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Detection of *Yersinia ruckeri* in rainbow trout blood by use of the polymerase chain reaction

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ABSTRACT: We evaluated a polymerase chain reaction (PCR) method for detecting *Yersinia ruckeri*, the bacterial pathogen causing enteric redmouth disease (ERM), in blood of rainbow trout *Oncorhynchus mykiss*. Identification of the PCR product was confirmed by Southern blot hybridization with a ^{32}P-labeled oligonucleotide probe matching a sequence within the small subunit ribosomal RNA gene of *Y. ruckeri*. Following a 1 h immersion of rainbow trout in water with 4.5×10^6 colony-forming units of *Y. ruckeri* l^{-1}, the PCR was positive for all blood samples from 1 h (first sample) to 5 d and was negative from 9 to 30 d (last sample). Fish in this experiment did not show signs of disease, probably because they had been vaccinated against *Y. ruckeri*. To test this method with naturally infected fish, 42 rainbow trout from hatcheries were examined. Four of these fish had clinical signs of ERM and were infected with *Y. ruckeri* based on bacteriological culture. The PCR method detected *Y. ruckeri* in blood, intestine, liver, and trunk kidney from the 4 fish with ERM and from 5 additional rainbow trout that were bacteriologically negative for *Y. ruckeri*. Three of 5 rainbow trout from streams receiving effluent from hatcheries were positive for *Y. ruckeri* when tested with PCR, although there was no growth of *Y. ruckeri* on culture plates inoculated with the same samples. Samples were successfully stored for 1 wk in lysis buffer at 25°C. This study demonstrated that a nonlethal blood sample can be used with PCR to detect *Y. ruckeri*.

KEY WORDS: Bacteria · Enteric redmouth disease · Detection method

INTRODUCTION

Yersinia ruckeri causes enteric redmouth disease (ERM), which is sometimes a problem in salmonid aquaculture. This bacterial species most commonly infects salmonids but has also been isolated from several non-salmonid fishes, earthworms, birds, and mammals (Willumsen 1989, Horne & Barnes 1999), including one isolation from human bile (Farmer et al. 1985). Plate culture is commonly used to isolate *Y. ruckeri*, which is then identified by biochemical tests or immunological assays (Toranzo et al. 1987, Romalde et al. 1995).

The polymerase chain reaction (PCR) can also be used to detect *Yersinia ruckeri* in spleen, liver, kidney, and feces of infected fish (Argenton et al. 1996, Gibello et al. 1999). However, Argenton et al. (1996) failed to detect *Y. ruckeri* in blood of infected rainbow trout *Oncorhynchus mykiss*. In the present study, primers described by Gibello et al. (1999) were used to detect and identify *Y. ruckeri* in blood of rainbow trout. Our primary objective was to evaluate PCR with blood as a nonlethal method to detect *Y. ruckeri* in naturally and experimentally infected rainbow trout. In addition, we compared room-temperature lysis buffer and freezing as methods for storing samples.

MATERIALS AND METHODS

Source of bacteria. *Yersinia ruckeri* was isolated from diseased rainbow trout from Georgia, USA (Southeastern Cooperative Fish Disease Laboratory case number: GA-97-016) and stored at –70°C. Before use in experi-

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mental infections, bacteria were subcultured on trypticase soy agar (TSA) to check purity, then cultured in trypticase soy broth (TSB) for 24 h at 22°C. Before experimental trials, these bacteria were used to infect rainbow trout by immersion in bacterial suspension and then reisolated. This procedure was repeated 4 times to insure that this isolate would reliably cause ERM. Characteristics used to identify *Y. ruckeri* were negative Gram stain; negative cytochrome oxidase and urease; alkaline slant, acid butt, and lack of H_2S and gas production in triple sugar iron agar; fermentation of glucose and non-fermentation of sucrose and lactose; non-utilization of rhamnose and arabinose; and lack of indole production (Ross et al. 1966, Holt et al. 1994, Horne & Barnes 1999). The isolate used in this study hydrolyzed Tween 80.

