Yersiniosis in Fish

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SUMMARY

Yersiniosis is a contagious bacterial disease of salmonids, eels, goldfish, sole, sturgeon and turbot caused by *Yersinia ruckeri*, a member of the family *Enterobacteriaceae*. The bacterium is found in fish populations throughout Europe, North and South America, Australia and New Zealand.

Infection with *Yersinia ruckeri* results in a bacterial septicaemia without disease specific signs but is most commonly detected due to exophthalmos and blood spots in the eye. The severity of the disease is dependant upon the biotype of the bacterium involved. Acute infections in trout with the 'Hagerman' strain are usually florid and the disease is referred to as enteric red mouth. A milder form of the disease occurring in Atlanitic salmon is termed Yersiniosis.

Identification of the agent: A diagnosis of Yersiniosis is based on clinical signs and isolation in culture of the bacterial pathogen from systemic sites such as head kidney or spleen. The bacterium is not fastidious and can be grown on simple culture media such as tryptone soya agar. The identification of *Y ruckeri* should be made by phenotyping. A polymerase chain reaction method is also available but has a prescribed application limited to confirmatory identification.

Status of Australia and New Zealand: *Yersinia ruckeri* is enzootic to both Australia and New Zealand. In Australia, two biotypes of *Y ruckeri* are known to occur: serotype O1b, biotype 1 and serotype O1, non-O1b, biotype 2. The virulent Hagerman strain, the cause of enteric red mouth in rainbow trout is exotic to both countries.

Part 1 – Diagnostic Overview

Introduction

Yersiniosis in fish is a significant bacterial septicaemia caused by *Yersinia ruckeri*. The organism appears to have a wide geographical distribution because it is found in many countries that raise salmonids under intensive conditions.

Y ruckeri has been reported to occur in fish in Australia, Bulgaria, Canada, Chile, Denmark, Finland, France, Germany, Greece, Iran, Italy, New Zealand, Norway, South Africa, Portugal, Spain, Sweden, Switzerland, Turkey, United Kingdom, United States of America and Venezuela. The number of countries in which *Y ruckeri* has been isolated is increasing and this list is indicative only.

The severity of the disease is dependant upon the biotype involved and the salmonid host. Acute infections in rainbow trout (*Oncorhynchus mykiss*) with the 'Hagerman' strain are usually florid and the disease is referred to by the universal epithet of enteric red mouth or ERM. A less severe form of the disease may also occur in Atlantic salmon (*Salmo salar*) involving a different serotype of *Y ruckeri*. This condition is referred to as yersiniosis[1] (also salmonid blood spot). The term yersiniosis is used to distinguish the less florid form of infection from the acute disease associated with ERM.

Data on epizootiology are based on rainbow trout infected with virulent strains. The disease can affect fish of all age classes but is most acute in small fish up to fingerling size. In larger fish, the disease is chronic. Fish most at risk are those subject to stress arising from poor management or environmental changes such as elevated temperatures or poor water quality.[2] Losses in juvenile rainbow trout may reach 2% per week with cumulative losses reaching 35%.

Asymptomatic carriage of the pathogen is known to occur in rainbow trout[3] and Atlantic salmon. Localisation of bacteria may occur in kidney and the distal portion of the gastro-intestinal tract, a site from which bacteria may be excreted to the water column.

A wide variety of fish species have been cited as susceptible hosts. Species of commercial importance include: Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), eel (*Anguilla anguilla*), goldfish (*Carassius auratus*), perch (*Perca fluviatilis*), channel catfish (*Ictalurus punctatus*), sole (*Solea solea*), sturgeon (*Acipenser baeri*) and turbot (*Scophthalmus maximus*).

In Australia, infection with *Y ruckeri* occurs predominantly in Atlantic salmon with very rare isolations from rainbow trout and brown trout.

While *Y ruckeri* is the pre-eminent species as a cause of disease, *Y enterocolitica Y intermedia* and *Y frederiksenii* have also been isolated from fish. Of the species listed, only *Y intermedia* has been associated with disease and is the cause of septicaemia in cold-compromised Atlantic salmon.[4]

Aetiology

Yersinia ruckeri is a member of the family *Enterobacteriaceae* and possesses general attributes associated with the taxon. The genus *Yersinia* forms a discrete cluster of species within the gamma subgroup of the *Proteobacteria*, based on 16S rRNA phylogenetic analysis.[5] Within the genus, 5 sub-lines can be identified, one of which contains a single species, *Y ruckeri*. The sequence homology for the genus is high ranging from 99.6% for *Y intermedia* and *Y mollarettii* to 96.6% for *Y ruckeri* and *Y enterocolitica*. The singularity of *Y ruckeri* is evident however by DNA-DNA hybridisation, which shows that *Y ruckeri* has only a 38% sequence homology with other species of the genus.

The phenotype of *Y ruckeri* is unlike other species of *Yersinia*, and instead appears to have characteristics of a number of other members of the *Enterobacteriaceae.* Early descriptions of the pathogen in Australia suggested that the organism had features of *Serratia liquefaciens*[1] while other studies have reported a similarity to *Salmonella arizonae*.[6] There is marked similarity in phenotypes between *Hafnia alvei* and *Y ruckeri* but not by serotype or genotype.[7] Current descriptions of the pathogen are more complete, and based on a wide range of

isolates from a number of geographic regions. *Y ruckeri* can be readily differentiated from other members of the *Enterobacteriaceae*.

The virulence factors of *Y ruckeri* have not been fully determined. It has been established that the pathogen can elaborate a siderophore, which is involved in sequestering iron under potentially growth limiting conditions.[8] Possession of an efficient iron uptake mechanism may form an important component of the virulence capacity of *Y ruckeri*. Survival of the pathogen within the host is also thought to be assisted by elaboration of a cell envelope lipid, a heat-sensitive factor (HSF+), which masks immuno-reactive surface antigens.[9] Extracellular factors including proteases and haemolysin[10] are known to be elaborated by *Y ruckeri,* which also possesses a Type III secretion system to transport exotoxins to host cells.[11]

A 62 megadalton (MDa) plasmid has been detected in European and American strains of *Y ruckeri*[12] while a 75 MDa plasmid has been found only in serogroup O1 strains[13] (*See serotyping below*). This plasmid appears to be significantly different to the 42-47 MDa virulence plasmid associated with other species of the genus *Yersinia*. The role of *Y ruckeri* plasmids as virulence factors remains unclear.

