Evaluation of a semi-nested PCR for detection of *Renibacterium salmoninarum* in samples from kidney, gill and ovarian fluid of Atlantic salmon broodfish

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ABSTRACT

A semi-nested PCR (snPCR) for detection of *Renibacterium salmoninarum* that causes bacterial kidney disease (BKD) in salmonids was constructed. The efficacy of the snPCR was evaluated by comparison with nested PCR (nPCR) and two ELISA methods on kidney, ovarian fluid and gill samples collected from Atlantic salmon broodfish with escalating disease. The PCR methods using a conventional isolation kit identified equal numbers of positive samples, or 30%, with acceptable agreement. The ratio of positive kidney samples in PCR increased significantly, 42.5% in snPCR and 45% in nPCR, when an FTA minicard was used for DNA isolation. ELISA, using polyclonal antibodies, detected the highest number of positive samples (65%) and ELISA using monoclonal antibodies the lowest (17.5%). Ovarian fluid and gills gave inadequate results and cannot replace kidney samples for determination of *R. salmoninarum* infection in Atlantic salmon broodfish.

Keywords: Atlantic salmon, bacterial kidney disease, ELISA, nested PCR, *Renibacterium salmoninarum*, semi-nested PCR.

YFIRLIT

Semi-nested PCR (snPCR) aðferð var þróuð til greiningar á nýrnaveikibakteríunni *Renibacterium salmoninarum*. Greiningargeta snPCR prófsins í nýrna-, tálkna- og hrognavökvasýnum var metin út frá samanburði við nested PCR (nPCR) og tvær ELISA aðferðir í Atlantshafsklaklöxum með virka sýkingu. PCR aðferðirnar greindu sama hlutfall af jákvæðum sýnum með hefðbundinni DNA einangrunaraðferð, eða 30%, með góðu innbyrðis samræmi. Einangrun DNA á FTA pappír stuðlaði að greiningu marktækt fleiri jákvæðra sýna, 42.5% fyrir snPCR og 45% fyrir nPCR. Fjölstofna ELISA aðferð (pELISA) greindi flest jákvæð sýni (65%), en einstofna aðferðin (mELISA) fæst (17.5%). Tálkn og hrognavökvi henta ekki til skimunar á *R. salmoninarum* í laxi.

INTRODUCTION

Bacterial kidney disease (BKD) in salmonid fish is caused by the gram positive intracellular bacterium, *R. salmoninarum* (Sanders & Fryer 1980). The disease causes losses in salmonid fish culture in fresh and marine waters and the bacterium is also detected in wild fish populations (VESO 2007).

Fish harbouring *R. salmoninarum* may display various internal and external signs of the disease such as exophthalmia, petechiae and granulomas in internal organs but can also be symptomless carriers (Fryer & Sanders 1981). The bacterium is transmitted horizontally from fish to fish through the water as well as vertically via the eggs (Evelyn et al. 1984).

The main strategy in fighting the disease is avoidance, as neither antibiotic treatment nor vaccines are sufficient for eradication (Rhodes et al. 2004, Fairgrieve et al. 2005). One such method is culling of ova from infected female broodfish (Gudmundsdottir et al. 2000). Screening for *R. salmoninarum* is carried out in various tissue samples using culture on agar, enzyme linked immunosorbent assays (ELISA), polymerase chain reactions (PCR) and fluorescent antibody techniques (FAT). The bacterium is slow growing and 12 weeks incubation time was found necessary for testing Atlantic salmon broodfish (*Salmo salar* L.) (Benediktsdottir et al. 1991). Therefore, polyclonal ELISA, a rapid and sensitive method, has been used for screening in Iceland since 1991 (Gudmundsdottir et al. 1993). According to OIE (2006), a positive culture, verified as *R. salmoninarum* by biochemical methods, specific antiserum or PCR can be used for diagnosis. When other techniques are used for detection, verification with a method based on a different biological principle is required, as for example, ELISA for screening and PCR for confirmation (OIE 2006).