Source of fish. Thirteen-month-old rainbow trout (Tasmanian strain) that weighed 165 ± 22 g (mean ± SD) and were 183 ± 21 mm in total length (TL) were obtained from Burton State Fish Hatchery, Rabun County, Georgia, USA. Fish were maintained in a 450 l tank supplied with aerated flow-through well water at an average temperature of 17°C. Fish were fed twice a day with commercial trout feed (Ziegler Brothers, Gardners, Pennsylvania). These fish had no history of untoward mortalities or abnormalities and had been vaccinated against *Yersinia ruckeri* (Alpharma, Fort Lee, New Jersey) when 1 mo old. Two weeks after transfer to the laboratory, 70 fish were exposed to *Y. ruckeri*; water flow was stopped and a 36 h culture of *Y. ruckeri* in TSB was added to the water to give a final concentration of 4.5×10^6 colony forming units (CFU) ml^{-1}. Bacterial CFU were counted by plating serial dilutions as described by APHA (1998). One hour after bacteria were added to the water, water flow to the tank was re-established. Sixty-five control fish from the same source were not exposed to *Y. ruckeri*.

Isolation of DNA from blood. Fish exposed to bacteria (4 fish per sample period) were bled 1 h after exposure, daily from 1 to 7 d, on alternate days through 21 d, and finally after 30 d. Four control fish were bled 1 h after exposure, daily from 1 to 7 d, and after 30 d. During the experiment, each fish was bled once and then transferred to a different tank for observation until 30 d after bacterial exposure. Blood samples (0.5 to 0.7 ml) were taken from the caudal vein with a syringe and 21-gauge needle and then dispersed into 0.6 ml lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5% sodium dodecyl sulfate [SDS], and 25 mM EDTA). Proteinase K (40 µg) was added and samples were incubated at 65°C overnight. After lysis, an equal volume of phenol was added to the sample, gently shaken for 5 min, and centrifuged at 9000 × *g* for 7 min. The aqueous phase was transferred to a sterile tube and extracted again with phenol. The extraction steps described for phenol were repeated twice with a phenol-chloroform mixture (1:1) followed by 2 extractions with chloroform. After the second chloroform extraction, DNA was precipitated at –20°C overnight by adding 0.6 ml of ice-cold isopropyl alcohol, then centrifuged at 9000 × *g* for 25 min. After the isopropyl alcohol was decanted, the DNA was centrifuged with 70% ethanol at 9000 × *g* for 25 min, dried overnight, suspended in 100 µl Tris EDTA (TE) buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8), quantified spectrophotometrically, and stored at 4°C until the PCR.

Isolation of DNA from cultured bacteria. *Yersinia ruckeri* was grown in TSB at room temperature for 24 h, transferred to 1.5 ml centrifuge tubes, and centrifuged at 9000 × *g* for 5 min. Supernatant was discarded and the pellet was dissolved in 567 µl TE buffer, 30 µl of 10% SDS, and 3 µl Proteinase K (60 µg). After 1 h of incubation at 37°C, 100 µl of 5 M NaCl was added and mixed thoroughly. Then 80 µl cetyltrimethylammonium bromide was mixed with the sample, which was incubated at 65°C for 10 min (Ausubel et al. 1987). The DNA was then purified as described above for blood samples except with a single extraction (phenol-chloroform).