The organism is oxidase negative, facultatively anaerobic, ferments glucose and motile (*See Phenotypic profile below for exceptions*) by means of a peritrichous arrangement of flagella; the G+C ratio of DNA has been determined at 48 ± 0.5 mol%. The species has a growth temperature optimum between 20 and 25°C but can grow at 37°C. Like other species of the genus, the response of *Y ruckeri* in characterisation tests can be significantly different at 37°C compared with 25°C[14]; the most consistent phenotypic descriptions of the species have been determined at 25°C.

On the basis of heat stable antigens (lipopolysaccharide), *Y ruckeri* can be divided into four major serotypes: O1, O2, O3 and O4.[15] Within serotype O1, two subgroups O1a and O1b can be distinguished while for O2, three subgroups O2a, O2b and O2c can be recognised. Australian isolates serotype as O1b (*See Serotyping below*). It should be noted that most isolates have been serotyped with the group specific O1 antiserum and only a few isolates have been tested with monospecific O1b antiserum. A serotype variant of the O1 group has emerged in Australia which *pro tem* is labelled non-O1b and has been reported to occur only in Tasmania.

Strains of *Y ruckeri* can be grouped into clonal types on the basis of biotype, serotype and outer membrane protein types.[16, 17,18,19] Most strains of *Y ruckeri* belong to serogroup O1 (*See Serotyping below*); within this serogroup, 6 clonal types designated 1-6 can be recognised. Australian isolates have been placed in clonal groups 1 and 3; by comparison, most strains from Europe, including the virulent 'Hagerman' strain from the United States have been placed in clonal group 5. Clonal groups 2 and 5 contain strains associated with major disease outbreaks and are considered to contain virulent strains. The remaining clonal groups are considered to be relatively avirulent. A further form of biotyping is recognised within the O1 serogroup. On the basis of motility and Tween 80 hydrolysis two distinct phenotypes are recognised.[16] Biotype 1 strains are motile and hydrolyse Tween 80 while biotype 2 is negative for both traits. In Australia, serotype O1b strains are biotype 1 while the non-O1b type is biotype 2.

Clinical Signs and Gross Pathology

There are no specific early signs of disease to indicate infection with *Y ruckeri* other than general indicators of bacterial septicaemia.

The first signs of the disease in juvenile salmonid fish are seen as an increase in mortalities above the normal attrition rate. Changes in fish behaviour may be observed including swimming near the surface, moving sluggishly, and darkening. Inappetence occurs in affected fish. A common feature of yersiniosis in Atlantic salmon is the development of a marked unilateral or bilateral exophthalmos often with frank patches of haemorrhagic congestion on the iris of the eye, a characteristic that gave rise to the epithet salmonid blood spot disease. In rainbow trout, subcutaneous haemorrhage in the mouth and throat is strongly indicative of the disease and hence the term enteric red mouth. This does not appear to be a characteristic of infection in Atlantic salmon in Australia.

Other external signs of the disease may include haemorrhagic congestion at the base of the pectoral and pelvic fins, a distended vent and in small fish especially, a pallor to the gills arising from bacterial induced anaemia. Small areas of muscle liquefaction resulting in skin lesions can occur but is not common.

Yersiniosis may occur in Atlantic salmon smolt, usually 3-6 weeks after their introduction to sea water. The number of fish affected is small, typically 0.1-0.75% per week, and infection is manifest by poor feeding response in recently transferred smolt, rising levels of mortality and appearance of exophthalmos and blood spots in the eye (Figure 1).

Figure 1. Atlantic salmon with exophthalmos and characteristic blood spot associated with *Yersinia ruckeri* serotype O1b. (*Courtesy Kevin Ellard*)

Post-spawning rainbow trout or egg-bound fish may develop a chronic peritonitis. In most instances this is due to *Maltaromaticum* (*Carnobacterium*) *piscicola*, but occasionally *Y ruckeri* may also be isolated. Since a number of pathogens may be associated with this condition, bacteriological examination should be undertaken to reach a definitive diagnosis.

Fish infected with *Y ruckeri* may have petechiation on the pyloric caecae, hypertrophy of the spleen, peritonitis, and the gastro-intestinal tract may be empty of food but filled with a clear to yellow mucus. In aggressive forms of the disease, erythema around the meninges may also be seen.

Histopathological findings in fish infected with *Y ruckeri* are those of a typical septicaemia. Bacteria are readily detected free in the blood and in circulating and sequestered macrophages; tissue localisation of bacteria at sites of haemorrhage may also be evident.

