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RESEARCH ARTICLE

Investigation of *Yersinia ruckeri* Infection in Rainbow Trout (*Oncorhynchus mykiss* Walbaum 1792) Farms by Polymerase Chain Reaction (PCR) and Bacteriological Culture [1]

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Summary

This study has been carried out to investigate the presence of *Yersinia ruckeri*, the causative agent of Enteric Red Mouth (ERM) disease, in rainbow trout fish breeding farms located in Elazig and Malatya provinces in Eastern Turkey. For this purpose, bacteriological culture and a specific polymerase chain reaction (PCR) have been compare with examine blood and intestine samples collected from adult and young rainbow trout fish as well as water samples collected from all the farms. *Y. ruckeri* was isolated and identified from eight of the 17 fish farms giving a proportion of 52.9% at fish farm. The disease was noted in five of the rainbow trout farms located in Elazig and three of the 11 rainbow trout farms located in Malatya. The agent was detected in five of the six young fish breeding farms. The isolation percentages of the causative agent at fish level were calculated as 25.7% (131/510) in adult and 31.7% (19/60) in young fish, giving an overall proportion of 26.3% (150/570). All the isolates have been successfully identified as *Y. ruckeri* by species-specific PCR. No isolation could be made from water samples. It is therefore concluded that *Y. ruckeri* infection poses a significant threat to the fish farms in the study area and necessary precautions should urgently be taken in order to minimize economical losses due to ERM disease. A specific PCR can be utilized successfully in aquaculture for rapid identification of bacterial agents which will help to prevent spread of infectious diseases and will therefore contribute to the productivity of fishery sector.

Keywords: *Rainbow trout, Yersinia ruckeri, Culture, PCR*

Gökkuşağı Alabalığı (*Oncorhynchus mykiss* Walbaum 1792) Çiftliklerinde *Yersinia ruckeri* İnfeksiyonunun Bakteriyolojik Kültür ve Polimeraz Zincir Reaksiyonu (PCR) ile İncelenmesi

Özet

Bu çalışma, Elazığ ve Malatya illerindeki gökkuşağı alabalığı yetiştirme çiftliklerinde Enterik Kızıl Ağız hastalığına neden olan *Yersinia ruckeri* etkeninin varlığını incelemek amacıyla gerçekleştirilmiştir. Bu amaçla, çiftliklerden toplanan genç ve yetişkin gökkuşağı alabalıklarının kan ve bağırsak örnekleri ile balıkların bulunduğu havuz suyu örnekleri bakteriyolojik kültür ve spesifik polimeraz zincir reaksiyonu ile kıyaslanarak incelenmiştir. *Y. ruckeri* 17 çiftliğin sekizinden izole ve identifiye edilirken, alabalık çiftliklerinde %52.9 oranında tespit edildi. Hastalık Elazığ'daki altı çiftliğin beşinde gözlenirken, Malatya'da lokalize olan 11 çiftliğin üçünde gözlendi. Etken sadece genç balık üretimi yapan altı çiftliğin beşinde tespit edildi. Balık düzeyinde etken izolasyon oranları; yetişkin balıklarda %25.7 (131/510), genç balıklarda %31.7 (19/60) olarak bulunurken, toplamda %26.3 (150/570) oranında saptandı. İzolatların tamamı tür spesifik PCR ile *Y. ruckeri* olarak başarılı bir şekilde identifiye edilmiş, su örneklerinde ise etken izolasyonu gerçekleştirilememiştir. Sonuç olarak, çalışma bölgesindeki balık çiftliklerinde *Y. ruckeri* infeksiyonunun balık çiftliklerinde önemli bir düzeyde seyrettiği tespit edildiğinden ERM hastalığından dolayı meydana gelebilecek ekonomik kayıpları azaltmak için acil olarak gerekli önlemler alınmalıdır. Bakteriyel etkenlerin hızlı bir şekilde identifikasyonu için başarılı bir şekilde kullanılabilen PCR sayesinde, su ürünlerinde infeksiyöz hastalıkların yayılmasının önlenmesine yardımcı olunabilecek ve balıkçılık sektörünün üretkenliğine katkıda bulunulabilecektir.

Anahtar sözcükler: *Gökkuşağı Alabalığı, Yersinia ruckeri, Kültür, PCR*

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INTRODUCTION

Enteric Red Mouth (ERM) or Yersiniosis is an acute/subacute disease of fish characterized with septicaemia and high mortality. ERM disease has first been reported in rainbow trout farms in the USA in 1950's and it is now widespread throughout the world. In Turkey, the disease was first detected in 1991 and economical losses due to ERM disease have increased steadily by the increase in the rainbow trout farms all over the country. ERM has become one of the most important bacterial diseases in rainbow trout breeding [1-8]. Although rainbow trout at young ages are more susceptible to infection, all the wild and cultural salmonid fish may be affected. The economical losses attributed to ERM disease may be enormous unless early and accurate diagnosis and necessary treatment strategies are applied. The mortality can be as high as 75% in untreated farms. Approximately 60-70% of the infected fish may become carrier without showing any clinical signs of the disease [9-11].