Primers and PCR. A forward primer YER8 (422F) (5′-GCGAGGAGGAAGGGTTAAGTG-3′) and a reverse primer YER10 (1010R) (5′-GAAGGCACCAAGGCATCTCTG-3′) for PCR amplification of a less conserved region of the small subunit (16S) ribosomal RNA (rRNA) gene sequence of *Yersinia ruckeri* (Gibello et al. 1999) were synthesized by Life Technologies (Grand Island, New York). The predicted size of the amplified product was (422F to 1010R) 589 base pairs (bp) based on the DNA sequence (GenBank accession number X75275). The amplification was performed in a 25 µl reaction mixture (Life Technologies) containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 3 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphate mixture, 100 ng of each primer, 40 to 100 ng of DNA template, and 0.1 U *Taq* polymerase. Initial denaturation for 5 min at 94°C (hot start) was performed before the *Taq* polymerase was added. A PTC-100 thermocycler (MJ Research, Waltham, Massachusetts) performed 35 cycles of denaturation at 94°C for 1 min, primer annealing at 61°C for 1 min, and primer extension at 72°C for 1 min followed by final extension at 72°C for 8 min. The PCR products were detected and their size estimated by electrophoresis of 10 µl of each amplification mixture in 1% agarose gels in 1% Tris-borate-EDTA with known molecular weight standards (Life Technologies). Gels were stained with 0.5 mg ml^{-1} ethidium bromide.

Southern blot hybridization. DNA was transferred from electrophoresis gels to nylon membranes (Luno,

Meriden, Connecticut) in 0.4 M NaOH and was cross-linked to the membranes by exposure to UV light (Stratagene, La Jolla, California) for 15 s. Membranes were washed with 0.5% SDS at 65°C for 2 h, followed by overnight incubation at 42°C in prehybridization buffer (per 100 ml: 6× saline sodium citrate [SSC; 0.9 M NaCl, 0.09 mM sodium citrate, pH 7], 5× Denhard's reagent [20 mg type 400 Ficoll, 20 mg polyvinylpyrrolidone, and 20 mg bovine serum albumin], 0.5% SDS, 50% formamide, and 2 mg salmon sperm DNA). A probe (5'-GCACTGAACATTGACGTTACTCGC-3'), designed to hybridize to nucleotides 449–472 within the amplified region of the 16S rRNA gene (GenBank accession number X75275), was end-labeled with ^{32}P according to the manufacturer's protocol (Life Technologies). The membrane was incubated overnight in probe and hybridization solution (same as prehybridization solution but without Denhard's reagent) at 42°C. After incubation, the membrane was washed with 2× SSC for 3 min and then washed with a mixture of 0.2× SSC and 0.2% SDS for 15 min at 55°C with agitation of 45 cycles min^{-1}. Labeled fragments were visualized by autoradiography.

Comparison of storage methods. Serial dilutions of a culture of *Yersinia ruckeri* were centrifuged at 9000 × *g* for 45 s. The pelleted bacteria from 1 ml of dilution were mixed with 0.6 ml lysis buffer to prevent bacterial replication during the experiment. Visceral organs, removed aseptically from goldfish *Carassius auratus*, were minced and 0.5 g was added to the lysed bacteria. Following addition of 60 µg Proteinase K, samples were stored at 25°C for 1 wk. For comparison, the same dilutions of *Y. ruckeri* were mixed with 0.5 g aliquots of minced viscera from goldfish and stored at –20°C for 1 wk. After storage, frozen samples were mixed with lysis buffer and Proteinase K. The DNA was extracted from samples, quantified spectrophotometrically, and diluted to 100 ng DNA for each PCR reaction. Methods for PCR and visualization of amplified DNA were as described above.