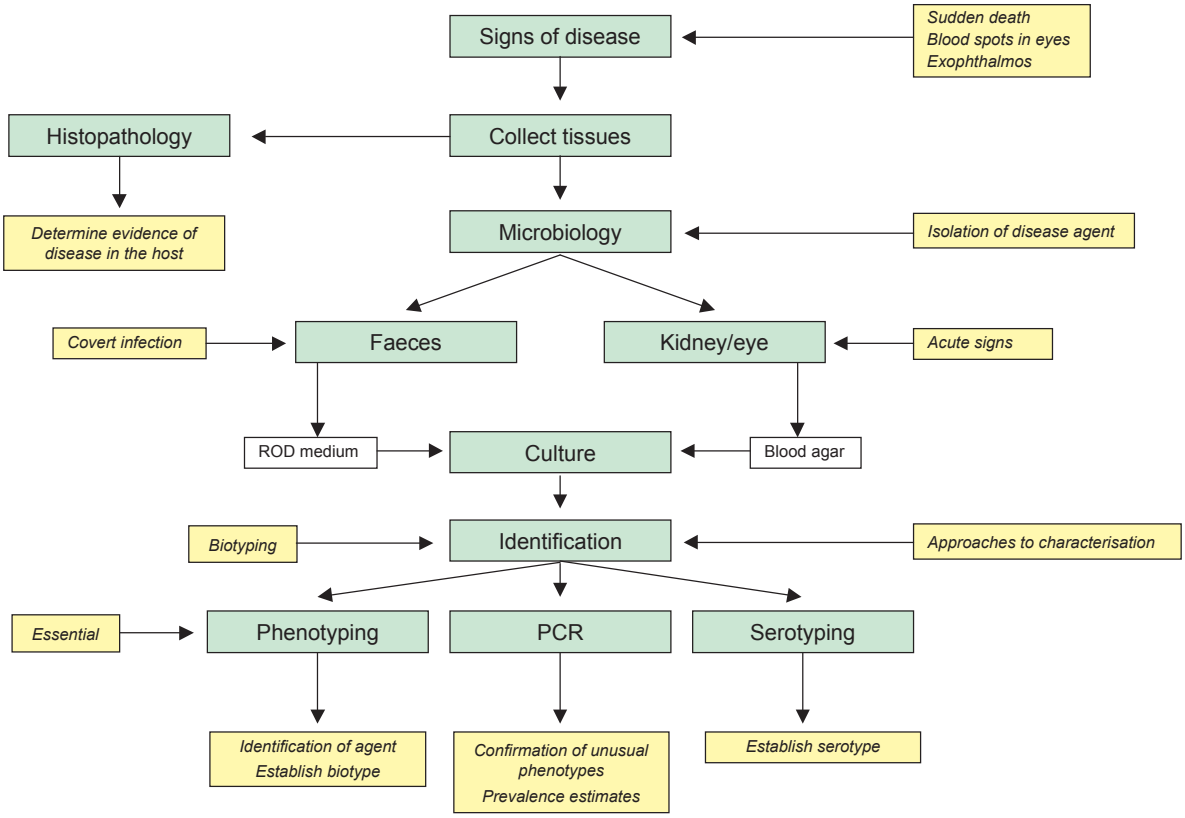
Histologically, salmon fry may contain overwhelming numbers of bacteria with high concentrations detectable in macrophages of kidney and liver sinusoids. Circulatory collapse is evident with oedema and apparent anaemia. These changes are most evident in the gills, which may show blood stasis and bacterial clumps.

Yersinia ruckeri infection in Atlantic salmon smolt during acclimatisation stress is characterised by fewer bacteria in blood, with congestion, haemorrhage and tissue localisation more apparent than acute inflammation. Localisation in the choroid and meninges is common, and encephalitis may be seen.

Diagnostic Tests Overview

Diagnosis of yersiniosis requires isolation and identification of *Y ruckeri* from tissue samples. More commonly it is based on the phenotypic profile and it can be readily differentiated from other taxa within the *Enterobacteriaceae*. In addition, a polymerase chain reaction technique is available but is best used to confirm the identity of ambiguous isolates. PCR should not be used on fish without apparent clinical signs.

Flow-diagram for investigating yersiniosis in fish



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Part 2 – Test Methods

Sample Collection

Acute infections

It is preferable to select fish on site and culture immediately. Where this is not possible, live fish should be submitted; if fish are too large to transport live, then they should be packed in ice and sent to the laboratory. To reduce adverse microbial changes, the time between collection and receipt by the laboratory should not exceed 12 hours.

Moribund fish or fish with apparent lesions such as exophthalmos, blood spots in the eyes or congestion at the base of the fins should be selected for culture. In the early stages of an outbreak, recovery of the pathogen from individual fish can be variable and it is necessary to culture at least 5 but preferably 10 fish to obtain a reliable diagnosis of infection.

The concentration of bacteria in organs of the fish may vary considerably particularly in the early stages of infection. To increase the probability of recovery, several sites must be cultured. It is essential to sample behind the eye and the kidney or spleen (or liver in small fish, if the spleen is too small). All these organs represent sites where bacteria are most likely to be concentrated within the host. Smears for Gram's stain should be prepared for each site sampled.

Eye – Lightly sear the surface of the eye and the surrounding skin. Excise the eye intact using a fine scalpel blade; collect a sample from the remnants of the choroid mass at the back of the orbit using a fine sterile glass pipette or a 10 µL pipette tip fitted to a pipettor if the fish are small. Alternatively, inoculate culture plates by touching the back of the eye on the surface of an agar plate.

Kidney – Observing aseptic precautions, dissect the fish to reveal the kidney. Collect a sample from the anterior region of the kidney using a sterile glass pipette. Use a 10 µL pipette tip fitted to a pipettor if the fish are small or use a drawn glass pipette.

Spleen – Working from the left hand side of the fish, aseptically remove the flank to reveal the spleen. Remove a portion of spleen and inoculate culture plates by touching the cut surface of the tissue on the surface of an agar plate. A liver sample may be collected in a similar fashion.

Testing for carriers

The absence of a reliable enrichment or highly selective medium for *Y ruckeri* can make testing for carriers by culture of faeces an unrewarding exercise. Where required, the following procedures are recommended for the collection of samples.

Euthanased fish Culture kidney by the method described above. In addition, remove the distal 1-2 cm of the gut and place in a sterile petri dish. Slit the gut along its length and, with a cotton swab, sample the walls of the gut.

Live fish Anaesthetise the fish to be tested. Collect a small quantity of faeces into a sterile tube by gently massaging the sides of the fish at the distal end of the gut. Alternatively, gently introduce a retropharyngeal swab mounted on a flexible wire into the distal part of the gut via the vent.

Culture

Yersinia ruckeri is not a fastidious organism and can be grown on simple culture media such as tryptone soya agar. In addition, the species can tolerate bile salts, the selective component in a number of media used for the isolation of enteropathogenic *Enterobacteriaceae*. *Y ruckeri* will grow readily on MacConkey agar and XLD agar (Xylose Lysine Desoxycholate).

Isolation from haematogenous sites

Inoculate a plate of blood agar base (Blood agar base no. 2, CM271, Oxoid) enriched with 7-10% defibrinated sheep's blood. Adequate growth can also be obtained on tryptone soya agar (Oxoid CM131), but colonial morphology on this medium is nondescript.

If secondary infection with other bacteria is suspected, samples should also be cultured on plates of XLD agar (Oxoid CM469) or preferably Ribose Ornithine Desoxycholate agar (ROD), a moderately selective indicator medium for *Y ruckeri*[20] (*See Appendix*).

Where signs of disease are apparent, typically *Y ruckeri* is recovered in pure culture from internal sites and, depending on the stage of infection, colony density will range from light to heavy.

Recovery from faeces

Recovery of *Y ruckeri* from faeces is only of value for the detection of asymptomatic carrier fish. Isolation is problematic in that no enrichment media or highly selective plate media are currently available.