Although clinical and pathological findings are valuable, definite diagnosis can only be made by isolation and identification of the causative agent from diseased organs. However, bacteriological culture has some drawbacks; it is laborious and requires a long time. It is therefore important to employ rapid diagnostic tools for the investigation of infectious diseases in fish farms in order to minimize economical losses. A polymerase chain reaction (PCR), which has successfully been used in human and veterinary medicine, can be a good alternative for this purpose [12-14].

The present study was conducted to investigate the presence of *Y. ruckeri* in blood and intestinal samples collected from rainbow trout (*Oncorhynchus mykıss* Walbaum 1792) farms located in Elazig and Malatya provinces by conventional culture and a specific PCR.

MATERIAL and METHODS

Sampling

Fish samples were collected from a total of 17 rainbow trout farms located in Elazig (n: 6) and Malatya (n: 11) provinces in eastern Turkey. Among these, six farms (five in Malatya and one in Elazig) were breeding young fish as well as adult fish. A number of 30 adult fish samples and 10 young fish samples randomly selected from each farm were examined by using bacteriological and molecular tools. 510 adult fish samples and 60 young fish samples were examined in total in this study. Water samples were also collected from all the farms studied here. Fish were anaesthetized with 50 ppm benzocaine, and blood samples were taken from each caudal vein using separate heparine solution (150 IU ml^{-1}) sterile injectors. Post-mortem examination of fish was carried out on sterile dissection table in plastic tubes using sterile scissors, forceps and scalpel. The samples taken from the internal organs of the fish were examined by conventional culture and PCR in the laboratories of the Department of Microbiology at the Faculty of Veterinary Medicine [15-17].

Bacteriological Isolation and Identification

Inoculations were made from intestine and blood samples of the fish onto Tryptic Soy Agar (TSA) (Difco) and Shotts-Waltman (SW) (Oxoid) culture media and the cultures were incubated at 24°C for two days. *Y. ruckeri* suspected colonies were examined by Gram Staining, and then pure cultures were prepared in nutrient broth and blood agar. Biochemical characteristics of the organisms were analyzed. For identification of *Y. ruckeri* suspected isolates; growth in Triple Sugar Iron agar, Urease, Citrate, Indole, Methyl Red, Voges Proskauer, Oxidase, Catalase, Lysine, Decarboxylase, ONPG, Arginine dihydrolase, Ornithine Decarboxylase, Tryptophan Deaminase and Lactose, Glucose, Sucrose, Maltose, Mannitol, Melibiose, Arabinose, Rhamnose, Inositol carbonhydrate fermentation tests were performed. Water samples (25 ml) were first mixed with peptone water, incubated at 24°C for 24 h and then, the above procedures for isolation and identification were followed [15,17,18].

DNA Extraction and Polymerase Chain Reaction (PCR)

For the DNA extraction from cultures; a few representative colonies from pure cultures were transferred into an Eppendorf tube containing 300 µl distilled water. The bacterial suspension was treated with 300 µl TNES buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and proteinase K (200 µg ml^{-1}), and was kept at 37°C for 2 h. Following 10 min of boiling, an equal amount of phenol: chloroform:isoamylalchol (25:24:1) was added to the suspension. The suspension was shaken vigorously by hand for 5 min and then centrifuged at 11 600 g for 10 min. The upper phase was carefully transferred into another Eppen-dorf tube and 3 M sodium acetate (0.1 volumes) and 95% ethanol (2.5 volumes) were added to the suspension, which was left at –20°C overnight to precipitate the DNA. The pellet, obtained following the centrifugation at high speed for 10 min was washed twice with 90 and 70% ethanol respectively, each step was followed by 5 min centrifugation. Finally, the pellet was dried and resuspended in 50 µl distilled water.

