Molecular identification of *Yersinia ruckeri* isolates by polymerase chain reaction test in rainbow trout, *Oncorhynchus mykiss*

Firooz Fadaeifard[*1], Ali Sharifzadeh[2], Mehdi Raissy[1], Hamed Mazrooi[3], Sohrab Safari[4] and Manouchehr Moumeni[4]

[1]*Department of Aquatic Animal Health and Disease, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*
[2]*Department of microbiology, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*
[3]*Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*
[4]*Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran*

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ABSTRACT

Yersinia ruckeri is a Gram-negative pathogen that causes the enteric redmouth disease, an important systemic fish infection. The aim of this study was to investigate the enteric redmouth disease or yersiniosis caused by Y. ruckeri in rainbow trout farms of Chaharmahal va Bakhtiari Province, in the western part of Iran. For this purpose, we evaluated fishes (8-12 cm in size) that showed clinical signs similar to yersiniosis from 10 farms on the subject of Y. ruckeri existence. At first, the bacteriological and biochemical tests were done, and then polymerase chain reaction (PCR) for detection of isolates *was used. Out of 50 evaluated specimens, 3 isolates were Y. ruckeri. PCR was applied to the detection of Y. ruckeri using primer pairs designed for the amplification of 16S rRNA gene fragment. The product of 573 bp was amplified from the DNA extracts of infected organs of suspected fish. DNA sequencing of the PCR products demonstrated specificity of the amplification. This is the first report of* molecular *identification of* Y. *ruckeri in this region.*

Keywords: *Yersinia ruckeri*, Enteric redmouth disease, polymerase chain reaction, Iran
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INTRODUCTION

Yersinia ruckeri is recognized as the etiological agent of enteric redmouth disease, a systemic infection of salmonid fishes, salmonids are the main fish species susceptible to Y. ruckeri[1,2]. Molecular techniques for detection of *Y.ruckeri* including restriction fragment- length polymorphism (RFLP) [3], PCR and multiplex PCR are often used. A PCR assay were developed based on the selective amplification of the 16S rRNA gene, for demonstration of Y. ruckeri in infected trout tissues[4]. Also *Y. ruckeri* was identified in the blood of rainbow trout using by PCR method [5]. Multiplex PCR assay were developed a based on the 16S rRNA genes for the simultaneous detection of three major fish pathogens, *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Y. ruckeri* [6]. During a 2-year period, bacterial fish pathogens were monitored on five rainbow trout, *Oncorhynchus mykiss* (Walbaum), freshwater farms in Denmark , the results led to identifying enteric redmouth disease and isolation of *Y. ruckeri* serotype O1 [7]. A new biogroup of *Y. ruckeri* recovered in England from diseased rainbow trout, which had been previously vaccinated with a commercial ERM vaccine. The bacterial isolates were confirmed as *Y. ruckeri* by the results of sequencing the 16S rRNA [8].

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In recent decade some studies have been done on the isolation and identification of *Y.ruckeri* in Iran. This bacteria was first recognized as a pathogen of rainbow trout in the west of Iran in the 1999 [9] , also isolation of highly virulent *Y.ruckeri*-like bacterium from diseased rainbow trout cultured in Tehran province with clinical signs of the disease was reported [10]. Study on the occurrence of *Y. ruckeri* infection from cultured rainbow trout in Fars province using conventional microbiological methods and polymerase chain reaction (PCR) assay was accomplished[11]. Salmonid production is growing in Iran in recent years. One of the important economic regions, with high production of rainbow trout, is located in Chaharmahal va Bakhtiari Province. By increasing of salmonids farming, the outbreak of infectious diseases, especially bacterial involvement, was revealed. Therefore, in the present study, we try to identify the infected fishes by PCR method in order to detect the *Y. ruckeri* bacterium.

MATERIALS AND METHODS

Sampling and isolation of bacterium
During spring and summer 2011, sampling from 10 rainbow trout (*Oncorhynchus mykiss*) farms in Chaharmahal-va-Bakhtiari Province (western part of Iran) was done. Infected fishes or suspected to diseases were randomly sampled from selected farms and then from some organs such as liver and intestine, Sterile swabs were streaked on TSA plate (Tryptic-soy-agar, Difco, Mi, USA) and plates were transferred to the lab beside the ice.

Isolation of pathogens and biochemical analysis
Plates transferred to the laboratory were incubated at 25°C for 48 h for growing the colonies. Single colonies from plates with pure culture growth were re-streaked on the blood agar media *(*Merck*, Germany)* to obtain pure isolates. In each of the grown colonies catalase, oxidase and gram staining tests were done and Gram-negative, catalase positive and oxidase negative bacilli were cultured in Waltman –Shatts medium, after 48h incubation at 25°C pure growth colonies used for PCR test. In each step of PCR testing, distilled water as negative control is used.

DNA extracting
For isolation, pure colonies were put in tubes beside 100 microliter distilled water. DNA was extracted according to kit of extracting DNA (Sinagen Co, Iran).

