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A Simplified PCR-Based Method for the Detection of *Renibacterium salmoninarum* Utilizing Preparations of Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) Lymphocytes

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A method for the detection of *Renibacterium salmoninarum* by PCR is described. A rapid, reliable procedure was developed for the extraction of DNA, which could be applied to infected kidney homogenates and head kidney lymphocyte preparations. The target for DNA amplification was a 376-bp region of the gene encoding the 57-kDa major surface antigen (MSA). The PCR was specific for *R. salmoninarum* and allowed the detection of 10 to 100 cells of the pathogen. Use of the PCR for the examination of experimentally infected rainbow trout showed it to be as reliable as plate culture methods for the detection of *R. salmoninarum* in infected kidneys.

Bacterial kidney disease (BKD), which is caused by the gram-positive diplococcus *Renibacterium salmoninarum*, is one of the most important infectious diseases of salmonids (12). The presence of chronic and asymptomatic infections, as well as the vertical transmission of the pathogen within eggs, is believed to be responsible for the persistence of the disease in cultured fish stocks (9). The organism is very difficult to culture, and, at present, no effective vaccine or chemotherapy regime exists for controlling the disease (11). Historically, the diagnosis of BKD has been made by a combination of culturing and serological methods, including the direct and indirect fluorescent-antibody techniques (FAT and iFAT) (1), the enzyme-linked immunosorbent assay (ELISA) (22), and Western blotting (immunoblotting) (13). However, it is generally accepted that these systems lack sufficient sensitivity to allow the detection of low levels of the pathogen, such as may be encountered in asymptomatically infected animals (10, 11). Moreover, the interpretation of results obtained by ELISA, FAT, and iFAT may be affected by cross-reactions with proteins from both fish tissues and other bacteria (2, 5, 8, 21).

Recently, the use of PCR has been advocated as a specific and sensitive means of diagnosing BKD. The PCR-based method involves the amplification of gene sequences unique to *R. salmoninarum* (6, 16, 17). The authors reported that the detection level of the PCR was at least 2 orders of magnitude greater than could be achieved by the most sensitive ELISA.

The purpose of this study was to develop a highly specific PCR-based detection system for *R. salmoninarum* and to assess the ability of this system to detect low levels of the pathogen in kidney tissue from experimentally infected rainbow trout (*Oncorhynchus mykiss*, Walbaum). Emphasis was placed on the simplification of the DNA extraction process and the use of lymphocytes to reduce the effects of host tissue components on the efficiency of the amplification reaction.

MATERIALS AND METHODS

Bacteria. Six strains of *R. salmoninarum*, isolated from a range of salmonid species and geographical locations (Table 1), were used. The isolates were maintained on plates of SKDM agar (3) at 15°C and subcultured every 6 weeks. Cultures of *Aeromonas hydrophila* ATCC 14715, *A. salmonicida* NCIMB 1102, *Carnobacterium piscicola* ATCC 35586, *Corynebacterium aquaticum* ATCC 14665, *Vibrio anguillarum* NCIMB 829, and *Yersinia ruckeri* (Norwegian isolate of serotype I; provided by P. D. Smith, Aquaculture Vaccines Limited, Saffron Walden, England) were grown on plates of brain heart infusion agar (Oxoid, Basingstoke, England) at 22°C. Cultures of *Arthrobacter globiformis* NCIMB 8907, *Micrococcus luteus* (NCIMB 9278), and *Rothia dentocariosa* (ATCC 17931) were cultured on brain heart infusion agar with incubation at 37°C.