For the PCR analysis of *Y. ruckeri* suspected isolates, the reaction mixture was prepared in a total volume of 50 µl, consisting of 5 µl DNA, 10x PCR buffer (750 mM Tris/HCl, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 5 µl 25 mM $MgCl_2$, 250 µM each of deoxynucleoside triphosphates, 1.25 U *Taq* DNA polymerase (MBI Fermentas) and 20 pmol each of primer pair derived from 16S rRNA gene of *Y. ruckeri* (16SFI-5′GCGAGGAGGAAGGGTTAAGTG-3′ and 16SFII-5′GAAGGCA CCAAGGCATCTCTG-3′) [19] and 5 µl target DNA. The PCR analysis was performed in a thermal cycler with an initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, then, a last step of extension at 72°C for 8 min. PCR products were separated by electrophoresis in 2% (w/v) agarose gels and visualized

by ethidium bromide staining. A 100 bp DNA ladder (MBI Fermentas) molecular mass marker was used to evaluate the size of bands.

A reference strain of *Y. ruckeri* (107/8C Serotype 1 Denmark) which was obtained from culture collection of Pendik Veterinary Control and Research Institute was used in order to determine the most suitable DNA extraction procedure and to optimize PCR.

Statistical Analysis

A chi square test was used to compare differences between the isolation rates by age and location, and $P<0.05$ was considered as statistically significant.

RESULTS

When the results were accounted at farm level; it was found out that the causative agent of ERM disease, *Y. ruckeri,* was isolated and identified from nine (52.9%) of the 17 rainbow trout farms located in Elazig and Malatya provinces. When adult fish were considered, the infection was determined in eight farms. Also, the agent was found in five of the six young fish breeding farms. In one farm, isolations were obtained from young fish only. When the results were accounted at fish level; the prevalence of infection was calculated as 25.7% (131/510) in adult fish and as 31.7% (19/60) in young fish, giving an overall proportion of 26.3% (150/570). The difference between the isolation rates by age was not statistically significant ($P>0.05$).

In bacteriological examination; while small, round, white and cream color colonies of 1-2 cm diameter were observed on TSA following 1-2 days of inoculation at 24°C, light green color colonies surrounded by a cloudy zone which was due to Tween 80 hydrolysis were produced following 4-5 days of inoculation on SW. The results of morphological, physiological and biochemical analyses showed that the isolates were Gram positive, motile, and positive for catalase, nitrate, citrate, methyl red (MR), lysine decarboxylase, ornithine decarboxylase, methyl red (MR) and Ortho-Nitrophenyl-Beta-D-Galactosidase (ONPG) tests, but were negative for oxidase, urease, indole, H_2S production, arginine dehydrolase, phenylalanine and tryptophan deaminase and Voges-Proskauer (VP) tests. Acid production from glucose and mannitol were positive, while it was negative from lactose, saccarose, arabinose, inositol, rhamnose and maltose.

Five of the six farms in Elazig were determined to be infected with *Y. ruckeri*. While the agent was isolated from both blood and intestines of the fish in two farms, it was found in body cavity of young fish in one farm. The isolation percentage of the causative agent in adult fish was 49.4% (89/180). While most of the isolations were obtained only from intestines (n: 80), two isolations were made from only blood samples. In seven fish, *Y. ruckeri* was isolated from both blood and intestines. On the other hand, the agent was isolated from body cavities in four of 10 fish in one young-fish breeding farm. The overall percentage of *Y. ruckeri* isolation was calculated as 48.9% (93/190).

In Malatya province, *Y. ruckeri* was isolated and identified from three (adult fish) of the 11 rainbow trout farms. Among the five young fish breeding farms, four were determined to be infected with the agent. The isolation percentage of the causative agent in adult fish was 12.7% (42/330). While 26 of the isolates were obtained from intestines only, three were made from blood samples only. In 13 fish, *Y. ruckeri* was isolated from both blood and intestines. On the other hand, the agent was isolated from 30% (15/50) of young fish in four farms. While four of the isolates were obtained from the gills only, two were originated from body cavity samples only. In nine young fish, *Y. ruckeri* was isolated from both gills and body cavities. The overall percentage of *Y. ruckeri* isolation in Malatya was calculated as 15.0% (57/380).

No isolations could be made from water samples collected from the farms in both provinces. When the isolation percentages were compared at location level, *Y. ruckeri* infection was observed to be significantly higher in farms located in Elazig than those in Malatya ($P<0.05$).