Primers
The oligonucleotide primers (Yer3, 5' CGAGGAGGAAGGGTTAAGT 3' and Yer4, 5'AAGGCACCAAGGCATCTCT 3') that specifically amplified 537-bp fragments were used for PCR amplification [6].

V-PCR amplification test
The amplification reactions were performed in 50 μl reaction mixtures containing 0.1 mM of each dexoynucteotide, 15 pmol of each primer, 50 mM KCl, 10 mM Tris-Hcl (pH = 9), 2 mM MgCl2,10%dimethyl sulfoxide (DMSO, Sigma), 1.5 U of Taq DNA polymerase (Sigma) and 40 ng of template DNA. The PCR reaction was carried out in a PCR programmed thermocycler (Eppendrof, Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany Co.) using the thermal profiles: initial cycle 94°C for 2 min, followed by a further 35 cycles: denaturation at 94°C for 40s; annealing at 60°C for 40s, extension by polymerase at 72°C for 40 s, and a final 5-min elongation period at 72Â°C. 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 (LifeTechnologies). Gels were stained with 0.5 mg ml–1 ethidium bromide.

RESULTS AND DISCUSSION

During this study, 100 samples of liver and intestine were investigated, after receiving the obtained samples, immediately they were cultured at Tripticase Soy Agar (TSA). Gram staining, catalase and oxidase tests were used for confirming primary isolated bacteria samples. Then 50 samples from suspected strains (Gram negative , catalase positive and oxidase negative) were isolated for molecular diagnosis and detection by PCR method, of which 3 cases were positive (Fig1). After PCR, the 573 bp fragment obtained bands, were blasted with other sequences associated with *Y. ruckeri* in the gene bank (NCBI, Gen Bank).

This study was carried out for molecular detection of *Y. ruckeri* bacterium in farmed rainbow trout cultured in western part of Iran. Results led to identification of these bacteria in the aforementioned fishes. This bacterium is the infectious agent of farmed fishes and the cause of high losses and mortality in different species of fishes. With regard to the growth of aquaculture in Iran, infectious diseases are also frequently reported in rainbow trout farms in recent years. Prevalence of Streptococcosis disease in Chaharmahal va Bakhtiari province, (the biggest producer of cold-water fish in Iran) is one of the cases. Identifying and controlling of infectious diseases, should be paid

attention to by sanitation programs. Therefore *Y. ruckeri* strains should be identified and all information about them should be collected. In the present study we succeed to detect of *Y. ruckeri* isolates, and these findings can be used in distinguishing of diseases and mortality suspected to yersiniosis in the future. We attempted to isolate and identify the native strains, and the virulence and resistance to antibiotics genes will be identified in the future.
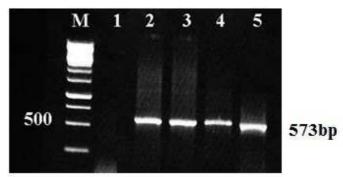

Figure1. Presentative amplification products from infected rainbow trout using PCR assay for the detection of *Yesinia ruckeri* lanes: M, 100 bp DNA Ladder, N: negative control, lane 5: positive control (*Yesinia ruckeri* No: 040811-1/3B), lanes 1-3 samples isolated from infected fishes

Y. ruckeri serovar I was identified by 16S rRNA sequencing together with serological tests. These isolates lacked motility and lipase activity and thus belonged to biotype 2, and were highly virulent for juvenile rainbow trout, both by intraperitoneal injection and bath challenge [12].Control of diseases as ERM has mainly focused on the use of antimicrobial compounds and on vaccination. Recent work, however, has concerned the application of probiotics [13]. the Yrp1 protease contributes to the virulence of the bacterium and is allegedly involved in the colonization and invasion of different tissues. Indeed, Yrp1 protease digests a wide variety of extracellular matrix and muscle proteins, and may lead to membrane alterations and pores in the capillary vessels. This may result in the leaking of blood from these vessels and hence cause the typical hemorrhages especially around the mouth and intestine [14].

Y. ruckeri strains were isolated from French fish farm .These strains were able to adhere to solid supports such as algae and sediment samples, and proved that surface colonization of fish farm tanks by *Y. ruckeri* biofilms is a potential source of recurrent infection for extended periods of time[15]. *Y. ruckeri* is readily isolated from kidney samples from clinically infected fish by using routine bacteriological media, but isolation from carrier fish is more difficult. In fact, such fish are difficult to recognize because they show no pathology and asymptomatic infection can be detected only in the lower intestine [16]. Outbreaks of yersiniosis are often associated with poor water quality, excessive stocking densities and the occurrence of environmental stressors. *Y. ruckeri* can also persist in an asymptomatic carrier state where infection through carrier fish is especially important under stress conditions. It is clear that *Y. ruckeri* is an economically important pathogen of cultured salmonids. Much more knowledge about the pathogenesis and the immune defense mechanisms of fish against *Y. ruckeri* is needed to develop more efficient approaches in the protection of fish. Severity of yersiniosis is dependent mainly on the virulence of the strain and the degree of environmental stress.

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