Polyclonal antibodies against whole bacterial cells or monoclonal antibodies against the 57kD protein, also known as MSA (major soluble antigen), of *R. salmoninarum* are used in double sandwich ELISA tests for antigen detection (Gudmundsdottir et al. 1993, Jansson et al. 1996). MSA is the predominant cell surface antigen of *R. salmoninarum* and comprises 70% of total surface proteins (Wood & Kaattari 1996). It is also secreted into the tissues and has been shown to be an important virulence factor with immunosuppressive functions (Grayson et al. 2002).

Several PCR methods have been developed for *R. salmoninarum*, most of them targeting the *msa* gene for amplification and a nested PCR (nPCR) detecting *msa* is recommended by OIE for screening purposes (Chase & Pascho 1998, OIE 2006).

The kidney, especially the posterior part, is the tissue most commonly sampled, but ovarian fluid is occasionally used for screening purposes (Pascho et al. 1998) and gills have been considered for non-lethal sampling (Elliott et al. 2011).

For culling and in control programs, sensitive and specific techniques for detection of *R. salmoninarum* are highly demanded. In the current study, one-tube semi-nested PCR (snPCR) was developed, tested on three different tissues and compared to the nPCR test (Chase & Pascho 1998) and two ELISA techniques. In addition, two methods for DNA isolation for PCR were evaluated.

MATERIALS AND METHODS

Fish

Samples were collected from two populations of Atlantic salmon female broodfish. In the first group, *R. salmoninarum* infection was escalating. Their life history was as follows: eggs were hatched in a land-based facility with pathogen free borehole water and moved to a different farm one year later, where they presumably got infected. Then they were transferred to sea cages and reared for two years. In the middle of the second summer fish were moved to land-based tanks where BKD was soon suspected. The following autumn 40 female broodfish were stripped and kidney, ovarian fluid and gill samples collected.

Control samples, from kidneys, were collect-

ed from 15 Atlantic salmon female broodfish that were reared in land-based tanks on a farm where *R. salmoninarum* had never been detected.

Bacterial strains

The *R. salmoninarum* strain S-182-90 (Grayson et al. 2000) was cultivated on selective kidney disease medium, S-KDM, to be used for the snPCR sensitivity testing and as a positive control for both PCR methods. This strain was initially isolated from farmed Atlantic salmon fry in Iceland and kept at -80°C.

Spiked kidney samples were prepared to estimate the sensitivity of snPCR. The DNA was prepared from cultured *R. salmoninarum* cells by a Genomic DNA purification kit (see below). Drop plate counting of serial tenfold dilutions of the bacterium was performed to estimate the number of bacteria in the original solution, which was used for DNA isolation. To include potential PCR inhibitors in the host kidney tissue, a fixed amount of isolated DNA from uninfected kidney tissue was added to each dilution of bacterial DNA before testing, to simulate field samples.

To test for cross-reactivity, ten bacterial species were grown on blood agar with and without addition of NaCl (1.5%) and a loopful of the growth suspended in 1 ml of sterile PBS. Samples of the solutions were placed on an FTA minicard (see below) and run in snPCR. The bacteria tested were the gram positive *Arthrobacter globiformis* NCIMB 8907, *Terrabacter tumescens* NCIMB 8914, *Nocardioides luteus* NCIMB 11455, *Carnobacter piscicola* ATCC 35586 and *Corynebacterium aquaticum* ATCC 14665. The five gram negative pathogens included were *Aeromonas salmonicida* subsp. *achromogene*s NCIMB 1110, *Vibrio salmonicida* NCIMB 2262, *Yersinia ruckeri* NCTC 10746, *Moritella viscosa* NCIMB 13584 and an Icelandic isolate, F-125-01, of *Vibrio anguillarum* of serotype O2β. *R. salmoninarum* strains NCIMB 1111 and NCIMB 1113 were also run in the test.

FTA minicard

An FTA minicard (Whatman) was used following the manufacturer's protocol. In short, 20 µl of homogenized kidney samples diluted 1:3 (w v^{-1}) in sterile Dulbecco's PBS (phosphate buffered saline) or undiluted ovarian fluid samples were placed on an FTA minicard and dried for one hour at room temperature. A 2 mm disc (in diameter) was punched out and placed in a PCR amplification tube. The disc was washed three times in the tube with 200 µl of FTA purification reagent and twice with 200 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA), with a 5 min interval between each washing step. The disc was air dried in the tube for 1 hour at room temperature and thereafter used as a template in the PCR reactions.