In the PCR analysis of DNA samples extracted from the suspected isolates that were grown onto TSA and SW media, positive results with molecular size of 589 bp were obtained from all the samples (131 adult fish and 19 young fish isolates). Negative *(E. coli)* and positive controls (reference *Y. ruckeri* strain 107/8C Serotype 1 Denmark) were used in all steps of the PCR amplification in order to check the presence of possible contamination and consistently negative results

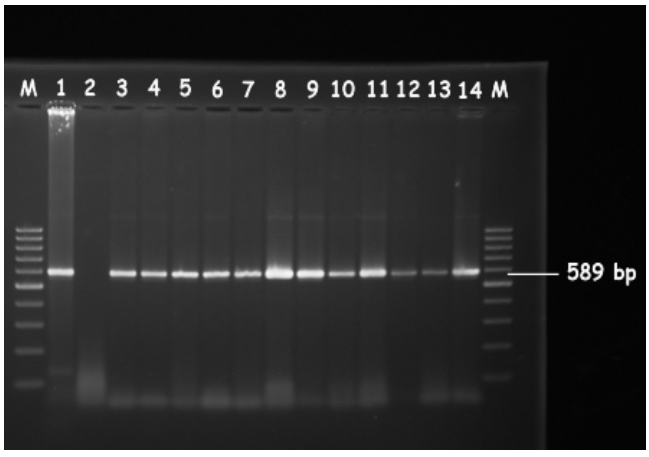

Fig 1. An 1.5% agarose gel electrophoresis of PCR products with molecular size of 589 bp of DNA samples extracted from *Y. ruckeri* suspected colonies originating from blood and intestinal contents of rainbow trout fish in farms located in Elazig and Malatya provinces. (1: positive control- reference *Y. ruckeri* strain, 2: negative control *(E. coli),* 3-9: *Y. ruckeri* suspected isolates obtained from blood and 10-13: intestinal content of fish, M: 100 bp DNA Ladder)

Şekil 1. Elazığ ve Malatya illerindeki gökkuşağı alabalığı tesislerindeki balıkların kan ve bağırsaklarından izole edilen *Y. ruckeri* şüpheli suşlardan elde edilen DNA'ların, PZR' da analizi sonucu oluşan 589 bp' lik bantları gösteren ethidium bromide ile boyanmış %1.5'luk bir agaroz jel (1: Pozitif control-referans *Y. ruckeri* suşu); 2: Negatif kontrol *(E.coli)*; 3-9: Balıkların kanından ve 10-13: Balıkların bağırsaklarından izole edilen *Y. ruckeri* şüpheli örnekler; M: 100 bp'lik moleküler marker)

obtained from *E. coli* strain suggested that no contamination took place throughout the PCR assays *(Fig. 1)*.

DISCUSSION

The aim of this study was to investigate the presence of *Y. ruckeri* infection in rainbow trout farms in eastern Turkey and the overall proportions of 52.9% and 26.3% were obtained at farm and fish levels, respectively. These percentages must be considered seriously, because none of the farms/fish sampled here showed any signs to suspect ERM disease. The results support the reports of other researchers which underline that fish might play significant role as carrier (but clinically healthy) in the transmission of natural infections to different water sources [2,11,20-22]. It is therefore important to check purchased fish and other materials for possible diseases and take necessary precautions before introducing them to the farm, in addition to other routine preventive applications which may cause to stress in fish such as dense stocking, pollution and insufficient oxygen. Otherwise, it would be impossible to talk about decreasing the risk of Yersiniosis and other diseases and eventually about economical losses.

The results of the current study showed that ERM infection was more prevalent in young fish, though the difference was not significant. Also, the number of young fish sampled here was not as big as to draw a general conclusion. However, previous studies have reported that young fish usually become infected at the beginning of spring (at the time when snow starts to melt) and show typical signs of ERM disease with the mortality rates rising up to 25-30%. On the other hand, no signs of disease have been observed in adult fish which suggests that the disease appears in early periods of life when the immune system is not effective enough and that fish recovering from this period usually become carriers and play role in the transmission to the larvae throughout eggs and sperms. This should therefore be taken into account when fighting against ERM disease.

The lack of early and accurate diagnosis is a major problem when dealing with contagious and devastating bacterial fish diseases, economical consequences of which are overwhelming. Although clinical and pathological observations are also useful, definite diagnosis of Yersiniosis is possible by isolating the agent *in vitro*. Bacterial culture is regarded as the most accurate method for diagnosis, but it has a number of drawbacks such as time consumption due to long incubation periods, labor and hazard for laboratory personnel. It is therefore crucial to develop rapid diagnostic tools for routine use and recent advances in molecular biology, particularly the use of PCR, have been promising. The suspicious isolates obtained from blood and intestinal contents of fish were identified as *Y. ruckeri* by both conventional biochemical tests and PCR in this study. As in the present study, the PCR has successfully been used by a number of researchers [22]. The routine use of PCR should therefore become common in diagnosis of fish diseases.

It is concluded that *Y. ruckeri* infection poses a significant threat to the fish farms in the study area and necessary precautions should urgently be taken in order to minimize economical losses due to ERM disease. A specific PCR can be utilized successfully in aquaculture for rapid identification of bacterial agents which will help to prevent spread of infectious diseases and will therefore contribute to the productivity of fishery sector.

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