Bull. Eur. Ass. Fish Pathol., 28(2) 2008, 66

Persistence of *Renibacterium salmoninarum* in experimentally infected rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The persistence of *Renibacterium salmoninarum* (*Rs*) in rainbow trout (*Oncorhynchus mykiss*) experimentally infected with either a virulent or non-virulent strain of *Rs* was examined in this study. Mortalities from bacterial kidney disease (BKD) peaked at 48 % 6 weeks post-challenge with the virulent strain, but no mortality from BKD occurred in fish infected with the avirulent strain. Culture on Selective Kidney Disease Medium (SKDM), a polymerase chain reaction (PCR) and an enzyme linked immunosorbent assay (ELISA) were used to monitor the duration of the infection. The presence of bacteria in challenged fish could be detected by PCR at 12 and 20 weeks post-challenge with non-virulent and virulent strains, respectively. On the other hand, it was only possible to detect *Rs* NCIMB 1113 by ELISA and by culture on SKDM up to 10 weeks and 12 weeks, respectively. Furthermore, the avirulent isolate was not detected in fish injected with this strain on SKDM or by ELISA.Using the methods of detection described, it would appear that the fish were able to clear the virulent *Rs* by week 22 post-challenge.

Introduction

The control of Bacterial Kidney Disease (BKD) in cultivated salmonids is hindered by the lack of effective chemotherapy and the absence of an effective vaccine for the disease. Detection of *Renibacterium salmoninarum* (*Rs*), the causative agent of BKD, in sub-clinically infected broodstock is necessary to instigate methods for controlling transmission of the pathogen (Pascho et al., 1991; Evenden et al., 1993; Bruno, 2004), however, this can be difficult to achieve. *Rs* is well known for its fastidious and slow growing nature, and this has made detection of the pathogen by culture particularly difficult due to the long incubation period needed before colonies can be seen (Austin et al., 1983).

A combination of culture and antibody-based methods, including direct and indirect fluorescent antibody techniques (FAT and IFAT) and enzyme-linked immunosorbent assays (ELISA) (Pascho et al., 1991) have been used to identify the pathogen in infected samples. The ELISA is considered more sensitive than bacteriological culture or IFAT for detecting *Rs* (Meyers et al., 1993). However, all of these methods lack the required sensitivity to detect sub-clinically infected fish. Highly sensitive methods, based on the polymerase chain reaction (PCR), have great potential for improving both the sensitivity and specificity for detection of *Rs* (Leon et al., 1994; McIntosh et al., 1996; Miriam et al., 1997; Chase & Pascho, 1998,

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Cook & Lynch, 1999; Königsson et al., 2005; Powel et al., 2005).

Although culture methods have revealed that *Rs* can establish an asymptomatic carrier state in fish (Evenden et al., 1993), no long-term studies have been performed to investigate how long after initial infection this state may persist. The persistence of *Rs* in rainbow trout experimentally infected with either a virulent or an avirulent isolate of *Rs* was monitored over a 24-week period using culture on SKDM, PCR and ELISA.

Material and methods

Preparation of bacterial strains

Strains of *Rs* used in the study were grown on Selective Kidney Disease Medium (SKDM) (Austin et al., 1983) for 3 weeks at 15°C. Bacteria were washed off of the plates with sterile deionized water (dH_2O). The concentration of the suspensions was adjusted to 1 $x10^6$ bacteria ml^{-1} (Farias, 1995) with dH_2O for PCR and with 0.02 M sterile phosphate buffered saline pH 7.2 (PBS) for the ELISA.

Experimental challenge with Rs

Eight hundred rainbow trout, *Oncorhynchus mykiss*, weighing between 30-50 g were divided into eight 200 L tanks (100 fish $tank^{-1}$) after acclimatizing to a water temperature of 12 ± 2°C for two weeks.

Isolates of *Rs*, previously determined as either virulent (isolate NCIMB 1113) or avirulent (isolate NCIMB 1111) (Turgut, 2002), were used for the experimental challenge. Four tanks of fish were challenged intra-peritoneally with 0.1 ml the virulent *Rs* isolate at a dose of 2.5 $x10^8$ cfu ml^{-1}, two tanks were challenged with the avirulent *Rs* isolate at a concentration of 0.6 $x10^8$ cfu ml^{-1}, and two tanks were injected with 0.1 ml PBS. The concentration of bacteria injected was determined retrospectively from cfu counts, which explains why the doses injected varied between the two strains. Injected fish were observed daily for gross clinical signs of BKD and dead fish removed.

