İstanbul Üniv. Vet. Fak. Derg.
32 (3), 21-30, 2006

J. Fac. Vet. Med. Istanbul Univ.
32 (3), 21-30, 2006

Araştırma Makalesi

DETECTION OF *YERSINIA RUCKERI* BY PCR IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS- WALBAUM*) HATCHERY FARMS IN THE WEST OF TURKEY

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Geliş Tarihi : 01.03.2006
Kabul Tarihi : 26.06.2006

Türkiye'nin Batısındaki Gökkuşağı Alabalık Yetiştirme Çiftliklerinde *Yersinia Ruckeri*'nin PZR Kullanılarak Tanımlanması

Özet: Bu araştırmada, Gökkuşağı Alabalığı çiftliklerindeki *Yersinia ruckeri* varlığı Polimeraz Zincir Reaksiyonu (PZR) metodu ile incelendi. Bu çalışmada Kızıl Ağız Hastalığı şüpheli 25 adet Gökkuşağı Alabalığı materyal olarak kullanıldı. 25 örneğin 8 (% 32)'inden *Yersinia ruckeri* identifiye edildi. 25 örneğin 10'unda PZR'da *Yersinia ruckeri* yönünden pozitiflik saptandı. PZR pozitif ürünlerin hepsinde, *Yersinia ruckeri* için spesifik 575 bp uzunluğunda bantlar elde edildi.

Anahtar Kelimeler: *Yersinia ruckeri*, Gökkuşağı Alabalığı, PZR, tanı.

Summary: The presence of *Yersinia ruckeri* in rainbow trout hatchery farms was investigated by PCR Assay. Twenty-five rainbow trout, suspected of Enteric Redmouth Disease, were examined in this study. *Yersinia ruckeri* strains were identified from 8 out of 25 samples (32%). Ten of 25 (40%) rainbow trout samples showed positive reactions for *Yersinia ruckeri* in the PCR. All PCR positive samples have shown specific amplification bands of the 575-bp fragment for *Yersinia ruckeri*.

Key Words: *Yersinia ruckeri*, Rainbow trout, PCR, diagnosis.

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Introduction

Yersiniosis, also known as Enteric Redmouth Disease (ERM), caused by *Yersinia ruckeri*, is one of the most common diseases, which can be peracute, acute or chronic, seen in the fish farms (**4, 23**).

Yersinia ruckeri was first isolated in Northern America in 1950s (**8**). Ewing et al (**5**) referred the causative agent as *Yersinia ruckeri* using DNA hybridisation techniques. The causative agent of the infection is spread by asymptomatic carriers to California, Nevada, Arizona, Colorado, many countries in Europe (**8**). *Yersinia ruckeri* was also isolated by several investigators in Turkish fish hatcheries (**15, 27**).

Y. ruckeri is an important pathogen in intensive aquaculture of trout and salmon. Disease outbreaks are related to stress and little information is available about the biology of the bacterium. Infected fish present characteristic red eyes and mouth as well as internal hemorrhages. Diagnostic methods include isolation of the agent, serology and molecular techniques (**10, 19, 22**). The disease is usually seen as an acute disease with high morbidity and mortality rates, which makes it advisable to have rapid and accurate methods for the specific detection of *Y. ruckeri* (**23**).

As *Y. ruckeri* is considered as an important fish pathogen, the development of sensitive and specific methods for rapid detection is needed. *Yersinia spp.* presents a high biochemical similarity and standard biochemical test systems have not been utilized (**20**). However, conventional biochemical testing, which was used as a "gold standard" for *Yersinia spp.* identification might fail to correctly identify some isolates (**14**).

Recently, 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 subline within the *Yersinia* five subline cluster (**14**). This fact suggests that by taking advantage of the differences at the level of 16S rRNA sequences, specific oligonucleotides that can be used in a PCR assay for diagnostic purposes can be designed. This approach has been used with different animal and fish pathogens (**21, 26, 28, 30**).

Amplification of specific DNA by PCR provides a highly sensitive and specific tool, as an alternative strategy, for detection and identification of microorganisms from different sources (**10, 16, 24**). Argenton et al (**1**) designed a PCR assay for diagnosis of *Y. ruckeri*. A PCR assay has also been developed for detection of *Y. ruckeri* in different tissues of infected trout based on amplification of unconserved rDNA regions (**10**).

The aim of the study was to detect *Yersinia ruckeri* in rainbow trout, suspected with ERM, by conventional method and by PCR.

