Detection of *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*) fry tissues, using bacterial culture, simple PCR and nested PCR

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**Abstract**
In this study the efficacy of bacterial culture, simple PCR and nested-PCR techniques for the detection of *Yersinia ruckeri* in tissues of naturally and experimentally infected rainbow trout (*Oncorhynchus mykiss*) fry was compared. In order to detect *Y. ruckeri* in naturally infected rainbow trout tissues, 30 fish suspected of enteric redmouth disease were randomly collected from 6 rainbow trout farms with a history of exposure to *Y. ruckeri*, the causative agent of enteric redmouth disease. Also experimental challenges were performed by immersing 30 healthy fish in a bacterial suspension contained 4×10⁸ CFU/ml *Y. ruckeri*. Results, from naturally and experimentally infected tissues respectively, showed that the culture method detected *Y. ruckeri* in 33.3% and 80% cases, simple PCR detected *Y. ruckeri* in 37% and 80% cases and nested PCR detected *Y. ruckeri* in 67% and 100% cases. Nested PCR using 2 species-specific sets of primers against the 16S ribosomal DNA gave more positive results (detection limit of 7×10³ CFU/ml) and did not cross-react with other microorganisms commonly found in haemorrhagic septicemic cases in rainbow trout. Nested PCR assay could be suggested as a powerful method for early detection of *Y. ruckeri* from tissue samples of asymptomatic carrier fry fish.

**Introduction**
Enteric redmouth disease (ERM) caused by *Yersinia ruckeri*, is an infectious disease in the rainbow trout farming industry that causes economic losses worldwide. The disease most commonly affects smaller rainbow trout (*Oncorhynchus mykiss*) and is less severe but more chronic in larger fish. Disease intensity is favored by water temperatures around 15-18°C while temperatures below 10°C suppressed the infection (Austin and Austin, 2007). Even though the disease has been diagnosed since 1981 in European countries and ERM is present in most of these countries (Furones et al., 1993), ERM has been reported from Iran only recently. Soltani et al. (1999) reported a yersiniosis- like infection in farmed rainbow trout, Tehran, Iran. Infectious diseases are emerging due to imposed stress factors. Since 2002 there were several suspected ERM cases in rainbow trout from northwest and northern rainbow trout farms of the Fars province, Iran. *Y. ruckeri* was detected and identified by conventional biochemical and by molecular methods (Akhlaghi and Sharifi Yazdi, 2008). Phylogenetic studies of the genus *Yersinia* based on 16S rRNA sequencing have shown...
that this genus represents a coherent tight cluster within the family enterobacteriaceae, with Y. ruckeri forming a separate group within the Yersinia five-subline cluster (Ibrahim et al., 1993). By taking advantage of the differences found in the 16S rDNA, specific oligonucleotides were designed and used in a PCR assay for detection of Y. ruckeri in tissues of inoculated and naturally infected trout (Gibello et al., 1999).

In order to issue a veterinary health certificate when asymptomatic small fish are tested, only culture method is now available. This study was undertaken to compare the performances of bacterial culture and simple PCR methods already described in the literature. We also used one nested-PCR, currently developed in our laboratory for molecular diagnosis of Y. ruckeri in tissues of naturally and experimentally infected rainbow trout fry.

Materials and methods

Fish

a) Samples from fish farms

Thirty rainbow trout (1-2 g weight range) suspected of carrying Y. ruckeri were obtained from 6 rainbow trout farms situated in the northwest and west of Fars province, Iran. Suspected fish were euthanized, dissected and samples from kidney, liver and spleen were collected both for microbiological examination and molecular assay. Fish tissues were cultured aseptically by streaking a loop onto brain hearth infusion agar (BHI) plates and incubated at 25°C for 48h. Bacterial colonies were subcultured onto BHI, identified using conventional biochemical (Austin and Austin, 2007) and biotyping systems (Davies and Frerichs, 1989). Fifteen milligrams of the fish tissues were also taken for genomic DNA extraction.

b) Experimental infection

In another experiment, 75 healthy rainbow trout (1-2 g weight range) were purchased from a recirculating system farm with no history of ERM and divided into two groups of experimental challenge (n:45) and negative control groups (n:30). A challenge protocol was designed according to Akhlaghi & Sharifi Yazdi (2008) using a bacterial suspension contained 4×10^8 Y. ruckeri CFU/ml (Strain YF87; FJ870985) as immersion for 30 min. Fish were kept in aerated aquaria with an average water temperature of 15°C and watched daily for two weeks. Moribund fish due to the ERM were examined and kidney, liver and spleen samples were taken as described above.

