y. ruckeri* infection. Rainbow trout were crowded (1 kg fish/28.3 dm^3) into a small section of trough and TSB cultures of *Y. ruckeri* were added continuously to the trough water at one or two drops per minute.

2.4.2.2 Injection challenge

Ip injection was used by Busch and Lingg (1975) in order to establish an asymptomatic carrier state of ERM in rainbow trout. The fish were challenged with an average of 18 bacteria from an 18 h TSA culture resuspended in 0.5% tryptone. Bullock *et al.* (1976) challenged 2.15 g Atlantic salmon with a 0.05 ml ip injection containing either 6.5×10^7 or 6.5×10^5 *Y. ruckeri* cells. Other workers have also preferred ip injection as a challenge method in vaccination studies using coho salmon and rainbow trout inoculated with 0.1 ml of a 20 h *Y. ruckeri* TSB culture (Newman and Majnarich, 1982); brown trout injected with 2×10^5 *Y. ruckeri* cells representing 0.02 ml of a 10^7 c.f.u. ml^{-1} TSB culture grown for 24 h at 20°C (Tatner and Horne, 1985); rainbow trout injected with 0.1 ml of a 1.2×10^8 c.f.u. ml^{-1} TSB culture incubated for 24 h at 22°C (Cossarini-Dunier, 1986b).

Subcutaneous inoculation of rainbow trout was reported by Anderson and Ross (1972) in a comparative study of efficacy of oral vaccines. A series of dilutions, calibrated to contain 4×10^8 to 4×10^3 *Y. ruckeri* in 0.1 ml of an unknown diluent was used. A similar method was reported by Anderson and Nelson (1974).

The testing of chemotherapeutic compounds for control of ERM, by ip injection challenge of rainbow trout, has been reported by Bosse and Post (1983). An LD_{75} of *Y. ruckeri* was established by growing an isolate in TSB and preparing suspensions of 0.0001 mg dry weight of bacteria ml^{-1}. This quantity of bacteria was subsequently used to induce mortalities in fish, within a 14-day period, after injecting a 1.0 ml suspension. Unfortunately it is not possible to compare this method with those in other studies. Since the expression mg dry weight of bacteria ml^{-1}, for challenge purposes, is unconventional and cannot be equated with the more normal c.f.u. ml^{-1}. Rodgers and Austin (1983) also used artificial challenge by ip injection but with 1×10^6 c.f.u. ml^{-1} of *Y. ruckeri* to determine the efficacy of oxolinic acid for prophylaxis and therapy of ERM in rainbow trout.

The pathogenicity of field isolates of *Y. ruckeri* has been demonstrated in several studies by using ip injection. Dalsgaard *et al.* (1984) inoculated 200 g

rainbow trout with 1 ml of a saline suspension containing approximately 1 x 10^8 c.f.u. ml^{-1} originally obtained from a disease problem on a Danish fish farm. McArdle and Dooley-Martyn (1985) reported challenging rainbow trout with 2.5 x 10^6 *Y. ruckeri* c.f.u. ml^{-1}, the strain having been originally isolated from goldfish imported into Ireland. Bragg and Henton (1986) injected 300 g rainbow trout with a strain obtained from an outbreak of ERM in South Africa.

Lewis (1981) used either intramuscular (im) injection of 10^3 *Y. ruckeri* cells or bath challenge for 6 h with 10^5 c.f.u. ml^{-1} to expose channel catfish (*Ictalurus punctatus*) in a study investigating immunoenzyme microscopy as a technique for detecting subclinical infections of *Y. ruckeri.*

There appears to have been a wide variety of culture conditions, bacterial concentrations and strains reported for artificial challenge testing to determine the potential virulence of *Y. ruckeri.* There is a clear need for standardization of techniques and methodologies so that different studies can be compared equally. This is especially so when the efficacy of vaccines and antimicrobial agents is being determined.

2.5 IDENTIFICATION

The isolation and subsequent identification of *Y. ruckeri* play a key role in the correct diagnosis and control of ERM. Moribund, dark looking fish with obvious clinical disease signs provide the best source of potentially infected material. Samples can be taken aseptically from posterior kidney, spleen, liver, heart and lower intestine. In addition, the movement history of a potentially infected group of fish and the occurrence of previous ERM epizootics in particular river catchments can provide useful background information for disease investigations.

2.5.1 Presumptive identification

2.5.1.1 Fluorescent antibody technique (FAT)

Presumptive identification of *Y. ruckeri* can be made by using the fluorescent antibody technique (FAT) on tissue smears (e.g. kidney or liver) (Johnson *et al.*,

1974; Lewis, 1981; Bullock, 1984; Smith *et al.*, 1987) or on smears prepared from a plate culture (Bullock *et al.*, 1978; Stevenson and Daly, 1982; McArdle and Dooley-Martyn, 1985; Pyle *et al.*, 1987; Smith *et al.*, 1987). Although FAT can give a rapid result at the time of initial clinical examination, the test can be susceptible to non-specific fluorescence and cross reactivity which could lead to mis-identification in the absence of other confirmatory tests (Johnson *et al.*, 1974; Lewis, 1981; Busch, 1983; Pyle *et al.*, 1987). However, Smith *et al.* (1987) showed that FAT could be a useful and rapid confirmatory technique in its own right although the authors also advised performing standard bacteriological tests in conjunction with FAT.

2.5.1.2 Whole cell agglutination (WCA)

Simple whole cell slide agglutination (WCA) can also be used for presumptive or serotypic identification of *Y. ruckeri* (Busch and Lingg, 1975; Knittel, 1981; O'Leary *et al.*, 1982; Stevenson and Daly, 1982; McArdle and Dooley-Martyn, 1985; Rodgers and Hudson, 1985; Meier, 1986; Michel *et al.*, 1986; Rintamäki *et al.*, 1986; Sparboe *et al.*, 1986; Dwilow *et al.*, 1987; Toranzo *et al.*, 1987; Willumsen, 1989). More precise microtitre plate agglutination, for determination of end point titres in specific serotype identification, has also been used. This is usually performed following isolation or in characterization studies of *Y. ruckeri* (Bullock *et al.*, 1978; Stevenson and Daly, 1982; Green and Austin, 1983; Pyle and Schill, 1985; Dwilow *et al.*, 1987; Dear, 1988). However, to reduce possible cross reactions, any antisera used in this type of test should be cross-adsorbed with antigens representing the other known serotypes of *Y. ruckeri* (Stevenson and Daly, 1982). In addition, antisera and the isolate under test should be diluted to predetermined optimal levels in order to minimise any possible cross reactivity with other fish pathogens or with other members of the Enterobacteriaceae (Busch, 1983).

2.5.1.3 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) has also been described for fisheries use. Neumann and Tripp (1986) used the method to test for antibody levels in a study on the influence of the route of administration of *Y. ruckeri* on the

humoral response of channel catfish. Whereas Olesen (1991) showed that ELISA was more sensitive than the agglutination test for detection of antibodies in rainbow trout. The rapid diagnosis of clinical cases of ERM using a monoclonal antibody-based ELISA was shown to be of value by Austin *et al.* (1986). In a novel approach, the authors used a dipstick-ELISA which, it was claimed, had advantages over the more usual microtube technique making the method easily adaptable for field use. However, the technique was designed for the confirmation of clinical cases of ERM and was not suitable for the detection of subclinically diseased or carrier fish. This lack of sensitivity may be one reason why the ELISA technique has not become more widely used in the diagnostic field. In addition, well tried traditional methods are more cost effective despite taking longer to reach confirmation.

2.5.1.4 Immunoenzyme microscopy

The use of immunoenzyme microscopy for differentiating systemic bacterial pathogens of fish, including *Y. ruckeri*, was described by Lewis (1981). This technique was comparable to immunofluorescence and cultural procedures for detecting subclinical ERM infections in channel catfish. Problems attributed to cross-reactivity and non-specific staining were similar to immunofluorescence. Although the immunoenzyme reaction gave less contrast than the immunofluorescence method this presented no difficulties in interpreting the results. However, the author further suggested that counter-staining could be used if diminished contrast became a problem in future studies.

2.5.2 Biochemical confirmation

The isolation of *Y. ruckeri* on laboratory media and subsequent growth characteristics are outlined in section 2.1 concerning aetiology and classification. However, definitive identification is achieved by examining specific biochemical characteristics using classical methods and these are detailed in Table 2.1.

2.5.2.1 API tests

The use of the API 20E (API-bioMérieux Ltd, Basingstoke, UK) system of biochemical testing for identification of *Y. ruckeri* has been used in many studies.

However, the test was originally developed for use in the medical field and the strips of micro-tubules normally require incubation at a temperature of 37°C for 24 hours. Most Gram-negative bacteria isolated from fish have an optimum growth temperature of less than 30°C and consequently the criteria for using API 20E have been modified accordingly. Some workers have reported using an incubation temperature of 20°C for 72 h (Dwilow *et al.*, 1987), although Knittel (1981) preferred 48 h. However, most studies have used 22°C for 48 h (Frerichs *et al.*, 1985; Hastings and Bruno, 1985; Rintamäki *et al.*, 1986; Dear, 1988; Bruno and Munro, 1989; Davies and Frerichs, 1989). A slightly higher incubation temperature of 25°C but for 24 h has also been tried (Stevenson and Daly, 1982; Daly *et al.*, 1986). There have been other reports of 25°C being used but the length of incubation is not always stipulated (Stevenson and Airdrie, 1984; De Grandis *et al.*, 1988). Ceschia *et al.* (1984) used API 20E but did not report any conditions of incubation.

Although the API 20E system of biochemical testing is rapid and convenient for identification of *Y. ruckeri* the resultant profiles need careful interpretation since certain reactions differ from those obtained with conventional tests (Stevenson and Daly, 1982; Dear, 1988; Davies and Frerichs, 1989). Davies and Frerichs (1989) indicated that incubation at 22°C for 24 h tended to result in false negative citrate utilisation and gelatin hydrolysis tests. Dear (1988) reported similar false negative reactions at the same temperature but after 48 h incubation. In addition, Davies and Frerichs (1989) also reported a higher number of VP positive results with the API 20E system compared with a conventional test method. Stevenson and Daly (1982) found that the lysine decarboxylase API reaction could be negative after 24 h at 25°C. A list of the possible API 20E profiles for *Y. ruckeri* is given in Table 2.2.

As a result an operational definition of *Y. ruckeri* was used throughout the following chapters. Presumptive identification used a simple whole cell slide agglutination technique (see sections 3.2.10.2 and 4.2.2.3). Confirmation using API 20E compared resultant profiles with those in previously published studies (Table 2.2) and by screening laboratory strains (Tables 3.1 and 3.2).

Table 2.1. **Biochemical characteristics of *Y. ruckeri*-conventional methods** (Extracted from published studies)[1]

Gram: negative	Methyl red: +
Shape: rod	Voges Proskauer: variable
Motility: variable	Citrate utilization: +
G+C ratio: 47.5-48.5%	Gelatin hydrolysis: variable
Catalase: +	Tween 80: variable
Oxidase: -	Urease: -
O/F reaction: fermentative	Acid from:
ß-galactosidase: +	arabinose: -
Arginine dihydrolase: -	fructose: +
Lysine decarboxylase: +	glucose: +
Ornithine decarboxylase: +	lactose: -
Indole: -	maltose: +
Hydrogen sulphide: -	ribose: +
Tryptophan deaminase: -	sorbitol: variable
Casein hydrolysis: variable	sucrose: -
Nitrate reduction: +	trehalose: +

[1]Ewing *et al.* (1978); O'Leary *et al.* (1979); Green and Austin (1983); Bercovier and Mollaret (1984); Davies and Frerichs, (1989)

Table 2.2. **Biochemical characteristics of *Y. ruckeri*-API 20E profiles** (Extracted from published studies)[1]

	5104100	5105100	5106100	5107100
	5104500	5105500	5106500	5107500
1307100	5304100	5305100	5306100	5307100
	5304500	5305500	5306500	5307500

[1]Ceschia *et al.* (1984); Davies and Frerichs (1989); Dear, 1988; Frerichs *et al.* (1985); Vuillaume *et al.* (1987).

A surprising report of apparent isolation of *Y. ruckeri* from sewage sludge after anaerobic digestion for 20-30 days, was made by Dudley *et al.* (1980). Serial dilutions of sludge were plated onto MacConkey and xylose lysine deoxycholate agars incubated at 37°C for 24 h, with subsequent identification using API 20E, under the same incubation conditions. This report should be treated with caution in view of the problems reported above by fisheries workers.

The use of the API 50CH system of carbohydrate testing has also been reported. O'Leary *et al.* (1979) incubated the system at 9, 18, 22, 27 and 37°C in a characterisation study of 17 isolates of *Y. ruckeri.* However, Dear (1988) used 22°C for 48 h to complement the API 20E test in order to confirm the first UK isolate from Atlantic salmon. Willumsen (1989) also used the system for identification purposes in a study of birds and wild fish as potential vectors of *Y. ruckeri,* although no further details were published.

2.5.3 Serotype differences

O'Leary (1977; cited by Busch, 1983) described sorbitol fermentation as a characteristic associated with only serotype II strains of *Y. ruckeri.* However, subsequent work has indicated that some serotype I strains can give sorbitol positive reactions (Stevenson and Airdrie, 1984; Michel *et al.*, 1986; Rintamäki *et al.*, 1986). In fact, Busch (1983) states that no significant biochemical differences occur between the recognised serotypes and this indicates the existence of only a single biotype. In a characterisation study of *Y. ruckeri* from different geographic areas, Pyle *et al.* (1987) concluded that, since the serology of this fish pathogen is complex, identification by biochemical testing should be continued and serological testing should only be a confirmatory test. Davies (1991) showed that a combination of biotyping, serotyping and outer membrane protein typing was useful for discriminating between strains of *Y. ruckeri.* In addition, serotype I isolates were thought to comprise six clonal groups.

2.6 CONTROL OF ERM

The prevention, control and treatment of ERM is normally achieved by good husbandry or management practices, vaccination and the use of antimicrobial agents (Table 2.4). Fish health inspection programmes can be important in order to help prevent the spread of disease (McDaniel, 1979). In addition, continual monitoring of mortalities can add to the disease history of a fish farm, especially when apparently healthy fish are being reared in good environmental conditions (Warren, 1983). Disinfection of fertilised ova could also prevent the transmission of *Y. ruckeri* from infected broodstock. Treatment with a 25 mg l^{-1} iodophor solution (Betadine or Wescodyne) for 5 min has been shown to be an effective preventive measure in laboratory tests (Ross and Smith, 1972).

2.6.1 Vaccination

2.6.1.1 Oral administration

The early experimental work on *Y. ruckeri* vaccine development concentrated on oral immunisation of fish. Ross and Klontz (1965) reported a 90% survival rate in rainbow trout 70 days after being fed on a diet incorporating phenol-killed *Y. ruckeri* whole cells. This compared to a 20% survival in corresponding control fish, after both groups had received an injection challenge. Anderson and Ross (1972) compared the effectiveness of several preparations, including wet cell packs of *Y. ruckeri* treated with 0.5 and 3% phenol, 3% chloroform and the addition of 1% formalin following sonication. The groups were fed each vaccine for 2 weeks, followed by a 3-week rest period, with a further one week on the bacterin-containing diet. All groups were then challenged, one week later, by subcutaneous injections of serial dilutions from 4×10^8 to 4×10^3 *Y. ruckeri* c.f.u. ml^{-1}. Essentially, all experimental groups had lower mortality patterns than the controls. However, the chloroform preparation gave greater protection to fish, but there were no differences in humoral antibody levels between the test and control fish. Subsequent work compared the levels and durations of protection in rainbow trout held at 17-18°C, given vaccines (1 mg of chloroform lyophilised cells) by both oral and subcutaneous

injection methods (Anderson and Nelson, 1974). Specific antibody was only found in the inoculated group and this was sustained, albeit decreasing, until the end of the 3-month experiment. Although protection occurred in both vaccinated groups, the inoculated fish had higher levels of protection which also lasted longer than those in the oral group. A later study by Johnson and Amend (1983) looked at the efficacy of vaccines of *Y. ruckeri* intubated into the stomach or into the lower intestine (via the anus) of rainbow trout. The fish were challenged by bath exposure 31 days after a single 0.05 ml application of a preparation containing gelatin. The study indicated that the anally-vaccinated fish were significantly better protected than those vaccinated by oral intubation or a comparative immersion method. Interestingly, the results obtained by these workers showed no differences in protection between immersion or oral vaccination. The authors concluded that bacterins administered orally were partially inactivated in the upper intestine and suggested that this method of application could be successful if the antigen could be protected through the upper intestine. The poor protection levels following oral vaccination are possibly caused by the action of gastric secretions and resultant antigenic destruction. In addition, the actual uptake of antigens may be less efficient from the digestive tract than via the gills and other external body surfaces. The poor protection offered by orally-administered vaccines and the logistical problem and increased cost of the injection method means that neither method is commercially viable for mass immunisation of fish, particularly those under 30 g (Horne and Robertson, 1987). Consequently, alternative delivery methods, largely developed using *V. anguillarum* antigens, have also been evaluated for ERM vaccines. These include the shower system (Amend and Eshenour, 1980) and direct immersion (Amend and Eshenour, 1980; Johnson *et al.*, 1982a, 1982b; Newman and Majnarich, 1982).

2.6.1.2 Shower and immersion administration

Amend and Eshenour (1980) reported that the shower vaccination system allowed fish from 10 to 45 g to be immunised quickly and economically. However, for smaller fish of 100/lb a simple 20 second one-step immersion system enabled

over 500,000 fish to be immunised in one day. Newman and Majnarich (1982) showed that significant levels of short- and intermediate-term protection occurred in juvenile coho salmon and rainbow trout using an immersion *Y. ruckeri* vaccine, at various dilutions. However, the vaccine, prepared from a molecular sieve filtration technique detailed elsewhere (Anon, BioMed Technical Bulletin), gave a higher relative level of protection in the trout compared to the salmon. The authors indicated that the dilution corresponding to 8 μg dry material ml^{-1} gave significant protection.

A large-scale mass immunisation programme for the control of ERM was reported by Tebbit *et al.* (1981). Almost 23 million rainbow trout were used in the trial over 2.5 years. The fish were held in spring water at 14-15°C and most of the fish were vaccinated at 4-5 g by immersion for 90 sec. The results indicated that at 3 farm sites there was a significant reduction in mortality, attributable to ERM, in vaccinated fish.

The efficacy of several delivery methods for a *Y. ruckeri* commercial vaccine were evaluated by Johnson and Amend (1983). The study showed that injection was the most efficient method, followed by direct immersion, shower and spray delivery. The authors concluded that this sequence followed the pattern of decreased exposure time to the vaccine.

The commercial vaccines currently available in UK are listed in Table 2.3.

2.6.2 Factors affecting efficacy of protection

2.6.2.1 Size of fish

The efficacy of early vaccine preparations, as measured by the level and length of duration, varied between field trials. One of the primary causes was shown to be the size of the fish at vaccination (Amend and Eshenour, 1980). Work at the time indicated that fish needed to be 1 g, or larger, before responding to vaccination and that the larger fish retained their immunity longer. However, 4-4.5 g was considered to be the most desirable size to vaccinate, after taking into account on-farm management practices.

Table 2.3 Currently licenced ERM vaccines

Vaccine	Application method	Dosage	Exposure time	Distributor
Ermogen	Immersion, spray and injection	100 kg l^{-1}	30 sec	Aqua Health (Europe) Ltd, Dunblane, Perthshire and Vetrepharm Ltd, Downton, Wiltshire
Enteric redmouth vaccine (ERB)	Immersion or auto-immersion	1 g fish and larger 100 kg l^{-1}	5-30 sec	AVL, Saffron Walden, Essex

These findings were confirmed by Johnson *et al.* (1982a, 1982b), since 1-2.5 g chinook salmon and rainbow trout were the minimum size at which maximum protective immunity occurred (Johnson *et al.*, 1982a). The study indicated that immunity appeared to be a function of size and not age. In addition, protective immunity developed after about 15 days, depending on the water temperature, and fish appeared to be immunised after less than 5 sec immersion. Parallel experiments showed that trout and salmon under 1 g had longer protection, of about 120 days, when the most concentrated bacterin was used. Moreover, immunity in 2 g fish lasted 180 days, but a year or longer in 4 g and larger fish (Johnson *et al.*, 1982b).

Field trials with an existing commercially-available ERM vaccine were reported from north-west Germany by Schlotfeldt *et al.* (1986), prior to registration and licensing of the vaccine. Although the relative percentage survival (RPS) between vaccinated and non-vaccinated groups of rainbow trout was significant, there were no essential differences between 30 sec or 1 h immersions. In addition, the study confirmed other reports that protection appeared incomplete in fry of less than 2 g.

2.6.2.2 Preparation of bacterin

Amend *et al.* (1983) considered several factors that could potentially affect the potency of *Y. ruckeri* bacterins administered by the immersion method. The study indicated that *Y. ruckeri* could be grown in TSB over a pH range of 6.5 to 7.7 and a time span of 12 to 96 h without affecting potency.

In addition, there were no apparent differences in protection between chloroform or formalin inactivated cells and extraction with either butanol or phenol. However, bacterins prepared from pH lysed cells resulted in a significant increase of protective immunity.

2.6.3 Economics of vaccination

Vaccines used against ERM can be an effective management practice, provided that they are administered correctly and are not considered as "cure-alls". The economic benefits of vaccinating fish stocks can be reflected in lower mortalities attributed to ERM, improved conversion rates in vaccinated fish, earlier marketability and savings in the cost of medicated feed (Amend and Eshenour, 1980; Tebbit *et al.*, 1981; Horne and Robertson, 1987).

2.6.3.1 Mortality patterns

Amend and Eshenour (1980) showed that the mortality figures in fish vaccinated against ERM were reduced to the point where they were not a factor (i.e. 0%) and yet they averaged 6.35% per month in control fish, from the time of vaccination to marketing. A similar situation was recorded by Tebbit *et al.* (1981) with mortalities of 1.3% in vaccinated stocks and 8.1% in non-vaccinated controls. This represented a reduction in mortality of 83.9%. Horne and Robertson (1987) reported unpublished work attributed to Paterson which indicated losses of 4.7% and 21.8%, respectively.

2.6.3.2 Conversion rates

Improved conversion rates can arise from better fish health following vaccination of stocks potentially exposed to ERM. Amend and Eshenour (1980) reported, simply, that overall conversion rates were significantly better in vaccinated

fish. This referred to a rate of 1.71 in vaccinates and 1.9 in control fish. However, these figures should be treated with caution since statistical analysis was not presented. In addition, the vaccinated fish were 10.5 months of age and weighed 248 g whereas a corresponding control group weighed 176 g. Tebbit *et al.* (1981) noticed that feed conversion rates improved in vaccinated fish by 10 to 22% across 3 farm sites and considered that this factor alone was economically important.

2.6.3.3 Medicated feed

The savings in the cost of medication, in a vaccinated group of fish were considered by Amend and Eshenour (1980) to have more than paid for the vaccine used. Tebbit *et al.* (1981) also considered that medicated feed requirements were substantially reduced in trout vaccinated against ERM.

Even though vaccines have been extensively used for some time to control *Y. ruckeri* infections in fish, they do not completely eliminate the disease or losses attributable to ERM (Busch, 1983). No published information exists as to whether vaccination can eliminate or prevent the carrier state. Hence, the transfer of apparently healthy, vaccinated but carrier fish has been proposed as a route by which ERM could spread into non-endemic areas (Ellis, 1988). However, Horne and Robertson (1987) concluded that the benefits of using vaccines as opposed to antibiotics could be highly cost-effective, resulting in more resources being devoted to overall improvements in husbandry. In addition, antibiotics tend to be used several times, leading to increased costs, particularly as the fish grow larger. Vaccines, however, are normally only used once at the beginning of the growth cycle and their efficacy can last until the fish reach market size. Vaccination of course is not 100% effective and the usage of antimicrobial agents may still be required in certain circumstances.

2.6.4 Antimicrobial agents

Several studies have indicated that a range of antimicrobial compounds can be used to successfully treat infections due to *Y. ruckeri.* The early work of Rucker (1966) recommended treatment with sulphamethazine, using 200 mg kg^{-1} of body

weight/day for 5 days, followed by 3 days on chloramphenicol or oxytetracycline at 50 mg kg^{-1}. However, this author also reported variations in dosage and length of treatment.

A slightly different type of experiment was performed by Wasteson *et al.* (1989) and showed that *Y. ruckeri* produced an antibacterial factor. The factor inhibited a wide spectrum of Gram negative and Gram positive, though not other strains of *Y. ruckeri.*

2.6.4.1 Laboratory evaluation

Subsequent laboratory experiments have shown the value of potentiated sulphonamide therapy for both artificial and natural infections of ERM. Bullock *et al.* (1983) used a combination of 5 parts sulphadimethoxine and one part ormetoprim (Ro5-0037) fed at 50 mg kg^{-1} of fish for 5 days to control ERM in rainbow trout challenged by the bath method. In addition, trout were similarly treated at a hatchery with an epizootic of ERM. A similar compound, Tribrissen (80 parts trimethoprim and 400 parts sulphadiazine), was used by Bosse and Post (1983) at 1 mg kg^{-1} of fish for 14 days to control *Y. ruckeri in vivo,* but under laboratory conditions. In addition, tiamulin, a semi-synthetic derivative of pleuromulin, was effective when administered orally for 14 days at 5 mg kg^{-1} day. The authors also reported that external signs of ERM virtually disappeared within 4 days of drug administration and that there was no recurrence of the disease during a 14-day post-medication observation period.

The potential of oxolinic acid, a member of the 4-quinolone group, for the control of ERM in fish was first reported by Rodgers and Austin (1983). Results from an *in vitro* study showed that the drug was bacteriostatic at 1 mg l^{-1} to 25 isolates of *Y. ruckeri* and bactericidal to 23 of the isolates at 5 mg l^{-1}. Data from a subsequent *in vivo* experiment demonstrated the success of oxolinic acid for prophylaxis and therapy, with no losses among fish receiving the drug as a medicated diet at 10 mg kg^{-1} for 10 days.

2.6.4.2 Susceptibility

The *in vitro* sensitivity of *Y. ruckeri* to specific antibiotics was reported by Ceschia *et al.* (1987). Flumequine, nalidixic acid, and also 4-quinolones, were the most active substances with oxytetracycline, gentamicin, nitrofurantoin, tetracycline, neomycin, chloramphenicol, chlortetracycline, erythromycin and furazolidone ranked in order of decreasing susceptibility. Additional experiments, not reported fully by the authors, indicated that flumequine was the most efficacious chemotherapeutant of those tested against ERM in farmed Italian trout.

The susceptibility patterns of 50 strains of *Y. ruckeri* to 23 antimicrobial agents were determined by De Grandis and Stevenson (1985). The most active compounds were oxolinic acid, potentiated sulphonamide (Ro5-0037), trimethoprim, nalidixic acid, gentamicin, oxytetracycline, tetracycline, carbenicillin and chloramphenicol. Baath (1986) showed all isolated strains of *Y. ruckeri* to be sensitive to chloramphenicol, tetracycline, and trimethoprim whereas a few strains were not sensitive to furazolidone, erythromycin, neomycin and sulphamerazine.

2.6.4.3 Resistance

The potential hazards of misuse of antimicrobial agents, particularly those caused by finishing treatment as soon as mortalities cease, has been highlighted by Post (1987). In addition, repeated short-term treatment may also be responsible for the appearance of drug resistance in strains of *Y. ruckeri* (Tebbit *et al.*, 1981). There are some areas of the USA, where ERM is enzootic, that now have isolates completely resistant to both sulphamerazine and oxytetracycline (Post, 1987).

De Grandis and Stevenson (1985) revealed that two isolates of *Y. ruckeri* showed high-level resistance to the tetracycline derivatives and sulphonamides. In addition, whereas 81% of the serovar I strains were highly susceptible to polymyxin B, the strains belonging to serovars II, III and V were highly resistant.

Bowser and House (1990) reported that enrofloxacin outperformed oxolinic acid in laboratory trials. The MIC values, determined in brain heart infusion broth against a strain of *Y. ruckeri,* were 0.16 and 0.8 $\mu g\ ml^{-1}$ respectively.

2.6.4.4 Clinical outbreaks

The treatment of clinical outbreaks of ERM, usually following laboratory antimicrobial sensitivity testing, has been reported by many workers. The dosages in rainbow trout for oxytetracycline have been variously reported as 80 mg kg^{-1} of fish/day (Frerichs *et al.*, 1985) and 50 mg kg^{-1} of fish/day (Bragg and Henton, 1986). Rübsamen and Weis (1985) indicated that a treatment of 2000 mg kg^{-1} of prepared feed for 7 days could also be effective. Alternate treatment with methylene blue (1000 mg kg^{-1} of food for 5 days), tetracycline (67 mg kg^{-1} of fish/day for 10 days), and a further 5 days treatment with methylene blue was outlined by Llewellyn (1980) for the salmonid blood spot bacterium infection. Wobeser (1973) reported treatment with oxytetracycline at 3500 mg kg^{-1} of feed for 5-7 days followed by a change to sulphamerazine at 200 mg kg^{-1} of feed for 14 days. Other recorded treatments include furizalidone fed with the diet at the rate of 44 mg kg^{-1} of fish, Apurone (flumequine) at the rate of 80000 mg kg^{-1} of food for 5 to 6 days (Lesel *et al.*, 1983a), chloramphenicol at 1000-1500 mg kg^{-1} of prepared feed for 7 days (Rübsamen and Weis, 1985), and 70 mg kg^{-1} for 7 days followed by flubactin at a dose of 120 mg kg^{-1} of fish for 3 days (Ocvirk *et al.*, 1988). In addition, an unspecified dose of flumequine was used by De La Cruz *et al.* (1986).

Treatment of ERM in other salmonid species has used similar antimicrobial compounds. Oxytetracycline at 88 mg kg^{-1} of fish/day for 10 days in chinook salmon was reported by Cipriano *et al.* (1986), whereas 75 mg kg^{-1} of fish/day for 10 days has been effective in Atlantic salmon (Sparboe *et al.*, 1986). Although oxytetracycline was used to treat the same species in a study by Willumsen (1989), the dosage was not recorded. Therapy with oxolinic acid at 10 mg kg^{-1} of fish for 10 days has also been used in Atlantic salmon (Dear, 1988).

Table 2.4 lists the recommended prevention and control measures for ERM (MAFF, 1984).

Table 2.4. Recommended prevention and treatment measures for ERM[1]

Prevention	Treatment
1. Sensible hygiene precautions	1. Oxolinic acid (10 mg kg day^{-1} for 10 days)
2. Buy from ERM free stocks	2. Potentiated sulphonamide (30 mg kg day^{-1} for 5-7 days)
3. Vaccinate own stocks	3. Oxytetracycline (50-75 mg kg day^{-1} for 10 days)
4. Buy pre-vaccinated fish	
5. Reduce stress, e.g. handling and grading	
6. Reduce or stop feeding during high risk periods	
7. Prophylactic use of external bactericides	

[1]Taken from MAFF (1984) ERM - topic of the moment. *Fish Farmer* Nov/Dec. 15-16.

2.6.4.5 Recurrence of disease

The problem of ERM recurring following the cessation of antimicrobial treatment has been highlighted by several workers. De La Cruz *et al.* (1986) indicated that, although treatment with flumequine resulted in a lowering of the mortality rate by 80%, there was an outbreak of the disease again approximately 3 weeks later. Rübsamen and Weis (1985) noticed that, with overcrowed stock, losses declined gradually after the use of an antibiotic, but only reduced still further when the fish were transferred to a larger pond with resultant reduced stocking density. However, they still noticed a few sick fish some weeks later, although these were not post mortemed to determine whether *Y. ruckeri* was still causing problems. Bragg and Henton (1986) reported that 4 months after treatment there were still some isolated cases of ERM in fish previously affected and *Y. ruckeri* was re-isolated from samples of spleen, liver and kidney. Cipriano *et al.* (1986) indicated that a second outbreak of ERM occurred 5-6 weeks after the onset of initial mortalities and subsequent successful therapy.

2.7 IMMUNE RESPONSE TO *Yersinia ruckeri*

The general immune response of fish to the antigenic components of disease agents is well documented (Corbel, 1975; Dorson, 1981). Consequently, this section deals primarily with the stimulation of the humoral or antibody response by *Y. ruckeri.*

2.7.1 Humoral response

Normally, the humoral response is measured by either titration of the circulating antibody levels or by determining the number of antibody producing cells.

2.7.1.1 Circulating antibody

Busch (1978) used a passive agglutination technique to monitor the fish stocks at sites with histories of ERM and at a site undergoing an epizootic. High antibody titres were thought to correlate with subacute-to-chronic clinical infections, whereas low-titred sera indicated the presence of an established carrier population. Additional work indicated that rainbow trout could develop agglutinating antibodies against protein-based soluble antigens after 13 days. The response to particulate antigens was detected after 21 to 28 days. In addition, the equivalent of an anamnestic response was noted after 106 days.

Anderson *et al.* (1979a) indicated that a measurable antibody response occurred to 10 $\mu g\ ml^{-1}$ 'O'-antigen 19 days after flush exposure and was highest by day 28, but declined afterwards to day 77. Ip injection with 100 $\mu g\ ml^{-1}$ antigen at 17°C gave no detectable antibody response after 7 days (Anderson *et al.*, 1979d), but 500 $\mu g\ ml^{-1}$ gave the highest response after 21 days. Additional work indicated that temperature affected the production of humoral antibody (Anderson *et al.*, 1979b). When rainbow trout were exposed to a similar antigen at 17°C, by either injection or immersion, antibody was detected after 14 days but not at 13°C. However, in dosage experiments antigen was administered by immersion at 13°C, and the minimal concentration producing humoral antibody was found to be 5 $\mu g\ ml^{-1}$.

A study using common carp held at 20-22°C, following administration of *Y. ruckeri* 'O'-antigen, indicated that antibody levels increased from day 12 and reached the highest level between 17 and 34 days (Lamers and Pilarczyk, 1982). The titres after bath immunisation were considerably lower than those after im or ip injection. Carp were also used by Cossarini-Dunier *et al.* (1988) to show that contamination of water with 50 mg l^{-1} of manganese ions did not decrease antibody production against an ip-injected *Y. ruckeri* vaccine at 20°C.

The humoral response of channel catfish to *Y. ruckeri* was studied by Neumann and Tripp (1986) using ip, im and intraoesophageal (io) routes of administration. Although the response varied greatly between individual fish, high antibody titres were found to a range of antigen doses irrespective of the administration method. However, the group of fish inoculated io experienced a substantial mortality and unfortunately, the study indicated that control fish also had moderately high titres against *Y. ruckeri.* The authors concluded that, although the presence of specific anti-*Y. ruckeri* antibody could not be ruled out, it was more likely that cross-reacting antibody was present. In addition, the response to *Y. ruckeri* appeared to be rapid and to be associated with high titres, which was indicative of a secondary immune response.

A secondary immune response was also noted by Cossarini-Dunier (1986a) in rainbow trout immunised by ip injection of either 1 mg dinitrophenol coupled to keyhole limpet (*Megathura crenulata*) haemocyanin (DNP-KLH) or a *Y. ruckeri* vaccine. Although DNP-KLH gave no anamnestic response, the *Y. ruckeri* antigen promoted a secondary response that was higher and quicker than the primary one. Herraez and Zapata (1987) showed that goldfish, immunised by ip injection of formalin-killed *Y. ruckeri* cells, elicited a peak primary antibody titre after 14 days with higher titres after secondary immunisation.

The protection afforded to rainbow trout by an injected *Y. ruckeri* vaccine mixed with saline or adjuvant was studied by Cossarini-Dunier (1986b). Although only 5.5% mortality occurred after challenge, among vaccinated groups, the levels of

circulating antibody were variable. This led to the conclusion that protection was independent of agglutinating antibody and probably due to cell-mediated immunity instead. Olesen (1991) did not detect an antibody response in immersion-vaccinated rainbow trout using ELISA although high titres were observed in sera of trout immunized with formalin-killed *Y. ruckeri* and in trout after experimental infection.

Cross-protection following immunisation with serotype I or II isolates of *Y. ruckeri* was studied by Cipriano and Ruppenthal (1987). The results showed that brook trout only produced highly-specific antibody against the serotype used in vaccine preparation. However, the fish were protected against both serotypes after bath challenge. The specificity of antibody production against *Y. ruckeri* confirmed the findings of other workers and indicated a lack of correlation between protection and circulating antibodies.

2.7.1.2 Antibody producing cells

The immune response of rainbow trout was recorded after 9 days by Anderson *et al.* (1979a), following immunisation with *Y. ruckeri* 'O'-antigen at 10 μg ml^{-1}, administered by flush exposure. Corresponding numbers of antibody-producing cells were highest on days 14 and 16, but had declined to low numbers after 28 days. First observation of cells was seen after 7 days by Anderson *et al.* (1979d) at 17°C, following ip injection, with maximum numbers occurring on day 11. A study by Anderson *et al.* (1979b) indicated that prior immersion in 5.6 or 2.6% NaCl did not affect the immune response, but a 2-minute exposure to the anaesthetic MS-222 resulted in a reduced level of immune response. A flush exposure technique showed that 5 μg ml^{-1} of *Y. ruckeri* 'O'-antigen still produced an immune response at 11°C, but a dosage of 0.5 μg or less did not elicit a response (Anderson *et al.*, 1979c). However, a study by Anderson (1978, cited by Anderson *et al.*, 1979c) indicated that ip injection of the 'O'-antigen did give an immune response with a dose as low as 0.5 μg ml^{-1}. This effect was also reported by Anderson *et al.* (1979d) at 17°C. In addition, the maximum detectable immune response occurred one week earlier than the maximum antibody titre using 500 μg

ml^{-1} antigen. Flush exposure to a range of 'O'-antigen concentrations (0.5-500 μg ml^{-1}) indicated that the number of antibody-producing cells could be directly related to the dose of antigen (Anderson *et al.*, 1982). This was confirmed by administration of DNP-*Y. ruckeri* 'O'-antigen conjugates by either ip injection or flush exposure (Anderson *et al.*, 1983).

The uptake of *Y. ruckeri* 'O'-antigen by Atlantic salmon fry, after bath immunisation, also indicated that there was a direct dose-response correlation in the immune response (Zapata *et al.*, 1987). The fish had high numbers of splenic antibody-producing cells when given an antigenic dose of 100 and 1000 μg ml^{-1}, but the numbers were lower with the 10 μg ml^{-1} dose. In addition, the antibody titres correlated with the number of cells.

Lamers and Pilarczyk (1982) showed that the prenephros in carp had an antibody-producing cell response between days 4 and 17 at 22°C, following administration of 'O'-antigen. The maximum response, however, was found on day 10.

Siwicki *et al.* (1989) investigated the possibility of immunomodulation by oxolinic acid, oxytetracycline and levamisole in rainbow trout. The results indicated that the number of splenic antibody-producing cells, in fish injected with oxolinic acid before ip injection of *Y. ruckeri* 'O'-antigen, were not significantly different than the controls. However, fish given oxytetracycline at 10 mg kg^{-1} or levamisole at 5 mg kg^{-1} had a marked reduction in such cells.

2.7.1.3 Passive protection

Cipriano and Ruppenthal (1987) obtained serum from brook trout already vaccinated with *Y. ruckeri* serotype I or II isolates. This was then injected into other brook trout and serum antibody levels were monitored 24 h later. Although circulating antibodies were detected, the study showed that they conferred no protection since the mortalities, after a bath challenge, equalled or exceeded those of the control group, which had been injected with normal serum. The authors concluded that serum antibody was not necessary for development of protection

against *Y. ruckeri*. They suggested, as other workers had done, that a localised or cell-mediated immune response may be more involved in the protective mechanism against *Y. ruckeri*. Olesen (1991), however, showed that the injection of sera from vaccinated rainbow trout partially protected fry against ERM but that protection was more pronounced with serum from vaccinated and infected trout.

2.7.2 Cellular response

The role of the melano-macrophage centres (MMC) of goldfish, after primary and secondary ip injection of formalin-killed *Y. ruckeri* cells, was investigated by Herraez and Zapata (1987). The results suggested a role for the splenic and nephric MMC, although there did not appear to be any variations in number, size or area occupied by MMC in the lymphoid organs. In addition, these results seemed to be independent of antibody titres and antigen doses.

Phagocytosis of *Y. ruckeri* by rainbow trout leucocytes was studied by Griffin (1983). In particular, the work investigated the role of specific antibody in opsonisation of phagocytic ingestion of antigen. The results indicated that ingestion of live *Y. ruckeri* cells was greatly enhanced by antibody raised specifically against formalin-killed homologous cells. However, the rate of intracellular killing was not affected. The effect of manganese ions on the phagocytosis of opsonised *Y. ruckeri* vaccine was studied by Cossarini-Dunier *et al.* (1988) and was found to have a stimulating effect.

Herraez and Zapata (1987) noted that goldfish had intense phagocytosis in renal tubule epithelial cells following secondary immunisation with formalin-killed *Y. ruckeri* cells.

Zapata *et al.* (1987) reported that mononuclear phagocytes were involved in the uptake of *Y. ruckeri* 'O'-antigen by the gills of Atlantic salmon. The results suggested that phagocytosis occurred after the antigen was taken up by the gill epithelial cells.

2.7.3 Immunosuppression

A synthetic corticosteroid and an alkylating agent were used by Anderson *et al.* (1982) to induce immunosuppression in rainbow trout following flush exposure to 'O'- antigen. Essentially, fish given the corticosteroid had reduced numbers of antibody-producing cells, lower antibody titres and numbers of splenic lymphocytes. On the contrary, fish given the alkylating agent had equal or reduced numbers of lymphocytes, but increased numbers of antibody-producing cells. The authors indicated that a similar situation exists in mammals where the corticosteroid can reduce all types of lymphocyte. However, the alkylating agent was more specific for the suppressor T-cell population of lymphocytes which control the antibody-producing B-cells.

It is recognised that the some of the terminology used in epidemiological studies can not only be confusing but it can also be misused. Nevertheless, the interpretation of states of infection, in particular, can be guided only by the published literature. Consequently, certain operational definitions are used in the following chapters:

a) Carrier, carrier status and asymptomatic are interchangable and are defined as the condition within a given population that can provide a constant reservoir for the infection of healthy fish or a recurrent infection of already diseased fish. Since *Y. ruckeri* is an enteric infection these terms apply only to faecal isolation.

b) Acute infection refers to the isolation of *Y. ruckeri* from internal organs (e.g. kidney and/or spleen) over a short period of time (up to several weeks), in conjunction with minimum disease signs.

c) Chronic infection refers to the isolation of *Y. ruckeri* from internal organs (e.g. kidney and/or spleen) over a longer period of time (e.g. several months), in conjunction with maximum disease signs.

CHAPTER 3

THE DEVELOPMENT OF A SELECTIVE MEDIUM FOR THE ISOLATION AND CHARACTERIZATION OF *YERSINIA RUCKERI*

3.1 INTRODUCTION

The primary isolation of the causal agents of bacterial disease is an essential first step in the diagnostic process and involves choosing the most appropriate culture medium. There are many general purpose media which can support a wide variety of bacteria. Nevertheless, in certain circumstances a selective medium may be required in order to suppress the growth of some bacteria while allowing the growth of others from a mixed flora. Modification of primary isolation media to achieve a degree of selectivity can be made simply by altering the pH or by adding an inhibitory specific chemical.

The nutritional requirements of bacteria are wide ranging and as a result differential media can also be used to help identify isolates amongst mixed populations. This type of medium normally contains one or more fermentable carbohydrates and a suitable indicator for the detection of acid or alkali production (Difco Manual, 1985).

MacConkey's agar, used for the isolation of enteric bacterial pathogens, is an example of this type of specialized medium. It acts as a selective medium since it contains crystal violet, which inhibits growth of Gram positive bacteria, and bile salts, which inhibit growth of non-enteric bacteria. In addition, MacConkey's agar is also a differential medium since lactose fermenting bacteria produce red colonies in contrast to enteric pathogens which are non-lactose fermenters (Sokatch and Ferretti, 1976).

The intestine of man and other animals harbours a complex mixed culture of bacteria (Drasar and Barrow, 1985). In addition, the composition of the normal flora is variable and can differ from one group of animals to another even in the healthy intestine. However, in the diseased gut complex interrelationships can exist between the host and microorganisms so that the balance of the flora is altered in favour of the

pathogenic bacteria (Drasar and Barrow, 1985). This situation would lead to relative ease of isolation since the causal agent of disease would predominante. Unfortunately the opposite would be true when the carrier state occurred.

Yersinia ruckeri is readily isolated from kidney samples of clinically infected fish using routine bacteriological media but isolation is more difficult from carrier fish. In fact, such fish are difficult to recognise because they show no pathology, with asymptomatic infection only detectable in the lower intestine (Rucker, 1966). Busch and Lingg (1975) demonstrated that up to 25% of rainbow trout (*Oncorhynchus mykiss*) could carry *Y. ruckeri* in their lower intestine. In addition, periodic shedding of the organism in the faeces acts as a source of infection to other fish (Busch, 1973).

Isolation of *Y. ruckeri* from faecal material using standard bacteriological media is difficult because of the number and variety of background contaminating bacteria. A differential medium to isolate *Y. ruckeri* has been described by Waltman and Shotts (1984) but its effectiveness in field trials in Scotland (Hastings and Bruno, 1985) and in Southern England (Rodgers and Hudson, 1985) has been found to be limited. The development and use of an improved differential medium was therefore undertaken not only to aid the detection of carrier fish but also to facilitate further studies on the epidemiology of ERM.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals, reagents and media

All chemicals and reagents, unless otherwise stated, were obtained from either BDH (Poole, U.K.) or Sigma Chemical Company Ltd. (Poole, U.K.). All media were obtained from either Difco (East Molesey, U.K.), LabM (Bury, U.K.) or Oxoid Ltd. (Basingstoke, U.K.).

3.2.2 Bacteriology

3.2.2.1 Bacterial strains

The origin of the strains of *Y. ruckeri* and the strains of comparative bacteria used in the initial media development and evaluation are listed in Table 3.1.

Table 3.1. Origin and API 20E profiles of bacterial strains used in this study

Strain Ref. No.	Origin[1]	Source of Isolate	API 20E Code	Chapter No.[2]
Aeromonas hydrophila				
F78	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1047524	3
F81	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1047524	3
95/70	NCTC 8049	Unknown source	7247126	3
Aeromonas salmonicida				
84/79	FDL isolate	*Salmo trutta* kidney (England)	ND[3]	5
Citrobacter freundii				
F57	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1345573	3
F70	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1404513	3
F85	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1405513	3
Enterobacter sp.				
F61	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1305173	3
F80	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1105173	3
GOUT	FDL isolate	Fish farm outlet water (England)	1105553	3
Escherichia coli				
158/76	NCTC 9001	*Homo sapiens* urine (Denmark)	ND	5
Hafnia alvei				
F55	FDL isolate	*Oncorhynchus mykiss* faeces (England)	4105102	3
F76	FDL isolate	*Oncorhynchus mykiss* faeces (England)	5105112	3
Pseudomonas sp.				
F68	FDL isolate	*Oncorhynchus mykiss* faeces (England)	ND	3
F86	FDL isolate	*Oncorhynchus mykiss* faeces (England)	ND	3
Unidentified species				
F65	FDL isolate	*Oncorhynchus mykiss* faeces (England)	ND	3

Strain Ref. No.	Origin[1]	Source of Isolate	API 20E Code	Chapter No.[2]
Yersinia ruckeri-Serotype 01				
42	FDL isolate	*Esox lucius* kidney (England)	1107100	3
86/61	FDL isolate	*Oncorhynchus mykiss* faeces (Wales)	1105100	3
29/75	NCMB1316	*Oncorhynchus mykiss* kidney (USA)	1104100	5
31/75	NCMB1315	*Oncorhynchus mykiss* kidney (USA)	5104100	5
137/76	Tavolek	*Oncorhynchus mykiss* kidney (USA)	5104100	5
138/76	Tavolek	*Oncorhynchus mykiss* kidney (Canada)	5105100	5
140/76	Tavolek	*Oncorhynchus mykiss* kidney (USA)	5107100	5
141/76	Tavolek	*Oncorhynchus mykiss* kidney (USA)	5106100	5
165/76	Tavolek	*Oncorhynchus mykiss* kidney (USA)	5105100	3, 5
166/76	Tavolek	*Oncorhynchus mykiss* kidney (USA)	5106100	5
167/76	Tavolek	*Oncorhynchus mykiss* kidney (USA)	5107100	5
724.77.1	CDC	*Homo sapiens* bile (USA)	5104500	5
724.77.2	CDC	*Homo sapiens* bile (USA)	5104500	5
23/81	NCTC10476	*Oncorhynchus mykiss* kidney (USA)	1305100	5
24/81	NCTC10477	*Oncorhynchus mykiss* kidney (USA)	1305100	5
25/81	NCTC 10478	*Oncorhynchus mykiss* kidney (USA)	5305100	3, 5
39/81	L. Llewellyn	*Oncorhynchus mykiss* (Australia)	1305100	5
40/81	L. Llewellyn	*Oncorhynchus mykiss* (Australia)	1305100	5
41/81	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5305100	5
42/81	J. Wood	*Oncorhynchus mykiss* kidney (England)	5104100	5
75/81	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5305100	5
1/82	M. Roberts	*Oncorhynchus mykiss* kidney (England)	1305100	5
18/83	G. Giorgetti	*Oncorhynchus mykiss* kidney (Italy)	1105100	5
30/83	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5
15/84	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5
16/84	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5

Strain Ref. No.	Origin[1]	Source of Isolate	API 20E Code	Chapter No.[2]
17/84	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5
18/84	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5
4/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1104100	5
5/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5
6/85	FDL isolate	*Oncorhynchus mykiss* faeces (England)	5305100	5
9/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5
10/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	ND	5
11/85	FDL isolate	Fish farm outlet water (England)	5107100	5
12/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5305100	5
17/85	FDL isolate	*Esox lucius* kidney (England)	1305100	5
18/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5305100	5
20/85	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1305100	5
26/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5305100	5
28/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5105100	5
29/85	FDL isolate	*Thymallus thymallus* kidney (England)	5305100	5
30/85	FDL isolate	*Gobio gobio* kidney (England)	5305100	5
31/85	FDL isolate	*Rutilus rutilus* kidney (England)	5105100	5
34/85	FDL isolate	*Abramis brama* kidney (England)	1107100	5
35/85	I. Dalsgaard	*Oncorhynchus mykiss* (Denmark)	1105100	5
37/85	I. Dalsgaard	*Salmo trutta* (Denmark)	1107100	5
39/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1107100	5
50/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5105100	5
54/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1107100	5
57/85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1305100	5
59/85	FDL isolate	Fish farm outlet water (England)	5105100	5
3/86	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1305100	5
5/86	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1305100	5

Strain Ref. No.	Origin[1]	Source of Isolate	API 20E Code	Chapter No.[2]
8/86	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5305100	5
10/86	R. Bragg	*Oncorhynchus mykiss* kidney (S Africa)	1105100	3, 5
12/86	R. Davies	*Oncorhynchus mykiss* kidney (Scotland)	5305100	3, 5
20/86	FDL isolate	*Oncorhynchus mykiss* faeces (Wales)	5305100	5
21/86	FDL isolate	*Oncorhynchus mykiss* spleen (Wales)	5107100	5
24/86	FDL isolate	*Oncorhynchus mykiss* kidney (Wales)	5107100	3
25/86	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5107100	5
29/86	FDL isolate	Fish farm inlet water (England)	5107100	3, 5
30/86	FDL isolate	Fish farm outlet water (England)	5105100	5
34/86	FDL isolate	*Oncorhynchus mykiss* spleen (England)	1107100	3, 5
35/86	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1105100	3, 5
36/86	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5107100	3
43/86	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5107100	5
46/86	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5305100	5
47/86	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1305100	5
48/86	FDL isolate	*Oncorhynchus mykiss* liver (England)	5107100	3
57/86	E. Janssen	*Oncorhynchus mykiss* (Sweden)	1307100	5
62/86	FDL isolate	*Thymallus thymallus* faeces (England)	5107100	3, 5
68/86	FDL isolate	*Salmo salar* (England)	1305100	5
1/87	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1305100	5
2/87	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1307100	5
3/87	E. Janssen	*Salmo salar* faeces (Sweden)	1305100	5
4/87	E. Janssen	*Salmo trutta* kidney (Sweden)	ND	5
BA3	B. Austin	*Oncorhynchus mykiss* kidney (England)	ND	5
DF11	M. D. Furones	*Oncorhynchus mykiss* kidney (Spain)	5105100	5
DF12	M. D. Furones	*Oncorhynchus mykiss* kidney (Spain)	5107100	5
DF25	M. D. Furones	*Oncorhynchus mykiss* liver (Spain)	ND	5

Strain Ref. No.	Origin[1]	Source of Isolate	API 20E Code	Chapter No.[2]
DF51	NCMB 2194	*Oncorhynchus mykiss* kidney (USA)	5105100	5
DF57	M. D. Furones	Unknown fish source (Norway)	ND	5
DF58	M. D. Furones	*Salmo salar* kidney (Scotland)	ND	5
DF59	M. D. Furones	*Salmo salar* kidney (Scotland)	ND	5
DF60	M. D. Furones	*Salmo salar* kidney (Scotland)	ND	5
DF61	M. D. Furones	*Salmo salar* kidney (Scotland)	ND	5
G1/1F	FDL isolate	*Oncorhynchus mykiss* faeces (England)	5105100	3
G6/7S	FDL isolate	*Oncorhynchus mykiss* spleen (England)	5107100	3
GIN2	FDL isolate	Fish farm inlet water (England)	1105100	3
GOUT	FDL isolate	Fish farm outlet water (England)	5105100	3
LT3/7	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1305100	3
LT7/9F	FDL isolate	*Oncorhynchus mykiss* faeces (England)	1105100	3
LT7/9K	FDL isolate	*Oncorhynchus mykiss* kidney (England)	1107100	3
LT11/4	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5106100	3
LT12/6	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5104100	3, 6
R10/5S	FDL isolate	*Oncorhynchus mykiss* spleen (England)	5107100	3
R10/13F	FDL isolate	*Oncorhynchus mykiss* faeces (England)	5107100	3
TVT85	FDL isolate	*Oncorhynchus mykiss* kidney (England)	5104100	3
MR1	M. Roberts	*Oncorhynchus mykiss* kidney (France)	5104100	5
MR5	M. Roberts	*Oncorhynchus mykiss* kidney (England)	1107100	3
MR6	M. Roberts	*Oncorhynchus mykiss* faeces (England)	1305100	5
MR19	M. Roberts	*Oncorhynchus mykiss* kidney (Germany)	5107100	5
MR20	M. Roberts	*Oncorhynchus mykiss* kidney (W Germany)	1305100	3
MR21	M. Roberts	*Carrasius auratus* kidney (Eire)	1305100	3, 5
MR22	M. Roberts	*Oncorhynchus mykiss* kidney (Italy)	1105100	3, 5
MR33	M. Roberts	*Oncorhynchus mykiss* kidney (England)	5105100	5
MR36	M. Roberts	*Oncorhynchus mykiss* kidney (England)	5105100	5

Strain Ref. No.	Origin[1]	Source of Isolate	API 20E Code	Chapter No.[2]
MR38	M. Roberts	*Oncorhynchus mykiss* kidney (England)	5305100	5
MR41	M. Roberts	*Oncorhynchus mykiss* kidney (England)	5107100	5
MR43	M. Roberts	*Oncorhynchus mykiss* kidney (England)	1305100	5
MR45	M. Roberts	*Oncorhynchus mykiss* kidney (England)	1305100	5
MR46	M. Roberts	*Oncorhynchus mykiss* kidney (England)	1305100	5
MR49	M. Roberts	*Oncorhynchus mykiss* kidney (England)	5305100	5
MR51	M. Roberts	*Oncorhynchus mykiss* kidney (England)	1305100	5
MR52	M. Roberts	*Oncorhynchus mykiss* kidney (England)	5105100	5
MR55	M. Roberts	*Anguilla anguilla* faeces (England)	1105100	3, 5
MR57	M. Roberts	*Esox lucius* faeces (England)	1305100	3, 5
MR58	M. Roberts	*Thymallus thymallus* faeces (England)	1105100	3, 5
MR60	M. Roberts	*Salmo trutta* kidney (England)	1105100	3, 5
MR61	M. Roberts	*Salmo trutta* kidney (England)	1105100	5
MR62	M. Roberts	*Perca fluviatilus* kidney (England)	1107100	3, 5
MR63	M. Roberts	*Abramis brama* kidney (England)	1105100	3, 5
MR64	M. Roberts	*Rutilus rutilus* kidney (England)	1105100	3, 5
MR66	M. Roberts	*Gobio gobio* kidney (England)	1105100	3, 5
RD38	R. Davies	*Micropterus salmoides* (USA)	5104100	5
Yersinia ruckeri-Serotype 02				
136/76	Oregon Univ.	*Oncorhynchus tshawytscha* kidney (USA)	5305500	3, 5
139/76	Tavolek	*Oncorhynchus tshawytscha* kidney (USA)	5105500	5
29/78	R. Sweeting	*Salmo trutta* kidney (England)	5105500	3, 5
2/85	P. D. Smith	*Oncorhynchus mykiss* kidney (USA)	1307500	3, 5
38/85	I. Dalsgaard	*Anguilla anguilla* (Denmark)	5105500	5
59/86	E. Janssen	*Oncorhynchus mykiss* (Sweden)	5305500	5
254/89/1	E. Janssen	Unknown fish source (Sweden)	5304500	5
254/89/2	E. Janssen	Unknown fish source (Sweden)	5304500	5

Strain Ref. No.	Origin[1]	Source of Isolate	API 20E Code	Chapter No.[2]
DF18	M. D. Furones	Unknown fish source (Canada)	5305500	5
DF53	M. D. Furones	Unknown fish source (USA)	ND	5
RD54	R. Davies	*Coregonus artedii* (USA)	ND	5
***Yersinia ruckeri*-Serotype 03**				
DF54	M. D. Furones	Unknown fish source (Norway)	ND	5
DF55	M. D. Furones	Unknown fish source (Norway)	ND	5
DF56	M. D. Furones	Unknown fish source (Norway)	ND	5
***Yersinia ruckeri*-Serotype 05**				
RD50	R. Davies	*Oncorhynchus mykiss* kidney (USA)	ND	5
***Yersinia ruckeri*-Serotype 06**				
RD194	R. Davies	Unknown fish source (Canada)	ND	5
***Yersinia ruckeri*-Serotype 07**				
RD36	R. Davies	*Oncorhynchus mykiss* kidney (USA)	ND	5

Footnotes

[1]B. Austin, Heriot Watt University, Department of Brewing, Edinburgh, Scotland.
R. Bragg, Fish Disease Unit, Veterinary Research Institute, Onderstepoort, Republic of South Africa.
Comunicable Disease Centre (CDC), Atlanta, Georgia, USA.
I. Dalsgaard, Danish Institute for Fisheries Research, Frederiksberg, Denmark
R. Davies, Stirling University, Institute of Aquaculture, Stirling, Scotland.
FDL isolate-M.A.F.F., Fish Diseases Laboratory, Weymouth, Dorset, England.
M. D. Furones, Polytechnic South West, Department of Biological Sciences, Plymouth, England.
G. Giogetti, Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy.
E. Janssen, Swedish Veterinary Institute, Upsala, Sweden.
L. Llewellyn, NSW State Fisheries, Kent Street, Sydney, New South Wales, Australia.
National Collection of Marine Bacteria (NCMB), Aberdeen, Scotland.
National Collection of Type Cultures (NCTC), Colindale, London, England.
Oregon State University, Department of Microbiology, Corvallis, Oregon, USA.
M. Roberts, Southern Water Authority, Worthing, Sussex, England
P.D. Smith, Aquaculture Vaccines Ltd., Saffron Walden, Essex, England.
R. Sweeting, Thames Water Authority, Reading, Berkshire, England.
Tavolek Inc., Redmond, Washington, USA.
J. Wood, Fishcare, Brighton, England.

[2]Chapter number where each strain was used

[3]API 20E test not done-no code available

3.2.2.2 Bacterial cultivation and storage

All strains were incubated at 26°C and subcultured every five days onto Tryptone Soya Agar (TSA; Oxoid) while being used for experimental work. Duplicates of each isolate were maintained on a nutrient agar (NA) slope at 4°C and at -20°C in 1ml aliquots of freeze-drying broth (3 parts 7.5% w/v glucose in horse serum:1 part nutrient broth) with the addition of 0.1% w/v glycerol (FDBG).

3.2.2.3 Total viable counts

Total viable counts (TVC) were undertaken using a spread plate technique. Each appropriate serial dilution of *Y. ruckeri*, in 0.9% saline, was plated (0.1 ml) onto TSA and incubated at 26°C for 48 h. Plates showing individual colonies (i.e. <300) were recorded and the TVC was calculated as colony forming units (c.f.u.) ml^{-1}. Each TVC was performed in duplicate.

3.2.3 Biochemical characterization of strains

Representative strains of Enterobacteriaceae, including *Y. ruckeri*, were identified using the API 20E (API-bioMérieux UK Ltd.) system of biochemical testing and selected representatives were further characterised using API 50CH carbohydrate strips, to indicate compounds which could be used further for growth studies in a differential medium. The strips were inoculated according to the manufacturers instructions but were incubated at 26°C and the results were recorded at 24, 48 (API 20E and API 50CH) and 72 h (only API 50CH). Other isolates were characterized by recording their Gram, O/F (Hugh and Leifson), catalase, oxidase, motility and growth at 37°C characteristics following the methods in Austin and Austin (1987).

3.2.4 Comparison of commercial selective media

In an initial screening to assess their suitability for differentiating between *Y. ruckeri* and other members of the Enterobacteriaceae, a comparison was made between selected commercially-available media. Media evaluated included Brilliant Green agar (BG), Desoxycholate agar (DESOX), Desoxycholate Citrate Lactose Sucrose agar (DCLS), Eosin Methylene Blue agar (EMB), Hektoen Enteric agar (HE), McConkey agar (McC), Violet Red Bile agar (VRB), and Xylose Lysine Deoxycholate agar (XLD)

(all Oxoid). Both commercial and subsequent laboratory formulated media were inoculated from TSA culture plates using routine sub-culture and spread plate techniques where necessary. Growth characteristics were recorded after 2, 4, 7, 10 and 14 days incubation at 26°C.

Morphological characteristics were recorded following incubation at 26°C for 4 and 7 days. The sensitivity of the laboratory formulated medium was determined by comparing the viable count of a standardised inoculum with that obtained using TSA.

3.2.5 Antibiotic sensitivity tests

Suspensions of each strain to be tested were made in 0.9% saline and standardized (A_{625}=1) using a spectrophotometer SP 1800 (Pye Unicam, Cambridge, UK). The suspensions were then used to surface inoculate (100 µl) sensitivity agar (LabM) using a spread plate technique which was designed to give 1 x 10^5 c.f.u. ml^{-1}. Multi-sensitivity discs were then placed on each plate. Zones of inhibition were recorded and measured in mm after incubation at 26°C for 48 h. Discs used included Mastring (Mast Laboratories Ltd, Liverpool, U.K.) and Oxoid Multodiscs (Oxoid). This enabled the following antibiotics to be tested: amikacin (30 µg ml^{-1}), ampicillin (2, 10 and 25 µg ml^{-1}), carbenicillin (100 µg ml^{-1}), cephalexin (30 µg ml^{-1}), chloramphenicol (50 µg ml^{-1}), clindamycin (2 µg ml^{-1}), colistin sulphate (25 and 100 µg ml^{-1}), cotrimoxazole (25 µg ml^{-1}), erythromycin (5 µg ml^{-1}), gentamycin (10 µg ml^{-1}), kanamycin (30 µg ml^{-1}), lincomycin (2 µg ml-1), mezlocillin (30 µg ml^{-1}), nalidixic acid (30 µg ml^{-1}), neomycin (30 µg ml^{-1}), nitrofurantoin (50 µg ml^{-1}), novobiocin (5 µg ml^{-1}), penicillin G (1 and 4 µg ml^{-1}), polymyxin B (250 µg ml^{-1}), streptomycin (25 µg ml^{-1}), sulphamethizole (200 µg ml^{-1}), sulphamethoxazole (25 µg ml^{-1}), tetracycline (10, 25 and 100 µg ml^{-1}), tobramycin (10 µg ml^{-1}) and trimethoprim (1.25 µg ml^{-1}).

3.2.6 Base media formulations

A broth comprised of 0.3% yeast extract, 0.1% sodium deoxycholate, 0.5% sodium chloride, 0.68% sodium thiosulphate and 0.08% ferric ammonium citrate was used to assess the effect of pH, the carbohydrates maltose and ribose on growth and the action of ornithine decarboxylase.

3.2.6.1 Effect of pH

The formulation in 3.2.6 was used as a base to follow the performance of the broth at pH 7.4, 6.5 and 5.5, for up to 48 h, by using changing absorbance at 560nm (SP1800; Pye Unicam) and by Total Viable Count (TVC). In addition, the change in pH for each broth was determined over the same time period.

3.2.6.2 Effect of nutritional compounds

A 10% solution of each carbohydrate or ornithine was prepared in distilled water and 50 µl was used to obtain serial doubling dilutions in 96 well microtitre trays (Sterilin). Each tray tested the effect of combinations of broth base and the compounds listed above. This method enabled a range of dilutions between 2.5% and 0.003% to be covered and used the final well as a negative control.

The bacterial strains tested were first inoculated onto TSA and incubated at 26°C for 48 h before being suspended in plate count broth (PCB; Difco) to an A_{560} of 0.1. Fifty µl of media were pipetted into each micro-titre well and the trays were then placed in a moist chamber and incubated at 26°C for 24 h. The well that showed no further increase in growth, as denoted by the size of the deposit, was recorded as the optimum growth concentration (OGC). This method allowed a rapid screening of nutritional compounds and in addition showed which combinations gave the best formulation prior to the addition of an indicator and antimicrobial agents.

3.2.6.3 Bacterial growth curves

The growth of *Y. ruckeri* in peptone water at pH values between 6 and 10, inoculated as above, was also determined. Growth was assessed by changing absorbance at 560 nm over a 48 h period.

3.2.7 Indicators

The pH range of indicator compounds was tested to select one with a decisive colour change between pH 5-7. A 1% w/v solution of each indicator was assessed by titration with 0.1M hydrochloric acid. The following indicators were tested: phenol red, bromothymol blue, bromophenol red, bromocresol purple, methyl red, bromocresol green, bromophenol blue, lacmoid (resorcin blue), alizarin red S, methyl red thymol

blue, gallein, BDH 4.5, BDH 4080, BDH 4460, ethyl red, chlorophenol red, cochineal and resazurin (all BDH).

3.2.8 Antimicrobial compounds

Compounds with potential antimicrobial action were screened in order to find those which would inhibit unwanted isolates from fish faecal material but still allow *Y. ruckeri* to grow with reduced competition. Compounds tested included ascorbic acid, crystal violet, cysteine hydrochloride, ethylene diamine-tetra acetic acid (EDTA), isoniazid, potassium iodide, sodium chloride, sodium deoxycholate, sodium dodecyl sulphate (SDS), phenol red, toluidine blue, urea (all BDH), bay oil, cycloserine, evening primrose oil, oil of origanum, sodium selenite, sodium tetrathionate, sodium thiosulphate, thymol, Tweens 20, 40, 60, and 80 (all Sigma) and bile salts (Oxoid).

A 10% solution of each compound was prepared in either PCB or absolute alcohol (aromatic oils only) and 50 µl aliquots were used to obtain serial doubling dilutions in U-well microtitre trays (Sterilin Ltd, Teddington, UK). This method enabled a range of dilutions between 2.5% and 0.003% to be tested in each tray and used the final well as a negative control. In addition the 'selectatab' Yersinia supplements (Diamed/Mast) and 'adatabs' aztreonam, cefotaxime, ceftazidime, ceftizoxime, cefsulodin and moxalactam (Diamed/Mast) were evaluated using the method described above for the antimicrobial compounds. The range of concentrations tested for the 'selectatabs' was 100-0.3 mg ml^{-1} and 32-0.01 mg ml^{-1} for the 'adatabs'. Further evaluation of individual antimicrobial compounds was undertaken by the agar dilution method (see section 5.2.6b).

The bacterial strains were tested as already described for base media formulations (section 3.2.6). However, the last well to show absence of growth was recorded as the minimum inhibitory concentration (MIC).

3.2.9 Laboratory trials

Once the initial medium formulation had been developed, sensitivity was determined in the laboratory by performing TVC's using a standardised inoculum of

representative strains of *Y. ruckeri* and other members of the Enterobacteriaceae. Colony colour and other cultural characteristics were also noted.

3.2.10 Field trials

Media formulations from the laboratory study were evaluated at two fish farms on the same river system in southern England (Farm A; Test Valley Trout Farm, Greatbridge, Romsey, Hampshire and Farm B; Test Valley Trout Farm, The Island, Romsey, Hampshire). Farm A is constructed essentially as a tank system (Plate 3.1a and 3b) which produces up to 360 tonnes of rainbow trout a year and where there is a significant chronic infection of ERM. Farm B, however, is a raceway unit (Plate 3.2a and 3.2b) producing 250 tonnes a year with only an occasional acute infection of ERM.

3.2.10.1 Fish

The fish used in the field trials represented the normal deliveries of rainbow trout to each farm. Consequently, different batches were continually monitored throughout this experiment. Sites supplying farms A and B included Broad Chalke Trout Farm, Wiltshire; Chalke Valley Trout Farm, Wiltshire; Cloan Hatchery, Tayside; Upwey Trout Farm, Dorset and Warnford Trout Farm, Hampshire. All fish were immunized with an anti-*Y. ruckeri* vaccine (AVL, Saffron Walden, UK) before delivery and varied in size on arrival from 4.5 g to 17 g (100/lb to 27/lb).

3.2.10.2 Bacteriological sampling

Fish populations were sampled every five weeks by taking a random sample of 15 fish from each of three groups. At post-mortem, kidney material was plated onto TSA and faecal material was plated onto each medium formulation under test. In addition, 25 ml samples of river water were collected aseptically in glass universal bottles from the inflow and outflow to each farm (Plates 3.3a, 3.3b, 3.4a and 3.4b). From each sample 0.1 ml was inoculated onto duplicate test medium plates using a spread plate technique.

The inoculated plates were incubated in the laboratory at 26°C and the TSA samples were checked for growth from kidney at two and four days. Growth on the test media from faecal material or water was checked after 2, 4, 7, 10 and 14 days. Colonies suspected of being *Y. ruckeri* were presumptively identified by whole cell slide agglutination (Toranzo *et al.*, 1987) using antiserum to serotypes 01 and 02 prepared in rabbits. Agglutination within 10 sec was considered to be positive, whereas no agglutination after 5 min was recorded as negative. Saline negative controls were used throughout. Isolates giving a positive whole cell agglutination reaction were confirmed as *Y. ruckeri* by the API 20E (API-bioMérieux, UK Ltd.) system of biochemical testing (see section 3.2.3).

The final medium formulation was evaluated throughout 12 months of field trials.

3.2.11 Statistics

A two-sample T-test was used to compare the sensitivity of media for bacterial growth. A probability value of 0.05 (5%) was considered to be the significance level.

Plate 3.1a **Small tank units for rearing fry at Farm A.**

Plate 3.1b **Large tank units for on-growing fish at Farm A.**

Plate 3.2a **Raceways for rearing fry (foreground) and for on-growing (background) at Farm B.**

Plate 3.2b **Detail of raceways for on-growing fish at Farm B.**

Plate 3.3a **Inflow water sampling point (site 1; I1) and automatic weed screen (WS) at Farm A.**

Plate 3.3b **Outflow water sampling point (O) at Farm A.**


WS
11

Plate 3.4a **Inflow water sampling point (I) and manual weed screen (WS) at Farm B.**

Plate 3.4b **Outflow water sampling point (O) at Farm B.**


I
WS


O

3.3 RESULTS

3.3.1 Biochemical comparison of isolates

The biochemical characteristics of representative Enterobacteriaceae and strains of *Y. ruckeri* were tested using API 20E and API 50CH carbohydrate strips. The results are given in Tables 3.1, 3.2 and 3.3.

Although there were ten different API 20E profiles for the strains of *Y. ruckeri* serotype 01 tested, 78% of the isolates gave one of the following 5 profiles: 1105100, 1305100, 5105100, 5107100 and 5305100. Strains 724.77.1 and 724.77.1 (human isolates) gave an additional profile but it was not possible to confirm these isolates as serotype 01 using simple whole cell slide agglutination. Consequently, the profile of 5104500 may represent one of the lesser serotypes not type 01 as indicated in Table 3.1. The serotype 02 strains gave one of 4 profiles.

Table 3.2. **Distribution of API 20E profiles for *Yersinia ruckeri*** (serotypes 01 and 02)

***Yersinia ruckeri* serotype 01** (n=107) (excluding human isolates)	
1104100 (1.9%)	5104100 (6.5%)
1105100 (14.0%)	5105100 (13.1%)
	5106100 (2.8%)
1107100 (8.4%)	5107100 (15.9%)
1305100 (21.5%)	5305100 (14.0%)
1307100 (1.9%)	
***Yersinia ruckeri* serotype 02** (n=9)	
	5105500 (33.3%)
	5304500 (22.2%)
	5305500 (33.3)
1307500 (11.1%)	

Table 3.3. Biochemical characteristics of bacteria used in this study -API 50CH

Bacterial Species	API 50CH Carbohydrate Positive Characters
Aeromonas hydrophila	Glycerol, L-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, N-acetyl glucosamine, arbutin, esculin, salicin, maltose, saccharose, trehalose, amidon, glycogen, gluconate.
Citrobacter freundii	Glycerol, D-arabinose, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, rhamnose, mannitol, sorbitol, N-acetyl glucosamine, cellobiose, maltose, lactose, melibiose, trehalose, L-fucose, gluconate, 2-ceto-gluconate, 5-ceto-gluconate.
Enterobacter sp.	Glycerol, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, rhamnose, dulcitol, mannitol, sorbitol, a-methyl-D-glucoside, N-acetyl glucosamine, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, trehalose, D-raffinose, b-gentiobiose, D-turanose, D-tagatose, gluconate, 2-ceto-gluconate, 5-ceto-gluconate.
Hafnia alvei	Glycerol, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, rhamnose, mannitol, N-acetyl glucosamine, arbutine, esculin, salicin, maltose, saccharose, trehalose, b-gentiobiose, L-fucose, gluconate.
Yersinia ruckeri (Serotype 01)	Glycerol, ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, N-acetyl glucosamine, maltose, trehalose.
Yersinia ruckeri (Serotype 02)	Glycerol, ribose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, N-acetyl glucosamine, maltose, trehalose.

3.3.2 Comparison of commercial selective media

Of the eight commercially available media tested, only Xylose Lysine Desoxycholate agar (XLD) had any potential diagnostic value in this study. This medium allowed *Y. ruckeri* to grow but at the same time reduced the amount of competing bacterial growth obtained from fish faecal material. However, *Y. ruckeri* was difficult to detect in the resultant mixed cultures because it did not exhibit any positive differentiating characteristics on XLD. The colonial characteristics of representive isolates are shown in Table 3.4.

Since all isolates of *Y. ruckeri* were unable to ferment the monosaccharide xylose and not all strains can utilise lysine, these compounds were replaced by alternatives. A comparison of the results for each strain tested showed therefore that ribose and ornithine hydrochloride could be useful substitutes (Tables 3.1, 3.2 and 3.3).