The Shotts-Waltman medium[21] is a semi-selective indicator medium for *Y ruckeri*, with inhibitory properties equivalent to MacConkey agar. The indicator is based on the ability of *Y ruckeri* to hydrolyse Tween 80, and inability to produce acid from sucrose. The concentration of bromothymol blue was incorrectly stated in the original formulation and was subsequently amended to 0.03 g/L.[22] The value of this medium has been questioned[23,24] since biotype 2 strains of *Y ruckeri* are unable to hydrolyze Tween 80.[16] Use of the medium is not recommended.

Ribose ornithine desoxycholate agar (ROD) can be useful for the detection of *Y ruckeri* in faeces of carrier fish[20] and can detect both biotypes 1 and 2. A key differential feature of the medium is formation of zones of precipitation around colonies of *Y ruckeri*. This feature however is seen only with serotypes O1 and O4[20] and the medium is of limited value for the detection of other serotypes of *Y ruckeri*. Given that serotype O1 is the most commonly isolated form of *Y ruckeri*, and is the only serotype encountered in Australia so far, the medium has practical application. The medium does not suppress the growth of all *Enterobacteriaceae*, and *Citrobacter, Hafnia* and *Enterobacter* may predominate in some faecal samples. If ROD is not available, XLD agar can be used but is less discriminating than ROD.

Faeces Plate out a sample directly on plates of blood agar and ROD medium. Prepare a 1:10 dilution of the faeces in phosphate buffered saline, pH 7.2, 0.1M. Homogenise the sample by aspirating with a pipette and inoculate single plates of blood agar and ROD medium with 1 µL of suspension using a calibrated loop.

Swabs Inoculate plates of blood agar and ROD medium directly with the swab and streak for isolated colonies with a loop.

The density of *Y ruckeri* in faecal samples tends to be low in carriers and, typically, few colonies of *Y ruckeri* will be evident on culture plates.

Incubation

Cultures should be incubated in air at 25°C for up to 72 hours, and examined daily. If isolation is attempted from faecal samples, suspect colonies should be subcultured on appearance to blood agar, to ensure that colonies of *Y ruckeri* are not overgrown. Samples cultured on ROD medium should be incubated for at least 96 hours so that the differential properties of the medium are fully expressed (*See Colonial Morphology*).

Identification

Smears

In smears from tissues such as kidney or from the retro-bulbar region of the eye, cells of *Y ruckeri* appear as short rods approximately 1.5 µm long and 0.75 µm wide. The cells stain well with dilute carbol-fuchsin as the counter stain, and a marked bipolar staining may be evident. Frequently, the cells of *Y ruckeri* in tissue smears appear rectangular with square ends. This unusual appearance is not evident when the bacteria are grown on culture media.

Care must be exercised when examining smears made from the eye and surrounding tissue. If retinal tissue forms part of the smear, casual examination may mistake retinal rods for bacillary bacteria. Retinal rods can be differentiated from bacteria by their regular, too angular appearance and brown pigmentation, features that can be observed when the condenser, field and stage iris of the microscope are set critically.

Colonial morphology

On blood agar, well separated colonies of *Y ruckeri* after incubation at 25°C for 48 hours appear off-white, opaque with a marked bull's eye (see Figure 2). Colonies are approximately 2-3 mm in diameter, smooth, entire edged with a low convex profile. Older cultures develop a highly characteristic slightly acrid odour reminiscent of stale mushrooms.

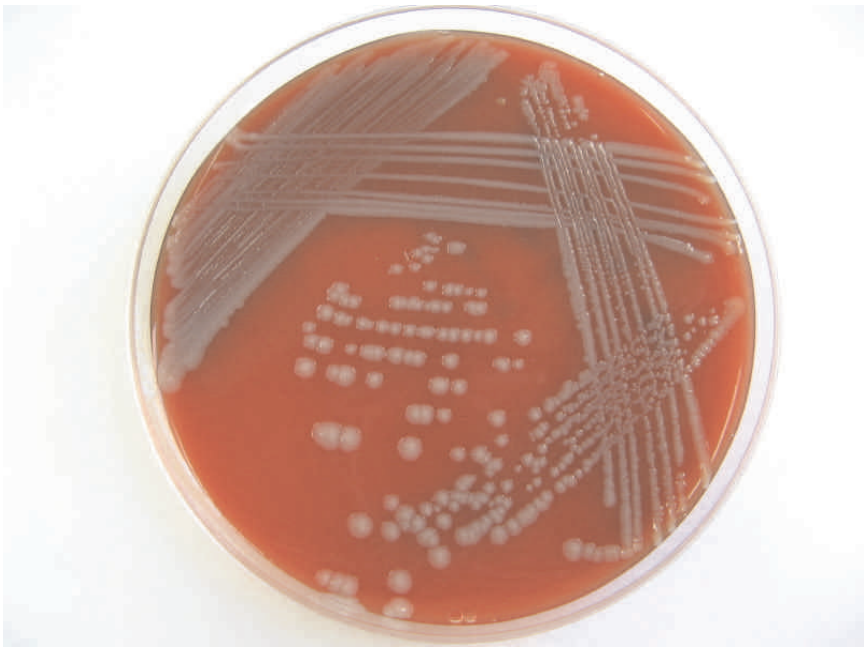


Figure 2. Pure culture of *Yersinia ruckeri* on sheep's blood agar after incubation at 25°C for 48 hours. Colonies showing characteristic bull's eye appearance.