Sampling

As well as sampling dead fish throughout the trial, four live fish were randomly selected from each group every 2 weeks post-inoculation. Kidney was streaked onto SKDM agar and incubated at 15°C. Plates were examined 6-8 weeks later for bacterial growth, and *Rs* was confirmed by Gram stain and ELISA.

Tissue samples, including kidney, spleen, liver, heart and brain were aseptically removed from the fish and placed into sterile microcentrifuge tubes and stored at -70°C until analysed. Sampling on SKDM and for ELISA and PCR continued until the *Rs* appeared to have cleared from the fish at 24 weeks.

Polymerase Chain Reaction

Extraction of DNA. DNA was extracted from 20-25 mg of tissues using a DNA extraction and purification genomic DNA kit (Nucleon Bacc2 extraction and purification kit, Nucleon Biosciences, Amersham, UK) as described by the manufacturer. The extracted DNA was dissolved in 100 µl dH_2O and stored at –20°C until analysed by PCR.

Polymerase Chain Reaction. The PCR was performed using primers designed to amplify a 149 DNA fragment (pMAM29

sequence) [forward primer GL1 (5-GATCGTGAAATACATCAAGG-3) and reverse primer GL2 (5'GGATCGTGTTTTATCCACCC-3)] (Leon et al., 1994). The PCR was carried out using PCR ready to go beads (Amersham Pharmacia Biotech, UK) and a thermal cycle profile as described by Leon et al. (1994). Thirty-five reaction cycles of 1.5 min (94°C), 1 min (60°C) and 1 min (72°C), were completed and thereafter the samples were incubated at 72°C for 5 min. PCR products (10 µl) were subjected to electrophoresis in conjunction with DNA size standards through a 1.5 % agarose gel which was stained with ethidium bromide and examined under a UV transilluminator. Samples were considered positive if an amplified 149 bp product was observed.

Sensitivity of PCR. DNA was extracted from *Rs* NCIMB 1113 using the DNA extraction and purification genomic DNA kit. The concentration and purity of extracted DNA was determined spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. An absorbance of 1.0 at 260 nm corresponded to 50 µg DNA ml^{-1}. The extracted DNA was then stored at –20°C until required.

Extracted DNA with an initial concentration of 50 µg ml^{-1} was serially diluted ten fold to 5 $x10^{-6}$ µg DNA ml^{-1} using TE Buffer (10 mM Tris-Cl, pH 7.5, 1mM EDTA), to give correspond bacterial concentrations of 1.25 $x10^9$, 1.25 $x10^7$, 1.25 $x10^6$, 1.25 $x10^5$, 1.25 $x10^4$, 1.25 $x10^3$ bacteria ml^{-1} (4 fg bacterial genome is equal to 1 bacterial cell). Samples were then amplified by PCR.

Sensitivity of PCR using kidney homogenates spiked with *Rs*. Kidney (100 mg), sampled from uninfected control fish, was homogenised in Reagent A buffer from the Nucleon Bacc2 extraction and purification kit. A ten fold serial dilution of *Rs* 1113 with a starting concentration of 60 µg ml^{-1} (corresponding to 1.5 $x10^{10}$ bacteria ml^{-1}) was prepared in dH_20 to give concentrations of 1.5 $x10^{10}$, 1.5 $x10^8$, 1.5 $x10^6$, 1.5 $x10^5$, 1.5 $x10^4$ and 1.5 $x10^3$ bacteria ml^{-1}. One hundred µl aliquots of bacterial suspension were then mixed with 900 µl of either PBS or kidney homogenate. DNA was extracted from both sets of samples (1 ml of each bacterial suspension and 1 ml of the spiked kidney tissue), and the resulting pellet dissolved in 100 µl of dH_2O and stored at –20°C until analysed. Samples of dH_2O and unspiked kidney were used as negative controls.