Table 3.4. Colonial characteristics of *Y. ruckeri* and other bacteria on commercial media and ROD

Medium	Bacterial isolate				
	A. hydrophila	*C. freundii*	*Enterobacter*	*H. alvei*	*Y. ruckeri* (Serotypes 01&02)
BG	Pale cream/ pink colonies and bright pink medium	Cream colonies and bright pink medium	Pale yellow colinies and red/ pink medium	Pale cream/ pink colonies and bright pink medium	Pale cream colonies and bright pink medium
DESOX	Pale orange colonies and medium.	Deep pink colonies and pink medium.	Deep pink centred colonies and pink medium.	Pale orange colonies and medium.	Pale orange colonies and medium.
DCLS	Pale yellow colonies and medium	Pale yellow colonies and medium	Pale yellow/orange colonies and yellow medium	Pale yellow/ orange colonies and medium	Pale yellow colonies and medium
EMB	Pale pink colonies and pale orange medium	Pale pink/red colonies and pale pink medium	Pale pink colonies and pale orange medium	Cream colonies and pale pink medium	Cream colonies and pale orange medium

Table 3.4. Colonial characteristics of *Y. ruckeri* and other bacteria on commercial media and ROD (continued)

Medium	Bacterial isolate				
	A. hydrophila	*C. freundii*	*Enterobacter*	*H. alvei*	*Y. ruckeri* (Serotypes 01&02)
HE	Green colonies and pale green medium	Bright yellow colonies and pale red medium with pale red agar deposit	Green colonies and pale green medium	Pale yellow/ green colonies and medium	Green colonies and blue/green medium
McC	Pale orange colonies and orange medium	Pink colonies and orange medium	Bright pink colonies and orange medium	Pale orange colonies and orange medium	Pale orange colonies and orange medium
VRB	Pale yellow/ brown colonies and medium	Mauve colonies and medium	Mauve colonies and deep mauve medium	Pale mauve colonies and pale yellow medium	Deep mauve colonies and mauve medium
XLD	Pale yellow/ brown colonies and red medium	Pale yellow colonies and yellow medium	Yellow colonies and medium	Cream/yellow colonies and red medium	Cream colonies and orange medium
ROD	Pale yellow colonies and medium	Pale yellow colonies and medium	Bright yellow colonies and yellow medium	Pale yellow colonies and medium	Bright yellow colonies and red medium. Yellow agar deposit with serotype 01.

3.3.3 Antibiotic sensitivity tests

The results for antibiotic sensitivities are shown in Table 3.5.

Table 3.5 Antibiotic sensitivities

Bacterial Strain	Sensitive to:
Aeromonas hydrophila	Amikacin, chloramphenicol, colistin sulphate, cotrimoxazole, erythromycin, gentamicin, kanamycin, mezlocillin, nalidixic acid, nitrofurantoin, polymyxin B, streptomycin, sulphamethizole, sulphamethoxazole, tetracycline, tobramycin and trimethoprim.
Citrobacter freundii	Amikacin, ampicillin, carbenicillin, chloramphenicol, colistin sulphate, cotrimoxazole, gentamicin, kanamycin, mezlocillin, nalidixic acid, nitrofurantoin, polymyxin B, streptomycin, sulphamethizole, sulphamethoxazole, tetracycline, tobramycin and trimethoprim.
Enterobacter sp.	Amikacin, carbenicillin, chloramphenicol, colistin sulphate, cotrimoxazole, gentamicin, kanamycin, mezlocillin, nalidixic acid, nitrofurantoin, polymyxin B, streptomycin, sulphamethizole, sulphamethoxazole, tetracycline, tobramycin and trimethoprim.
Hafnia alvei	Amikacin, carbenicillin, chloramphenicol, colistin sulphate, cotrimoxazole, gentamicin, kanamycin, mezlocillin, nalidixic acid, nitrofurantoin, polymyxin B, streptomycin, sulphamethizole, sulphamethoxazole, tetracycline, tobramycin and trimethoprim.
Yersinia ruckeri (01 & 02)	Amikacin, chloramphenicol, colistin sulphate, cotrimoxazole, erythromycin, gentamicin, kanamycin, nalidixic acid, neomycin, nitrofurantoin, polymyxin B, streptomycin, sulphamethizole, sulphamethoxazole, tetracycline, tobramycin and trimethoprim.

3.3.4 Base media formulations

The concentrations of ornithine, maltose and ribose required to support optimal growth (OGC) of *Y. ruckeri*, in combination, were 0.375, 0.75 and 0.375% respectively. This contrasted with the minimum concentration of 0.006, 0.04 and 0.09% respectively (Table 3.6).

Table 3.6 Stimulation of growth of *Y. ruckeri* by various medium additives

Compound Combination	Concentrations needed to support growth (%) Minimum	Optimum
Ornithine only	0.79	1.65
Maltose only	0.2	1.27
Ribose only	0.004	1.27
Ornithine/Maltose	0.09/0.14	0.22/0.22
Ornithine/Ribose	0.09/0.18	0.22/0.22
Maltose/Ribose	0.95/0.73	1.27/1.27
Ornithine/Maltose/Ribose	0.006/0.04/0.09	0.375/0.75/0.375

The optimum concentration for maltose and ribose as single source carbohydrates (1.27%) was lowered to 0.22% only when in combination with ornithine. The concentration remained at 1.27% when they were combined together.

Initial experiments, using inoculated peptone water at a range of pH, indicated that during the first 24 h of growth the log phase occurred from four to eight h at all values tested. However, the range of pH 6-8 gave the highest OD and indicated maximum growth, whilst less growth took place at pH 9-10 in comparison to these other values (Figure 3.1).

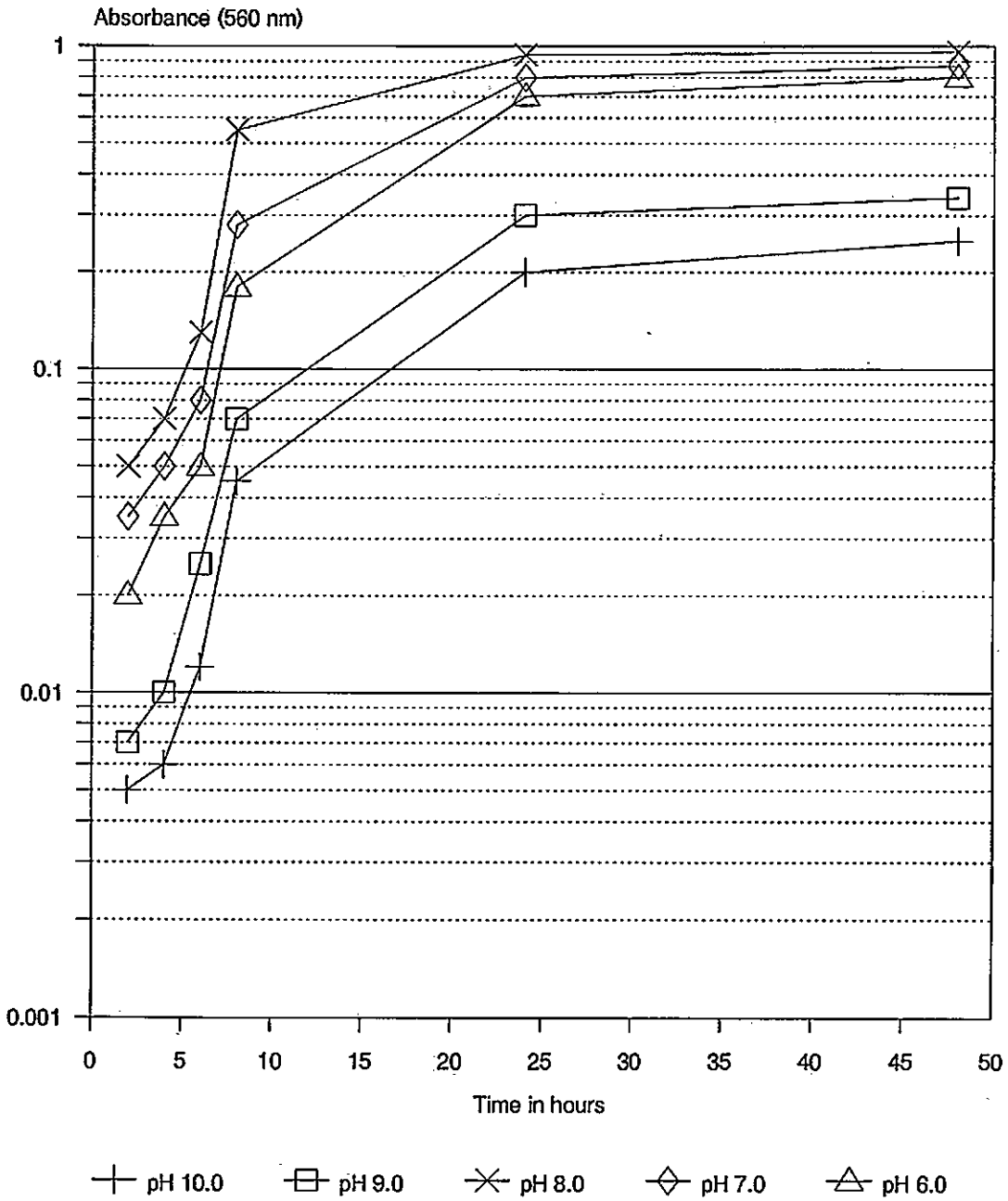


Figure 3.1 Growth in peptone water at different pH

A closer analysis of the values 5.5, 6.5 and 7.4, by using TVC from inoculated broth base, showed that both the lower values gave fewer cells between eight and 48 h (i.e. after the log phase) compared to pH 7.4. However, absorbance readings, obtained in parallel, indicated that all pH values gave similar values after a 48 h period (Figure 3.2a and 3.2b).

Examination of the shift in pH with time, using the same three starting values, showed that after 12 h all broths, irrespective of the initial value, had a pH of 6.4. However, the pH of each broth reduced to 5.1 after a further 12 h and only reached 4.9 over the subsequent 24 h period (Figure 3.3).

As a result of these preliminary experiments the above combinations of ribose, maltose and ornithine at pH 7.4 were used in further work to develop a corresponding agar based medium.

3.3.5 Indicators

Prospective indicators were selected for further trial on the basis of the above results.

A laboratory and field study was therefore used to evaluate phenol red, bromophenol red, bromocresol purple and resazurin for possible inclusion in the medium. These trials showed that phenol red gave the clearest colour change from red to yellow as the pH of the medium was reduced after inoculation either with laboratory strains of bacteria or with material from fish.

3.3.6 Antimicrobial compounds

Screening of antimicrobial compounds indicated that SDS and sodium deoxycholate, at 1% and 0.1% respectively, reduced background growth due to *Aeromonas hydrophila*, *Enterobacter* sp, *Pseudomonas* sp. and unidentified Gram negative rods, originally isolated from faecal material (Table 3.7). These compounds were therefore included in the formulation for testing in field trials.

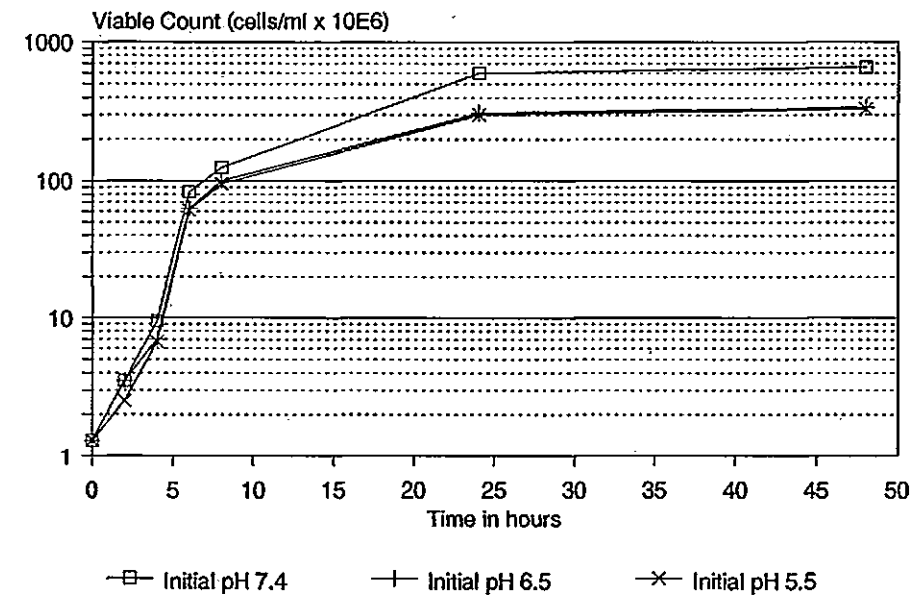


Figure 3.2a Total Viable Count at three pH values

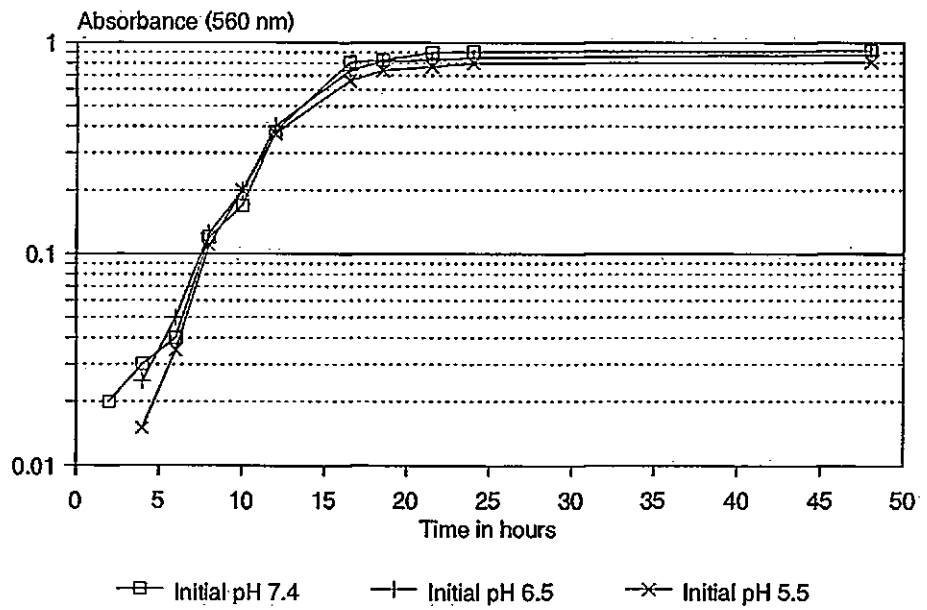


Figure 3.2b Broth base absorbance at three pH values

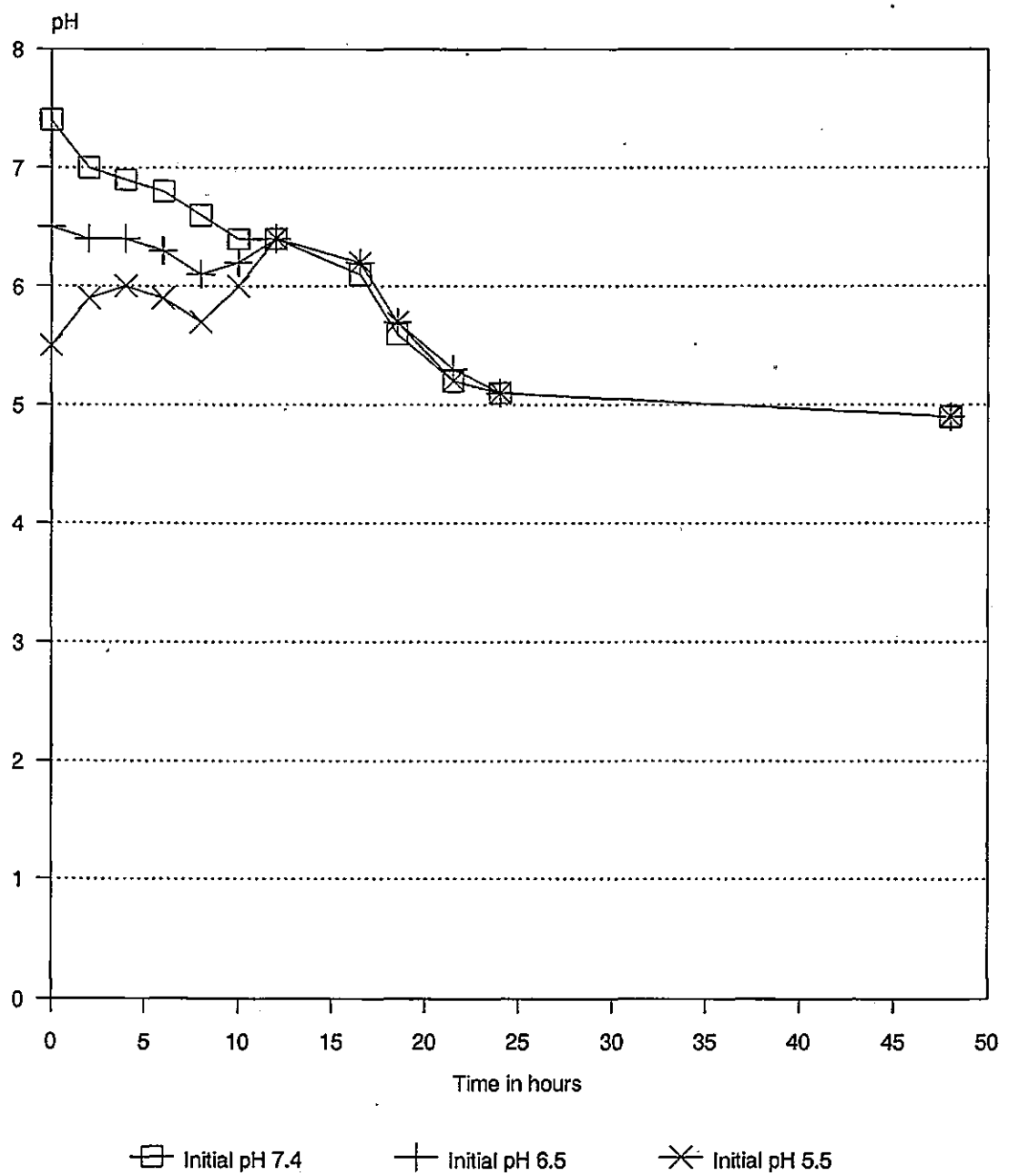


Figure 3.3 Broth base-changes in pH with time

Other antimicrobial compounds, namely bile salts, crystal violet, cycloserine, cysteine hydrochloride, EDTA and evening primrose oil, were also considered further. However, the differences in the MIC end points between *A. hydrophila*, *Enterobacter* sp. and *Y. ruckeri* were not as great as those shown by sodium deoxycholate and SDS. These compounds were therefore subsequently discounted. There were no compounds capable of differentiating between *C. freundii*, *H. alvei* and *Y. ruckeri* except phenol red but the inclusion of this agent is dealt with in section 3.3.5.

Table 3.7 Inhibition of growth of *Y. ruckeri* by various antimicrobial compounds

Compound	Isolate MIC (% concentration)				
	A. hydrophila	*C. freundi*	*Enterobacter*	*H. alvei*	*Y. ruckeri*
Ascorbic acid	>2.5	>2.5	>2.5	>2.5	>2.5
Bay oil	0.005	<0.003	<0.003	<0.003	<0.003
Bile salts	0.039	1.25	1.25	>2.5	2.5
Crystal violet	0.039	1.25	1.25	>2.5	2.5
Cycloserine	0.078	>2.5	1.25	1.25	1.25
Cysteine HCL	0.01	1.25	0.313	>2.5	1.25
EDTA	0.01	1.25	0.625	>2.5	1.25
Evening primrose oil	0.313	2.5	2.5	>2.5	1.25
Isoniazid	>2.5	2.5	1.25	2.5	0.125
Oil of origanum	<0.003	<0.003	<0.003	0.01	<0.003
Phenol red	0.125	0.125	0.125	0.005	0.125
Potassium iodide	>2.5	>2.5	>2.5	>2.5	>2.5
Sodium chloride	>2.5	>2.5	>2.5	>2.5	2.5

Table 3.7 Inhibition of growth of *Y. ruckeri* by various antimicrobial compounds (continued)

Compound	Isolate MIC (% concentration)				
	A. hydrophila	*C. freundi*	*Enterobacter*	*H. alvei*	*Y. ruckeri*
Sodium deoxycholate	0.003	1.25	0.01	>2.5	1.25
SDS	0.078	2.5	2.5	1.25	1.25
Sodium selenite	0.008	0.003	0.003	<0.003	0.005
Sodium tetrathionate	0.313	0.156	0.313	0.313	0.313
Sodium thiosulphate	>2.5	>2.5	>2.5	>2.5	>2.5
Thymol	<0.003	<0.003	<0.003	<0.003	<0.003
Toluidine blue	0.059	>2.5	0.078	>2.5	0.008
Tween 20	>2.5	>2.5	>2.5	>2.5	>2.5
Tween 40	>2.5	>2.5	>2.5	>2.5	>2.5
Tween 60	>2.5	>2.5	>2.5	>2.5	>2.5
Tween 80	>2.5	>2.5	>2.5	>2.5	>2.5
Urea	2.5	>2.5	2.5	>2.5	2.5
Yersinia supplement	0.313	0.039	>2.5	0.02	0.039

N.B. The MIC values of sodium deoxycholate and SDS for *Pseudomonas* sp. were 0.01 and 0.003% respectively whereas the values for the unidentified species were 0.039 and 0.313%.

In addition, the antibiotics aztreonam and ceftazidime were also selected for further laboratory evaluation based on the results shown in Table 3.8. However the additional experiments indicated that only aztreonam should be included in field trials. These trials gave inconsistent results over an 11 month period but showed that *Y.*

ruckeri was isolated from faecal samples of 46/156 (29.5%) fish using media without antibiotic. This compared with 30/156 (19.2%) on media containing 2.5 μg ml^{-1} aztreonam. However, the yellow colour change around the colonies and the red colouration within the medium were more sharply defined, resulting in plates which were easier to interpret. In addition the general background growth was reduced. Nevertheless, for maximum sensitivity, aztreonam was subsequently omitted from the final formulation.

Table 3.8 Adatab antibiotic sensitivities

Antibiotic	Isolate MIC (μg ml^{-1})				
	A. hydrophila	*C. freundii*	*Enterobacter*	*H. alvei*	*Y. ruckeri*
Aztreonam	0.2	>80.0	80.0	>80.0	80.0
Cefotaxime	6.3	0.2	6.3	6.3	6.3
Cefsulodin	2.5	40.0	80.0	80.0	40.0
Ceftazidime	0.3	40.0	80.0	80.0	80.0
Ceftizoxime	2.5	0.1	40.0	40.0	1.3
Moxalactam	0.1	0.2	1.3	0.6	0.6

3.3.7 Formulation of medium

From the results described in the above sections, the formulation for a novel medium, designated Ribose Ornithine Desoxycholate agar (ROD), was determined. 3 g yeast extract, 1 g sodium deoxycholate, 5 g sodium chloride, 6.8 g sodium thiosulphate, 0.8 g ferric ammonium citrate, 3.75 g ribose, 7.5 g maltose, 5 g ornithine hydrochloride, 1% sodium dodecyl sulphate (SDS), 0.08 g phenol red, and 12.5 g agar No. 1 (Oxoid), in 1 litre distilled water, at pH 7.4. The medium was initially prepared by bringing the ingredients to the boil without adding the antimicrobial agent SDS, taking care to avoid overheating of the medium. SDS was added by filtration through a 0.2 μm disposable filter unit (Millipore) after cooling the medium to 55°C in a water bath.

3.3.8 Laboratory trials

Y. ruckeri serotype 01 (biotypes 1 and 2; Davies and Frerichs 1989), and serotype 06 (Davies 1990) grown on ROD, produced yellow colonies against a red background with yellow crystalline deposits which diffused into the surrounding agar after 72 to 96 hours incubation at 26°C (Plate 3.5a). Further incubation, for up to 10 days, resulted in the yellow deposits becoming more intense with the surrounding medium also turning yellow. *Y. ruckeri* (serotypes 02, 05 and 07), however, did not produce a yellow deposit in the agar but did produce a pale yellow colony colouration (Plate 3.5a) which was similar to *C. freundii* (Plate 3.5b), *H. alvei* (Plate 3.6a) and *Enterobacter* sp. (Plate 3.6b).

The sensitivity of ROD compared to TSA showed that growth of *A. hydrophila* and the unidentified Gram negative rods was reduced on ROD whereas *Pseudomonas* sp. was eliminated (two sample t-test, $p<0.05$). The growth of the Enterobacteriaceae on ROD and TSA, however, was not significantly different ($p>0.05$; Table 3.9).

Table 3.9 Growth of *Yersinia ruckeri* and other bacteria on TSA and ROD

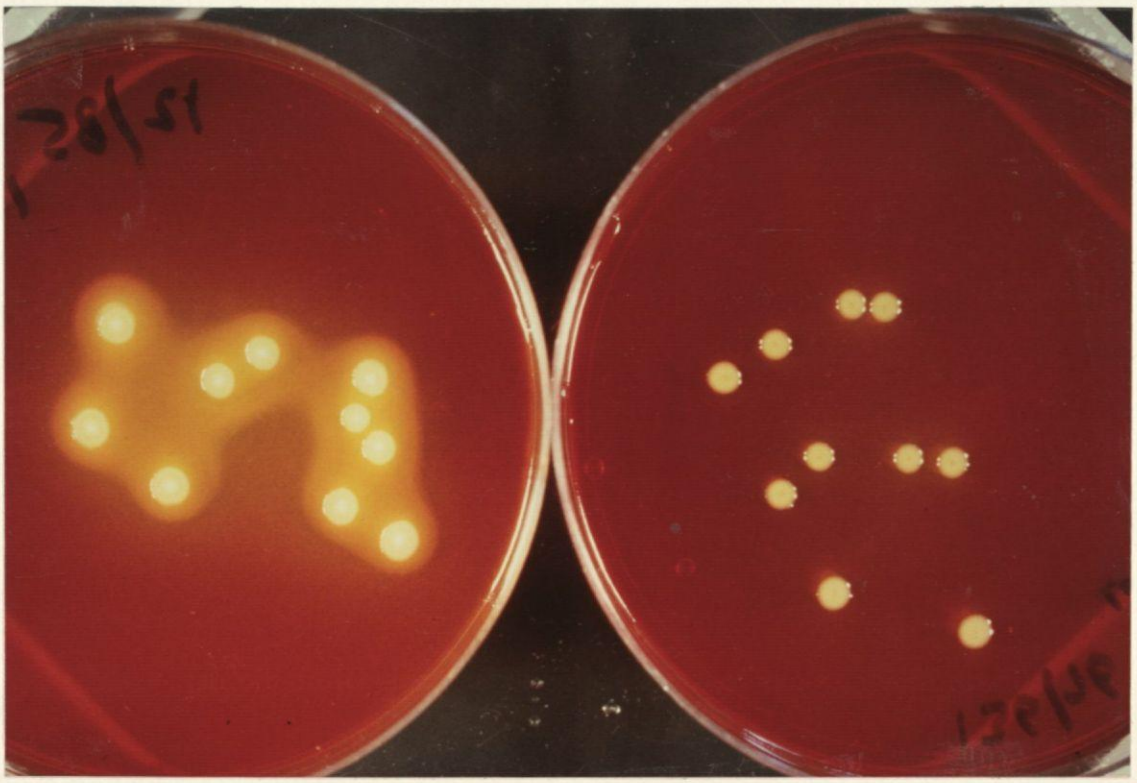
Bacterial strain	Total Viable Count (c.f.u. ml^{-1} x 10^6)* TSA	ROD
Y. ruckeri-01	3.04 (SD-1.02)+	4.08 (0.47)
Y. ruckeri-02	6.76 (3.14)	11.80 (1.50)
A. hydrophila	0.85(0.16)	0.03 (0.01)
H. alvei	3.24 (1.51)	3.70 (2.01)
C. freundii	2.35 (0.70)	4.10 (2.02)
Enterobacter sp.	3.98 (2.96)	2.90 (1.69)
Pseudomonas sp.	1.10 (0.10)	0.00 (-)
Unidentified species	3.09 (0.12)	0.03 (0.85)

* Mean of four replicates

+ Standard deviation is shown in parenthesis

Plate 3.5a Colonies of *Yersinia ruckeri* on ROD medium. Serotype 01 (left) and serotype 02 (right)

Plate 3.5b Colonies of *Citrobacter freundii* on ROD medium.



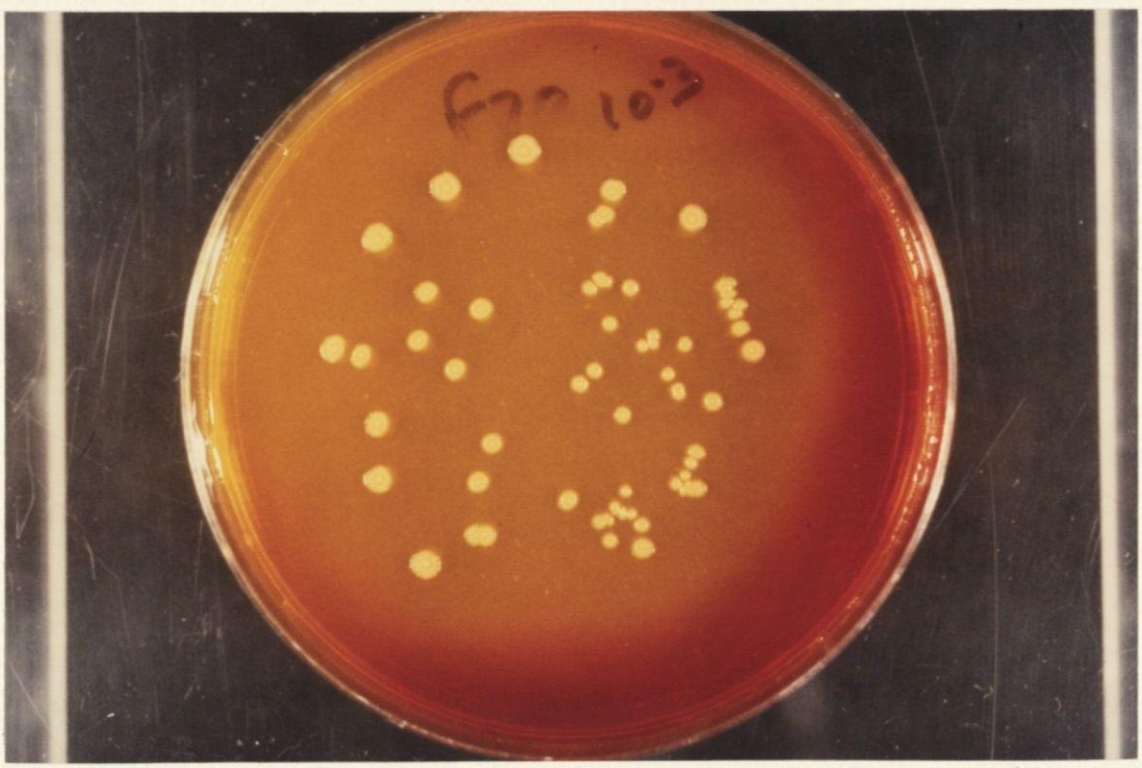
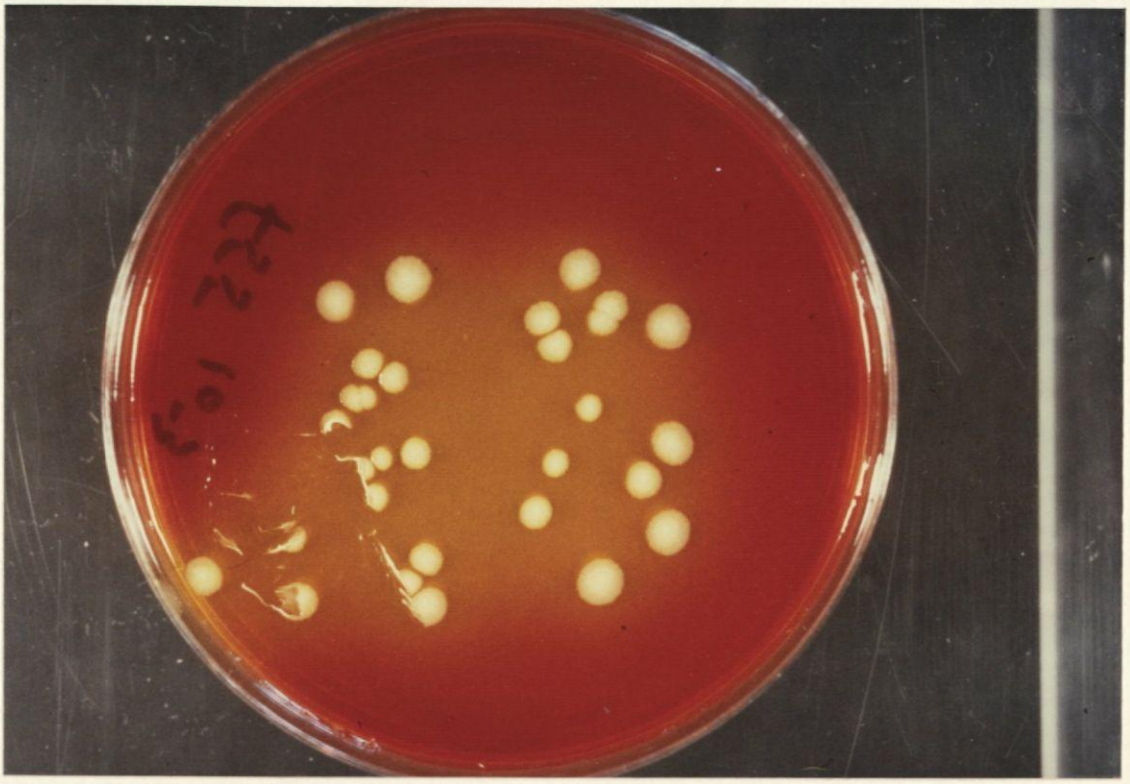
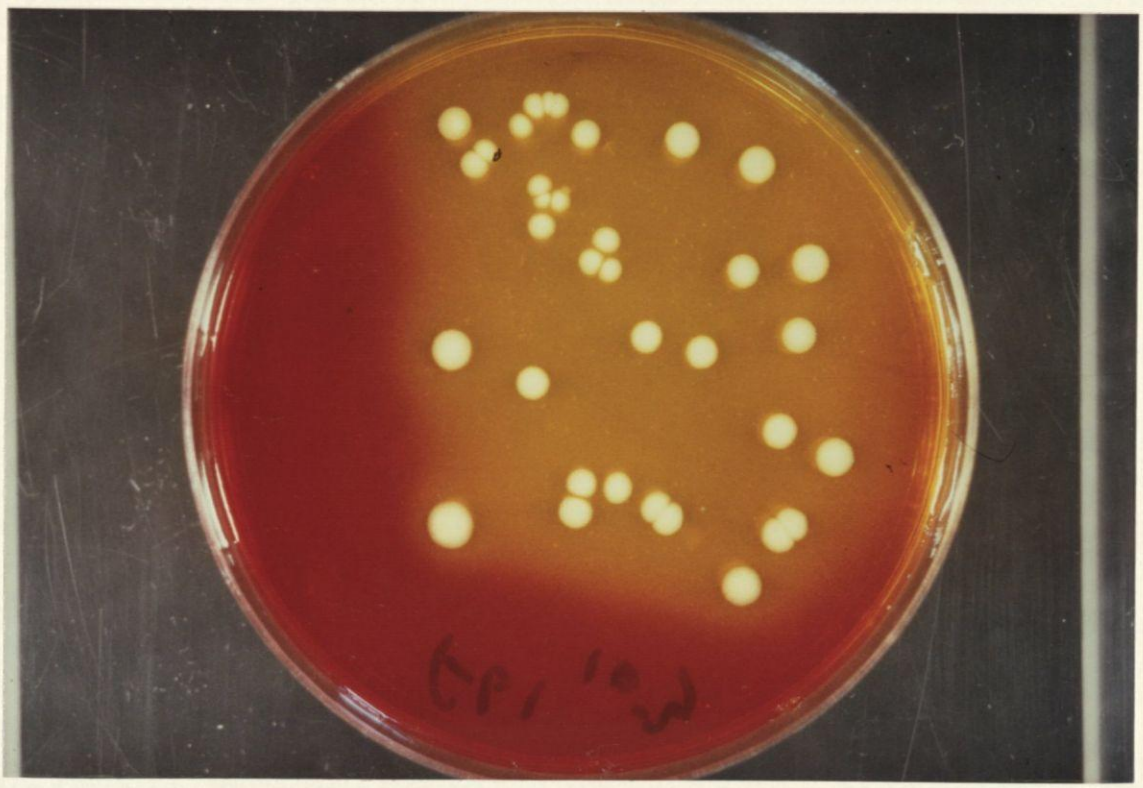


Plate 3.6a **Colonies of *Hafnia alvei* on ROD medium.**

Plate 3.6b **Colonies of *Enterobacter* sp. on ROD medium.**





3.3.9 Field trials

Field trials using the new medium were undertaken and the results showed that 90% of the presumptive colonies were confirmed as *Y. ruckeri*. The remainder resulted from an occasional general background growth which did not exhibit discrete, countable colonies. In particular, *Citrobacter, Hafnia,* and *Enterobacter* strains were isolated at Farm A especially between July and November in 8% of the faecal samples. This situation occurred less often at Farm B in only 3% of samples between September and October. Simple whole cell agglutination and confirmatory biochemical tests indicated that all the field isolates of *Y. ruckeri* were serotype 01.

3.3.9.1 Isolation of *Y. ruckeri* from faeces and kidney

The ability to isolate *Y. ruckeri* by the use of ROD medium indicated that the organism could occur from samples of fish faeces four to six weeks before it appeared in kidney samples. This seemed to show that infection in the intestinal tract occurred prior to the onset of clinical infection (Figure 3.4a and 3.4b). This is more clearly demonstrated at Farm B (Figure 3.4b) where increasing faecal infection occurred from January to May, when acute kidney infection became apparent. This pattern was repeated later in the sampling period with faecal infection in October being followed by kidney infection in December.

The situation at Farm A was more complex, but Figure 3.4a suggests that the infection at this site had a more chronic nature which persisted for a longer period in the kidney than from fish at Farm B.

The appearance of *Y. ruckeri* on ROD compared with TSA, after isolation from faecal material, is shown by Plates 3.7a and 3.7b.

3.3.9.2 Inflow and outflow water samples

An immediate improvement in the ability to isolate *Y. ruckeri* from inlet and outlet water samples at the two farms was achieved by using the new medium when compared to TSA. During the 12 month sampling period, *Y. ruckeri* could be detected in the inlet at Farm A as peaks of isolation in May and December but appeared in the outflow from April to the end of July and in November to January (Figure 3.5a).

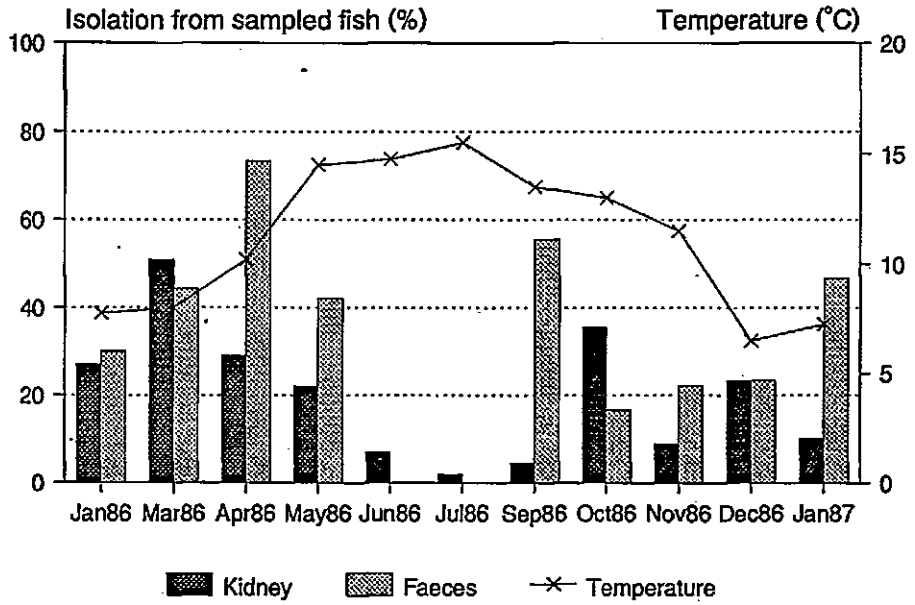


Figure 3.4a Seasonal variation in isolation of *Y. ruckeri* from fish faeces and kidney at Farm A

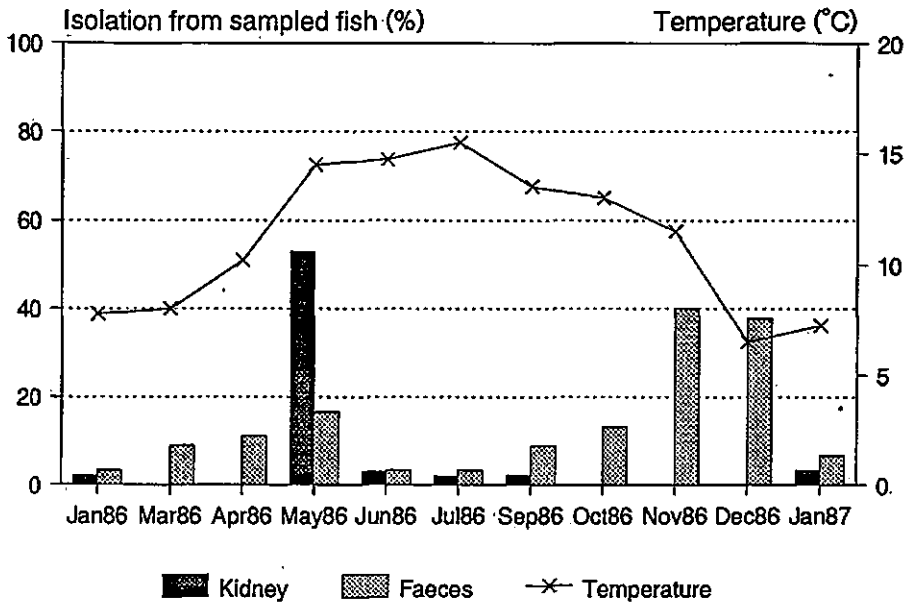
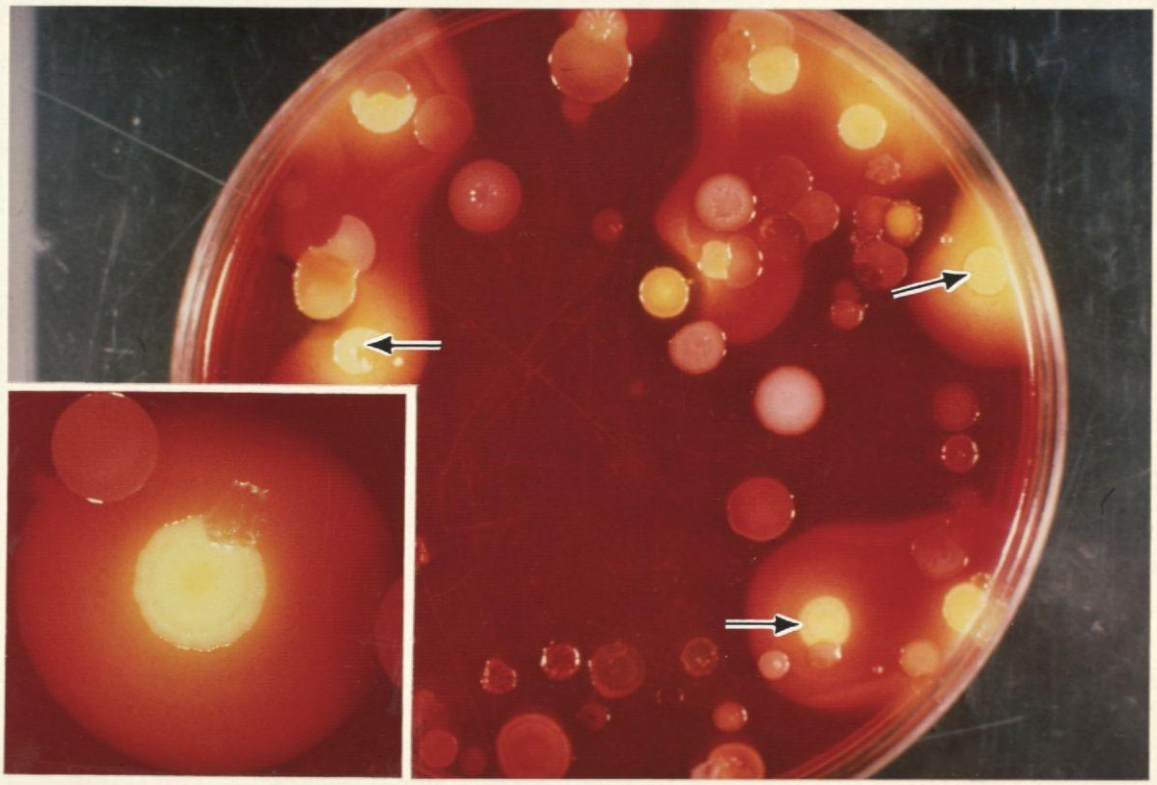
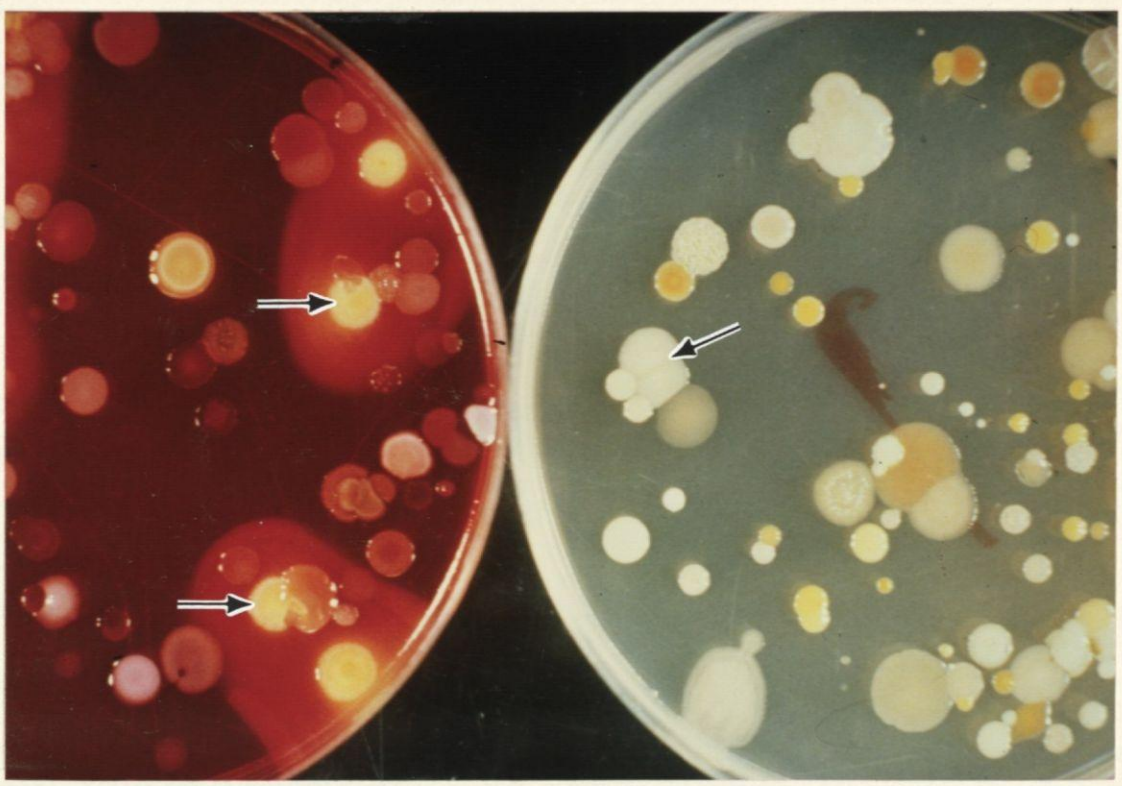


Figure 3.4b Seasonal variation in isolation of *Y. ruckeri* from fish faeces and kidney at Farm B

Plate 3.7a **Colonies of *Y. ruckeri* (arrowed) on ROD. Inset shows colony detail.**

Plate 3.7b **Colonies of *Y. ruckeri* (arrowed) on ROD (left) compared with TSA (right) after isolation from fish faecal material.**





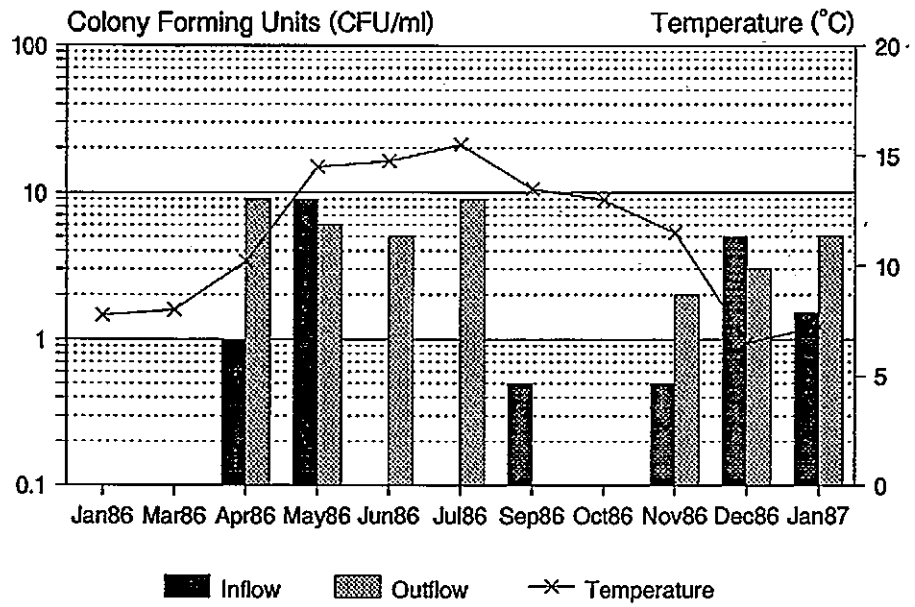


Figure 3.5a **Seasonal variation in isolation of *Y. ruckeri* from inflow and outflow water at Farm A**

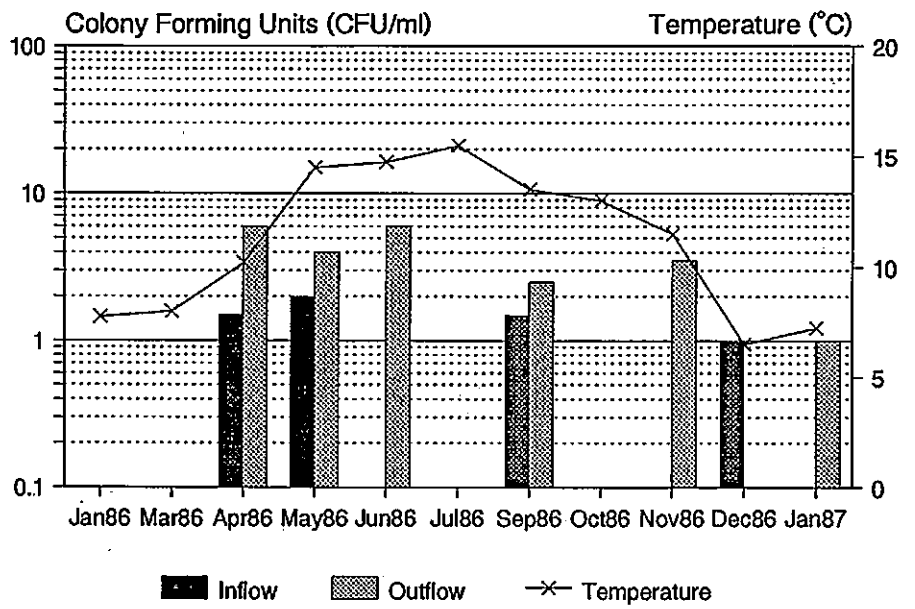


Figure 3.5b **Seasonal variation in isolation of *Y. ruckeri* from inflow and outflow water at Farm B**

A similar pattern emerged at Farm B except that *Y. ruckeri* was not present in the inflow during November nor the outflow in July but did occur in the outflow in September (Figure 3.5b). On the whole, the isolation of *Y. ruckeri* from water compared favourably with isolation from fish at both farms.

3.3.9.3 Water temperature

Figures 3.4a, 3.4b, 3.5a and 3.5b show the mean water temperature for the river during the period of the field experiments at Farms A and B. Clinical ERM occurred mostly when the water temperature was between 8-12°C. Isolation of *Y. ruckeri*, from the inflow water supply to both sites, showed a similar pattern. However, isolation from the outflows also occurred up to 15°C and this seemed to reflect the pattern of faecal isolation.

3.4 DISCUSSION

When a specific bacterium needs to be isolated from an area that has a natural microbial flora (e.g. the intestine), it is necessary to limit or suppress this background population but at the same time encourage the growth of the wanted organisms (Cowan, 1974). For this, selective or inhibitory media need to be developed and therefore a comprehensive understanding of the biochemical requirements of the bacterium is required.

The ten different API 20E profiles obtained for the strains of *Y. ruckeri* (serotype 01) used in this study (Table 3.2) and the 17 profiles reported in the literature (Table 2.2) indicate the variability of this system of biochemical testing with fisheries isolates. It has already been reported that the use of this rapid identification technique requires care since certain reactions can give different results when compared with the more traditional tests (Stevenson and Daly, 1982; Dear, 1988; Davies and Frerichs, 1989). The same authors indicated that variations in the time and temperature of incubation can lead to false negative results in tests such as citrate utilisation, lysine decarboxylase, VP and gelatin hydrolysis. The variations noted in this study were also in these tests despite the use of standardized inoculum preparation and incubation

protocols. However, the majority of strains (78%) gave one of only five profiles indicating the advantage of uniform sample processing leading to more accurate end results.

There are other published reports which indicate that API 20E tests such as gelatin liquifaction, VP, lysine decarboxylase and some carbohydrate fermentation reactions can also give variable reactions with non-fisheries isolates. *Yersinia pestis* and *Y. enterocolitica* (Ewing *et al.*, 1977), *Vibrio alginolyticus* and *Vibrio* sp. lac+ (Ewing *et al.*, 1979), various marine bacteria (Rüger, 1981) and environmental *Aeromonas* strains (Toranzo *et al.*, 1986) have all been shown to give either false-negative or false-positive reactions with the API 20E rapid identification system.

There are several reports in the literature of the API 50CH system of carbohydrate testing being used to characterize isolates of *Y. ruckeri* (O'Leary *et al.*, 1979; Dear, 1988 and Willumsen, 1989). However, only O'Leary *et al.* (1979) reported the results in any detail. Nevertheless, this present study is the first one to compare *Y. ruckeri* with other strains of Enterobacteriaceae by using their API 50CH reactions. The results showed that *Y. ruckeri* could utilize fewer carbohydrates than *C. freundii*, *Enterobacter* sp. or *H. alvei* which is a potential disadvantage with the formulation of a selective differential medium of the type reported here. The use of the API methods, however, indicated which compounds could be utilized in combination to form the basis of such a medium.

The early development of ROD medium was undertaken by using a broth formulation in order to assess the effect of pH, antimicrobial compounds, the carbohydrates maltose and ribose on growth and the action of ornithine decarboxylase. Although the dynamics of growth between liquid and solid media are different the former method was chosen to provide more quantitative data and also allowed a rapid screening of nutritional compounds. However, the final formulation, based on a solid medium underwent extensive laboratory and field trials to confirm that the characteristics recorded in the developmental phase had remained constant.

The growth of *Y. ruckeri* (serotypes 01 and 06), using the developed formulation as an agar medium, relies on ribose fermentation which lowers the pH from an initial value of 7.4 to approximately 6.8 within the first hours of incubation. This reaction is followed by decarboxylation of ornithine which stabilises the pH leading to a slight rise before maltose fermentation results in a drop in pH to around 5.0 after several days. At this pH, the phenol red indicator turns yellow and sodium deoxycholate precipitates around the bacterial colonies giving the characteristic differential properties of the medium. In addition, there is a slight precipitation due to the presence of SDS.

Both biotypes of *Y. ruckeri* serotype 01 gave the same characteristic reaction on ROD which indicates that the hydrolysis of Tween 80 and fermentation of sucrose are not important criteria for expression of the reaction. This is in contrast to the medium developed by Waltman and Shotts (1984) which has been shown to be most useful for only biotype 1 (Hastings and Bruno 1985; Davies and Frerichs 1989). A more recent publication (Shotts, 1991) has revealed an unfortunate printing error in the original Waltman and Shotts (1984) paper that led to a bromothymol blue concentration of 0.003 g l^{-1} instead of 0.03 g l^{-1}. In addition, pH stabilization was made more difficult by the use of rancid Tween 80. Nevertheless, the use of this medium, in the UK at least, despite originally having an incorrect formulation would still be very limited since the majority of UK isolates of *Y. ruckeri* are Tween-negative and non-motile (Davies and Frerichs, 1989) This is in contrast to strains isolated in USA (Waltman and Shotts, 1984; Shotts, 1991).

ROD medium was developed for a specific epidemiological study involving clinical outbreaks of ERM caused by *Y. ruckeri* at two fish farms in southern England. The reaction of both serotype 01 biotypes, however, indicates its potential usefulness in other countries where these isolates predominate, particularly in Europe where 91% of the outbreaks are caused by this serotype (Davies 1990).

The inability of *Y. ruckeri* (serotypes 02, 03, 05 and 07) to produce a yellow agar deposit and yet be able to exhibit pale yellow colonies on ROD may be linked to acidity but the exact reason is not fully understood. It should be pointed out that the

reaction of strains representing these serotypes was evaluated in a laboratory study. Nevertheless, it is quite possible that detection of this type of isolate from faecal material would have been more difficult without the appearance of the characteristic reaction shown by serotype 01 strains.

Three of the main constituents of ROD, namely ribose, ornithine and maltose are integral components of bacterial energy metabolism. The pathways involved in these reactions are extremely complex. They are essentially used, however, to provide direct energy or energy via intermediates which are oxidized in the tricarboxylic acid (TCA) cycle and to provide carbon skeletons for the biosynthesis of cellular components. For instance, ribose is a constituent of purine nucleotides which are key compounds in the translation of DNA into protein. In addition, ribose is degraded via the pentose phosphate pathway and provides pyruvate to the TCA cycle. This cycle in turn produces L-glutamate which is a precursor for ornithine decarboxylation. The presence of maltose results in high acid levels within the ROD medium and prevents the pH reverting to an alkaline value after the decarboxylation of ornithine.

The close taxonomic relationship between *Y. ruckeri* and other bacterial strains, namely, *H. alvei, C. freundii,* and *Enterobacter* sp., isolated in a low percentage of samples in this study, indicates a potential problem with this type of selective differential medium. It was not possible during this study to find an antimicrobial compound which could eliminate these strains of Enterobacteriaceae and also allow *Y. ruckeri* to grow without competition. Members of these taxa have been isolated from fish intestinal samples by other workers (Lesel *et al.*, 1985) and represent the general microbial flora harboured by rainbow trout. However, the proportions of each species vary depending on the fish culture conditions and the nature of the ERM infection level (Rodgers, unpublished data; Lesel *et al.*, 1985). Therefore the presence of these other organisms is not unusual and does not necessarily represent suppression by competition of *Y. ruckeri* from faecal material. However, this was only a low level problem at the two fish farms where the study took place.

The inclusion of an antibiotic in the medium, such as azthreonam or ceftazidime, is worthy of reevaluation. Additional experiments were outside the scope of this project and any further work would need to take into account any potential inhibitory or synergistic action between SDS, deoxycholic acid and an antibiotic. The inclusion of all three compounds may be unnecessary and could lead to delayed or reduced growth, particularly of damaged bacterial cells from intestinal samples. Replacement of these compounds by a single, specific inhibitory agent, at the correct concentration, would not only make the formulation less complex but could lead to better differentiation between *Y. ruckeri* and other members of the Enterobacteriaceae.

When *Y. ruckeri* was present in fish intestinal samples in very low numbers, the presence of other members of the Enterobacteriaceae masked the characteristic reaction of *Y. ruckeri* on ROD and the normal bright yellow colony colouration appeared yellow/brown. However, when higher numbers were present the reaction developed fully after five to seven days. Nevertheless, it was found that the plates should be reincubated then rechecked after 10 days to give maximum sensitivity. Extended incubation beyond 10 days was avoided, since the presence of other bacterial strains seemed to result in a rise in medium pH and a consequent failure or reversal in the formation of the yellow agar deposit. During the present study, it was found that after storage at 4°C the ROD plates needed drying (30 min at 45°C) before use, particularly when they were used for water sampling. This helped to reduce surface condensation and aided resuspension of SDS which appeared to precipitate at the lower temperatures of storage. When plates were not dried the presence of excess moisture on the plates occasionally led to difficulty in recording results from water sampling since confluent growth occurred and the ability to detect *Y. ruckeri* was impaired.

There was a markedly higher overall level of infection at Farm A compared to Farm B (Figure 3.4a and 3.4b). This could be due to several variables. The two most obvious differences between the farms lie in the design of the rearing units and in the on-site operation. Farm A largely uses circular tanks whilst Farm B consists of an integrated raceway system. Although both farms are intensively stocked, the layout of

Farm B results in fewer grading operations and therefore movements of fish during the time on site are consequently less stressful. This can be an important factor in the progression of ERM through individual groups of fish. Cyclical intestinal shedding of *Y. ruckeri* from asymptomatic carrier fish has previously been reported by Busch and Lingg (1975). However, the actual occurrence of shedding followed by subsequent transmission can depend on seasonal variations in water temperature (Busch, 1973). In fact in this study, increased infection levels occurred when water temperatures were rising or falling between 8-12°C. This infection pattern was similar at Farm B but with much less intense levels occurring over a shorter period of time, particularly from the kidney, and up to six to eight weeks later.

In conclusion, although the medium developed in this study is not totally selective, the ability to produce differential characteristics has shown the potential of the medium for epidemiological studies. In particular, the use of ROD medium has allowed *Y. ruckeri* to be isolated reliably from fish intestinal samples and water. This has enabled the transmission and carrier status of ERM to be studied under field conditions. In addition, ROD medium is now routinely used in the Diagnostics department at M.A.F.F., Fish Diseases Laboratory, Weymouth for screening fish against ERM. The sensitivity of the medium means that it is also used regularly in disease monitoring programmes to confirm the absence of *Y. ruckeri* at sub clinical levels. However, further developmental work is needed in order to resolve occasional selection problems with other members of the Enterobacteriaceae.

CHAPTER 4

EPIDEMIOLOGICAL FIELD STUDIES

4.1 INTRODUCTION

Epidemiology may be defined as the study of the patterns of disease that exist under field conditions and as such is closely allied with microbiology in the battle against disease (Martin *et al.*, 1988). The main purpose of epidemiology is to provide data which helps in the prevention and/or control of disease in animal populations (i.e. epizootiology). Consequently, epidemiological studies can help to estimate the frequency of disease or to identify factors that might cause a particular disease (Martin *et al.*, 1988). The amount of a disease which is present in animal populations needs to be measured in order to determine its importance and the efficacy of control strategies (Thrusfield, 1985).

In fish, the process by which an infection proceeds to a clinical outbreak of disease involves an interaction between the host, the pathogenic microbe and the environment (Shepherd, 1988b). Essentially, therefore, the first part of this study concentrated on the incidence of Enteric Redmouth disease (ERM) in vaccinated rainbow trout (*Oncorhynchus mykiss*) under field conditions and its correlation with the presence of *Yersinia ruckeri*. An attempt was made to correlate the incidence of ERM with various environmental and husbandry factors over a 12 month period. A key component of this study was the use of the selective differential medium (ROD), the development of which was described in chapter 3.

Immersion vaccination against *Y. ruckeri* is now an effective and widespread method for the control of ERM. Field trials indicating higher survival rates for vaccinated fish, expressed as the percentage relative protection (PRP), vary between reported studies. Horne and Robertson (1987) summarised other workers' results and showed PRP values, from five trials, of 67% to 100%. However, the level and frequency of isolation of *Y. ruckeri* from vaccinated fish during repeated natural

challenges has not previously been reported.

Essentially therefore, the aim of the second part of this study was to make a comparison of the ERM infection between vaccinated and non-vaccinated fish. This involved taking kidney, spleen, faeces and water samples over a 10 month period, during which time data was also collected in order to calculate condition indices, the conversion rate and the cumulative mortality.

4.2 MATERIALS AND METHODS

The epidemiology of *Y. ruckeri* was studied at two fish farms (A and B) on the River Test near Romsey in Hampshire, U.K. (see section 3.2.10) Farm A, the larger of the two sites, is essentially a tank system whereas Farm B, the smaller site, is an all raceway system. Farm B is approximately one mile downstream of farm A but, because of the complex nature of the river at this point, does not receive its effluent. However, there are natural stocks of cyprinid and salmonid fish along this stretch of the river.

4.2.1 Fish

The sources of rainbow trout used in this component of the research are listed in section 3.2.10.1. In addition, during the period September 1987 to June 1988 a group of non-vaccinated and a group of previously vaccinated fish were sampled every five weeks. Vaccination was carried out according to the manufacturers instructions (Aquaculture Vaccines Ltd., Saffron Waldon, England) at a hatchery site (Upwey Trout Farm), receiving a spring water supply, with no history of ERM. Each group was comprised of 33,000 all female fingerling rainbow trout (*O. mykiss*) and they were monitored from delivery to the point where they were of marketable size. Initially both groups were maintained in identical circular tanks (26.9m^3) with three water changes per hour before being moved after 15 weeks into larger circular tanks (79.3m^3) with two water changes per hour. All tanks were supplied with gravity-fed river water. Water temperature and any mortalities were monitored daily. At every five-weekly visit a random sample of

15 fish from each group was weighed, measured and post-mortemed, effectively from delivery to the point where they were of marketable size.

In the laboratory infection experiment (section 4.2.4) small groups (30) of other fish species were used for transmission work. Salmon parr (Dengrove Springs Hatchery, Shaftesbury, Dorset), grayling, dace (both National River Authority, Wessex Region, Corfe Mullen, Dorset), mirror and crucian carp (both Trafalgar Fish Farm, Salisbury, Wiltshire) were obtained from spring or borehole fed sites free of ERM. All fish were maintained in 30 l tanks at FDL Weymouth for 2 weeks prior to being used (see section 6.2.4.1).

4.2.2 Bacteriological sampling

4.2.2.1 Fish sampling

At post-mortem kidney and spleen material was collected by swab and plated onto tryptone soya agar (TSA, Oxoid). A small sample of faecal material was similarly collected and plated onto a previously developed selective-differential medium, Ribose Ornithine Deoxycholate (ROD) agar (see chapter 3.). In addition, a careful note was made of all the disease signs that occurred in post-mortemed fish and these were compared with subsequent isolation of *Y. ruckeri*. All figures were plotted by using a 3-sample running mean.

4.2.2.2 Water sampling

Although the outflow from Farm A was sampled at a single point close to the main discharge (Plate 3.3b), the inflow water was sampled at four points. Site 1 was closest to the inlet (Plate 3.3a), whereas sites 2 to 4 (Plates 4.1a and 4.1b) were at approximately 25 metre intervals, away from the farm, along a supply leat. In addition, the water at the inflow and outflow points to the raceways and tanks were also monitored. At Farm B, only the main inflow and outflow points were sampled. The water temperature at both farms was recorded daily.

Samples of river water (25 ml) were collected as in section 3.2.10.2 and ROD medium was inoculated accordingly. In addition, viable counts (TVC) were determined from these samples using a spread plate technique onto TSA and ROD.

Plate 4.1a **Inflow water sampling point Farm A-site 2 (I2)**

Plate 4.1b **Inflow water sampling points Farm A-sites 3 (I3) and 4 (I4)**


12


13
14

The resulting plates from sections 4.2.3.1 and 4.2.3.2 were incubated in the laboratory at 26°C and TSA plates, inoculated from kidney, were checked for growth after 4 days. Growth on ROD plates, from faecal material, was checked after 5 and 10 days. The water sample plates were read after 5, 10 and 14 days. All figures were plotted by using a 3-sample running mean.

4.2.2.3 Slide agglutination test

Suspected colonies of *Y. ruckeri* obtained in sections 4.2.2.1 and 4.2.2.2 were presumtively identified by simple whole cell slide agglutinaton using antiserum to serotypes 1 and 2 previously prepared in New Zealand white rabbits. A small amount (10-20 µl) of the relevant antiserum, diluted 1:10 in PBSa, was mixed with a portion of a bacterial colony from either TSA or ROD on a clean glass microscope slide with a microbiological loop. Agglutination within 10 sec was recorded as a positive reaction. The antiserum was replaced with PBSa for the negative control reactions.

4.2.2.4 Identification of isolates

Isolates giving a positive reaction in section 4.2.2.3 were confirmed as *Y. ruckeri* by the API 20E test, inoculated according to the manufacturers instructions but incubated at 26°C for 48 h (see section 3.2.3).

4.2.3 Laboratory infection

Fifty non-vaccinated rainbow trout (Upwey Trout Farm; 50 g average weight) were immersion challenged with 5×10^7 cells ml^{-1} of a *Y. ruckeri* strain (LT12/6) isolated from a clinical outbreak of ERM at Farm A. The inoculum was prepared as in section 6.2.5.1 and fish were immersed in the above concentration for 30 min with aeration provided. After 72 h water from the tank (720 l) containing the trout was piped directly to six smaller tanks (30 l) containing separate groups of 30 rainbow trout (30 g), salmon parr (8 g), grayling (15 g), mirror carp (10 g), crucian carp (10 g) and dace (15 g).

Five fish were sampled from each small tank at regular intervals by culturing kidney material onto TSA and faeces onto ROD medium. In addition, any

moribund or dead fish from the challenged group were also post-mortemed in a similar way. The resulting plates were incubated as in section 4.2.2.2. Suspected colonies of *Y. ruckeri* were presumtively identified by slide agglutinaton (see section 4.2.2.3) and confirmed as *Y. ruckeri* by the API 20E test (see section 3.2.3).

Water samples were also taken at the same time as the fish samples, in the same way as previously described in section 4.2.2.2. and the temperature was maintained at 13°C throughout the experiment.

4.2.4 Condition index

The measurements taken in section 4.2.1 every five weeks from fish at Farms A and B were used to calculate a condition index based on the relationship between length and weight (eg 100 x [weight/length^3]). Figures were plotted by using a 3-sample running mean.

4.2.5 Statistics

The chi-square test was used to compare the recovery rate of *Y. ruckeri* from faecal, kidney and spleen material in section 4.3.4.

The possible association between length, weight, temperature and the levels of isolation of *Y. ruckeri,* in sections 4.3.4 and 4.3.8, was determined by correlation and linear regression. In addition, a twosample T-test was used to determine the significance of weight in section 4.3.8.

A probability of less than 5% was considered significant throughout.

4.3 RESULTS

4.3.1 Artificial laboratory infection

Successful transmission of *Y. ruckeri* was achieved between different species of fish in a laboratory experiment. The condition index, however, was not determined since the samples were taken at more regular intervals than in the field situation. In addition the numbers of fish used in each group were lower and removal of a small sample size for post-mortem affected the population balance in each tank. Therefore weight and length measurements were considered unreliable

parameters to use in comparing performances. However, this laboratory work indicated that it was possible to isolate *Y. ruckeri* from faecal material of rainbow trout, mirror carp and dace 16 days after exposure to water from a tank of infected rainbow trout. Salmon parr, on the other hand, became infected only after 38 days. It was not possible to isolate *Y. ruckeri* from either the faecal material of grayling and crucian carp or water samples from the recipient tanks of fish. *Y. ruckeri* was confirmed from the kidney material of artificially infected fish after 72 h and 10 days but not from any recipient fish. In addition, it was possible to isolate *Y. ruckeri* from the water of the donor tank after 72 h, 7 and 28 days.

4.3.2 Comparison between two fish farms

4.3.2.1 Total isolation

The isolation of *Y. ruckeri* from kidney and faecal material during the early part of the field trials is dealt with in chapter 3 (section 3.3.9.1; Figures 3.4a and 3.4b). However, the following results include these early isolation levels and are shown for comparative purposes as 3-sample running maens.

There was a higher overall level of infection in both kidney and faeces at Farm A compared to Farm B throughout the initial sampling period (December 1985 to November 1987; Figures 4.1a and 4.1b). Isolation of *Y. ruckeri* from the kidney of fish at Farm A occurred as peaks of infection around March and November 1986 and January to March 1987. Peaks at Farm B occurred between April and June 1986 with much smaller peaks in January and September 1987. The peaks in faecal isolation were offset compared to kidney. At Farm A they occurred in April, October 1986 and January to March 1987. At Farm B maximum isolation from faeces was around April, November 1986 and May 1987.

It appeared that in the summer months fish at both farms had a reduced level of *Y. ruckeri* in their faeces and kidney. In fact at Farm B, *Y. ruckeri* became undetectable from kidney. This low level of infection coincided with an increase in fish condition and performance at both sites and an inability to isolate *Y. ruckeri* from inflow water samples (see sections 4.3.2.2 and 4.3.8).

4.3.2.2 Condition index

The condition indices for both farms (Figure 4.2) seemed to correlate largely with the recovery of *Y. ruckeri* from kidney and showed that after peaks of infection the performance of fish was adversely affected. Since the peaks of isolation for faeces were offset compared with kidney isolation they seemed to be more closely correlated with the peaks of condition.

4.3.2.3 Water temperature

Figures 4.1a and 4.1b also show the mean water temperature for the River Test during the period of the field experiments. Clinical ERM, represented by isolation of *Y. ruckeri* from kidney material, existed in the temperature range 8-12°C which coincided with rising spring temperatures and falling autumn temperatures.