Samples of kidney, liver and spleen from healthy fish (the negative control group) were also obtained aseptically for both culture onto BHI plates and PCR assays. A tissue homogenate were prepared from the fish organs after blending with appropriate volume of saline solution. The absence of Y. ruckeri in each tissue homogenate was determined according to the method used by Gibello et al. (1999).

DNA extraction from isolated bacteria

The method described by Holmes and Quigley (1981) was employed for genomic DNAs extraction and molecular confirmation from bacterial colonies with some modifications. Briefly, a medium-size bacterial colony was taken from bacterial cultures and suspended in 200 μl of sterile distilled water, incubated in a boiling water bath for 15 min,
centrifuged for 5 min at 10,000 ×g and 2 μl of the supernatant was used as template for the PCR amplification.

**DNA extraction from tissues**

DNA from tissue was extracted using a commercially available kit (Qiagen DNeasy Kit, Valencia, CA, USA) according to the manufacturer's protocol. Genomic DNA was extracted from tissue homogenate (kidney, liver and spleen) of fish.

**Simple PCR assay**

The simple PCR assay used was previously developed for definitive identification of *Y. ruckeri* by LeJeune and Rurangirwa (2000). Primer sequences were as Ruck1, 5´- CAG CGG AAA GTA GCT TG-3´ and Ruck2, 5´-TGT TCA GTG CTA TTA ACA CTT AA-3´ (Lejeune and Rurangirwa, 2000). The specificity of these primers was checked on all sequences available from the GenBank database using the BLAST program (Akhlaghi and Sharifi Yazdi, 2008). Primers were commercially synthesized by Cinnagen Company (Iran).

The following PCR conditions were applied to each assay; 50 mM KCL, 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl₂, 200 μM dNTPs, 10 pmol of each primer, and 1 U Taq DNA polymerase (Fermentas, Glen Burnie, MD, USA) per 25 μl reaction using 2 μl of DNA extracted as template. For amplification, samples were cycled in a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) with an initial denaturation at 95°C for 5 min, followed by 30 cycles, denaturation at 94°C for 45 sec, annealing at 55.5°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 7 min at the end of the amplification cycles was included. Sterile water and DNA of *Y. ruckeri* were used as the negative and positive control respectively. Each sample was tested at least in duplicate. A product of 409 base pairs (bp) was obtained from this first round PCR.

**Nested-PCR**

A nested-PCR was designed using Ruck1 and Ruck2, previously designed by Lejeune and Rurangirwa (2000) as outside forward and reverse oligonucleotide primers, and Ruck3 (5´-CGG ACG GGT GAG TAA TGT CT-3´) and Ruck4 (5´-CTT AAC CCT TCC TCC TCG CT-3´) as inside forward and reverse primers (Cinnagen Inc., Tehran, Iran). During the nested PCR reaction, the same simple PCR conditions were used with the following exceptions: 1) 2 μl of the first PCR product served as template in a 25 μl reaction; 2) primers Ruck3 and Ruck4 were used as inner primers; and 3) annealing temperature was at 55.5°C. PCR procedures were performed by using three physically separated rooms. PCR master mixtures were prepared in a laminar flow hood in an outer PCR room and recommended standard precautions against PCR contamination were taken (Orrego, 1990), with multiple negative controls included in each PCR run. The presence of PCR products was determined by electrophoresis.