Materials and Methods

Processing of rainbow trout

Twenty-five suspected rainbow trouts were obtained from two farms, where were experiencing cases of Yersiniosis. Fish were sacrificed, and tissues were removed and processed.

Identification of *Yersinia ruckeri*

Tissue (liver, kidney) homogenates (1 ml) were added to 9 ml of enrichment broth. The enrichment broth was prepared by allowing the agar of yersinia-selective agar base (Oxoid) to sediment, and the supernatant was recovered. The selective supplement, SR109, was not added to the broth. After 48 h of incubation at 22 °C, 0.1 ml of the enrichment broth was streaked onto TSA agar plates. The plates were incubated and *Y. ruckeri* colonies were identified by using API 20E system (**12**).

Reference strains

Y. ruckeri ATCC 29473 were purchased from the Pasteur Institute Collection.

DNA extraction from tissues

Tissue homogenates, 100 to 300 µl, previously filtered, were diluted to 500 µl with TES buffer and centrifuged. Bacterial chromosomal DNA was extracted by a modification of the method described by Lawson et al (**17**). The pellet was resuspended in 500 µl of TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer containing 50 mg of lysozyme (Sigma) and incubated at 37 °C for 30 min. Then, 5 µl of a 10-mg/ml solution of proteinase K (Sigma) was added, and the mixture was incubated at 65 °C in a water bath for 2 h. Subsequently, 50 µl of 20% sodium dodecyl sulfate (SDS) was added to the mixture, and it was returned immediately to the 65 °C water bath for a further 10 min. The solution was treated twice with an equal volume of phenol-chloroform (Sigma), and the aqueous fraction was recovered. The DNA was precipitated with 2 volumes of 100% ethanol, centrifuged and dried under vacuum, and finally, dissolved in 50 µl of sterile distilled water containing 2 U of RNase (Boehringer Mannheim). All DNA samples were stored at -20 °C (**10**).

Primers design

The forward primers, YER8 (5'-GCGAGGAGGAAGGGTTAAGTG-3') and the reverse primers, YER10 (5' GAAGGCACCAAGGCATCTCTG-3') were designed from unconserved regions of the *Y. ruckeri* 16S rRNA gene sequence (accession no. X75275) (**10**).

Preparation of PCR samples and PCR amplification

The PCR amplification was conducted in a total volume reaction of 50 µl containing: 50 to 70 ng of genomic bacterial DNA or 10-20 µl of DNA extracted from fish tissue, 1 mM each primer, 2 mM each deoxynucleotide triphosphate, 10 µl of *Taq* polymerase buffer (MBI Fermentas), and 1,5 U of *Taq* polymerase (MBI Fermentas). The amplification reaction was performed in a Mastercycler personal (Eppendorf AG) thermal cycler by using 25 cycles of denaturation for 1 min at 92 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C, followed by a final extension step of 72 °C for 5 min. Negative (no template DNA) and positive (*Y. ruckeri* ATCC 29473) controls were included in each batch of PCR mixtures. PCR-generated products were detected by using 1% agarose gels, with 10 ml of each amplification mixture subjected to electrophoresis (**6, 10**).

Determination of specificity of the PCR test

Y. ruckeri ATCC 29473 and all suspected DNA samples were tested to determine specificity of the primers used in the PCR procedure. In addition, a broad group of organisms, including *Aeromonas salmonicida* (clinical isolates in Turkey), *Hafnia alvei* ATCC 13337 *and Escherichia coli* ATCC 25922 were used.

Determination of sensitivity of the PCR test

To determine of sensitivity of the PCR, a suspension of *Y. ruckeri* ATCC 29473 containing $3x10^4$ CFU/ml was serially diluted twofold to 3 CFU/ml. Twenty microliters of each dilution was boiled for 10 min and added directly to the PCR mixture. The bacterial concentration, were verified by inoculating 20 µl of each dilution onto TSA plates.

Results

Yersinia ruckeri strains were isolated from 8 out of 25 samples (32%), which were including 3 liver samples, 5 kidney samples obtained from different rainbow trouts.

In the PCR, 10 rainbow trout DNA samples have shown positive reactions. All PCR positive samples produced a strong PCR amplification product. Amplification revealed a band at approximately 575 bp, in agreement with the expected size. Control experiments performed

with *Y. ruckeri* ATCC 29473 yielded the same PCR product, but no template PCR aliquot yielded any PCR product, which confirmed the specificity of the YER8 and YER10 primers. Although 17 samples from suspected rainbow trout were negative by the culture method, the numbers of negative samples obtained from PCR were 15. Some PCR amplification-product demonstrated in the Fig. 1.