Enzyme Linked immunosorbent assay (ELISA)

The presence of *Rs* in the kidney of challenged fish was determined from samples taken at two weekly intervals post-challenge using a sandwich ELISA. The procedure used to prepare samples for the ELISA was as described by Meyer et al. (1993). The sandwich ELISA was performed following a modification of the method of Adams (1992). ELISA plates coating with 100 µl $well^{-1}$ acid treated rabbit antisera (Turgut, 2002). Samples and controls were added to the wells (100 µl $well^{-1}$) in duplicate. Wells with only PBS-T20 or kidney from non-infected rainbow trout were used as negative controls, while heat killed *Rs* (strain 1113) was used as a positive control. A standard curve was prepared for the ELISA using ten fold dilutions of the

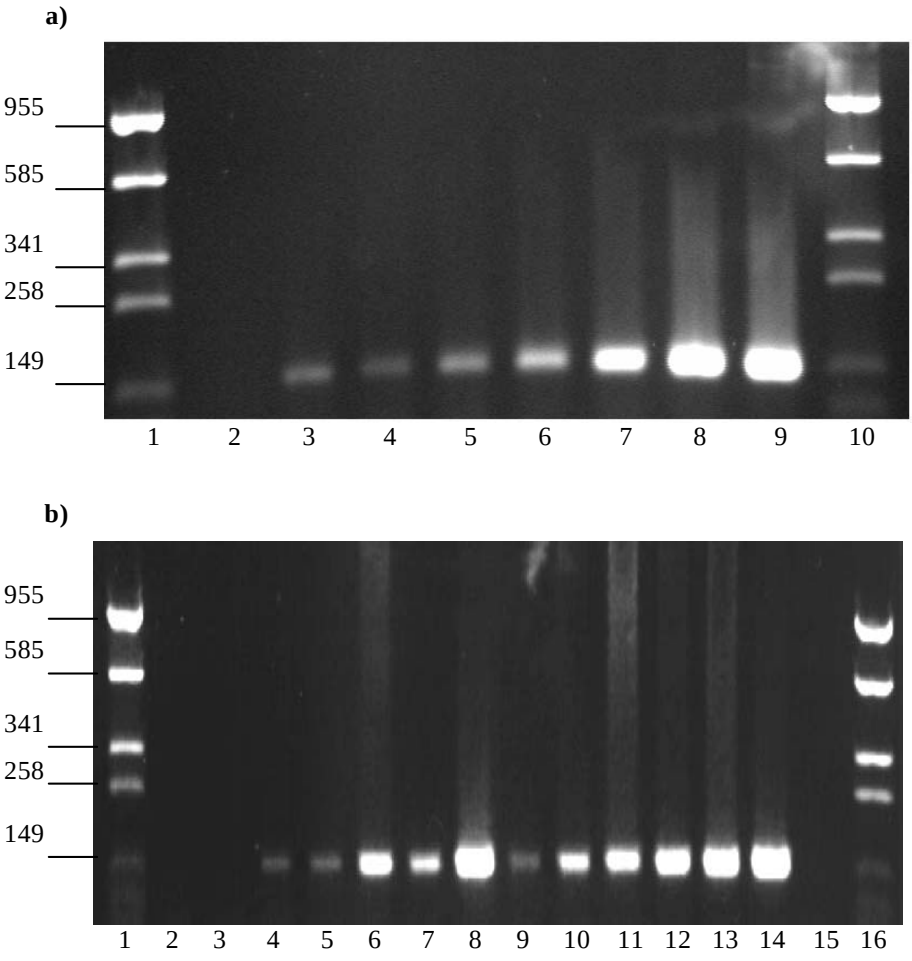

Figure 1. Sensitivity of PCR on a 1.5 % (w/v) agarose gel for (a) DNA extracted from *Renibacterium salmoninarum* NCIMB 1113. Lanes: (1) and (10) Molecular size markers; (2) water - negative control, (3) 12 fg DNA, (4) 120 fg DNA, (5) 1.2 pg DNA, (6) 12 pg DNA, (7) 120 fg DNA, (8) 1.2 ng DNA, (9) 12 ng DNA. (b) DNA extracted from kidney spiked with *R. salmoninarum* NCIMB 1113 Lanes: (1) and (16) Molecular size markers; (2) water - negative control; (3-8) serial diluted *Rs*: (3) 3.7 cells, (4) 3.7 x 10^1 cells, (5) 3.7 x 10^2 cells, (6) 3.7 x 10^3 cells, (7) 3.7 x 10^5 cells, (8) 3.7 x 10^7 cells, (9-14) kidney spiked with serial diluted *Rs*: (9) 3.7 cells, (10) 3.7 x 10^1 cells, (11) 3.7 x 10^2 cells, (12) 3.7 x 10^3 cells, (13) 3.7 x 10^5 cells, (14) 3.7 x 10^7 cells; (15) unspiked kidney tissue.

