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Directorate B - Scientific Health Opinions
Unit B3 - Management of scientific committees II

Bacterial Kidney Disease

Report of the

Scientific Committee on Animal Health and Animal Welfare

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Contents

1. REQUEST FOR OPINION

The Scientific Committee on Animal Health and Animal Welfare has been requested to advise on:

1.   standards for eradication programmes for Bacterial Kidney Disease (BKD)

2.   the criteria for determining zones free of the disease

3.   the risk of introducing the disease into such areas and

4.   methods for reducing this risk.

2. THE DISEASE

2.1 Introduction

Bacterial Kidney Disease (BKD) is a systemic infection of salmonids that is normally slowly progressive and frequently fatal. It seldom shows up in fish until they are 6-12 months old.

The pathogen that causes BKD is threatening juvenile salmonids worldwide and cultured broodstocks of endangered salmon species.

Bacterial Kidney Disease was first described in the early 1930's in wild Atlantic salmon, *Salmo salar* L., caught in two Scottish rivers (Dee disease). In 1935, similar lesions were described in rainbow trout (*Oncorhyncus mykiss)* in a North American hatchery, but it was not until 1956 that the causative bacterium was cultured. The disease has a wide geographic range (see section 2.5),

The disease, initially recorded in freshwater situations, is now recognised as a costly problem in the seawater farming of salmonids.

To date, natural outbreaks of BKD have been reported only in salmonids.

2.2 The pathogen

The gram-positive diplobacillus, *Renibacterium salmoninarum*, has been identified as the causative pathogen of BKD in cultivated and wild salmonids. It is a small (0.5x1.0µm), non-acid-fast, periodic-acid-Schiff positive, non-sporulating, non-motile rod that grows best at 15-18°C and not at all at 25°C.

It was observed that after a sufficiently long incubation period on Kidney Disease Medium 2 (KDM2), Kidney Disease Medium Charcoal (KDMC) or Selective Kidney Disease Medium (SKDM), *R. salmoninarum* produces white or creamy, shiny, smooth, round, raised, entire colonies that are pinpoint to 2 mm in size. Bacteria isolated from diseased fish will produce visible colonies after 2-3 weeks on average. However, up to 8 weeks have been reported for initial growth on KDM2, and 12 weeks on SKDM. Old cultures

may achieve a granular or crystalline appearance. Microscopic observation of transverse sections through such colonies will reveal the presence of Gram-positive rods in a crystalline matrix. The crystalline material is thought to be cysteine precipitated from the medium. Growth does not occur on blood agar medium without cysteine supplement or on trypticase yeast agar. For some strains, a uniformly turbid growth occurs in broth, but for others a sediment may develop. Microscopically *R. salmoninarum* appears frequently in pairs, short chains or pleomorphic forms as "Chinese letters" especially in fish tissue.

The organism is likely to be mistaken for only one other group of fish-pathogenic bacilli, and then only if the diagnosis is based exclusively on an examination of Gram-stained tissues smears. Under such circumstances it could be confused with a *Lactobacillus* that is responsible for pseudokidney disease (Ross and Toth, 1974). The pseudokidney disease bacillus does not appear to be highly virulent and has been reported as pathogenic only in fish that have undergone stress, such as that due to handling or spawning.

The slow-growing properties of *R. salmoninarum*, as well as the chronic nature of the disease that it usually causes, have contributed to the slow pace with which information on it has been gathered. In addition, other properties of the pathogen, particularly its ability to enter and survive inside eggs and phagocytic cells, have greatly complicated the task of developing methods for controlling renibacterial infections. In fact, *in vitro* infections of primary cultures of leukocytes (from kidneys) of rainbow trout (*Oncorhyncus mykiss)* showed that *R. salmoninarum* survived within the mononuclear phagocytes. Transmission electron microscopy revealed that intracellular survival of the bacterium partially depends on its ability to move from the phagosome into the cytoplasm. Formalin-killed *R. salmoninarum* also entered the cytoplasm, albeit at a slower rate and after sustaining greater cell wall damage, suggesting that the extracellular protein of the bacteria plays a role in intracellular survival.

Based on quantitative slide agglutination and Western blot assays, it has been suggested that there are two antigenically distinct groups (Bandín *et. al.* 1992).

2.3   Incubation periods, clinical signs and pathology

The duration of the incubation period is generally long (up to several months, even years) The length depends on environmental factors such as temperature, on characteristics of the host and on the bacterium strain**. (see section 4.2.).

After infection clinical signs may not be evident for a considerable period..

Fish severely infected with the agent may show no obvious external clinical/pathological signs, or may show one or more of the following: pale gills indicative of anaemia, exophthalmia, abdominal distension (due to the accumulation of ascitic fluid), skin blisters filled with clear or turbid fluid, shallow ulcers (the results of broken skin blisters), haemorrhages (particularly around the vent) and, infrequently, intramuscular cavities, filled with blood-tinged caseous or necrotic material. Turbid fluid may be collected into the abdominal and pericardial cavities, haemorrhages are seen on the walls of the abdominal cavity and on the viscera, fibrinous layer may cover one or more of the visceral organs, and most characteristically, creamy-white granulomatous lesions develop in the kidney and, less frequently, in the spleen and the liver.

Interestingly, these latter lesions can be well encapsulated and may even be resolved in species such as Atlantic salmon (*Salmo salar*), which appear to be fairly resistant to BKD. In the more susceptible Pacific salmon (Genus *Oncorhynchus*), however, the granulomas are rarely encapsulated.

Histologically, the lesions are chronic granulomas, mainly in haemopoietic tissue, but extending to the liver, the cardiac and skeletal muscles and virtually to any organ in the final stage of the disease. Histologically the granulomas contain a necrotic nucleus surrounded by epithelioid cells and infiltrating lymphocytes. The presence of a fibrous capsule is variable and lack of encapsulation is often associated with more aggressive infections or more susceptible species.