Genomic DNA purification kit

The "solid tissue protocol" of the Genomic DNA purification kit (Puregene), was used as described by the manufacturer, except that proteinase K was replaced by achromopeptidase (Sigma-Aldrich) (Magnusson et al. 1994). In brief, approximately 10 mg of kidney tissue or 20 mg of gill tissue were homogenized and incubated with 70 U of achromopeptidase for one hour at 37°C. Subsequently the samples were treated with RNase A solution, protein precipitation solution, isopropanol and ethanol, as described in the protocol. Finally, 50 µl of DNA hydration solution were used to dissolve the precipitate.

One-tube semi-nested PCR

Three primers, with different melting temperatures, were designed to carry out two reactions in one PCR tube, amplifying a sequence from the *msa* gene of *R. salmoninarum*. The primers were designed from the published sequence of the *msa* gene using Primer3 program (GenBank accession number: AY986794.1). Two primers, For_msa (5'-AGATGGAGCAACTCCGGTTA-3') and Rev_msa (5'-GGGATTACCAAAAGCAACGA-3'), amplified the first fragment of 271 base pairs. The third primer,

nRev_msa (5'-TCTCTCAACGCCAATAC-3'), was used to amplify the second fragment of 196 base pairs within the first fragment along with the For_msa primer. The melting temperatures for the primers are 57.3°C for the For_msa primer, 55.3°C for the Rev_msa primer and 50.4°C for the nRev_msa primer. The reaction mixture, in a total volume of 25 µl, contained sterile water, 0.24 mM of each nucleotide, 2 mM of MgCl, 24 mM of Tris-HCl (pH 8.4), 60 mM of KCl, 1.6 µM of For_msa and nRev_msa primers, 0.8 µM of Rev_msa primer, and 0.625 U of platinum taq DNA polymerase (Invitrogen). The template for the reaction mixture was either one µl of the elute from the DNA kit diluted tenfold, the dilution giving the best results in a previous testing (data not shown), or one disc punched out from an FTA minicard. The thermal cycling was done with a Peltier thermal cycler (PTC-200 MJ research, Bio-Rad) under the following conditions: twelve cycles of amplification (denaturation at 94°C for 30 sec, annealing at 61°C for 2 min (annealing temperature lowered by 0.5°C for each cycle), and extension at 72°C for 30 sec), followed by another 14 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 55°C for 2 min, and extension at 72°C for 30 sec). Yet another 17 cycles of amplification were carried out (denaturation at 94°C for 15 sec, annealing at 45°C for 15 sec, and extension at 72°C for 15 sec) along with a final 10 min elongation period at 72°C. Prior to the thermal cycling, the samples were heated up to 94°C for 10 min as required for antibody-mediated hot-start of platinum taq DNA polymerase.

Nested PCR

The protocol used for nested PCR was as previously described (Chase & Pascho 1998) with some modifications. Briefly, the reaction mixture for both the first and the second reaction had a total volume of 25 µl. The reaction mixture contained sterile water, 0.2 mM of each nucleotide, 2 mM of MgCl, 20 mM of Tris-HCl (pH 8.4), and 50 mM of KCl, 1 µM of each primers, and 0.625 U of platinum taq DNA polymerase. The template used in the reaction mixture was the same as was described in the snPCR protocol. Thermal cycling was carried out for both the first and second reactions with the initial denaturation step at 94°C for 10 min and then with 30 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min.

ELISA tests

Kidney tissue was diluted 1:3 (w v^{-1}) in Dulbecco's PBS, homogenized in Stomacher 80 micro-Biomaster (Seward) and poured into a tube, with the addition of 25 µl of Tween-20 for each ml of homogenate. The samples were heated at 100°C for 15 min, centrifuged at 2200g and 4°C for 20 min and the supernatant collected. Ovarian fluid was diluted 1:1 (v v^{-1}) in Dulbecco's PBS adding 25 µl of Tween-20 for each ml of solution. The solution was heated at 100°C for 15 min, centrifuged at 2200g at 4°C for 15 min and the supernatant collected for the ELISA.