Comparison of PCR and plate culture. Rainbow trout from 4 hatcheries (10 to 12 fish per hatchery) in Tennessee and South Carolina were necropsied. These fish were about 10 mo old and averaged 210 ± 22 mm TL. In addition, we necropsied 5 rainbow trout (137 ± 22 mm TL) from streams receiving effluent from 2 of the above hatcheries. Liver, posterior intestine and its contents, trunk kidney, and blood from the caudal vein were streaked on Shotts-Waltman (SW) agar (Waltman & Shotts 1984). Brain heart infusion (BHI) agar was also used to attempt isolation of bacteria from liver and trunk kidney. Each fish was examined for protozoan parasites, viruses, and other bacterial pathogens (Thoesen 1994). Bacteria that grew on BHI and cytochrome oxidase-negative bacteria that grew on SW agar were identified biochemically (Holt et al. 1994). Identification of *Citrobacter* sp. was confirmed with API 20E strips (Analytab Products, Plainview, New York). For PCR, 0.4 to 0.5 g of blood, liver, intestine, and trunk kidney were sampled from each fish. After organs were minced, samples were placed in 0.6 ml of lysis buffer with 60 µg of Proteinase K and stored at 25°C. Within 1 wk, samples were processed for PCR as described above.

RESULTS

During the 30 d following exposure to *Yersinia ruckeri*, none of the fish died and there were no signs of disease. Also, there was no indication that blood sampling adversely affected the fish.

The PCR procedure amplified a 600 bp (estimated from agarose gel) DNA fragment from blood of all rainbow trout from 1 h through 5 d following immersion exposure to *Yersinia ruckeri* (Fig. 1). After 6 d, 75% of the exposed fish were positive, 50% were positive after 7 d, and none of the fish were positive during the remaining 23 d of observation. No amplification product was obtained from control fish. Bacterial DNA extracted from a pure culture of *Y. ruckeri* was also amplified, and the PCR product co-migrated with the PCR product from samples of infected fish (Fig. 1). In a Southern blot, the PCR product hybridized with a probe matching a sequence within the 16S rRNA of *Y. ruckeri*.

After samples had been stored for 7 d at –20°C or in lysis buffer at 25°C, the PCR amplified *Yersinia ruckeri* DNA from 1:50 and 1:500 dilutions but not from 1:5000 dilutions (Fig. 2). This indicates that within the limits of 10-fold dilutions there was no greater loss of *Y. ruckeri* DNA from samples stored at 25°C in lysis buffer than in unprocessed samples stored frozen at –20°C.

Of 42 rainbow trout examined from 4 hatcheries, 4 fish from one hatchery had clinical signs typical of ERM. *Yersinia ruckeri* was isolated from the blood, liver, trunk kidney, and intestine of these 4 fish but not from any of the other fish examined. The PCR assay detected *Y. ruckeri* in blood, liver, trunk kidney, and intestine of all 4 fish with clinical signs of ERM and in 5 additional fish from the hatcheries. Three of the 5 fish that appeared healthy but were positive for *Y. ruckeri* based on PCR results were from the same hatchery as the fish with signs of ERM. The PCR method also detected *Y. ruckeri* in blood, liver, trunk kidney and intestine of 3 of 5 wild rainbow trout examined, although there was no growth of *Y. ruckeri* on SW or BHI agar. *Citrobacter* sp., usually from the intestine, was isolated from 11 hatchery fish and 2 wild fish. *Pseudomonas fluorescens* was isolated from 1 hatchery