2.4   Occurrence

Although BKD occurs mainly in freshwater, significant mortality also occurs in saltwater (Banner *et al*. 1983). As a consequence of infection, juvenile anadromous salmonids are unable to acclimatise to seawater and die. The mortality of infected smolts of Coho salmon was respectively of 17.2% in freshwater and 4% in saltwater (Fryer and Sanders 1981). Even if salmon are lightly infected when they enter saltwater, the disease continues to progress and deaths occur (Banner *et al.* 1986).

2.5   Host and geographic range

Bacterial kidney disease has been reported only in salmonids: Pacific and Atlantic salmon, grayling (*Thymallus thymallus*), and several species of trout and char. BKD continues to cause serious mortalities in lake trout (*Salvelinus namaycush*) and in Pacific and Atlantic salmon.

The organism has also been isolated, in the absence of disease, from several non-salmonid fish species such as greenling (*Heragrammos otakii*), flathead (*Platycephalus indicus*) and Pacific herring (*Clupea pallasi pallasi)* (Traxler and Bell, 1988).

Reports on the geographical range of BKD have originated from Canada, Chile, Japan, USA and most European countries, effectively wherever susceptible salmonids are kept.

2.6   Susceptibility to BKD

Suzumoto *et al*. (1977, cited in Fryer and Sanders, 1981) reported that juvenile Coho salmons of three different transferrin genotypes differed in resistance to experimental infection of BKD. Whitler and Evelyn (1987) compared five strains of the same species, but were not able to show differences in susceptibility to BKD. However, Beacham and Evelyn (1992) showed experimentally that three populations of Chinook salmon, originating from different rivers, presented various mortality rates to BKD . They and others (Gjedrem and Gjøen, 1995) were able to calculate the heritability for the mortality rate factor.

3. DIAGNOSTIC METHODS

The OIE Diagnostic Manual, 1997 (OIE, 1977), chapter 3.1.6, may be considered as the reference text for the detection and identification of *Renibacterium salmoninarum*. Most of the techniques which can be used are described with sufficient details from a well documented bibliography though this is complete only to 1993. More recent references are included in this report.

3.1 Isolation and bacteriological identification of the agent

The isolation and identification of *R. salmoninarum* is relatively easy in samples from clinically infected fish. The overt disease may readily be suspected from the clinical signs and lesions, and the microscopic observation in smears imprints or histological slides, of small gram-positive (PAS-positive and metachromatic) diplobacilli is sufficient to distinguish BKD from other chronic pathologies (PKD, mycosis, nephrocalcinosis). Other gram-positive bacteria (lactobacilli) may be responsible for similar conditions, but they are easily distinguished from *R. salmoninarum*. They are morphologically different and unlike *R. salmoninarum* they grow readily on standard laboratory media, at 30°C, react differently with a number of substrates and indeed fail to react with anti *R. salmoninarum* sera.

R. salmoninarum is a fastidiously growing organism that requires prolonged incubation (2-3 weeks on average, up to 8 to 16 weeks) at 15°C to produce colonies. Cystein and serum or serum substitutes are requisite factors. The OIE Diagnostic Manual details the media (KDM or SKDM) which are used for growing *R. salmoninarum* and some innovative ways which can increase isolation sensitivity (tissue suspensions prepared in isotonic saline, removal of colonies produced by fast growing organisms, use of the satellitism phenomenon, addition of "spent KDM broth" to the medium). The phenotypic characteristics of *R. salmoninarum* have been established using the API-zym system and other conventional tests. However, as stated in the Manual, "the slow growth of the organism does not render such tests very useful in practice and serological methods are more usually employed to confirm the identity of the isolated strains".

3.2   Antigen detection and identification

The antigenic "homogeneity" of *R. salmoninarum* has been usually considered as a characteristic that favours the use of specific antisera in identification procedures. This is explained by the existence of soluble, heat stable, antigenic factors, abundantly released in infected tissues, of which the most important is called p57 protein. However, Bandín et al. (1992) have demonstrated the existence of another antigenic group with a major 30kDA protein. Subsequently, quantitative agglutination tests and Western blotting assays indicate the existence of quantitative antigenic diversity which could be of a great interest from an epidemiological point of view. Nevertheless, in slide agglutination tests all *R. salmoninarum* isolates reacted positively with antiserum raised against the reference strain of the species. In addition, even in the strains characterised by the major 30kDA protein, a weak reaction against the p57 protein was obtained by Western Blotting

Both polyclonal antisera and mAbs can be readily obtained by immunising rabbits with *R. salmoninarum* preparations, preferably purified. However, it has been demonstrated (Bandín *et al.*1993) that the use of such antisera for immunodiagnostic procedures can give false positive reactions. Monoclonal antibodies (mAbs) have also been produced by a number of laboratories and, some of these mAbs are currently used in commercially available ELISA kits. Both polyclonal sera, and mAbs for *R. salmoninarum* can now be obtained from commercial sources.

3.2.1 Agglutination test

Although easy and rapid to perform, the test requires that the bacteria are first cultured. A coagglutination technique, using *Staphylococcus aureus* sensitised with specific immunoglobulins, appears suitable for the direct detection of *R. salmoninarum* in kidney tissue (Kimura and Yoshimizu, 1981). However, it does not give as high a sensitivity as immunofluorescence or ELISA tests (OIE 1997).

3.2.2 Immunofluorescence

Direct and indirect immunofluorescence antibody tests (FAT) have commonly been used for demonstrating the presence of *R. salmoninarum* in infected tissues including fixed and paraffin embedded tissue. Elliott and McKibben (1997) compared two fluorescent antibody techniques (FATs) (membrane filtration FAT or MF-FAT and smear-FAT or S-

FAT) for detection of *R. salmoninarum* in ovarian fluid from naturally infected spawning Chinook salmon (Pascho et al., 1998). They reported sensitivities in the region of 8.7 X 10^3 cells / ml (S-FAT) and 9.8 X 10^3 cells / ml (MF-FAT). Starliper and Teska (1995) screened ovarian fluid from asymptomatic brook trout populations using membrane filtration-FAT and ELISA and reported that twice as many positives were detected using this FAT method than by ELISA. However, interlaboratory comparisons have revealed that FAT reproducibility is poor for very low levels of infection, (Armstrong *et al.* 1989b) resulting in some infections being missed.