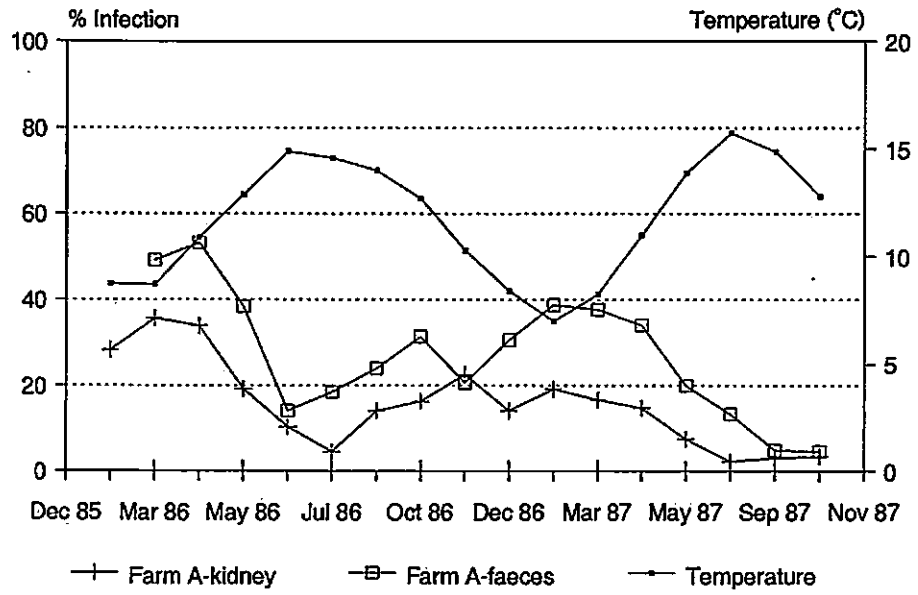


Figure 4.1a Overall level of kidney and faecal isolation from Farm A

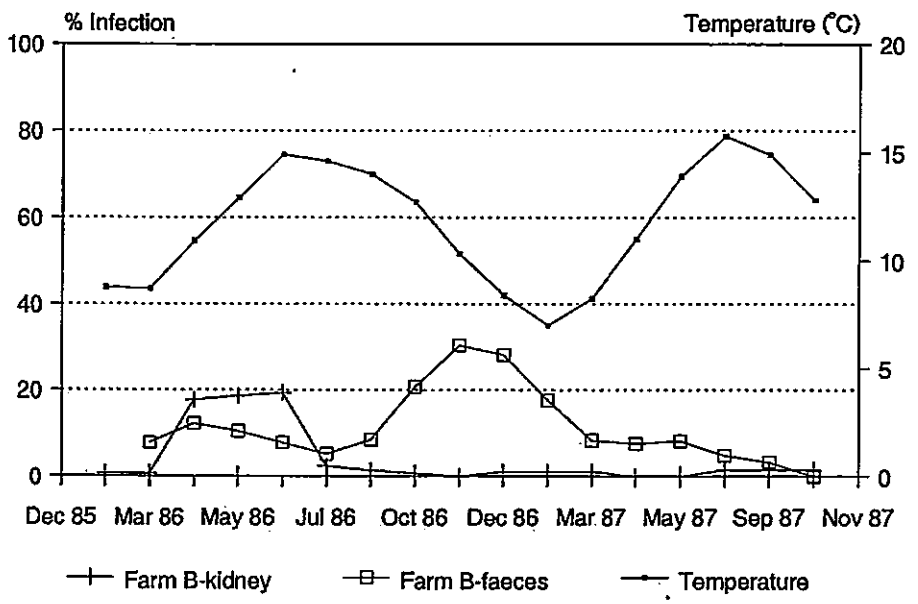


Figure 4.1b Overall level of kidney and faecal isolation from Farm B

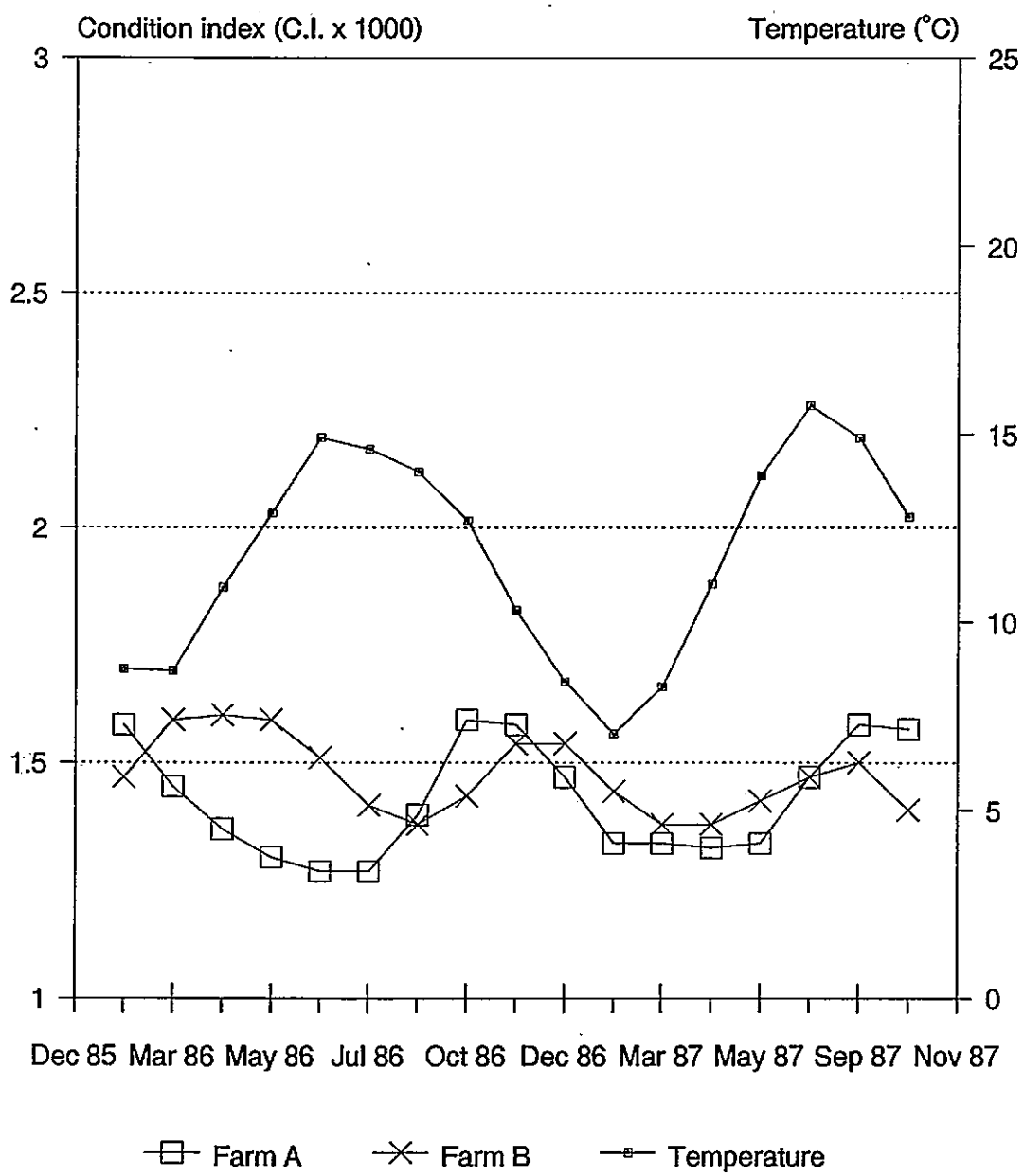


Figure 4.2 Overall condition index at Farms A and B

4.3.3 Seasonality of fish introduction

The month that fish were introduced to a site with chronic ERM appeared to be important to their subsequent performance and hence their early marketability. Fish that arrived at Farm A in autumn 1985 (November or December; groups 1 and 2), either side of a peak of ERM infection (Figure 4.1a), gradually lost condition through the winter (Figures 4.3a, 4.3b, 4.3c and 4.3d) until after the early spring peak of infection. At this point the condition of the earlier arrivals (group 2) improved throughout the summer as the water temperature increased and the ERM incidence decreased despite a late recrudescence of the disease (Figures 4.3c and 4.3d). Group 1, on the other hand, did not show this improved condition and continued to decline (Figures 4.3a and 4.3b). A corresponding group at Farm B, where the overall infection level was lower, gained in condition until it was possible to isolate *Y. ruckeri* from the kidney at a low level (Figures 4.3e and 4.3f).

Fish that arrived in spring at Farm A (March; group 4) during the second peak of infection lost condition only slightly and then seemed to improve in a similar way to the earlier arrivals (Figures 4.3g and 4.3h). However, the corresponding group at Farm B, arriving in April (group 4), did not show the same improvement until the end of the sample period (Figures 4.3i and 4.3j).

A group arriving at Farm A in early autumn 1986 (October; group 5) gained condition throughout the following winter despite having ERM (Figures 4.3k and 4.3l). Loss of condition only seemed to occur after the spring with a rise in water temperature. The chronic infection in this group is shown by the consistant isolation of *Y. ruckeri* from the spleen throughout the period of sampling (Figure 4.3k). A slightly later arrival (November; group 6) however only showed a very small reduction in condition over a similar period (Figures 4.3m and 4.3n). This group also had chronic ERM.

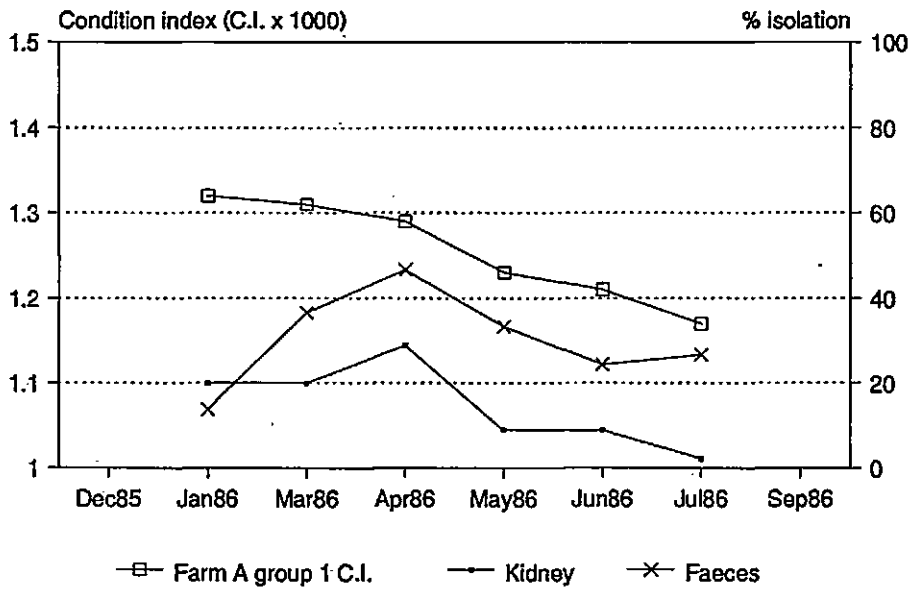


Figure 4.3a Farm A, group 1 condition index with kidney and faecal isolation

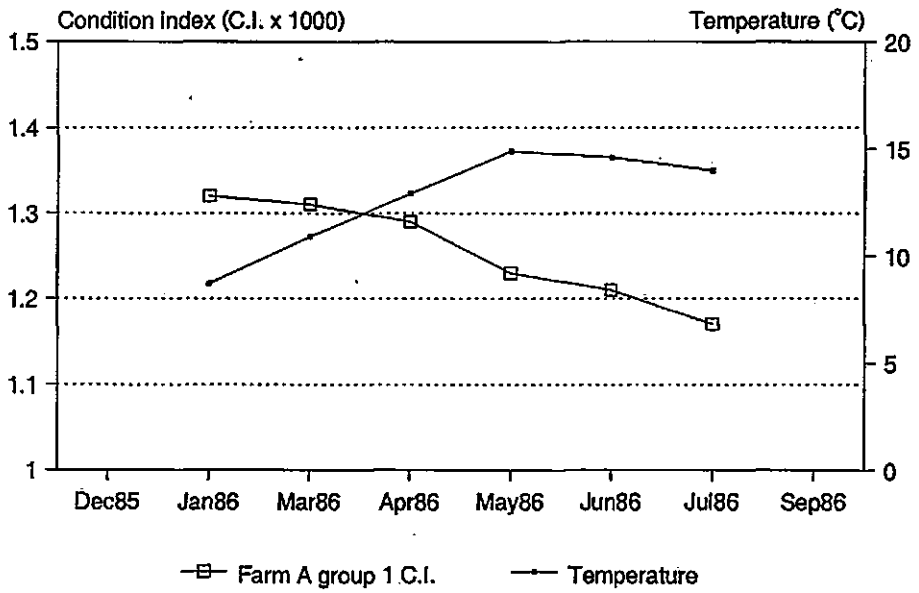


Figure 4.3b Farm A, group 1 condition index with temperature

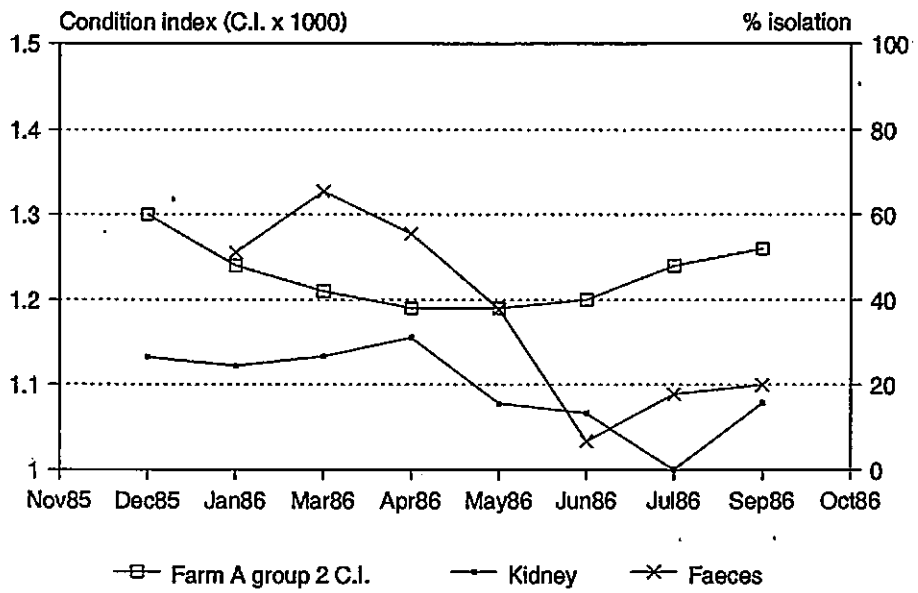


Figure 4.3c Farm A, group 2 condition index with kidney and faecal isolation

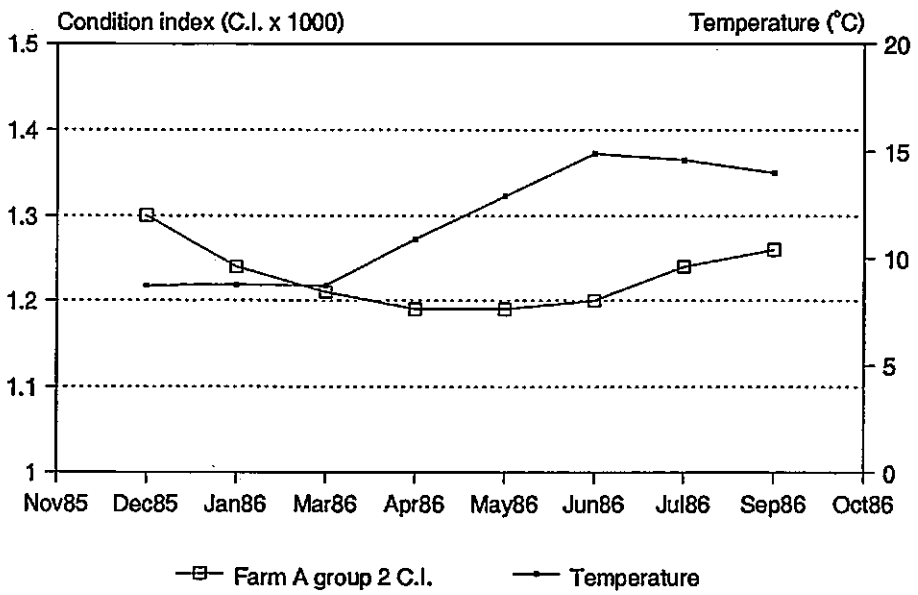


Figure 4.3d Farm A, group 2 condition index with temperature

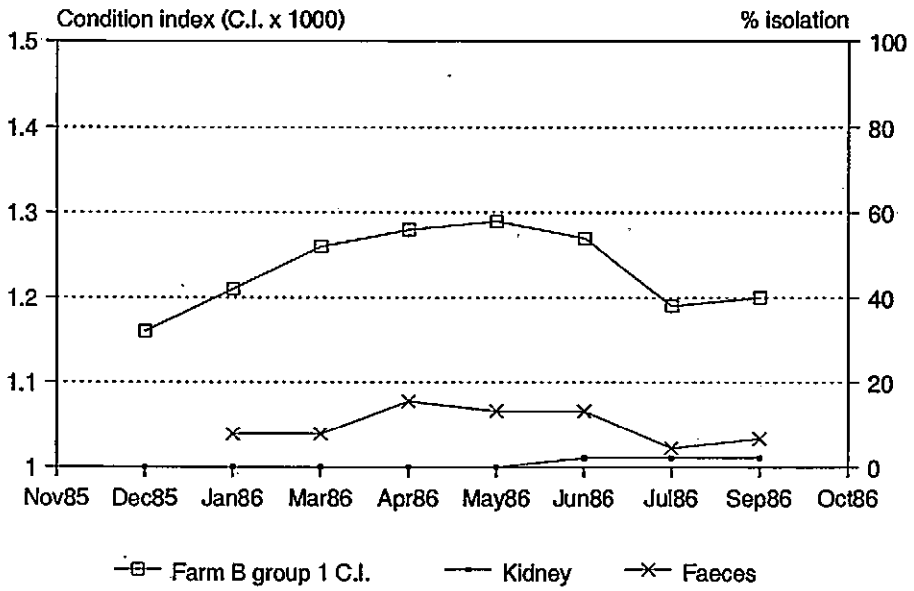


Figure 4.3e Farm B, group 1 condition index with kidney and faecal isolation

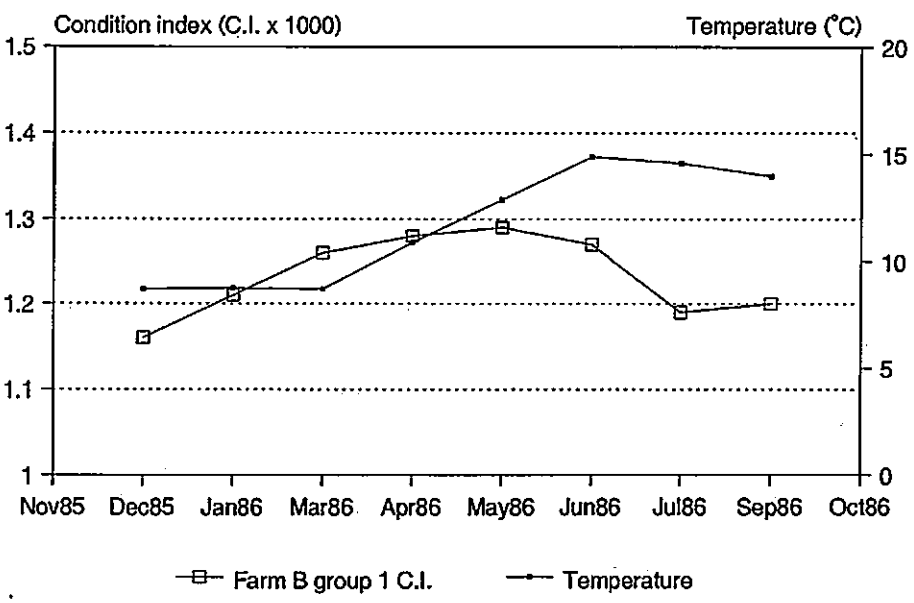


Figure 4.3f Farm B, group 1 condition index with temperature

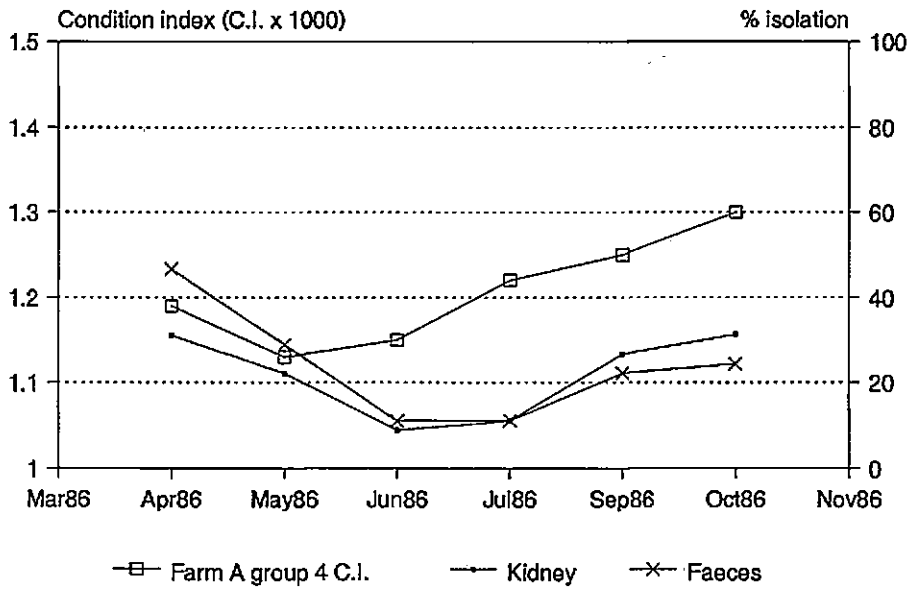


Figure 4.3g Farm A, group 4 condition index with kidney and faecal isolation

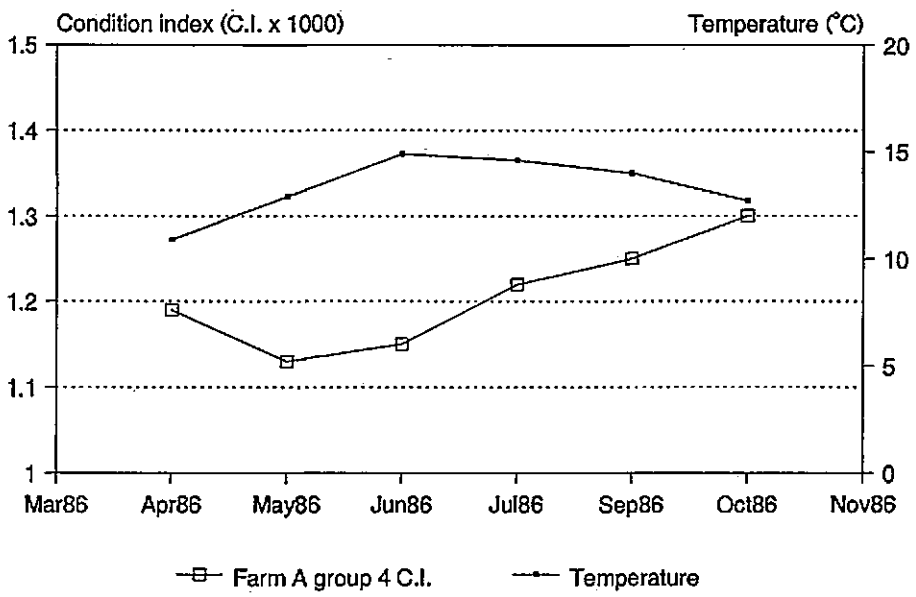


Figure 4.3h Farm A, group 4 condition index with temperature

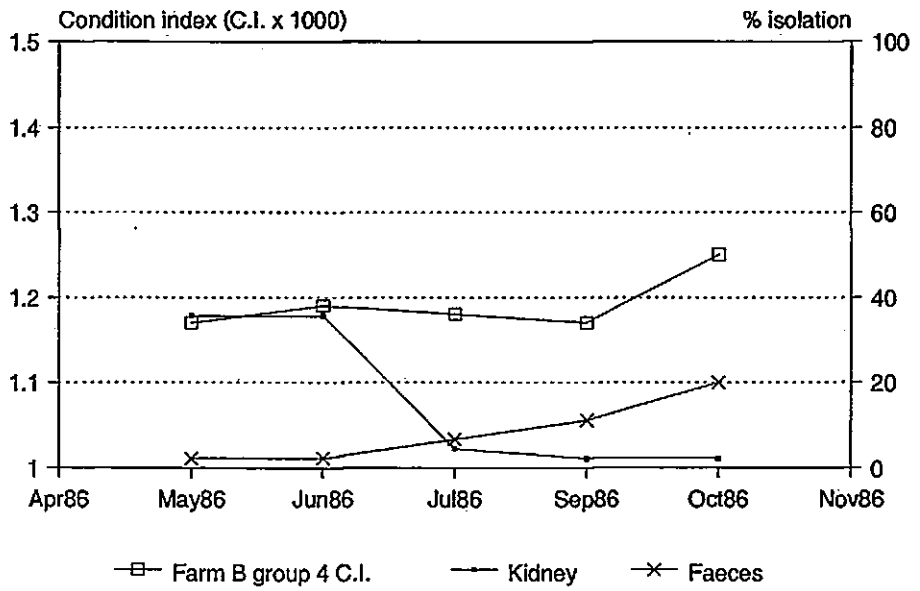


Figure 4.3i Farm B, group 4 condition index with kidney and faecal isolation

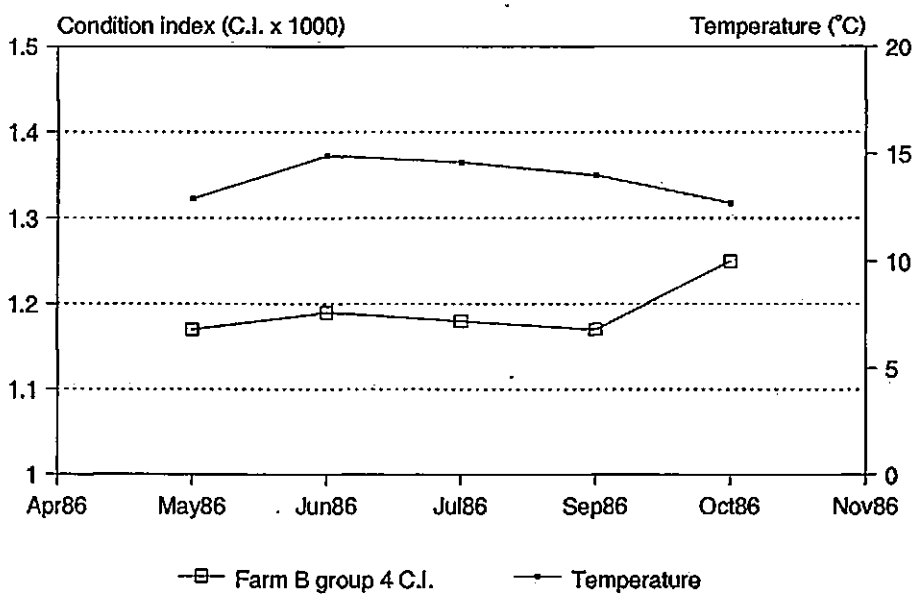


Figure 4.3j Farm B, group 4 condition index with temperature

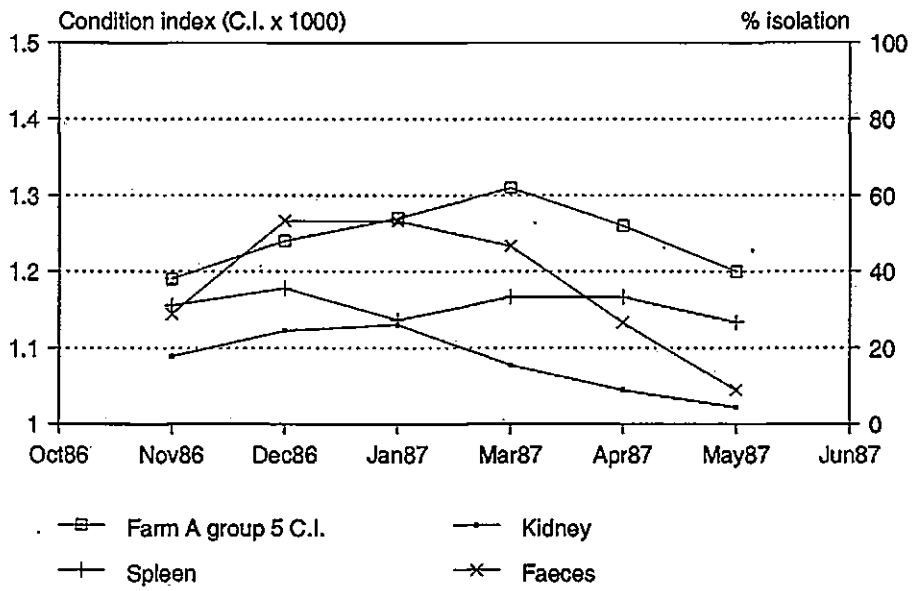


Figure 4.3k Farm A, group 5 condition index with kidney, spleen and faecal isolation

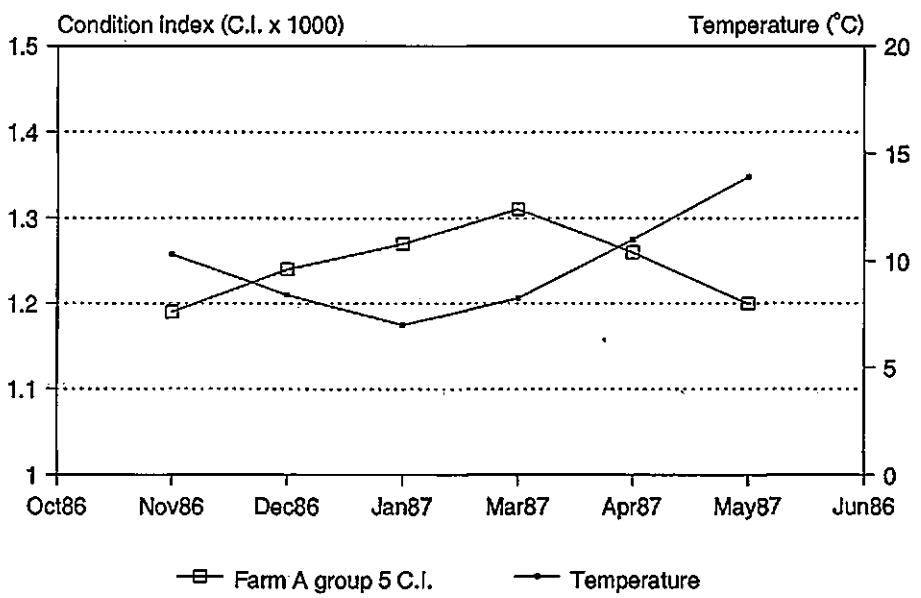


Figure 4.3l Farm A, group 5 condition index with temperature

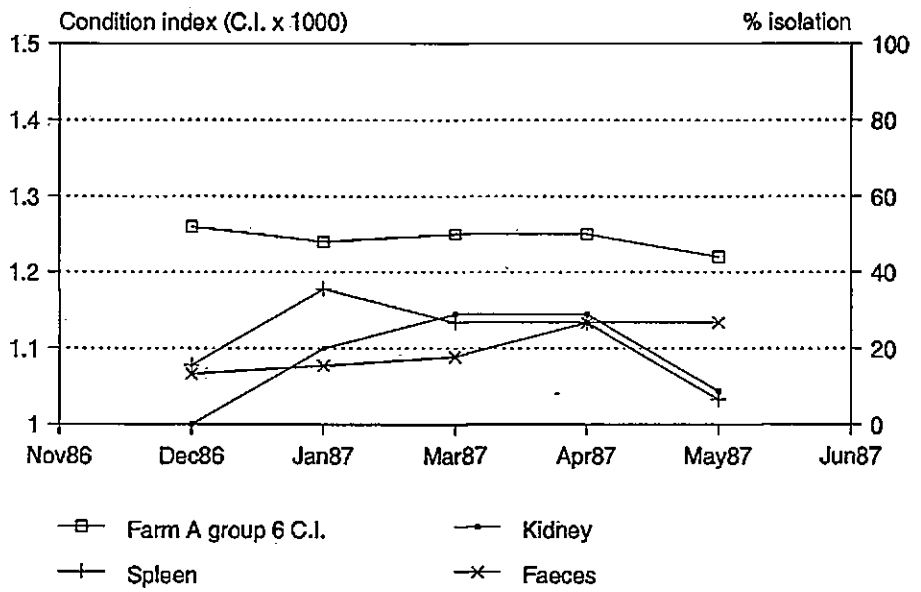


Figure 4.3m Farm A, group 6 condition index with kidney, spleen and faecal isolation

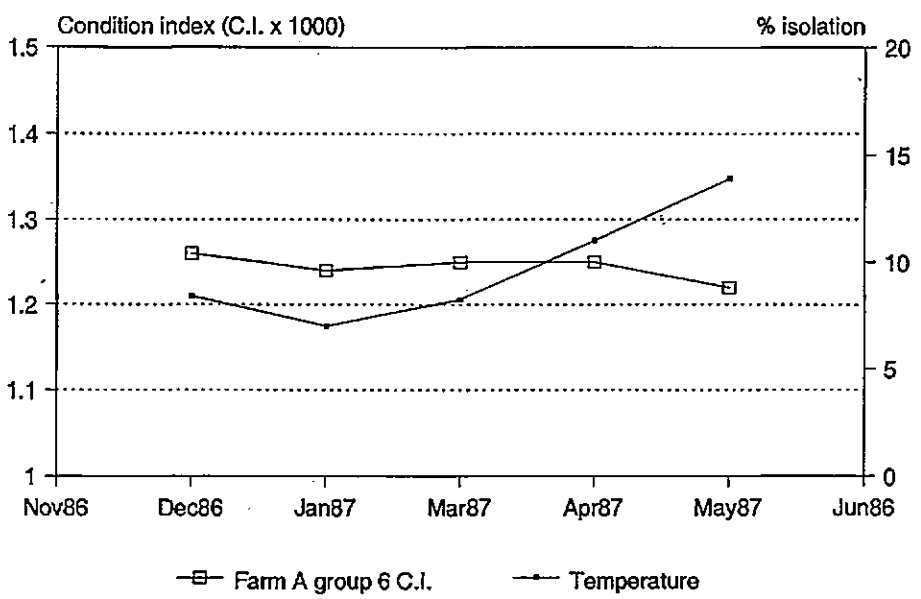


Figure 4.3n Farm A, group 6 condition index with temperature

Two of the remaining groups at Farm B (8 and 9) were brought onto the farm in March but it was only possible to isolate *Y. ruckeri* at a low level from faecal material of group 8 (Figures 4.3o). The condition index for this group reflects both this isolation and the water temperature over the sampling period (Figure 4.3p). Group 9 was the only batch of fish from either farm to have no recoverable *Y. ruckeri* from kidney, spleen or faeces (Figure 4.3q). As a result the condition index improves throughout the time on site. Although the water temperature was also rising for the first half of the exposure period (Figure 4.3r).

The only summer arrival group to be monitored was at Farm B (July; group 5) but was only sampled for a short time. Although this group had a low level of ERM from both faeces and kidney the condition index rose despite falling temperatures (Figures 4.3s and 4.3t).

Peaks of infection due to ERM, at Farm B, seemed overall to be less intense than those at Farm A (Figures 4.1a and 4.1b). In fact the rate of infection at both farms appeared to become less over the whole experimental period (December 1985 to November 1987). The fish at Farm B had no exposure to a chronic infection of ERM since the isolation of *Y. ruckeri* from kidney was only at a low level particularly after July 1986. However, *Y. ruckeri* was recovered from faeces throughout.

The results indicate that fish initially reared in raceways at Farm A (group 6) had a lower incidence of ERM than those reared in round tanks (group 5). However, group 5 fish arrived one month earlier in late September, slightly larger for growing on. Nevertheless, the isolation of *Y. ruckeri* from kidney, spleen and faecal material was reduced in fish from group 6 throughout the time on site (Figures 4.3i and 4.3k). The condition index for group 5, on the other hand, was inversely proportional to the water temperature and improved until early spring after which it decreased (Figure 4.3j). The condition index for group 6, also larger grow-on fish, remained fairly constant until the fish were moved into tanks in March, at which point the index began to decline slowly (Figure 4.3l).

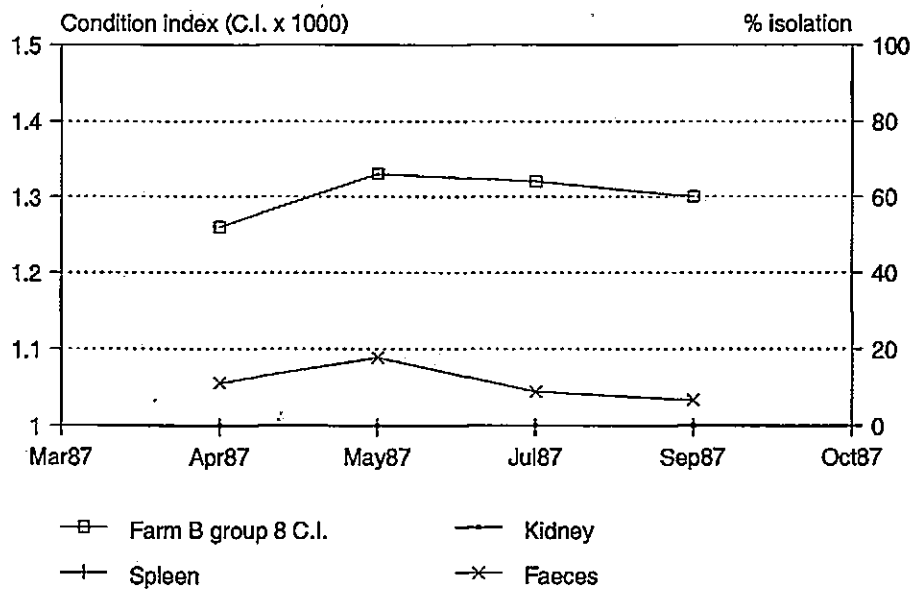


Figure 4.3o Farm B, group 8 condition index with kidney, spleen and faecal isolation

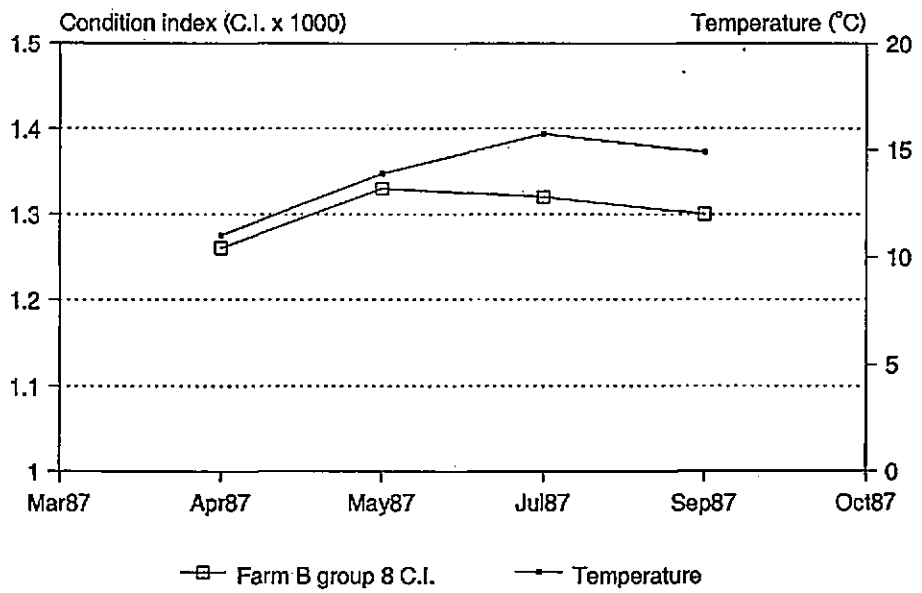


Figure 4.3p Farm B, group 8 condition index with temperature

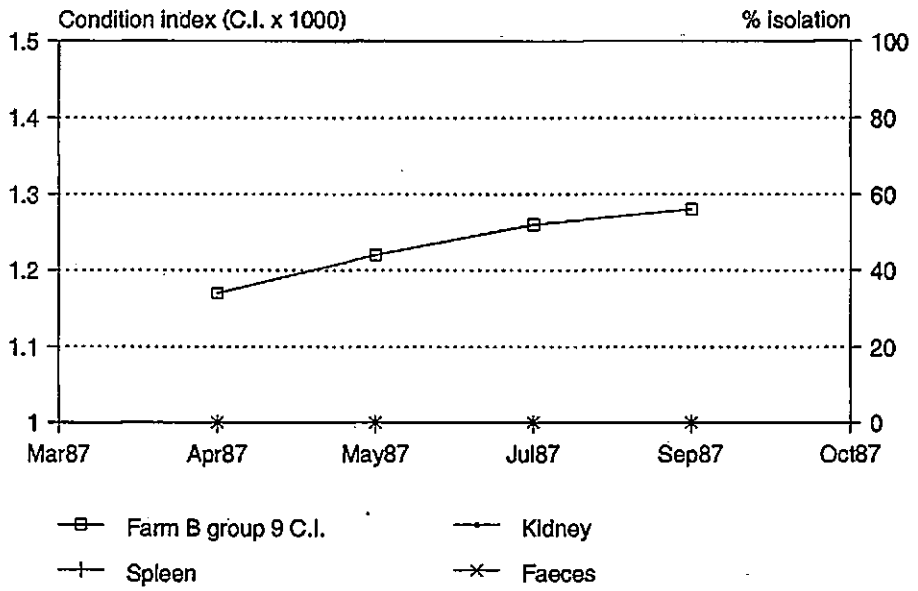


Figure 4.3q Farm B, group 9 condition index with kidney, spleen and faecal isolation

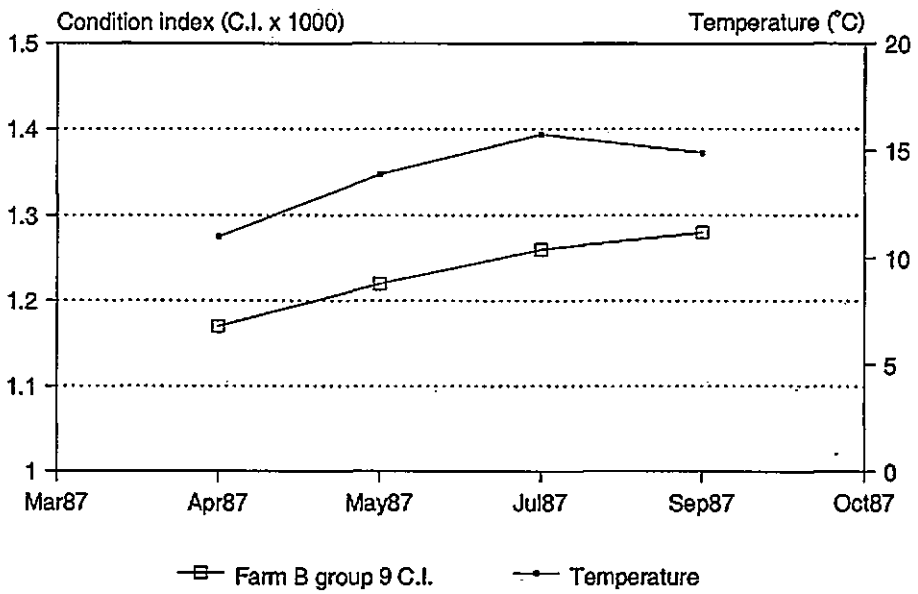


Figure 4.3r Farm B, group 9 condition index with temperature

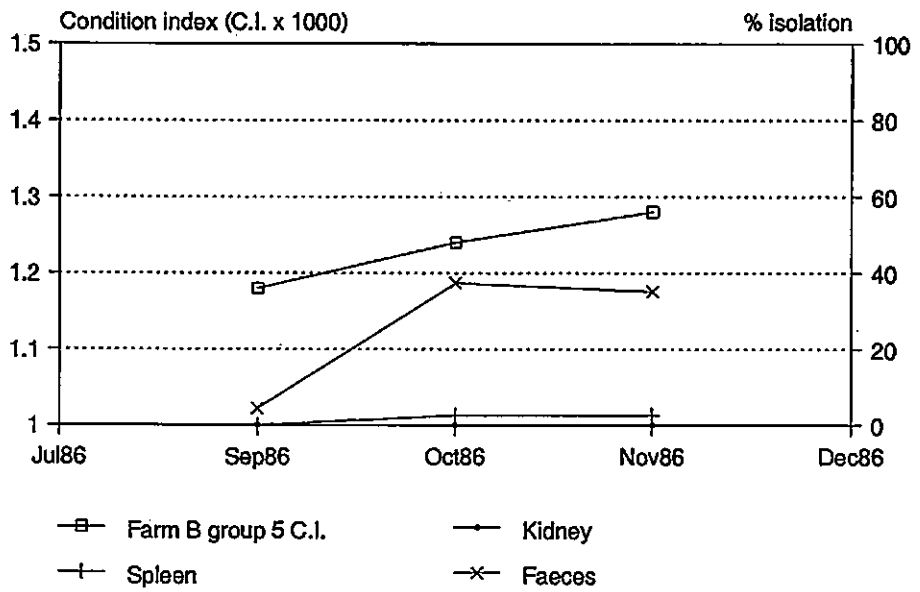


Figure 4.3s Farm B, group 5 condition index with kidney, spleen and faecal isolation

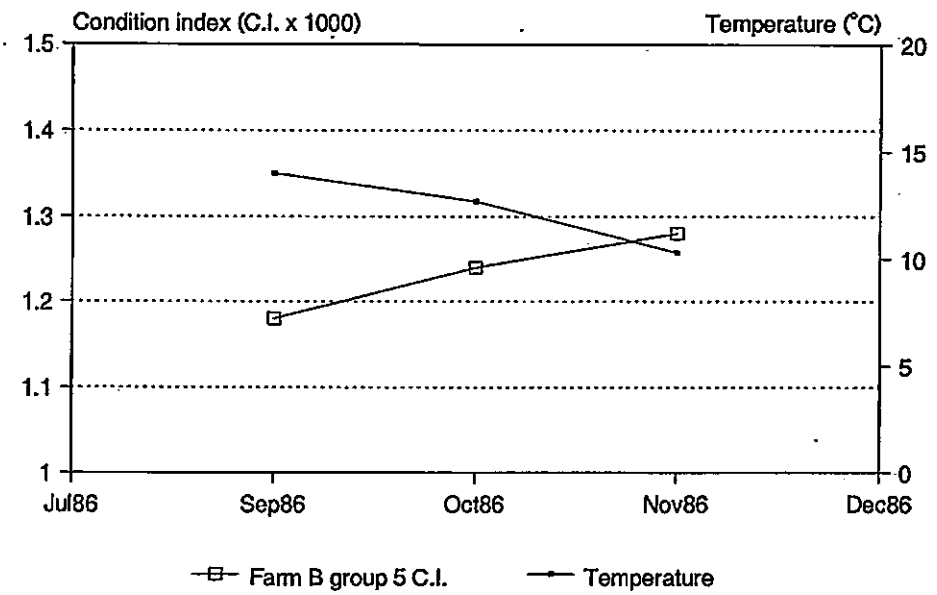


Figure 4.3t Farm B, group 5 condition index with temperature

4.3.4 Length, weight, temperature and infection relationship

a) Farm A

The measured variables namely; weight, length, water temperature, body (kidney and spleen) isolation and faecal isolation proved difficult to interpret statistically. Since all the variables would be expected to interact, multiple regression and subsequent predictive modelling could have provided an insight into their effect on each other. However, the effects of seasonality, other diseases (e.g. PKD) and on-farm husbandry conditions could not be quantified, since all the groups were vaccinated. Consequently, to reduce the options, the variables were arbitarily ranked in order of importance so that possible linear relationships could be determined. Therefore weight was chosen as the most important variable followed by length and temperature. The significance of these two latter variables on the weight of fish from Farm A is shown in Table 4.1. To simplify the relationships it was found necessary to perform a square root transformation on the weight variable and only consider single stepwise regession.

Table 4.1 Variables selected by stepwise regression-Farm A

Group	Regressor	Predictor	Model	r^{2*}	T-ratio
A1	√W*	L*	√W=-2.76+0.06L	99.3	28.33^{+}
	√W	T*	√W=-4.28+1.02T	73.3	4.06^{+}
A2	√W	L	√W=-3.34+0.07L	99.1	29.52^{+}
	√W	T	√W=-3.99+1.11T	72.6	4.60^{+}
A4	√W	L	√W=-3.45+0.07L	98.4	19.26^{+}
	√W	T	√W=-0.43+0.64T	25.1	1.42
A5	√W	L	√W=-3.65+0.07L	99.6	36.87^{+}
	√W	T	√W=5.35+0.29T	9.3	0.78
A6	√W	L	√W=-3.47+0.07L	99.7	40.55^{+}
	√W	T	√W=0.53+0.74T	57.2	2.58^{+}

*r^2=% variation (i.e. coefficient of determination); √W=square root weight; L=length; T=temperature

$^{+}$=p<0.05

When the differences between the observations and the fitted values (i.e. the residuals) were plotted against the data from each group of fish they did not show evident patterns and are therefore not shown. This indicates, for instance, that the effect of infection level on weight, with the influence of length removed, was minimal since length predicted weight in all groups (r^2>98%). The effect with temperature removed, was greater (r^2=9.3-73.3).

b) Farm B

There was a similar pattern at Farm B (Table 4.2) where length was able to predict weight, again in all groups (r^2>96%). The effect with temperature removed showed a wider range than at Farm A (r^2=0.8-82.8).

Table 4.2 Variables selected by stepwise regression-Farm B

Group	Regressor	Predictor	Model	r^{2*}	T-ratio
B1	√W*	L*	√W=-3.17+0.07L	99.7	48.36+
	√W	T*	√W=-5.0+1.11T	71.5	4.47+
B4	√W	L	√W=-3.76+0.07L	97.9	15.33+
	√W	T	√W=8.93-0.14T	0.8	-0.20
B5	√W	L	√W=-4.19+0.07L	99.2	19.41+
	√W	T	√W=19.2-0.94T	82.8	-3.80+
B8	√W	L	√W=-2.99+0.07L	96.8	11.01+
	√W	T	√W=-1.92+0.75T	47.4	1.90
B9	√W	L	√W=-3.53+0.07L	99.2	22.32+
	√W	T	√W=-1.30+0.64T	35.9	1.50

*r^2=% variation (i.e. coefficient of determination); √W=square root weight; L=length; T=temperature

+=p<0.05

Further multiple regression analysis and predictive modelling on the raw data obtained from Farms A and B is beyond the scope of this project since the interaction of all the known variables would be complex. In addition, it would not be possible to determine the effect of the unknown variables.

4.3.5 Isolation of *Y. ruckeri* from faeces, kidney and spleen

The sensitivity of ROD medium for detection of ERM again showed that *Y. ruckeri* could be isolated from fish faeces up to 4-6 weeks before appearing in the kidney. The results confirmed the earlier observation in section 3.3.9.1 that the carrier status developed prior to the onset of clinical infection (Figures 4.1a and 4.1b). Although the Figures represent 3-sample running means, which tend to smooth out the plots, groups 1, 2 and 6 from Farm A and groups 1 and 5 from Farm B show this early faecal infection.

In addition, Table 4.3 shows the incidence of *Y. ruckeri* isolated from faeces, spleen and kidney respectively over the whole sampling period. The results indicate that kidney is not as consistent for isolation as spleen and faeces.

Table 4.3 Incidence of *Y. ruckeri* isolated from faeces, spleen and kidney

Farm/Group	Number of fish	Sample period	Incidence (%) of *Y. ruckeri* Kidney	Spleen	Faeces
A1	120	Dec 85-Sep 86	12	ND*	25
A2	135	Dec 85-Oct 86	18	ND	29
A4	120	Mar 86-Nov 86	27	ND	27
A5	120	Oct 86-Jul 87	12	26	30
A6	105	Nov 86-Jul 87	12	18	19
B1	135	Dec 85-Oct 86	1	ND	7
B4	105	Apr 86-Nov 86	16	ND	10
B5	75	Jul 86-Dec 86	0	1	19
B8	90	Mar 87-Oct 87	0	0	9
B9	75	Mar 87-Oct 87	0	0	0

*ND=Not Done

The higher incidence of isolation from faeces compared with kidney was significant for groups A1, A2, A5, B1, B5 and B8 (chi-square test; $p<0.05$). Compared with spleen, isolation from faeces was significant for group B5 (chi-square test; $p<0.05$). However, the numbers of fish with faecal isolation does not include those where it was also possible to isolate *Y. ruckeri* from kidney or spleen. Faecal isolation from groups A4 and A6 was significant compared with kidney and groups A5 and A6 were significant compared with spleen when these additional fish were considered (chi-square test; $p<0.05$). The isolation of *Y. ruckeri* from spleen was significantly higher than kidney from group A5 (chi-square test; $p<0.01$) but not from group A6 despite the greater recovery (chi-square test; $p>0.05$).

4.3.6 Incidence of disease signs

There were some minor differences in the incidence of disease signs between the various groups of fish. Groups 1 and 2 from Farm A had fewer fish with no signs and no isolation of *Y. ruckeri* than group 6 (chi-square test; $p<0.01$). In addition, group 1 had more fish that had *Y. ruckeri* in the kidney but with no visible signs than either group 5 or 6 (chi-square test; $p<0.05$). Fluid or yellow faeces in conjunction with kidney isolation was noted more frequently in group 5 compared to group 1 (chi-square test; $p<0.05$). There were even fewer differences at Farm B, with only group 1 having less fish with no signs and no isolation of *Y. ruckeri* than either group 5 or 8 (chi-square test; $p<0.001$).

The disease signs exhibited by fish with ERM infection at Farms A and B are shown in Tables 4.4 and 4.5 respectively. Since there were only minor differences between groups of fish from Farm A, groups 1, 2, 4, 5 and 6 are considered together. Essentially, at Farm A, there was no significant difference between the signs exhibited by the fish with *Y. ruckeri* in the intestine and those with no isolation. However, there were fewer fish with clinical (i.e. body isolation) infection and no signs. In addition, there were more fish with *Y. ruckeri* in the kidney that had associated exophthalmia, haemorrhages in the eyes, peritoneal cavity and intestine (chi-square test, $p<0.01$).

Table 4.4 Incidence of signs associated with isolation of *Y. ruckeri* at Farm A

Disease Signs	% of fish showing signs		
	Body isolation*	Faecal isolation	No isolation
None	57+	81+	75
Fluid or yellow faeces	18	11	15
Haemorrhaged eyes/exophthalmia	6+	2	2
Haemorrhaged buccal cavity	2	0	0
Enlarged spleen	9	4	6
Haemorrhaged intestine or peritoneal cavity	8+	2	2

*Kidney or spleen isolation +Chi-square test (compared to no isolation), $p<0.01$

Table 4.5 Incidence of signs associated with isolation of *Y. ruckeri* at Farm B

Disease Signs	% of fish showing signs		
	Body isolation*	Faecal isolation	No isolation
None	50+	81	74
Fluid or yellow faeces	25+	14	16
Haemorrhaged eyes/exophthalmia	0	5	1
Haemorrhaged buccal cavity	0	0	1
Enlarged spleen	25+	0	6
Haemorrhaged intestine or peritoneal cavity	0	0	2

*Kidney or spleen isolation +Chi-square test-insufficient observations

Groups 1, 5, 8 and 9 from Farm B were also considered together. However, although there were similar trends compared with Farm A there was insufficient data to determine the statistical significance.

Plates 4.2a, 4.2b, 4.3a and 4.3b show some typical external and internal disease signs seen at Farms A and B.

4.3.7 Inflow and outflow water samples

The improvement in the ability to isolate *Y. ruckeri* from inlet and outlet water samples at the two farms in the first 12 months, by using ROD medium, has already been detailed in chapter 3 (section 3.3.9.2; Figures 3.5a and 3.5b). Isolation during the second experimental period (January-December 1987) was slightly more erratic with the pattern indicating an additional presence of *Y. ruckeri* in late summer but reduced levels in winter (Figures 4.4a and 4.4b). The patterns, however, corresponded with isolation from fish faecal material at both farms and indicated that *Y. ruckeri* mostly occurred in higher numbers in the outflow.

Water samples from four sites along the inlet channel to Farm A showed that the two sites (1 and 2) closest to the farm gave slightly higher total background levels of bacterial growth on ROD than the sites furthest away (sites 3 and 4). However, more importantly, they also had higher and more frequent levels of *Y. ruckeri* (Figures 4.5a, 4.5b, 4.5c and 4.5d). This seemed to indicate a potential reservoir of infection in the inflow water. The river at this point is a mill leat which only supplies the farm and visual observation of these sites indicated that there were more escapee fish at sites 1 and 2 than higher up at sites 3 and 4.

Plate 4.2a Dark, lethargic looking fish at the tank outflow point

Plate 4.2b Dark, lethargic looking fish exhibiting severe bi-lateral exophthalmia

Plate 4.2c Haemorrhaging in the eye (arrow)

Plate 4.2d Haemorrhaging in the lower intestine (arrow)

A

B

C

D

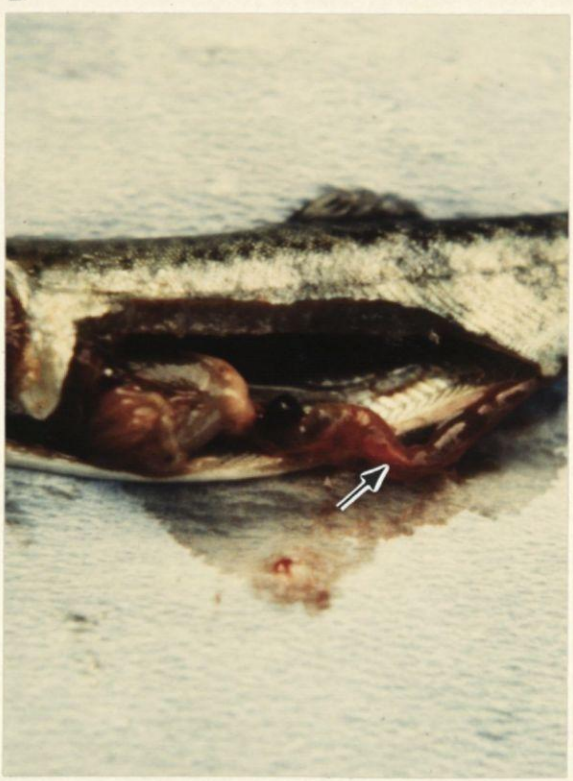


Plate 4.3a **Fluid, yellow faeces**

Plate 4.3b **Haemorrhaging in the pyloric region**

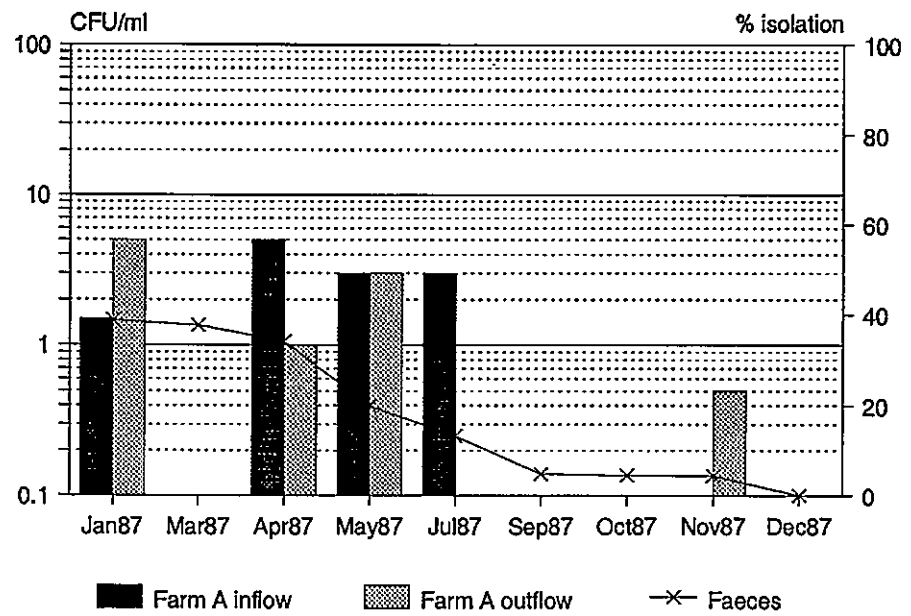


Figure 4.4a Isolation of *Yersinia ruckeri* from inflow and outflow water at Farm A

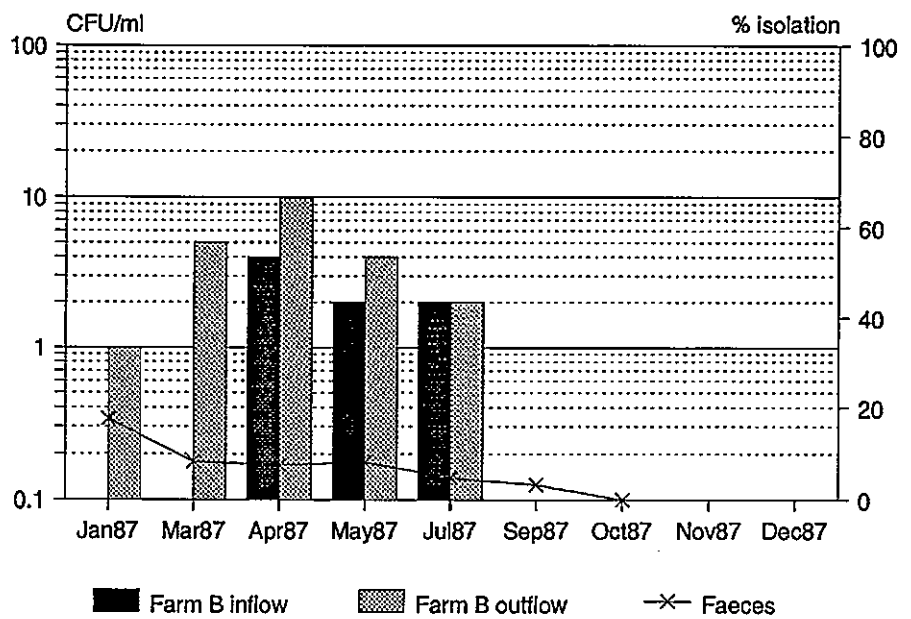


Figure 4.4b Isolation of *Yersinia ruckeri* from inflow and outflow water at Farm B

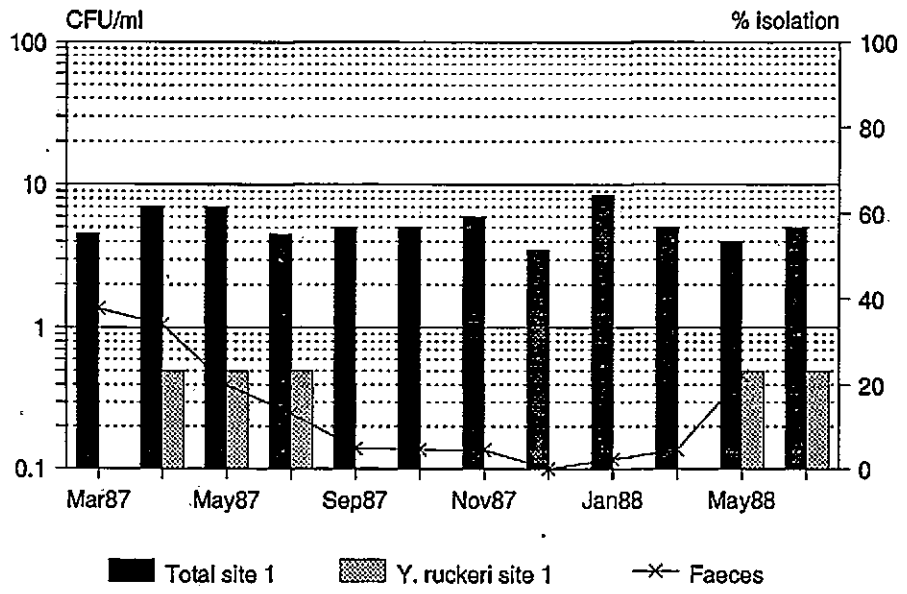


Figure 4.5a Total counts of all isolates and *Yersinia ruckeri* from inflow site 1

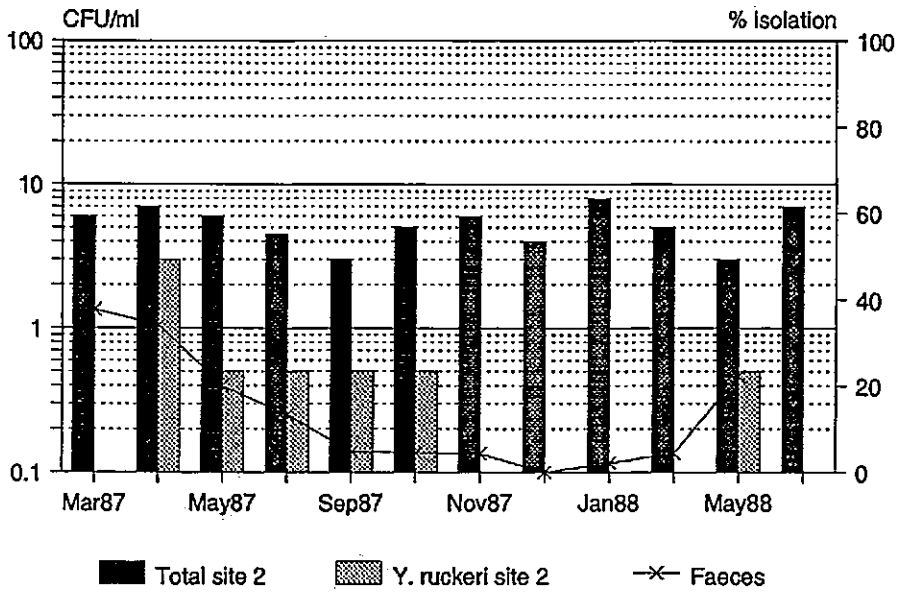


Figure 4.5b Total counts of all isolates and *Yersinia ruckeri* from inflow site 2

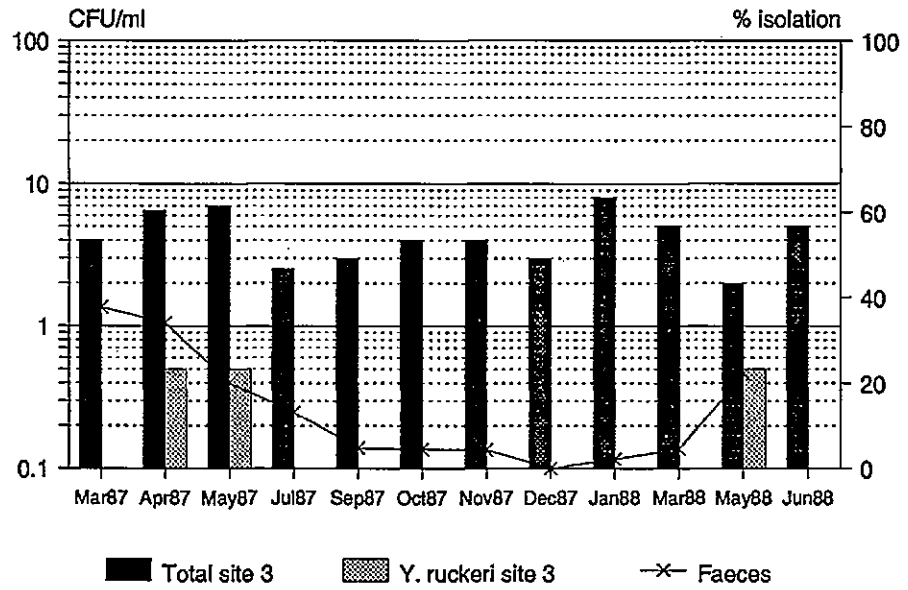


Figure 4.5c Total counts of all isolates and *Yersinia ruckeri* from inflow site 3

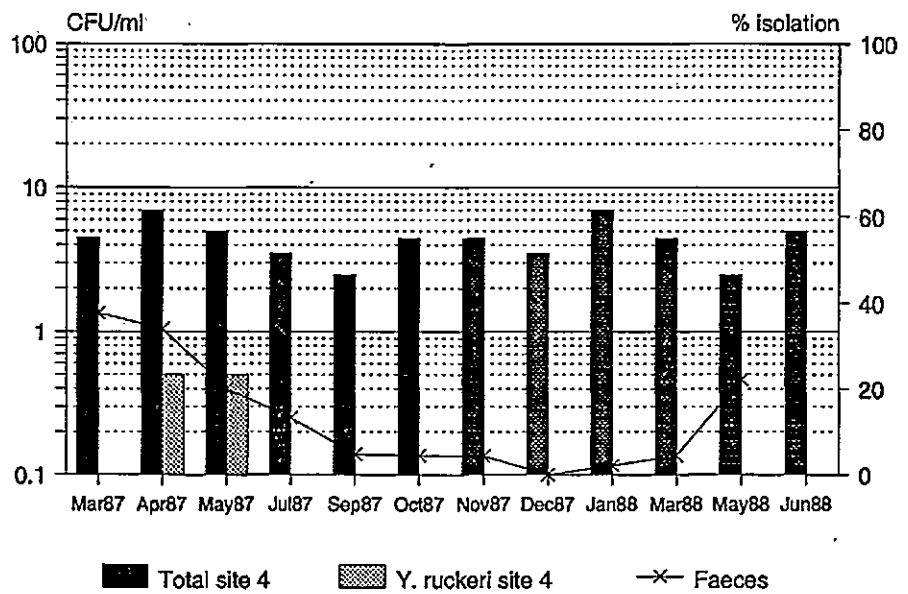


Figure 4.5d Total counts of all isolates and *Yersinia ruckeri* from inflow site 4

4.3.8 Vaccinated and non-vaccinated fish

A comparison between a vaccinated and a non-vaccinated group of fish at Farm A formed the third part of the field experiments. The figures represent 3-sample running means and indicate trends whereas the quoted values are taken from the actual data.

The isolation of *Y. ruckeri* from faeces showed that at 6 weeks (October sample) the non-vaccinated (NV) group had a 47% infection (Figure 4.6a). The corresponding level in the vaccinated (V) group (Figure 4.6b), however, only showed a 12% faecal infection at the same time. During the subsequent 2 month period the V group appeared to lose this low level faecal infection, within 4 weeks, whereas the NV group only had a 20% reduction in carriage before being undetectable at the December sample.

A second natural challenge, shown by a sudden rise in the faecal infection, then occurred within the following 4 weeks which resulted in a 100% NV faecal infection. In the V group however the infection remained undetectable. The following 5 week period through to March indicated a reduction in infection for the NV group but with the V group now having low levels of *Y. ruckeri*.

The next samples were taken in early May and indicated that a third peak of infection had taken place. Faecal infection in the NV group again reached 100% whereas the V group had only 5%.

The final samples after a subsequent 6 week period showed a minor reduction of infection in the NV group but interestingly the V group actually showed its first major rise in faecal carriage.

Figures 4.6a and 4.6b represent faecal infection irrespective of whether *Y. ruckeri* was also isolated from the kidney or spleen. Figure 4.7 considers those fish with only faecal carriage and shows that the V group maintained similar isolation levels, as shown in Figure 4.6b, except towards the end of the 10 month period. Whereas infection in the NV group was seemingly reduced throughout. This, and the level of isolation from kidney or spleen, indicates that the ERM vaccine not only

reduced faecal carriage of *Y. ruckeri* but also reduced clinical infection as well.

Figures 4.6a and 4.6b indicate that isolation from kidney was not as sensitive as that from spleen in either group. Clinical body infection in the V group, as measured by spleen isolation, confirms the previous pattern revealed by faecal isolation with a suppression of infection throughout the 10 month period, particularly in the first half of the trial.

It was noticeable that in the NV group wherever there was faecal carriage there was also corresponding clinical infection (Figure 4.6a). In the V group however there was a very different almost irregular pattern with faecal and clinical infection only occurring together towards the end of the sample period, from January 1988 onwards. It is also worth noting that in the V group an early warning of clinical infection was seen by using the selective-differential medium.

Peaks of infection in both groups during the 10 month exposure period occurred in the temperature range 8-12°C (Figures 4.6a and 4.6b). In addition the late summer and spring peaks correlated with the isolation of *Y. ruckeri* from inflow/outflow water samples (Figure 4.8). All strains isolated during the study were characterised as serotype 01.

The condition index for both groups (Figures 4.9 and 4.10) correlated with the infection levels and showed that after peaks of infection the performance of fish was adversely affected. In addition, apart from the beginning and end of the study the condition index was consistently lower in the NV group. The peaks in this group occurred several weeks before the corresponding peaks in the V group, indicating quicker loss of condition, whereas the situation was reversed at the troughs indicating a quicker recovery in the V group after natural ERM challenges.

The disease signs associated with each group during the trial period are summarised by Tables 4.6 and 4.7. Unbracketed figures refer to numbers of fish showing a particular symptom whereas bracketed figures refer to the numbers of fish showing a particular symptom but that also had *Y. ruckeri* isolated. Essentially more NV fish exhibited signs and a greater number had detectable ERM.