Occasionally, plaques can be seen on primary plates (Figure 3) arising from bacteriophage activity. The significance of this finding is uncertain other than bacteriophages specific for *Y. ruckeri* are known to exist.[25]

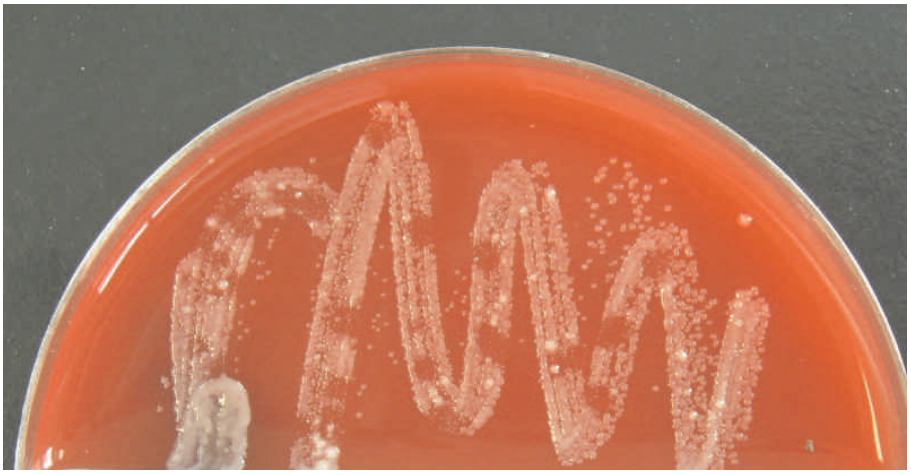


Figure 3. Primary culture (farm submission) from kidney of Atlantic salmon, showing plaques caused by bacteriophage.

On ROD medium, colonies of *Y ruckeri* serotype O1, biotype 1 and 2 and serotype O4 appear as yellow colonies on a red background with zones of precipitation around the colonies after incubation for 96 hours (see Figure 4). *Y ruckeri* other than serotypes O1 or O4 appear as yellow colonies but without the characteristic zone of precipitation.

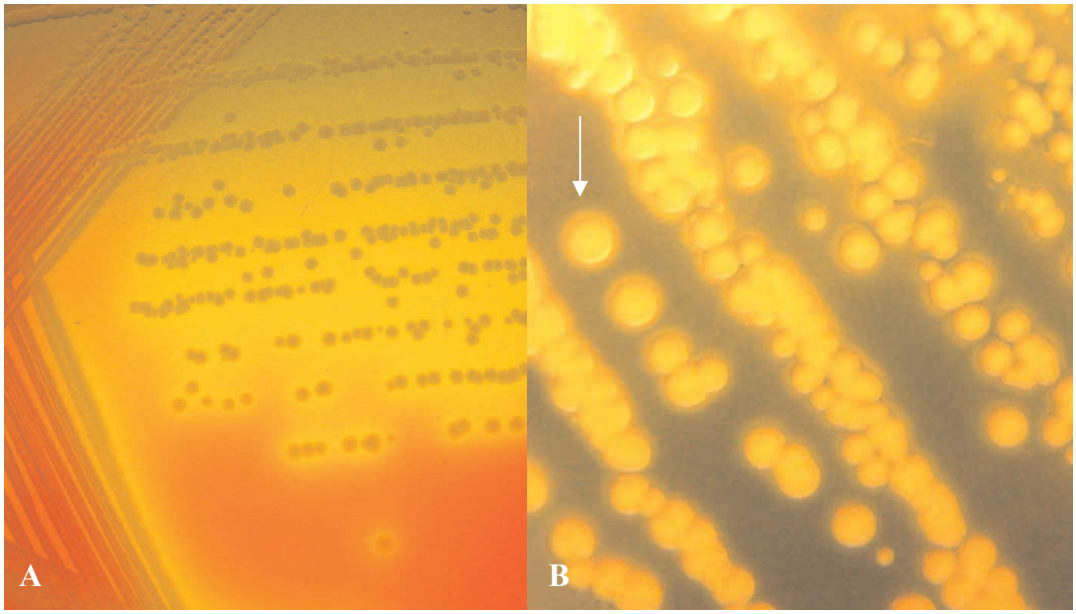


Figure 4. Colonies of *Yersinia ruckeri* on ribose ornithine desoxycholate agar.

A: yellow colonies of *Y ruckeri* arising from ribose fermentation.

B: After four days incubation at 25°C, zones of precipitation (arrowed) are evident with serotype O1 isolates.

Y ruckeri on XLD appear as bright pink colonies, 1-2 mm in diameter, often with a zone of diffuse pink colouration (see Figure 5).

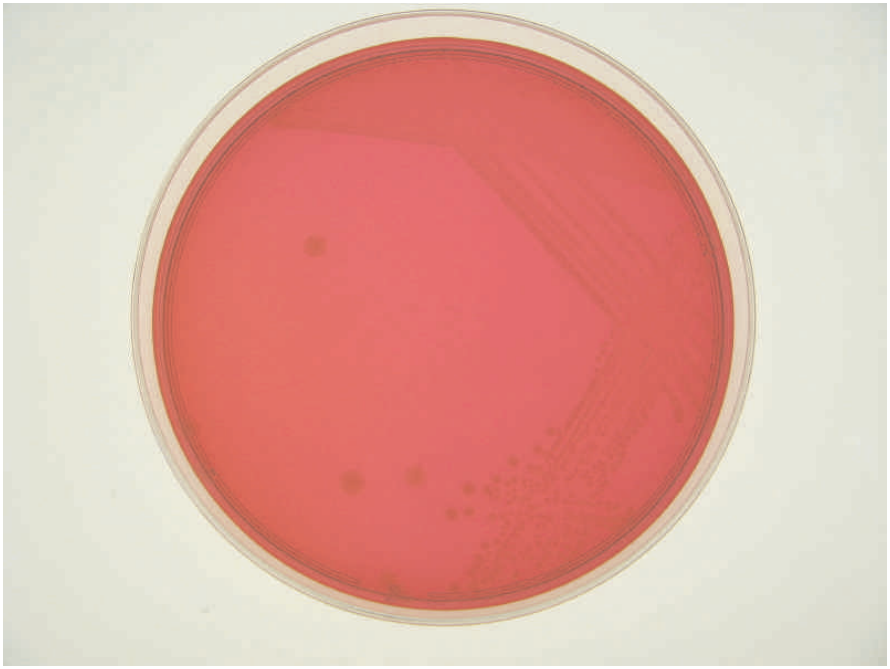


Figure 5. Yersinia ruckeri on XLD medium after 48 hours incubation at 25°C. Nondescript pink colonies arising from lysine decarboxylation and failure to ferment either xylose, lactose or sucrose.

Differential reactions on the medium rely on the ability to ferment xylose, lactose or sucrose and/or decarboxylate lysine. Most coliforms appear as yellow colonies, the result of acid production. *Salmonella, Shigella, Edwardsiella* and *Providencia* species appear as pink colonies, due either to lysine decarboxylation or no fermentation action at all. *Y ruckeri* is unable to ferment any of the 3 sugars present but is able to decarboxylate lysine. Pseudomonads may also appear as pink or pink/orange colonies and should be differentiated from *Y ruckeri*.