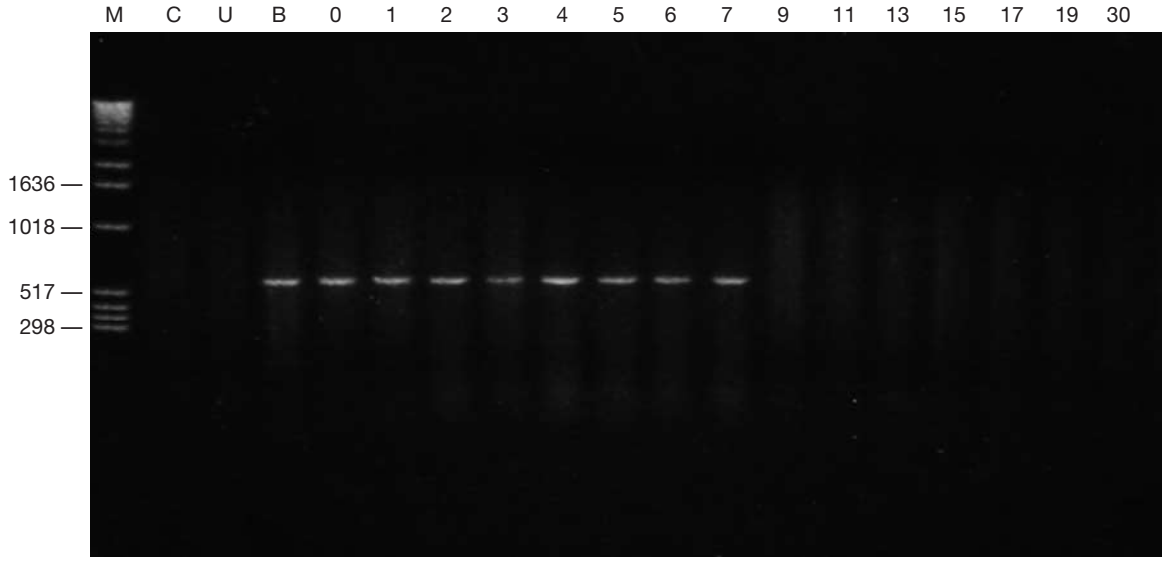

Fig. 1. Polymerase chain reaction (PCR) DNA products generated with primers specific for *Yersinia ruckeri*. Lane M, 1 kb pair DNA ladder standard; Lane C, PCR reagents (no DNA template); Lane U, DNA from a control fish; and Lane B, DNA from *Y. ruckeri*. The remaining lanes contain PCR products produced from blood of selected rainbow trout exposed to *Y. ruckeri*. The lane number corresponds to the number of days after exposure except Lane 0, which contains a sample collected 1 h after exposure

fish and 2 wild fish, and *Aeromonas* sp. was isolated from 1 hatchery fish. Only 1 of the fish infected with bacteria other than *Y. ruckeri* was PCR positive. All fish were negative for viruses, *Renibacterium salmoninarum*, and pathogenic parasites.

DISCUSSION

Detection of *Yersinia ruckeri* in fish is important for diagnosis of ERM because the clinical signs of this disease are variable and not distinctive (Horne & Barnes 1999). For this purpose, culture of organs obtained during necropsy is usually satisfactory; however, for detection of fish that are carriers of *Y. ruckeri*, but not necessarily diseased, a more rapid, nonlethal method is desirable.

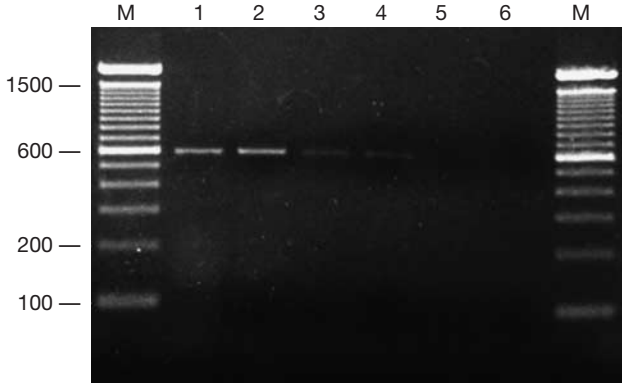

Fig. 2. Comparison of PCR products from samples stored for 1 wk at 25°C in lysis buffer (Lanes 1, 3, and 5) to unprocessed samples stored at –20°C (Lanes 2, 4, and 6). Serial dilutions of *Yersinia ruckeri* were added to minced visceral organs of goldfish. Dilutions were 1:50 (Lanes 1 and 2), 1:500 (Lanes 3 and 4), and 1:5000 (Lanes 5 and 6). Lane M is a 100 base pair DNA ladder standard