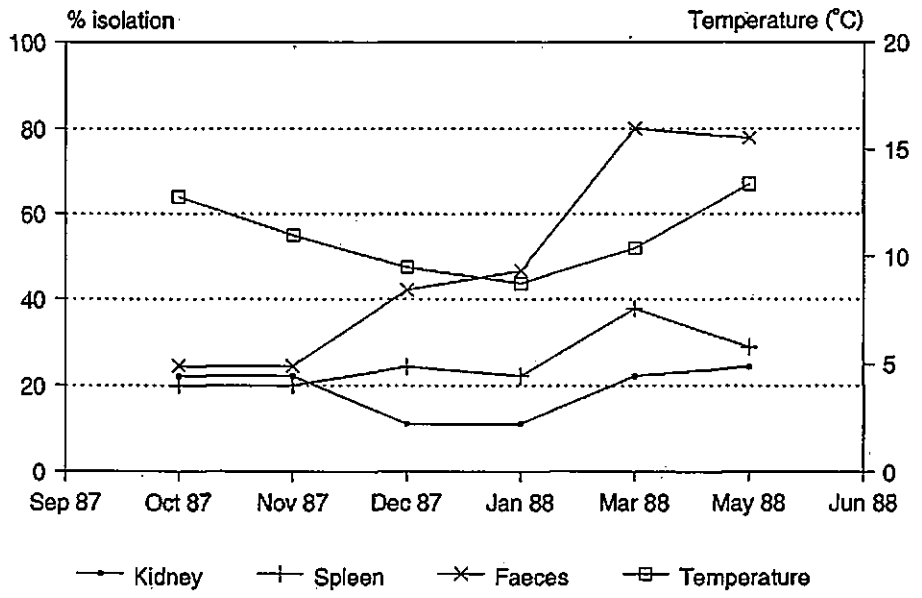


Figure 4.6a **Isolation of *Yersinia ruckeri* from non-vaccinated fish**

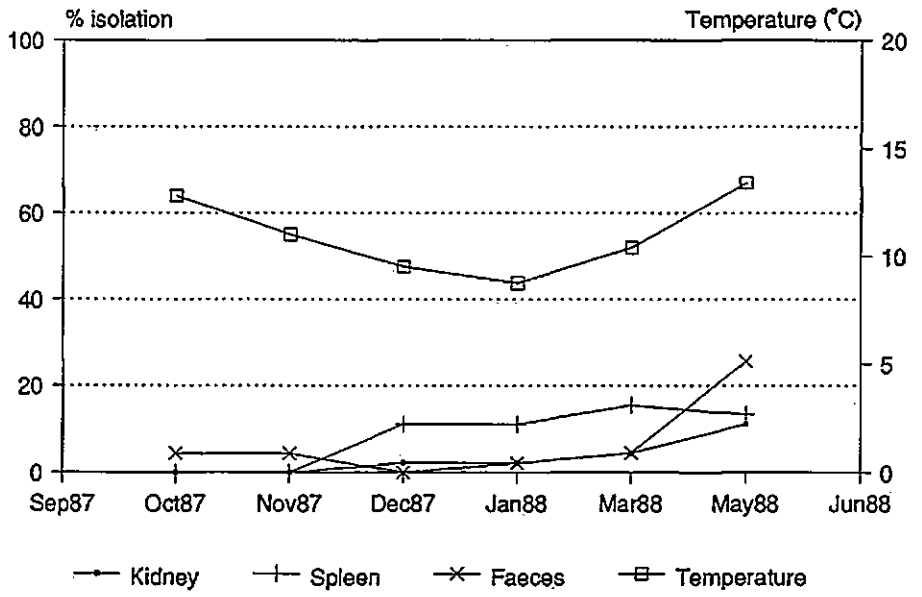


Figure 4.6b **Isolation of *Yersinia ruckeri* from vaccinated fish**

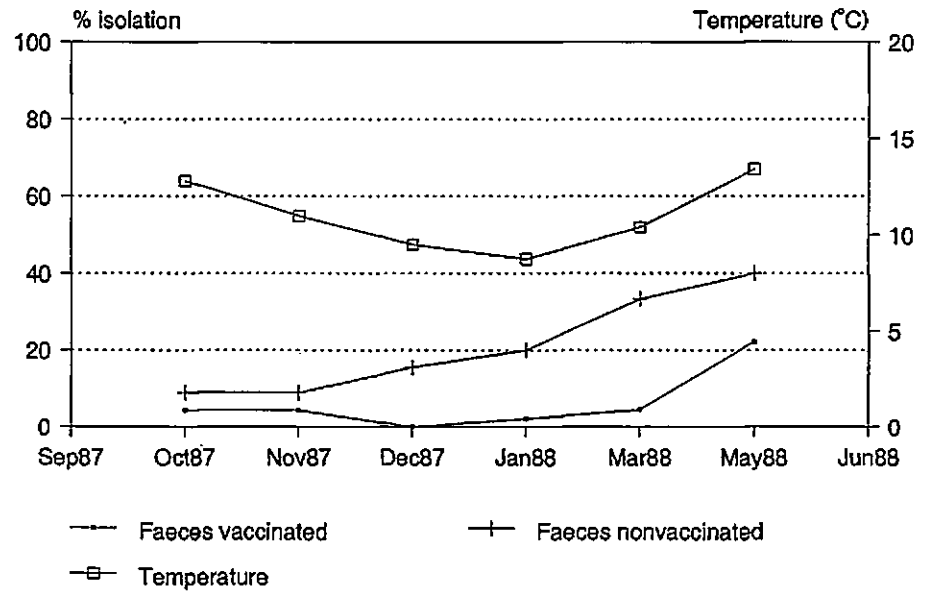


Figure 4.7 **Only faecal isolation from non-vaccinated fish and vaccinated fish**

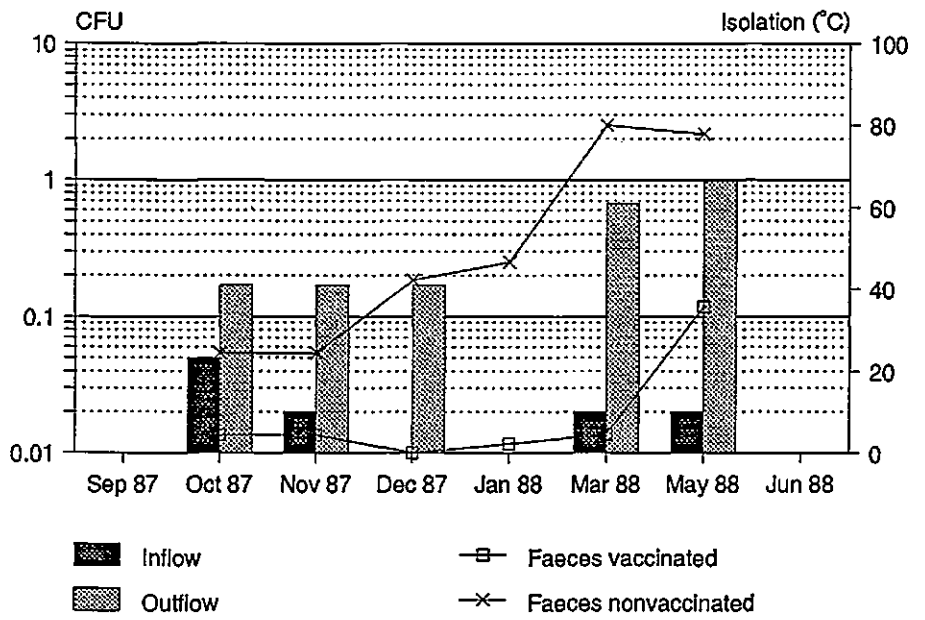


Figure 4.8 **Isolation of *Yersinia ruckeri* from inflow and outflow water and from non-vaccinated and vaccinated fish faeces**

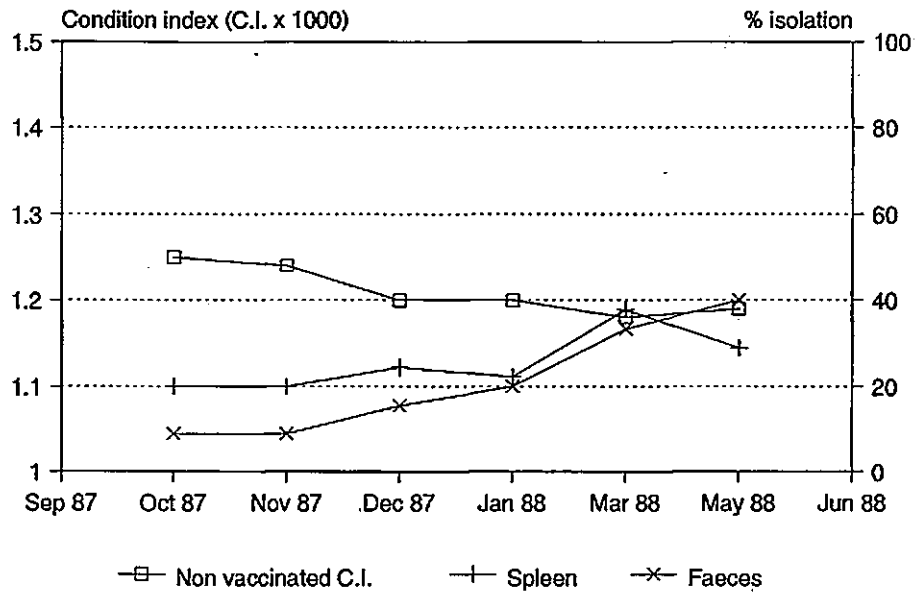


Figure 4.9 **Non-vaccinated group condition index with spleen and faecal isolation**

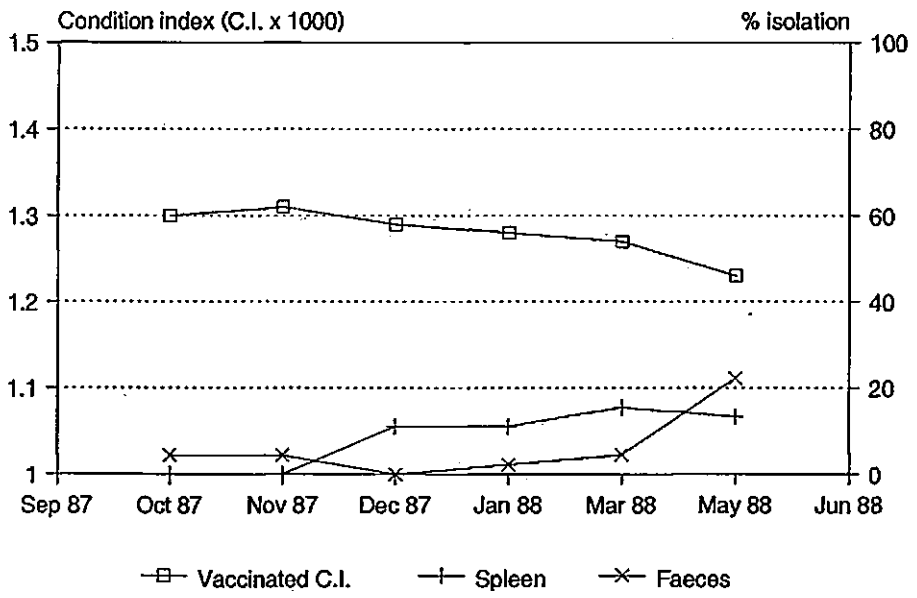


Figure 4.10 **Vaccinated group condition index with spleen and faecal isolation**

Table 4.6 Incidence of signs associated with isolation of *Y. ruckeri* in non-vaccinated fish at Farm A

Disease Signs	% of fish showing signs		
	Body isolation*	Faecal isolation	No isolation
None	16^{+}	24^{+}	76
Fluid or yellow faeces	34^{++}	35^{++}	17
Haemorrhaged eyes/exophthalmia	4	2	0
Haemorrhaged buccal cavity	1	0	0
Enlarged spleen	19^{+}	21^{+}	3
Haemorrhaged intestine or peritoneal cavity	26^{+}	16^{+}	2

*Kidney or spleen isolation

Chi-square test (compared to no isolation), $p<0.01^{+}$ and $<0.05^{++}$

Table 4.7 Incidence of signs associated with isolation of *Y. ruckeri* in vaccinated fish at Farm A

Disease Signs	% of fish showing signs		
	Body isolation*	Faecal isolation	No isolation
None	37^{+}	20^{+}	84
Fluid or yellow faeces	26^{++}	53^{+}	8
Haemorrhaged eyes/exophthalmia	0	0	0
Haemorrhaged buccal cavity	0	0	0
Enlarged spleen	21^{+}	13	4
Haemorrhaged intestine or peritoneal cavity	16^{++}	14	4

*Kidney or spleen isolation

Chi-square test (compared to no isolation), $p<0.01^{+}$ and $<0.05^{++}$

The conversion rates for both groups of fish are shown in Table 4.8. The rates indicated that the NV group had an increasingly worse conversion whereas the V group, although deteriorating initially, increased towards the end and was better than the NV group for 2/3 of the trial. In addition, after 11 months there were 3725 more fish in the V group (chi-square; p = <0.001) and at market size this represents approximately 0.75 tonne of fish (current English market value = £1653 @ UK £1/lb).

The results seem to indicate that vaccination against ERM significantly reduces the infection resulting from a natural challenge of *Y. ruckeri*. Nevertheless, repeated challenges over a 10 month period led to an increase in subsequent infection despite vaccination. However, this increase is greater for non-vaccinated fish.

Table 4.8 Conversion rate and mortality of non-vaccinated and vaccinated fish

Sample period	Conversion rate	Cumulative mortality
Sept-Dec		
Non-vaccinated	0.85	
Vaccinated	0.85	
Jan-May		
Non-vaccinated	1.51	
Vaccinated	1.44	
May-July		
Non-vaccinated	1.96	10550 (32%)
Vaccinated	1.25	*6825 (21%)

*Difference of 3725 fish (Chi-square test, p<0.001)=0.75 Tonne

The measured variables in groups A9 and A10 were the same as groups A1-A6 and B1-B9 except that A10 was comprised of non-vaccinated fish. Consequently, in order to determine whether there were any differences in the interaction between length, weight, temperature and isolation, partial multiple regression analysis was carried out. Weight was again chosen as the most important variable and was used following a square root transformation. The significance of the length and temperature variables, considered singly and in tandem, on the weight of fish is shown in Table 4.9.

Table 4.9 Variables selected by stepwise multiple regression

Group	Regressor	Predictor	Model	r^{2*}	T-ratio
A9	$\sqrt{W}$	L	$\sqrt{W}$=-3.13+0.07L	99.8	50.91^{+}
	$\sqrt{W}$	T	$\sqrt{W}$=3.31+0.38T	14.5	1.01
	$\sqrt{W}$	L, T	$\sqrt{W}$=-3.5+0.06L+0.05T	100	--
A10	$\sqrt{W}$	L	$\sqrt{W}$=-3.03+0.06L	99.3	30.19^{+}
	$\sqrt{W}$	T	$\sqrt{W}$=3.49+0.33T	11.6	0.89
	$\sqrt{W}$	L, T	$\sqrt{W}$=-3.65+0.06L+0.07T	99.9	--

*r^2=% variation (i.e. coefficient of determination); $\sqrt{W}$=square root weight; L=length; T=temperature

$^{+}$=$p<0.05$

As can be seen from Table 4.9 length and temperature totally explain the variations in weight in vaccinated group A9 (r^2=100%) but not in non-vaccinated group A10 (r^2=99.9%). A plot of the residuals from the equation obtained by using both length and temperature to predict weight, for group A10, indicated that there was a reduction in weight during kidney and spleen infection (data not shown). In addition, there was a significant difference between the standard deviations of the fitted values for square root weight from groups A9 and A10 (Twosample T-test, $p<0.001$). This indicates a greater variation in weight for non-vaccinated fish compared with vaccinated fish.

4.4 DISCUSSION

Yersinia ruckeri was successfully transmitted from a donor tank of artificially infected rainbow trout to mirror carp, dace, salmon parr and other rainbow trout. This finding supports work reported in other studies. Busch (1983) indicated that transmission of *Y. ruckeri* was primarily horizontal through the water and this has also been shown by other workers (Rucker, 1966; Dulin *et al.*, 1976-cited by Bullock, 1984; Hunter *et al.*, 1980). In addition, water which has been inhabited by rainbow trout with ERM can also infect salmon and other rainbow trout (Ross *et al.*, 1966; Rucker, 1966; Bullock *et al.*, 1976). These reports, however, were using mortality and subsequent isolation of *Y. ruckeri* as criteria to assess transmission. Bullock *et al.* (1976) observed the first mortality in salmon receiving infected effluent after 10 days. The study reported here did not have any mortalities in any of the species of recipient fish. In fact, the salmon only had detectable *Y. ruckeri* in faecal material after 38 days. There has been a variation in reported time to first death of rainbow trout exposed to infected effluent. Ross *et al.* (1966) found that the first death occurred after 5 days whereas Bullock *et al.* (1976) reported that this occurred after only 48 h. Nevertheless, it was shown here that although strain LT12/6 (unpassaged) did not kill any rainbow trout receiving infected water, they became carriers within 16 days. Unreported differences in virulence may be the main reason to explain these variations since water temperatures were comparable (12.5 and 13°C) between this study and the one reported by Bullock *et al.* (1976). The immersion challenge doses were also similar (1 and 5 x 10^7 bacteria ml^{-1}).

Asymptomatic carrier fish have been known to lead to recurrent ERM problems (Bullock, 1984; Busch, 1978, 1983) since they have the potential to excrete *Y. ruckeri* into the water via the faeces. This was shown by Busch and Lingg (1975) when up to 25% of rainbow trout carried *Y. ruckeri* in the intestine 45 days after artificial infection. Cyclical shedding after 36-40 days, leading to 50-

75% of survivors harbouring *Y. ruckeri* for up to 65 days following infection, was also indicated. The rainbow trout used in this experiment only became carriers after 16 days and it was not possible to isolate *Y. ruckeri* after this time, indicating the subsequent removal of the carrier status at this stocking density (1 x 30 g fish l^{-1}). A cyclical pattern was also indicated in this experiment since the salmon did not become carriers for 36 days. This presumably reflects the continuing carrier status and shedding of *Y. ruckeri* from the donor fish, although this was not verified by isolation.

The recovery of *Y. ruckeri* from mirror carp and dace indicates the potential of these species of fish to act as reservoirs of infection. Other reports have also referred to isolation from carp (Fuhrmann *et al.*, 1984; Enriquez and Zamora, 1987) and dace (Roberts, pers com.). In addition, it was not possible in this study to isolate *Y. ruckeri* from grayling, although Ceschia *et al.* (1984) have reported isolation from farmed species.

The experimental field trials indicated that there was a higher level of detectable *Y. ruckeri* from Farm A than Farm B, throughout the period of monitoring. The farms are constructed and consequently managed differently. Farm A is largely a tank system whereas Farm B is a total raceway unit. As a result fish at Farm A tend to be handled more than at Farm B since they are moved from tank to tank as they grow. Farm B is much less labour intensive and the stocking densities can be more easily managed. The results indicated that this was an important factor in the progression of ERM through individual groups of fish. It was shown that this type of farm practise could lead subsequently to ERM problems for several weeks afterwards. The stress caused by handling has been reported to trigger outbreaks of ERM in infected fish (Bullock, 1984; Sparboe *et al.*, 1986).

However, the actual occurrence of shedding followed by subsequent transmission can depend on seasonal variations in water temperature, density of fish and handling stress (Busch, 1973). Certainly fish at Farm A were pre-disposed to acute outbreaks of ERM, particularly after grading during the spring and autumn

months.

Unfavourable on-farm conditions such as low dissolved oxygen, over-crowding, increased suspended solids, elevated levels of ammonia or other waste products and fluctuating water temperatures have also been linked to acute epizootics, particularly with the chronic form of the disease (Busch, 1978, 1983; Austin, 1982; Bullock, 1984; Frerichs *et al.*, 1985). The peaks of infection, at both farms, seemed to occur largely in late winter through to spring and again in the autumn. This coincided with a water temperature of 8-12°C. Most published reports indicate that ERM is most severe at 15-18°C or higher (Dalsgaard *et al.,* 1984; Rübsamen and Weis, 1985; Ocvirk *et al.*, 1988). In fact, Rucker (1966) indicated that ERM could be less severe at 10°C or below. Nevertheless, various combinations of the previously mentioned stress factors could lead to outbreaks of ERM when water temperatures are fluctuating quickly in the spring and autumn.

These stress factors, which occur at specific times of the year, also seemed to affect the performance and marketability of groups of fish. Given good on-farm conditions and an appropriate feeding regime water temperature is the key factor controlling the rate of fish growth (Bromage and Shepherd, 1988). The complicating factor of isolation of *Y. ruckeri* from fish at the two farms seemed to suppress the condition index of fish, particularly those with chronic ERM. Consequently, over wintering fish subjected to two natural challenges from *Y. ruckeri* performed poorly compared to fish brought onto the farms in the spring or summer. However, it was difficult to compare individual groups of fish, particularly from year to year, even though the fish were brought onto the farm in comparable months. Though a comparison between groups of fish from the respective farms indicated that the lower levels of acute infection at Farm B had only a short term effect on fish despite regular recovery of *Y. ruckeri* from faecal material. Fish at Farm A, on the other hand, that experienced chronic ERM with regular peaks of infection were affected for several months afterwards. It also seemed that fish reared initially in raceways performed better than those reared in

round tanks, until they themselves were graded and moved into round tanks too. The use of the circular design has been criticised for causing greater contact between the fish, disease organisms and waste materials compared to raceways (Bromage *et al.*, 1988).

At its simplest the condition index, based on an analysis of length-weight data, assumes that heavier fish of a given length are in a better condition (Bolger and Connolly, 1989). The use of condition factors raises problems in their interpretation because the weight of fish is not always proportional to the cube of its length (Le Cren, 1951). A study by Elliott (1976) showed that there was some variation in the condition factors for brown trout of similar length in a feeding experiment, even amongst control fish. Consequently, the author concluded that the simplest interpretation of the condition factor was to regard it not as an absolute index but as a relative index of condition. The statistical interpretation of the relationship between weight, length, temperature and isolation proved extremely complex. As a result it was not possible to prove that only isolation had a significant effect on weight, when groups of vaccinated fish were compared. Nevertheless, it was shown that there was a greater variation in weight in non-vaccinated fish compared with vaccinated fish. A poor conversion following infection has been shown in fish infected with ERM (Horne *et al.*, 1984).

The isolation of *Y. ruckeri* from fish faeces up to 4-6 weeks before appearing in the kidney, using ROD medium, indicated the intestinal nature of chronic ERM and showed that the carrier status occurred prior to the onset of clinical infection. In addition, the results indicated that kidney was not as consistent for isolation as spleen and faeces. Most published reports of ERM diagnosis rely on the isolation of *Y. ruckeri* from kidney material although isolation from other organs has also been reported from clinically affected fish (Busch, 1983; Ceschia *et al.*, 1984; Baath, 1986; Bragg and Henton, 1986). Busch and Lingg (1975) reported the relative pathogen recovery rates from intestine, kidney, liver and spleen in an experimental laboratory study of the carrier status. They concluded that fish health

programmes should include intestinal sampling and not just the classical methods from kidney or spleen. However, although the data indicated that isolation from spleen was slightly more sensitive than from kidney, following ip infection, the significance of this finding was not discussed. There are no other comparative studies of differences in isolation between kidney, spleen and faeces. Consequently, the greater sensitivity of isolation from spleen and faeces, reported here for the first time in a field trial, could be an important finding that would aid further epidemiological studies of ERM. In addition, there would be potential for improved diagnosis, particularly in the early stages of the chronic form of the disease, by taking spleen and faeces samples instead of only kidney.

The signs associated with ERM indicated that a haemorrhaged intestine or peritoneal cavity and haemorrhaged eyes or exophthalmia were significant in fish with body isolation of *Y. ruckeri* at Farm A. In addition, fluid or yellow faeces and an enlarged spleen were also significant in fish from Farm B. The absence of intestinal and eye haemorrhages coupled with an increased percentage of enlarged spleen and yellow faeces at Farm B indicates the more acute nature of ERM at this site. The reduced stress levels, mentioned earlier, at Farm B seem to allow the immune response in vaccinated fish to not only reduce the levels of *Y. ruckeri* but also to reduce the visual signs of the disease. Interestingly, there was a significant number of fish at both farms showing no signs but that had *Y. ruckeri* isolated from faecal material, compared to the number of fish with no isolation. This could represent the limits of detection for ROD medium, particularly in the early stages of chronic infection at Farm A when *Y. ruckeri* would be expected to be at low levels in the gut. The signs observed in the field study were consistent with the published reports of Rucker (1966), Busch (1978, 1983), Warren (1983) and Sparboe *et al.* (1986).

ROD medium continued to prove useful to isolate *Y. ruckeri* from inlet and outlet water samples at both fish farms. The isolation from water corresponded with isolation from fish faecal material with higher levels in the outflow. In

addition, a potential reservoir of infection due to escapee fish was shown to occur in the inflow supply. Busch (1978) reported that *Y. ruckeri* can survive for long periods of time in organically rich waters and that transmission occurs through the water from faeces of infected fish. Bromage *et al.* (1988) recommended that water channels should be kept free from fish to avoid potential sources of infection.

The results indicated that vaccination against ERM reduced the infection resulting from a natural challenge of *Y. ruckeri* but that repeated challenges over a 10 month period seemed to increase the susceptibility of even vaccinated fish. The ability of the vaccinated fish to resist the challenge of *Y. ruckeri* appeared to be good. They also had an increased ability to maintain reduced levels of the bacterium from their systems. This does not support the theory that vaccination against ERM may increase the incidence of carrier fish. In fact the trend is for a reduction of detectable infection. The sensitivity of ROD medium, used for faecal detection, cannot detect very low levels of *Y.ruckeri* carried in the intestine, hence the elimination of the carrier state cannot be proven by non-isolation of *Y. ruckeri.* However, this applies equally to vaccinated and non-vaccinated fish.

The indication was that as ERM progressed in a group of fish the smaller fish contracted the disease first. However, with repeated natural challenge the larger more vigorous fish ultimately succumbed and became infected. In addition, the fish contracting early infection under went an acute form of ERM whereas later infection had a more chronic debilitating nature. The signs associated with disease progression tended to support this finding particularly for the vaccinated fish. There were more fish with yellow faeces, haemorrhaged eyes, intestine and 'redmouth' that also had *Y. ruckeri* isolated from the body (kidney or spleen) in non-vaccinated fish compared to vaccinated fish. Conversely, isolation from faeces was associated with yellow faeces in more vaccinated fish. The same was true for body isolation and no signs. This indicates that vaccination reduces not only the appearence of signs but also the infection levels as well. Cyclical shedding, represented by peaks of infection, was apparent during this study in the NV group

but to a lesser extent in the V group. The primary reservoir for ERM seems to be asymptomatic carrier fish that regularly shed large numbers of *Y. ruckeri* into the water through the faeces (Rucker, 1966; Busch, 1983). Moreover, Busch and Lingg (1975) demonstrated that the carrier state in the lower intestine was followed by regular intestinal shedding causing recurrent infection and mortality on a cyclical basis. This has also been noted by McDaniel (1971) who demonstrated cyclical mortality patterns throughout the year in a large hatchery population chronically infected with ERM. Certainly fish at Farm A were pre-disposed to acute peaks of ERM infection, particularly after grading during the spring and autumn months. This was in addition to the more chronic nature of the disease at other times.

Although the levels of circulating antibody were not measured in the field study, the results suggested that natural challenge may not act as an effective 'booster' nor as a primary stimulation for the immune system. However, the role of on-farm stress related factors may contribute to suppression of the immune response. This finding has also been reported by Busch (1983) who stated that natural epizootic infections of ERM do not appear to confer complete protection within a population. In addition, recurrent outbreaks have been correlated with the stress of handling, grading, hauling, excessive loading densities and periods of high water temperatures. Moreover, vaccines used to control *Y. ruckeri* infections in fish do not completely eliminate the disease or losses. When immunised fish are held for longer periods (e.g. over a year) or when they are exposed to some of the above stress creating factors, significant mortality can still occur. The fish in this study were monitored for 10 months and during that time were graded twice. This resulted in increased infection and mortality within 10 days afterwards, particularly in the NV group. In addition, towards the end of the trial the fish were held in over-stocked tanks (vaccinated fish-330/m^3; non-vaccinated fish-238/m^3) at a water temperature of 14-16°C, which may have contributed to increased isolation of *Y. ruckeri* in both groups of fish. Nevertheless, the V group had significantly fewer mortalities and a better conversion rate despite higher stocking levels. This

supports the observation by Horne *et al.* (1984) that unvaccinated fish that recover from ERM show a recurrent, low level mortality and a poor conversion rate.

CHAPTER 5

IN VITRO RESISTANCE TO ANTIMICROBIAL AGENTS

5.1 INTRODUCTION

The discovery and development of antimicrobial agents to treat systemic bacterial infections is one of the most fascinating stories in the history of microbiology (Sokatch and Ferretti, 1974). Unfortunately, the problem of drug resistance has been known for almost as long as the occurrence of such agents and has been recognized as a severe limitation to their use. With few exceptions it has been found that drug resistance can occur in any organism and against any antimicrobial agent (Maas, 1986).

Early workers examined the property of azo dyes for possible antimicrobial action. Chemical modification of a synthesized compound called prontosil led directly to another series of compounds, the sulphonamides (reviewed by Hammond and Lambert, 1978a). Penicillin was the first natural antibacterial agent which was clinically successful. Although Fleming observed the inhibition of *Staphylococcus aureus* on an agar plate by a contaminating *Penicillium* it was some years later when other workers purified enough penicillin for clinical trials (reviewed by Sokatch and Ferretti, 1974). Since this early work in the 1940's there have been many reported studies on chemotherapeutic agents, with the result that there are now antibiotics available for virtually all bacterial infections (Sokatch and Ferretti, 1974).

The inherent ability of microorganisms to adapt to hostile environments leads directly to the development of antibiotic resistance in microbial strains (Hammond and Lambert, 1978d). Antibiotic resistance can be divided into two categories: intrinsic resistance due to the physiology, biochemistry or morphology of an isolate, and resistance acquired by microbial strains following exposure to antibiotics.

The usage of antimicrobial compounds in the successful treatment of infections due to *Yersinia ruckeri* has been reported in several studies. For instance, the early

work of Rucker (1966) recommended treatment with sulphamethazine for 5 days, followed by 3 days on chloramphenicol or oxytetracycline. Subsequent laboratory experiments have shown the value of potentiated sulphonamide therapy for both artificial and natural infections of ERM (Bullock *et al.,* 1983). Oxolinic acid, a member of the 4-quinolone group, was used for prophylaxis and therapy of ERM in rainbow trout by Rodgers and Austin (1983). However, the hazards of misuse of antimicrobial agents have been highlighted by Post (1987). The selection of drug resistance in strains of *Y. ruckeri* can be encouraged by inadequate, variable dosage, by finishing treatment too early or by repeated short-term treatment (Tebbit *et al.*, 1981). In fact, Post (1987) has reported that some areas of the USA, where ERM is enzootic, now yield isolates completely resistant to normal levels of both sulphamerazine and oxytetracycline. This type of multiple resistance to tetracyclines and sulphonamides has also been demonstrated by De Grandis and Stevenson (1985). Therefore the potential of *Y. ruckeri* to develop *in vitro* resistance to the three main fisheries antimicrobial compounds was investigated in this project. The subsequent loss of induced resistance was also followed and the minimum inhibitory concentration for each compound was studied in greater detail.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals, Reagents and Media

The origins of all chemicals, reagents and media are detailed in section 3.2.1.

5.2.2 Bacteriology

5.2.2.1 Bacterial strains

The strains of *Y. ruckeri* used in this study are listed in Table 3.1.

5.2.2.2 Bacterial cultivation and storage

The routine cultivation and storage of strains are detailed in section 3.2.2.2. Strains derived from parent strain 39/81 at each stage in sections 5.2.9 and 5.2.10 were stored in FDBG at -20°C but with the addition of an appropriate concentration of either oxolinic acid, oxytetracycline or a potentiated sulphonamide (v/v).

5.2.3 Biochemical characterization of strains

Strains of *Y. ruckeri* used in section 5.2.6 were characterized using API 20E strips as detailed in section 3.2.3. In addition, strains derived from parent strain 39/81 were similarly identified at each step in the increase and decrease of *in vitro* resistance experiments (sections 5.2.9 and 5.2.10 respectively).

5.2.4 Preparation of antimicrobial agents and incorporation into media

Three antimicrobial compounds commonly used for the treatment of ERM (see chapter 7) were prepared as stock solutions as follows:

a) Oxolinic acid (OA; Microbiologicals, Fordingbridge, U.K.) was dissolved in distilled water to give a working solution of 1024 μg ml^{-1}. To aid dissolution the pH was adjusted to 9.3 with 1M sodium hydroxide.

b) Oxytetracycline (OT; Sigma) was dissolved in distilled water to give a working solution of 1024 μg ml^{-1}.

c) A potentiated sulphonamide (PSu; sulphadiazine:trimethoprim, 5:1 w/w; Sigma) was dissolved in distilled water to give a working solution of 1024 μg ml^{-1}. To aid dissolution the pH was adjusted to 10.5 with 1M sodium hydroxide.

All working solutions were freshly prepared as required and filter sterilized before use with a 0.22 μm Millipore disposable filter.

5.2.5 Standardization of bacterial inoculum

Standard inocula were prepared by taking growth from TSA plates (26°C; 48 h), mixing in phosphate buffered saline (PBSa) and measuring the absorbance (A_{625}=0.1; SP-1800 Spectrophotometer, Pye-Unicam, Cambridge, U.K.), to give a final concentration of approximately 1 x 10^5 bacteria ml^{-1}.

5.2.6 Estimation of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) were determined for 124 strains of *Y. ruckeri* using two methods for comparative purposes:

a) Microtitre plate dilution

A micro-dilution technique in 96 U-well microtitre plates (Sterilin,U.K.) using 50 μl of antibiotic medium 3 (AM3; Difco) was used (Tsoumas *et al.*, 1989). Serial

two-fold dilutions (Thrupp, 1986) were achieved from the working solutions (section 5.2.4) giving final concentrations for each antimicrobial agent of 256 μg ml^{-1} to 0.00012 μg ml^{-1}. Each well was then mixed with 50 μl of a standard inoculum (section 5.2.5) followed by incubation at 26°C for 48 h. Two microtitre plates were used for each isolate with one row of 24 wells being used to test one antimicrobial agent against one strain of *Y. ruckeri*. The final well contained no antimicrobial agent and was used as a negative control.

The MIC for each strain was estimated visually and was determined as the last well to show absence of growth when compared to the negative control well. In addition, particularly where the end point was not clear cut, the microtitre plates were read with a Uniskan reader at 405 nm.

b) Agar incorporation

An agar dilution technique was also used by incorporating each antimicrobial compound into sensitivity agar (LabM) in 90 mm petri dishes (Sterilin). The range of concentrations in the medium was similar to that in section 5.2.6a. Each plate was inoculated with 28 strains of *Y. ruckeri* by using a Lidwell applicator and was incubated at 26°C for 48 h. The last plate to show absence of growth was recorded as the MIC. Plates containing no antimicrobial agent were used as negative controls.

All MIC determinations (sections 5.2.6 a and b) were performed in duplicate.

In addition to the strains of *Y. ruckeri*, one strain each of *Aeromonas hydrophila, A. salmonicida* and *E. coli* were used as positive controls in order to determine whether MIC values previously determined by other studies were repeatable.

5.2.7 Action of antimicrobial agents at the MIC

Following the MIC experiment (section 5.2.6) one strain of *Y. ruckeri* (39/81) was chosen for further work to determine whether each antimicrobial compound at the actual MIC was bacteriostatic or not within 72 h.

A serial dilution technique was again used (section 5.2.6a) in order to obtain the MIC of each antimicrobial agent but with larger volumes of AM3 (2.5 ml) in sterile glass universal containers. A further 2.5 ml of AM3 was added from a culture

of strain 39/81 (26 °C; 48 h) which had been washed once in PBSa (2600 rpm; 4°C; 20 min) and resuspended in AM3 to give a final concentration of approximately 1 x 10^5 bacteria ml^{-1}. Containers of AM3 without any of the three antimicrobial agents were used as controls.

The universal containers, in duplicate, were then incubated at 26°C for 8, 16, 24, 32, 48 and 72 h after which each 5 ml of AM3 was filtered through a 0.22 μm Millipore filter unit. The bacteria retained within the filter were then washed with an additional 5 ml of AM3 in order to remove any residual antimicrobial agent and a further 5 ml of AM3 was used to back wash the cells. This action resuspended the bacteria so that total viable counts could be undertaken by performing serial 1:10 dilutions in 0.9% saline. A spread plate technique was then used to count individual colonies after incubation at 26°C for 48 h.

Since this method could have resulted in a loss of bacterial cells following back washing, particularly by adsorption to the filter membrane, an additional series of control cultures was also prepared. Various concentrations of strain 39/81 (6.4 x 10^1 to 6.4 x 10^8 c.f.u. ml^{-1}) in triplicate (TVC1) were filtered and back washed in the same way but with no antimicrobial agent. Regression analysis of the post filtration total viable counts (TVC2) against TVC1 was then carried out to provide a loss from filtration equation.

5.2.8 Estimation of minimum bactericidal concentration (MBC)

In addition, 39/81 was also used to determine the minimum bactericidal concentration (MBC) necessary to give a 99.9% kill rate (Thrupp, 1986) after 48 h.

The same serial dilution technique was used (section 5.2.6a) but with the larger volumes of AM3 (section 5.2.7). Eleven doubling dilutions were used for OA, whereas nine each were used for OT and PSu. The MIC was included as the highest dilution and each dilution was inoculated as in section 5.2.7 to a final concentration of approximately 1 x 10^5 c.f.u. ml^{-1}.

The universal containers, in duplicate, were then incubated at 26°C for 48 h. Each universal of AM3 was then filtered and washed before performing TVC's (see

section 5.2.7). Control cultures similar to those used in section 5.2.7 were also prepared.

5.2.9 Increase of *in vitro* resistance

One of the most sensitive strains of *Y. ruckeri* (39/81) to OA, OT and PSu was used in order to determine the potential for increased resistance. Each antimicrobial agent was incorporated into cooled (56°C) sensitivity agar (LabM) before pouring by hand into 55 mm petri dishes (Microbiological Supply Co., Toddington, UK). This method gave a range of concentrations from half the previously determined MIC value to 512 μg ml^{-1}. Duplicate plates with the lowest concentration (i.e. ½x MIC) were then inoculated as spread plates by taking 10 μl of a standard suspension in PBSa (section 5.2.5; A_{625}=0.1) obtained from a 48 h TSA plate culture. This was designed to give an inoculum of 1 x 10^5 c.f.u. ml^{-1}. Following incubation at 26°C for 48 h a further suspension was made from these plates in the same way and this was used to inoculate the next highest plate concentration (i.e. 1 x MIC). The procedure was repeated subsequently for 15 subcultures. The transference to the next highest concentration of antimicrobial agent was arbitarily determined to be the point where growth of any kind occurred at the lower concentration and this was recorded as the next step in selected resistance. However, where growth was not confluent (i.e. individual countable colonies) plates of the same concentration were also inoculated. Consequently this sometimes involved more than one subculture at the same concentration as well as subculture to the higher concentration. Sensitivity agar plates containing no antimicrobial agent were inoculated at each stage and served as controls.

5.2.10 Decrease of *in vitro* resistance

Following the attempt to increase resistance in section 5.2.9 the resultant strain of 39/81 with the highest MIC for each antimicrobial agent was used to determine whether repeated subculture, without the presence of any antimicrobial agent, could decrease any previously induced resistance. The strain was therefore routinely subcultured onto TSA plates and incubated at 26°C for 48 h. After each subculture the MIC to each respective antimicrobial agent (OA, OT or PSu) was determined by

the method detailed in section 5.2.6b but using simple streak culture instead of a Lidwell applicator. This procedure was repeated subsequently for 15 subcultures.

5.2.11 Statistics

A two-sample T-test was used to compare the bacterial growth, as determined by TVC, obtained in sections 5.3.3 and 5.3.4. A probability value of up to 0.05 (5%) was considered to be significant.

5.3 RESULTS

5.3.1 Biochemical characterization of strains

At each stage of sections 5.3.3, 5.3.4 and 5.3.5 all strains were confirmed as *Y. ruckeri*. The profiles obtained included: 1105100, 5104100, 5304100 and 5307100.

5.3.2 Estimation of minimum inhibitory concentration (MIC)

A comparison of the minimum inhibitory concentration (MIC) values for 124 strains of *Yersinia ruckeri* indicated that the three chosen methods gave contrasting results.

5.3.2.1 Microtitre plate dilution

This method indicated a MIC range of <0.0001-0.25 $\mu g\ ml^{-1}$ (mode=0.0078 $\mu g\ ml^{-1}$) for oxolinic acid; 0.125-2 $\mu g\ ml^{-1}$ (mode=1 $\mu g\ ml^{-1}$) for oxytetracycline and 0.0156-64 $\mu g\ ml^{-1}$ (mode=0.125 $\mu g\ ml^{-1}$) for a potentiated sulphonamide (Figure 5.1a). The actual end point was difficult to determine accurately with this method since turbidity due to cell growth occurred over a wide range of concentrations.

5.3.2.2 Absorbance measurements

This method indicated a MIC range of <0.0001-4 $\mu g\ ml^{-1}$ (mode=0.0078 $\mu g\ ml^{-1}$) for oxolinic acid and 4-64 $\mu g\ ml^{-1}$ (mode=16 $\mu g\ ml^{-1}$) for oxytetracycline (Figure 5.1b). The growth and consequent heavy cell suspension meant that an absorbance reading could not be taken for a potentiated sulphonamide using this method.

5.3.2.3 Agar incorporation

This method indicated a MIC range of 0.0156-0.25 μg ml^{-1} (mode=0.0625 μg ml^{-1}) for oxolinic acid; 1-4 μg ml^{-1} (mode=4 μg ml^{-1}) for oxytetracycline and 1-64 μg ml^{-1} (mode=4 μg ml^{-1}) for a potentiated sulphonamide (Figure 5.1c). The end point with this method was clear cut and sharply defined and consequently was easier to record than the MIC's determined in sections 5.3.2.1 and 5.3.2.2.

The strains of *A. hydrophila, A. salmonicida* and *E. coli* used as positive controls gave MIC values which were similar to other published studies.

A summary of the results for the estimation of the MIC values for the strains of *Y. ruckeri* examined is given in Table 5.1.

Table 5.1 Estimation of minimum inhibitory concentration (MIC)

	MIC50	MIC90	MIC range (μg ml^{-1})
Oxolinic acid			
Microtitre plate dilution	0.0039	0.0313	0.0001-0.25
Absorbance measurement	0.0313	0.5	0.0001-4.0
Agar incorporation	0.0625	0.125	0.0156-0.25
Oxytetracycline			
Microtitre plate dilution	1.0	1.0	0.125-2.0
Absorbance measurement	16.0	32.0	4.0-64.0
Agar incorporation	4.0	4.0	1.0-4.0
Pot. sulphonamide			
Microtitre plate dilution	0.5	4.0	0.0156-64.0
Absorbance measurement	----	----	--------
Agar incorporation	4.0	4.0	1.0-64.0

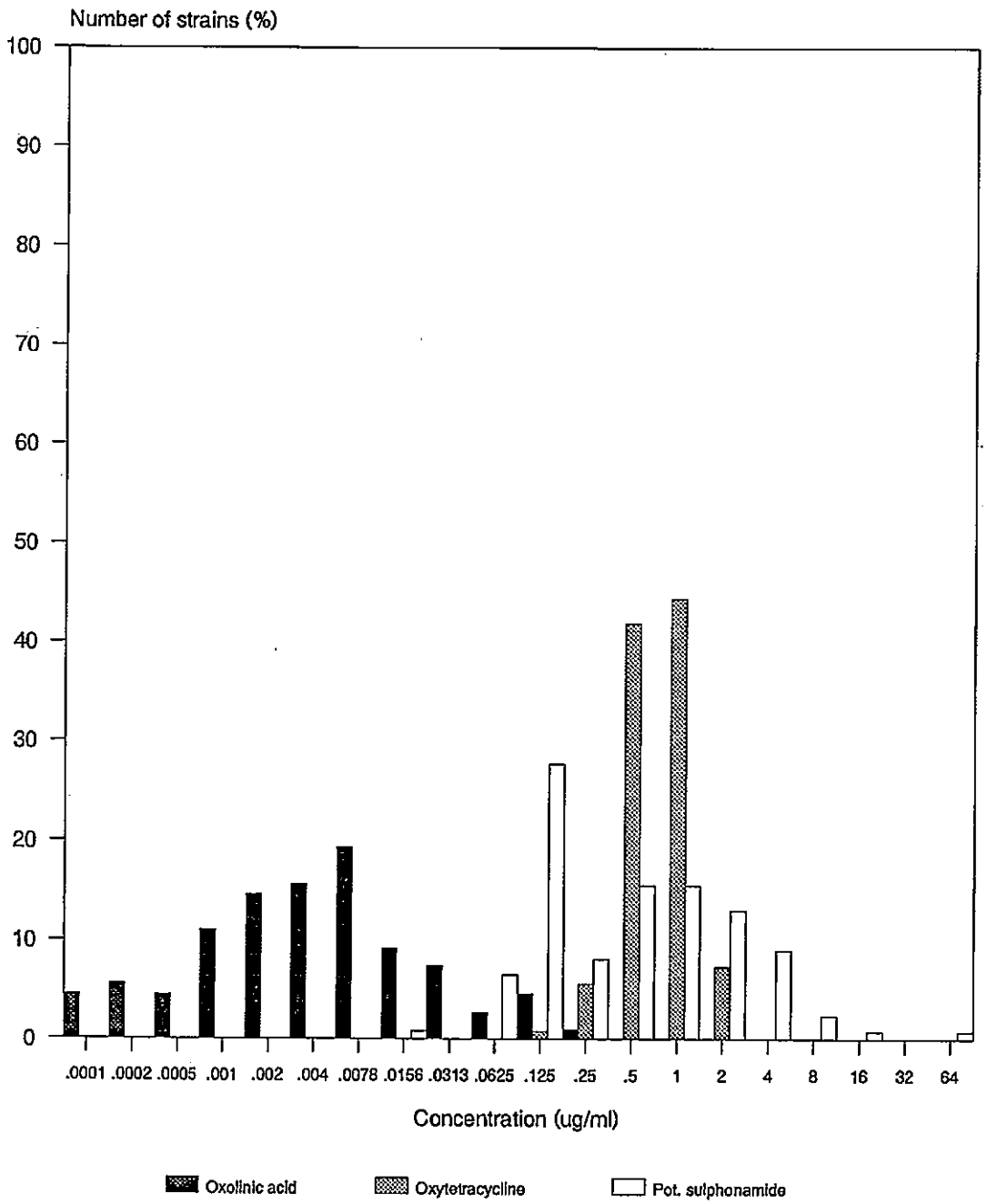


Figure 5.1a Distribution of M.I.C. values-microtitre plate dilution

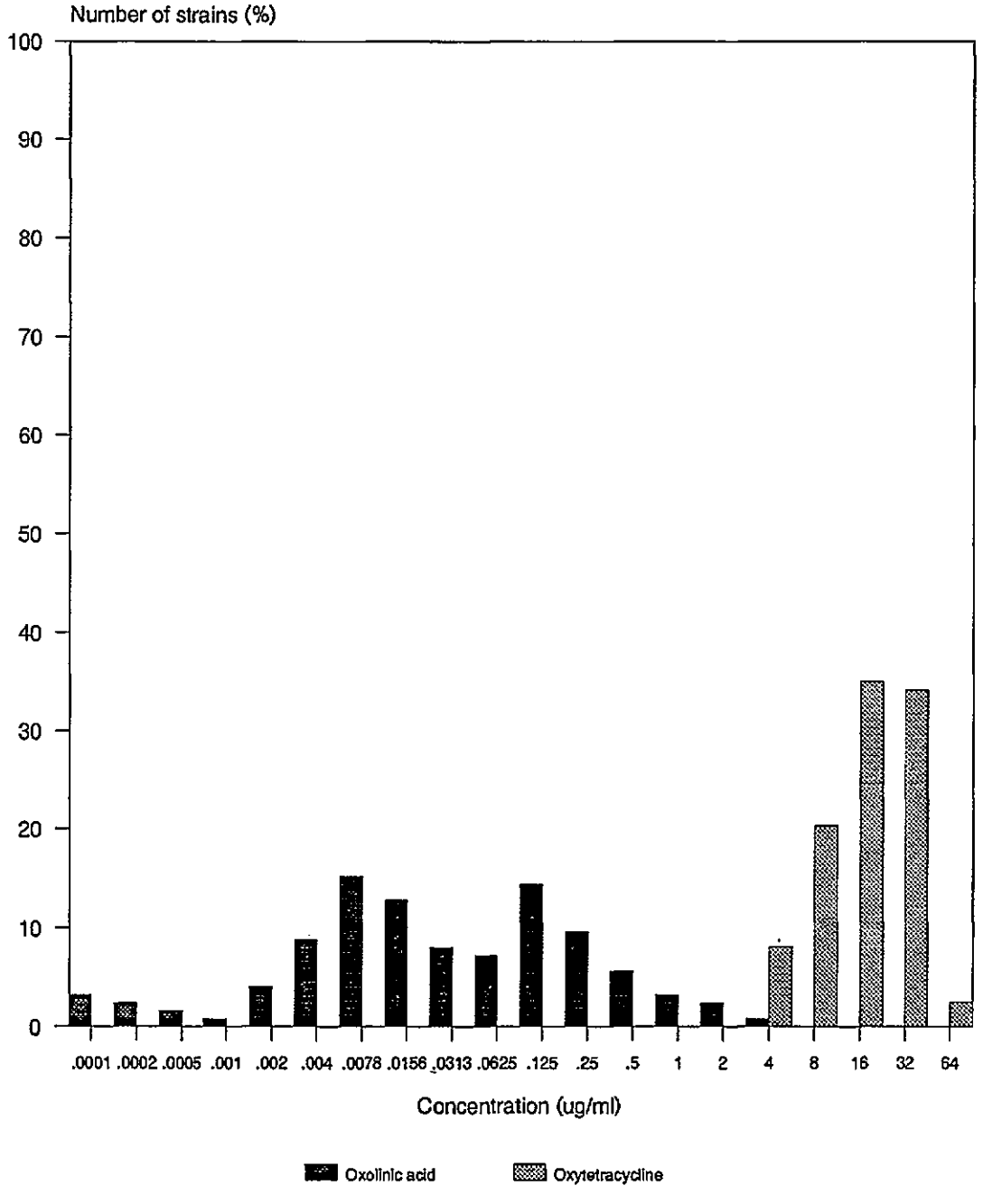


Figure 5.1b Distribution of M.I.C. values-absorbance measurements

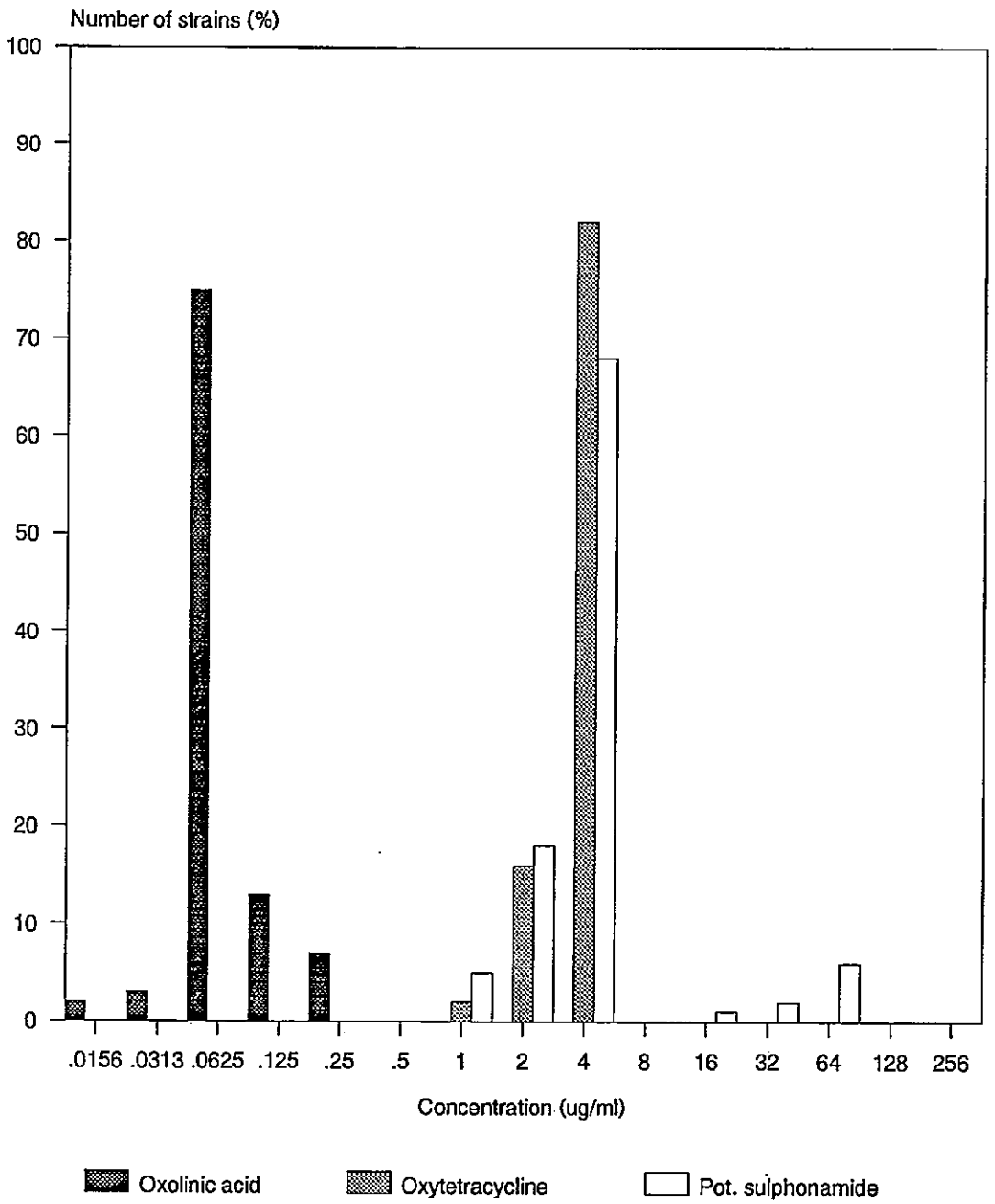


Figure 5.1c Distribution of M.I.C. values-agar incorporation

From the results of the MIC study, using agar incorporation, strains 39/81 and MR21 were found to be most sensitive to all three antimicrobial agents, i.e. oxolinic acid-0.0156 μg ml^{-1}; oxytetracycline-1.0 μg ml^{-1} and potentiated sulphonamide-1.0 μg ml^{-1}. Although there were another four isolates with similar MIC values that were equally as sensitive, only strains 39/81 and MR21 also gave the characteristic reaction on ROD medium. Both isolates were also serotype 01. Strain 39/81 was chosen for further work however because it had been isolated from an outbreak of ERM in rainbow trout, whereas strain MR21 had been isolated from a goldfish showing no clinical signs. Consequently, strain 39/81 was selected as being more representative of the type of situation experienced at the field trials sites that provided much of the background data for chapters 3 and 4.

5.3.3 Action of antimicrobial agents at the MIC

Oxolinic acid was bacteriostatic at the MIC (0.0156 μg ml^{-1}) for strain 39/81 after 8, 32 and 48 h incubation when compared with a control culture (two sample t-test, $p<0.05$) and the initial inoculum (two sample t-test, $p<0.001$). However, incubation periods of 16, 24 and 72 h appeared to be bactericidal (Figure 5.2a) since there was no viable recoverable cell growth. Oxytetracycline was bacteriostatic at the MIC (1 μg ml^{-1}) for the whole incubation period of 8-72 h. It was only at 72 h, however, that there was an increase in cell numbers (Figure 5.2b) but the amount of growth was still significantly less than the control (two sample t-test, $p<0.05$) and slightly more than the original inoculum (two sample t-test, $p<0.01$). The potentiated sulphonamide on the other hand showed a growth pattern that was similar to the control with only a slight reduction in cell numbers for each sampling point between 8 and 72 h (Figure 5.2c). However, growth at 8 and 16 h showed a significant decrease compared to the control (two sample t-test, $p<0.05$) but an increase when compared with the initial inoculum (two sample t-test, $p<0.01$). There was no significant difference between the cell numbers of the potentiated sulphonamide and the control between 24 and 72 h (two sample t-test, $p>0.05$). This was also the case over the same incubation period for the potentiated sulphonamide and the control alone (two

sample t-test, $p>0.05$). Consequently, possible bacteriostasis was only observed within the first 16 h of incubation despite the fact that growth was still greater than the initial inoculum (Figure 5.2c).

Figure 5.2d compares the action of each antimicrobial agent but without the control.

The filtration method, used to remove any residues of the antimicrobial agents from the bacterial cells, was shown to be efficient for TVC's (TVC1 and TVC2) of 6.4 x 10^3 to 6.4 x 10^8 c.f.u. ml^{-1} by linear regression (log TVC2 = -0.22 + 0.5 log TVC1; r^2=100%, $p<0.001$).

5.3.4 Estimation of minimum bactericidal concentration (MBC)

A single strain of *Y. ruckeri* was grown for 48 hours at increasing concentrations of each antimicrobial agent, starting with the relevant MIC. Results confirmed the earlier experiment (see section 5.3.3) that oxolinic acid was bacteriostatic at the MIC (0.0156 μg ml^{-1}) with a 3-fold logarithmic decrease in cell numbers after 48 h, compared with the inoculum (two sample t-test, $p<0.001$). In addition, it appeared to be bactericidal at intermediate concentrations (0.0313-1 μg ml^{-1}) but bacteriostatic again at higher concentrations (2-16 μg ml^{-1}) when compared with the control culture (two sample t-test, $p<0.05$; Figure 5.3a). Oxytetracycline was not only bacteriostatic again at the MIC of 1 μg ml^{-1} (two sample t-test, $p<0.001$) but also at the intermediate concentrations of 2-32 μg ml^{-1} (two sample t-test, $p<0.05$). It was only bactericidal at the higher concentrations (64-256 μg ml^{-1}) (Figure 5.3b). The potentiated sulphonamide reduced growth most between 16-256 μg ml^{-1} (two sample t-test, $p<0.05$) but was not bactericidal at all (Figure 5.3c). However, although growth at the MIC (1 μg ml^{-1}) was significantly higher than the inoculum (two sample t-test, $p<0.01$) it was not significantly different to that of the control after 48 h (two sample t-test, $p>0.05$).

The MBC (99.9% kill of the inoculum) figures for oxolinic acid, oxytetracycline and a potentiated sulphonamide were 0.0156, 64 and >256 μg ml^{-1} respectively. These values gave MBC/MIC90 ratios of 0.06, 8 and >32 respectively, using the more accurate MIC's obtained with the agar dilution method.

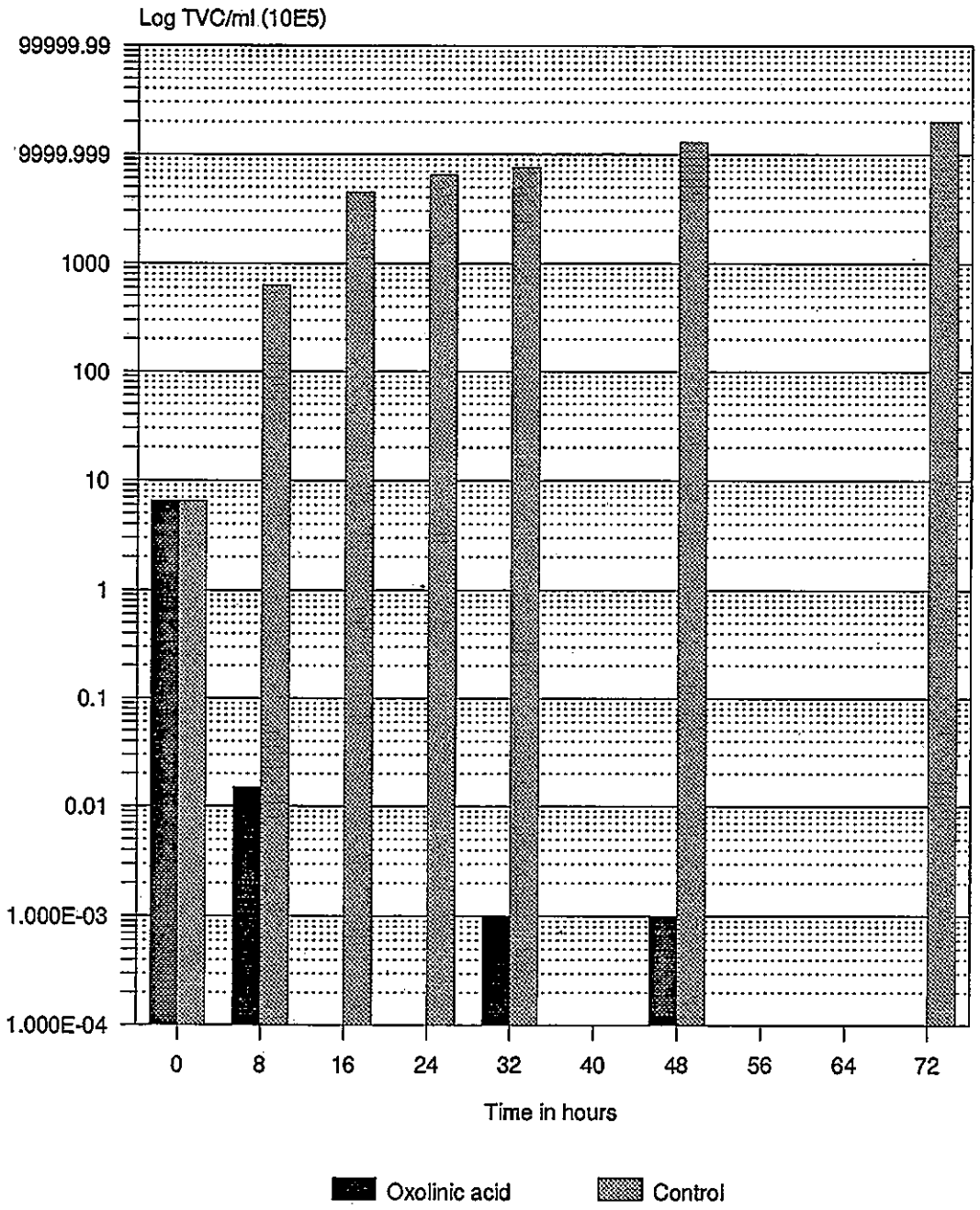


Figure 5.2a Action of oxolinic acid at the M.I.C

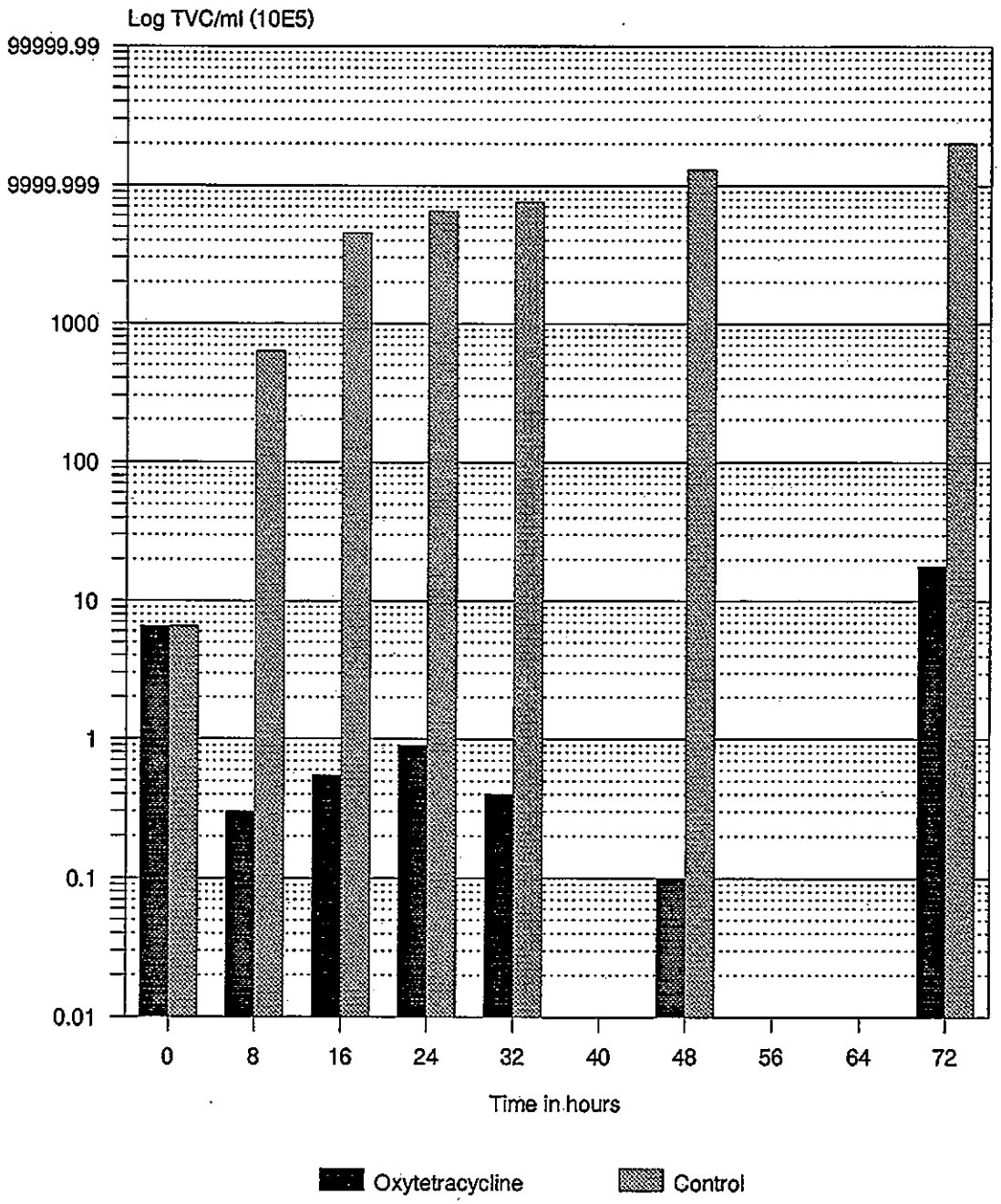


Figure 5.2b Action of oxytetracycline at the M.I.C

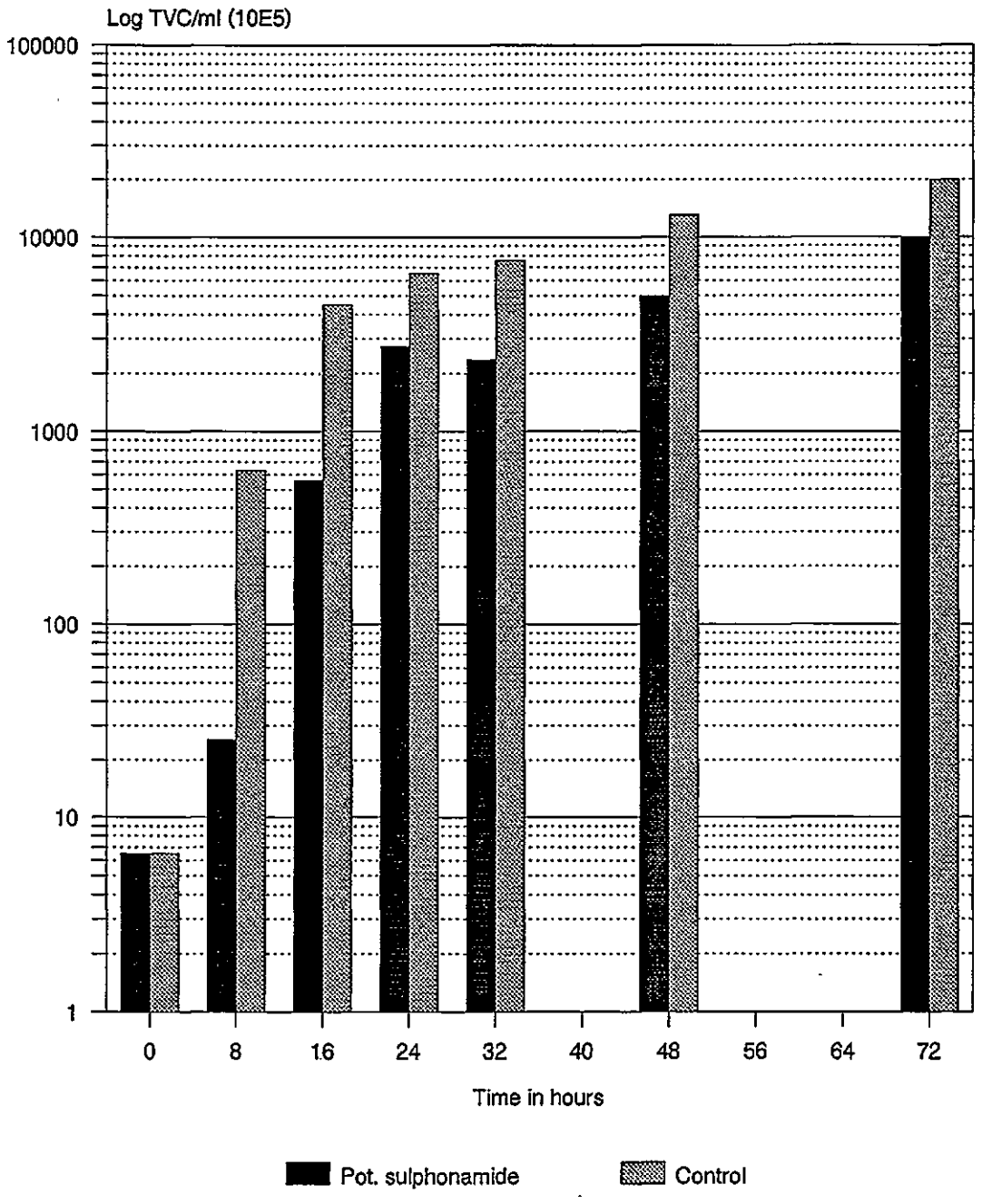


Figure 5.2c Action of a potentiated sulphonamide at the M.I.C

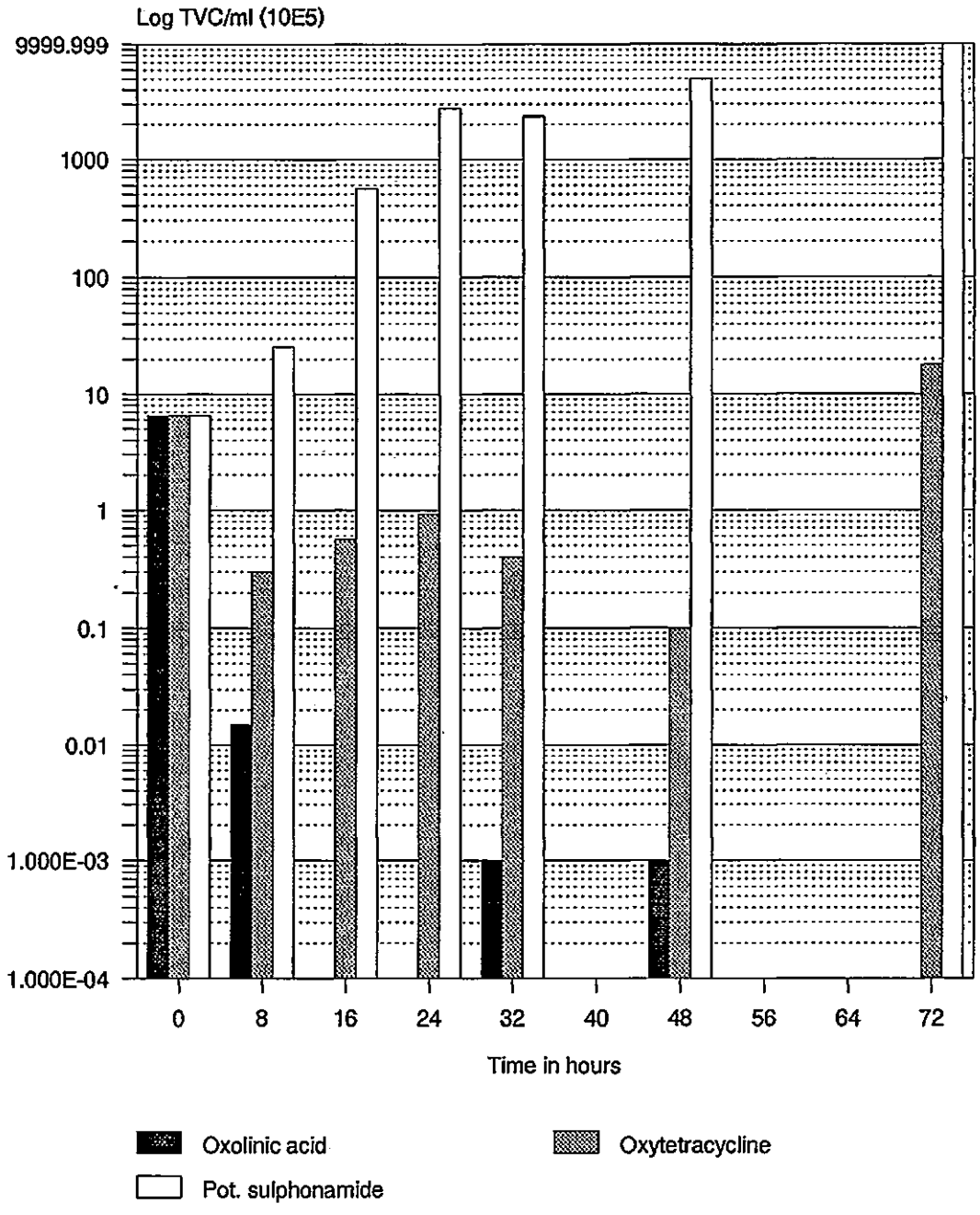


Figure 5.2d Action of the antimicrobial agents at the M.I.C

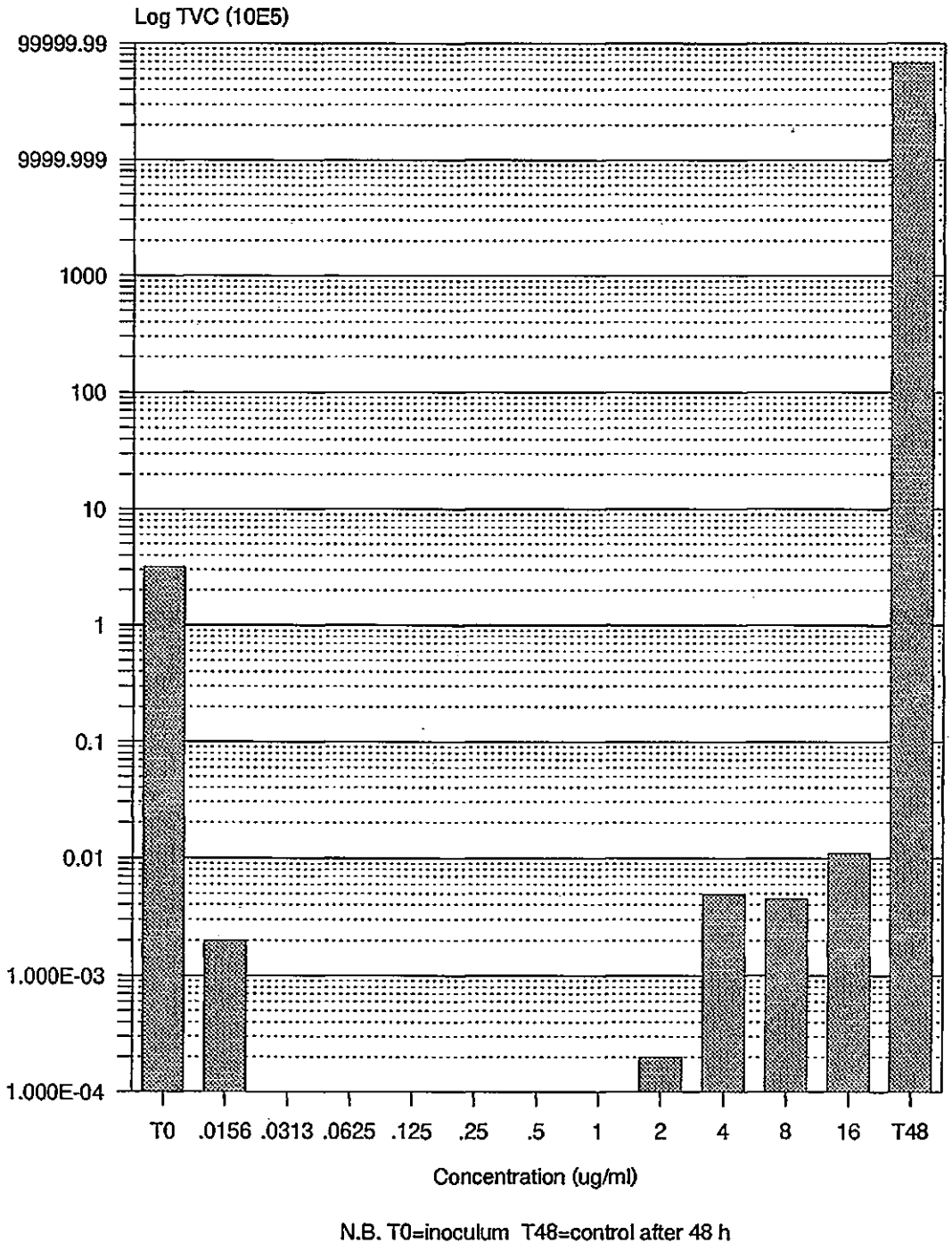


Figure 5.3a **Action of oxolinic acid over a range of concentrations**

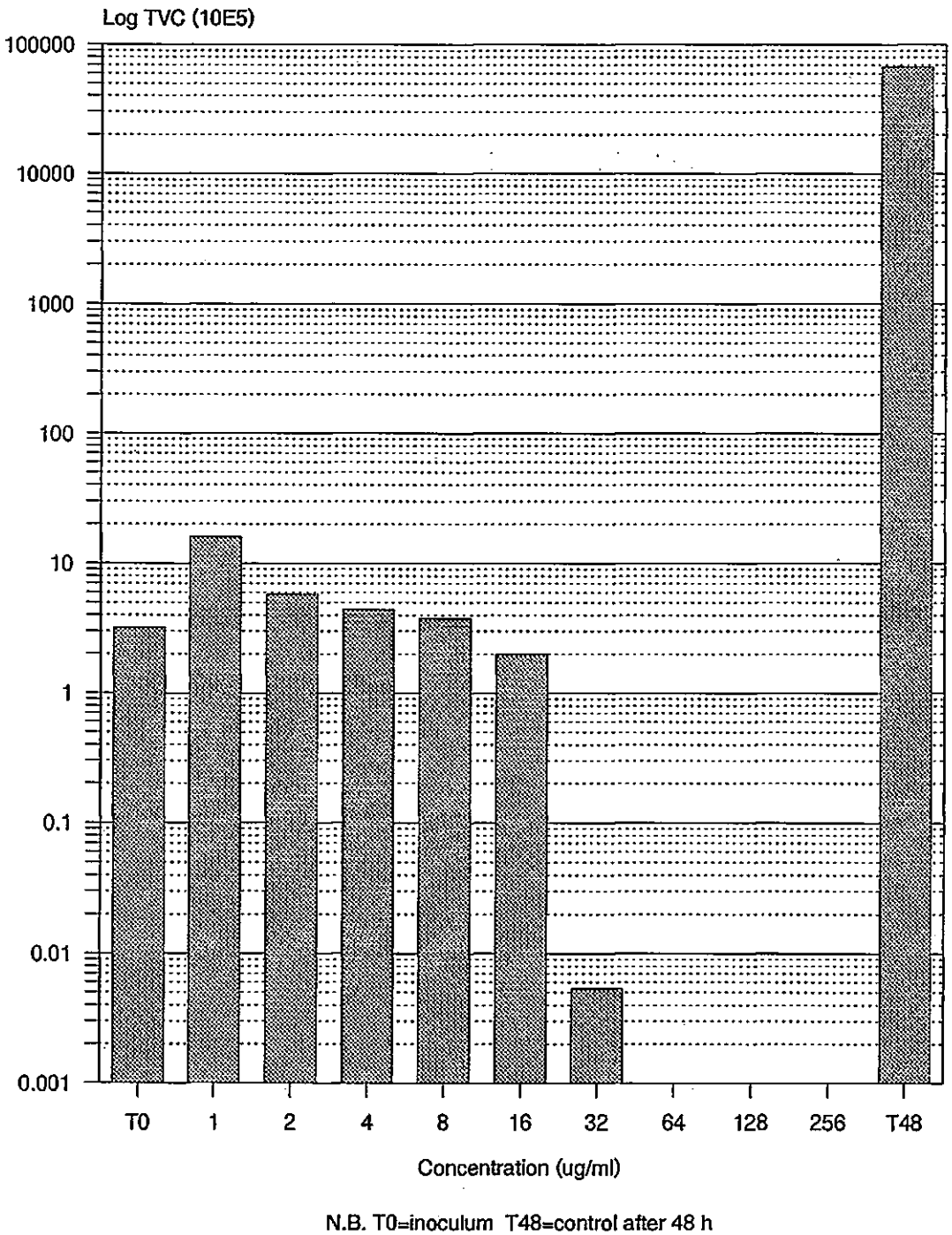


Figure 5.3b Action of oxytetracycline over a range of concentrations

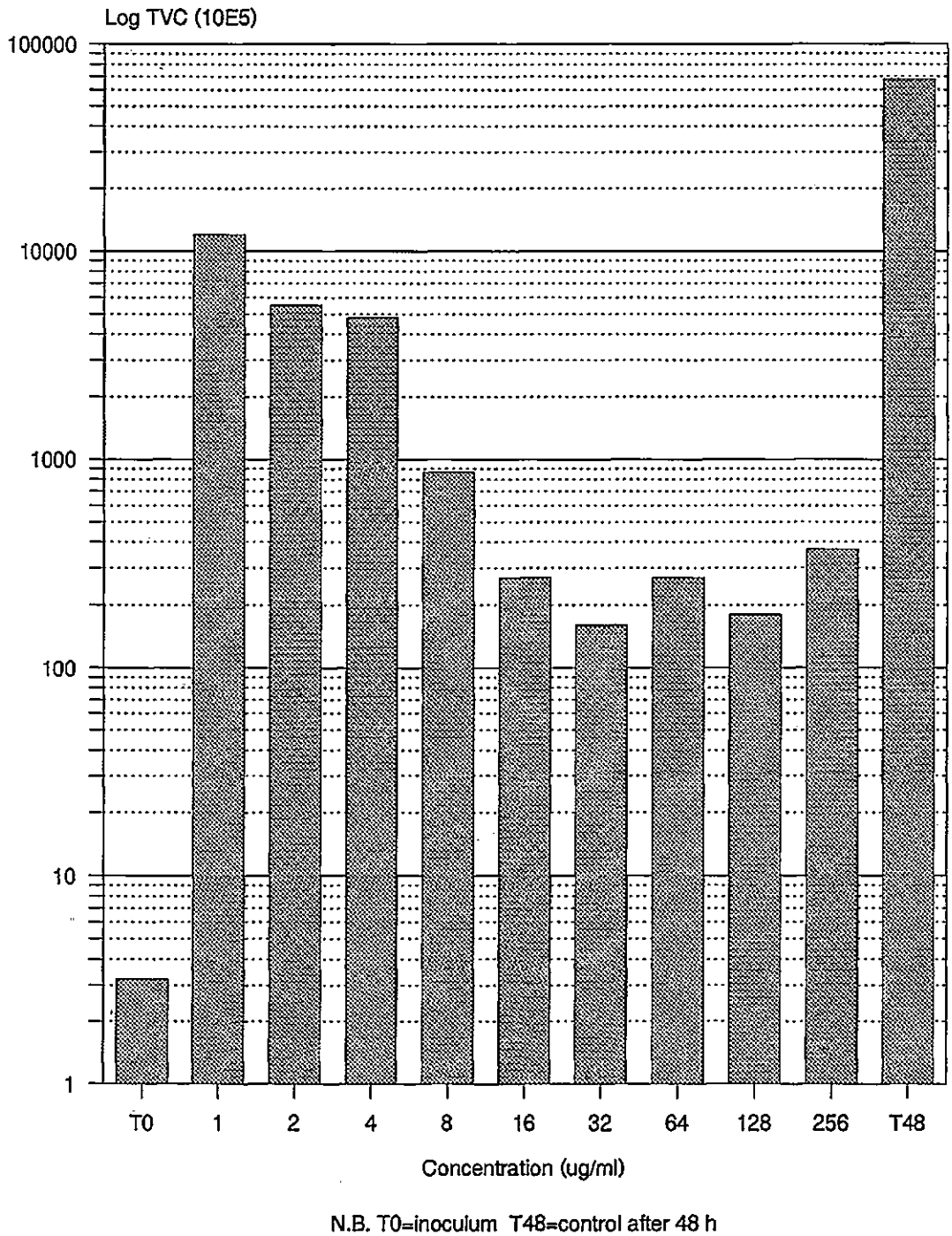


Figure 5.3c Action of a potentiated sulphonamide over a range of concentrations

5.3.5 Increase of *in vitro* resistance

For OA the growth at each step-wise increase in resistance occurred as individual countable colonies varying from three to approximately 500 up to an induced MIC of 0.5 μg ml^{-1}. This represented an overall reduction in growth compared to the inoculum (1 x 10^5 c.f.u. ml^{-1}) of three to five fold at each step. It was also noticed that the colonies were very small, pale and translucent giving the appearence of vague, hazy growth on the plates. Nevertheless this type of colony gave growth at the next highest concentration in 11 out of 15 of the following subcultures. The remaining four subcultures (7, 8, 11 and 12) would only transfer successfully to a higher concentration after confluent growth had been achieved. After 15 subcultures the MIC for OA had increased 20-fold (i.e. 0.0156-16 μg ml^{-1}). This step-wise increase is shown in Figure 5.4.