Fish infectivity experiments and sample processing. Rainbow trout (average weight, 40 g), in groups of 25, were maintained in 50-liter-capacity covered polypropylene tanks supplied with aerated through-flowing, dechlorinated freshwater at an average temperature of 11°C. Each animal was given an intraperitoneal injection of 10^6 cells of *R. salmoninarum* FT10. Twenty-five fish were inoculated with 0.1 ml of sterile (110°C for 10 min) phosphate-buffered saline (PBS; pH 7.2), as controls. Five fish from each group were removed at 4, 7, 14, 21, and 28 days after challenge and were killed by exposure to an overdose of anesthetic (MS222; Sigma, Poole, England). The entire head kidney of each fish was removed aseptically, and each kidney was placed in a separate 5-ml volume of ice-cold holding medium (RPMI 1640; Gibco, Paisley, Scotland) supplemented with 0.1% fetal bovine serum (Gibco). Each tissue sample was drained of excess medium, weighed, and divided into two approximately equal sections before being transferred to fresh 5-ml volumes of ice-cold holding medium. Half of each kidney was used to produce a tissue homogenate (10%, wt/vol) in PBS. Homogenates were prepared rapidly in microcentrifuge tubes with sterile (121°C for 15 min) micropestles (Eppendorf; Greiner, Stunehouse, England) and then returned to the ice for 10 min to allow the settlement of any undisrupted tissue fragments. Then 200 μl of each homogenate was transferred to a fresh microcentrifuge tube. All manipulations were performed with filter pipette tips (Northumbria Biologicals Ltd., Cramlington, England) to reduce the risk of cross-contamination between samples. Duplicate volumes (20 μl) of each homogenate were used to prepare serial 10-fold dilutions to 10^{-7} in PBS, and two 20-μl volumes of each dilution served as inocula for plates of SKDM by a drop plate method (13). Plates were sealed with laboratory film and incubated at 15°C for up to 12 weeks, with weekly examination for evidence of colony formation. Representatives of any colonies suspected of being *R. salmoninarum* were Gram stained and subcultured onto quarter plates of SKDM or tryptic soy agar (TSA, a medium that does not support the growth of *R. salmoninarum*). The remaining 160 μl of homogenate was frozen at −20°C before being processed for use in the PCR.

Preparation of lymphocyte lysates. The second half of each head kidney was used to prepare a crude lymphocyte suspension. The tissue was macerated crudely with a sterile scalpel in 2 ml of ice-cold holding medium in a petri dish, fully dissociated by 10 passages through a 2-ml disposable syringe, and transferred to a 15-ml centrifuge tube containing 5 ml of ice-cold holding medium. The tubes were held on ice for 10 min to allow larger tissue fragments to settle, 6 ml of cell suspension was removed with sterile disposable 10-ml pipettes into a fresh 15-ml centrifuge tube, and the cells were sedimented by centrifugation at 400 × *g* for 10 min at 4°C. The supernatant was removed, and cells were resuspended in 2 ml of fresh holding medium and then centrifuged as described above. Thereafter, the holding medium was removed by aspiration with a Pasteur pipette, and cells were lysed by the addition of 200 μl of ice-cold, tissue culture quality distilled water (Gibco), with vigorous vortexing for 2 min. The lysates were transferred to sterile microcentrifuge tubes and held on ice for 10 min to allow the sedimentation of particulate material. Finally, 150 μl of each lysate was transferred to fresh tubes and processed for bacteriologic testing in an identical

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TABLE 1. *R. salmoninarum* strains used in this study

Strain	Host	Country of origin
ATCC 33209	*Oncorhynchus tshawytscha*	United States
NCIMB 1112	*Salvelinus fontinalis*	Scotland
FT10	*Salmo salar*	Scotland
K57	*Salmo salar*	Scotland
K70	*Salmo salar*	Scotland
K84	*Oncorhynchus mykiss*	England

manner to the tissue homogenates, with the remaining lysate being stored at −20°C.

DNA extraction. The extraction of DNA was based on a method suggested by S. Griffiths (Research and Productivity Council, Fredericton, New Brunswick, Canada). Tissue homogenates and lymphocyte lysates were thawed on ice, and 50-μl volumes were applied to individual fiberglass discs (0.6 cm in diameter) prepared from GF/C glass microfiber filters (Whatman, Maidstone, England), which were held in the wells of flat-bottomed 96-well microtiter plates (Dynatech, Billinghurst, England). The saturated filters were left to air dry at room temperature overnight and then transferred to separate microcentrifuge tubes each containing 100 ml of Instagene matrix (Bio-Rad, Hemel Hempstead, England). After being vortexed for 10 s, the tubes were placed in a water bath at 55°C for 30 min, boiled for 8 min, and centrifuged at 10,000 × *g* for 5 min to sediment the resin and the fiberglass discs. Then, 70-μl volumes of DNA-containing supernatants were transferred to fresh microcentrifuge tubes.