The PCR method was used to detect DNA of *Yersinia ruckeri* in blood of experimentally and naturally infected rainbow trout. Use of blood for PCR provides a nonlethal method to detect infection with this pathogen. Previously available nonlethal methods to detect *Y. ruckeri* infections are culture of feces from the posterior intestine (Busch & Lingg 1975, Rodgers 1992) and biopsy of head kidney (Noga et al. 1988). Isolation of *Y. ruckeri* from feces is often complicated by the large number and diversity of other bacteria in the sample. Although selective media (Waltman & Shotts 1984, Rodgers 1992) simplify the isolation of *Y. ruckeri* from feces, these media may be less sensitive than the media typically used for this species (Horne & Barnes 1999). The biopsy method described by Noga et al. (1988) included isolation of the bacteria on agar plates followed by biochemical identification of the isolated bacteria. Potentially, PCR of biopsy samples could reduce the time required to obtain results and increase the sensitivity.

In the present study, DNA of *Yersinia ruckeri* was detected in the blood of both clinically ill and apparently healthy rainbow trout. Although we did not determine the detection limit with PCR, this method was more sensitive than plate culture; more infected

fish were identified with the PCR method than by plate culture. Gibello et al. (1999) also found rainbow trout that tested positive for *Y. ruckeri* by PCR but were negative based on the absence of bacterial growth on TSA and MacConkey agar plates; however, *Y. ruckeri* was isolated on TSA after tissue from these fish was cultured in enrichment broth for 48 h.

A previously published PCR method did not detect *Yersinia ruckeri* in the blood of rainbow trout (Argenton et al. 1996). Similar problems detecting other pathogens in blood have been attributed to PCR inhibitors (Iralu et al. 1993, Klein et al. 1997). Although we did not systematically determine which procedural modification prevented PCR inhibition, the changes most likely resulting in success were overnight incubation at 65°C in lysis buffer with Proteinase K and an extraction method yielding high purity DNA. Future studies should consider simplified DNA extraction methods that would be more conducive to rapid processing of large numbers of samples but still overcome the problem of PCR inhibition by blood.

Fish with *Yersinia ruckeri* in the posterior intestine but not in other locations have been described based on results of bacteriological culture, and this condition is considered indicative of a carrier state (Busch & Lingg 1975). Based on bacterial growth, fish with infection limited to the intestine were not found during the present study; therefore, we were not able to determine whether this type of carrier had sufficient *Y. ruckeri* cells in the blood to be detected by PCR.

To monitor *Yersinia ruckeri* in experimentally exposed fish, we used rainbow trout that had been vaccinated against *Y. ruckeri*. Without vaccination, 95% of the rainbow trout died within 1 wk of exposure to the bacterial isolate used in this study (authors' unpubl. data). However, vaccinated rainbow trout survived and, based on PCR results, were able to clear *Y. ruckeri* from their blood. The sensitivity of the PCR method allowed detection of *Y. ruckeri* 1 h after immersion exposure.

Although all the control fish and experimentally infected fish with *Yersinia ruckeri* were vaccinated against *Y. ruckeri*, none of the exposed fish after 9 d and none of the control fish and were positive for *Y. ruckeri*. These results indicate that killed bacteria used for vaccination were not amplified.

Although the rainbow trout from one of the hatcheries had been vaccinated against *Yersinia ruckeri*, 4 of the 10 fish we examined had ERM, and an additional 3 fish were infected with *Y. ruckeri* but did not have signs of disease. The reasons why vaccination was not successful in this case are unknown.

The greater speed and sensitivity of PCR compared to bacteriological culture of *Yersinia ruckeri* were discussed by Argenton et al. (1996) and Gibello et al. (1999). The use of blood for PCR does not require necropsy, and for experimental study of pathogenesis would allow repeated sampling of individual fish. Samples for PCR can be placed in lysis buffer with Proteinase K and stored at room temperature for at least 1 wk without loss of DNA integrity compared to samples frozen for the same length of time. These advantages of PCR suggest that additional consideration should be given to this method for detecting *Y. ruckeri*.

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