With practice, colonies of *Y ruckeri* can be readily identified, but care must be exercised, as *Hafnia alvei* has an almost identical colonial morphology on blood agar. On XLD however, *H alvei* forms yellow colonies because of xylose fermentation while on ROD it forms yellow colonies due to ribose fermentation; no halo of precipitation is apparent as is seen with some serotypes of *Y ruckeri*. Where suspicious colonies are evident, confirmatory identification based on phenotype or genotype must be undertaken. Where *H alvei* is identified in fish with signs of clinical disease, the finding is considered significant, as the species is a recognised pathogen of brown trout and rainbow trout.[26, 27]

Identification tests

Standard tests for the *Enterobacteriaceae* can be used for the identification of *Y ruckeri*. Recommended procedures may be found in Cowan[28] and MacFaddin.[29] Tests should be incubated at 25°C and results recorded at 48 hours. Tests should be in conventional tube format or in miniaturised form so long as they have direct equivalence to the conventional test. Identification can be achieved by computer-assisted probabilistic methods (*See Phenotypic profile*).

Use of API 20E for identification is not recommended as it is considered unreliable.[30, 31]

Phenotypic profile

The biochemical profile of *Y ruckeri* is well described and the species may be differentiated readily from other taxa within the genus *Yersinia* and the family *Enterobacteriaceae* (see Table 1). *Hafnia alvei* is similar

morphologically and serologically to *Y ruckeri* but can be readily differentiated by phenotype. Key differential tests are motility at 37°C, citrate utilisation, gluconate oxidation, fermentation of rhamnose and xylose.

The range of tests given in Table 1 can be used for computer-assisted probabilistic identification using the software package PIBWin[32], which is freely available for download and use. A probabilistic database for *Enterobacteriaceae* based on data compiled from Holmes and Costas[33] and Farmer[34], is freely available for from the author. MicroSys E24, a panel of miniaturised tests for *Enterobacteriaceae*, validated for use with *Y ruckeri* is available (DPIW, Launceston, Tasmania) and is designed for use with probabilistic identification systems.

Table 1. Phenotypic characteristics of *Yersinia ruckeri* and *Hafnia alvei*

Test		***Y ruckeri***	***H alvei***
Oxidase		0*	0
Motility 25C		82	70
Motility 37C		0	85
MacConkey		100	100
Arginine dihydrolase[a]		0	10
Lysine decarboxylase[b]		100	98
Ornithine decarboxylase[b]		100	100
Urease[c]		0	0
H_2S		0	0
Citrate[d]		100	16
ONPG[e]		100	90
Phenylalanine deaminase[f]		0	0
Gluconate oxidation[g]		0	93
Acetoin[h] 25°C		100	88
Indole[i]		0	0
Acid:[J]	Cellobiose	0	45
	Inositol	0	0
	Lactose	0	7
	Maltose	100	100
	Mannitol	100	100
	Raffinose	0	4
	Rhamnose	0	96
	Salicin	0	19
	Sorbitol	20	17
	Sucrose	0	2
	Trehalose	100	98
	Xylose	0	98

***** % strains positive

Test type: [a]Thornely, [b]Møller, [c]Christensen, [d]Simmons, [e]Lowe, [f]Ewing, Davis & Reavis, [g]Haynes, [h]Clark & Lubs + Coblentz reagents, [i]Kovác, [J]Bromocresol purple broth base.

There is little phenotypic variation within the species other than the two biotypes within serogroup O1.[16] Biotype I contains most known strains, which are motile and hydrolyse Tween 80, while biotype 2 consists of non-motile, Tween 80 hydrolysis negative strains, most of which have been found only in the United Kingdom and Norway and as an emerging type in Australia. Strain variation is seen with respect to sorbitol fermentation. Nearly all representatives of serotype O1 are sorbitol negative while isolates of serotype O2 and O4 are sorbitol positive. This characteristic, while not considered a reliable indicator of serotype, is nevertheless a useful marker. Strains that possess the virulence-associated, heat-sensitive factor (HSF) can be identified using Coomassie Blue-TSA-SDS agar (*See Appendix*). On this medium HSF^+ strains appear as blue colonies with pale centres with surrounding zones of precipitation, while HSF^- colonies are dark blue and have no zones of precipitation.[9] The virulence factor is associated only with strains of serotype O1 and is not found in any of the other serotypes.

PCR Identification

Direct detection of pathogens in overtly and covertly infected fish by means of PCR must be approached with caution. Despite the exquisite sensitivity inherent in PCR, when used for direct detection in fish, the test may not be sufficiently sensitive. Typical lower limits of detection are rarely less than $1x10^3$ cells g^{-1} of tissue, which limits the usefulness of the test as a means of detecting low levels of infection in fish. Problems also lie in regard to assigning significance to positive PCR reactions. Since the test signifies only presence of amplifiable DNA, in the absence of clinical signs or a previous clinical history, a positive PCR reaction is not sufficient evidence of disease.

Despite the limitation of PCR as a means of direct detection it can be used reliably as a means of confirming the identity of isolates with ambiguous phenotypes or, with caution, can be used for rapid and specific detection of *Y ruckeri* in fish with overt infection and an established history of disease. Direct detection PCR should not be considered for fish without apparent clinical signs or a history of disease.

Primer set – A well-characterised primer set (Table 2) has been developed for *Y ruckeri* based on the 16S rRNA gene of the bacterial genome[35] and has a high level of intra- and inter-species specificity.

Table 2 *Yersinia ruckeri* PCR primer set

Primer	Direction	Sequence 5'→ 3'
Yrf	forward	AAC CCA GAT GGG ATT AGC TAG TAA
Yrr	reverse	GTT CAG TGC TAT TAA CAC TTA ACC C

DNA Extraction – Rapid DNA extraction from colonies, suitable for confirming bacterial identity, is achieved simply by boiling. To 100 µL of 18MΩ water in a 1.5 mL microfuge tube, suspend sufficient cells to a density equal to McFarland 0.5 (~5 x 10^8 cells mL^{-1}). Hold the tube at 100°C in a dry-heat block for 10 minutes and then cool rapidly in ice for 5 minutes. Centrifuge the tube at 15,000 *g* for 5 minutes and collect the supernatant containing released DNA. The extracted DNA is suitable for amplification without purification.