Unlike OA the step-wise increase in resistance for OT occurred as confluent growth without any noticable decrease in cell numbers compared to the inoculum. However, the growth occasionally appeared pale and translucent also giving a similar appearence to the indvidual colonies seen for OA of vague, hazy growth on the plates. However, this type of confluent growth only gave further confluent growth at the next highest concentration in 8 out of 15 subcultures. The remaining steps to a higher concentration only occurred after one, two or three subcultures at the lower concentration. After 15 subcultures the MIC for OT had increased 16-fold (i.e. 1-256 μg ml^{-1}). This step-wise increase is shown in Figure 5.4.

A similar step-wise increase in resistance occurred with PSu but growth at each step also appeared in a similar way to OA as individual countable colonies up to an induced MIC of 16 μg ml^{-1}. This also represented an overall reduction in growth compared to the inoculum (1 x 10^5 c.f.u. ml^{-1}) of three to five fold. Colony appearance was again noted to be very small, pale and translucent. This type of colony gave growth at the next highest concentration in 8 out of 15 subcultures. The remainder only transferred successfully to a higher concentration after confluent growth had been achieved. After 15 subcultures the MIC for PSu had increased 16-

fold (i.e. 1-256 μg ml^{-1}). This step-wise increase is shown in Figure 5.4.

The number of stepped increases in MIC was two, three and four for OA, OT and PSu respectively.

5.3.6 Decrease of *in vitro* resistance

After 15 subcultures attempts to decrease resistance to oxolinic acid were unsuccessful. However, the MIC for oxytetracycline was reduced 6-fold (i.e. 256-32 μg ml^{-1}) whereas the MIC for a potentiated sulphonamide was reduced only 4-fold (i.e. 256-64 μg ml^{-1}). Both compounds had a two step decrease, as shown in Figure 5.5.

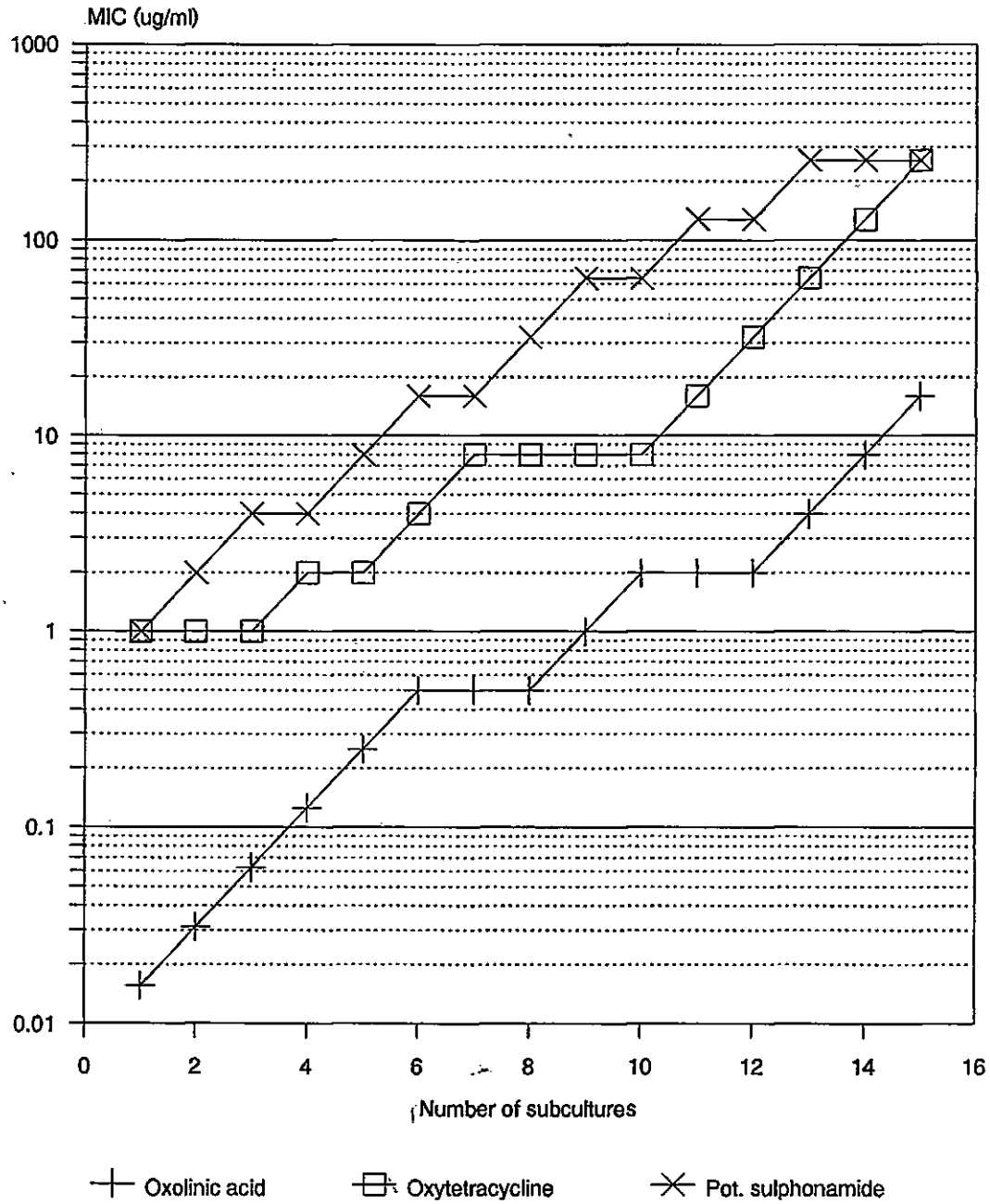


Figure 5.4 Increase of resistance to antimicrobial agents

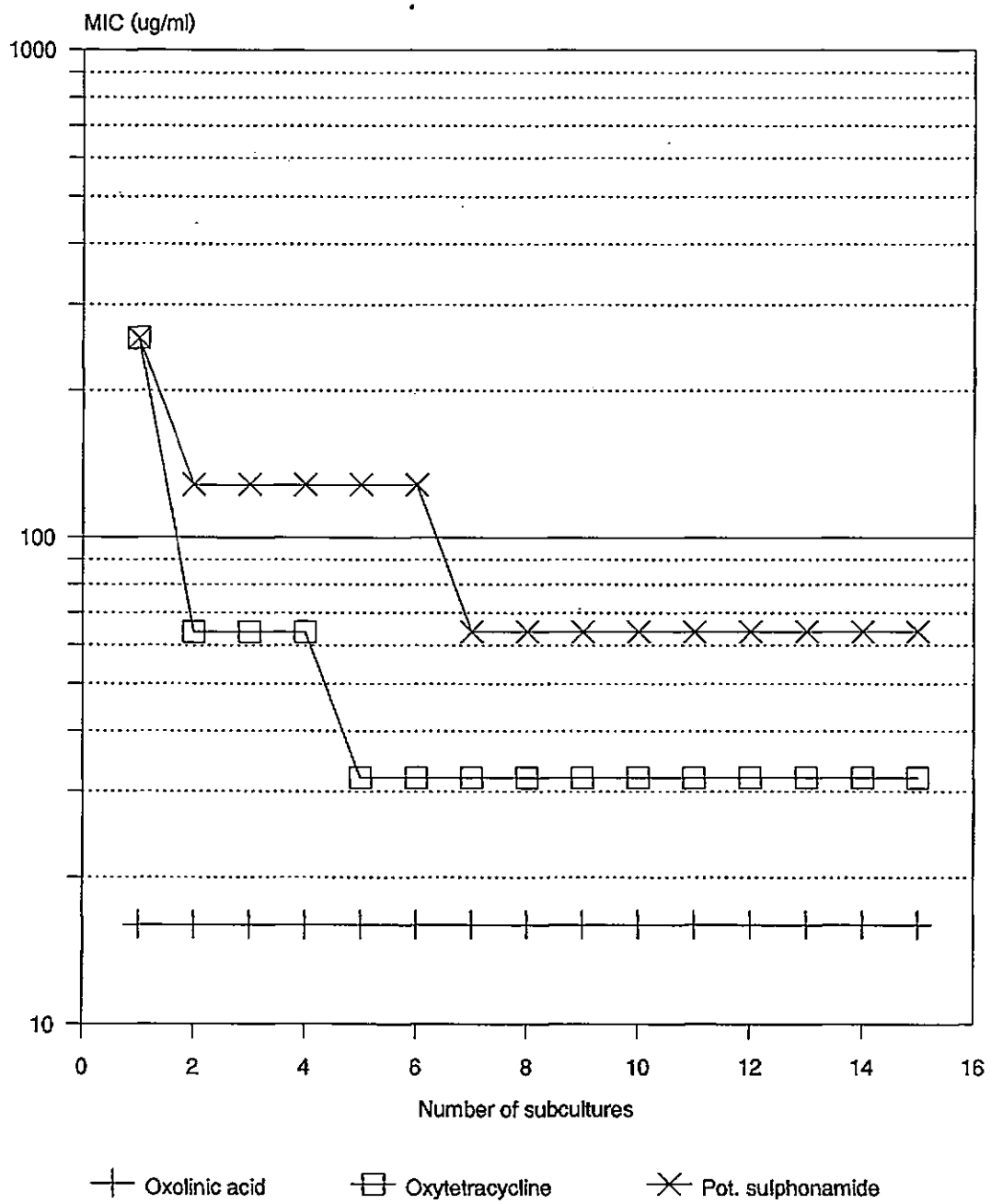


Figure 5.5 Decrease of resistance to antimicrobial agents

5.4 DISCUSSION

Although the API 20E profile numbers changed slightly during the experiments conducted in sections 5.3.3, 5.3.4 and 5.3.5 they were all within the acceptable variation of this test with respect to the citrate, VP and gelatin reactions (Stevenson and Daly, 1982; Dear, 1988; Davies and Frerichs, 1989). Consequently, exposure to the three antimicrobial agents and attempts to induce resistance did not alter the standard biochemical profile of strain 39/81.

The estimation of the minimum inhibitory concentration (MIC) using three methods gave contrasting results. The microtitre plate dilution technique, also used by Tsoumas *et al.* (1989) to determine the MIC's for seventy strains of *Aeromonas salmonicida*, gave a wide range of values when compared with an agar dilution method. However, the measurement of absorbance gave an even wider range, particularly for oxolinic acid, and it was not possible to take readings for the potentiated sulphonamide because of a heavy cell suspension at each concentration. The agar dilution technique was therefore considered to be more accurate since it gave a more sharply defined result that indicated a relatively narrow MIC range for each of the antimicrobial agents. The range for each compound included three concentrations for oxytetracycline, five for oxolinic acid and six for a potentiated sulphonamide.

The measurement of absorbance and microtitre plate dilution techniques relied on growth in broth to determine an end point. However, it proved very difficult to take readings because of the diffuse nature of such growth, particularly with the optical method. This type of problem was pointed out by Thrupp (1986) in a comparison of data from interlaboratory studies. Essentially, an agar dilution method gave a narrower MIC range than a broth dilution method. The agar technique also gave slightly better reproducibility although this tended to vary depending on the drug itself. The mode of action of oxolinic acid is by interference with bacterial DNA gyrase hence preventing the completion of the negative supercoiling of the bacterial chromosome (Alderman, 1988; Smith and Lewin, 1988). One curious feature of the mode of action is that while sensitive cells are dying they also elongate abnormally

(Smith, 1985). Consequently, optical methods can not be relied on to determine the bacterial killing by oxolinic acid (Smith, 1985).

In this current study 100% of the results for oxolinic acid using agar dilution fell within two dilutions of the modal result, compared to 66.1% and 48.8% for the microtitre plate and absorbance methods respectively. Conversely, 100% of the results for oxytetracycline were within ±2 dilutions for both the agar method and the absorbance technique, whilst the microtitre plate gave only slightly less (99.2%). For the potentiated sulphonamide the figures were 92.8% for the agar technique and 57.7% for the microtitre method.

The MIC50, MIC90 and MIC range results for the agar incorporation method were largely comparable with those obtained by De Grandis and Stevenson (1985) for 50 strains of *Y. ruckeri*. The exceptions were a wider but lower MIC range for oxolinic acid in this study, 0.0156-0.25 $\mu g\ ml^{-1}$ as opposed to $\leq 0.5\ \mu g\ ml^{-1}$ and a narrower range for oxytetracycline, 1-4 $\mu g\ ml^{-1}$ as opposed to 1-256 $\mu g\ ml^{-1}$.

The action of oxolinic acid at the MIC after 8, 32 and 48 h incubation appeared to be bacteriostatic whereas incubation periods of 16, 24 and 72 h appeared to be bactericidal. Since the filtration method was shown to be inaccurate for cell counts of 6.4×10^2 c.f.u. ml^{-1} and below, bacteriostasis could have occurred throughout despite the apparent non-recovery of cells. However, the transfer of washed cells to agar plates containing no oxolinic acid and subsequent non-recovery indicates the potential existance of the bactericidal state at these incubation times. This type of drug is known to be bactericidal (Smith, 1985) and it is most likely therefore that the bactericidal state only occurred after 72 h since recoverable cells were obtained at 32 and 48 h. The non-recoverable stages at 16 and 24 h could therefore have resulted from damaged but unculturable bacterial cells arising from the unique mode of action of oxolinic acid.

The action of oxytetracycline at the MIC between 8 and 48 h incubation shows the bacteriostatic nature of this compound. Even the higher growth after 72 h was not significantly different than the initial inoculum. All tetracyclines depend on

accumulation within bacterial cells for their action but once in the cytoplasm they inhibit protein synthesis by the ribosomes (Hammond and Lambert, 1978b). Consequently, since this is an active process, removal of the antimicrobial agent before cell killing has taken place will allow growth to reoccur. Although the tetracyclines have a broad spectrum of antimicrobial activity they are mainly bacteriostatic (Martindale, 1989)

The action of the potentiated sulphonamide at the MIC between 8 and 72 h incubation showed a growth pattern that was similar to the control. Only incubation up to 16 h gave a significant decrease in cell numbers. This early indication of potential bacteriostasis has been mentioned with reference to the duration of treatment of fish (Michel, 1986). The same author indicated that these types of antimicrobial compounds do not exert their full effect unless treatment is prolonged and that there is a high risk of rapid disease recurrence with shorter treatment periods. Potentiated sulphonamides normally act at two points in folic acid synthesis by competing with the natural metabolite *p*-aminobenzoic acid and by sequential blocking of dihydrofolate reductase (Hammond and Lambert, 1978c; Alderman, 1988). As a result they are generally considered to be bacteriostatic (Michel, 1986).

The estimation of a minimum bactericidal concentration (MBC) is a means of determining the level of drug required to achieve irreversible inhibition (i.e. killing) of an inoculum after a defined period of incubation (Thruup, 1986). Most clinical laboratories use a standard endpoint, which represents a 99.9% killing of the original inoculum, to indicate the MBC (Pearson *et al.*, 1980). The results in this study showed that oxolinic acid was bacteriostatic at the MIC, bactericidal at intermediate concentrations but bacteriostatic again at higher concentrations. This gave a MBC figure of 0.0156 $\mu g\ ml^{-1}$ which was the same as the MIC. The quinolones in general are rapidly bactericidal at concentrations 1-4 times the MIC (Norris and Mandell, 1988). Thruup (1986) indicates that antimicrobial agents which are relatively bactericidal in their mode of action will be lethal at a level equal or close to the MIC. This in turn would give an MBC endpoint that was identical with or only slightly

above the MIC. In contrast the reappearance of the bacteriostatic state at the higher concentrations of oxolinic acid is a typical paradoxical (or Eagle) effect. This phenomenon is not well understood but it has been suggested that it may represent differential inhibition of lytic systems (Thruup, 1986) or interference with protein synthesis (Taylor *et al.*, 1983) which become evident only at high drug concentrations. Consequently, the MBC in such cases is usually defined as the point where the first sharp drop to the cutoff occurred (Anhalt *et al.*, 1980; Thruup, 1986) as indicated by Figure 5.3a. Oxytetracycline was bacteriostatic at the MIC and at intermediate concentrations with only the higher concentrations proving bactericidal. This gave a MBC of 64 $\mu g\ ml^{-1}$ which was six doubling dilutions higher than the MIC. The antimicrobials which are relatively bacteriostatic in their mode of action show MBC endpoints many dilutions higher than the MIC (Thruup, 1986). Although the potentiated sulphonamide reduced growth it was not bactericidal. It was therefore not possible to achieve a MBC.

The MBC/MIC ratio provides a useful guide to the potential tolerance of strains of bacteria. Such organisms which are inhibited but not killed by an antimicrobial agent can be regarded as tolerant (Thruup, 1986). It has been suggested that MBC endpoints five or more two-fold dilutions greater than the MIC, as occurred in this study with oxytetracycline and a potentiated sulphonamide, indicates tolerance (Anhalt *et al.*, 1980; Sabath *et al.*, 1977).

The attempts to induce *in vitro* resistance in *Y. ruckeri* indicated step-wise increases for all three antimicrobial agents. After 15 subcultures the MIC had increased 20, 16, 16-fold for oxolinic acid, oxytetracycline and a potentiated sulphonamide respectively. This type of development has also been shown in *Y. ruckeri* by Stamm (1989) with reported multiple increases in MIC of 16, 16 and 2-4 for the same three antimicrobials. However, the actual increases in MIC in this current study were much larger at 1025, 256 and 256 respectively.

There are two ways in which drug resistance can occur in a particular pathogen: intrinsic and acquired. The term intrinsic resistance is used to imply that

inherent features of the cell, such as the presence of an outer membrane in Gram-negative bacteria, are responsible for preventing antibiotic action (Hammond and Lambert, 1978d; Chopra, 1988). Acquired resistance on the other hand occurs when resistant strains emerge from previously sensitive bacterial populations. This type of resistance can be a result of mutation or as a result of gene transfer from another bacterium (Maas, 1986; Chopra, 1988). The mutational or acquired form of resistance can occur rapidly in a single step or more gradually in a step-wise manner (Martindale, 1989). Antibiotics act as screening agents preventing the growth of sensitive cells and allowing the outgrowth of such mutants (Hammond and Lambert, 1978d). However it is important to understand that antimicrobial agents do not induce resistance, but that mutations appear spontaneously as a result of damage to DNA and the antibiotics simply provide the selection pressure for such drug-resistant mutants (Wray, 1986). Single mutants can confer a high degree of resistance and increases of up to a thousand-fold over the parent strain have been reported after a single exposure to streptomycin or erythromycin (Hammond and Lambert, 1978d). However, the same authors point out that more commonly high levels of resistance develop as the consequence of a series of small two to five-fold increments. It has been shown that mutations occur spontaneously and in a random manner with or without the presence of an antimicrobial agent (Maas, 1986). An antibiotic exerts its effect by acting on a specific target in the bacterial cell. Mutants prevent the lethal effect of the antibiotic by either altering the target (e.g an enzyme or ribosomal site) or preventing the target being reached (e.g. inhibition or inactivation) (Maas, 1986).

The resistance to quinolones appears to result from a reduced affinity to a DNA gyrase subunit or alteration in cell wall permeability resulting from a loss of outer membrane proteins (Olsson-Lilejequist *et al.*, 1985; cited by Norris and Mandell, 1988) and not from plasmids (Smith and Lewin, 1988). Rapid resistance to ofloxacin, also a quinolone, has been reported to occur in clinical situations with another member of the Enterobacteriaceae, *Citrobacter freundii* (Cheng *et al.*, 1987). A worrying finding by some studies is that mutational resistance to any 4-quinolone also confers at

least some resistance to all other 4-quinolones (Smith and Lewin, 1988). However, the treatment of goldfish with oxolinic acid (20 mg kg^{-1} fish for 7 days) has been reported not to affect the resistance of intestinal microflora, including *Aeromonas hydrophila* and unspecified members of the Enterobacteriaceae (Sugita *et al.*, 1989). Barry and Jones (1984) reported a gradual stepwise increase in MIC's for Gram-negative bacilli against seven quinolones, including oxolinic acid, and at the same time demonstrated cross-resistance between the drugs. Tenney *et al.* (1983) documented the rapid stepwise emergence of resistance of *E. coli* to three quinolones by serial transfer on agar plates containing subinhibitory concentrations of each drug.

Resistance to the tetracyclines is often inducible and appears to be associated with the ability to prevent accumulation of the antibiotic within the bacterial cell (Martindale, 1989). The appearance of resistance in many different bacterial species has limited the selection of the tetracyclines as the drug of choice in the treatment of many clinical infections for which they were previously effective (Levy, 1984). In fact, tetracycline resistance is now the most common resistance determinant among all bacterial species and is the result of selection of resistant strains by the widespread use of these drugs throughout the world (Levy, 1984). Needless to say resistance to the tetracyclines is found in the majority of the Enterobacteriaceae (Chopra, 1985). The active uptake of tetracycline during therapy is necessary in order to reach the inhibitory site. This has been shown in *E. coli* to be a combination of rapid energy-independent transport and a slow energy-dependent uptake (McMurry and Levy, 1978). Since the ribosome is the target of tetracycline activity resistance should be an alteration in the ribosomal binding site. However, some reports indicate that resistant cells actively prevent entry of tetracycline or actually efflux the drug (Levy and McMurry, 1978; Ball *et al.*, 1980; McMurry *et al.*, 1980). Nevertheless, the resistance of intestinal microflora of goldfish has not been reported to be significantly influenced by the oral administration of oxytetracycline (50 mg kg^{-1} fish for 7 days) (Fukumoto *et al.*, 1987; cited by Sugita *et al.*, 1989). However, in the USA some outbreaks of ERM have been unresponsive to treatment with oxytetracycline and this

has been attributed to antibiotic resistant strains of *Y. ruckeri* (Post, 1987) In addition, De Grandis and Stevenson (1985) demonstrated that two out of 50 isolates of *Y. ruckeri* were highly resistant to tetracyclines but this was due to a transferable 36-megadalton plasmid. Several natural isolates of *Aeromonas salmonicida* have also been shown to have significantly reduced susceptibilities to oxytetracycline following the use of the antibiotic to treat furunculosis outbreaks on fish farm facilities (Wood *et al.*, 1986).

Resistance to potentiated sulphonamides (sulphonamide and trimethoprim) involves a by-pass of the inhibition of the two enzymes involved in folate metabolism by duplication of each respective target enzyme (Chopra, 1988). In addition, another mechanism of resistance to the sulphonamide alone is hyperproduction of *p*-aminobenzoate which will overcome the metabolic block imposed by the inhibition of the first enzyme (dihydropteroate synthetase) necessary in folic acid synthesis (Hamilton-Miller, 1984). This type of sulphonamide resistance can be the result of a single point mutation or the presence of a plasmid which causes synthesis of a new enzyme (Neu, 1986). Similar resistance problems to those reported for oxytetracycline in the USA and Canada have also been noted for potentiated sulphonamide (De Grandis and Stevenson, 1985; Post, 1987) or sulphonamide therapy (Wood *et al.*, 1986) in some outbreaks of ERM. In addition, some epizootics have not responded to the combined treatment of oxytetracycline and sulphamerazine (Post, 1987).

The increase in drug resistance as a result of induction of a drug-inactivating enzyme is a transitory phenomenon and the level of resistance will decrease when the bacteria are returned to growth in the absence of the drug (Maas, 1986), such as happened with oxytetracycline and a potentiated sulphonamide in this study. The increase in drug resistance as a result of selective growth of resistant mutants is a permanent phenomenon, such as occurred with oxolinic acid. Such mutants will persist during subsequent growth in the absence of the drug, unless they are at a selective disadvantage under such conditions (Maas, 1986).

The potential appearance of naturally resistant strains is obviously dependant on the achievable serum concentrations in fish during therapy. A mean level of 0.9 $\mu g\ ml^{-1}$ for oxolinic acid has been reported in Atlantic salmon fed 10 mg kg^{-1} body weight per day (O'Grady *et al.*, 1986). Although the MIC values for all the strains tested in this study fell within 0.0156-0.25 $\mu g\ ml^{-1}$ the paradoxical effect seen at concentrations higher than the MIC and the potential for increased resistance indicate that careful administration of such drugs is vital. A plasma tetracycline level of 1.9 $\mu g\ ml^{-1}$ has been reported in channel catfish 4 h after fish received 80 mg kg^{-1} orally in the feed (Plakas *et al.*, 1988). The MIC levels for strains of *Y. ruckeri* against oxytetracycline in this study were 1-4 $\mu g\ ml^{-1}$ which clearly indicates that the development and survival of resistant mutants is possible after using this type of bacteriostatic antibiotic. Any misuse of antibiotics therefore may lead to the emergence of selected resistant strains which are not inhibited by the drug of choice.

The experiments detailed in this study have clearly shown the potential for decreased susceptibility of *Y. ruckeri* to oxolinic acid, oxytetracycline and a potentiated sulphonamide under *in vitro* conditions. It is important therefore that the emergence of antimicrobial resistance among bacterial fish pathogens, such as *Y. ruckeri,* is minimized by continual monitoring, careful drug use, optimisation of treatment regimes and the cycled use of the available chemotherapeutants.

CHAPTER 6

LOCALISATION OF *YERSINIA RUCKERI* ANTIGENS

6.1 INTRODUCTION

All multicellular animals are equipped with defensive mechanisms which afford protection against invasion of the body by microorganisms (Inchley, 1981). At its simplest this defence is almost solely dependent on phagocytic cells which are designed to engulf and digest foreign organisms (Inchley, 1981). In vertebrates, however, the immune response to an antigen may be either humoral or cell mediated (Ingram and Alexander, 1981a). Fish have non-specific, natural, humoral and cell mediated immune mechanisms similar to, but not identical with, those of higher vertebrates in order to protect themselves against invasive disease (Ingram and Alexander, 1981b; Anderson and Siwicki, 1989). The main types of cell involved in the immune response of fish are the phagocytic macrophages and the lymphocytes which are involved in antibody synthesis (Dorson, 1984). The principal lymphoid organs of fish are the anterior kidney, spleen, thymus, blood and the lymphoid tissue of the digestive tract (Weinberg, 1975; Davina *et al.*, 1980; Dorson, 1984; Doggett and Harris, 1987).

Corbel (1975) and Dorson (1981) have already reviewed the immune response of fish. There are indications that although antibodies can develop against either soluble or particulate antigens (Busch, 1978) protection can result from cell-mediated immunity (Cossarini-Dunier, 1986b). Results from the earlier epidemiological component in this study showed sufficient differences between vaccinated and non-vaccinated fish to warrant a further investigation of the intestinal mucosal immune response. Consequently, the localisation of two concentrations of whole cell antigens from viable *Y. ruckeri* after artificial anal infection were followed over a ten day period in vaccinated and non-vaccinated fish.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals, reagents and media

The origins of all chemicals, reagents and media are detailed in section 3.2.1.

6.2.2 Bacteriology

6.2.2.1 Bacterial strains

The strain of *Y. ruckeri* used in the localization work was isolated originally as LT12/6 from a rainbow trout kidney at Farm A during the field trials described in chapter 3 (section 3.3.7). Subsequently, the strain was used in a laboratory challenge experiment and was passaged 5 times through trout in order to maintain virulence. The passaged strain (LT12/6P) was kindly donated by Dr. M. D. Furones.

6.2.2.2 Bacterial cultivation and storage

See section 3.2.2.2.

6.2.3 Biochemical characterization of strains

Strain LT12/6P was confirmed as *Y. ruckeri* during the infection experiments by using the API20E (API-bioMérieux UK Ltd.) system of biochemical testing following bacteriological sampling from fish kidney and faeces. The strips were inoculated according to the manufacturers instructions but were incubated at 26°C and the results were recorded at 48 h.

6.2.4 Experimental animals

6.2.4.1 Fish

Unvaccinated rainbow trout weighing 25-30 g were obtained from Upwey Trout Farm (Weymouth, Dorset) which is a known ERM-free hatchery site. They were held in circular, glass-fibre tanks (720 l) at 13°C until required for experimental work and were supplied with flow-through dechlorinated mains water. The fish were fed twice a day on a maintenance diet with commercially available pellets supplied by EWOS (Bathgate, UK). For experimental work the fish were transferred to smaller rectangular, glass-fibre tanks (30 l) 2 to 3 days before required. The water supply and temperature were identical to the larger tanks. Although the feeding regime was similar to the holding group of fish, some experimental groups were not fed for 2 days

before sampling, particularly where intestinal mucus samples were required. Before each experiment the main stock of fish was sampled to ensure that they were not carriers of ERM as described in section 6.2.5.6.

6.2.5 Infection experiments

6.2.5.1 Preparation of bacteria for infection

Strain LT12/6P was grown on TSA for 48 h (26°C) and individual colonies were used to inoculate 100 ml conical flasks of TSB. Following further overnight incubation (26°C), the broths were centrifuged (3000 rpm; 4°C) for 30 min and the precipitate was resuspended in 20 ml of 0.9% saline in order to wash the cells. This was repeated and the cells were then resuspended to an approximate A_{625} of 0.5. The approximate viable count (TVC) of this suspension was then estimated from a calibration graph using the formula log TVC=8.1787+1.267xA_{625}. The graph had been previously obtained by using a series of different concentrations of a similar suspension. A TVC estimation was also performed (see section 3.2.2.3) in order to determine the exact viable count.

6.2.5.2 Anaesthesia

Where required, fish were anaesthetized with MS222 (Sandoz, Switzerland) which had been diluted 1:10000 (w/v) with dechlorinated mains water. This dilution was designed to anaesthetize fish within 30 sec to 1 min.

6.2.5.3 LD_{50} estimation

Dilutions of the bacterial suspension were prepared for use in the infection experiments to cover the range 3.3×10^2 to 3.3×10^7 c.f.u. ml^{-1}. The susceptibility of the fish stock to infection with strain LT12/6P was assessed by LD_{50} estimation in one of two ways, for comparative purposes:

a) Intraperitoneal (ip) injection

Duplicate groups of 5 rainbow trout were anaesthetized and injected ip with 0.1 ml of one of the prepared dilutions. Control groups were injected ip with 0.1 ml of phosphate buffered saline (PBSa). Any subsequent mortalities were noted daily for up to 14 days with kidney and faecal samples being taken from dead fish to

confirm that *Y. ruckeri* was the cause of death. Kidney material was plated onto TSA and faecal material onto ROD medium and incubated as previously described (section 3.2.10.2). Any disease signs were also recorded.

b) Anal intubation

Duplicate groups of 5 rainbow trout were anaesthetized (section 6.2.5.2) and intubated anally with 0.1 ml of one of the prepared dilutions. Control groups were intubated with 0.1 ml of PBSa. A silicone rubber tube (1 mm diameter; BDH Ltd., Eastleigh, U.K.) attached to a 2 ml plastic syringe (Becton and Dickinson, Plymouth, UK) was inserted 2 cm into the lower intestine via the vent and slowly withdrawn after injection. Subsequent mortalities were treated in the same way as in section 6.2.5.3a.

6.2.5.4 Vaccination

Two duplicate groups of 50 rainbow trout were vaccinated with a commercially available anti-*Y. ruckeri* vaccine which was kindly donated by Dr. P. Smith, AVL Ltd (Saffron Walden, U.K.). Vaccination was carried out according to the manufacturer's instructions (1:10 dilution, 30 sec immersion). Following immersion the fish were divided into groups of 25 and returned to the experimental 30 l tanks. Control groups were immersed in dechlorinated mains water for 30 sec.

6.2.5.5 Laboratory infection

Following the LD_{50} experiment (section 6.2.5.3) groups of 50 vaccinated and non-vaccinated fish were infected with two separate concentrations of *Y. ruckeri* cells by anal intubation. The actual doses were chosen to give artificial challenges less than the LD_{50}. These concentrations enabled fish to exhibit disease signs whilst inducing the fewest mortalities, particularly in the non-vaccinated, unprotected fish. The two infective doses were 2 $\log_{10}$ dilutions different and were designated high (H) and low (L) challenges. Consequently, the following groups were used in the experiment:

a) Non-vaccinated and not infected (NVNI-pre and post infection control)

b) Non-vaccinated and infected with high and low doses (NVH; NVL)

c) Vaccinated and not infected (V-pre infection control; VNI-post infection control)

d) Vaccinated and infected with high and low doses (VH; VL)

All infected groups were intubated 14 days after vaccination.

6.2.5.6 Bacteriological sampling

Five fish from each designated group (see section 6.2.5.5) were sampled after 1, 8, 24, 48 h and 4, 7, and 10 days. Kidney and faecal material were plated onto TSA and ROD medium respectively. Potential growth of *Y. ruckeri* was recorded after incubation (26°C) for 72 h (TSA) and 10 days (ROD). Suspected colonies were presumptively identified by slide agglutination (see section 3.2.10.2) and characterized by API 20E (see section 3.2.3). The growth of *Y. ruckeri* from either medium was noted as either pure growth or countable colonies of less than 300.

6.2.5.7 Serum samples

Five fish from each designated group (see section 6.2.5.5) were anaesthetized (section 6.2.5.2) and blood obtained by severing the caudal peduncle. Samples from individual fish were collected in 1 ml microcentrifuge tubes (BDH Ltd.) and allowed to clot at room temperature for 1 h. The clot was then allowed to retract overnight at 4°C and the serum was separated by centrifugation for 25 min (3000 rpm, 4°C). All samples were then stored at -20°C until required.

6.2.5.8 Intestinal mucus collection and storage

Following bacteriological sampling of faecal material (section 6.2.5.6) a ventral incision was made in each of the 5 fish from the designated groups. The intestine (upper and lower) was then cut transversly at a point 2 cm from the anus. This length of intestine was then carefully teased away from any fatty tissue but was left attached at the vent end. A 1 ml Gilson pipette (Anachem Ltd., Luton, U.K.) containing 0.5 ml PBSa was then inserted into the vent and the intestinal contents were washed into a 1 ml microcentrifuge tube (BDH Ltd.) held in iced water (4°C). The washing was made homogeneous by repeated use of the pipette in the tube. Each sample was then centrifuged (2500 rpm, 4°C) for 5 min and the supernatant

removed to a second 1 ml tube. Ten microlitres of phenylmethylsulphonylfluoride (PMSF; Sigma) was then added, followed by 10 µl 0.5% (w/v) bovine serum albumin (BSA; Sigma) and incubation at room temperature for 20 min. Finally, 10 µl 1% (w/v) sodium azide (Sigma) was added and all intestinal samples were freeze-dried (Edwards Modulyo Freeze Dryer) and reconstituted to 250 µl with PBSa containing 0.5% v/v tween 20 (PBST). The samples were then stored at -20°C until required.

6.2.6 Histology

Following the collection of intestinal contents the 2 cm lengths of intestine were cut into equal 1 cm portions and designated hind (nearest the vent) and fore (nearest the stomach). These portions were further sub-divided into 1-2 mm sections and placed in 4% (w/v) paraformaldehyde (BDH) buffered to pH 7.6 for 24 h. In addition, small portions (approximately 0.5 cm in length) of kidney, spleen and liver were similarly treated. These fixed tissues were processed to paraffin wax blocks on an automatic vacuum infiltration processor (Tissue-Tek VIP, Miles Scientific, Stoke Poges, U.K.) and dispensing consoles (Tissue-Tek). Serial 4-5 µm sections were cut on a rotary microtome (Leitz 1512) and the sections were floated on warm distilled water (45°C) containing 0.05% egg albumin. The sections were then taken onto clean microscope slides, dried overnight at 45°C, and stained as required.

6.2.6.1 Differential staining

The following staining techniques were used:

a) Haematoxylin and eosin (H and E)-used for general morphological and histopathological studies.

b) Gram-Twort-used for the localization of bacterial cells.

c) Alcian blue/periodic acid Schiff (AB/PAS)-used for mucopolysaccharide determination and as a counterstain at the end of the immunoperoxidase technique.

d) Carazzi's haematoxylin-used as a light counterstain in conjunction with AB/PAS at the end of the immunoperoxidase technique.

6.2.7 Photomicroscopy

Stained material was examined under a Reichart-Jung Polyvar microscope and photographs were taken with an automatic photomicrographic camera. Colour prints were commercially developed from Kodacolor Gold 200 film corrected for tungsten light with a blue filter.

6.2.8 Immunological procedures

6.2.8.1 Production of anti-*Y. ruckeri* rabbit sera

Serum used for the immunoperoxidase technique was prepared from strain LT12/6P initially grown on TSA (26°C, 48 h). A suspension (approximately 1 x 10^9 c.f.u. ml^{-1}) was then prepared from a TSB culture (section 6.2.5.1). One ml of this suspension was then thoroughly mixed with an equal volume of Freund's complete adjuvant (FCA) and used to inject (2 x 0.5 ml) intramuscularly the flanks of a New Zealand white rabbit. Another four injections were then administered weekly by using subcutaneous injection and a similar concentration of bacterial cells but mixed in Freund's incomplete adjuvant (FIA). A final booster injection (0.1 ml) was made into the marginal ear vein using a suspension of 1 x 10^7 c.f.u. ml^{-1} in PBSa. After a further 5 days the rabbit was bled from the marginal ear vein and the blood was then allowed to clot at 37°C for 1 h. The blood samples were then treated as in section 6.2.5.7. Additional samples of serum prepared in this way were kindly donated by Dr. A. Wrathmell (Polytechnic South West, Plymouth).

6.2.8.2 Production of anti-*Y. ruckeri* trout sera

Samples of anti-*Y. ruckeri* trout sera were kindly donated by Dr. A. Wrathmell (Polytechnic South West, Plymouth). Essentially, 0.1 ml suspensions of strain LT12/6P (see section 6.2.5.1) were injected ip into rainbow trout. After 4 weeks the fish were bled from the caudal vein and the blood samples were then treated as in section 6.2.5.7.

6.2.8.3 Titration of antibody

A standard doubling dilution titration using U-well microtitre plates (96 well; Sterilin, Teddington, UK) was used for both fish and rabbit serum samples and fish

intestinal mucus samples. Each well was filled with 50 µl of PBSa followed by 50 µl of neat antiserum to the first well only. Serial dilutions were then made in all the remaining wells except for the final one which served as a negative control and only contained PBSa. A suspension of *Y. ruckeri* strain LT12/6P was then prepared (5 x 10^7 cells ml^{-1}; see section 6.2.5.1) and 50 µl added to all wells. Each microtitre plate was incubated at 37°C for 4 h and then transferred to 4°C overnight. The last well showing a diffuse deposit of cells was noted as the end point titration and was recorded as the reciprocal of the highest doubling dilution of serum. Wells showing a 'button' deposit were recorded as negative.

6.2.8.4 Enzyme-linked immunosorbent assay (ELISA)

The ELISA method was developed by R. Barton (Polytechnic South West, Plymouth) for use in another project concerning *Y. ruckeri*. Essentially, the wells of flat-bottomed plates (Falcon Pro-Bind; Becton and Dickinson) were coated with sonicated whole cells of strain LT12/6P (50 µl; 1 µg ml^{-1}) in coating buffer (carbonate/bicarbonate, pH 9.7). Plates were either incubated for 3 h at room temperature or overnight at 4°C in a damp chamber and then washed (3 x 3 min) with PBST. Serum or mucus samples, diluted with PBST where necessary, were added to the wells (50 µl) and the plates incubated for 2 h at room temperature. The plates were then washed as before. Rabbit anti-trout immunoglobulin, diluted 1:2000 with PBST, was added (50 µl/well) and the plates were incubated for 1 h at room temperature. This was followed by further washing (i.e. 3 x 3 min with PBST). Swine anti-rabbit peroxidase conjugate (Dako Ltd., High Wycombe, U.K.), diluted 1:1000 with PBST, was then added to each well (50 µl) and the plates were incubated for 1 h at 37°C. After incubation the plates were washed again (3 x 3 min with PBST). Fresh substrate solution (20 mg o-phenylenediamine, OPD, Sigma; + 20 µl hydrogen peroxide) was prepared in citrate/phosphate buffer (50 ml, pH 5.0) and added (50 µl) to all wells and left for approximately 10 min. The reaction was stopped by adding 50 µl of 1M sulphuric acid. The results were read immediately on a Multiskan reader (Labsystems, Basingstoke, UK) at 492 nm (A_{492}). Known

positive and negative serum samples, diluted 1:2000 to 1:64000 in PBST, were used throughout as controls. In addition, the outer wells of plates were not coated in order to prevent temperature effects and duplicate samples were tested in different well positions in different plates. Some randomly selected coated wells only received buffer, not serum samples, in order to determine whether there was any background absorbance problems.

Test results were interpreted by dividing the A_{492} value of a mucus or serum sample by the A_{492} value of the negative control. This figure was referred to as the positive-negative ratio (pnr).

6.2.8.5 Immunoperoxidase technique

Sections of fixed tissue, prepared as in section 6.2.6, were dewaxed and rehydrated following routine histological procedures. Before the immunoperoxidase staining technique each section was placed in Tris buffered saline (8 g sodium chloride, 0.605 g Tris, 1 l distilled water, pH 7.6) for 5 min, rinsed with distilled water and treated with 3% hydrogen peroxide (5 min, room temperature). This step was included in order to quench any endogenous peroxidase activity present in the tissues.

Following quenching, the tissues were washed (3 min running tap water followed by 2 min distilled water and 5 min TBS) and incubated for 20 min with normal swine serum (Dako Ltd., High Wycombe, UK) diluted 1:5 in TBS in order to block any non-specific background. The serum was then rinsed briefly in TBS and any residual was tapped off onto absorbant tissue. Each section was then incubated (primary incubation) with rabbit anti-*Y. ruckeri* serum diluted optimally in TBS and then washed as above. Secondary incubation with swine anti-rabbit immunoglobulins (Dako Ltd.) diluted in TBS was then undertaken and was followed by further washing. A final tertiary incubation was then performed with rabbit peroxidase-anti-peroxidase (PAP; Dako Ltd.) also diluted in TBS and the sections were washed again. At this point the tissues were treated with freshly prepared chromogenic reagent 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma; 0.07%

w/v + 0.03% v/v hydrogen peroxide in TBS) and incubated for 1.5-2 min. The sections were then immediately washed in running tap water for 5 min and rinsed in distilled water for 5 min. Counterstaining was then performed with alcian blue/periodic acid Schiff (AB/PAS) and Carazzi's haematoxylin following routine histological procedures.

All incubation steps were performed at room temperature.

Particular attention was paid to the provision of control procedures and the following were used, where appropriate, on known positive and negative tissue sections:

a) Omission of hydrogen peroxide quenching step.

b) Omission of non-specific blocking with normal swine serum.

c) Omission of primary incubation with rabbit anti-*Y. ruckeri* serum.

d) Use of rabbit anti-*Y. ruckeri* serum in the primary incubation step, preabsorbed with homogolous strain LT12/6P.

e) Use of normal rabbit serum in the primary incubation step.

f) Use of normal trout serum in the primary incubation step.

g) Omission of secondary incubation with swine anti-rabbit immunoglobulins.

h) Omission of tertiary incubation with PAP.

i) Omission of DAB.

j) Omission of all incubation steps between quenching with hydrogen peroxide and incubation with DAB.

Fish from group A6 (i.e. Farm A) were also sampled for the immunoperoxidase technique.

6.2.9 Statistics

The chi-square test was used to compare the recovery rate of *Y. ruckeri* from faecal and kidney material in section 6.3.2.2. A two-sample T-test was used to compare the pnr values in section 6.3.4. A probability value of up to 0.05 (5%) was considered to be significant for both types of test.

6.3 RESULTS

6.3.1 Biochemical characterization of strains

During the infection experiments (section 6.3.2) all reisolated strains of LT12/6P from either kidney or faecal material were confirmed as *Y. ruckeri*. The API20E profile obtained throughout was 5104100.

6.3.2 Infection experiments

6.3.2.1 LD_{50} estimation

a) Ip injection

The LD_{50} estimation by ip injection indicated that 1 3 x 10^4 c.f.u. ml^{-1} gave the relevant value.

b) Anal intubation

In contrast to the ip injection method, anal intubation gave a LD_{50} value of 1.3 x 10^7 c.f.u. ml^{-1}. Consequently, the groups of fish receiving a high (H) infective dose (e.g. NVH and VH) were given 4.5 x 10^6 c.f.u. ml^{-1} *Y. ruckeri* by anal intubation. The groups receiving a low (L) dose (e.g. NVL and VL) were given 4.5 x 10^4 c.f.u. ml^{-1}.

6.3.2.2 Recovery of *Y. ruckeri*

a) High dose (H) infection

The recovery of *Y. ruckeri* from the faecal material of non-vaccinated (NV) and vaccinated (V) fish following a high dose (H) infection is shown by Figures 6.1a and 6.1b. Peak recovery appeared to be after 1 h and 4 days from group NVH compared with 2 days from group VH. Both groups showed a decline in recovery within the first few hours of the experiment; 8 h for group NVH and 24 h for group VH. However, after 4 days it was not possible to recover *Y. ruckeri* from the faecal material of group VH. This compared with 10 days for group NVH. Essentially there was significantly less recovery overall from group VH after 10 days (chi-square test; $p<0.05$).

There were ten fish from group NVH that gave pure growth of *Y. ruckeri* on ROD compared with only three from group VH. This indicated a significant

reduction in recovery from the faecal material of vaccinated fish (chi-square test; $p<0.05$). Although there were also less countable colonies of *Y. ruckeri* from the VH group (six as opposed to ten) the difference was not significant (chi-square test; $p>0.1$).

The recovery of *Y. ruckeri* from the kidney material of groups NVH and VH is shown by Figures 6.2a and 6.2b. Group VH gave a peak recovery after 24 h with a secondary, lower peak after 7 days. This compared with a peak for group NVH of between 2-4 days. *Y. ruckeri* was also isolated from three fish in group VH one hour after challenge whereas the first isolation from group NVH (four fish) occurred after eight hours. Apart from group NVH after one hour, *Y. ruckeri* was isolated from the kidney material of fish in both groups throughout the 10 day sampling period. However, although *Y. ruckeri* was recovered from fewer fish in group VH the difference was not significant (chi-square test; $p>0.1$).

Fifteen fish from group NVH gave pure growth of *Y. ruckeri* on TSA compared with seven from group VH. This indicated a significant reduction in recovery from the kidney material of vaccinated fish (chi-square test; $p<0.05$). Interestingly there were less countable colonies of *Y. ruckeri* from the NVH group (seven as opposed to eleven) but the difference was not significant (chi-square test; $p>0.1$).

b) Low dose (L) infection

The recovery of *Y. ruckeri* from the faecal material of non-vaccinated (NV) and vaccinated (V) fish following a low dose (L) infection is shown by Figures 6.3a and 6.3b. Peak recovery from both groups appeared to be after one hour although there were secondary, lower peaks after 24 hours. It was not possible to recover *Y. ruckeri* from group VL after 8 hours and 2-10 days whereas this only occurred at 4 days from group NVL. There was a further peak of isolation from group NVL between 7-10 days. There was significantly less overall recovery from group VL after 10 days (chi-square test; $p<0.05$).

There were only two fish from group NVL that gave pure growth of *Y. ruckeri* on ROD compared with none from group VL. In addition, there were also less countable colonies from group VL (3 as opposed to 9) but the difference was not significant (chi-square test; $p<0.05$).

The recovery of *Y. ruckeri* from the kidney material of groups NVL and VL is shown by Figures 6.4a and 6.4b. Peak recovery occurred in group VL after 8 hours compared to 7 days for group NVL. Interestingly, *Y. ruckeri* was isolated from a single fish in group VL, again one hour after challenge, whereas the first isolation from group NVL, in two fish, occurred after eight hours. This was a similar pattern to the previous experiment that used a high infection challenge. However, it was not possible to isolate *Y. ruckeri* from group VL after 2 days whereas there was sustained recovery from group NVL throughout the 10 day sampling period, apart from the first sample after one hour. Although there was less overall recovery from group VL, after 10 days, the difference was not significant (chi-square test; $p<0.05$). However this was only marginal since the found value of x^2 (3.54) was only slightly lower than the 5% probability level (3.84). This represented a difference of only one more positive or negative fish during the experiment.

Although there were five fish from group NVL that gave pure growth of *Y. ruckeri* on TSA compared to none from the VL group, there were six fish in each group that gave countable colonies.

6.3.3. Titration of antibody

6.3.3.1 Mucus samples

The intestinal mucus antibody levels for groups NVH and VH were <1/2-1/2 and <1/2-1/16 respectively when determined by whole cell agglutination. The highest recorded antibody levels (1/4 and 1/16) occurred in two fish in group VH 10 days after infection but 24 days after vaccination.

The levels of mucus antibody from fish in groups NVL and VL were <1/2-1/4 and <1/2-1/8 respectively. The level of 1/8 was recorded in a single fish 10 days after infection.

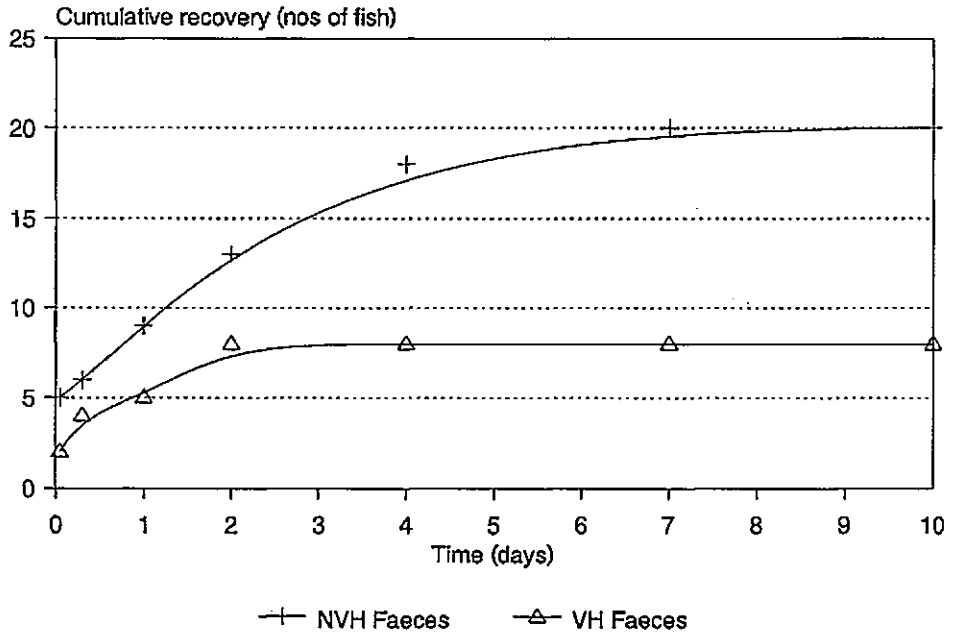


Figure 6.1a **Cumulative recovery of *Y. ruckeri* from faecal material of non-vaccinated (NV) and vaccinated (V) fish following a high (H) dose infection**

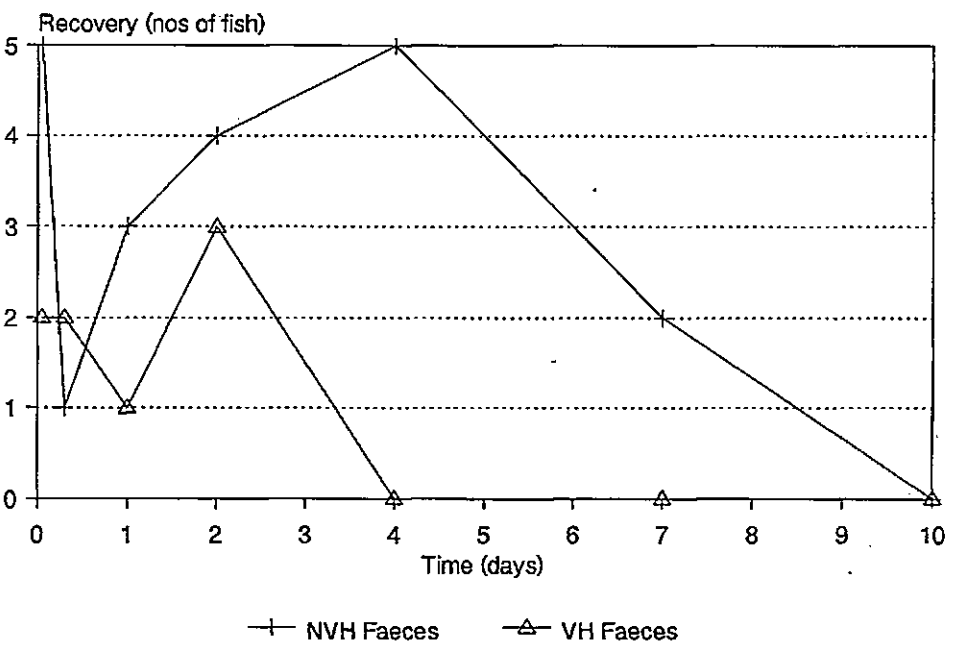


Figure 6.1b **Recovery of *Y. ruckeri* from faecal material of non-vaccinated (NV) and vaccinated (V) fish following a high (H) dose infection**

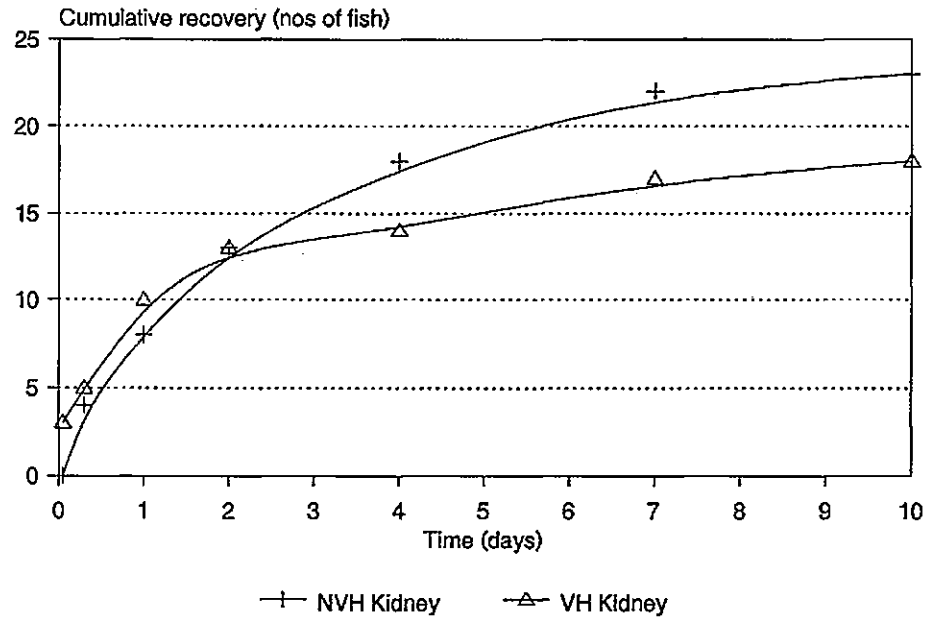


Figure 6.2a **Cumulative recovery of *Y. ruckeri* from kidney material of non-vaccinated (NV) and vaccinated (V) fish following a high (H) dose infection**

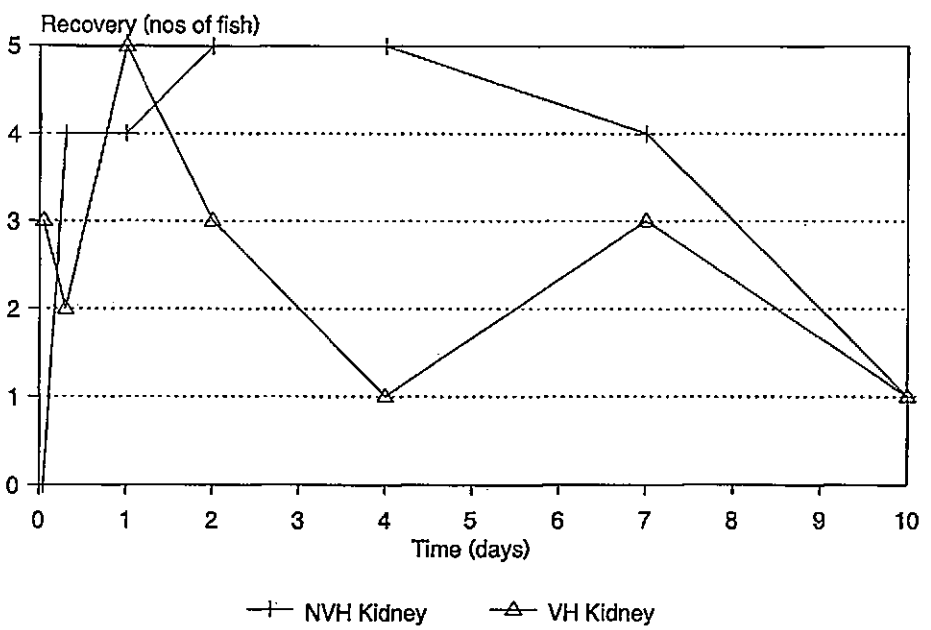


Figure 6.2b **Recovery of *Y. ruckeri* from kidney material of non-vaccinated (NV) and vaccinated (V) fish following a high (H) dose infection**

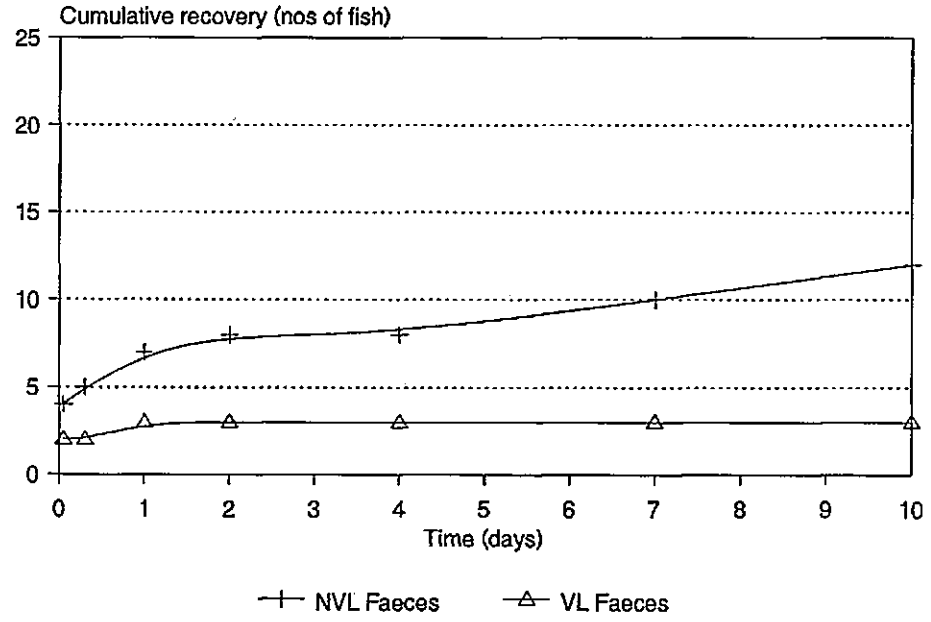


Figure 6.3a **Cumulative recovery of *Y. ruckeri* from faecal material of non-vaccinated (NV) and vaccinated (V) fish following a low (L) dose infection**

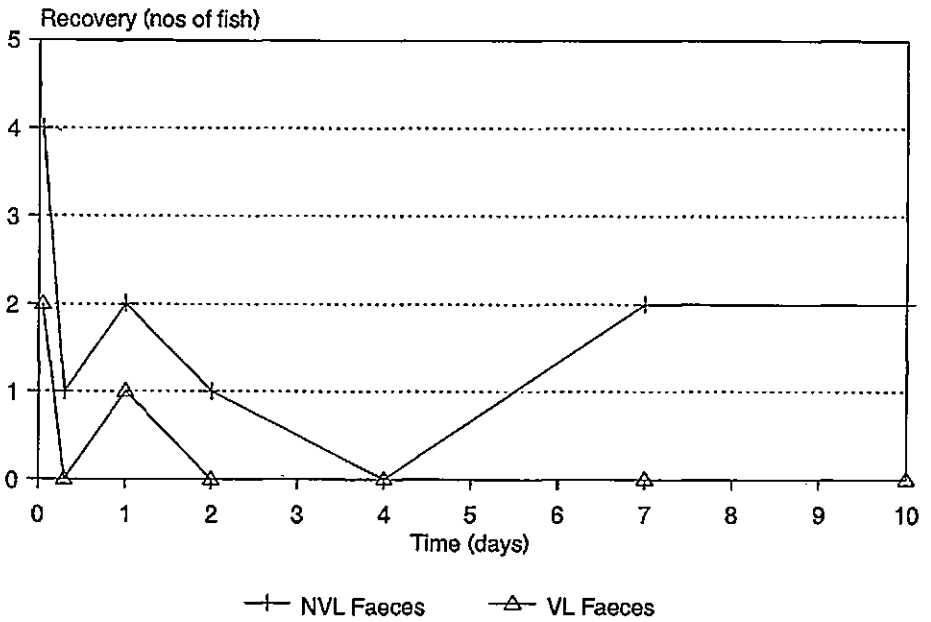


Figure 6.3b **Recovery of *Y. ruckeri* from faecal material of non-vaccinated (NV) and vaccinated (V) fish following a low (L) dose infection**

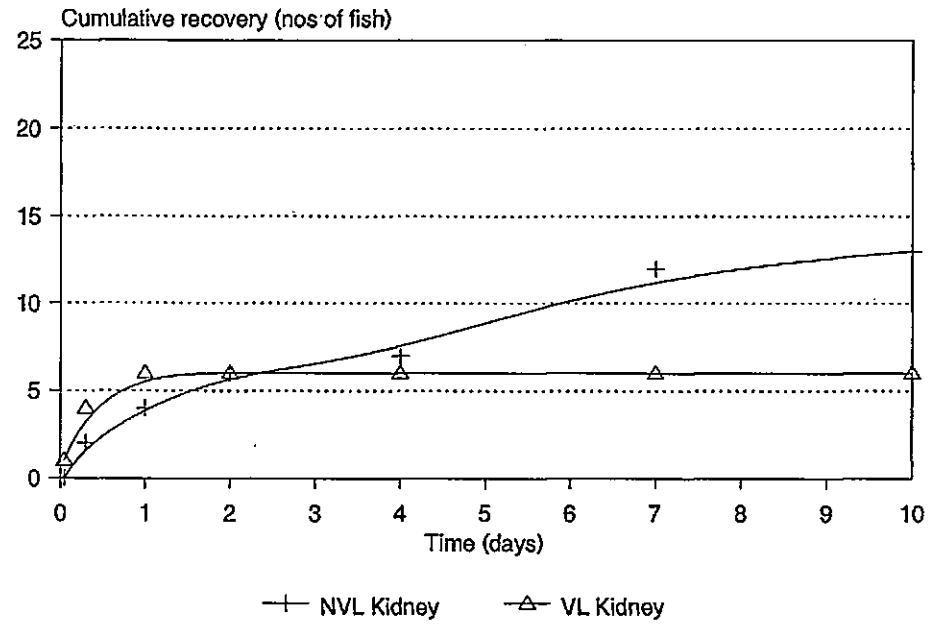


Figure 6.4a **Cumulative recovery of *Y. ruckeri* from kidney material of non-vaccinated (NV) and vaccinated (V) fish following a low (L) dose infection**

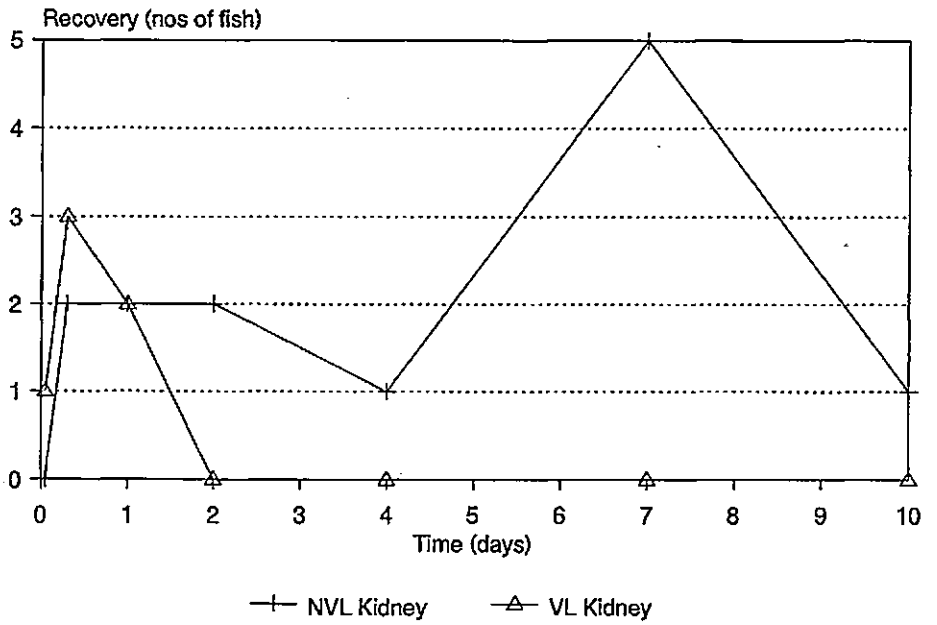


Figure 6.4b **Recovery of *Y. ruckeri* from kidney material of non-vaccinated (NV) and vaccinated (V) fish following a low (L) dose infection**

In the 14 day period after vaccination (i.e. pre-infection group V) the levels of intestinal mucosal antibody were <1/2-1/2.

6.3.3.2 Serum samples

The serum antibody levels for groups NVH and VH, measured by whole cell agglutination, were <1/4-1/4 and <1/4-1/8 respectively. The highest level of 1/8 was recorded in five fish 2, 4, 7 and 10 days after infection which corresponded to 16, 18, 21 and 24 days after vaccination.

The levels of serum antibody from fish in groups NVL and VL were <1/4-1/4 and <1/4-1/64. Levels of 1/16 and 1/64 were recorded in individual fish 8 hours and 10 days after infection.

In the 14 day period after vaccination (i.e. pre-infection group V) the levels of serum antibody were <1/4-1/4.

The antibody titres for the rabbit and trout serum used as positive controls in sections 6.3.3 and 6.3.4 were 1/8192 and 1/1024 respectively.

6.3.4 Enzyme-linked immunosorbent assay (ELISA)

6.3.4.1 Mucus samples

The positive-negative ratios (pnr) for intestinal mucus samples from groups VH and NVH are shown plotted against their respective A_{492} values in Figures 6.5a and 6.5b. There was no apparent pattern to the pnr for either group during the 10 day sampling period. Therefore all the values are plotted together in their respective groups. In addition, dilutions of the control rabbit (1/100-1/6400) and trout (1/100-1/800) sera are included for reference.

Both groups of mucus samples gave a similar pattern between pnr 0.4 and 4.29 but group VH had an additional 10 fish with a pnr between 4.66 and 9.01. This difference was considered significant (two sample T-test; $p<0.05$).

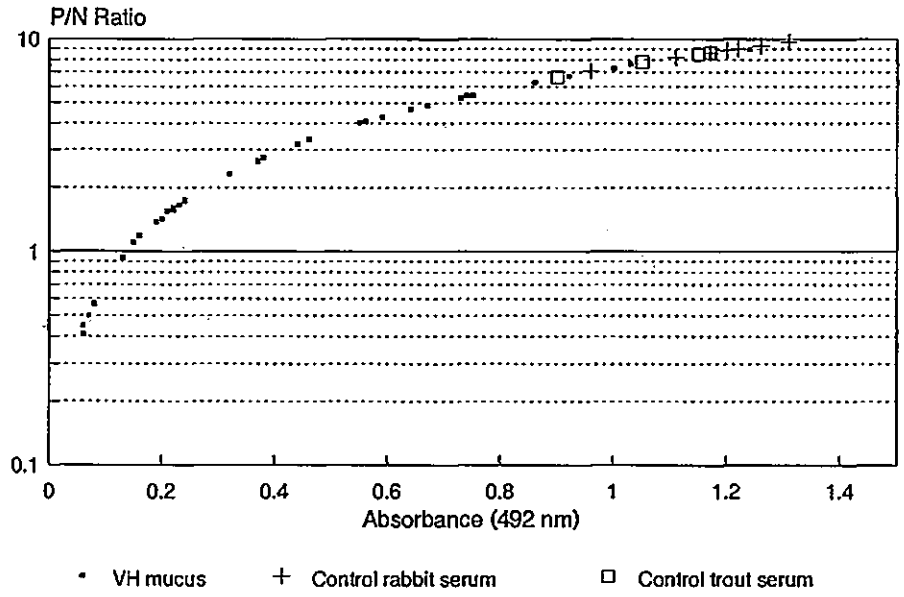


Figure 6.5a Intestinal antibody levels (ELISA) of vaccinated (V) fish following a high (H) dose infection of *Y. ruckeri*

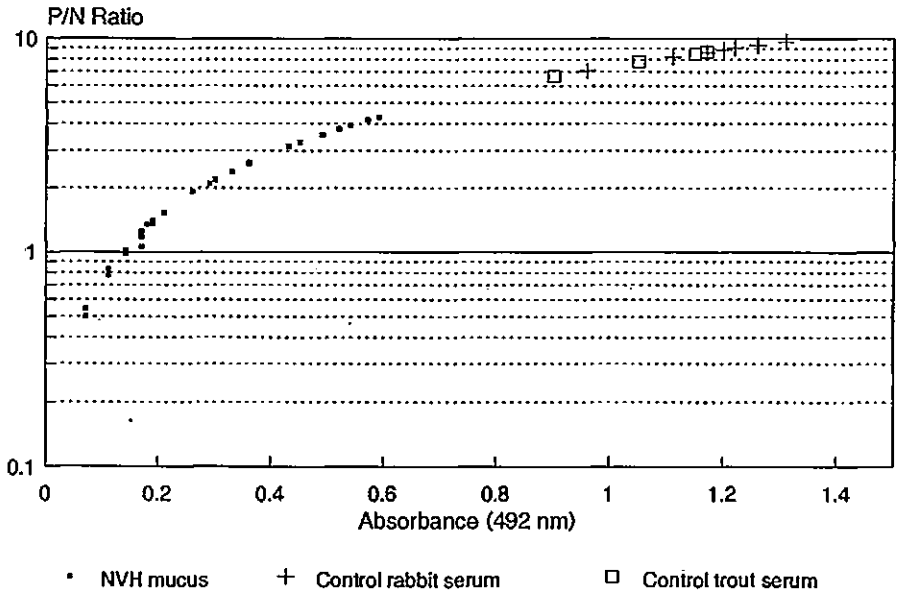


Figure 6.5b Intestinal antibody levels (ELISA) of non-vaccinated (NV) fish following a high (H) dose infection of *Y. ruckeri*

Only one fish from group VH, 10 days after infection, gave a high pnr value (7.31) combined with slightly elevated mucosal (1/16) and serum (1/8) antibody levels as measured by whole cell agglutination (see sections 6.3.3.1 and 6.3.3.2). One additional fish gave a pnr of 6.28 and a serum antibody level of 1/8 but with no apparent increase in the mucosal level measured by agglutination. There was no correlation between ELISA and agglutination for the other nine fish that gave pnr values higher than those in infected control group NVH.