**PCR test specificity**

*Y. ruckeri* (FJ870985) DNA was used to determine specificity of the primers used in the PCR procedure. In addition, DNAs from other bacterial fish pathogens including *Y. enterocolitica* O:9, *Vibrio anguillarum*, *Flavobacterium psychrophilum*, *Streptococcus iniae*, *Streptococcus agalactiae* and *Lactococcus garvieae* were used.
PCR test detection limit
To determine the lowest concentration of *Y. ruckeri* DNA that can be detected in tissue homogenates by PCR assays, the method previously described by Gibello et al. (1999) was used. Briefly, samples of the spleen, liver and kidney were aseptically obtained from healthy rainbow trout. The organs were weighed and blended with the volume of 0.9% saline solution to obtain a 1/10 dilution of each organ. The tissue homogenates were then filtered with sterile cheesecloth and stored at -22°C until processing. The absence of *Y. ruckeri* in the tissue homogenates was determined by plating 100 μl onto MacConkey agar plates, which were incubated at 22°C for 3 days. Samples of 1 ml of tissue homogenate were inoculated with *Y. ruckeri* to obtain a final concentration of 10^6 CFU/ml. Then, 0.1 ml of the appropriate 10-fold dilutions of these homogenates was plated onto MacConkey agar for the enumeration of *Y. ruckeri* in the artificially contaminated tissues. Non-inoculated tissue homogenates were used as controls (Gibello et al., 1999).

**Results**
*Y. ruckeri* (biotype 1) was isolated from 33.3% (10/30) cases of naturally and 80% (24/30) cases of experimentally infected fish respectively (Table 1a). No bacteria were detected from fish kidney, liver and spleen in the control group. In the experimental infection challenge, immersed fish in the bacterial suspension showed 70% mortality during 14 days post-infection. Experimentally infected fish swam at the water surface, reluctant to eat feed and displayed typical haemorrhages, blackening of skin, exophthalmia, wide haemorrhages within the oral cavity and on the internal organs. *Y. ruckeri* colonies were detected on BHI plates from all the dead fish kidneys. No mortality was observed in the control group.

In the direct PCR, 37% (11/30) of naturally and 80% (24/30) of experimentally infected rainbow trout DNA from tissue homogenates respectively (Table 1) produced the expected amplicons of 409 bp specific to *Y. ruckeri* (Figure 1A). PCR detected 2 additional positive cases than culture method. However, all culture positive samples were positive by PCR, except for one naturally infected sample that was negative by PCR (Table 1b).

In the nested PCR, 67% (20/30) of naturally and 100% (30/30) of experimentally infected rainbow trout DNA from tissue homogenates respectively (Table 1a) produced amplicons of 355 bp fragment of 16S rDNA sequences specific to *Y. ruckeri* (Figure 1B).

Specificity of direct and nested PCR used in this research was verified as the expected 409 bp and 355 bp bands were not observed in the negative control tubes, including no-template DNA, DNA genomic from healthy rainbow trout and DNA obtained from non-*Y. ruckeri* bacteria.

Sensitivity of the direct PCR method used in this study had a detection limit of 7×10^4 CFU/ml while this was 7×10^3 CFU/ml in tissue homogenates for the nested PCR.

**Discussion**
The fast developments occurring in fish farming causes the increase in the number of farms and therefore increases the risk of the
Table 1. Comparison between culture method, PCR and nested PCR for the detection of *Y. ruckeri* in naturally and experimentally infected rainbow trout fry.

(a)

<table>
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<th>Detection method</th>
<th>Positive</th>
<th>Negative</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>Naturally infected fish (30)</td>
<td>Culture</td>
<td>10 (33)</td>
<td>20 (67)</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
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<td>19 (63)</td>
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<tr>
<td></td>
<td>Nested PCR</td>
<td>20 (67)</td>
<td>10 (33)</td>
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<tr>
<td>Experimentally infected fish (30)</td>
<td>Culture</td>
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</tr>
<tr>
<td></td>
<td>PCR</td>
<td>24 (80)</td>
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<tr>
<td></td>
<td>Nested PCR</td>
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(b)