All of the conventional methods samples (a total of 8 samples) were also positive for PCR assay.

Specificity of primers- The PCR test was specific for *Yersinia ruckeri* strains. The amplification of all *Y. ruckeri* yielded the expected 575 bp amplicon. However, none of the other genera (*Aeromonas salmonicida*, *Hafnia alvei*, *Escherichia coli)* isolates yielded a PCR product when tested with the YER8 and YER10 primers (Fig. 1).

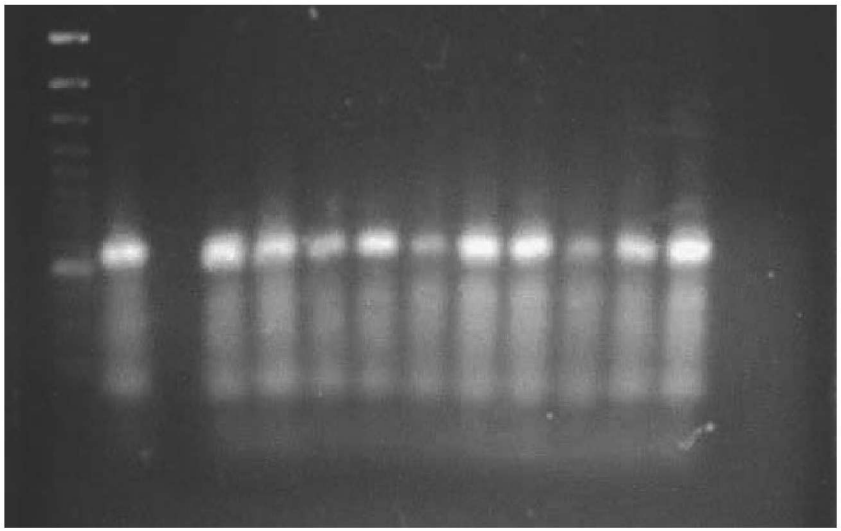

Figure 1. PCR assay for *Y. ruckeri* (specific amplification of the 575-bp fragment from the total DNA of *Y. ruckeri*). Lane 1, no template (negative control); lane 2, 1-kb plus DNA ladder (MBI Fermentas); lane 3, *Y. ruckeri* ATCC 29473 (positive control); lane 4, *Aeromonas salmonicida*; lane 5 to 14, some suspected DNA from kidney and liver homogenates from suspected infected rainbow trout; lane 15, *Hafnia alvei* ATCC 13337; and lane 16, *Escherichia coli* ATCC 25922.

Şekil 1. *Y. ruckeri* için PZR analiz sonuçları (*Y. ruckeri*'nin DNA'larından 575 baz çifti aralığının spesifik amplifikasyonu). Sütun 1, DNA yok (negative kontrol); sütun 2, 1-kb DNA işaretleyicisi (MBI Fermentas); sütun 3, *Y. ruckeri* ATCC 29473 (pozitif kontrol); sütun 4, *Aeromonas salmonicida*; sütun 5-14, infeksiyon şüpheli alabalıkların böbrek ve karaciğerlerinden hazırlanan bazı DNA homojenizatları; sütun 15, *Hafnia alvei* ATCC 13337; ve sütun 16, *Escherichia coli* ATCC 25922.

Sensitivity of the PCR test- The PCR assay had a detection limit of 30-35 cells per ml. PCR mixture (Fig. 2), assuming that the lysate procedure was completed, since no viable cells were detected after the boiling treatment. This level equals $3x10^1$ CFU/ml.