The pnr for mucus samples from groups VNI and NVNI are shown in Figures 6.6a and 6.6b, with all values again being plotted together. A similar pattern and extended range, compared to groups VH and NVH, was observed. Both non-infected groups had similar values between 0.20 and 4.34 with group VNI having an additional 7 fish with a pnr between 4.60 and 9.44. A two sample T-test on these figures indicated a probability of only 0.049 which is considered significant since the cut-off value was taken as 0.05. However, although the significance is marginal, there appears to be a higher mucosal immune response in vaccinated infected fish (group VH) than in non-vaccinated infected fish (group VNI) compared to the respective control groups.

Only three fish from group VNI gave high pnr values 1 (4.66 and 6.84) and 4 (5.87) days after infection, and slightly elevated mucosal antibody levels (1/4, 1/8 and 1/8 respectively) as measured by whole cell agglutination. None of the fish had elevated serum antibody levels. There was no correlation between ELISA and agglutination for the other four fish that gave pnr values higher than those in non-infected control group NVNI. Figures 6.6a and 6.6b also include the values for mucus samples taken from fish in group V, i.e. the first 14 days of the experiment. There was no pattern to the pnr values for group V during this period but there were six fish with pnr values between 4.37 and 8.20. None of these fish had elevated mucosal or serum antibody levels measured by whole cell agglutination.

The levels of antibody in intestinal mucus samples for groups VL and NVL were not determined.

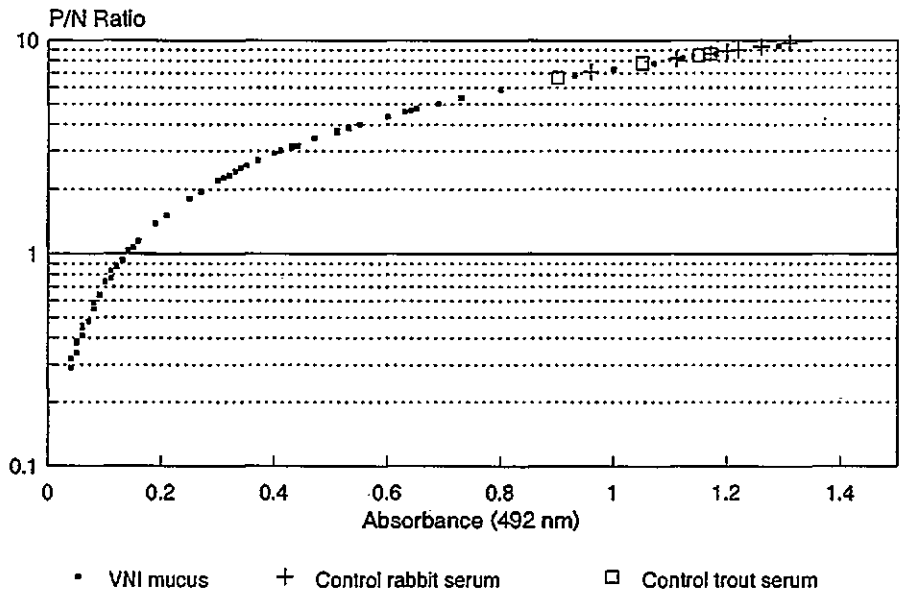


Figure 6.6a Intestinal antibody levels (ELISA) of vaccinated (V) fish not receiving (NI) an infection of *Y. ruckeri*

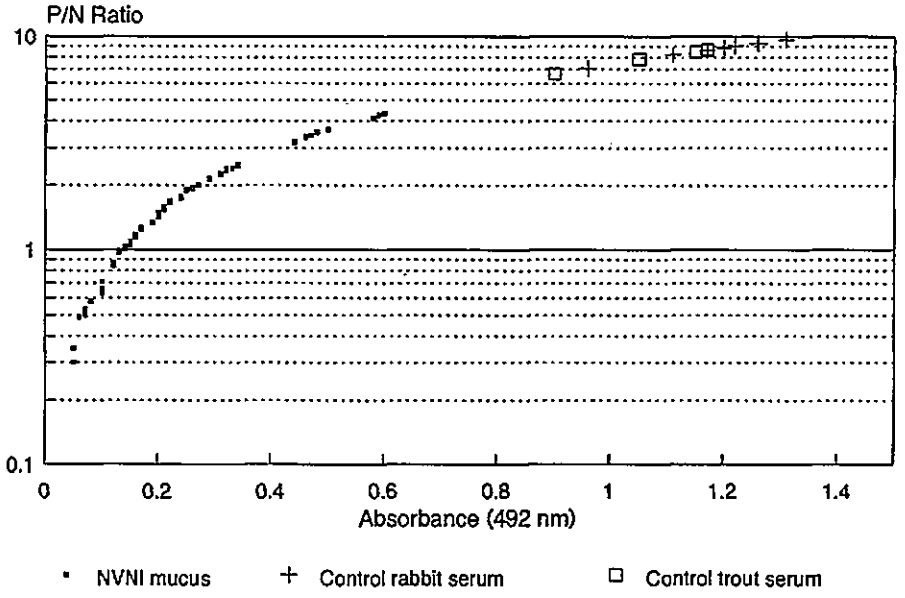


Figure 6.6b Intestinal antibody levels (ELISA) of non-vaccinated (NV) fish not receiving (NI) an infection of *Y. ruckeri*

The increased number of fish with pnr values above the 4-5 range in groups VH and VNI compared to their respective controls is shown in Figures 6.7a and 6.7b. In addition, there appears to be a slight trend towards the higher values in group VH.

6.3.4.2 Serum samples

There was no apparent difference between the serum antibody levels of groups VH and NVH as measured by ELISA (Figures 6.8a and 6.8b).

The serum antibody levels for groups V and VNI are plotted together (Figure 6.9a) since there were no apparent differences during the 24 days of the experiment. Although there was one fish after 4 days with a raised pnr (4.97) in group V, it was not significant (two sample T-test; $p>0.05$). There was no significant difference between this combined group and the control group NVNI (Figure 6.9b; two sample T-test; $p>0.05$).

The number of fish and their respective pnr values for groups VH, NVH, VNI and NVNI are shown in Figures 6.10a and 6.10b.

The levels of antibody in serum samples for groups VL and NVL were not determined.

The pnr values for rabbit anti-*Y. ruckeri* serum diluted 1/100-1/6400 ranged from 9.71 to 7.11 whereas those for trout anti-*Y. ruckeri* serum diluted 1/100-1/800 ranged from 8.65 to 6.65. These values are shown on Figures 6.5a, 6.5b, 6.6a, 6.6b, 6.8a, 6.8b, 6.9a and 6.9b for comparative purposes.

Figure 6.7a **Numbers of fish and P/N Ratio for intestinal antibody levels of vaccinated (V) and non-vaccinated (NV) fish following a high (H) dose infection of *Y. ruckeri***

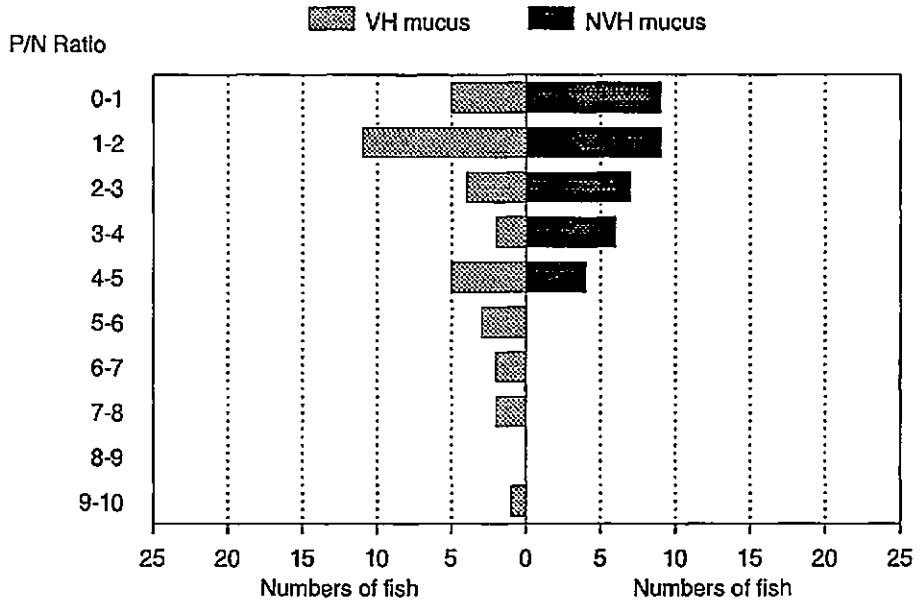
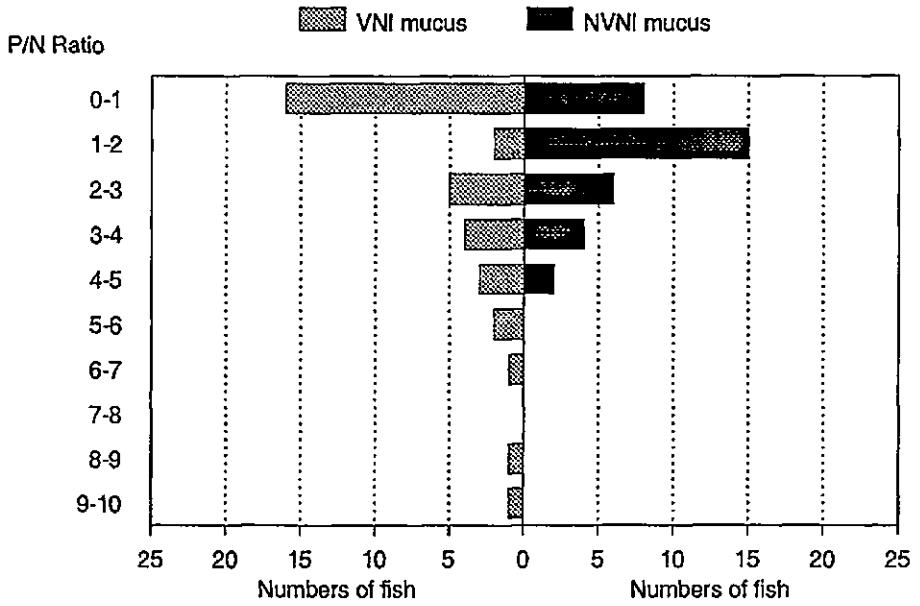


Figure 6.7b **Numbers of fish and P/N Ratio for intestinal antibody levels of vaccinated (V) and non-vaccinated (NV) fish not receiving (NI) an infection of *Y. ruckeri***



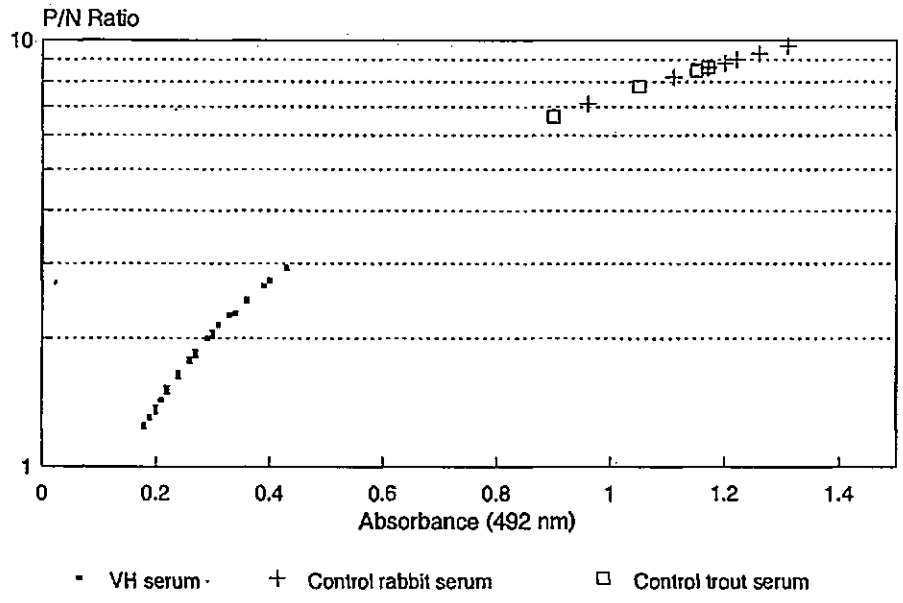


Figure 6.8a Serum antibody levels (ELISA) of vaccinated (V) fish following a high (H) dose infection of *Y. ruckeri*

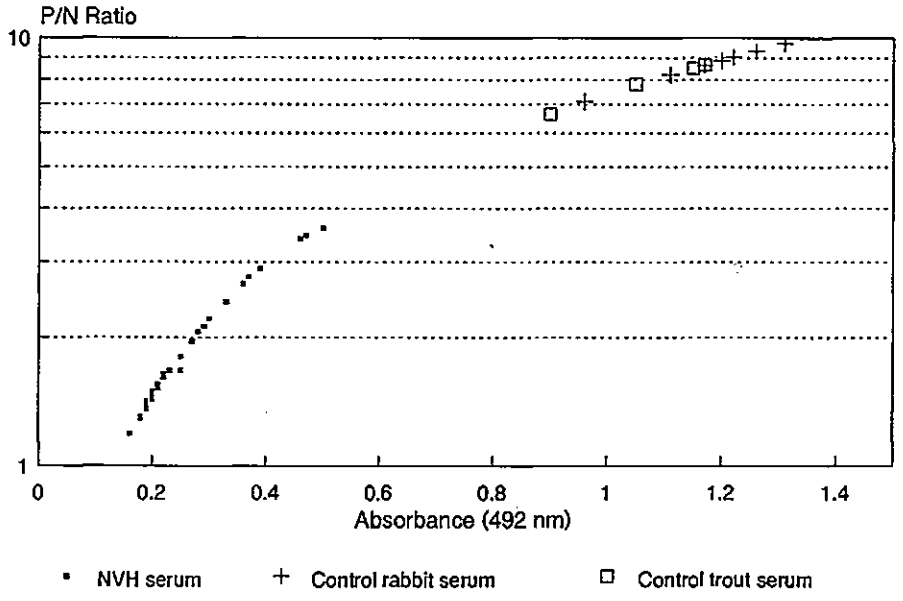


Figure 6.8b Serum antibody levels (ELISA) of non-vaccinated (NV) fish following a high (H) dose infection of *Y. ruckeri*

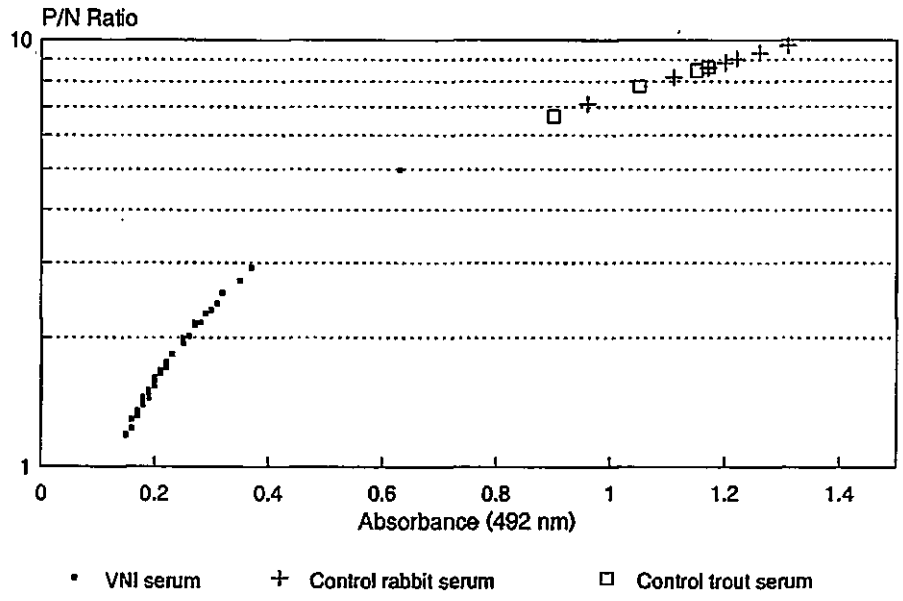


Figure 6.9a Serum antibody levels (ELISA) of vaccinated (V) fish not receiving (NI) an infection of *Y. ruckeri*

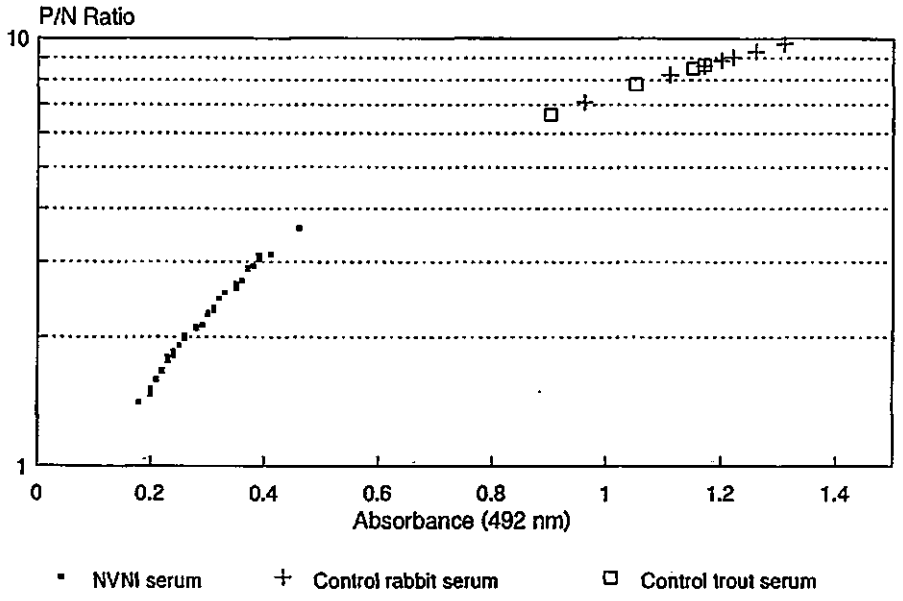


Figure 6.9b Serum antibody levels (ELISA) of non-vaccinated (NV) fish not receiving (NI) an infection of *Y. ruckeri*

Figure 6.10a **Numbers of fish and P/N Ratio for serum antibody levels of vaccinated (V) and non-vaccinated (NV) fish following a high (H) dose infection of *Y. ruckeri***

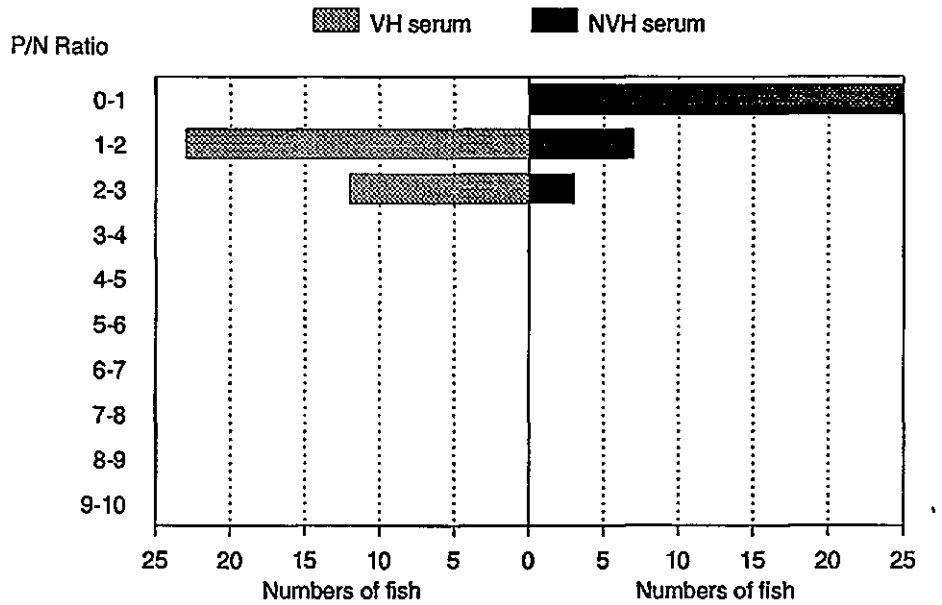
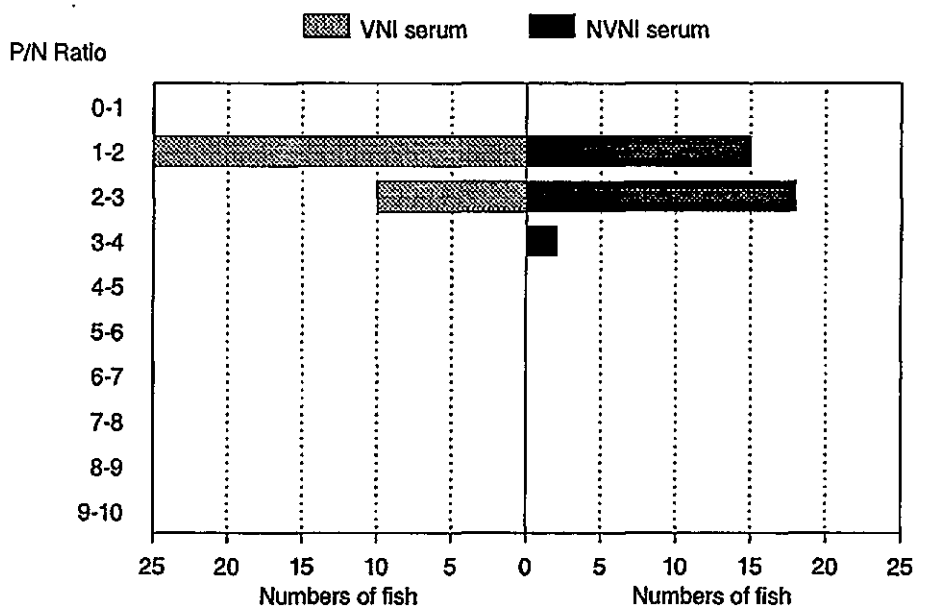


Figure 6.10b **Numbers of fish and P/N Ratio for serum antibody levels of vaccinated (V) and non-vaccinated (NV) fish not receiving (NI) an infection of *Y. ruckeri***



6.3.5 Immunoperoxidase technique

Following extensive preliminary experiments, on known positive and negative control tissues, the following reagent dilutions and incubation times were used throughout the immunoperoxidase technique:

a) Primary incubation-1/500 dilution of rabbit anti-*Y. ruckeri* serum for 30 min.

b) Secondary incubation-1/100 dilution of swine anti rabbit immunoglobulins for 30 min.

c) Tertiary incubation-1/200 dilution of PAP for 30 min.

The results of antigen localisation, following anal challange, for non-vaccinated and vaccinated fish are shown in Plates 6.1-6.6 and 6.7-6.9 respectively. Similar localisation for fish sampled from Farm A is shown in Plates 6.10-6.12.

The immunoperoxidase technique demonstrated that uptake of *Y. ruckeri* antigen occurred in vaccinated and non-vaccinated fish. However, vaccinated fish appeared to take up the antigen by an active process, within 1-8 h after infection. This seemed to occur only in posterior sections of the intestine (i.e. the final 1 cm). Although adherence to the mucosal lining was demonstrated in non-vaccinated fish, active uptake was not seen. The antigen appeared in the spleen and kidney more quickly than in non-vaccinated fish. Accumulations of antigen appeared to be associated with melanomacrophage centres (MMC) and became focal in non-vaccinated fish after 4 and 7 days.

The fish from Farm A showed similar localisation of antigen to the non-vaccinated fish at the laboratory. In particular, *Y. ruckeri* was associated with vascular intestinal tissue, glomerular capillary tissue and focal deposition around splenic MMC. Interestingly, complete sloughing of the intestinal epithelial layer was demonstrated in both a moribund, chronically infected fish from Farm A and a recently dead fish from the non-vaccinated laboratory group, 4 days after challenge.

6.3.6 Histopathology

6.3.6.1 Differential staining

Although H and E demonstrated a slight loss of haemapoietic tissue in the kidney of non-vaccinated fish after 7 days it was not possible to determine whether this had been caused by necrosis or by processing shrinkage. Increased intestinal distension and vacuolation was seen in the intestinal tissue after 4-7 days, again in non-vaccinated fish.

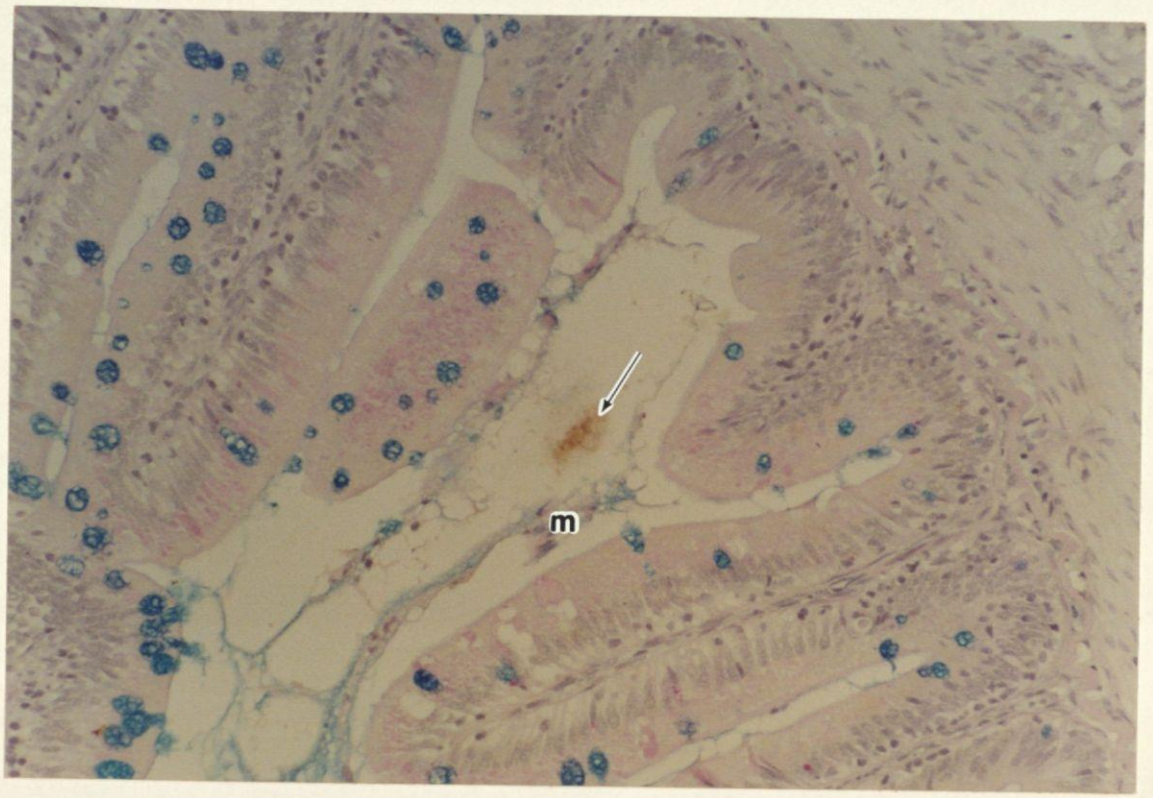
The Gram-Twort stain confirmed the presence of Gram negative rods, largely in non-vaccinated fish after 4 days.

Plate 6.1 **Sections of lower intestine from non-vaccinated fish following a high dose infection (NVHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Characteristic brown deposit denoting cells of *Yersinia ruckeri* (arrow) within intestinal mucus (m), 1 h after infection (x190).

B: *Y. ruckeri* (arrow) along the intestinal wall, 24 h after infection (x300). The inset shows a close association with the mucosal lining and microvilli border (b), in the vicinity of an acidic mucus producing goblet cell (g) (x750).

A



B

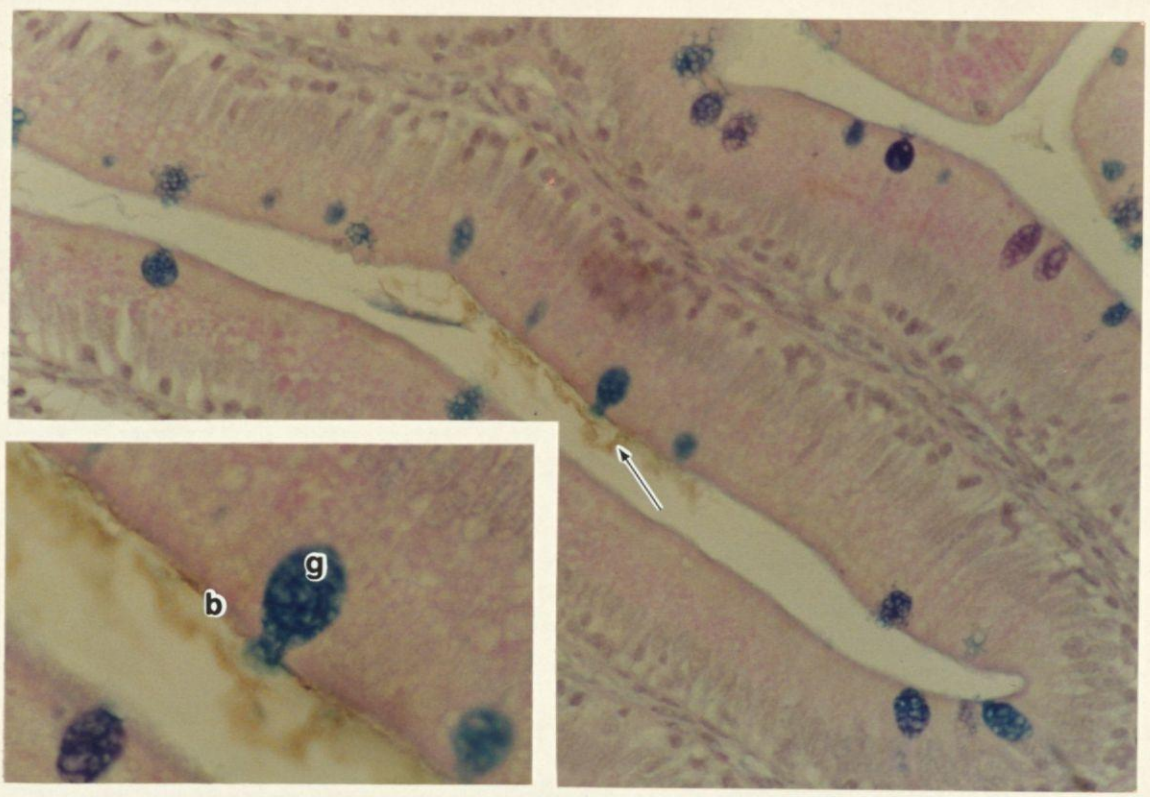
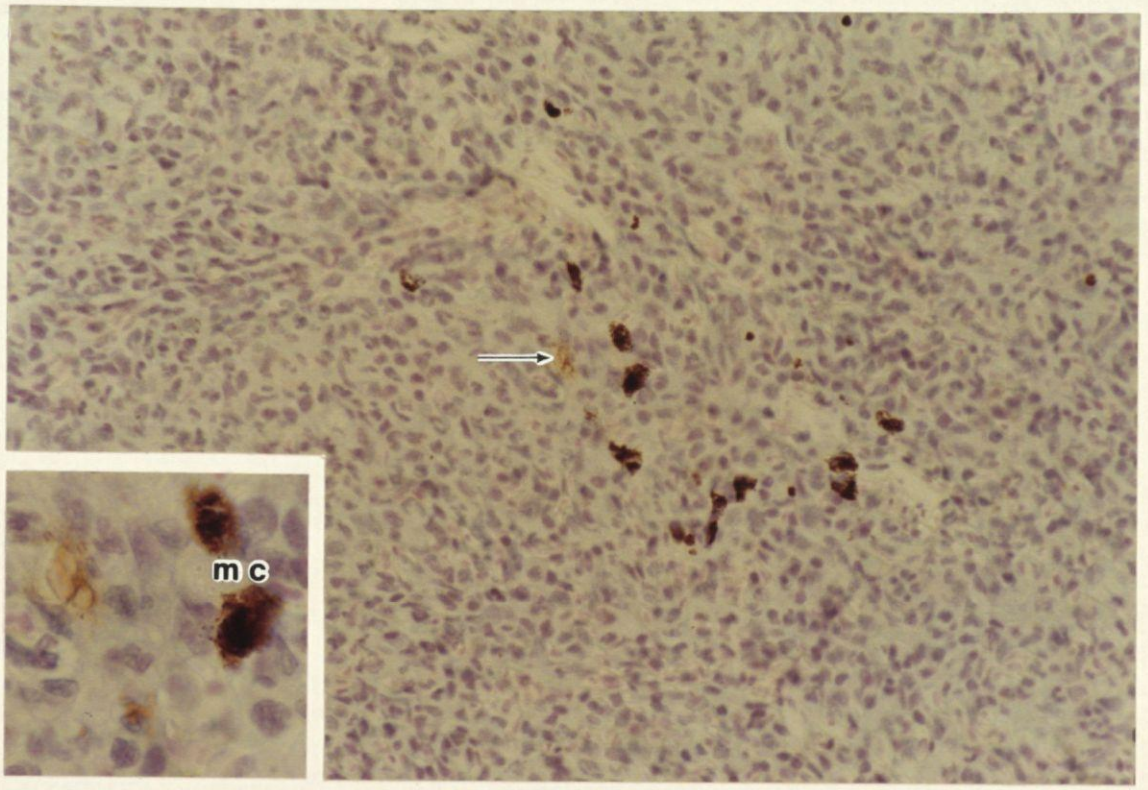


Plate 6.2 **Sections of spleen and lower intestine from non-vaccinated fish following a high dose infection (NVHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Small deposit of DAB indicating the presence of *Y. ruckeri* antigen (arrow) in spleen, 24 h after infection (x300). The inset shows the close association with melano-macrophage centres (mc) (x750).

B and C: *Y. ruckeri* antigen, inside a macrophage, within the vascular tissue of the lower intestine (arrow), 48 h after infection. The columnar epithelial cells (e) lining the intestinal wall are also shown (x750).

A



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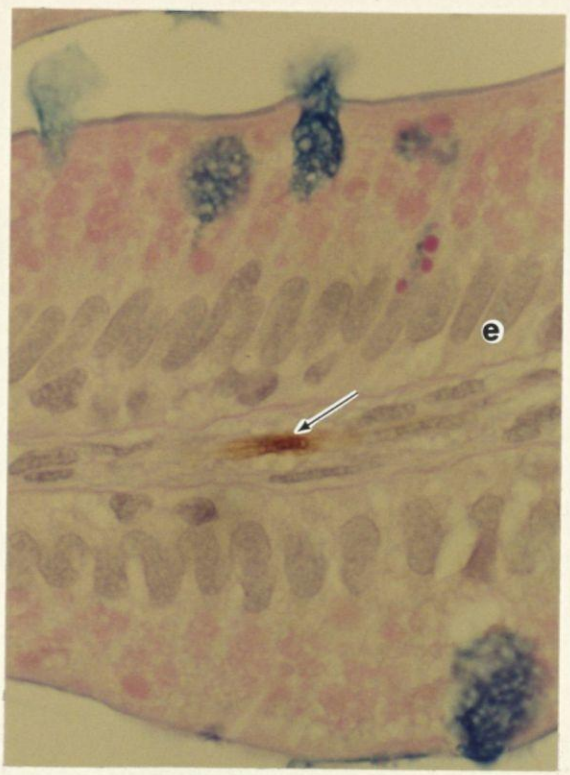

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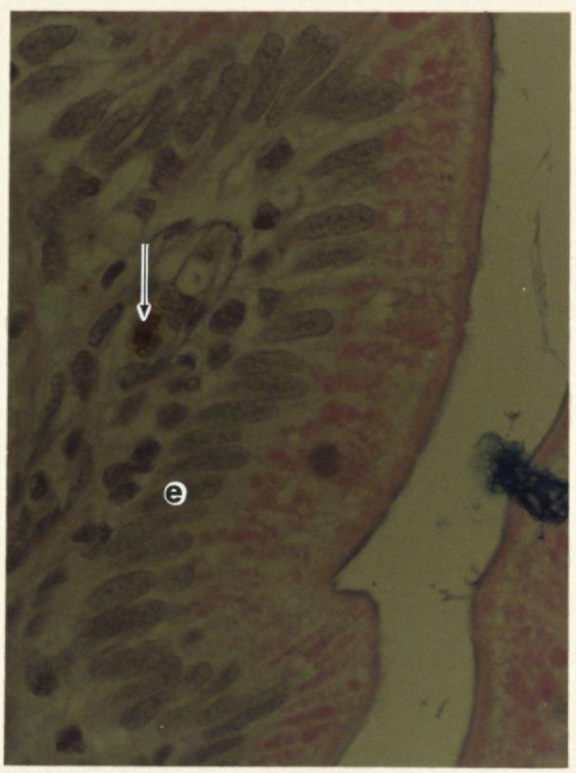

Plate 6.3 **Sections of kidney and spleen from non-vaccinated fish following a high dose infection (NVHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Small area of DAB indicating the presence of *Y. ruckeri* in kidney (arrow), 48 h after infection (x300).

B: Small areas of DAB indicating the presence of *Y. ruckeri* in spleen (arrow), 48 h after infection (x300).

C: Increased deposition of DAB in kidney glomerular capillary tissue (g) and interstitial tissue (arrow) due to *Y. ruckeri,* 4 days after infection (x300).

D: Increased deposition of DAB in splenic artery (arrow) and white pulp areas (w) due to *Y. ruckeri,* 4 days after infection (x300).

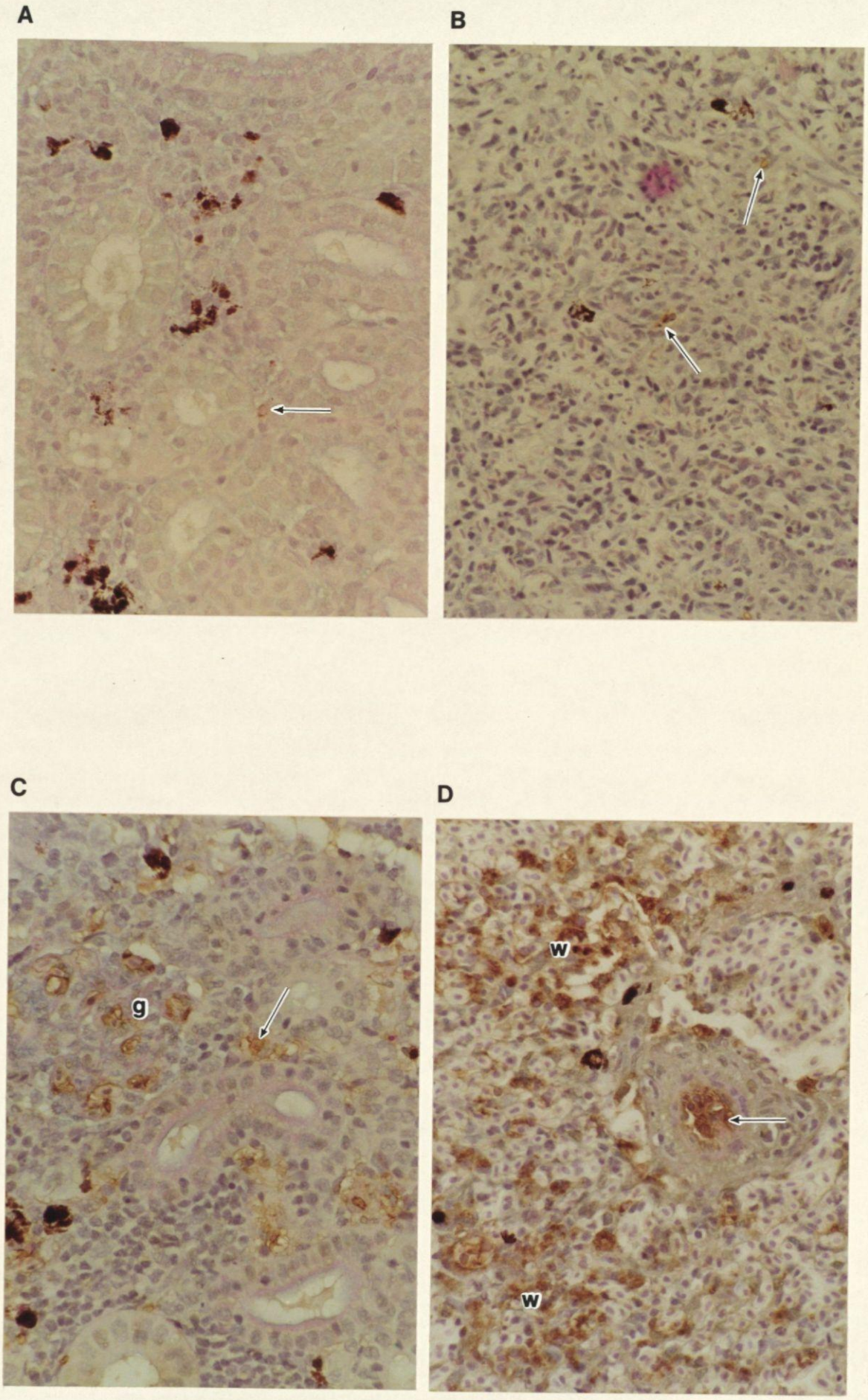
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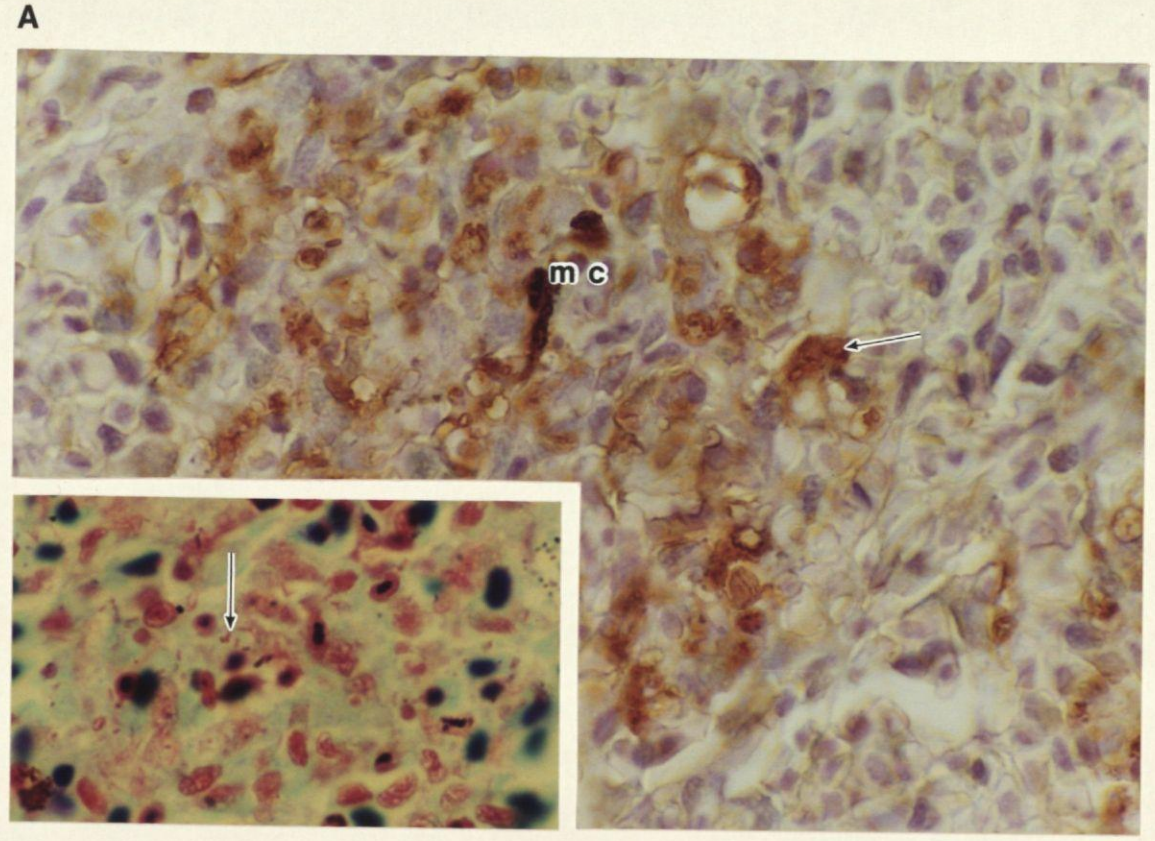
Plate 6.4 **Sections of spleen, lower intestine and kidney from non-vaccinated fish following a high dose infection (NVHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Focal area of DAB deposit in spleen showing the presence of *Y. ruckeri* antigen (arrow) surrounding a melano-macrophage centre (mc), 4 days after infection (x750). The inset shows the presence of Gram negative rods (arrow) in a spleen section from the same fish (Gram-Twort, x750).

B: Lower intestine of fish approximately 5 h after dying from *Y. ruckeri* infection (4 days after challenge). Note the total absence of epithelial cells which have been sloughed away into the intestinal lumen (e). There is also evidence of intestinal distension, possible tissue oedema and autolytic changes(x300).

C: Kidney of fish approximately 5 h after dying from *Y. ruckeri* infection (4 days after challenge) showing presence of antigen in a capillary (arrow) of the interstitial tissue and a glomerulus (x300).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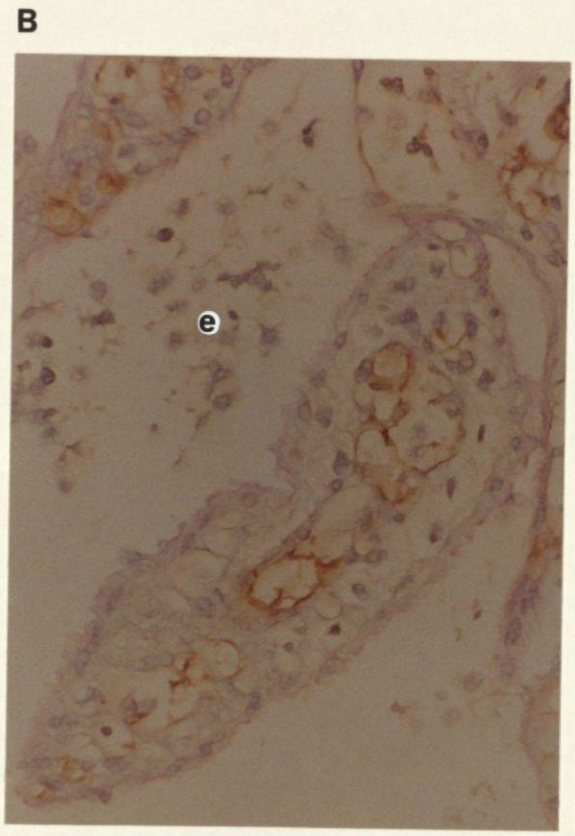
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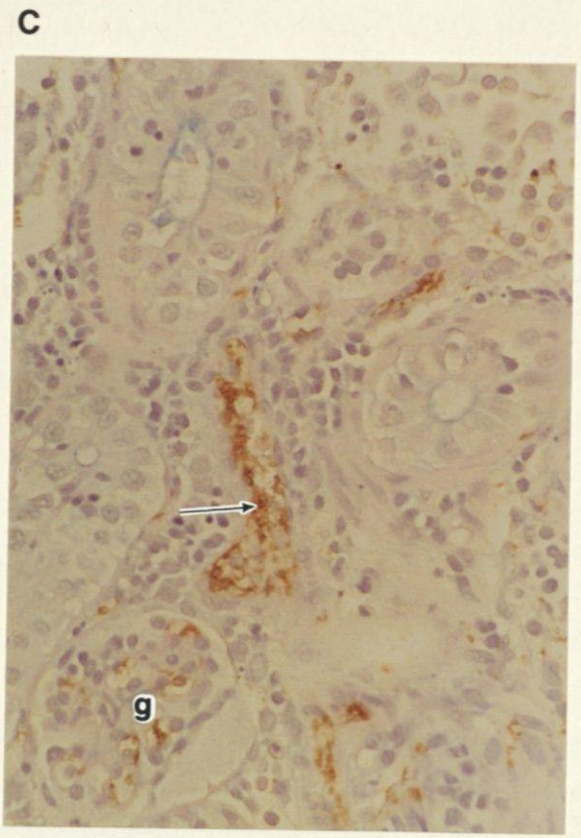
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g

Plate 6.5 **Sections of spleen, liver and lower intestine from non-vaccinated fish following a high dose infection (NVHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

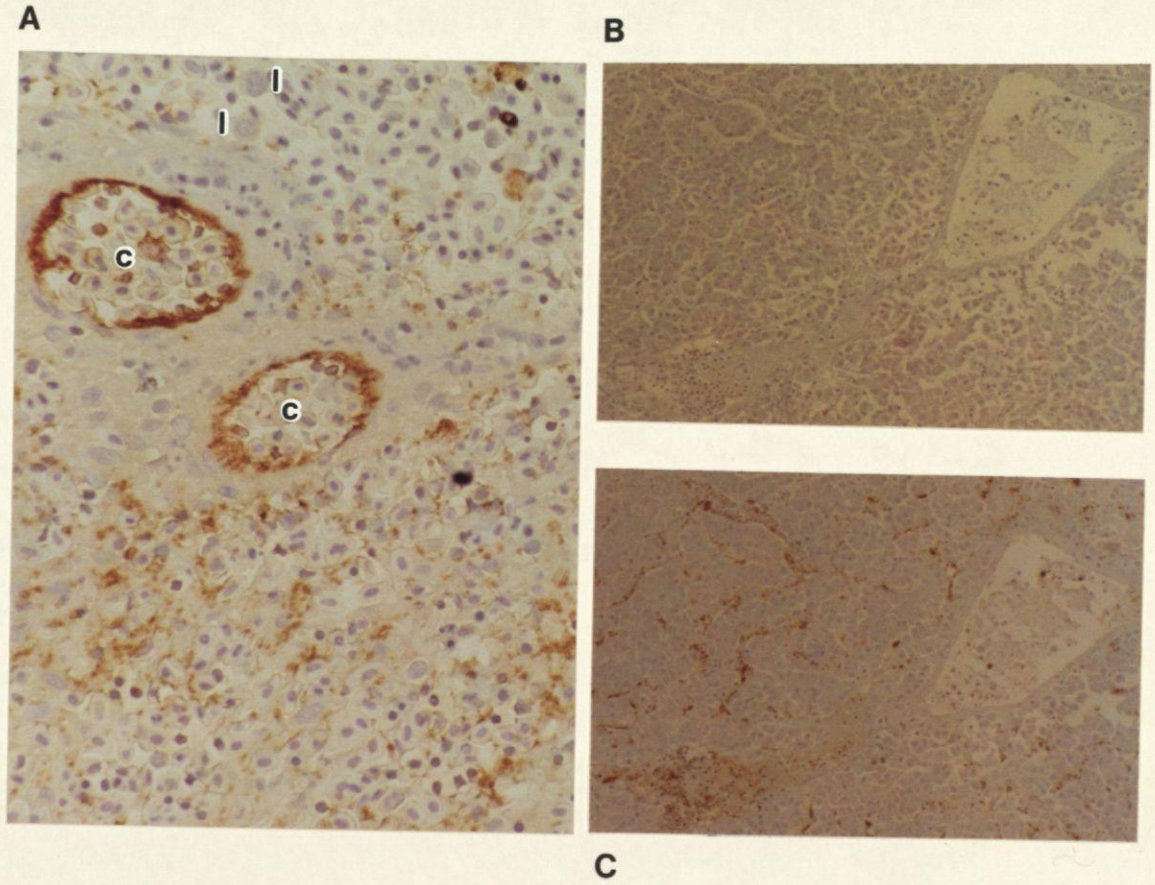
A: Spleen of fish approximately 5 h after dying from *Y. ruckeri* infection (4 days after challenge) showing lymphocytes (l) and the presence of antigen in capillaries (c) (x300).

B: Liver of fish approximately 5 h after dying from *Y. ruckeri* infection (4 days after challenge). Negative control (x75).

C: Liver of fish approximately 5 h after dying from *Y. ruckeri* infection (4 days after challenge) showing presence of antigen in vascular tissue (x75).

D: Transverse section through intestinal fold showing antigen (arrow) in the lumen (l) and vascular tissue of the lamina propria (v) 7 days after infection (x750). A lymphocyte (ly) is also present.

E: Macrophages (m) in the vascular tissue of the intestine 7 days after infection (x750). Note the deposits of DAB, indicating presence of antigen, within the macrophages.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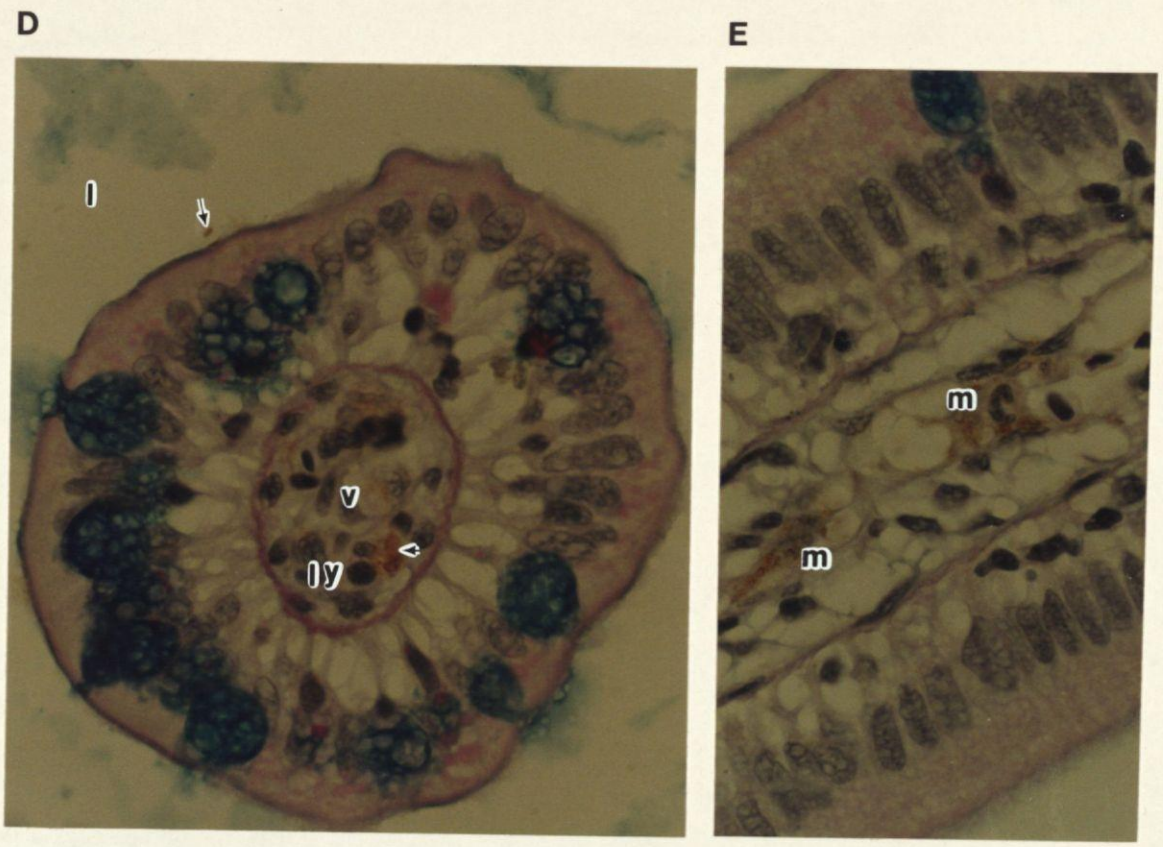
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m

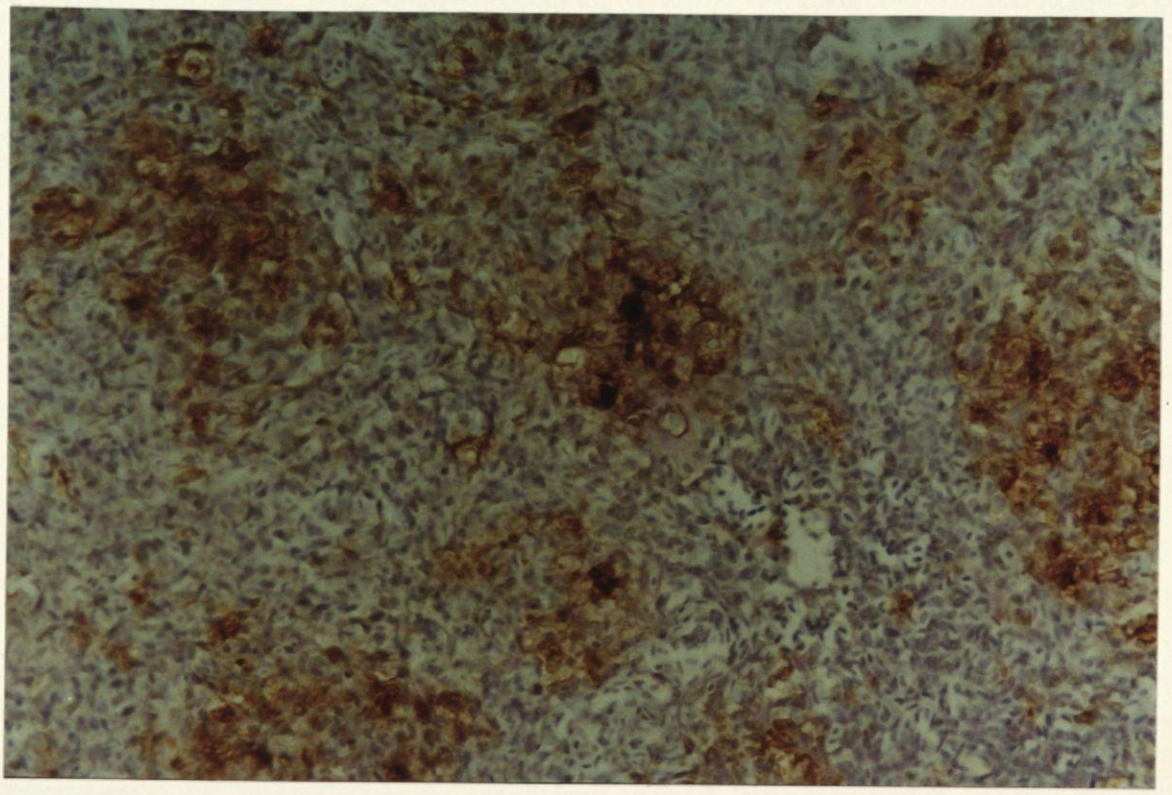
Plate 6.6 **Sections of spleen and kidney from non-vaccinated fish following a high dose infection (NVHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Focal deposition of brown DAB in spleen tissue, 7 days after infection indicating accumulation and migration of antigen into the areas of melano-macrophages (x300).

B: Detail of focal antigen localisation in spleen associated with a melano-macrophage centre (mc) (x750).

C: Focal deposition of brown DAB in kidney tissue 7 days after infection (x300). The antigen is also associated with melano-macrophage centres (mc).

A



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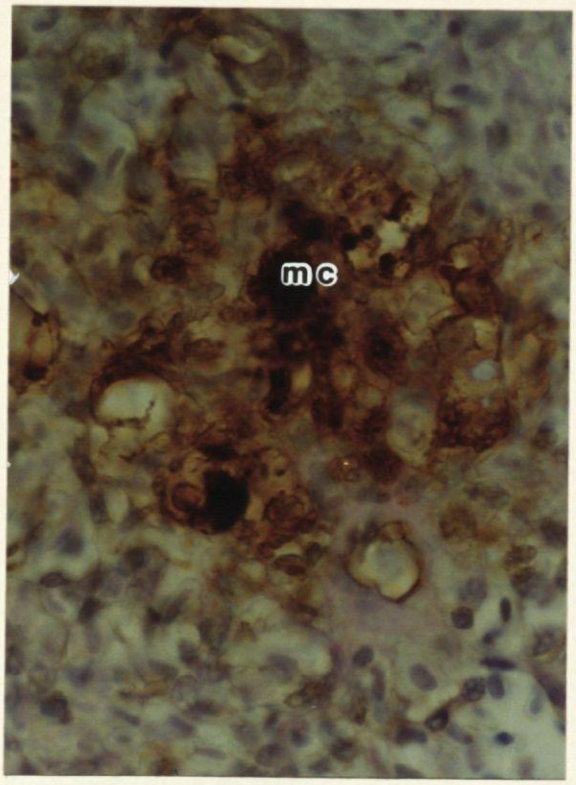

C

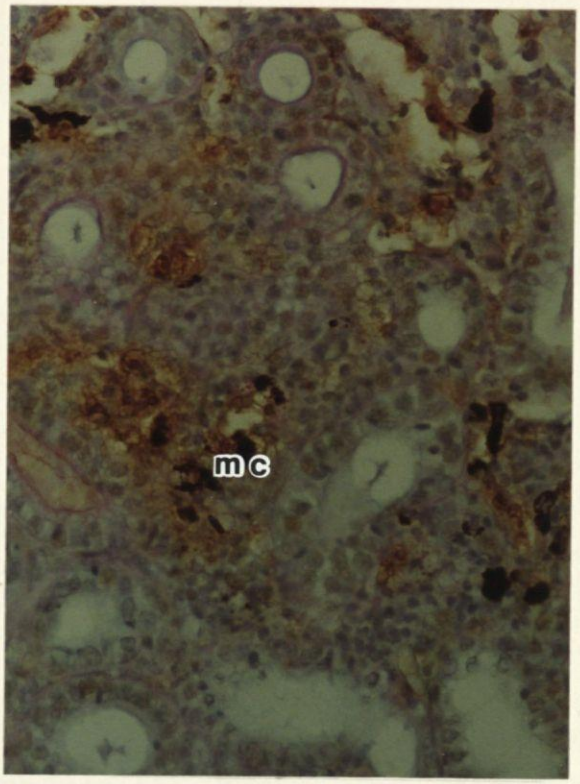

Plate 6.7 **Sections of lower intestine from vaccinated fish following a high dose infection (VHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: *Y. ruckeri* (arrow) associated with the intestinal mucus (m) 1 h after infection (x300).

B: Apparent uptake (pinocytosis) of antigen at the edge of an intestinal fold (arrow) just below the microvilli border (b) in the vicinity of a goblet cell, 1 h after infection (x750).

C and D: Further uptake (pinocytosis) of antigen, within vacuoles, at the edge of an intestinal fold (arrow), 8 h after infection (x750). This type of uptake was only noted in the hind portion of the lower intestine in vaccinated fish.

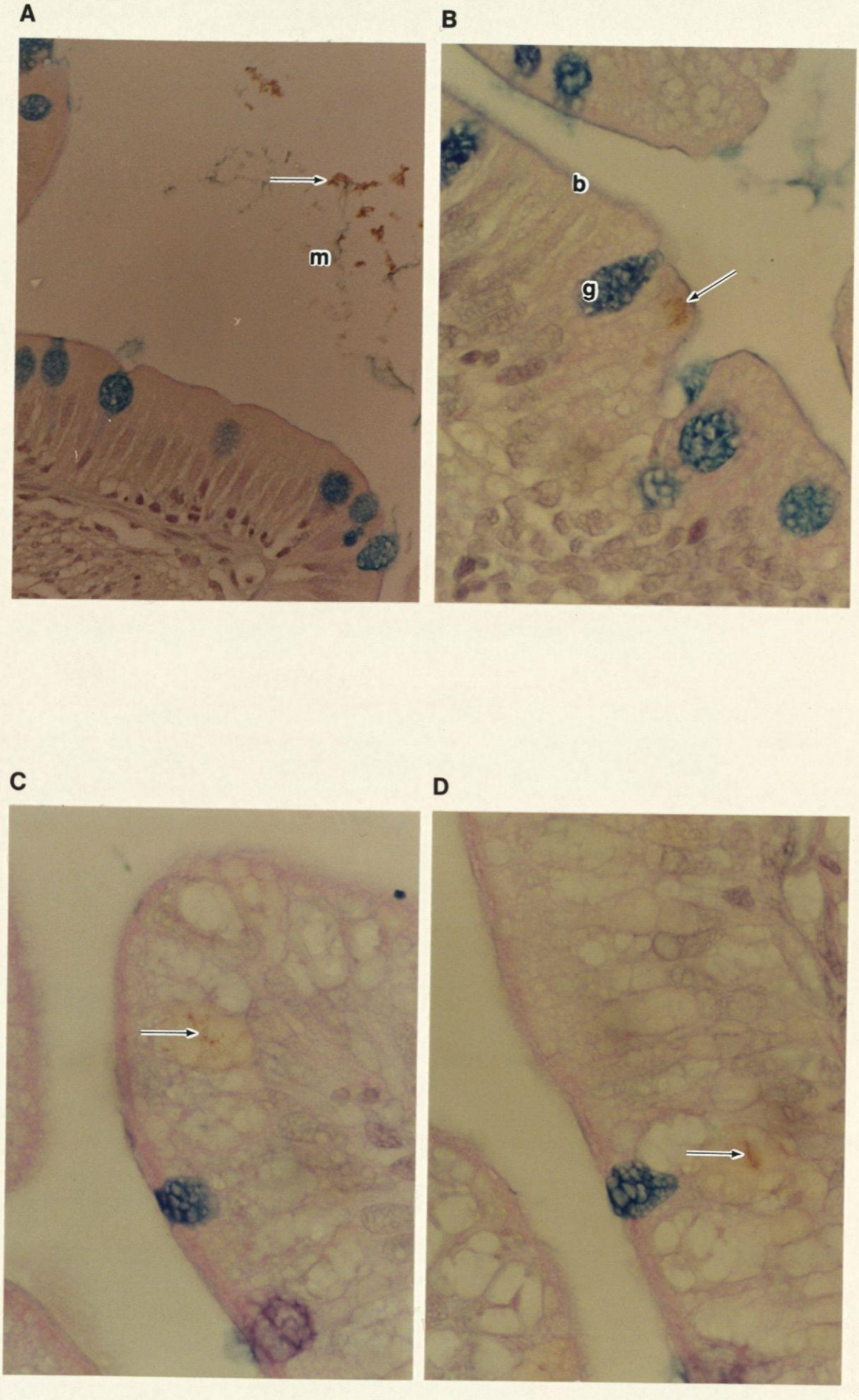
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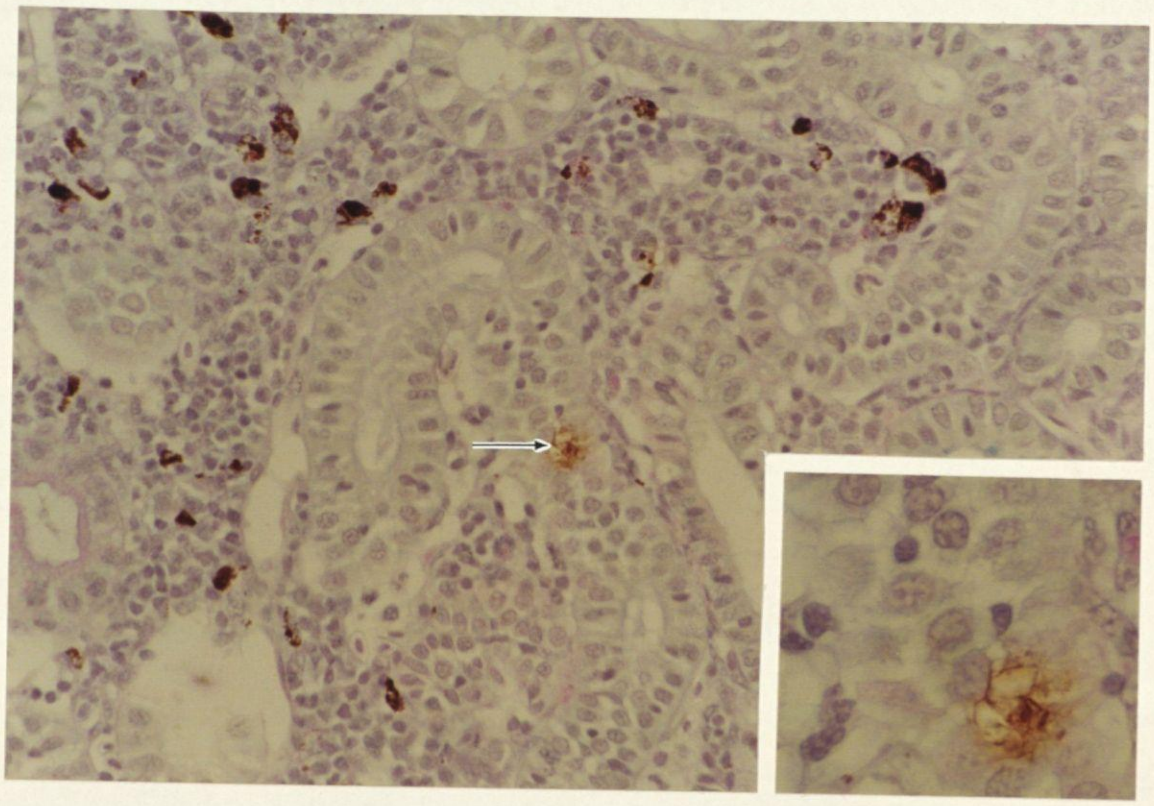
Plate 6.8 **Sections of kidney, spleen and lower intestine from vaccinated fish following a high dose infection (VHI).. DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Area of antigen deposition in kidney (arrow) but possibly within a macrophage, 8 h after infection (x300). Inset shows detail (x750).

B: Area of antigen deposition in spleen (arrow), 1 h after infection (x750).

C: Area of antigen deposition in vascular tissue of the lower intestine (arrow), 24 h after infection (x300).

A



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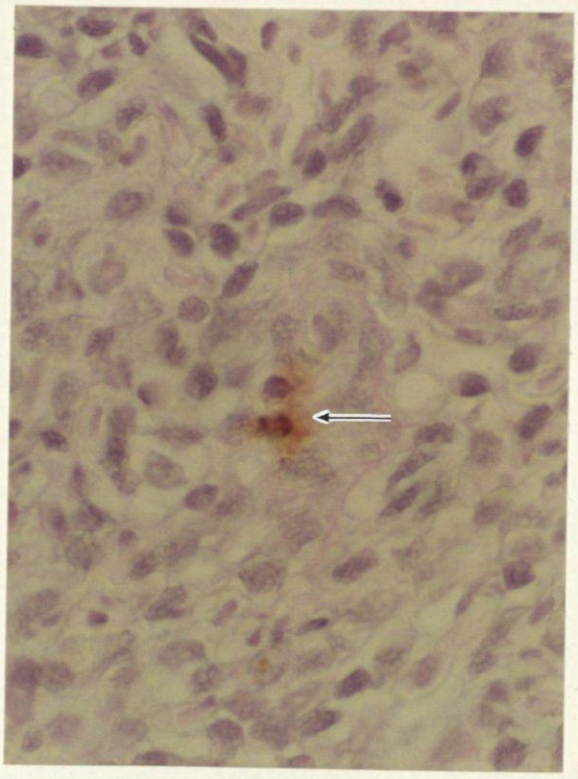

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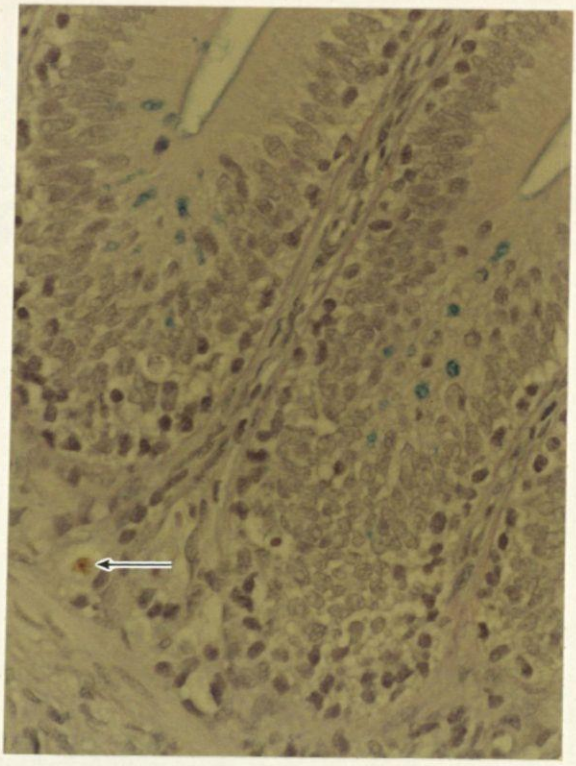
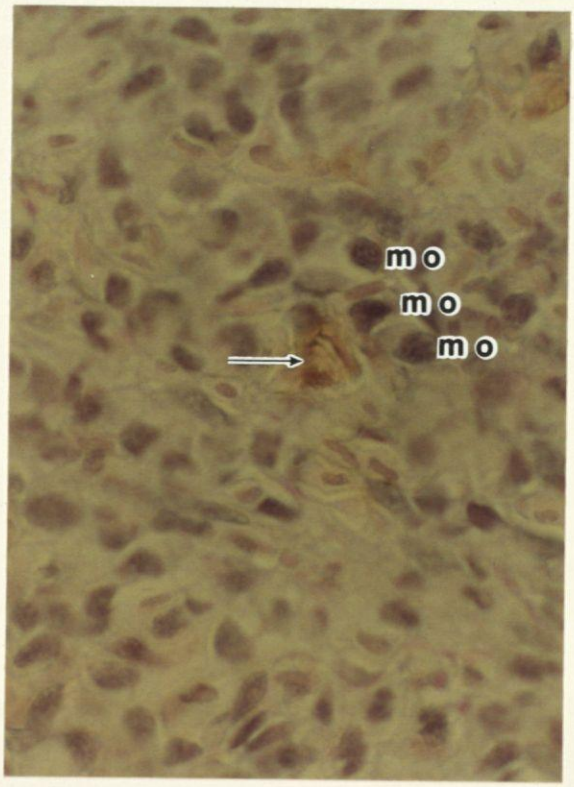


Plate 6.9 **Sections of spleen and kidney from vaccinated fish following a high dose infection (VHI). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Area of *Y. ruckeri* antigen in spleen (arrow) with an accumulation of monocytes (mo), 24 h after infection (x750).

B: Area of *Y. ruckeri* antigen in kidney (arrow), associated with a melano-macrophage centre (mc) and lymphocytes (ly), 7 days after infection (x750).