Bacterial DNA can be extracted from blood and tissue of fish using QIAmp (Qiagen) or PureGene (Gentra Systems) tissue digestion and extraction kits. DNA prepared using either system is substantially free of PCR inhibitors and can be used for amplification without further purification. For the PCR conditions described (*See above*), the concentration of purified DNA should be adjusted to a concentration of 10-20 ng μL^{-1} for PCR; template volume is 1 µL.

Master mix – There are no specific reagent standards required for the test other than the use of a hot-start DNA polymerase. Good amplicon yields have been achieved using Platinum *Taq*™ (Invitrogen, Life Technologies). The reaction mix based on this polymerase is given in Table 3. The concentrations specified for dNTPs, magnesium and primers are critical for maintaining specificity and should not be altered unless validated.

Table 3 Amplification reagents

Reagent	Stock concentration	Reaction volume	Reaction concentration
x10 buffer	As supplied	2 µL	
dNTPs	2.5 mM ea	1.6 µL	200 µM
$MgCl_2$	50 mM	0.8 µL	2.0 mM
Primer Yrf	10 µM	4 µL	2.0 µM
Primer Yrr	10 µM	4 µL	2.0 µM
Taq	5U μL^{-1}	0.1 µL	0.5U $20\mu L^{-1}$
Water		6.5 µL	
Template	10-20 ng μL^{-1}	1 µL	10-20ng DNA
Total		**20 µL**	

Cycle – Amplification is achieved using an initial denaturation of 94°C for 3 minutes followed by annealing at 60°C for 30 s, extension at 72°C for 30 s and denaturation at 94°C for 30 s over 35 cycles, with a final extension at 72°C for 4 min.

Controls – A positive control of purified DNA from a known isolate of *Y ruckeri* must be included with each PCR test. The DNA concentration of the positive control should be in the range of 50-100fg. A negative control using water only as the template must also be included in each test.

Where tissue extraction is undertaken, a spiked positive control sample must be included to control the extraction procedure. Kidney tissue from specific pathogen-free fish should be homogenised and seeded with $1x10^5$ cells g^{-1} of *Y ruckeri*. The seeded tissue should be dispensed in 100 mg quantities in sterile cryotubes and frozen at –20°C.

Detection – Amplicon should be visualised by electrophoresis using 2% agarose gel containing 0.5µg mL^{-1} ethidium bromide and TAE buffer (*See Appendix*). Use a 100bp ladder as a comparative index of amplicon size. A single amplicon of 247bp length is the expected result following amplification with primer pair Yrf and Yrr.

If PCR is used for a critical diagnosis, the amplicon must be verified using an internal probe or, preferably, sequenced. The sequence of the internal verification probe for the amplicon is GCA CTT TCA GCG AGG AGG AAG GGT TAA and has a melting point of 64ºC. The probe can be biotin labelled and used in conventional dot-blot or Southern hybridisation procedures.

Amplicon is bound to a nylon membrane using a UV crosslinker at 5000 µJoules/cm^2. The membrane should be blocked at 55°C for 30 minutes using prehybridisation buffer (*See Appendix*) containing 0.2 mg mL^{-1} salmon sperm

Figure 6. Amplicon sequence, 5'→3' for 16S rRNA gene fragment diagnostic of *Yersinia ruckeri*. The underlined section is the probe sequence.

AACCCAGATG GGATTAGCTA GTAAGTGGGG TAATGGCTCA CCTAGGCGAC
GATCCCTAGC TGGTCTGAGA GGATGACCAG CCACACTGGA ACTGAGACAC
GGTCCAGACT CCTACGGGAG GCAGCAGTGG GGAATATTGC ACAATGGGCG
CAAGCCTGAT GCAGCCATGC CGCGTGTGTG AAGAAGGCCT TCGGGTTGTA
AAGCACTTTC AGCGAGGAGG AA**GGGTTAAG TGTTAATAGC ACTGAAC**

DNA hybridisation – The biotin-labelled probe should be applied at a final concentration of 500 ng mL^{-1} in hybridisation buffer (*See Appendix*) at 56°C for 18 hours. Following hybridisation, the membrane is washed at 59°C for 5 minutes in 2 x SSC buffer (*See Appendix*) and at 59°C for 20 minutes in 0.1 x SSC buffer. Amplicon-probe hybrids can be visualised thereafter using streptavidin-alkaline phosphatase conjugate and NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate) substrate, available in convenient kits from commercial sources.

Sequencing. The expected sequence for the amplicon is given in Figure 6. Sequences in bold represent the forward and reverse primers Yrf and Yrr respectively; nucleotides underlined are the internal probe site.

Lower limit. Using purified DNA as template the limit of detection using 1 µL of template is between 5-10 fg, which approximates to 1-2 bacterial genome equivalents. Amplification should be sufficiently robust to produce clearly visible bands by agarose gel electrophoresis.

Limitations – Despite a lower level of detection approaching a single bacterium, PCR for direct detection in tissue is not sensitive. Lower limits of detection are typically not less than 10^3 cells g^{-1} and more frequently are closer to 10^4 cells g^{-1}. This apparent lack of sensitivity arises from the small tissue samples that can be processed coupled with small template volumes of low concentration. As a consequence, direct detection PCR is only of utility where fish are septicaemic and *Y ruckeri* is present in tissue or fluids in high numbers.

Serotyping

Davies established a serotyping scheme based on heat-stable O-antigens.[17] In this scheme 5 serotypes are recognised designated O1, O2, O5, O6 and O7. On the basis of 6 representative isolates, Australian strains are considered to be rough-type mutants of serogroup O1. This scheme was revised by Romalde *et al*[15] who recognised serotypes O1a, O1b, O2a, O2b, O2c, O3 and O4. Using this serotyping scheme Australian isolates of biotype 1 belong to serogroup O1b. Australian isolates of biotype 2 are currently designated O1:non-O1b.