The polyclonal ELISA (pELISA) used is a double sandwich test (Gudmundsdottir et al. 1993). The cut-off value for determination of positive samples was 2.3 times the average $OD_{492\ nm}$ value of three negative control samples.

The monoclonal double sandwich ELISA (mELISA) was performed according to the manufacturer's instructions (GrupoBios, Chile). Based on these instructions, the cut-off value using the negative control samples was determined as 0.260 at OD_{450nm}.

Statistical analysis

Chi-square, testing for homogeneity or multinominal distribution, was used for statistical analysis of the differences recorded for different diagnostic methods in the infected sample group. $P<0.05$ was the critical value of significance.

RESULTS

One-tube semi-nested PCR

The products of the snPCR, bands of sizes 271 and 196 base pairs, are shown in Figure 1. The lower band was sequenced, confirming that the amplified product belongs to the *msa* gene of *R. salmoninarum* (NCBI Reference Sequence: NC_010168.1). The detection limit was estimated to be 5 *R. salmoninarum* CFU's per reaction. Samples from ten bacterial species tested negative in snPCR and *R. salmoninarum* NCIMB-1111 and NCIMB-1113 yielded bands of correct sizes.

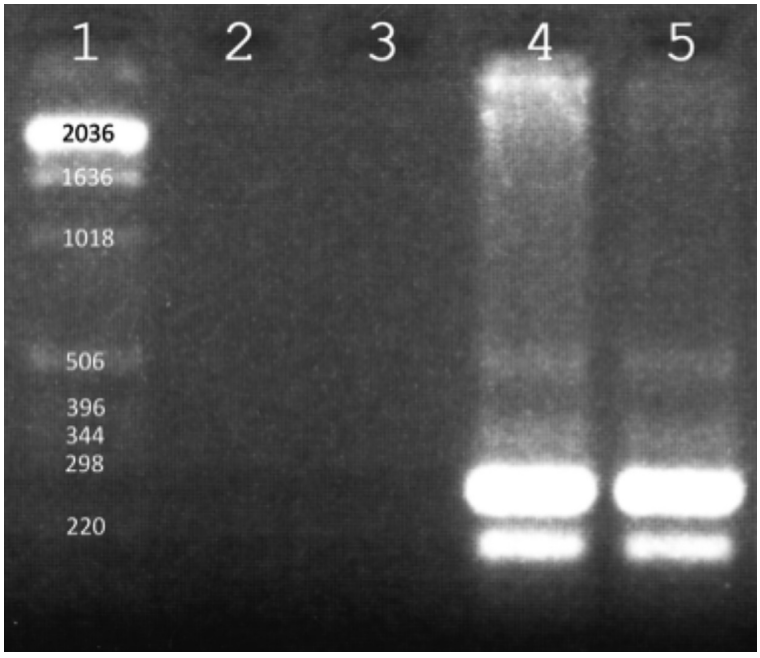

Figure 1. Gel electrophoresis of snPCR showing two products of snPCR, 271 bp and 196 bp in size. Lane 1: Ladder (1Kb DNA standard, Invitrogen). Lanes 2 & 3: Negative control sample. Lanes 4 & 5: DNA from *R. salmoninarum* cells.

Field samples

All results for the infected group are presented in Figure 2. A total of 65% of the kidney samples were positive in pELISA, the highest ratio for all methods run and statistically significant in comparison to all other tests ($p<0.05$). The mELISA detected the lowest number of positive samples (17.5%) and this was also statistically significant in comparison to all other methods applied ($p<0.05$). The average OD value in pELISA was 1.140 compared to 0.327 in mELISA. All samples positive in mELISA were also positive in pELISA. The results further showed that when the OD value for pELISA increased, more and more detection methods were in agreement. When the OD value for pELISA reached approximately 0.75, one or more PCR methods showed positive results for all samples except for one.

Of the 40 kidney samples tested using DNA kit for isolation, 38 samples were in agreement by the two PCR methods. Twelve samples (30%) tested positive in each PCR method, and of these 11 were in agreement. When FTA was used for DNA isolation, 37 samples of the 40 were in agreement. Seventeen samples (42.5%) were positive in snPCR and 18 samples (45%) positive in nPCR, of which 16 were in agreement. The difference between the isolation methods was significant when the samples were run in nPCR ($p<0.05$) and close to significance for snPCR ($0.05<p<0.1$).