A



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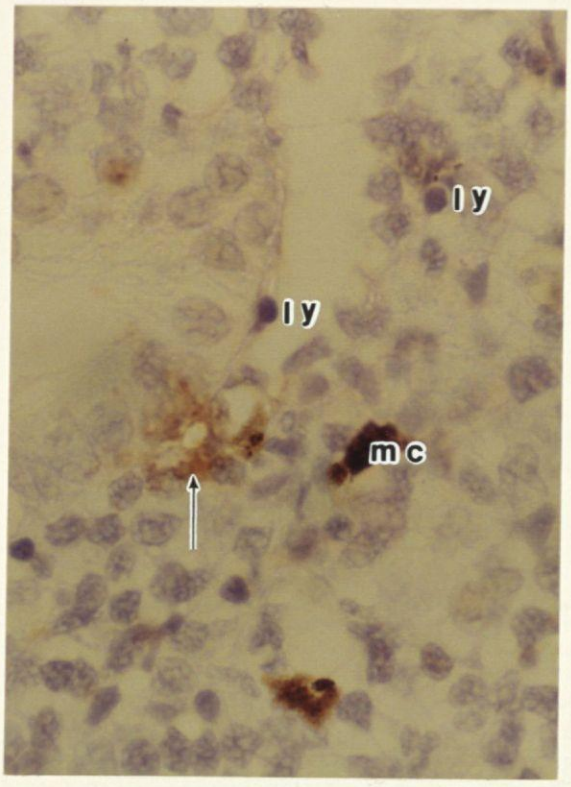

Plate 6.10 **Sections of intestine from fish, reared at Farm A, with chronic enteric redmouth disease (ERM). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Brown deposition of DAB showing *Y.ruckeri* antigen (a) located in the intestinal lumen (x300).

B: Antigen located in the vascular tissue of an intestinal fold (arrow) (x190). Note the absence of epithelial cells which have been sloughed away into the intestinal lumen (e). There is also evidence of possible necrotic tissue.

C and D: Antigen located in the vascular tissue of an intestinal fold (arrow) possibly associated with macrophages (x750). There is also extensive loss of mucosal epithelium and the presence of a monocyte (mo).

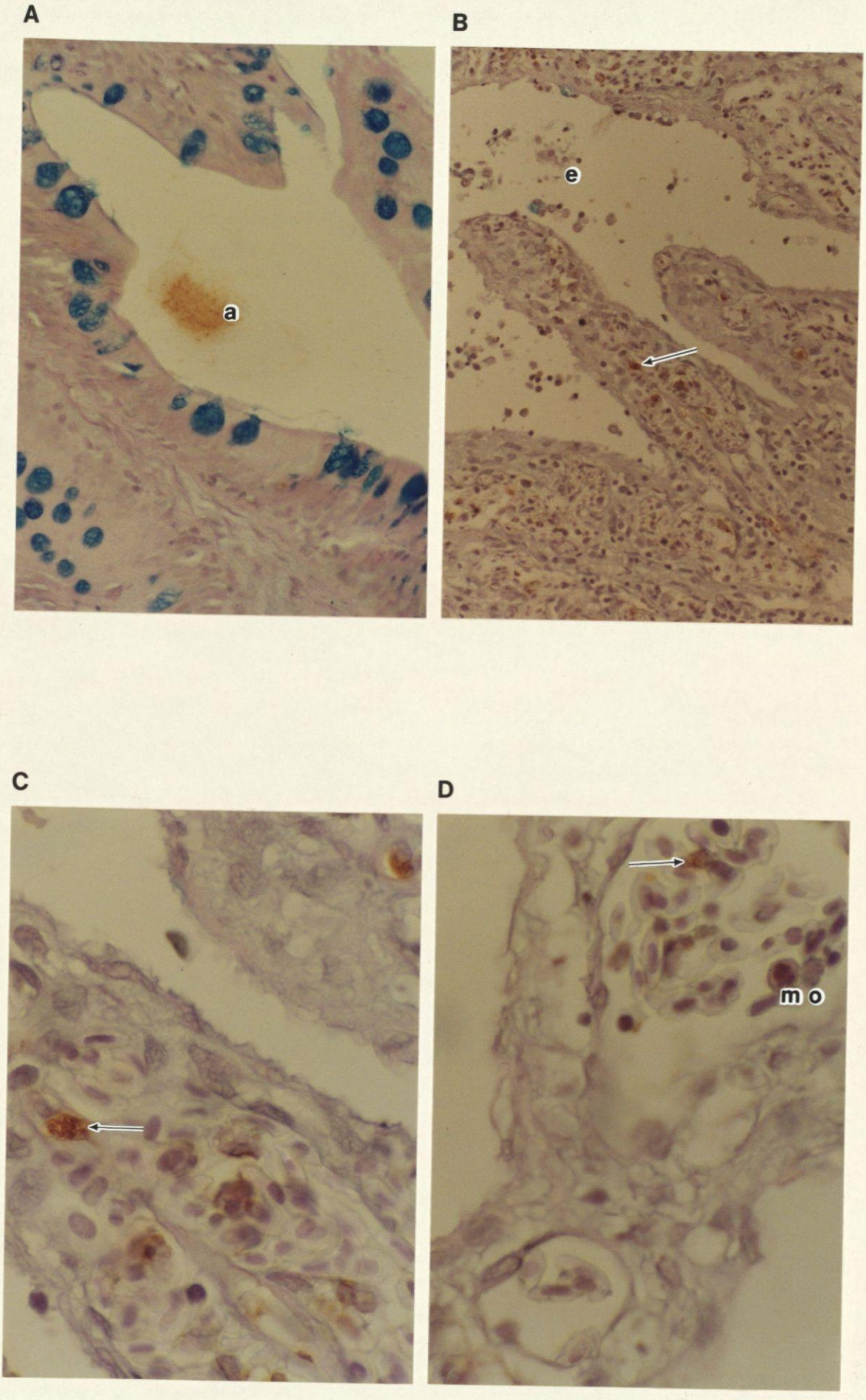
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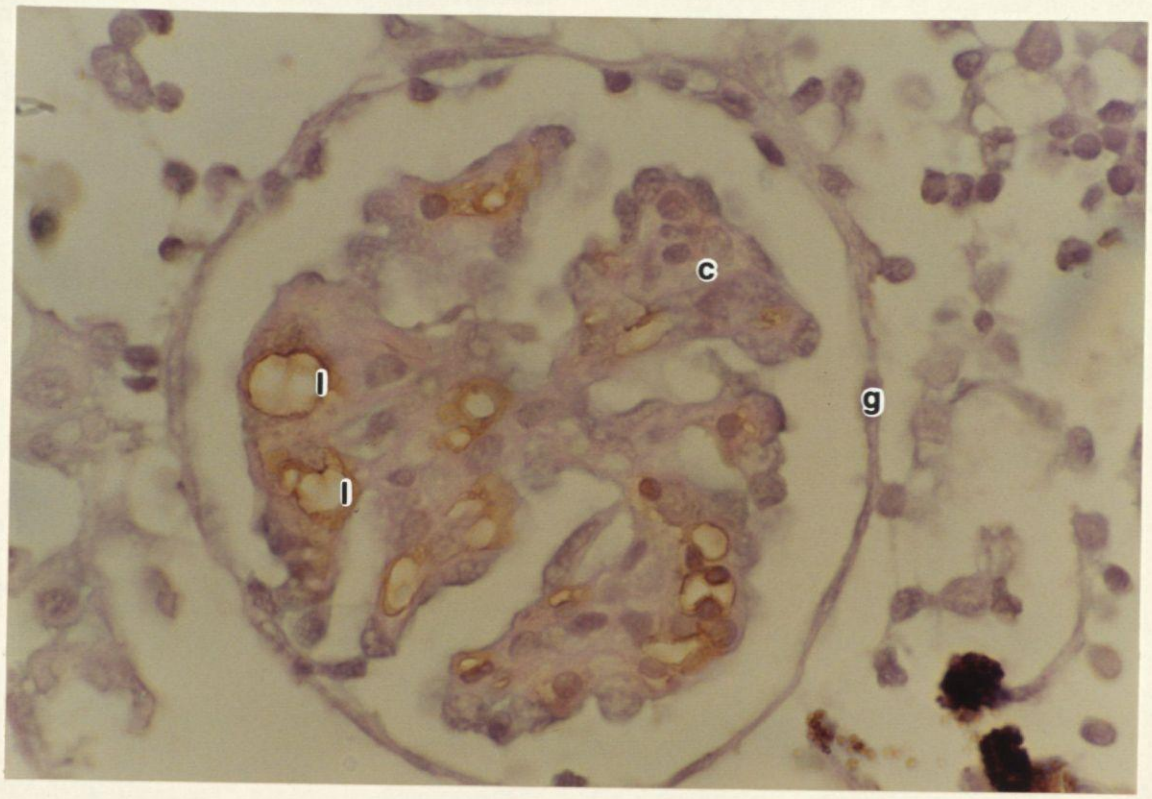
Plate 6.11 **Sections of kidney and spleen from fish, reared at Farm A, with chronic enteric redmouth disease (ERM). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Kidney section showing glomerular capsule (g) with brown deposit of DAB, denoting the presence of antigen, associated with capillary tissue (c) and storage vacuoles, e.g. areas of lipid (l) (x750).

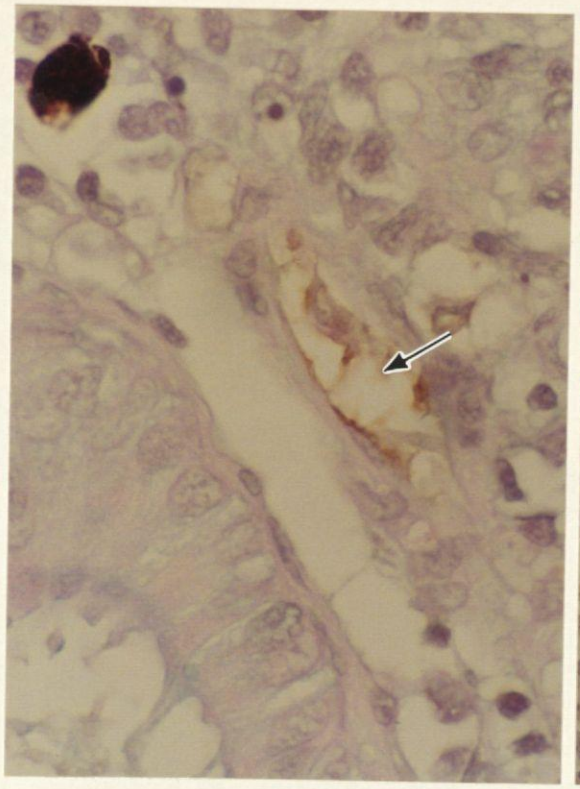
B: Area of *Y. ruckeri* antigen (arrow) in kidney (x750).

C: Focal areas of antigen in spleen, associated with melano-macrophages (mc) and the white pulp lymphoid tissue (v) (x300).

A



B



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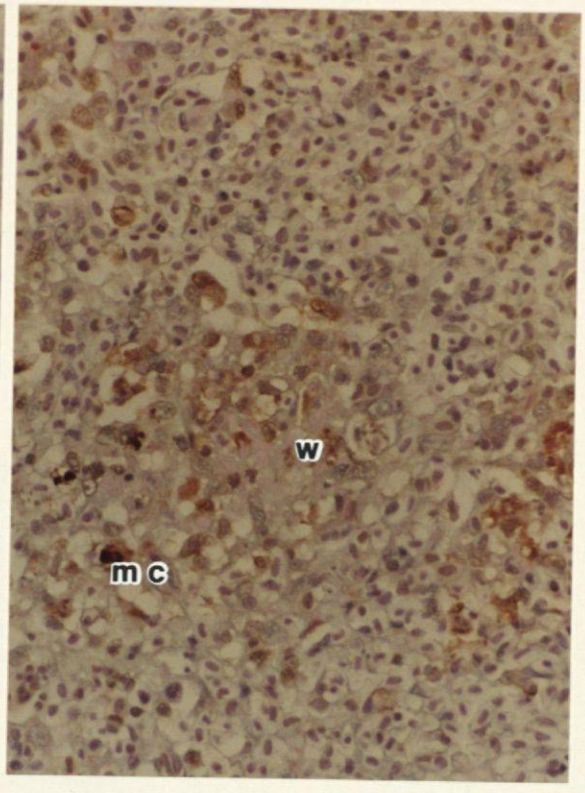
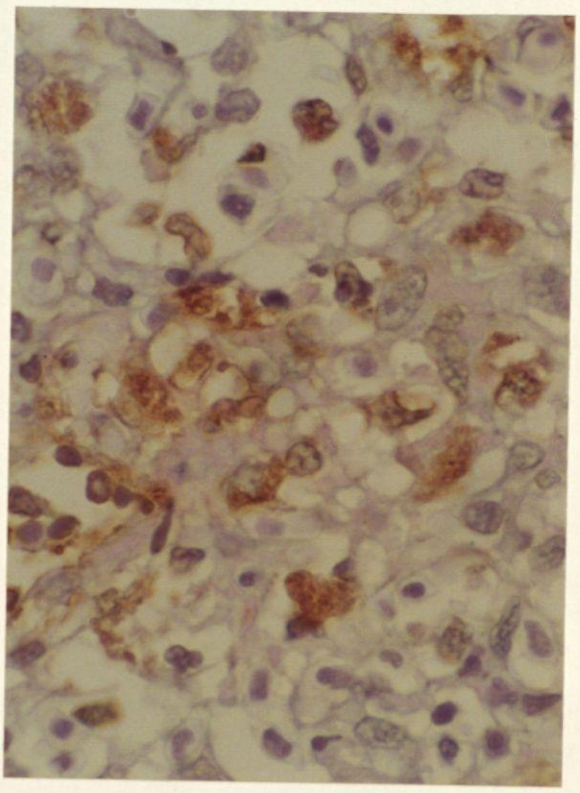


Plate 6.12 **Sections of spleen and liver from fish, reared at Farm A, with chronic enteric redmouth disease (ERM). DAB, AB/PAS and Carazzi's haematoxylin treated.**

A: Brown deposit of DAB showing localisation of *Y. ruckeri* antigen in spleen (x750).

B: *Y. ruckeri* antigen (arrow) in capillary tissue within the hepatic sinusoids (x750). Note the two cord structure of the hepatocytes and a lymphocyte (ly).

A



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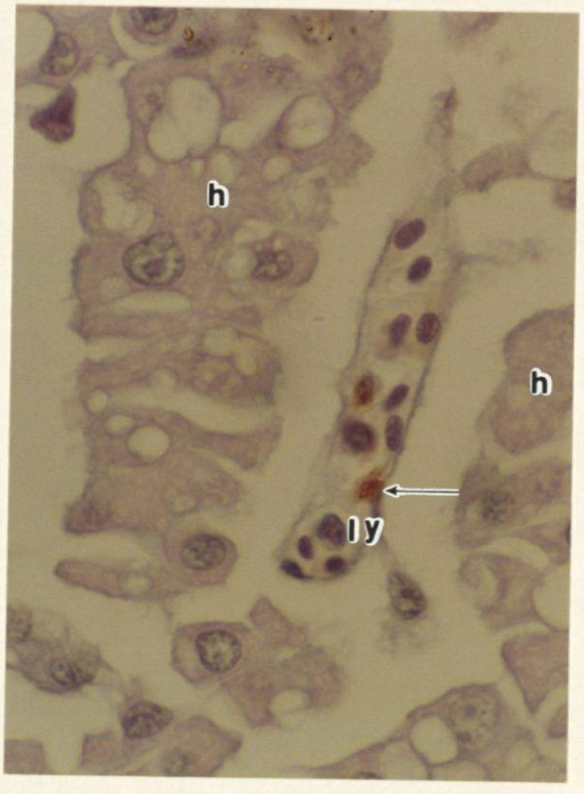

6.4 DISCUSSION

Artificial challenge by immersion or injection, to determine the virulence of *Y. ruckeri* serotype 01, has been reported in many published studies (Busch and Lingg, 1975; Bullock *et al.,* 1976; Johnson *et al.,* 1982b; Newman and Majnarich, 1982; Amend *et al.,* 1983; Bullock *et al.,* 1983; Rodgers and Austin, 1983; Cipriano and Ruppenthal, 1987). However, a comparison between ip injection and anal intubation is reported here for the first time. The LD_{50} by ip injection was 1.3×10^4 c.f.u. ml^{-1} which compares favourably with a 68% mortality in 10 days, after a challenge of 1.3×10^3 c.f.u./fish (Amend *et al.,* 1983) and up to a 60% mortality, after injection of 3×10^3 c.f.u./fish (Chilmonczyk and Oui, 1988). The LD_{50} by anal intubation, used as a challenge in the infection experiments, was 1.3×10^7 c.f.u. ml^{-1} which compares most closely with 8.5×10^5 c.f.u./fish, by subcutaneous injection (Anderson and Ross, 1972), 3×10^5 c.f.u. ml^{-1} by immersion (Bullock and Anderson, 1984) and 1.1×10^6 c.f.u. ml^{-1} by immersion (Knittel, 1981). Challenge by anal intubation proved to be a reliable and reproducible method for studying the progression of *Y. ruckeri* infection in vaccinated and non-vaccinated rainbow trout but was less sensitive than ip injection. There were no mortalities in vaccinated fish and only 3/50 (6%) in non-vaccinated fish challenged anally by 4.5×10^6 c.f.u. ml^{-1}. The lower challenge dose did not result in any mortalities.

The decline in faecal recovery from both groups, using either level of initial challenge, may simply reflect the characteristic bacterial lag phase induced immediately after infection by the harsh intestinal environment. However the subsequent log phase, shown by an increase in recovery, was both delayed and lower in vaccinated fish. In fact it was not possible to isolate *Y. ruckeri* after 4 days using a high dose infection or after 2 days with a low dose infection. In contrast to these findings there was immediate recovery of *Y. ruckeri* from the kidney material of vaccinated fish between 1 and 24 h after challenge. This on the whole also coincided with less plate growth as defined by more countable colonies, as opposed to profuse

growth. Although the low dose challenge gave similar results the overall effect was lower and less marked.

The bacteriological results indicating recovery are supported by the visualisation of *Y. ruckeri* antigen using the immunoperoxidase technique. Both methods were complimentary and seemed to indicate that uptake of *Y. ruckeri* occurred in vaccinated and non-vaccinated fish. However, uptake happened much more quickly in vaccinated fish and appeared to be an active process. In addition, the course of infection was largely over within the first 4 days compared with the full 10 days for non-vaccinated fish. Undoubtedly, therefore, use of the anti-*Y. ruckeri* vaccine protected the rainbow trout against ERM.

The fish sampled from a fish farm gave a similar result for antigen localisation using immunoperoxidase. However, the fish were under going chronic ERM and had similar localisation patterns to the non-vaccinated fish in the laboratory experiment. Anderson and Ross (1972) concluded that the immune response could be overwhelmed by large numbers of virulent bacteria which could happen in poorly managed fish hatcheries where sanitary precautions are not taken. This would also be true for continual exposure to *Y. ruckeri* over a period of weeks or repeated exposure over a period of months, such as happened at Farm A.

Apart from individual fish with slightly raised serum antibody levels there were no appreciable differences between vaccinated and non-vaccinated fish. This indicates that although an antibody response may have played a role in later infection it had not developed sufficiently to protect fish 14 days after vaccination or in the subsequent 10 day period after challenge. However, the raised levels in intestinal mucus, as measured only by ELISA, indicated that there may have been a local secretory response in vaccinated fish. These levels in conjunction with the bacteriological results and the immunoperoxidase technique, clearly demonstrated a certain level of protection soon after challenge (i.e. 1-48 h). This apparent contradiction between antibody levels in the serum and recovery indicates that there was either quicker uptake in vaccinated fish, at the gut mucosal level, or the same

level of uptake, compared with non-vaccinated fish, but followed by a more rapid immune (i.e. phagocytic) response after vaccination.

There has been conflicting evidence, in published studies, concerning the presence of circulating antibody during ERM infection. Busch (1978) reported that rainbow trout developed antibodies against protein-based soluble antigens after 13 days whereas a response to particulate antigens, as used in this study, was only detected after 21 to 28 days. Cossarini-Dunier (1986b) concluded that protection was independent of agglutinating antibody and probably due to cell-mediated immunity instead. Olesen (1991) did not detect an antibody response in immersion-vaccinated rainbow trout using ELISA. Cipriano and Ruppenthal (1987), in a study of cross-protection, also concluded that there was a lack of correlation between protection and circulating antibodies. In addition, apart from a cell-mediated immune response, they thought that a localised response could be involved in protection against *Y. ruckeri.*

Temperature has also been shown to affect the production of a humoral response with antibody being detected after 14 days at 17°C but not at 13°C (Anderson *et al.*, 1979b), as used throughout this study.

The role of the cellular reponse was indicated by Herraez and Zapata (1987) by studying the melano-macrophage centres of goldfish. Their results suggested a non-defined involvement for the splenic and nephric cells after exposure to *Y. ruckeri* but interestingly they also concluded that the results were independent of antibody titres. In addition, they noted phagocytosis in renal tubule epithelial cells. Phagocytosis was also seen by Zapata *et al.* (1987) after *Y. ruckeri* antigen was taken up by the gill epithelial cells of Atlantic salmon.

These published findings tend to support the results of this study in so far as the immediate protection afforded by vaccination against *Y. ruckeri* was not due to the production of circulating antibodies but to the possibility of a local intestinal response and an alternative pathway of the immune response. Unfortunately it was only possible to use *Y. ruckeri* as an antigen in these experiments. Therefore is was not

possible to determine whether the heightened immune response observed in vaccinated fish was specific for *Y. ruckeri* or non-specific for other bacterial antigens.

The sequential uptake of peroxidase in the mouse intestine has been studied by Owen (1977). Essentially, aggregations of lymphoid follicles (i.e. Peyer's patches) contain membranous epithelial or 'M' cells and these were shown to take up peroxidase within 1 h by pinocytosis. Uptake of *Salmonella typhimurium* from mouse intestine with subsequent distribution between Peyer's patches and the mucosal layer has been reported by Hohmann *et al.* (1978). Hanski *et al.* (1989) showed that *Yersinia enterocolitica* could be found in the lamina propria of the mouse intestine within 20 h after oral challenge and then in the Peyer's patches. The authors concluded that this could have been partly due to ingestion by migrating macrophages. However, the number of phagocytosed bacteria was small in relation to the challenge dose and transport via the lymphatic channels was suggested as an additional mechanism.

Although Peyer's patches are not present in the intestine of fish a similar uptake and transport mechanism has been reported. Rombout and Van den Berg (1989) showed the uptake of ferritin and *V. anguillarum* from the intestinal lumen of carp into the epithelial cells of the gut. In turn the ferritin then reached the supranuclear vacuoles and was finally transported within intraepithelial macrophages. The authors postulated that small mobile macrophages were capable of inducing a systemic immune response whereas the larger macrophages stayed in the intestinal epithelium and could be involved in a local or mucosal response.

In order to infect a host the virulence mechanisms of an invading bacterium, in this case *Y. ruckeri,* must allow the organism to adhere, gain entry and avoid the immune system for long enough to cause disease. Adherence of *Y. ruckeri* to the intestinal wall was seen in non-vaccinated fish after 24 h in the immunoperoxidase experiment but not in vaccinated fish. This has also been reported for *Vibrio anguillarum* within 100 min of exposure (Horne and Baxendale, 1983), with the authors also showing less adherence in vaccinated fish. In contrast to the non-

vaccinated group, vaccinated fish in this study showed active uptake of *Y. ruckeri* within the first 8 h after challenge, by a process similar to pinocytosis. Davina *et al.* (1982) showed that ingested but inactivated *V. anguillarum* antigen administered to the cyprinid *Barbus conchonius* was taken up by pinocytosis in the epithelial cells of the posterior intestine, within 30 min of exposure. Uptake was localised in supranuclear vacuoles and intraepithelial macrophages. A more recent study reported rapid intestinal uptake of a *V. anguillarum* bacterin in turbot and sea bass following administration by anal intubation (Vigneulle and Baudin Laurencin, 1991). An immunofluorescence study showed that when the bacterin disappeared from the lumen, by day 1, it became distributed within the intestinal epithelium and subepithelial connective tissue. Aoki *et al.* (1986) showed that vaccinated ayu, artificially infected with *V. anguillarum*, had the ability to eliminate the bacteria from the intestinal tract. They concluded that elimination could be related to lymphocytes, neutrophils and macrophages. Fujino *et al.* (1987) used immunoperoxidase to show the uptake of rabbit immunoglobulin into the columnar epithelial cells of the intestine of rainbow trout. The study indicated that the most active uptake was in the posterior part of the intestine.

The close association between lymphoid cells and the absorptive epithelia of the intestine in teleosts has been shown by several studies (Rombout *et al.*, 1985; Temkin and McMillan, 1986; Doggett and Harris, 1987; Doggett and Harris, 1991). In addition, Noaillac-Depeyre and Gas (1973) indicated that bioactive proteins could be engulfed by macrophages located at the base of the epithelia. Georgopoulou *et al.* (1988) showed that ingested horseradish peroxidase passed through the intestinal epithelium of rainbow trout into the circulatory system. The work further demonstrated that once the antigen came into contact with lymphoid cells, particularly macrophages, it could be internalised and the process could induce a local immune response. Johnson and Amend (1983) showed that anal intubation gave better protection against ERM than either bath or oral vaccination. The work reported here and the previously published studies have obvious implications for the oral

vaccination of fish using particulate antigens. However, targetting of the antigen to achieve the most efficient response but at the same time minimising potential denaturation within the intestine would be important. Therefore, the use of an adjuvant or a micro-encapsulation technique could lead to improved fish vaccines.

There seems little doubt that antigens within the intestine of fish, which are associated with the mucosal layer, can be taken up by an active process and pass through the epithelial layer where they can form the basis of an immune response. This was shown in the present study despite the lack of detectable serum antibody or a significant mucosal response. Nevertheless, the protection demonstrated in vaccinated fish points to the priming of that response.

Phagocytosis of foreign material both serves as a defence mechanism in itself and as an initial step in the onset of the specific immune response (Rijkers, 1982). Following ip injection of particulate matter into plaice Ellis *et al.* (1976) demonstrated subsequent phagocytosis by macrophages in the pronephros, mesonephros and spleen. In addition, some macrophages were shown to form aggregates with melanomacrophages. In the present study areas of *Y. ruckeri* antigen deposition were seen in non-vaccinated fish associated with spleenic melanomacrophages. This seemed to occur, particularly in later infection, as focal aggregates. Although the same type of deposition was noticed in vaccinated fish it did not progress to being focal and appeared at a lower level. Lymphocytes and monocytes were also seen in greater numbers, associated with areas of antigen, around melanomacrophages in vaccinated fish.

Complement is an enzyme cascade system composed of about 12 protein components (Roberts, 1989). It can be involved in the clearance of foreign substances by mediating inflammatory vasodilation, chemotactically attracting leucocytes and promoting ingestion by phagocytes (Roberts, 1989). Complement can be activated by the classical pathway which requires the antigen to be coated by antibody or the alternative pathway which is activated by contact with bacterial cell wall polysaccharides (e.g. LPS) (Rijkers, 1982). Certain Gram-negative bacteria

activate the complement system in the absence of antibody (Fletcher, 1981) but cell lysis, mediated for instance by complement in intestinal mucus, also requires lysozyme in order to disrupt the polysaccharide wall (Ellis, 1981). C-reactive protein (CRP) is also capable of activating complement in mammals but its defensive role in fish has not been elucidated, although there is evidence that it participates in immediate hypersensitivity reactions (Roberts, 1989) and as a modulator of the inflammatory response (Fletcher, 1981). It has also been suggested that complement mediates immune adherence and enhances phagocytosis and killing of bacterial cells (Ellis, 1981). CRP has been shown in the plaice (Pepys *et al.*, 1978; White *et al.*, 1981) and the lumpsucker (Fletcher *et al.*, 1981).

During an inflammatory response macrophages become activated by developing increased phagocytic and killing properties (Ellis, 1981). Although they occur in many tissues, such as the peritoneum, they are mainly found as reticuloendothelial cells in the kidney and spleen (Roberts, 1989) but also occur as wandering cells (Ellis, 1981). Monocytes, also found in the kidney, are considered to be the precursors of tissue macrophages and are capable of migrating to an area of inflammation where they become actively involved in phagocytosis (Rowley *et al.*, 1988). The involvement of a complement-mediated phagocytic process has been shown in channel catfish by Scott *et al.* (1985) with the phagocytosis of *Edwardsiella ictaluri*, another enteric fish pathogen like *Y. ruckeri*. This type of activation is often termed opsonisation (Roberts, 1989). Some macrophages contain melanin and are termed melanomacrophages. The role of the melanin is not certain but it may play a role in bactericidal mechanisms or as a modulator of bactericidal mechanisms (Roberts, 1989). In addition, this may involve the production of free radicals by phagocytic cells (Ellis, 1981). Rowley *et al.* (1988) consider that there is circumstantial evidence pointing to migration of cells to the melanomacrophage centres (MMC), since diseased fish often show an increase in size of MMC resulting from the addition of macrophages from other sites, such as the splenic ellipsoids. The authors further suggested that MMC function by accumulating antigen which is

then released to immunocompetent cells in a controlled manner so as to avoid immunological paralysis. This type of accumulation was noticed in this current study in non-vaccinated fish, particularly in spleen 7 days after infection.

The specific aspects of the immune response in fish, namely, humoral (antibody), cell-mediated and memory are initiated and mediated by lymphocytes (Roberts, 1989). Since serum antibodies were not detected in this study and the experiment, which lasted 10 days, could not have shown an immunological memory in vaccinated fish, the involvement of a cell-mediated response (CMR) was possible. In higher vertebrates there are two distinct populations of lymphocytes: T (thymus-derived) and B (bone marrow-derived) (Rowley *et al.* 1988). The T cells are responsible for the CMR whereas the B cells are responsible for antibody production (Roberts, 1989) but it is still not known whether the exact equivalents of these cells occur in fish (Ellis, 1986; Rowley *et al.*, 1988). However, the general characteristics of immunity related to T and B lymphocytes are exhibited by fish (Ellis, 1986). Nevertheless, very little, if any thing, is known about the involvement of the CMR with bacterial fish pathogens. Most studies, to date, have dealt with transplantation immunity and lymphocytes have been observed in the rejection process (Roberts, 1989).

Unfortunately, the present study could not address all the issues concerning the reasons for short term protection associated with an intestinal mucosal immune response in vaccinated rainbow trout. However, the results indicated that there was more rapid uptake of *Y. ruckeri* antigen, by an active process, following vaccination. The actual process involved clearly needs further study but it is most likely that vaccination using formalin-killed whole cells (LPS) of *Y. ruckeri* stimulates the complement system which, in conjunction with lysozyme, is involved in subsequent bacterial lysis following artificial anal challenge. In addition, a local intestinal, mucosal secretory response may be involved initially. Those bacterial cells which remain unlysed are possibly then transported by monocytes and macrophages, via the lymphoid system, to the melanomacrophage centres, particularly in the ellipsoids of

spleen. Once trapped in this way the antigen could form an immune complex involving the equivalent of mammalian T lymphocytes and be slowly degraded. It is also likely that a serum humoral response would then take place but at a much later stage in infection.

CHAPTER 7

SURVEY TO DETERMINE THE USE OF VACCINATION AND ANTIMICROBIAL AGENTS FOR CONTROL OF *YERSINIA RUCKERI*

7.1 INTRODUCTION

Although the effects of ERM on farmed susceptible species are well documented (Rucker, 1966; Wobeser, 1973; Bentley, 1982; Fuhrmann *et al.*, 1983; Busch, 1983; Warren, 1983; Bullock, 1984; Horne *et al.*, 1984; Rübsamen and Weis, 1985; De La Cruz *et al.*, 1986; Meier, 1986 and Sparboe *et al.*, 1986) the collection of basic data concerning the incidence of the disease in England and Wales has never been attempted. Consequently, a postal survey was devised in order to gather information relating not only to the occurrence of ERM, but also to the use of vaccination and antimicrobial agents for the control of *Y. ruckeri*.

7.2 MATERIALS AND METHODS

A total of 217 salmonid fish farms in England and Wales were selected to receive a postal questionnaire, comprised of 20 main questions, between August and September 1989. The form, accompanied by an explanatory letter (Table 7.1) and brief completion notes (Table 7.2), was divided into 5 sections. This meant that data could be collected on general farm production figures, on-farm facilities, details of enteric redmouth disease, vaccination particulars and the usage of antimicrobial agents (Table 7.3). In order to preserve confidentiality and to improve the response farms were not required to identify themselves when returning the questionnaire. However, 30 farms known to have had ERM diagnosed in the past received questionnaires with a simple identification mark. Although it was not possible to identify which farm returned which questionnaire, it was possible to determine whether the section on ERM had been filled in correctly. These forms therefore acted as controls.

Fish farms were chosen using a commercially available database of British fish farms (Clearwater, Oxon). Farm selection was based on whether or not rainbow trout, brown trout or Atlantic salmon were held on site.

Table 7.1 Explanatory letter for questionnaire

Dear Fish Farmer,

SURVEY OF ENTERIC REDMOUTH VACCINATION

I am enclosing a questionnaire with this letter which I hope you will find time to complete. The forms are being sent to most salmonid fish farms and are intended to help provide information about the use of vaccines against enteric redmouth disease (ERM). They have been designed for ease of completion and most questions only require a simple tick at a correct choice. The remainder, however, only need the minimum of input.

The survey does not require you to fill in your name and address which means therefore that your reply will be completely confidential. The information will be analysed for general trends and cannot be attributed to individual sites.

Once collated the results will form an integral part of a PhD thesis on the epidemiology of ERM. As such they will be a valuable addition not only to our current knowledge but also to future strategies concerning the economic affect and progress of this disease within the industry. A good response to the questionnaire is therefore absolutely essential and I would be grateful if you can return the survey forms in the **FREE POST** envelope within 14 days of receipt.

Thank you in advance for taking the trouble to complete the questionnaire and I hope the exercise does not prove too time consuming.

Yours sincerely,

C. J. RODGERS

Table 7.2 Notes for questionnaire

NOTES ON FORM COMPLETION

The method for completing the forms is reasonably self explanatory. However, the following short notes can be used for guidance.

1. All recipients should complete Section A (question 1 **or** 2) **and** Section B.

2. If the answer to question 4 in Section C is **'No'** proceed to Section D.

3. If the answer to question 10 in Section D is **'No'** proceed to Section E.

Table 7.3 Questionnaire-page 1

ENTERIC REDMOUTH SURVEY

USE OF VACCINATION AND ANTIBIOTICS

SECTION A. PRODUCTION

1. TABLE FARMS

Production Figure (Tonnes per annum)

☐ 0-10 ☐ 11-25 ☐ 26-50 ☐ 51-100

☐ 101-150 ☐ 151-200 ☐ 201-250 ☐ 251-500 ☐ 501-1000

2. EGG FRY AND FINGERLING FARMS

OVA Production Quantity (Numbers per annum)

☐ 0-500,000 ☐ 500,000-1 million ☐ 1-2 million

☐ 2-5 million ☐ 5-10 million ☐ More than 10 million

FRY Production Quantity (Numbers per annum)

☐ 0-500,000 ☐ 500,000-1 million ☐ 1-2 million

☐ 2-5 million ☐ 5-10 million ☐ More than 10 million

FINGERLINGS Production Quantity (Numbers per annum)

☐ 0-500,000 ☐ 500,000-1 million ☐ 1-2 million

☐ 2-5 million ☐ 5-10 million ☐ More than 10 million

SECTION B. FACILITIES

3. Type of facilities on farm.

	Nos	Hold capacity Fish	Hold capacity Water	Average water temperature Jn-Mar	Apr-Jn	Jl-Sep	Oct-De	Water* Supply
Fry tanks								
Fry raceways								
Grow on tanks								
Grow on raceways								
Earth ponds								

*Choose from: Spring (SPR), borehole (BOR) or river (RIV)

4. Within which Water Authority area is the farm?

☐ Anglian ☐ Northumbrian ☐ North West ☐ Severn-Trent ☐ Southern
☐ South West ☐ Thames ☐ Welsh ☐ Wessex ☐ Yorkshire

Table 7.3 Questionnaire-page 2 (continued)

SECTION C. ENTERIC REDMOUTH DISEASE

5. Has ERM ever been diagnosed? ☐YES ☐NO. If Yes which was first year? 19__
which years since? 19__
19__
19__

6. Who diagnosed ERM? ☐ All

☐ MAFF ☐ VIC ☐ Vet ☐ Water Authority
☐ Consultant ☐ Other (specify):

7. What time of year does ERM occur? ☐ Jan-Mar ☐ Apr-June ☐ July-Sept ☐ Oct-Dec

8. Size of fish affected: ☐ 100/lb-7oz ☐ 8oz-1lb ☐ 1lb-5lb

9. Fish species affected: ☐ Rainbow ☐ Brown ☐ Salmon
☐ Other (specify):

10. Does ERM coincide with: ☐ Poor water quality (specify):
☐ Recent grading ☐ Over crowding ☐ River flooding
☐ Rise in temperature (specify range):
☐ Fall in temperature (specify range):
☐ Other (specify):

SECTION D. VACCINATION

11. Do you vaccinate your stocks? ☐ YES ☐ NO

If Yes:

	Rainbow	Brown	Salmon
Vaccination on site	☐YES ☐NO	☐YES ☐NO	☐YES ☐NO
Time from vaccination to transfer to other site	days	days	days
Buy fish already vaccinated	☐YES ☐NO	☐YES ☐NO	☐YES ☐NO
Age at vaccination	weeks	weeks	weeks
Use of recommended dilution	☐YES ☐NO	☐YES ☐NO	☐YES ☐NO
Year started vaccination	19__	19__	19__
Each year since start	☐YES ☐NO	☐YES ☐NO	☐YES ☐NO
Annual cost of vaccine	£	£	£
Source of vaccine*			

*Choose from: Aquaculture Vaccines Ltd (AVL), Stirling (STR), Vetrepharm (VET), Other (OTH)

12. Have there been any breakdowns or failures in vaccination? ☐ YES ☐ NO

13. If Yes, has this been attributed to:

☐ Poor fish condition at vaccination
☐ Low temperature at vaccination
☐ Natural challenge too close to vaccination
☐ Other (specify):

Table 7.3 Questionnaire-page 3 (continued)

14. Would you now contemplate NOT using vaccination? ☐ YES ☐ NO

15. If Yes, identify reason:
- ☐ Too expensive
- ☐ Ineffective
- ☐ Difficult to obtain
- ☐ Difficult to administer
- ☐ Other (specify):

SECTION E. ANTIBIOTIC USAGE

16. Which antibiotics do you use on site?

	Length of av. treatment	Number of treatments p.a.	Cost per annum	Treatment effective
☐ Oxolinic acid	days	☐1 ☐2 ☐3 ☐4	£	☐YES ☐NO
☐ Oxytetracycline	days	☐1 ☐2 ☐3 ☐4	£	☐YES ☐NO
☐ Terramycin	days	☐1 ☐2 ☐3 ☐4	£	☐YES ☐NO
☐ Methasul	days	☐1 ☐2 ☐3 ☐4	£	☐YES ☐NO
☐	days	☐1 ☐2 ☐3 ☐4	£	☐YES ☐NO
☐	days	☐1 ☐2 ☐3 ☐4	£	☐YES ☐NO
☐	days	☐1 ☐2 ☐3 ☐4	£	☐YES ☐NO

17. Do you use any of the above compounds as a prophylactic? (i.e. As a precaution before problems develop)

☐ Always ☐ Occasionally ☐ Never

18. Do you need to use another compound when the first treatment is completed?

☐ Always ☐ Occasionally ☐ Never

19. Where do you obtain antibiotic?

☐ Veterinary surgeon ☐ Wholesaler ☐ Manufacturer
☐ Other (specify):

20. Any further comments?

Thank you for your help in completing the form. Please return it to C.J.Rodgers at Polytechnic South West, Plymouth in the FREEPOST envelope.

7.3 RESULTS

Responses were received from 90 of the 217 farms (41.5%) supplied with a questionnaire. Since most main questions were sub-divided, the survey response level of 41.5% meant that over 3,400 responses needed collating. Consequently, the results appeared to be quite complex and are therefore presented not only as tabular data but also as figures.

7.3.1 Farm characteristics

Of the farms responding, 82% were involved in table farming, 30% in fingerling production, 29% in fry production and 21% in ova production.

Those farms that were involved in both table fish and egg, fry and fingerling production made up 26% of the total.

In addition, 58.9% of the table farms responding were producing up to 50 tonnes per annum. However, 47.4% of the responding farms produced up to 500,000 ova, 57.7% produced up to 500,000 fry, and 59.3% produced up to 500,000 fingerlings.

Essentially, the returns could be divided into 3 main sections for the purposes of evaluation (Figure 7.1):

(a) Those farms that have had ERM diagnosed at some time in the past represented 23% of the returns. The survey indicated that there had been no diagnosis prior to 1982.

(b) Those farms that have not had ERM diagnosed, but nevertheless had decided to vaccinate against *Y. ruckeri*, represented 31% of the returns.

(c) Those farms that have not had ERM diagnosed and were not vaccinating represented 46% of the returns.

It was noticeable that there was a tendency for those sites where ERM had been diagnosed, irrespective of vaccination, to be larger table farms with production ranging up to over 200 tonnes p.a. In contrast, farms without ERM were for the most part producing less than 100 tonnes p.a. (Figures 7.2a, 7.2b, 7.3a and 7.3b).

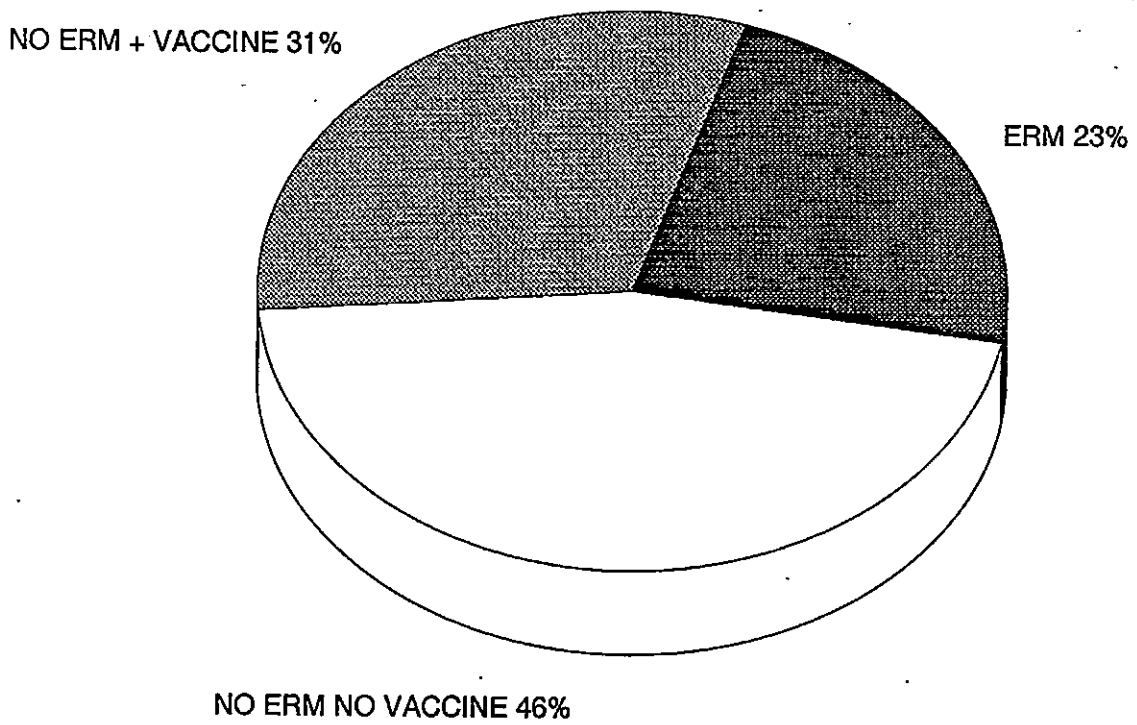

Figure 7.1 Distribution of responding fish farms

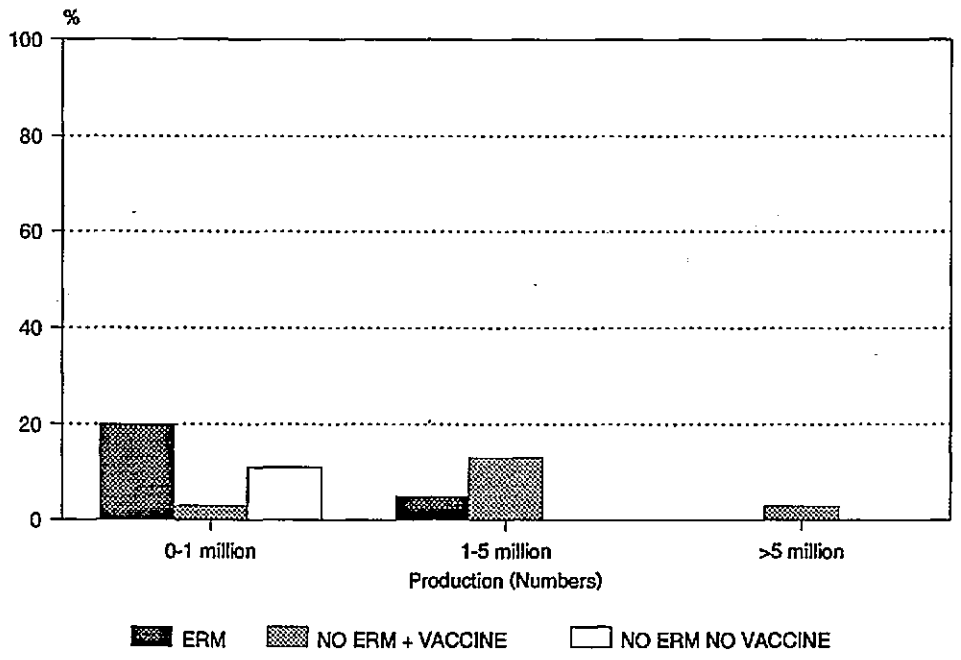

Figure 7.2a Production of ova farms

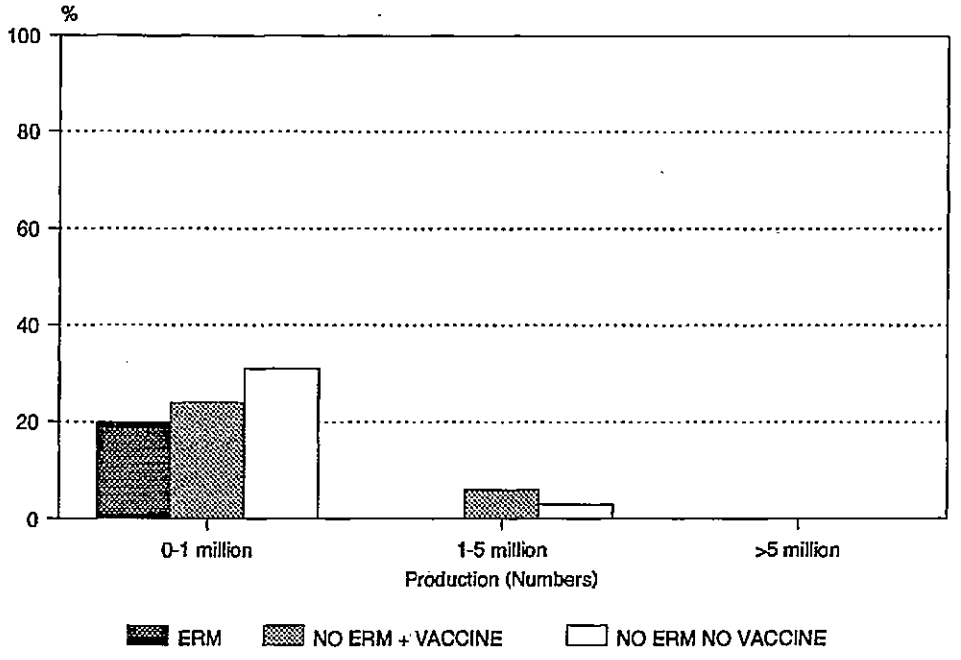

Figure 7.2b Production of fry farms

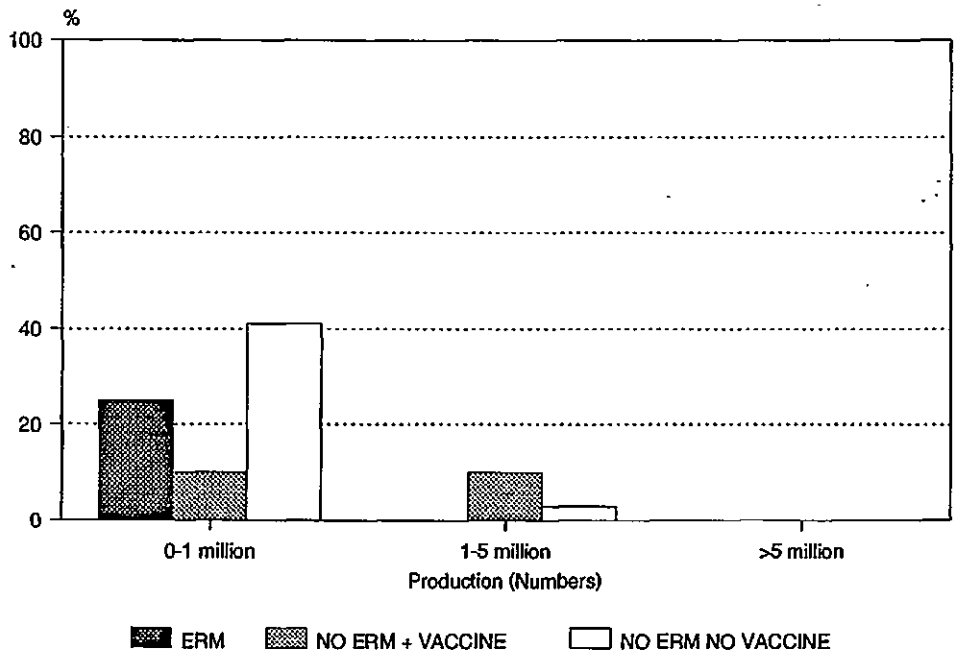

Figure 7.3a Production of fingerling farms

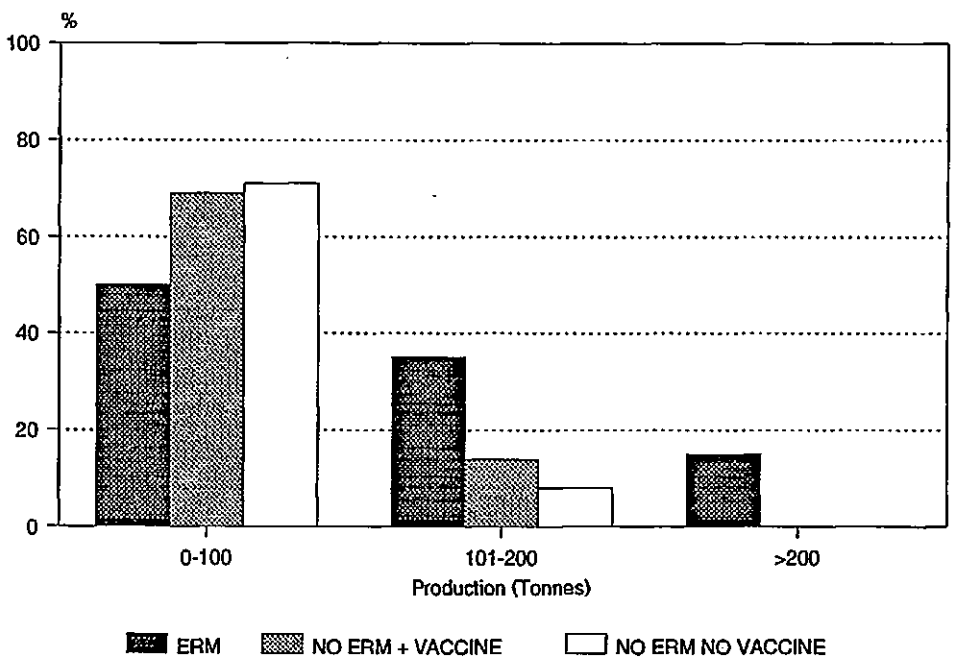

Figure 7.3b Production of table farms

The average number of tanks, raceways and earth ponds by farm type is detailed in Table 7.4. Essentially, the differences between farm types were small except for more than double the number of fry tanks on sites without ERM that were vaccinating.

Table 7.4 Type of on-farm facilities

Type of facility	ERM	No ERM and vaccinating	No ERM and not vaccinating
Fry tanks	14	31	14
Fry raceways	14	12	11
Grow-on tanks	11	11	14
Grow-on raceways	11	8	8
Earth ponds	9	8	14

The majority of the farms with ERM used river water, while those with no ERM were mainly on a borehole or spring supply. There were a few cage sites, most of which also had ERM (Figure 7.4).

The majority of responding fish farms were in the southern half of England and Wales. The pattern of farms by water authority area is shown in Table 7.5. Essentially, 47% of all farms were in Southern, South West and Wessex. The same percentage of farms with diagnosed ERM, however, occurred only in Southern and South West. Those farms that had not had ERM diagnosed but were vaccinating seemed to be mainly (44%) in South West and Wessex. The remainder of the farms, those not vaccinating, were fairly evenly divided among most of the water authorities.

Table 7.5 Fish farms by water authority area

Water authority	All farms	ERM	No ERM and vaccinating	No ERM and not vaccinating
Anglian	7%	5%	4%	13%
Northumbrian	0%	0%	0%	0%
North West	12%	10%	15%	11%
Severn-Trent	8%	10%	4%	11%
Southern	15%	25%	11%	8%
South West	17%	20%	22%	8%
Thames	9%	10%	4%	13%
Welsh	11%	5%	11%	18%
Wessex	15%	10%	22%	13%
Yorkshire	6%	5%	7%	5%

7.3.2 Time of ERM appearance

It was apparent that ERM was most prevalent on 69% of farms in the spring-to-autumn period (April-September) (Figure 7.5a). The only fish species affected by ERM in England and Wales appears to be rainbow trout, with 60% of sites identifying 222/kg-200 g (100/lb-7 oz) fish as being the most susceptible (Figure 7.5b). However, the survey showed that larger fish of up to 2.27 kg (5 lb) can also become infected. Most of the diagnosis of ERM was by MAFF (40%), a veterinary surgeon (30%) or a fisheries consultant (25%).

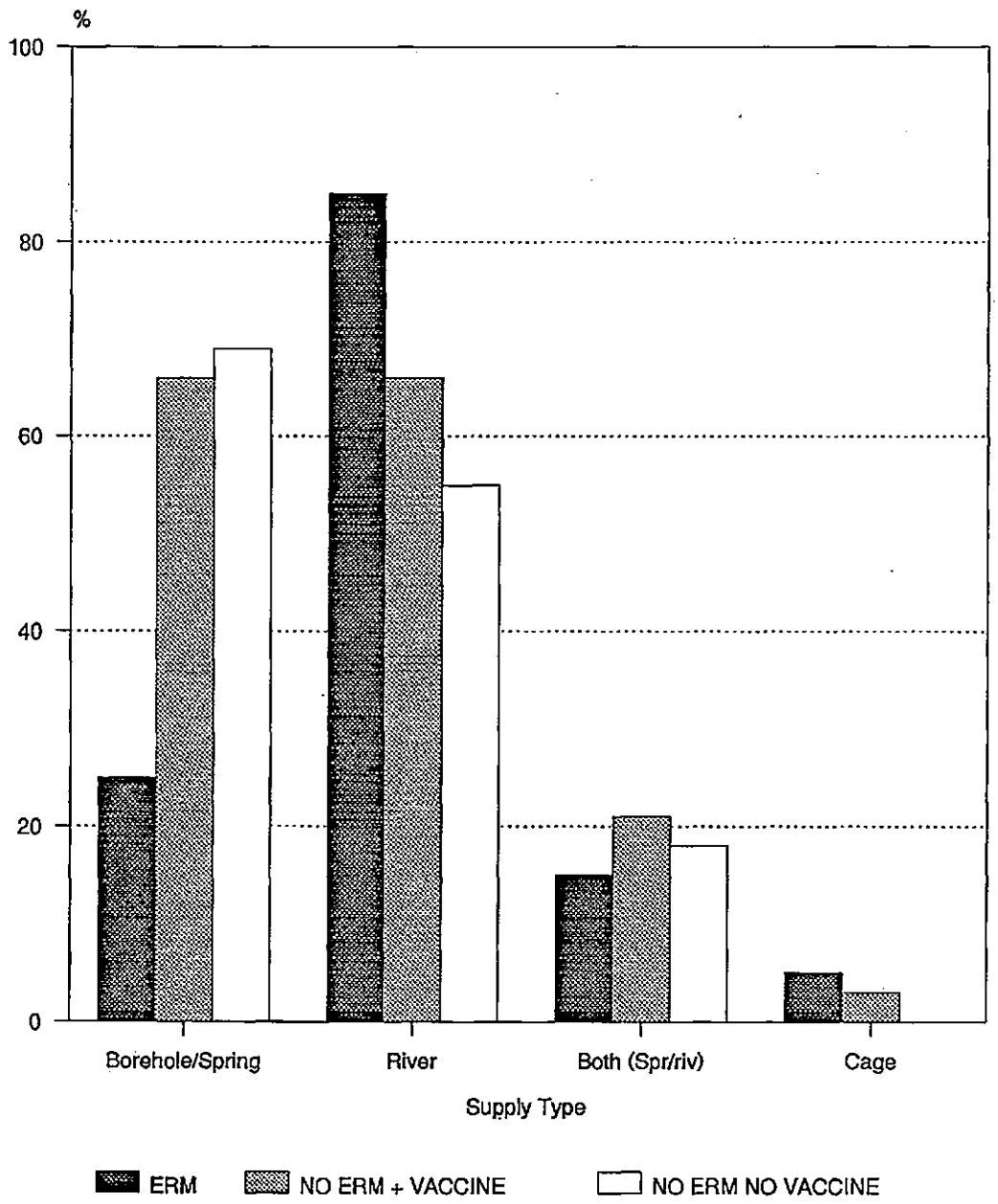

Figure 7.4 Type of water supply to responding farms

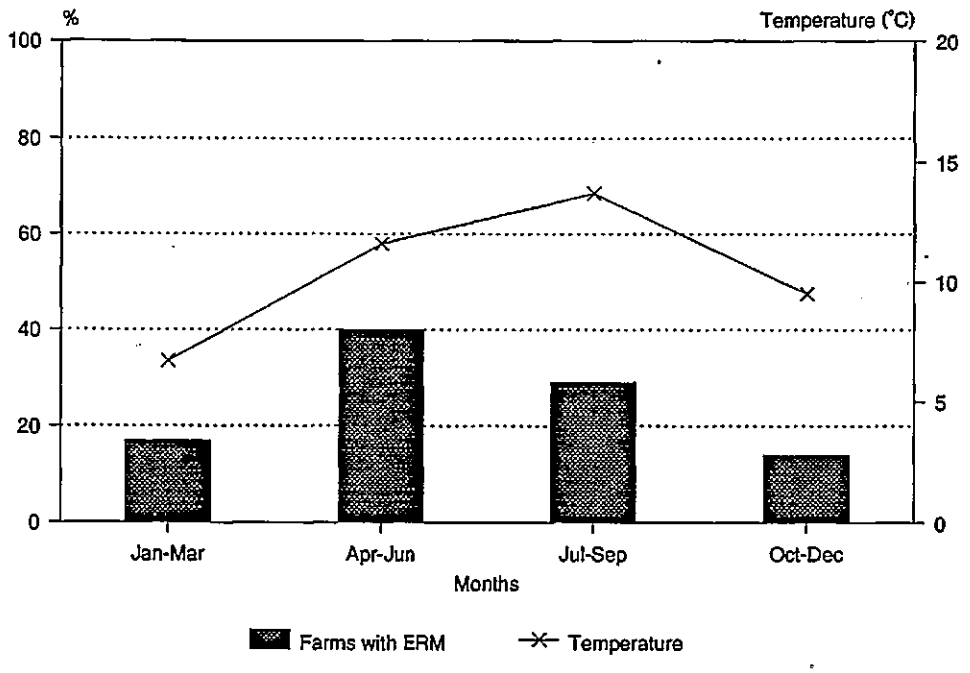

Figure 7.5a Seasonal incidence of ERM and average water temperature

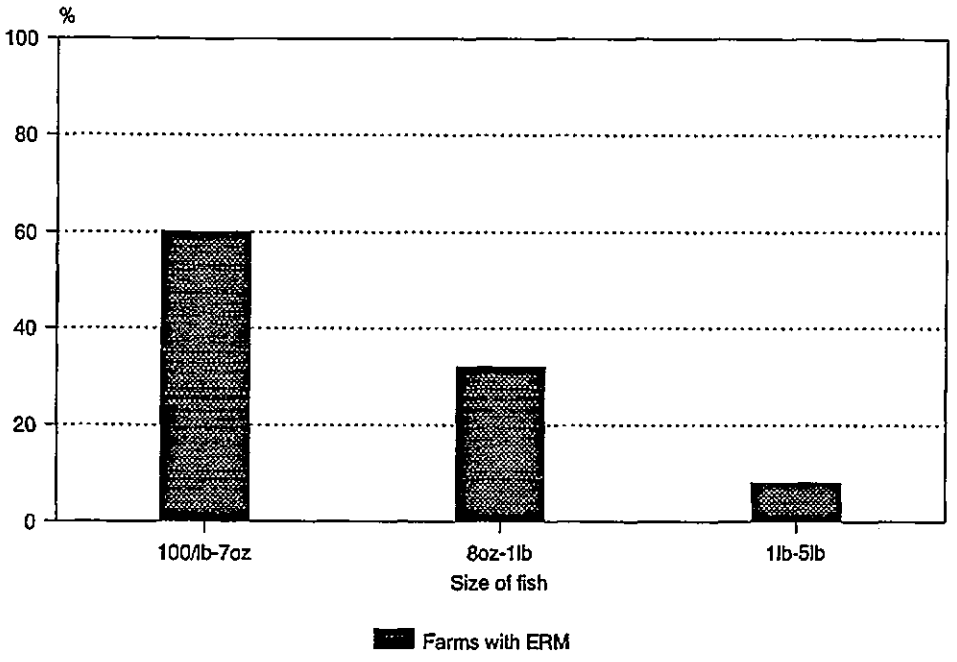

Figure 7.5b Size of affected rainbow trout

The fish farmers with rainbow trout affected by ERM considered that certain factors often coincided with the appearance of *Y. ruckeri.* These included, in order of importance, over-crowding of stocks, fluctuations in water temperature (particularly a rising temperature in the spring), recent grading, poor water quality (including low flow rates or low dissolved oxygen and high ammonia levels), river flooding and consequent silt build-up.

7.3.3 Use of vaccination

Of the farms choosing to vaccinate against ERM (53%) it was noticeable that the overwhelming majority (90%) of those with confirmed ERM were using an anti-*Y. ruckeri* vaccine, whereas farms without known ERM infection were fairly evenly divided with slightly more (57%) choosing not to vaccinate (Figure 7.6). Transfer of vaccinated fish to a second site and age at transfer are shown in Figure 7.7a and 7.7b. In addition, the survey showed that ERM vaccines have been in use, not surprisingly, since the date of first licencing in 1984, and that the average cost of vaccination for these farms at the time of the survey was £2,495 p.a. (range: £399-11,700), irrespective of whether the fish stocks had ERM or not. In fact, the amount spent on vaccination was slightly higher on farms with no ERM - £2,745 as opposed to £2,245.

Over 40% of sites with ERM also considered vaccination to be effective, but some 45% of such sites using a vaccine considered it to have failed in some way so that it did not offer adequate protection under prevailing farm management conditions (Figure 7.8a).

The reasons suggested for vaccine failure were poor fish condition at the time of vaccination, low water temperature at vaccination, fish being over-crowded or stressed after vaccination, 'pushing the fish too hard', or a suspect batch of vaccine.

As a result of these reported failures, 21% of all vaccinating farms (28% of farms with ERM) would consider abandoning vaccination. However, the majority intended to continue immunisation whether they have had ERM diagnosed or not (Figure 7.8b).

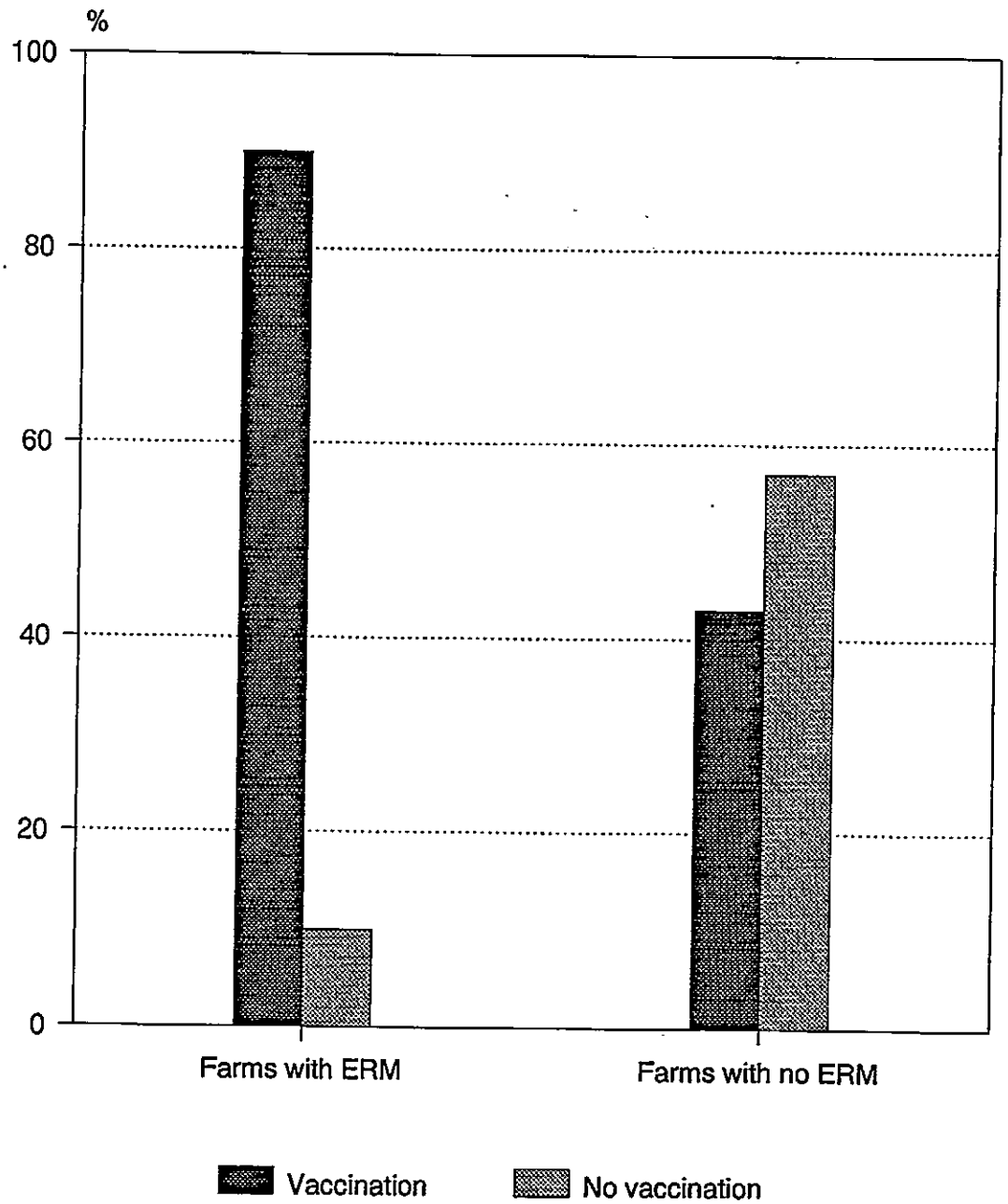

Figure 7.6 Vaccination on fish farms

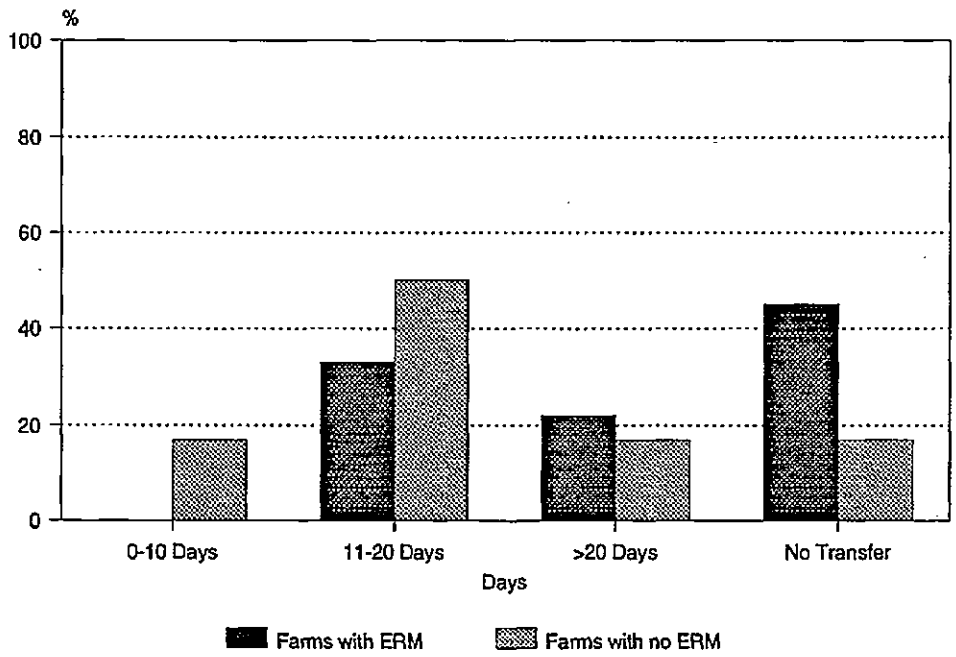

Figure 7.7a Transfer of vaccinated fish to a second site

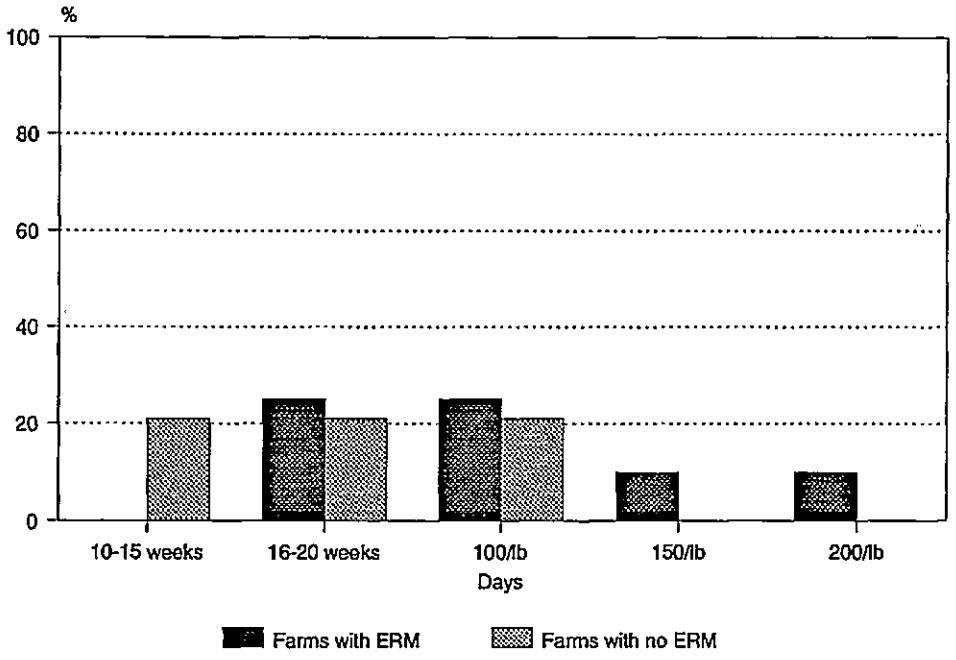

Figure 7.7b Age or size of vaccinated fish at transfer
(N.B. No attempt has been made to convert age in weeks to size by weight)

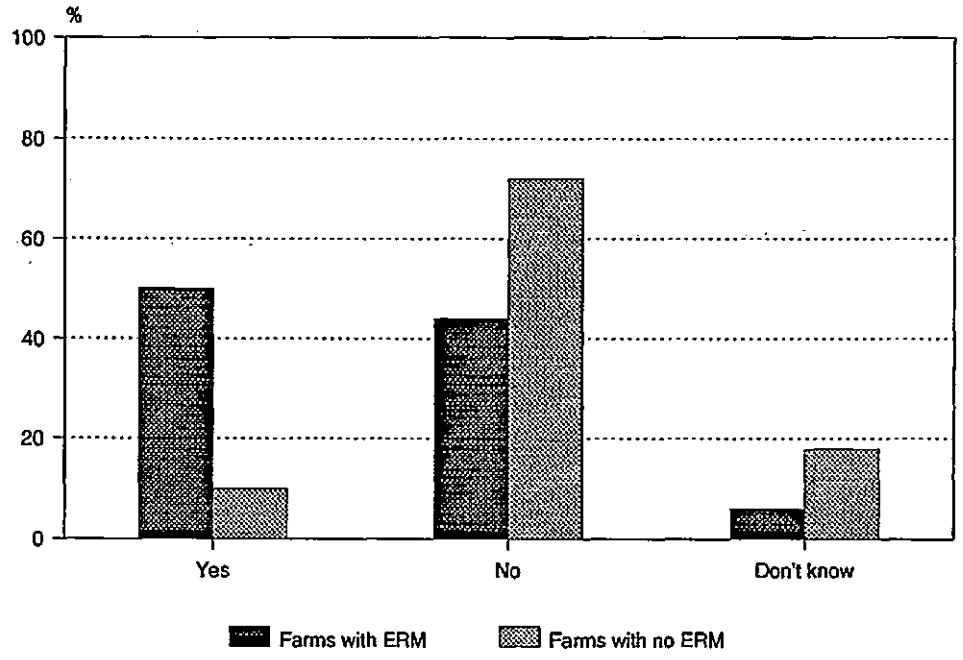

Figure 7.8a Failure of vaccination

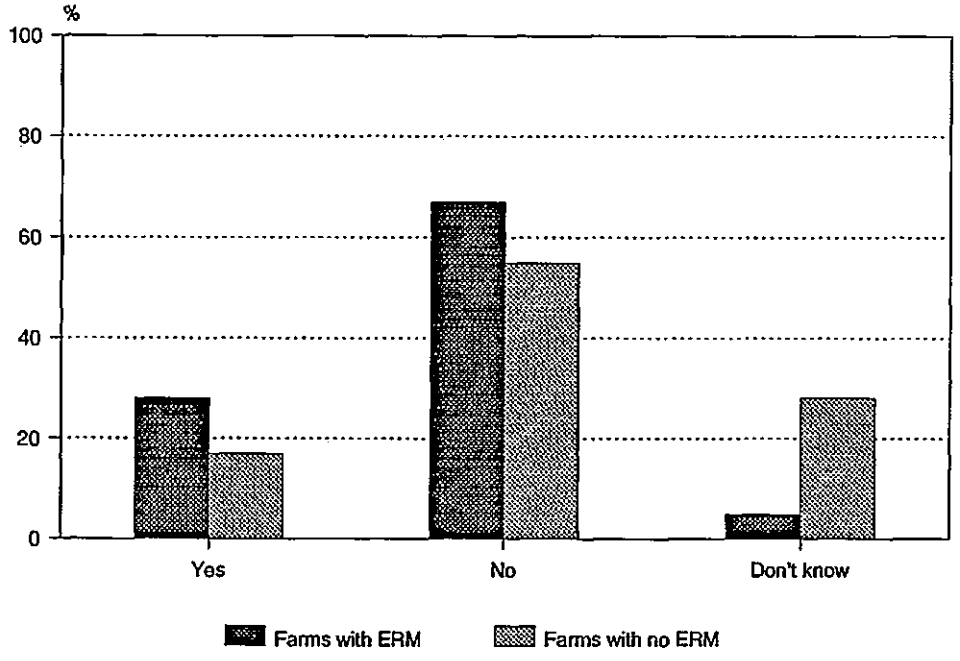

Figure 7.8b Continued use of vaccination

The reasons given for not vaccinating were that it could be ineffective or too expensive; additionally, some considered that good husbandry might mean that vaccination was unnecessary. Some producers would only vaccinate at the customer's request.