3.2.3 Enzyme-linked immunosorbent assay (ELISA).

ELISA is widely used for the detection of *R. salmoninarum* and several commercial kits are available. The most common technique used is a double antibody sandwich ELISA method. Polyclonal antiserum reacting with heat stable antigenic determinants may be used, But in order to reduce the risk of cross-reaction with other bacteria, the antisera are often absorbed or affinity purified. Some mAbs, however, are very specific. Samples of tissue (fish kidneys) collected for examination by ELISA must be kept cold or frozen after collection. Tissues from clinically and subclinically infected fish give ELISA reactions which are clearly distinct from those of uninfected fish. A positive threshold has been computed and proposed for ELISA results interpretation (Meyers *et al.* 1993). Griffiths *et al.* (1996) screened kidney tissue and ovarian fluid from sub-clinically infected Atlantic salmon broodstock using culture (SKDM), IFAT, ELISA and Western blot. Only qualitative data was gathered demonstrating that culture was the most sensitive technique followed by IFAT, whereas ELISA and Western blot were relatively insensitive. However, the authors adapted the Western blot approach by first pre-incubating samples in SKDM broth resulting in more positive samples than by the other techniques. It has been claimed (Jansson *et al* . 1996) that the sensitivity of ELISA is "comparable and possibly greater than cultivation under optimal conditions". It is commonly used for assessing the prevalence and the severity of *R. salmoninarum* in wild salmonids (e.g. Magnusson *et al*. 1994; Maule *et al.* 1996; Elliott *et al.* 1997). Jansson and Ljungberg (1998) screened naturally infected populations and experimentally challenged rainbow trout and Atlantic salmon for *R. salmoninarum* using both the ELISA method and culture (SKDM). Quantitative data were not given by the authors but overall, 4 weeks after experimental infection, ELISA and culture detected all fish as positive. However, 12 weeks after

challenge more experimentally infected fish were considered positive by ELISA than by culture. In contrast, culture appeared to be more sensitive than ELISA for screening naturally infected fish.

On the other hand, the detection of *R. salmoninarum* in kidney samples by ELISA and isolation on selective medium was compared and described by Gudmundsdottir *et al.* (1993). The study comprised 1239 kidney samples and showed that the ELISA test gave significantly higher numbers of positive samples.

Unfortunately ELISA does not work very well on ovarian or seminal fluid. The problems with tests on ovarian fluid are addressed by Pascho, Chase, and McKibben (1998). Seminal fluid turns into a glue-like substance when boiled, so it has not proven very satisfactory for ELISA either.

While several ELISA methods are available, it is important to note that the various evaluations as to their sensitivity and specificity have not been uniformly carried out. Indeed considerable information in this area is lacking.

3.2.4 Immunohistochemical detection

The essential for a successful immunohistochemical staining is the availabilityof antiserum/monoclonal antibodies (mAbs) able to give a reaction of high sensitivity and specificity (Heines and Chelack, 1991). Polyclonal antisera can lead to cross-reactivity as specifically observed with other bacteria (Rockey *et al*. 1991). Cross reactions are reported with *Carnobacterium piscicola*, *Corynebacterium aquaticum* (Dixon, 1985; Bandin et al., 1993; Toranzo et al., 1993; Leon et al., 1994; Wood et al., 1995), *Brevibacterium linens* (Magnusson et al., 1994), *Lactobacillus* sp., (Teska et al., 1995) *Pseudomonas* spp. (Brown et al., 1995; Yoshimizu et al.,1987), *Rothia dentocariosa*, *Bacillus sphaericus* (Dixon, 1987) and *Arthrobacter globiformis* (Jansson et al., 1996).

The most promising results were obtained using the mAbs 4D3 and 2G5 – both specific for the 57-58-kD outer membrane protein (p57) which is unique for *R. salmoninarum* (Wiens and Kaattari, 1989, 1991). Evensen *et al*. (1999) developed;

(1)   a method for immunohistochemical identification of R. salmoninarum in paraffin embedded tissue samples using the mAbs 4D3 and 2G5,

(2)   a method to evaluate the sensitivity for different fixatives using artificial test substrates

(3)   a double staining technique for the *in situ* identification of *R. salmoninarum.*

The possible problem of masking of antigens (Takamyia *et al*. 1978) depending on fixation solutions used is influenced by variables like pH and temperature. To assess these variables in front of a masking process of the formalin-sensitive epitopes (p57 and others) of *R. salmoninarum*, microwave (MW) treatment to unmask the antigenic determinants concealed during fixation was the most promising method. Evensen and Terland (1999) showed that for neutral and slightly acidic solutions there was a correlation between increased intensity score and temperature increase (up to 100°C). At low pH, exposure to 60-70°C gave staining intensity comparable to 100°C for the pH 5.5-solution. However, at temperatures above 70°C, the pH 3-solution resulted in loss of antigenicity (marked drop in staining intensity) and distorted tissue integrity. As can be seen, both pH and temperature will affect antigen unmasking and microwave treatment using low pH solutions will not allow exposure to high temperatures.

Taking into account the frame of these considerations, the immunohistochemical detection of *R. salmoninarum* can be considered as a highly specific detection method for this bacterium, with the advantages of being rapid and feasible in stored samples of tissues of BKD-suspected fish and therefore independent of time.