Four ovarian fluid samples of 40, tested positive with either snPCR or nPCR, of which three were positive in both tests and pELISA identified one positive ovarian fluid sample (data not shown). One gill sample tested positive in snPCR and two in nPCR (data not shown). All kidney samples from the control group were negative in pELISA, snPCR and nPCR.

DISCUSSION

In this study, two DNA isolation techniques,

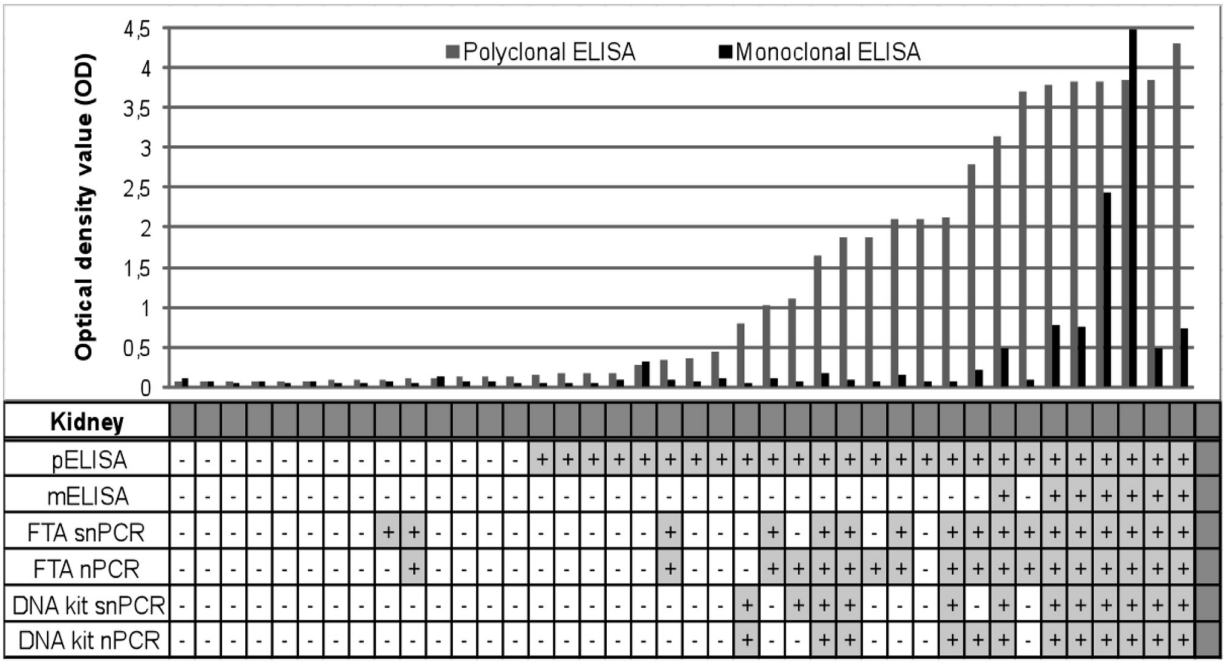

Figure 2. The columns on the chart represent OD values in kidney tissue samples for pELISA (grey columns) and mELISA (black columns). Every set of columns represents an individual fish. Samples are aligned on the chart from the left to the right with increasing OD values in pELISA. The results for the PCR methods tested on the kidney tissue for each fish are shown below the chart. Positive samples are marked with "+" and negative with "–". All the samples were tested in duplicate in the PCR's. If one of the reactions was positive and the other negative the sample was retested to obtain conclusive results.

two PCR methods and two ELISA methods were applied to three tissues of Atlantic salmon broodfish for detection of *R. salmoninarum.* The snPCR was developed in this study as an alternative to the OIE recommended nPCR. The nPCR has some advantages over traditional PCR but has some disadvantages as well. The use of two sets of primers for two-step amplification increases the specificity and sensitivity of the PCR method but it also increases the contamination risk due to the opening of tubes between reactions. Further, it doubles the time and cost needed to perform the reaction for each sample compared to traditional PCR. The snPCR is simpler than the nPCR as three primers are used to make two products in a single run, which reduces the risk of contamination and saves time as both reactions take place in a single tube. Only half of all reagents and plastic materials that are needed for nPCR are used in snPCR. The specificity of the developed snPCR was confirmed by running 10 bacterial species other than *R. salmoninarum*, all testing negative. The detection limit of snPCR is similar to that reported for nPCR (Chase & Pascho 1998). Additionally, a similar number of positive samples was identified in the current study, using either method.