Primer design. Oligonucleotide primers for PCR were designed from the published sequence of the gene encoding the p57 protein (major soluble antigen) of *R. salmoninarum* (GenBank accession number S46378) (7). The forward primer, named G6481, was 5′-GCGCGGATCCAAAATAAAAAAAATTTTAGCGCTG-3′; the underlined portion corresponds to nucleotides 51 to 75 of the published sequence. The reverse primer, G6480, was 5′-GCGCGGATCCTTGGCAGGACCATCTTTGT-3′, with the underlined portion being complementary to nucleotides 410 to 389 of the published sequence. Both primers contained a *Bam*HI recognition site (5′-G/GATCC-3′) at the 5′ end to facilitate the subcloning of the PCR product (in a separate experiment). The expected PCR product is 376 bp long, with an *Eco*RI site (5′-G/AATTC-3′) starting 100 nucleotides from the 5′ end.

PCR amplification. DNA from a 5-μl sample was amplified in a 25-μl reaction volume in a 0.5-ml thin-walled polypropylene tube containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dGTP, dCTP, and dTTP, and 1 μM each primer. This mixture was overlaid with 40 μl of mineral oil before the PCR was performed (in a Techne [Duxford, England] PHC-3 thermal cycler). After an initial denaturation step of 94°C for 10 min, 0.5 U of thermophilic DNA polymerase (Goldstar; Eurogentec, Saraing, Belgium) was added and 35 cycles of 94°C for 2 min, 55°C for 2 min, and 72°C for 2 min were performed, followed by a final extension step at 72°C for 5 min. Each batch of reaction mixtures (consisting of up to 25 samples) included both negative (no template) and positive (1 ng of DNA purified from *R. salmoninarum* ATCC 33209) controls.

Detection and identification of PCR products. Volumes (10 μl) of each PCR product were subjected to electrophoresis in conjunction with DNA size standards (1-kbp ladder; Gibco) through an agarose gel (1.5%, wt/vol) which was stained with ethidium bromide and examined under UV illumination.

To confirm that the PCR product was derived from the desired region of the major soluble antigen-encoding gene, a 10-μl sample of the remaining reaction mixture was subjected to digestion with *Eco*RI (Northumbria Biologicals Ltd. Gene Sciences) as directed by the manufacturer. The digested material was then separated on an agarose gel (2%, wt/vol), and the sizes of any fragments were determined as described above.

Sensitivity and specificity of the PCR. To determine the detection limit of the PCR, 10-μl volumes of suspensions of *R. salmoninarum* ATCC 33209 and FT10 in PBS were mixed with 90 μl of kidney homogenate or lymphocyte lysate produced from BKD-free rainbow trout to give final concentrations of 50,000 to 5 viable cells per ml. Each suspension was examined in duplicate by the extraction and amplification procedures outlined above. Similarly, to evaluate the specificity of the amplification procedure, DNA was extracted from duplicate 50-μl samples of PBS suspensions, containing 10^4 cells of each of the *Renibacterium* isolates per ml (Table 1) or from suspensions of each of the other bacteria listed above (10^8 cells per ml). This material was used as a template in the PCR.

RESULTS AND DISCUSSION

In common with previous studies (6, 16, 17), the purpose of these experiments was to develop a specific and sensitive PCR-based assay for the rapid detection of *R. salmoninarum* in salmonid tissues. The specificity of the primers used in the PCR was confirmed by the finding that a product with the expected size of 376 bp was generated when DNA extracted from the six isolates of *R. salmoninarum* was used as the template. Further evidence that the PCR product contained the correct sequence was provided by analysis of an *Eco*RI digest. In all cases, digestion of the PCR product resulted in the expected fragments, one of approximately 100 bp and the other of 270 bp (results not shown). Additional PCR experiments with DNA extracted from other common fish pathogens and bacteria, which have been reported to be serologically cross-reactive with or taxonomically related to *R. salmoninarum* (3), as well as DNA recovered from both kidney homogenates and lymphocyte lysates isolated from healthy rainbow trout, did not give any detectable product (Fig. 1).