<table>
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<th>Simple and Nested PCR</th>
<th>Number</th>
<th>Culture method</th>
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disease (Kirkan et al. 2000). Diagnosis for a preventive strategy is important, especially when small size fish are examined for issuing a health certificate. Dissecting small fry fish is difficult for a successful sample collection. This study attempted to compare the results of nested PCR assay with those of direct PCR and culture methods for the detection of *Y. ruckeri* in naturally and experimentally infected fry rainbow trout. Our results are comparable with those of Kirkan et al. (2006) who found *Y. ruckeri* in 32% (8/25) cases by culture method and 40% (10/25) cases by PCR from naturally infected rainbow trout. Gibello et al. (1999) also found rainbow trout positive for *Y. ruckeri* by PCR but negative on TSA and MacConkey agar plates based on the absence of bacterial growth. However, they isolated *Y. ruckeri* on TSA after the fish tissue was cultured in enrichment broth for 48 h. When we used nested PCR the
detection improved drastically in the naturally and the experimentally infected rainbow trout fry (Table 1).

In the present study, all of the culture positive samples were also positive for direct PCR assay, except only one naturally infected sample, which had a direct PCR negative result. According to previous reports this false negative PCR result may be explained by the presence of PCR inhibitors that are co-extracted from fish tissues and inhibit or reduce the sensitivity of the PCR (Coleman et al., 1996; Mooney et al., 1995; Saulnier and de Kinkelin, 1997; Gibello et al., 1999). Nested PCR technique reduces the effect of PCR inhibitors by sample dilution. Therefore, nested PCR evaluated in this study resulted more sensitive than the other assays, due to the double round of PCR amplification and also by potentially eliminating PCR inhibitors.

The specificity of the primers used in both direct PCR and nested PCR methods was verified in this research. In addition, Lejeune & Rurangirwa (2000) previously reported that Ruck1 and Ruck2 primers did not amplify other genetically related Yersinia or a wide variety of aquatic bacteria.

Classical diagnosis methods in microbiology involves culture, isolation and phenotypic identification that takes several days to complete. In addition Y. ruckeri identification based on biochemical profiles must be interpreted with caution (Furones et al., 1993). PCR-based detection methods tend to be more sensitive, specific and quicker than traditional microbiological approach, since they can be performed in an 8-h day (Gibello et al., 1999).

Gibello et al. (1999) reported a detection limit of 2×10^4 CFU/ml in tissue homogenates using a

**Figure 1.** Electrophoretic analysis (1.5 % agarose gel) of DNA amplified fragments by simple PCR (A) and nested PCR (B) from different Y. ruckeri strains, isolated in this study, compared with standard strain. Lane 11 (A) and 5 (B), Marker 100 base pair; Lane 12 (A) and 8 (B), Negative control; Lane 3, 4 5 6, 9 and 10 (A) positive samples in simple PCR; Lane 1, 2, 3, 4, 6 and 7 (B), Positive samples in nested PCR; Lane 14 (A) and 9 (B), Positive controls.
PCR assay. Results of this study using PCR and nested PCR procedures have high potential as a rapid screening test for *Y. ruckeri* detection in asymptomatic carrier rainbow trout fry. Our nested PCR results (with detection limit of $7 \times 10^3$ CFU/ml) outperformed direct PCR and conventional microbiological culture. We would recommend this nested PCR assay as a sensitive method for early detection of *Y. ruckeri* in trout. However, the main disadvantages of the nested PCR approach are the requirement for two rounds of PCR, which increases time and cost of the assay. In addition, carry-over contamination may occur during the second nested PCR step and post-PCR manipulation, leading to false positive results. So, care must be taken during each of these steps and negative controls must be included for each reaction (Apfalter et al., 2002; Mothershed and Whitney, 2006).

The results of previous studies showed that PCR analysis was the most sensitive assay for detecting positive fish using tissue samples (Gibello et al., 1999; Altinok et al., 2001). However results of the nested PCR compared with other methods obtained in the present study suggesting that the conventional microbiological culture and one step PCR is not sensitive enough for an accurate detection of the organism in asymptomatic carriers and subclinically infected fish.

In conclusion, nested PCR assay could be suggested as a powerful method for early detection of from tissue samples of asymptomatic carrier fry fish.

References