Using FTA for DNA isolation has several advantages when compared to the commonly used DNA kit. It is easier to use and a significantly higher number of positive samples was detected using FTA rather than conventional method for DNA isolation. According to the manufacturer, samples on FTA can be stored at room temperature for years without affecting the quality of the DNA.

In a few samples, PCR detected bacterial

DNA where ELISA was negative. In those samples the bacterium itself seems to have reached the tissue tested but the levels of the antigens are below the sensitivity of the ELISA tests. Furthermore, it can be speculated that in such cases the bacterium could have been inactive or even non-viable and therefore antigens were not produced and secreted. Similar results have been reported by others (Pascho *et al*. 1998, Faisal & Eissa 2009).

The pELISA detected a significantly higher number of positive samples than all other methods tested, while the mELISA detected a significantly lower number. The results for mELISA obtained in the present study are in harmony with results in a recent paper (Bruno et al. 2007) where mELISA showed less sensitivity than the qPCR applied. Positive ELISA in individuals with negative PCR can possibly arise when MSA antigen secreted by bacterial foci elsewhere in the body has reached and accumulated in the kidney ahead of the bacterium. Another possibility is that inhibitory components in kidney tissue repress the PCR reaction, as reported in some studies (Magnusson et al. 1994). Finally, the time it takes for the fish immune system to get rid of deposited bacterial antigens must be taken into consideration. A preliminary study was reported where positive ELISA results were obtained for several weeks in fish injected with ECP of *R. salmoninarum* (Arnason 2010). A further study on this aspect is currently in progress.

Using ovarian fluid and gill tissue for the detection of *R. salmoninarum* would be ideal if results for these samples were as informative as the results from kidney samples. Here, the use of ovarian fluid and gill tissue for the detection of *R. salmoninarum* gave poor results which are therefore not feasible for detection of the bacterium in samples from Atlantic salmon, not even those taken during an escalating outbreak.

When methodological studies for detection of pathogens are compared, many factors have to be considered. The material selected for testing is very important. Samples from natural infections should ensure a more variable material compared with samples collected after an experimental i.p. challenge, as all individuals are exposed at the same time. Furthermore, an i.p. infection bypasses important immunological mechanisms and it can be speculated that it may differ from a natural route of infection and hence provide less informative test results. Field samples may represent different stages of disease development (Bruno et al. 2007), being caught in the wild (Sandell & Jacobson 2011) or returning for spawning (Gudmundsdottir et al. 2000, Faisal & Eissa 2009). Individual fish sampled at the time of escalating infection in a group, as was the case in the present study, can also represent different stages of infection, thus yielding highly variable test results. Finally, it is very important to take into consideration the differences between salmonid species in their susceptibility to *R. salmoninarum* and to consider that there are differences between experiments pertaining to environmental parameters and husbandry practices. All the factors mentioned above underline the fact that different results reported for the same methods will to some extent reflect differences in modes of infection, experimental set-up and biological differences between the salmonid species studied.

CONCLUSION

In conclusion, the results from the snPCR method developed in the current study equaled results obtained by the nPCR method in sensitivity. The snPCR is less expensive and less time consuming than nPCR and reduces the risk of contamination. Samples prepared for snPCR and nPCR on an FTA minicard increased the number of positive samples in comparison to isolations done with a DNA kit. Ovarian fluid and gill tissue cannot replace kidney tissue as the organ of choice when screening for *R. salmoninarum* in Atlantic salmon. Kidney samples tested in pELISA will continue to be the main screening method for *R. salmoninarum* in Iceland and the novel snPCR, using the FTA card isolation method,

will be the first choice for a confirmation test in the nearest future.

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