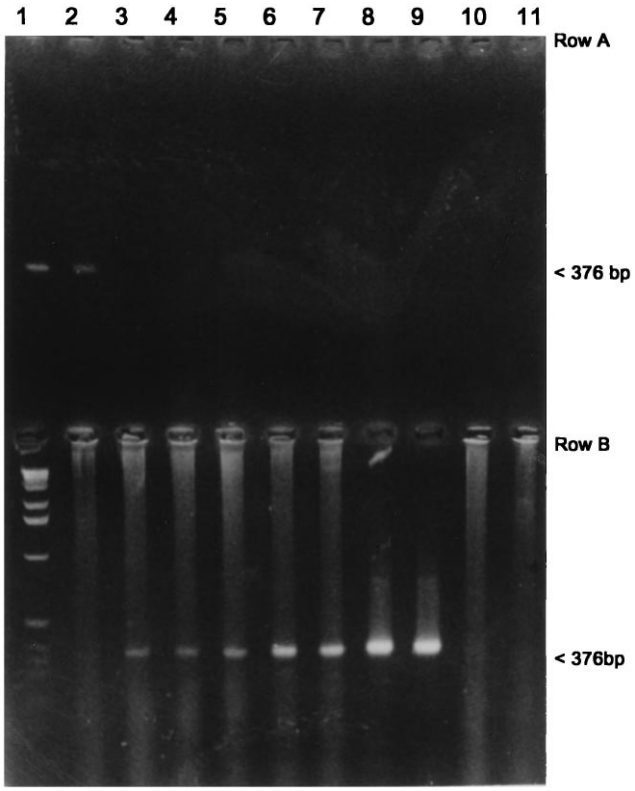

FIG. 1. Specificity and sensitivity of detection of *R. salmoninarum* by PCR. Row A: lane 1, 125 cells of *R. salmoninarum* ATCC 33209; lane 2, 12.5 cells of *R. salmoninarum* ATCC 33209; lanes 3 to 11, DNA extracted from 10^7 cells of, respectively, *A. hydrophila*, *A. salmonicida*, *Carnobacterium piscicola*, *Corynebacterium aquaticum*, *V. anguillarum*, *Y. ruckeri* (serotype I), *Arthrobacter globiformis*, *M. luteus*, and *Rothia dentocariosa*. Row B: lane 1, 1-kbp DNA ladder; lanes 2, 4, 6, and 8, kidney homogenate 4, 7, 14, and 21 days postchallenge, respectively; lanes 3, 5, 7, and 9, lymphocyte lysate 4, 7, 14, and 21 days postchallenge, respectively; lane 10, kidney homogenate from uninfected fish; lane 11, lymphocyte lysate from uninfected fish.

Initial experiments to determine the detection limit of the PCR assay (in terms of the number of viable cells per milliliter of starting material) were performed with cell suspensions in PBS of the type strain ATCC 33209 and strain FT10. Results from these experiments indicated that the assay had a detection limit of 5×10^3 cells per ml, which equates to 12.5 cells per PCR, assuming that the DNA extraction process has completely released all the bacterial DNA present in the sample. However, it was considered likely that the presence of host DNA and undefined inhibitors in tissue extracts would reduce the sensitivity of the assay. Indeed, similar inhibitory effects

TABLE 2. Progress of infection and detection of *R. salmoninarum* in tissue homogenates and lysates of head kidney lymphocytes

Time after infection (days)	CFU/ml of homogenate	CFU/ml of lysate	PCR detection in:	
			Homogenate	Lysate
4	3.5×10^2	6.0×10^2	−	+(w)[a]
7	2.5×10^4	5.5×10^4	+(w)	+
14	4.0×10^6	6.5×10^6	+	+
21	7.5×10^8	1.0×10^9	+	+
28	TNTC[b]	TNTC	+	+

