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DETECTION OF *YERSINIA RUCKERI* BY POLYMERASE CHAIN REACTION (PCR) IN INFECTED RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM 1792)

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ABSTRACT

This study was conducted to induce experimental infection in rainbow trout with *Y. ruckeri*, the causative agent of ERM disease and progress of the disease was then monitored over the time period. Application of PCR for early and accurate diagnosis of infection was also investigated. Total 100 fish were allocated to four experimental and a control group each containing 20 fish. There were 20 aquaria and five fish were housed in each aquaria. There were 4 replicates for each group. Fish, at an average weight of 75 g, were injected intramuscularly with 0.1 ml dilutions of *Y. ruckeri* at various doses ($4x10^8$, $1x10^8$, $4x10^4$ and $1x10^4$ per ml) in the experimental groups. Blood samples were collected from fish that showed typical signs of the disease. DNA's extracted from blood samples were subjected to PCR amplification based upon a pair of *Y. ruckeri* specific primers. Following agarose gel electrophoresis of the PCR products, positive bands with the molecular size of approximately 589 bp which were considered indicative for identification as *Y. ruckeri* were observed. In addition, isolation and identification of *Y. ruckeri* from various internal organs of infected fish were made by conventional culture method. The results suggested that early diagnosis of ERM disease can be made while fish are alive and diseased fish can be isolated and treated accordingly which will help decrease economic losses.

Key words: Enteric red mouth disease; rainbow trout, polymerase chain reaction.

INTRODUCTION

Enteric red mouth (ERM), also known as Yersiniosis is caused by *Yersinia ruckeri*. The disease appeared first in 1950's in rainbow trout enterprises in USA with high mortality (Lucangeli *et al*., 2000). Later on it spread throughout the world (Lesel *et al*., 1983). In Turkey, Yersiniosis was detected in rainbow trout fish first time in 1991 and the incidence of disease increased along with the increasing number of the enterprises. The disease is still widespread in rainbow trout farms and has become one of the most important bacterial disease of the country (Kubilay and Diler, 1999).

The infection is mainly observed in young rainbow trout. In addition, all wild and cultured salmonids are susceptible to disease (Horne and Barnes, 1999). Factors causing stress in fish such as excessive stocking, pollution, oxygen depletion play a key role in the outbreak of the disease. The infection spreads from fish to fish via water. Severe epidemics are observed at the temperatures between 15 -18ºC. The disease generally appears in per acute and acute forms in young fish during spring months when sudden increases in water temperatures appear; and in chronic form in fish of one year old during autumn months when water temperatures fall (Busch, 1978). The causative agent inhabits in small intestine, spleen and liver of fish without showing any clinical signs until after 60-65 days of infection (Busch, 1982).

Intime and accurate diagnosis is the major problem in the treatment of these diseases which result in massive deaths and economic losses. Although clinical and pathological findings are important, isolation and identification of agent from lesioned tissues is required for precise diagnosis. However, culture requires a long time in order to grow bacteria *in vitro*. For this reason, development of a rapid and accurate diagnostic tool has become increasingly important. In medicine and veterinary medicine, in addition to culture and biochemical analyses, serologic tests such as agglutination, immunodiffusion, immunofluorescence, hemagglutination, radioimmun assay, enzyme-linked immunosorbent assay (ELISA) and complement fixation test (CFT), and molecular tools such as polymerase chain reaction (PCR) are being used for the diagnosis of bacterial infections (Anderson, 1974; Plumb and Bowser, 1983; Bullock, 1989; Tanrıkulu *et al*., 1996). The employment of the latter method has provided promising results for early and reliable diagnosis with high sensitivity and specificity (Saiki *et al*., 1988; Mullis, 1990).

This study was conducted to induce experimental infection in rainbow trout with *Y. ruckeri*, the causative agent of ERM disease and progress of the disease was then monitored over the time period.

Application of PCR for early and accurate diagnosis of infection was also investigated.

MATERIALS AND METHODS

Fish: Total of 100 apparently healthy rainbow trout (*Oncorhynchus mykiss* Walbaum 1792), supplied from fish breeding farm, were used in this study. The average length and weight of the fish were 15 cm and 75 g, respectively. Following the adaptation of fish in aquarium for two weeks, a total of five groups; one control group and four experimental groups were formed. Since the rainbow trout is quite active hence was maintained in aquaria in low numbers. Each aquarium contained five fish for both experimental and control groups. The study was performed in the laboratories of Faculty of Aquaculture at Firat University.

After washing and cleaning, all the aquaria (70x30x25 cm) were filled with non-chloride fresh water and ventilation was provided through an air motor. During the study period, aquarium water was changed with refreshed water at ½ ratio two times a day. During aquarium cleaning, used water was transferred in a tank, filtered from potassium permanganate and then poured to sewage system in order to prevent a possible contamination.