3.3   Polymerase Chain Reaction (PCR)

PCR has recently been used for the detection and identification of *R. salmoninarum* directly within ovarian fluid or individual eggs. Brown *et al.* (1994) described a PCR method for detection of *R. salmoninarum* in Coho (*Oncorhynchus kisutch*) and Chinook (*O. tshawytscha*) salmon eggs. The amplified DNA region, product of 501 base pairs, belongs to the gene coding for the antigenic p57 protein. The reaction can be performed from only 100 microliters of homogenized suspect tissue and allows detection of as few as 2 bacterial cells per egg with a high degree of specificity (Brown *et al*. 1995). Magnusson *et al.* (1994) used a PCR method to detect *R. salmoninarum* in ovarian fluid samples compared to culture and ELISA methods from kidney. The PCR/ovarian fluid method did

not detect significantly more positives than the other techniques but was quicker (1-2 days) and was non-lethal, ovarian fluid being obtained when stripping eggs. The reported sensitivity of the technique was ~1 to 10 bacteria per ml.

More recently, a nested PCR has been developed by Chase and Pascho (1998) to detect a 320 bp DNA segment of the gene encoding the p57 protein. The sensitivity of the method was increased a hundredfold compared to a conventional PCR method. The specificity of the nested PCR was confirmed by testing DNA extracts of common bacterial fish pathogens and a panel of bacterial species reported to cause false-positive reactions in the ELISA and the FAT. Kidney samples from infected Chinook salmon were checked by the nested PCR, the ELISA and the FAT, and the detected prevalences of *R. salmoninarum* were 61%, 47% and 43% respectively. The results of a comparison of methods to detect *R. salmoninarum* in ovarian fluid (Pascho *et al*. 1998), and those of Brown *et al* (1994) suggest that the prevalence of *R. salmoninarum* among eggs of mating pairs from populations in which BKD is enzootic may be far greater than anticipated. Others (Miriam *et al*. 1997) have cautioned, however, that PCR-positive samples may contain some proportion of dead *R. salmoninarum* with detectable levels of DNA. Miriam *et al*. (1997) compared PCR, nested PCR and culture (SKDM) for detection of *R. salmoninarum* in ovarian fluid and kidney tissues in both Atlantic salmon broodstock and experimentally challenged salmon. They reported that PCR and SKDM culture were equally sensitive when identifying subclinically infected fish that were experimentally challenged (between 4 to 40 bacteria). However, PCR identified higher numbers of kidney tissues and ovarian fluid samples from commercially reared broodstock fish to be positive for *R. salmoninarum* than culture. However, the authors suggest this may be due to antibiotic therapy used in the past to control BKD and that PCR detected dead or unculturable bacterial cells. A nested PCR probe increased the sensitivity of detection to between one and four cells in ovarian fluid samples.

PCR is useful for the testing of ovarian fluid, but not for seminal fluid (the high DNA content of seminal fluid is a problem in the PCR). Whether PCR would be useful for a large-scale screening program is unclear and will be addressed in Chapter 7. The likelihood of DNA contamination becomes very high if a screening is done in field laboratories that are also conducting many other diagnostic procedures.

3.4   Serological tests

The development of the antibody response to *R. salmoninarum* in fish has not been clearly correlated with the course of the infection and consequently, serological tests do not appear to be suitable as a routine technique for the detection of infected fish.

3.5   Conclusions

In clinically infected fish, very simple techniques leading to the observation of morphologically characteristic Gram-positive bacteria, which do not grow rapidly on usual media, allow suspicion of BKD with a high degree of confidence. For confirmation, growing the bacteria on specific media, then looking for the phenotypic characteristics is the classical method but is slow and time consuming. Today, the more simple and rapid way for the identification of the bacteria as *R. salmoninarum* is to detect specific antigen, either by immunofluorescence or, preferably, by ELISA.

(For screening methods see chapter 7).

4. PATHOGENESIS AND ENVIRONMENTAL FACTORS

4.1 Pathogenesis

The development of the lesions of BKD is briefly described in chapter 2.3. The precise route of natural infection is not completely understood, but Richards *et al.* (1985) report that experimental infection occurs quicker through skin lesions than by the oral route. The pathogenesis of BKD in Atlantic salmon has been found to be temperature dependent: under +10ºC diffuse pseudomembranous peritonitis is typical, whereas at higher temperatures the main lesions are intraparenchymatous focal granulomas (Richards *et al.* 1985). Ferguson (1989) states that macrophages are mainly responsible for the phagocytosis of the infective organism. In addition, even cells which are not considered to be phagocytic or strongly phagocytic (e.g. thrombocytes, endothelial cells of the kidney and oocytes) engulf the bacteria, which does not produce acutely lethal toxins (Evelyn 1993). These properties together with the slow propagation of *R. salmoninarum* are probably important factors in determining the chronic course of BKD and difficulties in the treatment of the condition.

According to Evelyn (1993) the infection of eggs, which is responsible for vertical transmission, usually originates from the *Renibacterium*-infected coelomic fluid of the female brood fish. It has been shown, however, that intra-ovum and progeny infections may also occur prior to ovulation and directly from the ovarian tissue (Evelyn 1993).

4.2 Environmental factors

4.2.1 Effects of temperature and hardness of the water

BKD is a slowly developing infection which can cause mortality in a wide range of water temperatures. The onset and magnitude of mortality is, however, dependent on the water temperature and may vary depending on the species involved (Sanders, Pilcher and Fryer 1977). Among seven temperature levels, ranging from 4°C to 20.5°C, the mortality of

Coho salmon (*Oncorhynchus kisutch* Walbaum) and Steelhead trout (*Oncorhyncus mykiss)* was highest at 6.7°C, 9.4°C and 12.2° C, but in Sockeye salmon it was almost 100% at any water temperature varying from 6.7°C to 20.5 °C. After the observation of many hatchery epizootics, Wood (1972, cited in Fryer and Sanders (1981) reported that mortality occurred after 30-35 days following exposure at temperatures above 11°C and 60-90 days at 7.2-10°C.