positive control i.e. 4 x10^7 to 1.6 x10^2 bacteria ml^{-1} prepared in PBS-T20. The *Rs* were detected in the ELISA using a mixture of two anti-*Rs* monoclonal antibodies (MAbs: 4A20-17 and 6E11-12) (Turgut, 2002), by adding 100 $\mu lwell^{-1}$ of the cocktail for 1 h at 20°C. The remainder of the assay was preformed as described. The negative-positive threshold of the ELISA was taken as the mean OD_{450} value of the samples from clinically healthy non-infected fish plus 3 times the standard deviation of samples according to Jansson et al. (1996).

Weeks	PCR		ELISA				SKDM	
			1113		1111			
	1113	1111	OD_{450}	+/-	OD_{450}	+/-	1113	1111
2	+	+	5.5 x10^5	+	-	-	+	-
4	+	+	4.3 x10^5	+	-	-	+	-
6	+	+	8.8 x10^4	+	-	-	+	-
8	+	+	7 x10^4	+	-	-	+	-
10	+	+	3.4 x10^4	+	-	-	+	-
12	+	+	-	-	-	-	+	-
14	+	-	-	-	-	-	-	-
16	+	-	-	-	-	-	-	-
18	+	-	-	-	-	-	-	-
20	+	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-
Mortalities	+	+	1.6 x$10^{7\ a}$	+	-	-	-	-

n=4; (+): detection of *Rs*; (-): no detection of *Rs*
Kidney taken from fish injected with PBS had a mean OD_{450} value of 0.118 (n=2). Values of samples higher than negative positive threshold, 0.148 are considered positive for which bacterial concentrations are reported. [a]Average OD_{450} value of kidneys from mortalities sampled during challenge period.

Table 1. The presence of *Renibacterium salmoninarum* in the kidney of rainbow trout experimentally challenged with the bacterium determined by PCR, ELISA and culture on SKDM sampled at 2 weekly intervals post-challenge.

Results

Sensitivity of PCR

Using serial dilutions of DNA extracted from *Rs*. The lowest detectable concentration of *Rs* DNA was 12 fg of DNA per reaction (Figure 1a, Lane 3), which was equivalent to 3 renibacterial cells.

Using DNA extracted from kidney samples spiked with *Rs*. DNA amplified from kidney samples spiked with *Rs* produced more distinct bands than DNA extracted from bacteria (Figure 1b). The sensitivity threshold of the PCR with spiked kidney was estimated to be 3.7 cells reaction^{-1} (15 fg DNA reaction^{-1}) (Figure 1b, Lane 4), while an estimated 37 cells reaction^{-1} (150 fg reaction^{-1}) (Figure 1b, Lane 9) was the lowest level detected when only bacteria were used.

Detection of Rs *in experimentally infected rainbow trout*

Mortalities. Fish infected with the virulent *Rs* isolate displayed swollen, greyish kidneys and produced ascites in their peritoneal cavity. However, none of the fish injected with avirulent *Rs* isolate NCIMB 1111 showed signs of pathology or had gross lesions. From the results, BKD was confirmed as the cause of mortality in fish injected with the virulent *Rs* (48.2 %), while the mortalities which occurred in fish injected with *Rs* NCIMB 1111 (0.5 %) were not due to BKD. There were no mortalities in the control fish injected with PBS. No mortalities occurred in the group injected with *Rs* isolate NCIMB 1113 after 6 weeks post-challenge.

Detection of *Rs* from experimentally infected rainbow trout by culture on SKDM. It was possible to recover *Rs* on SKDM from kidneys of fish infected with *Rs* NCIMB 1113 for up to 12 weeks post-challenge, while the mortalities which had occurred in fish injected with *Rs* NCIMB 1111 (0.5%) were not due to overt BKD and *Rs* was not recovered from any of the fish injected with *Rs* NCIMB 1111 (Table 1).