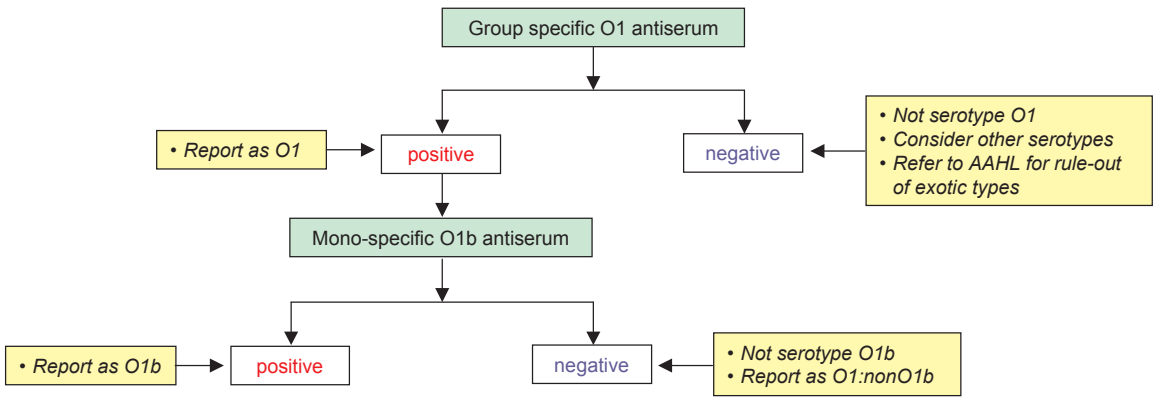
Both group specific O1 antiserum, and monospecific antiserum for serotype O1b, are available from the AAHL Fish Diseases Laboratory, CSIRO, Private Mail Bag 24, Geelong Victoria 3220. Tel 03 5227 5000, and can be used for serotyping by the slide agglutination test. A mono-specific antiserum for the O1:non-O1b type has not been developed.

Slide Agglutination Test

Test. Isolates for serotyping must be grown between 20-22°C to ensure adequate expression of group antigen (N Gudkovs, personal communication); 25°C cultures may result in weaker less easily read reactions. In two separate 20 µL volumes of saline on a glass slide, make a moderately dense suspension of the organism taken from blood agar or TSA. Add a loopful of O1 group specific antiserum to one of the suspensions and observe for agglutination against a black background. Check that the control suspension remains smooth and has not auto-agglutinated. Repeat the test using O1b monospecific antiserum.

Interpretation. A summary of reactions and interpretations is given in Figure 7. . Where an O1:non-O1b serotype is isolated for the first time the strain should be sent to AAHL to exclude the Hagerman O1a strain, the

Figure 7. Interpretation flow-chart for serotyping *Yersinia ruckeri* strains extant in Australia



cause of enteric redmouth. If an isolate with a phenotype consistent with *Y ruckeri* fails to agglutinate with the

group specific O1 antiserum, then an exotic serotype should be considered and the isolate referred to the AAHL Fish Diseases Laboratory, CSIRO.

Limitations. Cross-reactions with some strains of *Hafnia alvei* have been observed, particularly where the antiserum is used undiluted. Isolates should be identified by phenotype before serotyping.

The slide agglutination test must not be used as the sole means of identifying *Y ruckeri*. The purpose of the test is to determine whether isolates of *Y ruckeri* belong to serotype O1 or subgroups.

Appendix

Ribose Ornithine Desoxycholate (ROD) Agar[17]

Yeast extract	3.0 g
Sodium deoxycholate	1.0 g
NaCl	5.0 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	0.8 g
Ribose	3.75 g
Maltose	7.5 g
Ornithine hydrochloride	5.0 g
Phenol red	0.08 g
Agar	12.5 g
Distilled water	950.0 mL
pH	7.4

Dissolve the ingredients in the distilled water and bring to the boil; do not overheat; do not autoclave. Cool to 55°C.

Dissolve 10.0 g of sodium dodecyl sulphate in 50 mL of distilled water and filter sterilise; add aseptically to the cooled molten base. Pour as plates.

Coomassie Blue-TSA-SDS Agar[9]

Tryptone soya agar (Oxoid CM131)	40.0 g
Distilled water	940.0 mL
pH	7.3±0.2

Dissolve the ingredients, autoclave at 121°C for 15 minutes and cool to 55°C.

Dissolve 10.0 g of sodium dodecyl sulphate in 50 mL of distilled water and sterilise by filtration; add aseptically to the cooled molten base.

Prepare a 1% solution of Coomassie Brilliant Blue R250 (BioRad) in distilled water and sterilise by filtration. To 990 mL of cooled molten TSA+SDS aseptically add 10 mL of the sterile Coomassie Blue solution.
Pour the medium as plates.

TAE Buffer

(i) 0.5M EDTA

EDTA*	46.5 g
Distilled water	150.0 mL

*Ethylenediamine tetra-acetic acid, di-sodium dihydrate form

To dissolve the EDTA, raise the pH to 8.0 by adding approximately 5 g of NaOH. Make up to 250 mL with distilled water. Autoclave at 121° for 15 minutes

(ii) 50X stock

Tris base	242.0 g
Glacial acetic acid	57.1 mL

0.5M EDTA (pH 8.0) 100.0 mL
Distilled water to 1 litre

Dissolve the Tris base in 700 mL of distilled water. Add the glacial acetic acid followed by the EDTA solution; make up the volume to 1 L with distilled water.
To use, take 20 mL of x50 TAE stock and make up to 1 L with distilled water. The final concentration of the buffer is 0.04M Tris acetate and 0.001M EDTA.

Hybridisation Buffers

20xSSC: 3M NaCl, 0.3M Sodium citrate, pH 7.0
Wash 1: 2xSSC, 0.1%SDS
Wash 2: 0.1xSSC, 0.1%SDS

Sheared salmon sperm DNA: 10 mg/mL (Invitrogen Life Technologies)
100x Denhardt's solution: Amresco

Hybridisation Buffers

Component	Pre-hybridisation	Hybridisation
20x SSC	3.0 mL	3.0 mL
100x Denhardt's solution	0.5 mL	0.5 mL
Salmon Sperm DNA	0.1 mL	0.1 mL
10% SDS	0.5 mL	0.5 mL
0.5 M EDTA, pH 8.0	-	0.2 mL
Distilled water	5.9 mL	5.7 mL
Probe	-	5pg
Total Volume	**10 mL**	**10 mL**