On the other hand, according to various reports, the most severe clinical outbreaks occur in soft water (Warren 1963). Attempts to reproduce this phenomenon in the laboratory have, however, failed. Lall *et al*. (1985) while reporting their results on the control of BKD by dietary modification, put forward the suspicion that mineral content of the water rather than water hardness alone may be important in determining the prevalence of BKD.

4.2.2 Effects of diet

Ascorbate depletion was suspected to be responsible for an increased incidence of BKD (Wedemeyer and Ross, 1973, cited in Fryer and Sanders, 1981). Since then, several studies concluded that vitamins had no effect on incidence (Sakai *et al* 1986). Observations over a quarter of century ago (Wood, 1974, cited in Fryer and Sanders, 1981) pointed also that mortality rates were higher in fish fed with dry rather than moist diets, but this observation has not been confirmed by other workers.

Woodall and Laroche, 1964, (cited in Paterson *et al*., 1981b) demonstrated a reduction of BKD infections by feeding Chinook salmon a diet containing high levels of iodine (10.1µg/g). Paterson *et al*., (1981a) showed a considerably lower level of infection in Atlantic salmon fed with a diet containing high trace elements (Fe, Cu, Mn, Co, I and F) in comparison with six other types of diet. Later, Paterson *et al*. (1985) compared a diet rich in iodine (4.5 µg/g) and fluorine (4.5µg/g) with a commercial feed and five other types of diets and found a lower prevalence of clinical BKD (3.6%) compared to the commercial (34%) and the five other experimental diets (15-24%). Among the latter experiments it is remarkable that fish fed with a diet containing high levels of iodine and fluorine in the presence of high levels of zinc (75 mg/kg) and magnesium (0.06%) did not show the same magnitude of decrease of BKD infections.

4.3   Conclusions

Water temperature affects the incubation time and the levels of mortality, but this varies between fish species.   As many other bacterial fish diseases, a quick rise in water temperature seems to be associated with the development of mortality in the field.   The question of the effect of the hardness of the water on the morbidity and mortality has not been answered consistently.   There is evidence that diets having high concentration of iodine and/or fluorine can be used in the reduction of the development of clinical BKD.

5. DISEASE TRANSMISSION

5.1   Horizontal transmission

Fish can be infected by the oral route (Balfry *et al*. 1996) and also via skin erosions.

The source of the infection can be:

1.   contaminated feed (Richards *et al*., 1985; Frerichs and Roberts, 1989),

2.   contaminated water, although the pathogen does not survive well outside the host. (Inglis *et al*. 1993, Bullock and Herman, 1988).

3.   diseased or inapparently infected fish (Mitchum *et al*. 1979, 1981).

5.2   Vertical transmission

Vertical transmission of *R. salmoninarum* via eggs has been shown by many authors and various means (Fryer and Sanders,1981; Evelyn *et al*. 1984; Richards *et al*., 1985; Evelyn *et al*. 1986a, 1986b, 1986c; Bruno and Munro, 1986). Even disinfection of the eggs for 15 minutes in 250 ppm iodine solution does not prevent vertical transmission (Evelyn *et al*. 1984; Bruno and Munro, 1986).

Transmission through the sperm plays an unimportant role in the vertical transmission of *R.salmoninarum* (Klontz, 1983; Evelyn *et al*. 1986b).

The pathogen can become located within eggs, when uncontaminated eggs are immersed before fertilisation in infected coelomic fluid (Evelyn *et al*. 1986b). An increased incidence of *intraovum* infection, and subsequent infection of juvenile salmonids (e.g. smolts), is correlated with the presence of bacteria in coelomic fluid, even in low numbers (Lee and Evelyn, 1989).

5.3   Fish as possible vectors and carriers

All salmonids can act as carriers though with marked differences regarding efficiency of transmission. The rainbow trout is the most refractory salmonid species to BKD (Fryer and Sanders, 1981; Mitchum *et al*. 1981; *Richards et al*., 1985; Austin and Austin, 1987; Bullock and Herman, 1988; Frerichs, 1989; Frerichs and Roberts, 1989).

Pacific herring (*Clupea pallasi pallasi*) and other non-salmonids (e.g. the shiner perch *Cymatogaster aggregate*), which occur incidentally on salmon farms, could become accidental carriers of *R. salmoninarum*. However, they fail to reveal the presence of infection and shiner perch appears refractory to infection when held in tanks for periods up to 5 months with *R. salmoninarum*- infected fish (Paclibare *et al*. 1988; Traxler and Bell, 1988; Evelyn, 1993). It seems unlikely that the agent can multiply in non-salmonids**.**

Other fish mentioned in the literature as possible vectors are greenling (*Hexagrammos otakii*) and flathead (*Platycephalus indicus)* (Sakai and Kobayashi, 1992).

5.4   Possible vectors other than fish

Bivalve molluscs that reside around sea cages are capable of ingesting and destroying bacterial pathogens like *R. salmoninarum* without being themselves adversely affected. These bivalves, however, are unlikely to remove all pathogens from around a fish farm (Paclibare *et al*. 1988; Kent, Traxler and Evelyn, unpublished data). The Japanese scallop (*Patinopecten yessoensis)* has been mentioned in the literature as a possible vector (Sakai and Kobayashi, 1992).

It has also been postulated that salmon lice (*Lepeophteirus salmonis*) - like other blood-sucking ectoparasites - can act as vectors for *R. salmoninarum*. The lice can occasionally harbour the pathogen (Richards *et al*., 1985; Frerichs and Roberts, 1989)] - but until now there is no evidence for active BKD transmission caused by salmon lice recorded in the literature, as for example with the demonstrated active transmission of Spring Viraemia of Carp Virus by the carp lice (*Argulus folieaceus*) in freshwater (Ahne, 1978). Furthermore, an attached salmon louse seldom changes its host, unless it finds a much weaker or injured fish.