PCR of tissues sampled from fish challenged with *Rs* NCIMB 1113. At 2 weeks post-challenge, PCR products from DNA samples prepared from heart, liver, spleen and kidney of fish infected with *Rs* NCIMB 1113, all produced distinct bands at 149 bp (Figure 2, Lanes 9, 10, 11 and 12 respectively), and gave much more intense bands than found with brain samples (Figure 2, Lanes 8). Positive PCR products were obtained from kidney and spleen samples up to 20 weeks post challenge

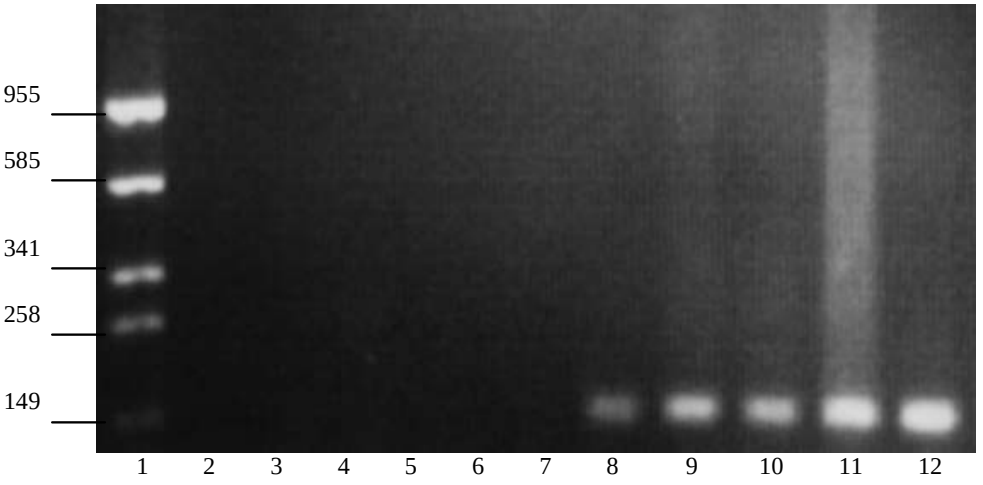

Figure 2. Confirmation of the presence of *R. salmoninarum* in fish infected with *Rs* NCIMB 1113, 2 weeks post-challenge, as detected by PCR on a 1.5 % (w/v) agarose gel. Lanes: (1) molecular marker; (2) negative control; Lanes (3-7) tissue sampled from challenged rainbow trout injected with PBS: (3) brain, (4) heart, (5) liver, (6) spleen, (7) kidney; Lanes (8- 12) tissue sampled from fish infected with *Rs* NCIMB 1113: (8) brain, (9) heart, (10) liver, (11) spleen, (12) kidney.

with NCIMB 1113, whereas positive results were only obtained with brain and heart tissue up to 8 weeks post-challenge (data not shown). No PCR products were detected in control fish (Figure 2, Lanes 3-7).

Persistence of *Rs* in rainbow trout challenged with *Rs* NCIMB 1113 determined by PCR and ELISA. PCR products could be detected in the kidneys of surviving fish infected with *Rs* NCIMB 1113 up to 20 weeks post-challenge (Table 1). Very strong bands were evident at Weeks 2, 6 and 10 post-challenge (Figure 3a, Lanes 8, 7 and 6). After this time, the intensity of the bands became much fainter and no PCR product was observed with samples taken at 22 weeks post-challenge (data not shown). With DNA extracted from fish injected with *Rs* NCIMB 1111, a strong band was obtained at Week 2, 6 and 10 (Figure 3b, Lane 8, 7 and 6) and thereafter decreased in intensity until it was no longer evident at Week 14 (Figure 3b, lane 5).

From the standard curve constructed for the ELISA, the detection limit of the assay was approximately 1.6 x10^4 bacteria ml^{-1} (data not shown). No bacteria were detected in kidney taken from fish injected with *Rs* NCIMB 1111 by ELISA, while *Rs* was detected up to Week 10 post-injection in fish injected with virulent *Rs* (Table 1).

Discussion

Kidney samples taken at the time of death from fish infected with *Rs* NCIMB 1113 had higher levels of bacteria present, detected by culture, PCR and ELISA, compared to fish sampled once mortalities had stopped. Many samples which were negative by culture and ELISA, were positive by PCR. Virulent *Rs* could still be detected in kidney from infected fish up to 20 weeks post-challenge by PCR,