The majority of farms (75%) with ERM were buying in already vaccinated fish but only 50% were using the recommended vaccine dilution. These farms (78%) have also found it necessary to vaccinate their fish stocks every year since ERM was diagnosed whereas this only occurred on 48% of the other farms. These remaining answers to the questions on vaccination are collated in Table 7.6.

Table 7.6 Remaining vaccination statistics

	Farms with ERM	Farms without ERM and vaccinating
Buying vaccinated fish	75%	66%
Use of recommended dilution	50%	41%
Vaccination each year since start	78%	48%
Source of vaccine:		
Aquaculture Vaccines Ltd.	61%	43%
Vetrepharm Ltd.	22%	7%

7.3.4 Use of antimicrobial treatments

As expected a close correlation between the usage of antimicrobial agents and diagnosis of ERM was observed. Over 80% of the sites with ERM indicated that they were using an antimicrobial agent despite the majority (90%) of them also using a vaccine. The majority of sites (67%) that have not had ERM and are not vaccinating considered it unnecessary to use an antimicrobial agent (Figure 7.9a). The preferred treatment on sites with ERM was oxolinic acid, whereas the other farms preferred oxytetracycline, presumably for other bacterial fish pathogens such as *Aeromonas salmonicida* (Figure 7.9b). Farms that used both accounted for 41% of the sites (Figure 7.9b). In addition, over 80% of the farms, irrespective of whether they have had ERM or not, considered antimicrobial treatment to be effective. The average length of treatment was given as 7 days, which was a once-a-year application that occurred on 45% of farms. However, some sites needed more than 4 treatments a year. Length of treatment for oxolinic acid and oxytetracycline is shown in Figures 7.10a and 7.10b. Potentiated sulphonamides were only used on sites without diagnosed ERM, with vaccinating farms needing less (6%) than non-vaccinating farms (21%).

Prophylaxis was occasionally practised on 41% of sites with ERM, but only on 16% of sites with no ERM, although 6% of these sites always used an antimicrobial compound as a precaution (Figure 7.11a).

A second treatment using a different antimicrobial compound was occasionally used on 12% of sites with ERM and 26% of sites without ERM (Figure 7.11b).

The majority of farms (88%) with diagnosed ERM obtained their antibiotic from a veterinary surgeon. The sites with no diagnosed ERM got their antibiotic in the same way, irrespective of whether they were vaccinating (76%) or not (79%).

Table 7.7 compares the average annual cost of vaccination and antimicrobial treatment. The cost of treating with an antimicrobial agent was approximately £675 on sites with ERM, but approximately £222 on farms without diagnosed ERM.

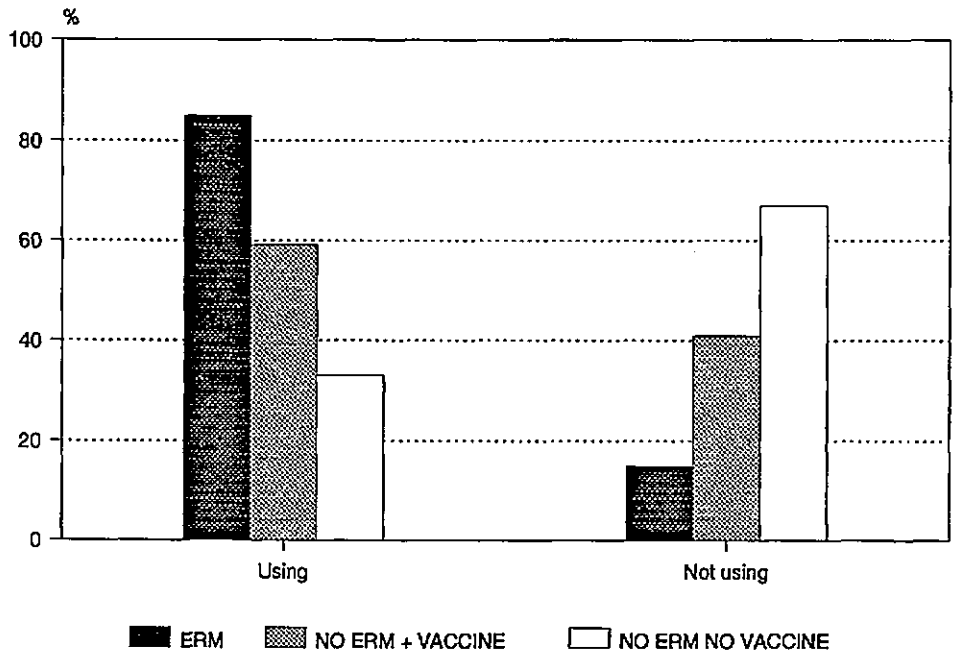

Figure 7.9a Use of antimicrobial agents

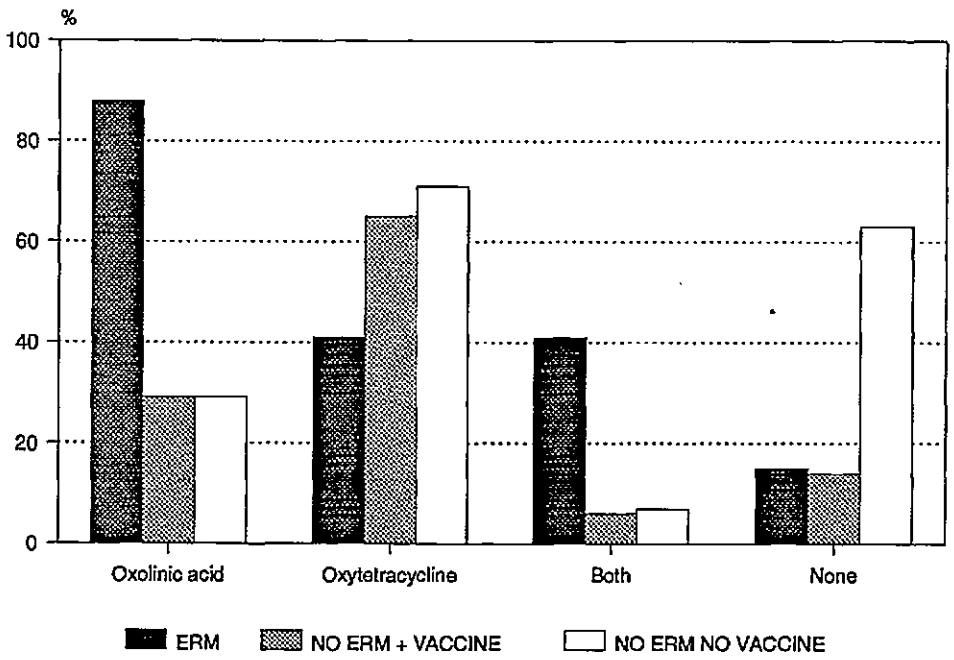

Figure 7.9b Type of antimicrobial agent

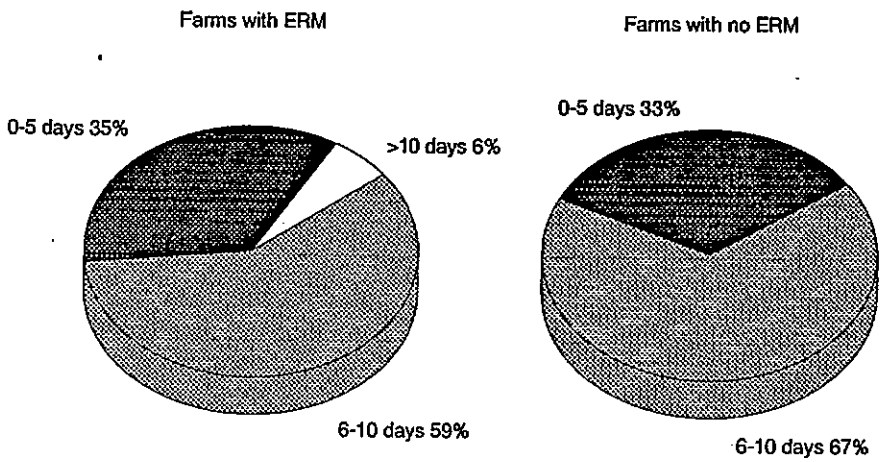

Figure 7.10a Length of treatment for oxolinic acid

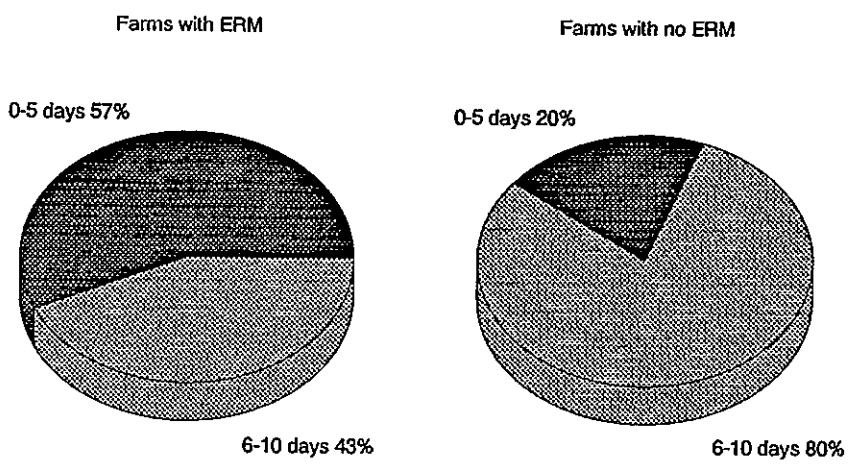

Figure 7.10b Length of treatment for oxytetracycline

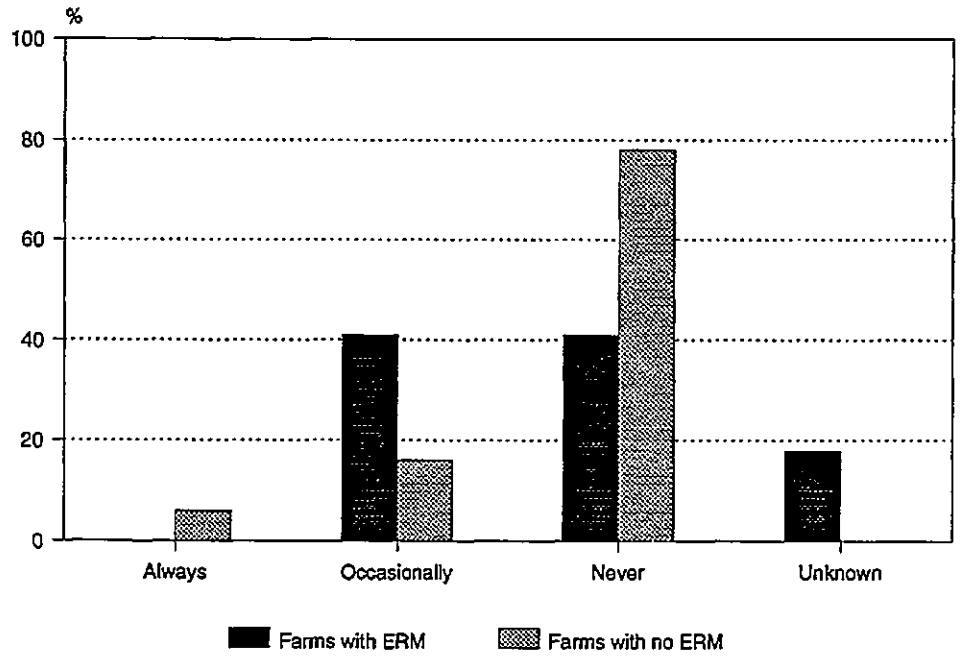

Figure 7.11a Use of prophylactic treatment

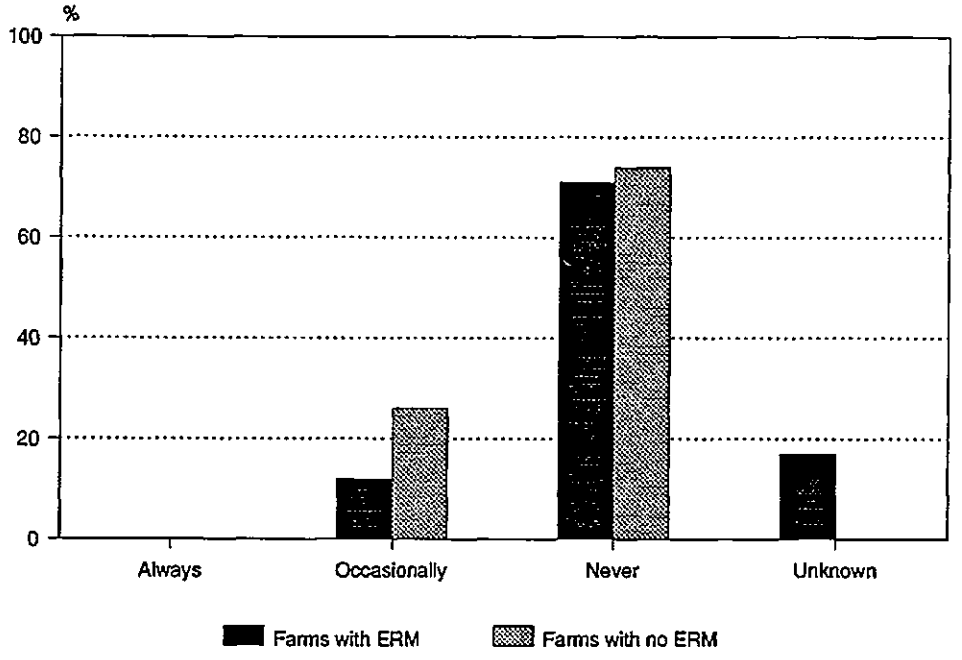

Figure 7.11b Use of second treatment

Table 7.7 Cost of treatments

Fish Farm Sites	Vaccine	Oxolinic acid	Oxytetracycline
With ERM	£2245	£711	£640
With no ERM but vaccinating	£2745	£110	£258
With no ERM and not vaccinating	--------	£415	£103

Of the 30 positive control farms that received questionnaires with an identification mark, eight were returned and they all indicated that they had had ERM previously diagnosed.

7.4 DISCUSSION

The majority of fish farms that returned questionnaires were in the southern half of England and Wales with 47% of all farms in the Southern, South West and Wessex water authority areas. This agees well with the MAFF statistics for annual farm returns which show that most farms benefit from the chalk streams and higher temperatures in the south.

Although the survey indicated that there had been no diagnosis of ERM on any responding farm in England and Wales prior to 1982, the first U.K. isolation of *Y. ruckeri* occurred in 1978 (R. Sweeting, pers. com.), and Roberts (1983) reported an outbreak in fingerling rainbow trout (*Oncorhynchus mykiss*) reared on a table farm during the spring of 1981. The fish farm involved in the 1978 outbreak was actually included in the survey, but is now no longer trading. The remainder of the farms with ERM represented some of the larger production sites that also received

river water supplies and most were vaccinating against ERM. The differences in tank numbers and earth ponds between farms did not vary a great deal. However, the production capacities were much higher on farms with ERM which suggests greater stocking densities at these units.

Farms without ERM that had decided to vaccinate represented smaller production units of which approximately half were on river water and half on borehole or spring water. These sites had more than twice the number of fry tanks compared to other farms which indicated their involvement in fry production for on-growing elsewhere. Therefore, vaccination was most likely intended either as a purely precautionary measure or as a service for customers.

Farms free of ERM and not vaccinating tended to be the smallest units using spring water or borehole supplies, indicating that *Y. ruckeri* infections were considered to be a low risk for these types of producers.

The additional stress factors imposed on fish stocks reared on the larger production units receiving river water supplies can lead to infections capable of causing sustained, low-level mortalities lasting several months (Bullock 1984). These have the potential to cause heavy losses given unfavourable on-farm conditions. This chronic form of the disease may result in an acute epizootic following stress arising from handling, low dissolved oxygen, over-crowding, fluctuating water temperatures, increased suspended solids, elevated levels of ammonia or other waste products (Busch 1978, 1983; Austin 1982; Bullock 1984; Frerichs *et al.*, 1985). These factors were identified by farmers in the survey as coinciding with ERM outbreaks.

The spring-to-autumn period was identified as the period when ERM was a problem and field studies in southern England tend to confirm that *Y. ruckeri* is most prevalent in early spring when the water temperature is rising quickly and early autumn when the temperature is beginning to fall (Roberts, pers. com.). The disease is most severe at a water temperature of 15-18°C and this is confirmed in outbreaks recorded by Dalsgaard *et al.* (1984) and Ocvirk *et al.* (1988), with Rübsamen and

Weis (1985) reporting rising mortalities at 20°C. Rucker (1966) indicated that ERM is less severe at 10°C or below. Handling stress can trigger outbreaks of ERM (Sparboe *et al.*, 1986), especially when water temperatures exceed 13°C (Busch and Lingg 1975; Dulin *et al.*, 1976). Hunter *et al.* (1980) showed in a laboratory study that heat stress can play an important part in triggering the release of *Y. ruckeri* from carrier fish. Their study indicated that only carriers stressed by raising the temperature to 25°C transmitted *Y. ruckeri* to healthy recipient fish. Unstressed carrier fish did not transmit the pathogen to healthy fish. Unfortunately it is not possible to extrapolate this finding to the UK situation since this type of heat stress does not happen. Nevertheless, carrier fish can undoubtedly transmit ERM, as the survey revealed, but during the fluctuating temperatures of spring and autumn.

In all, 60% of sites indicated that 222/kg-200 g (100/lb-7 oz) fish were the most susceptible to ERM but that larger fish of up to 2.27 kg (5 lb) were also at risk from infection. This agrees well with other published studies. Rucker (1966) reported that fingerling rainbow trout of approximately 7.5 cm in length are most commonly affected. Larger fish of 12.5 cm undergo a more chronic but less severe form of the disease. Busch (1978) reported that peracute to acute infection usually occurs in the spring and early summer in young-of-the-year fish, whereas acute-to-subacute infections usually occur in yearling fish in the fall and early winter. Mortalities in 25-30 cm fish in Europe have been reported by Bentley (1982), Fuhrmann *et al.* (1983) and Meier (1986). Outbreaks of ERM in smaller fish (between 5-20 cm) are noted by Rübsamen and Weis (1985), De La Cruz *et al.* (1986) and Meier (1986).

The reports of vaccine failure are difficult to interpret. Although the majority of sites claiming they were free of ERM reported 'no failures', this is not surprising because the fish on these sites do not receive a natural challenge of *Y. ruckeri*, since they were largely on spring or borehole supplies. Since there are more sites reporting vaccine failure than have diagnosed ERM, the criteria used for assessing possible 'failure' remain obscure. Therefore the sites in this group rearing

table fish would not have a basis for assessing the effectiveness of an anti-*Y. ruckeri* vaccine and these results can be discounted. However some of the farms were egg, fry and fingerling producers and would have been basing their response on feed-back from customers rearing fish using river water supplies. Consequently, these returns may lead to some duplication of data. Farms with ERM reporting 'failure' may consider that any losses attributed to *Y. ruckeri* as unacceptable. Surprisingly, only 50% were using the recommended vaccine dilution and this is a possible contributory factor to vaccine failure. Most of these units (78%) had also found it necessary to vaccinate against ERM every year since the original diagnosis compared with 48% of sites without ERM. This fact alone does not indicate dissatisfaction with vaccination. It has been recognised for some time that vaccines used against ERM can be an effective management practice, provided that they are administered correctly and are not considered as 'cure-alls'. The economic benefits of vaccinating fish stocks can be reflected in lower mortalities attributable to ERM, improved conversion rates in vaccinated fish, earlier marketability, and savings in the cost of medicated feed (Amend and Eshenour 1980; Tebbit *et al.*, 1981; Horne and Robertson 1987). The savings in the cost of medication, in a vaccinated group of fish, were considered by Amend and Eshenour (1980) to have more than paid for the vaccine used. Tebbit *et al.* (1981) also considered that medicated feed requirements were substantially reduced in trout vaccinated against ERM. In this survey, 90% of sites with diagnosed ERM were also using vaccination. Therefore it was not possible to assess whether there was a significant increase in medicated feed costs on farms choosing not to vaccinate. Even though vaccines are being extensively used to control *Y. ruckeri* infections in fish, they do not completely eliminate the disease or losses attributable to ERM (Busch, 1983). However, Horne and Robertson (1987) concluded that the benefits of using vaccines as opposed to antibiotics could be highly cost-effective, enabling more resources to be devoted to overall improvements in husbandry. In addition, antibiotics may need to be used more than once, leading to increased costs, particularly as the fish grow larger. Vaccines, in

contrast, are normally only used once at the beginning of the growth cycle and can remain effective until the fish reach market size. Unfortunately, vaccination is not 100% effective and the usage of antimicrobial agents as indicated in the survey may still be required under certain circumstances.

Prophylaxis and the use of a second treatment with a different antimicrobial compound was revealed by the survey. The potential problem of antimicrobial agent misuse, particularly by terminating treatment as soon as mortalities cease, has been highlighted by Post (1987) amongst others. In addition, repeated short-term treatments can also be responsible for the appearance of drug resistance in strains of *Y. ruckeri* (Tebbit *et al.*, 1981). The problem of ERM recurring following the cessation of antimicrobial treatment has been highlighted by several workers. De La Cruz *et al.* (1986) indicated that, although treatment with flumequine resulted in a lowering of the mortality rate by 80%, there was an outbreak of the disease again approximately 3 weeks later. Rübsamen and Weis (1985) noticed that losses declined only gradually after the use of an antibiotic, but only reduced still further when the fish were transferred to a larger pond with resultant reduced stocking density. Bragg and Henton (1986) reported that 4 months after treatment there were still some isolated cases of ERM in fish previously affected and *Y. ruckeri* was re-isolated from samples of spleen, liver and kidney. Cipriano *et al.* (1986) indicated that a second outbreak of ERM occurred 5-6 weeks after the onset of initial mortalities and subsequent successful therapy.

This survey was the first of it's type designed to collect background information on the occurrence of ERM in England and Wales. The results indicated that stress factors linked to the appearence of *Y. ruckeri* in fish stocks are similar to those reported from other countries. In addition, although the use of vaccination was shown to be widespread among the responding farms, there was an unfortunate tendency for farmers to consider that vaccines had failed in some way. A worrying trend towards prophylaxis was also revealed but it is hoped that the type of data collated from the survey returns will go some way towards highlighting the potential

misuse of antimicrobial agents. The collection of basic data concerning the incidence of ERM should enable the affect of the disease on the fish farming industry to be more fully understood.

CHAPTER 8

GENERAL CONCLUSIONS

1. A new medium, designated Ribose Ornithine Deoxycholate agar (ROD), was developed for isolation of *Yersinia ruckeri* from faecal material under field conditions.

The ability to isolate *Y. ruckeri* with this medium indicated that the organism could occur in samples of fish faeces four to six weeks before it appeared in kidney samples, particularly during chronic ERM. The medium also proved useful for isolation of *Y. ruckeri* from inlet and outlet water samples on two fish farms. Further epidemiological studies dealing with fish condition, performance and disease signs were also considered. Essentially, recovery of *Y. ruckeri* from kidney coincided with subsequent adverse performance but the timing of fish introduction was also important to marketability.

In addition, the results indicated that kidney was not as consistent for isolation as spleen and faeces. Water isolation indicated a potential reservoir of infection in the inflow water due to escapee fish.

Vaccination against ERM was shown to reduce both the infection levels and the visual signs resulting from a natural challenge of *Y. ruckeri*.

Although the medium developed in this study is not totally selective, the ability to produce differential characteristics has shown its potential for epidemiological studies. This has enabled the transmission and carrier status of ERM to be studied more fully. However, further developmental work is needed in order to resolve occasional selection problems with other members of the Enterobacteriaceae, notably strains of *Citrobacter, Hafnia* and *Enterobacter*.

2. Although oxolinic acid appeared to be bacteriostatic at its minimum inhibitory concentration (MIC) there were indications that it could also be bactericidal. Oxytetracycline on the other hand was only bacteriostatic. A potentiated sulphonamide was neither bacteriostatic nor bactericidal at the MIC.

Additional results, after increasing the concentrations of each antimicrobial agent, showed that oxolinic acid was bactericidal at intermediate values but bacteriostatic at higher concentrations. Oxytetracycline was bacteriostatic at intermediate concentrations but bactericidal at the higher concentrations. The potentiated sulphonamide reduced growth at higher concentrations but was not bactericidal at all.

It was possible, after 15 subcultures, to increase the MIC for oxolinic acid, oxytetracycline and a potentiated sulphonamide. However, attempts to decrease resistance to oxolinic acid were unsuccessful. Nevertheless, the MIC's for oxytetracycline and a potentiated sulphonamide were decreased.

The results indicate the ease with which antimicrobial resistance among bacterial fish pathogens, such as *Y. ruckeri,* can occur. It is concluded therefore that potential resistance should be minimized by continual monitoring, careful drug use, optimisation of treatment regimes and the cycled use of the available chemotherapeutants. The phenomenon of multiple resistance should also be investigated.

3. There was significantly less overall recovery of *Y. ruckeri* from the faecal and kidney material of a vaccinated group of fish, 10 days after anal challenge, compared with a non-vaccinated group. An ELISA technique indicated that although there was no detectable serum antibody there was a local mucosal response in vaccinated fish.

In addition, uptake of *Y. ruckeri* antigen was demonstrated in vaccinated and non-vaccinated fish. However, vaccinated fish appeared to take up the antigen by an active process which seemed to occur only in posterior sections of the intestine.

Bacteriological results, in conjunction with an immunoperoxidase technique, demonstrated a certain level of protection soon after challenge.

The actual process involved in the intestinal response and subsequent uptake needs further study. The role of the cellular immune response requires clarification and experiments concerning oral vaccination in conjunction with micro-encapsulation should prove valuable.

4. A survey of salmonid farms confirmed that certain factors often coincided with the appearance of *Y. ruckeri*. These included over-crowding of stocks, fluctuations in water temperature (particularly a rising temperature in the spring), recent grading, poor water quality (including low flow rates or low dissolved oxygen and high ammonia levels), river flooding and consequent silt build-up. It was further apparent that ERM was most prevalent in the spring-to-autumn period, more particularly on larger table farms with production ranging up to over 200 tonnes p.a.

In some cases vaccines were considered to have failed but the reasons were not clear. However, the majority intended to continue immunisation whether they have had ERM diagnosed or not.

The preferred treatment on sites with ERM was oxolinic acid, whereas the other farms preferred oxytetracycline. The average length of treatment was given as 7 days, which was a once-a-year application.

Additional follow up surveys, perhaps on a bi-annual basis, would provide useful background information for ERM control strategies.

REFERENCES

Alderman, D.J. (1988) Fisheries chemotherapy: a review. In: *Recent Advances in Aquaculture* (edited by J.F.Muir and R.J.Roberts). Croom Helm, Sydney and London, 3, chapter 1, 1-61.

Amend, D.F. and Eshenour, R.W. (1980) Development and use of commercial fish vaccines. *Salmonid* (March/April), 8-12.

Amend, D.F., Johnson, K.A., Croy, T.R. and McCarthy, D.H. (1983) Some factors affecting the potency of *Yersinia ruckeri* bacterins. *Journal of Fish Diseases* 6, 337-344.

Anderson, D.P. (1978) Passive haemolytic plaque assay as a means of detecting antibody-producing cells in rainbow trout immunized with the 'O'-antigen of enteric redmouth bacteria. Ph.D. Thesis, University of Maryland, College Park, Maryland. 96 pp.

Anderson, D.P. and Nelson, J.R. (1974) Comparison of protection in rainbow trout (*Salmo gairdneri*) inoculated with fed Hagerman redmouth bacterins. *Journal of the Fisheries Research Board of Canada* 31, 214-216.

Anderson, D.P. and Ross, A.J. (1972) Comparative study of Hagerman redmouth disease oral bacterins. *The Progressive Fish Culturist* 34, 226-228.

Anderson, D.P. and Siwicki, A.K. (1989) Nonspecific defense mechanisms and specific immune protection of trout against viral agents. In: *Viruses of Lower Vertebrates*. (Edited by W. Ahne and E. Kurstak), Springer-Verlag, Berlin, 479-486.

Anderson, D.P., Dixon, O.W. and Roberson, B.S. (1979a) Kinetics of the primary immune response in rainbow trout after flush exposure to *Yersinia ruckeri* 'O'-antigen. *Developmental and Comparative Immunology* 3, 739-744.

Anderson, D.P., Merchant, B., Dixon, O.W., Schott, C.F. and Lizzio, E.F. (1983) Flush exposure and injection immunization of rainbow trout to selected DNP conjugates. *Developmental and Comparative Immunology* 7, 261-268.

Anderson, D.P., Roberson, B.S. and Dixon, O.W. (1979b) Plaque-forming cells and humoral antibody in rainbow trout (*Salmo gairdneri*) induced by immersion in a *Yersinia ruckeri* 'O'-antigen preparation. *Journal of the Fisheries Research Board of Canada* **36**, 636-639.

Anderson, D.P., Roberson, B.S. and Dixon, O.W. (1979c) Induction of antibody-producing cells in rainbow trout, *Salmo gairdneri*, by flush exposure. *Journal of Fish Biology* **15**, 317-322.

Anderson, D.P., Roberson, B.S. and Dixon, O.W. (1979d) Cellular immune response in rainbow trout *Salmo gairdneri* Richardson to *Yersinia ruckeri* 'O'-antigen monitored by the passive haemolytic plaque assay test. *Journal of Fish Diseases* 2, 169-178.

Anderson, D.P., Roberson, B.S. and Dixon, O.W. (1982) Immunosuppression induced by a corticosteroid or an alkylating agent in rainbow trout (*Salmo gairdneri*) administered a *Yersinia ruckeri* bacterin. *Developmental and Comparative Immunology* Suppl.2, 197-204.

Anhalt, J.P., Sabath, L.D. and Barry, A.L. (1980) Special tests: bactericidal activity, activity of antimicrobics in combination, and detection of ß-lactamase production. In: *Manual of Clinical Microbiology*. (Edited by E.H.Lennette, E.H.Spaulding and J.P.Truant). American Society for Microbiology, Washington, D.C., 478-484.

Anon. Enteric redmouth bacterin. *Technical Bulletin No. 1, BioMed Research Laboratories Incorporated*, 5 pp.

Anon. (1990) Yersiniosis outbreaks in Venezuela. *Animal Pharm* No. 213, 15.

Aoki, T., Sakai, M., Kitao, T. and Takahashi, S. (1986) Elimination of challenge bacteria from the blood and intestines of ayu *Plecoglossus altivelis* vaccinated by immersion. *Bulletin of the Japanese Society of Scientific Fisheries* **52**, 971-975.

Austin, B. (1982) Close-up of a killer. *Fish Farmer* **5**, 30-31.

Austin, B. and Austin, D.A. (1987) *Bacterial fish pathogens: Disease in farmed and wild fish*. Ellis Horwood Ltd., Chichester.

Austin, B., Bishop, I., Gray, C., Watt, B. and Dawes, J. (1986) Monoclonal antibody-based enzyme-linked immunosorbent assays for the rapid diagnosis of clinical cases of enteric redmouth and furunculosis in fish farms. *Journal of Fish Diseases* **9**, 469-474.

Austin, B., Green, M. and Rodgers, C.J. (1982) Morphological diversity among strains of *Yersinia ruckeri*. *Aquaculture* **27**, 73-78.

Baath, C. (1986) The occurrence of redmouth in Bavaria. *Fischer und Teichwirt* **37**, 71-72.

Ball, P.R., Shales, S.W. and Chopra, I. (1980) Plasmid-mediated tetracyclin resistance in *Escherichia coli* involves increased efflux of the antibiotic. *Biochem. Biophys. Res. Commun.* **93**, 74-81.

Bangert, R.L., Ward, A.C.S., Stauber, E.H., Cho, B.R. and Widders, P.R. (1988) A survey of the aerobic bacteria in the feces of captive raptors. *Avian Diseases* **32**, 53-62.

Barry, A.L. and Jones, R.N. (1984) Cross-resistance among cinoxacin, ciprofloxacin, DJ-6783, enoxacin, nalidixic acid, norfloxacin and oxolinic acid after *in vitro* selection of resistant populations. *Antimicrobial Agents and Chemotherapy* **25**, 775-777.

Baudin-Laurencin, F. and Tixerant, G. (1985) Pathologie des poissons élevés en mer. *Recerche Méd. Vét.* **161**, 735-746.

Bentley, H. (1982). An English experience. *Fish Farmer* **5**, 31.

Bercovier, H and Mollaret, H.H. (1984) Genus XIV *Yersinia*. In: *Bergey's Manual of Systematic Bacteriology* (edited by N.R.Krieg). Williams and Wilkins, Baltimore, **1**, 489-506.

Blazer, V.S. and Wolke, R.E. (1984) Effect of diet on the immune response of rainbow trout (*Salmo gairdneri*). *Canadian Journal of Fisheries and Aquatic Science* **41**, 1244-1247.

Bolger, T. and Connolly, P.L. (1989) The selection of suitable indices for the measurement and analysis of fish condition. *Journal of Fish Biology* **34**, 171-182.

Bosse, M.P. and Post, G. (1983) Tribrissen and tiamulin for control of enteric redmouth disease. *Journal of Fish Diseases* **6**, 27-32.

Bowser, P.R. and House, M. (1990) *In vitro* sensitivity of some fish pathogens to the quinolones nalidixic acid and oxolinic acid and the fluoroquinolone enrofloxacin. *Bulletin of the European Association of Fish Pathologists* **10**, 48-49.

Bragg, R.R. and Henton, M.M. (1986) Isolation of *Yersinia ruckeri* from rainbow trout in South Africa. *Bulletin of the European Association of Fish Pathologists* **6**, 5-7.

Bromage, N.R. and Shepherd, C.J. (1988) Fish, their requirements and site evaluation. In: *Intensive Fish Farming*, BSP Books, (edited by C.J. Shepherd and N.R. Bromage). Chapter 2, 17-49.

Bromage, N.R., Shepherd, C.J. and Roberts, J. (1988) Farming systems and husbandry practice. In: *Intensive Fish Farming*, BSP Books, (edited by C.J. Shepherd and N.R. Bromage). Chapter 3, 50-102.

Bruno, D.W. and Munro, A.L.S. (1989) Immunity in Atlantic salmon, *Salmo salar* L., fry following vaccination against *Yersinia ruckeri* and the influence of body weight and infectious pancreatic necrosis virus (IPNV) on the detection of carriers. *Aquaculture* **81**, 205-211.

Bullock, G.L. (1984) Enteric redmouth disease of salmonids. *US Department of the Interior, Fish Disease Leaflet 67.* 14 p.

Bullock. G.L. and Anderson, D.P. (1984) Immunisation against *Yersinia ruckeri*, cause of enteric redmouth disease. Office International des Epizooties, Fish Diseases Commission, Symposium on Fish Vaccination, Paris. 151-166.

Bullock, G.L., Maestrone, G., Starliper, C. and Schill, B. (1983) Potentiated sulfonamide therapy of enteric redmouth disease. *Canadian Journal of Fisheries and Aquatic Science* **40**, 101-102.

Bullock, G.L. and Snieszko, S.F. (1979) Enteric redmouth disease of salmonids. *Dept. of the Interior, US Fish and Wildlife Service, FLD-57.* 7 pages.

Bullock, G.L., Stuckey, H.M., Herman, R.L. and Smith, C.E. (1976) Comparative susceptibility of Atlantic salmon (*Salmo salar*) to the enteric redmouth bacterium and *Aeromonas salmonicida*. *Journal of Wildlife Diseases* **12**, 376-379.

Bullock, G.L., Stuckey, H.M. and Shotts, E.B. (1978) Enteric redmouth bacterium: comparison of isolates from different geographical areas. *Journal of Fish Diseases* **1**, 351-356.

Busch, R.A. (1978) Enteric redmouth disease (Hagerman strain). *Marine Fisheries Review* **40**, 42-51.

Busch, R.A. (1983) Enteric Redmouth Disease (*Yersinia ruckeri*). In: *Les antigènes des micro-organismes pathogènes des poissons*. Symposium International de Talloires (10-12 May 1982). Collection Fondation Marcel Merieux Lyon. (edited by D.P.Anderson, M.Dorson and P.H.Dubourget), pages 201-223.

Busch, R.A. and Lingg, A.J. (1975) Establishment of an asymptomatic carrier state infection of enteric redmouth disease in rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* **32**, 2429-2432.

Ceschia, G., Giorgetti, G., Bertoldini, G. and Fontebasso, S. (1987) The *in vitro* sensitivity of *Yersinia ruckeri* to specific antibodies. *Journal of Fish Diseases* **10**, 65-67.

Ceschia, G., Giorgetti, G., Bovo, G. and Fontebasso, S. (1984) *Yersinia ruckeri* in Italian trout farms: epizootiological considerations. *Atti Della Societa Italiana Delle Scienze Veterinarie* 38, 716-719.

Cheng, A.F., Li, M.K.W., Ling, T.K.W. and French, G.L. (1987) Emergence of ofloxacin-resistant *Citrobacter freundii* and *Pseudomonas maltophilia* after ofloxacin therapy. *Journal of Antimicrobial Chemotherapy* **20**, 283-292.

Chilmonczyk, S. and Oui, E. (1988) The effects of gamma irradiation on the lymphoid organs of rainbow trout and subsequent susceptibility to fish pathogens. *Veterinary Immunology and Immunopathology* **18**, 173-180.

Chopra, I. (1985) Mode of action of the tetracyclines and the nature of bacterial resistance to them. In: *The Tetracyclines, Handbook of Experimental Pharmacology*. (Edited by J.J. Hlavka and J.H. Boothe), Springer-Verlag, Berlin **78**, 317-392.

Chopra, I. (1988) Mechanisms of resistance to antibiotics and other chemotherapeutic agents. *Journal of Applied Bacteriology Symposium Supplement 1988*, 149S-166S.

Cipriano, R.C. and Ruppenthal, T. (1987) Immunisation of salmonids against *Yersinia ruckeri*: significance of humoral immunity and cross-protection between serotypes. *Journal of Wildlife Diseases* **23**, 545-550.

Cipriano, R.C., Ruppenthal, T., Schill, W.B., Pyle, S.W. and Shotts, E.B. (1987) Susceptibility of brook trout (*Salvelinus fontinalis*) to experimental infections with different serotypes of *Yersinia ruckeri*. *Microbios Letters* **35**, 27-31.

Cipriano, R.C., Schill, W.B., Pyle, S.W. and Horner, R. (1986). An epizootic in chinook salmon (*Oncorhynchus tshawytsche*) caused by a sorbitol-positive serovar 2 strain of *Yersinia ruckeri*. *Journal of Wildlife Diseases* **22**, 488-492.

Corbel, M. J. (1975) The immune response in fish: a review. *Journal of Fish Biology* **7**, 539-563.

Cossarini-Dunier, M. (1986a) Secondary response of rainbow trout (*Salmo gairdneri*) to DNP-haemocynanin and *Yersinia ruckeri*. *Aquaculture* **52**, 81-86

Cossarini-Dunier, M. (1986b) Protection against enteric redmouth disease in rainbow trout. *Salmo gairdneri* Richardson, after vaccination with *Yersinia ruckeri* bacterin. *Journal of Fish Diseases* **9**, 27-33.

Cossarini-Dunier, M., Demael, A., Lepot, D. and Guerin, V. (1988) Effect of manganese ions on the immune response of carp (*Cyprinus carpio*) against *Yersinia ruckeri*. *Developmental and Comparative Immunology* **12**, 573-579.

Cowan, S.T. (1974) Media: constituents and sterilization. In: *Manual for the identification of medical bacteria*. Cambridge University Press, chapter 2, 7-14.

Dalsgaard, I., From, J. and Hørlyck, V. (1984) First observation of *Yersinia ruckeri* in Denmark. *Bulletin of the European Association of Fish Pathologists* **4**, 10.

Daly, J.G., Lindvik, B. and Stevenson, R.M.W. (1986) Serological heterogeneity of recent isolates of *Yersinia ruckeri* from Ontario and British Columbia. *Diseases of Aquatic Organisms* **1**, 151-153.

Davies, R.L. (1990) O-serotyping of *Yersinia ruckeri* with special emphasis on European isolates. *Veterinary Microbiology* **22**, 299-307.

Davies, R.L. (1991) Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein-types. *Journal of Fish Diseases* **14**, 221-228.

Davies, R.L. and Frerichs, G.N. (1989) Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. *Journal of Fish Diseases* **12**, 357-365.

Davina, J.H., Parmentier, H.K. and Timmermans, L.P.M. (1982) Effect of oral administration of *Vibrio* bacterin on the intestine of cyprinid fish. *Developmental and Comparative Immunology*, **2**, 157-166.

Davina, J.H., Rijkers, G.T., Rombout, J.H.W.M., Timmermans, L.P.M. and Van Muiswinkel, W.B. (1980) Lymphoid and non-lymphoid cells in the intestine of cyprinid fish. In: *Development and Differentiation of Vertebrate Lymphocytes*. (Edited by J.D.Horton), Elsevier/North Holland, 129-140.

Dear, G. (1988) *Yersinia ruckeri* isolated from Atlantic salmon in Scotland. *Bulletin of the European Association of Fish Pathologists* **8**, 18-20.

De Grandis, S.A., Krell, P.J., Flett, D.E. and Stevenson, R.W.M. (1988) Deoxyribonucleic acid relatedness of serovars of *Yersinia ruckeri*, the enteric redmouth bacterium. *International Journal of Systematic Bacteriology* 38, 49-55.

De Grandis, S.A. and Stevenson, R.W.M. (1985) Antimicrobial susceptibility patterns and R plasmid-mediated resistance of the fish pathogen *Yersinia ruckeri*. *Antimicrobial Agents and Chemotherapy*, **27**, 938-942.

De La Cruz, J.A., Rodriguez, A., Tejedor, C., De Lucas, E. and Orozco, L.R. (1986) Isolation and identification of *Yersinia ruckeri*, causal agent of enteric redmouth disease (ERM), for the first time in Spain. *Bulletin of the European Association of Fish Pathologists* **6**, 43-44.

Difco Manual. (1985) Microbiology for clinical laboratory testing. In: *Dehydrated culture media and reagents for microbiology*. Tenth edition, Difco Laboratories, Michigan, USA, 19-25.

Doggett, T.A. and Harris, J.E. (1987) Ontogeny of the gut-associated lymphoid tissue in *Oreochromis mossambicus*. *Journal of Fish Biology*, Supplement A, **31**, 23-27.

Doggett, T.A. and Harris, J.E. (1991) Morphology of the gut-associated lymphoid tissue of *Oreochromis mossambicus* and its role in antigen absorption. *Fish and Shellfish Immunology* **1**, 213-227.

Dorson, M. (1981) Role and characterization of fish antibody. *Developments in Biological Standardization.* International Symposium on Fish Biologics: Serodiagnostics and Vaccines, Leetown, W. Va., U.S.A. **49**, 307-319.

Dorson, M. (1984) Applied immunology of fish. In: *Symposium on Fish Vaccination*, Paris, (edited by P. de Kinkelin), O.I.E. Fish Diseases Commission, 39-74.

Drasar, B.S. and Barrow, P.A. (1985) Introduction and methods for investigation of the intestinal flora of man and animals. In: *Intestinal microbiology*. Aspects of Microbiology 10, Van Nostrand Reinhold (UK) Co. Ltd., 1-18.

Dudley, D.J., Guentzel, M.N., Ibarra, M.J., Moore, B.E. and Sagik, B.P. (1980) Enumeration of potentially-pathogenic bacteria from sewage sludges. *Applied and Environmental Microbiology* **39**, 118-126.

Dulin, M.P., Huddleston, T., Larson, R.E. and Klontz, G.W. (1976) Enteric redmouth disease. *Forest, Wildlife and Range Experimental Station Bulletin of the No.8. Univ ID.* Moscow ID. 15 p.

Dwilow, A.G., Souter, B.W. and Knight, K. (1987) Isolation of *Yersinia ruckeri* from burbot, *Lota lota* (L.), from the Mackenzie River, Canada. *Journal of Fish Diseases* **10**, 315-317.

Elliott, J.M. (1976) Body composition of brown trout (*Salmo trutta* L.) in relation to temperature and ration size. *Journal of Animal Ecology* **45**, 273-289.

Ellis, A.E., Munro, A.L.S. and Roberts, R.J. (1976) Defence mechanisms of fish. I. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice (*Pleuronectes platessa* L.). *Journal of Fish Biology* 8, 67-78.

Ellis, A.E. (1981) Non-specific defense mechanisms in fish and their role in disease processes. *Developments in Biological Standardization.* International Symposium on Fish Biologics: Serodiagnostics and Vaccines, Leetown, W. Va., U.S.A. **49**, 337-352.

Ellis, A.E. (1986) The function of teleost fish lymphocytes in relation to inflammation. *International Journal of Tissue Reaction* **VIII**, 263-270.

Ellis, A.E. (1988) Vaccination against enteric redmouth (ERM). In: *Fish Vaccination*, Academic Press (edited by A.E. Ellis). 85-92.

Enriquez, M.V. and Zamora, M.V. (1987) Isolation of *Yersinia ruckeri* from carps (*Cyprinus carpio*) in Valdivia. *Arch. Med. Vet.* **19**, 33-36.

Ewing, W.H., Ross, A.J., Brenner, D.J. and Fanning, G.R. (1978) *Yersinia ruckeri sp. nov.*, redmouth (RM) bacterium. *International Journal of Systematic Bacteriology* 28, 37-44.

Ewing, W.H., Tomfohrde, K.M. and Naudo, P.J. (1977) Isolation and differentiation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *API species*, **1**, 13-28.

Ewing, W.H., Tomfohrde, K.M. and Naudo, P.J. (1979) Isolation of *Vibrio cholerae* and certain related vibrios; an outline of methods. *API species*, **2**, 10-32.

Farmer III, J.J., Davis, B.R., Hickman-Brenner, F.W., McWhorter, A., Huntley-Carter, G.P., Asbury, M.A., Riddle, C., Wathen-Grady, H.G., Elias, C., Fanning, G.R., Steigerwalt, A.G., O'Hara, C.M., Morris, G.K., Smith, P.B. and Brenner, D.J. (1985) Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. *Journal of Clinical Microbiology* **21**, 46-76.

Fletcher, T.C. (1981) The identification of non-specific humoral factors in the plaice (*Pleuronectes platessa* L.) *Developments in Biological Standardization.* International Symposium on Fish Biologics: Serodiagnostics and Vaccines, Leetown, W. Va., U.S.A. **49**, 321-327.

Fletcher, T.C., White, A., Youngson, A., Pusztai, A. and Baldo, B.A. (1981) Characterization of C-reactive protein from the eggs of the marine teleost, *Cyclopterus lumpus* L. *Biochimica et Biophysica Acta* **671**, 44-49.

Frerichs, G.N., and Collins, R.O. (1984). Enteric redmouth in Scotland. *Veterinary Record* **115**, 45-46.

Frerichs, G.N., Stewart, J.A. and Collins, R.O. (1985) Atypical infection of rainbow trout, *Salmo gairdneri* Richardson, with *Yersinia ruckeri*. *Journal of Fish Diseases* 8, 383-387.

Fuhrmann, H., Böhm, K.H. and Schlotfeldt, H.-J. (1983) An outbreak of enteric redmouth disease in West Germany. *Journal of Fish Diseases* **6**, 309-311.

Fuhrmann, H., Böhm, K.H. and Schlotfeldt, H-J. (1984) On the importance of enteric bacteria in the bacteriology of freshwater fish. *Bulletin of the European Association of Fish Pathologists* 4, 42-46.

Fujino, Y., Ono, S. and Nagai, A. (1987) Studies on uptake of rabbit's immunoglobulin into the columnar epithelial cells in the gut of rainbow trout *Salmo gairdneri*. *Nippon Suisan Gakkaishi* **53**, 367-370.

Fukumoto, M., Sugita, H., Koyama, H. and Deguchi, Y. (1987) Changes in the fecal microflora of goldfish, Carassius auratus L., with the oral administration of oxytetracycline. *Abstracts of the Annual Meeting of the Japanese Society of Science and Fisheries*, 4-5 October 1987, Hakodate, Hokkaido, Japan, p.188.

Furones, M.D., Gilpin, M.J. Alderman, D.J. and Munn, C.B. (1990) Virulence of *Yersinia ruckeri* serotype I strains is associated with a heat sensitive factor (HSF) in cell extracts. *FEMS Microbiology Letters* **66**, 339-344.

Gelev, I., Khvoinev, A., Khristo, D. and Strashimirova, N. (1984). Haemorrhagic septicaemia in rainbow trout caused by *Yersinia ruckeri* sp. nov. *Veterinarnomeditsinski Nauki* **21**, 84-90.

Georgopoulou, U., Dabrowski, K., Sire, M.F. and Vernier, J.M. (1988) Absorption of intact proteins by the intestinal epithelium of trout, *Salmo gairdneri*. A luminescence enzyme immunoassay and cytochemical study. *Cell and Tissue Research* **251**, 145-152.

Giorgetti, G., Ceschia, G and Bovo, G. (1985) First isolation of *Yersinia ruckeri* in farmed rainbow trout in Italy. In: *Fish and Shellfish Pathology,* London and New York, Academic Press, (edited by A.E. Ellis), 161-166.

Green, M. and Austin, B. (1983) The identification of *Yersinia ruckeri* and its relationship to other representatives of the Enterobacteriaceae. *Aquaculture* **34**, 185-192.

Griffin, B.R. (1983) Opsonic effect of rainbow trout (*Salmo gairidneri*) antibody on phagocytosis of *Yersinia ruckeri* by trout leukocytes. *Developmental and Comparative Immunology* **7**, 253-259.

Hamilton-Miller, J.M.T. (1984) Resistance to antibacterial agents acting on antifolate metabolism. In: *Antimicrobial Drug Resistance.* (Edited by L.E. Bryan). Academic Press, London, chapter 6, 173-190.

Hammond, S.M. and Lambert, P.A. (1978a) Antibiotics as chemotherapeutic agents. In: *Antibiotics and Antimicrobial Action*. (Edited by S.M. Hammond and P.A. Lambert). Chapter 1, 1-7.

Hammond, S.M. and Lambert, P.A. (1978b) Inhibitors of ribosome function. In: *Antibiotics and Antimicrobial Action*. (Edited by S.M. Hammond and P.A. Lambert). Chapter 6, 45-49.

Hammond, S.M. and Lambert, P.A. (1978c) Metabolic inhibitors. In: *Antibiotics and Antimicrobial Action*. (Edited by S.M. Hammond and P.A. Lambert). Edward Arnold, London, Chapter 7, 50-52.

Hammond, S.M. and Lambert, P.A. (1978d) Prospects for chemotherapy. In: *Antibiotics and Antimicrobial Action*. (Edited by S.M. Hammond and P.A. Lambert). Edward Arnold, London, Chapter 8, 53-63.

Hanski, C., Kutschka, U., Schmoranzer, H.P., Naumann, M., Stallmach, A., Hahn, H., Menge, H. and Riecken, E.O. (1989) Immunohistochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype 08 with intestinal mucosa during experimental enteritis. *Infection and Immunity* **57**, 673-678.

Hastings, T.S. and Bruno, D.W. (1985) Enteric redmouth disease: survey in Scotland and evaluation of a new medium, Shotts-Waltman, for differentiating *Yersinia ruckeri*. *Bulletin of the European Association of Fish Pathologists* **5**, 32-35.

Herraez, M.P. and Zapata, A. (1987) Trapping of intraperitoneal-injected *Yersinia ruckeri* in the lymphoid organs of *Carassius auratus*: the role of melano-macrophage centres. *Journal of Fish Biology* **31A**, 235-237.

Hohmann, A.W., Schmidt, G. and Rowley, D. (1978) Intestinal colonization and virulence of *Salmonella* in mice. *Infection and Immunity* **22**, 763-770.

Horne, M.T. and Baxendale, A. (1983) The adhesion of *Vibrio anguillarum* to host tissues and its role in pathogenesis. *Journal of Fish Diseases* **6**, 461-471.

Horne, M.T. and Robertson, D.A. (1987) Economics of vaccination against enteric redmouth disease of salmonids. *Aquaculture and Fisheries Management* 18, 131-137.

Horne, M.T., Tatner, M.F. and Roberts, R.J. (1984) Enteric redmouth - a threat or a promise? *Fish Farming International* 2, 12.

Hunter, V.A., Knittel, M.D. and Fryer, J.L. (1980) Stress-induced transmission of *Yersinia ruckeri* infection from carriers to recipient steelhead trout *Salmo gairdneri* Richardson. *Journal of Fish Diseases* 3, 467-472.

Inchley, C.J. (1981) Immunological strategy. In: *Immunobiology*. (Edited by C.J. Inchley). Edward Arnold, London, Chapter 1, 1-5.

Ingram, G.A. and Alexander, J.B. (1981a) The primary immune response of brown trout (*Salmo trutta*) to cellular and soluble antigens: Enumeration of antibody-secreting and antigen-binding cells, and the production of antibody. *Acta biol. med. germ.* 40, 317-330.

Ingram, G.A. and Alexander, J.B. (1981b) A comparison of the methods used to detect the cellular and humoral immune response of brown trout. *Developments in Biological Standardization.* International Symposium on Fish Biologics: Serodiagnostics and Vaccines, Leetown, W. Va., U.S.A. 49, 295-299.

Johnson, G.R., Wobeser, G. and Rouse, B.T. (1974) Indirect fluorescent antibody technique for detection of RM bacterium of rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* 31, 1957-1959.

Johnson, K.A. and Amend, D.F. (1983) Comparison of efficacy of several delivery methods using *Yersinia ruckeri* bacterin on rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases* 6, 331-336.

Johnson, K.A., Flynn, J.K. and Amend, D.F. (1982a) Onset of immunity in salmonid fry vaccinated by direct immersion in *Vibrio anguillarum* and *Yersinia ruckeri* bacterins. *Journal of Fish Diseases* 5, 197-205.

Johnson, K.A., Flynn, J.K. and Amend, D.F. (1982b) Duration of immunity in salmonids vaccinated by direct immersion in *Yersinia ruckeri* and *Vibrio anguillarum* bacterins. *Journal of Fish Diseases* **5**, 207-213.

Knittel, M.D. (1981) Susceptibility of steelhead trout, *Salmo gairdneri* Richardson, to redmouth infection by *Yersinia ruckeri* following exposure to copper. *Journal of Fish Diseases* **4**, 33-40.

Lamers, C.H.J. and Pilarczyk, A. (1982) Immune response and antigen localization in carp (*Cyprinus carpio*) after administration of *Yersinia ruckeri* 'O'-antigen. *Developmental and Comparative Immunology* Suppl.**2**, 107-114.

Le Cren, E.D. (1951) The length-weight relationship and seasonal cycle in gonad weight and condition in the perch (*Perca fluviatilis*). *Journal of Animal Ecology* **20**, 201-219.

Lehmann, J., Mock. D., Stürenberg, H-J, Bernoth, E-M. (1989) Infection of eel (*Anguilla anguilla,* L.) with *Yersinia ruckeri*. *Bulletin of the European Association of Fish Pathologists* **9**, 45-46.

Lesel, R., Gavini, F., Lesel, M. and Darroze, S. (1985) Dynamics of rainbow trout bacterial flora during *Yersinia ruckeri*-induced enterosepticaemia. In: INSERM, International Bacteriology Symposium, Lille, May 25-27, 1983, *Canadian Translation of Fisheries and Aquatic Sciences No. 5194.*

Lesel, R., Lesel, M., Gavini, F. and Vuillaume, A. (1983a) Outbreak of enteric redmouth disease in rainbow trout, *Salmo gairdneri* Richardson in France. *Journal of Fish Diseases* **6**, 385-387.

Lesel, R., Lesel, M., Gavini, F. and Vuillaume, A. (1983b) Identification in France of a haemorrhagic enterosepticaemia caused by *Yersinia ruckeri* (enteric redmouth disease) in rainbow trout (*Salmo gairdneri* Richardson). *Bulletin Français de la Pisciculture* 288, 73-74.

Levy, S.B. (1984) Resistance to the tetracyclines. In: *Antimicrobial Drug Resistance*. (Edited by L.E. Bryan). Academic Press, London, chapter 7, 191-240.

Levy, S.B. and McMurry, L.M. (1978) Plasmid-determined tetracycline resistance involves new transport systems for tetracycline Nature, **276**, 90-92.

Lewis, D.H. (1981) Immunoenzyme microscopy for differentiating among systemic bacterial pathogens of fish. *Canadian Journal of Fisheries and Aquatic Science* **38**, 463-466.

Llewellyn, L.C. (1980) A bacterium with similarities to the redmouth bacterium and *Serratia liquifaciens* (Grimes and Hennerty) causing mortalities in hatchery reared salmonids in Australia. *Journal of Fish Diseases* **3**, 29-39.

Maas, W.K. (1986) Mutations to antibiotic resistance. In: *Antibiotics in Laboratory Medicine* (Edited by V.Lorian), Williams and Williams, Baltimore. Chapter 17, 669-682.

McArdle, J.F. and Dooley-Martyn, C. (1985) Isolation of *Yersinia ruckeri* type 1 (Hagerman strain) from goldfish *Carassius auratus*. (L.). *Bulletin of the European Association of Fish Pathologists* **5**, 10-11.

McCarthy, D.H. and Johnson, K.A. (1982) A serotypic survey and cross-protection test of North American field isolates of *Yersinia ruckeri*. *Journal of Fish Diseases* **5**, 323-328.

McDaniel, D.W. (1971) Hagerman redmouth: a new look at an old problem. *American Fish Farmer and US Trout News* **1**, 14.

McDaniel, D. (1979) Procedures for the detection and identification of certain fish pathogens. *American Fisheries Society*, Fish Health Section, 55-57.

McMurry, L.M. and Levy, S.B. (1978) Two transport systems for tetracycline in sensitive *Escherichia coli*: critical role for an initial rapid uptake system insensitive to energy inhibitors. *Antimicrobial Agents and Chemotherapy* **14**, 201-209.

McMurry, L.M., Petrucci, R. and Levy, S.B. (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the USA* **77**, 3974-3977.

MAFF. (1984) ERM - topic of the moment. *Fish Farmer* Nov/Dec., 15-16.

Martin, S.M., Meek, A.H. and Willeberg, P. (1988) Veterinary epidemiology - principles and methods. Iowa State University Press, Ames, Iowa, 343 pages.

Martindale. (1989) The extra pharmacopoeia. (Edited by J. Reynolds), 29th edition. The Pharmaceutical Press, London, 1896 pages.

Meier, W. (1986) Enteric redmouth disease outbreak in rainbow trout in Switzerland. *Diseases of Aquatic Organisms* 2, 81-82.

Michel, C. (1986) Practical value, potential dangers and methods of using antibacterial drugs in fish. *Rev. sci. tech. Off. int. Epiz.*, **5**, 659-675.

Michel, C., Faivre, B. and De Kinkelin, P. (1986) A clinical case of enteric redmouth in minnows (*Pimephales promelas*) imported in Europe as bait-fish. *Bulletin of the European Association of Fish Pathologists* **6**, 97-99.

Mitchum, D.L. (1981) Concurrent infections: ERM and furunculosis found in emerald shiners. *American Fisheries Society Newsletter/Fish Health Section* **9**, 2.

Neu, H.C. (1986) Antibiotic inactivating enzymes and bacterial resistance. In: *Antibiotics in Laboratory Medicine.* (Edited by V.Lorian), Williams and Williams, Baltimore. Chapter 20, 757-789.

Neumann, D.A. and Tripp, M.R. (1986) Influence of route of administration on the humoral response of channel catfish (*Ictalurus punctatus*) to *Yersinia ruckeri. Veterinary Immunology and Immunopathology* **12**, 163-174.

Newman, S.G. and Majnarich, J.J. (1982) Direct immersion vaccination of juvenile rainbow trout *Salmo gairdneri* Richardson, and juvenile coho salmon, *Oncorhynchus kisutch* (Walbaum), with a *Yersinia ruckeri* bacterin. *Journal of Fish Diseases* **5**, 34-37.

Noaillac-Depeyre, J. and Gas, N. (1973) Absorption of protein macromolecules by the enterocytes of the carp (*Cyprinus carpio* L.). Ultrastructural and histochemical study. *Zeitschrift fuer Zellforschung und Mikroskopische Anatomie* **146**, 525-541.

Norris, S. and Mandell, G.L. (1988) The quinolones: history and overview. In: *The Quinolones*. (Edited by V.T.Andriole), Academic Press, London, 1-22.

Ocvirk, J., Janc, M., Jeremic, S. and Skalin, B. (1988) The first case of enteric redmouth disease in Yugoslavia. *Ichthyos* **6**, 34-37.

O'Grady, P., Palmer, R., Hickey, C. and Smith, P. (1986) Antibiotic therapy of furunculosis in freshwater and seawater. In: *Pathology in marine aquaculture*, (edited by C.P. Civares, J.R. Bonami and B. Jaspers), European Aquaculture Society Special Publication, Bredene, Belgium, **9**, 407-415.

O'Leary, P.J. (1977) Enteric redmouth bacterium of salmonids: a biochemical and serological comparison of selected isolates. M.S. Thesis, Oregon State University, Corvallis, 93 pages.

O'Leary, P.J., Rohovec, J.S. and Fryer, J.L. (1979) A further characterisation of *Yersinia ruckeri* (enteric redmouth bacterium). *Fish Pathology* **14**, 71-78.

O'Leary, P.J., Rohovec, J.S., Sanders, J.E. and Fryer, J.L. (1982) Serotypes of *Yersinia ruckeri* and their immunogenic properties. *Technical paper No. 6235*. 15 p.

Olesen, N.J. (1991) Detection of the antibody response in rainbow trout following immersion vaccination with *Yersinia ruckeri* bacterins by ELISA and passive immunization. *Journal of Applied Ichthyology* **7**, 36-43.

Olsson-Lilejequist, B., Gezelius, L. and Svensson, S.B. (1985) Selection of multiple antibiotic resistance by norfloxacin and nalidixic acid in Klebsiella and Enterobacter. In: *Abstracts, 25th Interscience Conference on Antimicrobial Agents and Chemotherapy, Minneapolis*, Abstract 106.

Owen, R.L. (1977) Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology* **72**, 440-451.

Pearson, R.D., Steigbigel, R.T., Davis, H.T. and Chapman, S.W. (1980) Method for the reliable determination of minimal lethal antibiotic concentrations. *Antimicrobial Agents and Chemotherapy* **18**, 699-708.

Pepys, M.B., Dash, A.C., Fletcher, T.C., Richardson, N., Munn, E.A. and Feinstein, A. (1978) Analogues in other mammals and in fish of human plasma proteins, C-reactive protein and amyloid P-component. *Nature* 273, 168-170.

Plakas, S., McPhearson, R. and Guarino, A. (1988) Disposition and bioavailability of ^{3}H-tetracycline in the channel catfish (*Ictalurus punctatus*). *Xenobiotica*, 18, 83-93.

Post, G. (1987) Enteric redmouth disease (Yersiniosis). *Textbook of fish health.* TFH Publications Ltd., 47-51.

Pyle, S.W., Ruppenthal, T., Cipriano, R.C. and Shotts, E.B. (1987) Further characterization of biochemical and serological characteristics of *Yersinia ruckeri*, from different geographic areas. *Microbios Letters* 35, 87-93.

Pyle, S.W. and Schill, W.B. (1985) Rapid serological analysis of bacterial lipopolysaccharides by electrotransfer to nitrocellulose. *Journal of Immunological Methods* 85, 371-382.

Rijkers, G.T. (1982) Non-lymphoid defense mechanisms in fish. *Developmental and Comparative Immunology* 6, 1-13.

Rintamäki, P., Valtonen, E.T. and Frerichs, G.N. (1986) Occurrence of *Yersinia ruckeri* infection in farmed whitefish, *Coregonus peled* Gmelin and *Coregonus muksun* Pallas, and Atlantic Salmon, *Salmo salar* L, in northern Finland. *Journal of Fish Diseases* 9, 138-140.

Roberts, M.S. (1983) A report of an epizootic in hatchery rainbow trout *Salmo gairdneri* Richardson, at an English trout farm, caused by *Yersinia ruckeri*. *Journal of Fish Diseases* 6, 55-552.

Roberts, R.J. (1989) The immunology of teleosts. *Fish Pathology*, Baillière Tindall, 2nd edition. Chapter 4, 135-152.

Rodgers, C.J. and Austin, B. (1983) Oxolinic acid for control of enteric redmouth disease in rainbow trout. *Veterinary Record* 112, 83.

Rodgers, C.J. and Hudson, E.B. (1985) A comparison of two methods for isolation of *Yersinia ruckeri* from rainbow trout (*Salmo gairdneri*). *Bulletin of the European Association of Fish Pathologists* **5**, 92-93.

Rombout, J.H.W.M., Lamers, C.H.J., Helfrich, M.H., Dekker, A. and Taverne-Thiele, J.J. (1985) Uptake and transport of intact macromolecules in the intestinal epithelium of carp (*Cyprinus carpio* L.) and the possible immunological implications. *Cell and Tissue Research* **239**, 519-530.

Rombout, J.H.W.M. and Van den Berg, A.A. (1989) Immunological importance of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. *Journal of Fish Biology* **35**, 13-22.

Ross, A.J. and Klontz, G.W. (1965) Oral immunization of rainbow trout (*Salmo gairdneri*) against an etiologic agent of redmouth disease. *Journal of Fisheries Research Board of Canada* **22**, 713-719.

Ross, A.J., Rucker, R.R. and Ewing, W.H. (1966) Description of a bacterium associated with redmouth disease of rainbow trout (*Salmo gairdneri*). *Canadian Journal of Microbiology* **12**, 763-770.

Ross, A.J. and Smith, C.A. (1972) Effect of two iodophors on bacterial and fungal fish pathogens. *Journal of Fisheries Research Board of Canada* **29**, 1359-1361.

Rowley, A.F., Hunt, T.C., Page, M. and Mainwaring, G. (1988) Fish. In: *Vertebrate Blood Cells* (edited by A.F. Rowley and N.A. Ratcliffe), Cambridge University Press. Chapter 2, 19-127.

Rübsamen, S. and Weis, J. (1985) Detection of enteric redmouth disease in rainbow trout, *Salmo gairdneri* Richardson, in South Baden. *Tierärztliche Umschau* **40**, 995-998.

Rucker, R.R. (1966) Redmouth disease of rainbow trout (*Salmo gairdneri*). *Bulletin of the Office of International Epizootics* **65**, 825-830.

Rüger, H-J. (1981) Comparison of the API and Minitek identification systems with conventional methods for differentiating marine bacteria. *Veröff. Inst. Meeresforsch. Bremerh.* **19**, 21-34.

Sabath, L.D., Wheeler, N., Laverdiere, M., Blazevic, D. and Wilkinson, B. (1977) A new type of penicillin resistance of *Staphylococcus aureus*. *Lancet* **1**, 443-447.

Schlotfeldt, H-J., Böhm, K.H., Pfortmüller, F. and Pfortmüller, K. (1986) Enteric redmouth disease/ERM of trout and other farmed fish in north-west Germany. *Fischkrankheiten* **37**, 66-70.

Scott, A.L., Rogers, W.A. and Klesius, P.H. (1985) Chemiluminescence by peripheral blood phagocytes from channel catfish: function of opsonin and temperature. *Developmental and Comparative Immunology* **9**, 240-250.

Shepherd, C.J. (1988a) What is fish farming? In: *Intensive Fish Farming*, BSP Books, (edited by C.J. Shepherd and N.R. Bromage). Chapter 1, 1-16.

Shepherd, C.J. (1988b) Fish health and disease. In: *Intensive Fish Farming*, BSP Books, (edited by C.J. Shepherd and N.R. Bromage). Chapter 6, 198-238.

Shotts, E.B. (1991) Selective isolation methods for fish pathogens. In: *Pathogens in the environment.* Society of Applied Bacteriology Symposium Series No. 20 (edited by B. Austin), 75S-80S.

Shotts, E.B. and Bullock, G.L. (1975) Bacterial disease of fishes: diagnostic procedures for Gram-negative pathogens. *Journal of the Fisheries Research Board of Canada* **32**, 1243-1247.

Shotts, E.B. and Rimler, R. (1973) Medium for the isolation of *Aeromonas hydrophila*. *Applied Microbiology* **26**, 550-553.

Siwicki, A.K., Anderson, D.P. and Dixon, O.W. (1989) Comparisons of nonspecific and specific immunomodulation by oxolinic acid, oxytetracycline and levamisole in salmonids. *Veterinary Immunology and Immunopathology* **23**, 195-200.

Smith, A.M., Goldring, O.L. and Dear, G. (1987) The production and methods of use of polyclonal antisera to the pathogenic organisms *Aeromonas salmonicida, Yersinia ruckeri* and *Renibacterium salmoninarum*. *Journal of Fish Biology* **31A**, 225-226.

Smith, J.T. (1985) The 4-quinolone antibacterials. In: *Progress in Antimicrobial Chemotherapy*. (Edited by D.Greenwood and F.O'Grady), Society for General Microbiology Symposium, **38**, 69-94.

Smith, J.T. and Lewin, C.S. (1988) Chemistry and mechanisms of action of the quinolone antibacterials. In: *The Quinolones*. (Edited by V.T.Andriole), Academic Press, London, 23-82.

Sokatch, J.R. and Ferretti, J.J. (1976) Growth of bacteria. In: *Basic bacteriology and genetics*. Year Book Medical Publishers, Inc., Chicago. 40-43.

Sparboe, O., Koren, C., Håstein, T., Poppe, T. and Stenwig, H. (1986) The first isolation of *Yersinia ruckeri* from farmed Norwegian salmon. *Bulletin of the European Association of Fish Pathologists* **6**, 41-42.

Stamm, J.M. (1989) *In vitro* resistance by fish pathogens to aquacultural antibacterials, including the quinolones difloxacin (A-56619) and sarafloxacin (A-56620). *Journal of Aquatic Animal Health* **1**, 135-141.

Stevenson, R.M.W. and Airdrie, D.W. (1984) Isolation of *Yersinia ruckeri* bacteriophage. *Applied and Environmental Microbiology* **47**, 1201-1205.

Stevenson, R.M.W. and Daly, J.G. (1982) Biochemical and serological characteristics of Ontario isolates of *Yersinia ruckeri*. *Canadian Journal of Fisheries and Aquatic Science* **39**, 870-876.

Sugita, H., Miyajima, C. Fukumoto, M. Koyama, H. and Deguchi, Y. (1989) Effect of oxolinic acid on fecal microflora of goldfish (*Carassius auratus*). *Aquaculture*, **80**, 163-174.

Tatner, M.F. and Horne, M.T. (1985) The effects of vaccine dilution, length of immersion time, and booster vaccinations on the protection levels induced by direct immersion vaccination of brown trout, *Salmo trutta*, with *Yersinia ruckeri* (ERM) vaccine. *Aquaculture* **46**, 11-18.

Taylor, P.C., Schoenknecht, F.D., Sherris, J.C. and Linner, E.C. (1983) Determination of minimum bactericidal concentrations of oxacillin for *Staphylococcus aureus*: influence and significance of technical factors. *Antimicrobial Agents and Chemotherapy* **23**, 142-150.

Tebbit, G. L., Erickson, J.D. and Vande Water, R.B. (1981) Development and use of *Yersinia ruckeri* bacterins to control enteric redmouth disease. *Developments in Biological Standardization,* International Symposium on Fish Biologics: Serodiagnostics and Vaccines, Leetown, W. Va., U.S.A. **49**, 395-401.

Temkin, R.J. and McMillan, D.B. (1986) Gut-associated lymphoid tissue of the goldfish *Carassius auratus*. *Journal of Morphology* **190**, 9-26.

Tenney, J.H., Maack, R.W. and Chippendale, G.R. (1983) Rapid selection of organisms with increasing resistance on subinhibitory concentrations of norfloxacin in agar. *Antimicrobial Agents and Chemotherapy* **23**, 188-189.

Thrupp, L. D. (1986) Susceptibility testing of antibiotics in liquid media. In: *Antibiotics in Laboratory Medicine.* (Edited by V.Lorian), Williams and Williams, Baltimore. Chapter 4, 93-150.

Thrusfield, M. (1985) How to use a knowledge of epidemiology in practice. *Pig Veterinary Society Proceedings* **12**, 25-37.

Toranzo, A. E., Baya, A. M., Roberson, B. S., Barja, J. L., Grimes, D. J. and Hetrick, F. M. (1987) Specificity of slide agglutination test for detecting bacterial fish pathogens. *Aquaculture* **61**, 81-97.

Toranzo, A. E., Santos, Y., Nieto, T.P. and Barja, J. L. (1986) Evaluation of different assay systems for identification of environmental *Aeromonas* strains. *Applied and Environmental Microbiology* **51**, 652-656.

Tsoumas, A., Alderman, D.J. and Rodgers, C.J. (1989) *Aeromonas salmonicida*: development of resistance to 4-quinolone antimicrobials. *Journal of Fish Diseases* **12**, 493-507.

Vigneulle, M. and Baudin Laurencin, F. (1991) Uptake of *Vibrio anguillarum* bacterin in the posterior intestine of rainbow trout *Oncorhynchus mykiss*, sea bass *Dicentrarchus labrax* and turbot *Scophthalmus maximus* after oral administration or anal intubation. *Diseases of Aquatic Organisms* **11**, 85-92.

Vuillaume, A., Brun, R., Chene, P., Sochon, E. and Lesel, R. (1987) First isolation of *Yersinia ruckeri* from sturgeon, *Acipenser baeri* Brandt, in south west France. *Bulletin of the European Association of Fish Pathologists* **7**, 18-19.

Waltman, W.D. and Shotts, E.B. (1984) A medium for the isolation and differentiation of *Yersinia ruckeri*. *Canadian Journal of Fisheries and Aquatic Science* 41, 804-806.

Warren, J.W. (1983) Enteric Redmouth Disease. In: *A guide to integrated fish health management in the Great Lakes Basin*. Special Publication No. 83-2, Great Lakes Fishery Commission (edited by F.P.Meyer, J.W. Warren and R.G.Carey), Chapter 24.

Wasteson, U., Hvaal, A-B., Sφrum, H., Myhr, E. and Fossum, K. (1989) Antibacterial spectrum and some other characteristics of an antimicrobial factor produced by *Yersinia ruckeri*. *Acta veterinaria Scandinavica* **30**, 253-257.

Weinberg, S. (1975) Occurrence of lymphoid cells in the intestine of the goldfish. *Bijdragen tot de Dierkunde*, **45**, 195-204.

White, A., Fletcher, T.C., Pepys, M.B. and Baldo, B.A. (1981) The effect of inflammatory agents on C-reactive protein and serum amyloid P-component levels in plaice (*Pleuronectes platessa* L.) serum. *Comparative Biochemical Physiology* **69C**, 325-329.

Willumsen, B. (1989) Birds and wild fish as potential vectors of *Yersinia ruckeri*. *Journal of Fish Diseases* **12**, 275-277.

Wobeser, G. (1973) An outbreak of redmouth disease in rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* **30**, 571-575.

Wood, S.C., McCashion, R.N. and Lynch, W.H. (1986) Multiple low-level antibiotic resistance in *Aeromonas salmonicida*. *Antimicrobial Agents and Chemotherapy*, **29**, 992-996.

Wray, C. (1986) Some aspects of the occurrence of resistant bacteria in the normal animal flora. *Journal of Antimicrobial Chemotherapy* **18**, Supplement C, 141-147.

Zapata, A.G., Torroba, M., Alvarez, F., Anderson, D.P., Dixon, O.W. and Wisniewski, M. (1987) Electron microscopic examination of antigen uptake by salmonid gill cells after bath immunization with a bacterin. *Journal of Fish Biology* **31A**, 209-217.