R. salmoninarum is quite rapidly killed at the body temperature of birds. Thus, on the basis of temperature alone, the ingested bacterium would not be expected to survive passage through a birds intestinal tract. Evelyn (Unpublished data, pers. comm.) showed that the bacterium suspended in KDM was killed after 4.5 hours at 35°C, a temperature well below that normally found in birds and mammals).

It seems that the bacterium can be transmitted by birds and mammals as an external contaminant. However, at room temperature, which is approximating the bird's surface temperature, the bacterium survives poorly (at 25°C in KDM, 97% viability is lost after 71 hours). However, although the mechanical transmission of other pathogens between fish farms by birds has been described (Beveridge, 1989), there is no specific evidence of this occurring in BKD but it would appear possible.

5.5   Reservoirs

The main reservoirs of infection are infected salmonids. All subclinically infected or latent carrier salmonids may serve as reservoir of infection (Fryer and Sanders, 1981; Richards *et al*. 1985; Bullock and Herman,1988; Traxler and Bell, 1988). The survival of the pathogen in faeces and pond sediments for up to 21 days has been reported (Bullock and Herman, 1988).

6. TREATMENT AND VACCINATION

6.1 Treatment

Control of BKD with conventional treatment methods, such as chemotherapeutics remains problematical due to the intracellular nature of *R. salmoninarum* infection, so that currently there is no practical treatment for the disease. Austin (1985) tested more than 70 antimicrobial compounds both *in vivo* and *in vitro*. He found that the antibiotics clindamycin, erythromycin, kitasamycin, penicillin G and spiramycin were useful for combating early clinical cases of BKD and that cephradine, lincomycin and rifampicin were effective prophylactically but were of limited use therapeutically. However, none of the listed antimicrobials has an accepted maximum residue limit (MRL) established for use in fish and therefore all remain unlicensed in Europe. Elsewhere, there have been reports that injection of erythromycin phosphate into broodstock females prior to spawning significantly reduces the vertical transmission of BKD (Sakai *et al.* 1986, Evelyn *et a.l* 1986b, Lee and Gordon 1987, Armstrong *et al.* 1989a, Lee and Evelyn 1994). Indeed therapeutic levels of this antibiotic have been detected in the yolk of eggs and the yolk sac of alevins post hatching (Brown *et al.* 1990). Broodstock injection does not eliminate *R. salmoninarum* infection in tissues and eggs but combined with good husbandry techniques it is possible to significantly reduce the incidence of BKD in hatcheries by this means (Lee and Evelyn 1994).

Further work is required to make an effective treatment for BKD available. The use of such treatments, in practice, would have to take full account of the requirements of public health and environmental safety.

6.2 Vaccination

At present there are no commercially available vaccines for BKD. However, successful field trials of a prototype bacterin vaccine have recently been reported from Canada where a licence application is in the final review stages, and research on potential new vaccines is being carried out currently among several research groups worldwide. The approaches

being followed include the development of DNA-based vaccines which have resulted in patents being lodged in the State of Oregon (Christensen *et al.* 1999) for specific vaccine preparation techniques. Another approach was the use of whole cells *R. salmoninarum* in which p57 is decreased using 37°C treatment. Recent studies (Piganelli *et al*. 1999) suggest the efficacy of such treated cells as an oral BKD vaccine.

In the past, vaccination against BKD has been attempted with varying degrees of success and some produced conflicting results. Paterson *et al*. (1981b) reported that an inactivated suspension of *R. salmoninarum* mixed 1:1 with Freunds Complete Adjuvant (FCA) administered by i.p. injection, reduced the level of infection of *R. salmoninarum* in yearling salmon but did not completely eliminate it. In contrast, McCarthy *et al*. (1984) had more success with pH-lysed bacteria, without adjuvant, given by a single i.p. injection. They found that whilst >80% of their unvaccinated controls were affected by *R. salmoninarum* less than 10% of vaccinated fish showed signs of infection. Sakai *et al*. (1989, 1993 and 1995) found that although specific antibodies were produced against inoculated formalin-killed cells of *R. salmoninarum,* a challenge post vaccination showed there was no protective effect. One of the difficulties with developing efficient vaccines against *R. salmoninarum* is that the pathogen survives in the phagocytic cells, thus resisting phagocytosis. FCA can stimulate the bactericidal activity of macrophages, hence Sakai *et a.* (1993) found that by incorporating FCA into a BKD vaccine the level of killing by macrophages was increased, but to a level insufficient to eliminate all of the bacteria. Bruno and Brown (1993) reported that incorporating FCA or other adjuvants into vaccines – including vaccines against other fish pathogens - could lead to the production of intra-abdominal adhesions attributed to the adjuvant. If fish are subclinically infected with *R. salmoninarum,* bacteria can localise in the adhesions and remain undetected during subsequent examination. If the adhesions are sufficiently severe they can even mask overt clinical signs of BKD.

Thus, it is not possible to anticipate when (or if) a commercial vaccine which provides effective protection against BKD will become available and which offers a practical means for the aquaculture industry to reduce the significance of this disease for farmed salmonids in the way that protective vaccines have done for some of the other bacterial diseases of fish.

7. CONTROL METHODS IN AFFECTED FARMS/ZONES/COUNTRIES

7.1 Eradication of BKD

Because of the presence of the bacterium in wild fish, its ability to persist at low levels in farmed fish populations and the absence (in many cases) of obvious clinical signs in infected populations, BKD is considered as being very difficult, if not impossible, to completely eradicate by traditional methods (stamping out) from geographical areas where it is endemic.

In single fish farms, however, where the water supply is from a source free of fish, it may be possible to eradicate the disease by stamping out. In these cases the water supply should be from a borehole or from a spring where there is no possibility that there are living fish upstream. In addition a barrier should be in place to prevent any upstream migration of wild fish into the farm or its supply water.