[a] w, weak.
[b] TNTC, too numerous to count.

have been reported for a PCR-based system used for the detection of the fish pathogen *A. salmonicida* (19). This hypothesis proved to be correct, with 10- and 5-fold reductions in sensitivity being observed when dilutions were prepared in kidney homogenate and lymphocyte lysate, respectively. Thus, the reaction was deemed to have a detection limit between 62.5 and 125 cells depending on the level of host material present. This level of detection is still 1 order of magnitude greater than that which can be achieved by the most sensitive ELISA system reported to date (18). Seeding experiments, such as those described above, have limited value insofar as they do not test the ability of the extraction procedure to release DNA of pathogens from within a sample of infected host cells.

R. salmoninarum is well documented to be an intracellular pathogen (1, 11, 15) and is capable of surviving and multiplying within the cytoplasm of infected macrophages (15, 23). It was therefore of some value to investigate the PCR amplification of *Renibacterium* DNA from this particular cell type. To this end, infectivity experiments were designed to provide comparative data on the effects of sample preparation on detection of the pathogen at different stages of infection. The data presented in Table 2 show the progress of the infection over the 28-day experimental period, as well as the relationship between sample preparation and detection by PCR. Experiments were performed with two groups of fish, which were taken from the same stock, and comparable infection rates were observed in both groups (results not shown). In all samples examined, the use of the lymphocyte lysate resulted in an increase in the sensitivity of detection compared with kidney homogenates produced from the same sample (Table 2). This was not entirely unexpected in view of the results discussed above. It may be seen that in the early stages of infection, the pathogen was not detected by PCR when kidney homogenates were used as the starting material for DNA extraction (Fig. 1). It is clear that until the infection was firmly established at day 14, the intensity of the PCR product was always greater when lymphocyte lysates were used (Fig. 1). Interestingly, the level of detection observed in infected tissue samples surpassed the values recorded in the seeding experiments. Thus, at 4 days after challenge, only 17.5 (kidney homogenate) or 30 (lymphocyte lysate) viable cells of the pathogen were present in 50 μl of infected material used for the extraction of DNA, with only 5% of the extracted DNA being used in the PCR. This anomaly is most probably explained by the presence of a population of dead and/or damaged cells which failed to produce colonies on SKDM. This hypothesis is supported by the work of Bandin et al. (4), who demonstrated that *R. salmoninarum* is sensitive to intracellular killing by rainbow trout macrophages in vitro. The rapid progress of the infection in both groups used in these experiments is clearly not representative of the natural disease situation, and future research would benefit from the use of a more natural challenge method, such as cohabitation (20).

In the absence of data from naturally infected fish, it is impossible to determine whether the use of the extended tissue-processing procedure would be justified. However, it seems likely that any procedure that reduces the level of reaction inhibitors and host DNA present in the template, as well as targeting a known source of the pathogen, could only improve the efficiency of the assay. It is unknown how many cells of the pathogen are needed to initiate and maintain an infection; it may require only the presence of one infected macrophage. What is clear is that methods presently used for evaluating the incidence of low levels of infection are insufficient (10, 11).

One of the central objectives of this study was to reduce the need for a lengthy DNA extraction procedure, involving multiple organic extractions and alcohol precipitation steps, each of which offers the possibility of cross-contamination of samples and a reduction in template yield. A similar rationale was advocated in the processing of kidney tissue fragments for use in PCR-based detection of BKD in coho salmon (*O. kisutch*) (16). In the present study, the use of a convenient overnight drying stage involving fiberglass filters, coupled to a single-step extraction with a nontoxic, commercially available resin, resulted in a safe, rapid procedure with minimal likelihood of cross-contamination between samples.

In conclusion, the PCR assay developed here has the potential to detect low levels of the pathogen. The PCR allowed the processing of multiple samples, with a conclusive result being obtained within 48 h of receiving a sample, compared with up to 12 weeks for the detection of colonies from an asymptomatically infected animal on SKDM (14).

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