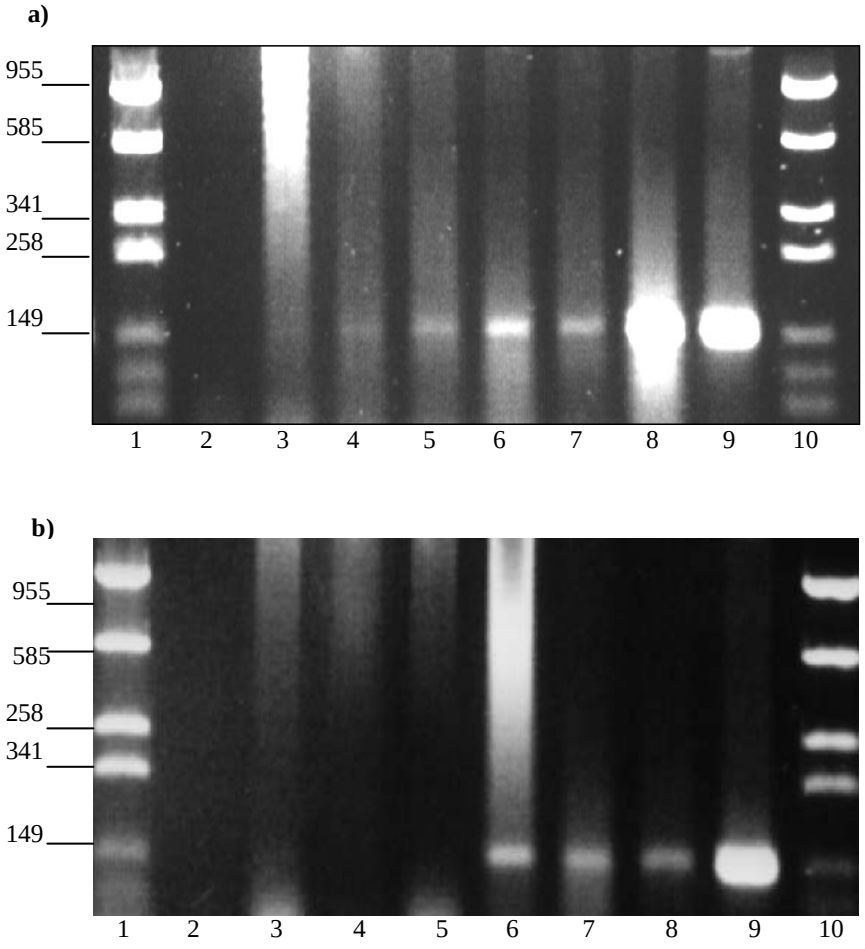

Figure 3. Presence of *R. salmoninarum* in kidney of rainbow trout as detected by PCR on a 1.5 % (w/v) agarose gel after challenging fish with (a) virulent *Rs* NCIMB 1113, (b) avirulent *Rs* NCIMB 1111. Lanes: (1 and 10) molecular weight markers; (2) water as negative control; (3) 22 weeks; (4) 18 weeks; (5) 14 weeks; (6) 10 weeks; (7) 6 weeks; (8) 2 weeks; (9) Positive control, *Rs*.

becoming negative at 22 weeks, while samples were only positive up until 10 weeks post-challenge by ELISA and 12 weeks by culture. Similarly, when fish were challenged with avirulent *Rs*, bacteria could be detected up to 12 weeks post-challenge by PCR. However, no bacteria were detected by ELISA or culture in any of the kidney samples of these fish.

There have been several reports relating to the detection limits of PCR for *Rs*, (Magnusson et al., 1994; McIntosh et al., 1996; Chase & Pascho, 1998; Rhodes et al., 1998; Königsson, 2005). In this study PCR products could be detected at concentrations as low as 12 fg *Rs* DNA (corresponds to 3 renibacterial cells) when extracted from a bacterial suspension. To determine the sensitivity of the PCR

reaction with kidney tissue, DNA was extracted from kidney spiked with the same dilution of *Rs* as the bacterial suspension and then amplified by PCR. The sensitivity of the PCR in this case was 37 bacterial cells. Clearly there is a difference in the sensitivity of the PCR depending on the nature of the sample from which the DNA was extracted. For some reason the detection level of the PCR appeared more sensitive with the spiked kidney compared to samples of pure bacteria, possibly due to the presence of the kidney tissue or homogenising the sample improved the extraction of the DNA. McIntosh et al. (1996) reported a reduction in sensitivity in PCR using kidney and lymphocytes spiked with *Rs* compared with bacteria suspended in PBS. Both Magnusson et al. (1994) and McIntosh et al. (1996) reported inhibitors in kidney tissue but not ovarian fluid, which reduced the sensitivity of the PCR. However, inhibition did not appear to be a problem in the present PCR assay but it should be noted that the methods of DNA extraction differed between the assays reported in the different studies, which possibly resulted in different yields of DNA.