Where eradication is attempted, measures to empty, clean, disinfect and fallow a fish farm should be standard and identical to those for other fish diseases and should follow the principles of eradication of VHS and IHN stamping-out. A fallowing period of a minimum of 30 days, preferably 60 days, during the summertime, is recommended before restocking takes place. According to Bullock and Hermann (1988), *R. salmoninarum* can survive in pond sediment for up to 21 days.

Although some attempts have been made to eradicate BKD from fish farms in open waters e.g. sea and lake cages, or from farms with water supply from rivers, such attempts have poorer chances of success primarily due to wild fish carrying *R. salmoninarum* in the environment. In Denmark, when BKD was first diagnosed in 1997 in a rainbow trout farm with severe mortality, the infected farm and a farm situated down stream were emptied, cleaned, disinfected and restocked after a fallowing period of 60 days. The farms are situated on a relatively large river. Unfortunately, the disease reappeared the following year (Olesen, pers.comm).

BKD is believed to have been introduced into Japan in the early 1970's with imports of eggs of Coho salmon which were certified free from infection at that time. The disease was

first confirmed in 1973/74 and soon affected and decimated the genuinely Japanese (and much appreciated) Cherry salmon (*O. masou*). A serious and very costly eradication campaign in the mid-1980's failed (Nomura 1994; Yoshimizu 1996; H. Wakabayashi, pers. comm 1999).

7.2   Restocking

Following an attempt at eradication by stamping out, the farm should be restocked with certified BKD-free stock (see section 7.4 below).

Following restocking the farm should be closely monitored to determine if the eradication programme has been successful. This monitoring should comprise regular inspections and laboratory examinations, e.g. 2 inspections and examinations per year. (see section 7.3 for a discussion on the numbers of samples necessary). Because of some past experiences with recurrence of the disease within one or two years after fallowing and restocking, it is suggested that this monitoring is continued for a period of at least two years before the farm is officially recognised as "free".

After restocking, measures should be taken to reduce the possibility of reinfection by the mechanical transmission of the disease agent. These measures should include restrictions on visitor access, site and vehicle cleanliness and bird control measures such as fencing with wires or netting in order to prevent birds from accessing the farm

7.3   Monitoring: sampling for the agent in previously affected areas

Sampling for the agent may be carried out for a number of purposes such as

- monitoring to determine that a farm, a zone or a country is free,

- monitoring after fallowing to determine that the agent has been eradicated or

- monitoring to ensure that a free farm/zone/country remains free.

The required number of samples to be examined in order to detect the agent depends on a number of factors such as the percentage of infected fish in the population and the degree of confidence required for the results.

Because *R. salmoninarum* can occur at low levels in the population the sampling of relatively large numbers of fish on a random basis would be required to detect the infection, e.g. it would be necessary to test 150 fish on a random basis to be 95% confident of detecting an infected fish if the infection prevalence is 2%. (Cannon and Roe, 1982). However, several steps can be taken to reduce these requirements to more practicable levels by optimising the selection of fish for examination and by extending the control period.

These steps involve;

- selection of sick or unhealthy fish

- selection of fish older than 1 year

- repeating the sampling and testing on a number of occasions

- collecting the most susceptible fish species

Nevertheless, as many fish as is practicable should be examined when monitoring following eradication or before declaring a farm free.

In addition, every unexplained morbidity and/or mortality giving rise to suspicion of BKD should be investigated by methods given in Section 3.

The most suitable laboratory tests used for confirmation of freedom from BKD are sandwich ELISA for organ material and PCR for ovarian fluids (Gudmundsdottir *et al*. 1993, Miriam *et al*. 1997) as described in chapter 3. Immunofluorescence based techniques are not suitable for screening of populations due to lack of sensitivity and difficulty in handling large numbers.

In the event that one of the above methods gives a positive result, particularly in the case of PCR, infection should be confirmed by using the culture method followed by bacterial identification.

The sampling strategy used could follow the figures given in Table 1, which are also the sample sizes recommended by the OIE (1997). These recommendations are based a publication by Ossiander and Wedemeyer (1973) for random sampling to provide 95% confidence that infected fish will be included in the sampling assuming a minimum prevalence of infection greater than 2%.

Table 1: Monitoring for absence of BKD, according to standard procedures			
Purpose	**Number of fish sampled***	**Frequency**	**Duration of sampling regime**
Following eradication	150	Every 6 Months	2 Years
Determining if free	150	6 Monthly	2 Years
Maintenance of freedom	30	6 Monthly	Indefinite

* Kidney material from no more than 2 fish should be pooled in one sample.

When targeted sampling (e.g. selecting moribund fish) is used on the above figures it would be possible to identify the agent even when present in a low percentage of the fish.

7.4 Criteria for confirming that farms/zones/countries are free from the BKD agent.

The following criteria need to be met to confirm that a farm or zone is free from the BKD agent.

1.   Historical evidence of freedom certified by the Competent Authorities

The disease should never have been confirmed in the zone or farm, except in cases where it has been successfully eradicated. It is essential that the disease would have been notifiable under the legislation in force for a considerable period (at least two years is suggested). In

that period, any notifications of suspected outbreaks of BKD should be fully investigated by the Competent Authorities.

2.   Epidemiological information

This information should comprise the boundaries of the zone, the water sources involved, fish species involved both in the farms and in the wild, migratory patterns, barriers to upstream movement etc. Control of movements into the farm or area must have been in place and investigation should have revealed no cases of movement from infected sources into the area.

3.   Results of sampling for the agent

The farm or farms in the zone should be regularly inspected for a period before the status is granted. Because of the ability of the organism to persist at low levels without necessarily causing obvious disease, this period should be as long as practicable. A minimum period of two years is suggested. During this period, fish from the farm should be regularly clinically examined, sampled and tested for the disease. See section 7.3 for a discussion on the numbers of samples necessary.