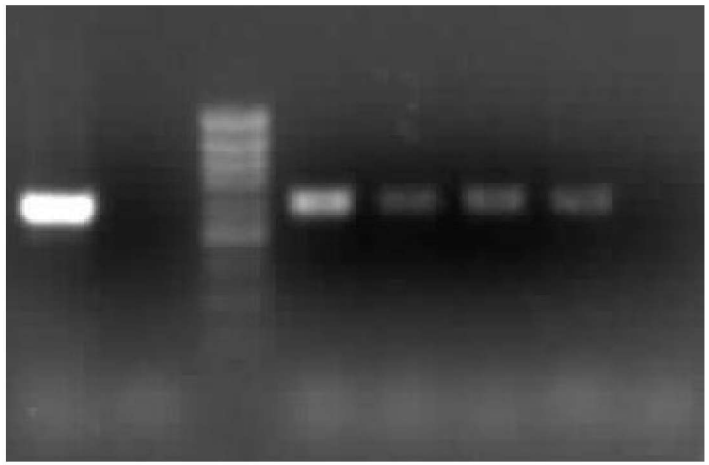

Figure 2. Sensitivity detection of *Yersinia ruckeri* by PCR. Lane 1, *Y. ruckeri* ATCC 29473 (positive control); lane 2, no template (negative control); lane 3, 1-kb plus DNA ladder (MBI Fermentas); lane 4 to 8, $3x10^4$, $3x10^3$, $3x10^2$, 30 and 3 cells of *Y. ruckeri* ATCC 29473, respectively.

Şekil 2. *Y. ruckeri*'nin PZR yoluyla duyarlılığının tespiti. Sütun 1, *Y. ruckeri* ATCC 29473 (pozitif kontrol); sütun 2, DNA yok (negative kontrol), sütun 3, 1-kb DNA işaretleyicisi (MBI Fermentas); sütun 4-8, sırasıyla $3x10^4$, $3x10^3$, $3x10^2$, 30 ve 3 tane *Y. ruckeri* ATCC 29473.

Discussion

ERM, causes high mortality (up to 60%) in fish, especially in rainbow trout grown in the farms, and high economical loses in fish industry. The fast developments occurring in fish farming causes the increase in the number of farms, and therefore increases the risk of the diseases (**2, 15**).

The fast diagnostic techniques have brought out new developments in the diagnosis of the infection. Santos et al (**25**) reported that API-20E could be used to identify *Yersinia ruckeri*. Gibello et al (**10**) stated that *Yersinia ruckeri* could be safely identified from naturally and experimentally infected rainbow trouts by using PCR. Leclercq et al (**18**) suggested that liquid-gaseous chromatography could be used to identify and to determine the pathogenicity of *Yersinia spp*.

In this study, *Yersinia rukeri* was identified from 8 rainbow trout samples by API 20E system. Seventeen samples were negative by conventional bacteriological method. However, 10 rainbow samples have shown positive reaction by PCR.

The specificity of our assay was verified by performing the PCR with *Aeromonas salmonicida, Hafnia alvei and Escherichia coli*. None of these related bacteria gave the corresponding amplification product. Therefore, the primers described here proved to be specific for *Yersinia ruckeri* under the conditions assayed. These results are similar with those obtained by Gibello et al (**10**) for *Y. ruckeri* when using PCR.

Our PCR assay could detect as few as $3x10^1$ CFU/ml of *Y. ruckeri* and could detect 30 to 35 cells/ml for *Y. ruckeri*. These results are similar to those previously reported in the literature, in which detection limits of 60 to 65 cells for *Y. ruckeri* (**10**). These results indicate that the procedure used in this study is a highly sensitive and specific method for detecting *Yesinia ruckeri* in fish farm outbreaks.

It was demonstrated by several researchers (**3, 13**) that PCR assay could also be useful for the detection of asymptomatic infected fish, which were difficult to identify by using traditional bacteriological approaches. PCR represents a widely used alternative to traditional identification methods (**29**). Although pathogenicity and virulence genes have been used as target regions, the rRNA molecule is now accepted as a very useful tool for identification purposes (**7, 9, 11**).

The detection of the asymptomatic carriers is very important in order to prevent the transmission and diffusion of ERM (**18**). The traditional microbiological approach (isolation plus identification) usually takes 2 to 3 days for the definitive identification of *Y. ruckeri*. In addition, different numerical profiles for *Y. ruckeri* can be obtained when commercial multisubstrate identification systems, particularly the API 20E system, are used; these profiles must be interpreted with caution (**8**). The entire PCR assay proposed in this work (tissue homogenization, DNA extraction, PCR amplification and detection of amplified products) is a very specific and sensitive alternative, and faster than the traditional microbiological approach, since it can be performed in an 8 h period of time.

In conclusion, *Yersinia ruckeri* was detected by using PCR and this PCR assay was found to be successful for the detection of *Y. ruckeri* in tissue homogenates. Polymerase chain reaction (PCR) offers a very powerful tool to elaborate specific, sensitive and rapid detection methods for bacterial pathogens in clinical and environmental samples. Although this study didn't cover asymptomatic fish, the results observed might suggest the ability of using PCR assay for asymptomatic fish.

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