The intensity of the PCR varied both with the challenge strains used and the type of tissue sampled. The PCR signal obtained from kidney, spleen and liver of infected fish always gave a stronger reaction than heart and brain, and intensity was always greatest with kidney. Furthermore, positive results by PCR were obtained with kidney and spleen up to 20 weeks post-challenge with fish challenged with virulent *Rs*, whereas positive results were only obtained with brain and heart tissue up to 8 weeks post-challenge, indicating that the number of bacteria present in the kidney was greater than in the other tissues examined, and reflects the distribution of the pathogen in the different fish tissues during infection. However, the intraperitoneal injection used in this study does not representative of a natural disease outbreak and a more natural challenge method such as a cohabitation challenge would be more suitable for future work.

Positive results were obtained up to 10 weeks post-challenge by ELISA with kidney samples from fish experimentally infected with *Rs* NCIMB 1113, compared to 20 weeks post-challenge by PCR. Kidneys sampled from fish infected with the avirulent *Rs* isolate were all negative by ELISA from the onset of the experiment, while positive samples were detected up to 12 weeks post-challenge by PCR. Other researchers have reported PCR-based assays to be one or more orders of magnitude more sensitive than conventional immunological tests (Miriam et al., 1997; Chase & Pascho, 1998; Pascho et al., 1998).

Although mortalities ceased after 6 weeks post-challenge with the virulent isolate, it was still possible to detect bacteria up to 20 weeks post-challenge by PCR. On the other hand, the PCR detected bacteria only up to 12 weeks post-challenge in fish infected with the avirulent isolate of *Rs*. Furthermore, no growth was observed on SKDM with samples taken from fish infected with the avirulent isolate. Thus, it would appear that the avirulent isolate did not establish a persistent infection in the fish, although non-culturable bacteria were still detected by PCR. On the other hand the results of the PCR and culture indicate that the clearance of virulent *Rs*

NCIMB 1113 can take a long time within convalescing fish, with bacterial DNA still being detected by PCR up to 20 weeks post-challenge. This may be due to nonviable bacteria as there was no growth on SKDM plates after the 12 week sample or persistent bacterial DNA. However an alternative culture medium, such as Ten-M, may have supported *Rs* growth more successfully (Chambers & Barker 2006). It has been shown that head kidney and spleen samples from Atlantic salmon vaccinated with formalin inactivated *A. salmonicida* (i.e. dead bacteria), were still positive by PCR 16 weeks after intraperitoneal injection with the bacterium (Hoie et al., 1996). This clearly indicates that bacterial DNA can persist for long periods in the fish tissues.

Senson & Stevenson (1999) also demonstrated the presence of *Rs* after ip challenge with *Rs* isolate JD24 (92 % mortalities in 45 days) or MT 239 (7 % mortalities). When the challenge was stopped after 14 weeks, 2 out of 8 remaining fish challenged with JD24 gave IFAT positive results, while those challenged with MT 239 did not show any sign of *Rs* when smears of kidney tissue were examined by IFAT.

One disadvantage with DNA-based methods is that it does not distinguish between living and dead organisms (Hoie et al., 1996) which has serious implications for using PCR to screen fish where antibiotic chemotherapy is used to treat disease outbreaks or as a routine treatment for broodstock prior to spawning, since dead bacteria resulting from the treatment will also be detected. Cook & Lynch (1999) described the potential of using mRNA for the specific detection of live *Rs*, since mRNA is a good indicator of viability due to the short life of bacterial mRNA with a half life of only a few minutes.

The apparent clearance of the bacterial infections is an important issue. It should be pointed out that very low levels of *Rs* infection can be detected in healthy populations of fish (0.1 – 0.5 %), possibly due to carriers sequestering the bacterium within a few randomly dispersed granulomas. It is feasible that these granulomas are missed while sampling and the tissue appears negative even though it is really positive. This means hundreds of fish need to be tested to detect a single positive fish.

In the present work, culturable *Rs* was detectable up to 12 weeks post-challenge with the virulent strain, 6 weeks after mortality had ceased. PCR continued to detect the bacterium for a further 8 weeks. The fish were PCR negative at 22 weeks post-challenge, suggesting that convalescent fish may eventually be able to clear the carrier state.

Acknowledgements

This research was supported by PhD program of Higher Educational Council (YÖK), Turkey.

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