8. REDUCING THE RISK OF INTRODUCTION OF BKD INTO FREE AREAS /ZONES/ COUNTRIES

The spread of the infection into previously free areas or farms can be limited by strict preventive measures. These measures primarily involve restrictions on the introduction of fish and eggs, special requirements for water supply, and general preventive measures such as bird and vehicle control. Consignments for introduction must be accompanied by a movement document issued by the Competent Authority certifying that the fish/eggs do not originate from an infected site.

8.1 Risk of disease introduction

The main risk of introducing BKD into free farms/zones/countries lies with the transfers of live (salmonid) fish and eggs, especially through unrestricted trade without health guarantees (Yoshimizu 1996). The greatest risks will, of course, occur from importing live fish (Mitchum *et al.* 1979) and eggs (Evelyn *et al.* 1984) from sites with a recognised history of BKD. However, it is possible that fish demonstrating no clinical signs of BKD, but infected with low levels of *R. salmoninarum*, could be imported from sites with no recorded history of BKD.

Equally, it is possible that imported eggs from broodstock with no external signs of BKD, even from sites with no clinical history of the disease, could be carrying significant numbers of *R. salmoninarum* on the egg surface or even within the egg itself (Lee and Evelyn 1989). Iodophor disinfectants are effective at eliminating many fish pathogens that can be transmitted vertically and are associated with egg surfaces. However, it is well documented within the scientific literature that although such disinfectants may reduce numbers of *R. salmoninarum* contaminating eggs from infected broodfish, they are unable to eliminate the bacterium completely (Evelyn *et al.* 1983, 1984) due to *intra-ovum* infection, and that fry subsequently hatching from treated eggs can be infected with the pathogen (Evelyn *et a.l* 1986b,c).

The risk of introducing BKD through the importation of uneviscerated killed salmonids from sources with no known history of BKD (but not 'officially free') is normally likely to be low. However, a significantly higher risk would occur if the fish originated from a source undergoing an active outbreak of BKD at the time of slaughter, since even those fish without clinical signs of the disease at the time may have high levels of *R. salmoninarum* in their tissues, particularly the anterior kidney. Processing of such carcasses in premises without treatment of effluent discharging to an aquatic environment, particularly one containing salmonid fish, could easily introduce *R. salmoninarum* infection.

8.2   Methods for reducing the risk

The main method to reduce the risk of spreading BKD is to introduce live fish and eggs only from sites which carry out well-regulated health screening programmes to confirm the absence of *R .salmoninarum*. Restricting imports to eggs will further reduce risks. Health screening programmes must be carried out over a prolonged period of time (2 years minimum) by the Competent Authorities using recognised techniques such as ELISA, PCR and standard bacteriological methods (OIE Diagnostic Manual 1997).

A particular problem arises when broodstocks cannot be held under secure conditions e.g. Atlantic salmon kept in sea cages or trout farms supplied by river water and thereby potentially exposed to infection from wild fish in the vicinity. In such cases, stocking of BKD free hatcheries could be with disinfected eggs from erythromycin treated females. The use of erythromycin as a mean of preventing vertical transmission of *R. salmoninarum* has been described by Evelyn *et al*. (1986b) and by Sakai *et al*. (1986). The method is useful in order to reduce the risk of transfer of the bacteria by the eggs into a free zone or farm. Alternatively, it is possible to individually test the brood fish and use eggs only from parents shown to be negative for *R. salmoninarum*. Broodstocks in some countries/areas are examined at stripping for the presence of *R.salmoninarum* by laboratory tests such as ELISA or PCR. This allows fertilised eggs from different parents to be kept in separate tanks and destroyed if they originate from *R. salmoninarum*-positive fish. This has been done for about 10 years on the Faroe Islands by testing kidney samples in ELISA. Unfortunately, the method did not seem to reduce the number of clinically BKD infected

fish in the farms. From 1999 the strategy was changed to use combined erythromycin treatment and individual testing.

Killed salmonids for importation into a free zone or country should never be derived from a source experiencing an active outbreak of the disease or from a population slaughtered as part of a BKD control measure by the official services. The risk will be further reduced if all killed salmonid fish from sources within a zone or country known to be affected by BKD are required to be eviscerated before shipping. Even carcasses of susceptible species from a source of unknown BKD status should, ideally, be eviscerated before despatch to a free zone or country.

9. CONCLUSION AND RECOMMENDATIONS

Standards for eradication programmes for Bacterial Kidney Disease (BKD) and the criteria for determining zones free of the disease are discussed in Section 7.1 to 7.4. of this report. The risk of introducing Bacterial Kidney Disease into such areas and methods for reducing this risk are covered in sections 8.1 and 8.2.

9.1 Overall Conclusion

Because of the presence of the bacterium in wild fish, its ability to persist at low levels in farmed fish populations and the absence (in many cases) of obvious clinical signs in infected populations, BKD is considered as being very difficult, if not impossible in many cases, to completely eradicate by traditional methods (stamping out) using the currently available tools, from geographical areas where it is endemic.

9.2 Recommendations

For those EU Member States or Regions who wish to eradicate this disease, it may be more appropriate to further develop the necessary tools such as screening tests and vaccines when new investigations and developments will provide more effective and practical protective measures. In this respect, there would be a need for a Community Reference Laboratory, or similar, which could harmonise and co-ordinate developments in this area.

The possibility of breeding salmonids for BKD resistance should be further investigated whilst paying full regard to preserving the biodiversity of wild fish.

There is much that individual farms can do to avoid becoming infected. Guidelines for prevention and control by the methods available at present should be circulated. These may be particularly useful for farms supplied by protected water supplies e.g. springs, wells, etc.

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11. ACKNOWLEDGEMENTS

Reports of the Scientific Committee on Animal Health and Animal Welfare of the European Union are available at the Committee Website;

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