Reference culture was incubated in tryptic soy broth for 24 hours and was suspended in phosphate buffer saline (PBS). Different dilutions were prepared from the reference strain of *Y. ruckeri* at various doses of ($4x10^8$, $1x10^8$, $4x10^4$ and $1x10^4$ per ml) by spectrophotometric method in order to induce infection in four experimental groups (Table 1).

Inoculation: 0.1 ml of the bacterial suspension from each dilution was injected intramuscularly to 20 fish in each group using separate sterile injectors. On the other hand, fish in the control group were injected with phosphate buffer saline (PBS) solution only (Table-1).

Monitoring of Fish: Following the inoculations, general condition of the fish was monitored daily and data were recorded. The fish with clinical signs of Yersiniosis were anaesthetized in 50 ppm benzocaine, and blood samples (150 IU ml^{-1}) were collected from caudal vein by heparine injectors. Postmortem examination of fish was carried out on sterile dissection table in plastic tubs using sterile scissors, forceps and scalpel. The samples taken from the internal organs of the fish were examined by conventional culture and by PCR in the Department of Microbiology in the Faculty of Veterinary Medicine.

Isolation and Identification of *Y. ruckeri*: Inoculations were made from peritoneal fluid, stomach content, liver, kidney and blood samples of the fish and aquarium water onto Tryptic Soy Agar (TSA) (Difco) and Shotts-Waltman (SW) (Oxoid) culture media and the cultures were incubated at 24 ^oC for two days. Suspected *Y. ruckeri* 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.

DNA Extraction and Polymerase Chain Reaction (PCR): Caudal fins of all the fish were cut. 125 of µl blood was taken into EDTA tubes and was transferred to sterile eppendorf tubes. Blood samples were mixed with 250 µl lysis buffer (0,32 M sucrose, 0,01 M TRIS, 0,005 M MgCl2, % 1 Triton X-100; pH 7.5) and centrifuged at 11.600 g for 1 min, and the resulting pellet was washed three times with lysis buffer. The final pellet was re-suspended in 250 µl PCR buffer (10 mM TRIS-HCl, 50 mM KCl, 0.1% Triton X-100; pH 8.3) containing 200 µg proteinase K/ml and was incubated at 56 °C for 1 h and then at 95 °C for 10 min. Five µl was taken from this suspension and was used as target DNA in PCR.

For the PCR analysis of *Y. ruckeri* 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.* 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 (Gibello *et al*., 1999).

RESULTS

Although typical signs of disease started to appear on the 3rd day in fish that received high dose of *Y. ruckeri*, the infection remained in subclinical form as dose of the agent decreased, and thus the symptoms appeared later (Table 2).

After first appearance of disease symptoms, infection was observed in all the fish in the experimental groups within 3-8 days. Following *Y. ruckeri* injection, darkening of color, unwillingness to eat food and inactivity was observed in fish. In infected fish, while

hemorrhage appeared in injection area, pectoral, pelvic, anal, caudal fins, eyes and anus, signs of Yersiniosis, (red mouth), were observed only in lower jaw, upper jaw and tongue. In postmortem examination of the experimentally infected fish, common hemorrhages in internal organs, petechial hemorrhages in liver, intestinal tissue and lateral body muscles, darkening in color of kidney and yellowish mucoid liquid accumulation in the intestines were observed.

Inoculations from blood and internal organs of fish in experimental groups onto TSA and SW culture media resulted in growth of *Y. ruckeri* which produced small, round, white and cream color colonies of 1-2 cm diameter on TSA following 1-2 days of inoculation at 24 ^{o}C and light green color colonies 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 dihydrolase, phenylalanine and tryptophan deaminase and Voges-Proskauer (VP) tests. Acid production from glucose and mannitol were positive, while it was negative in lactose, saccarose, arabinose, inositol, rhamnose and maltose.

The causative agent of Yersiniosis was isolated and identified from the internal organs and blood of all the fish. The isolation was also achieved from aquarium water samples.

The PCR amplification products which were obtained from DNAs extracted from blood samples of fish in experimental groups with disease signs, were assessed in agarose gel electrophoresis and specific bands with the molecular size of approximately 589 bp indicative of *Y. ruckeri* presence were observed (Figure 1).

DISCUSSION AND CONCLUSION

External and internal examination of rainbow trout, experimentally infected with injection different doses of *Y. ruckeri*, revealed that all the fish presented typical signs of ERM disease. Bragg and Henton, (1986); Arda *et al*., (2002) have reported the similar observations, such as general hemorrhagic septicemia, subcutanaeous hemorrhages in mouth cavity, around the chin, fin bases and anus, common petechial hemorrhages in internal organs such as spleen, liver and kidney and yellowish fluid in stomach and intestine. With the increase in dosage there was proportionate decrease in time required for the appearance of disease signs. In treatment group I, infected with high dose of bacteria, disease signs appeared on the 3rd day in almost half of the fish in Group IV which were infected with low dose of bacteria displayed disease symptoms on the 8th day. Austin and Austin, 1993) reported that first deaths appeared on the 6th day after infection, reached to maximum level on the 9th day and ended the 15th day in experimental studies. The disease usually occurs in epidemic form at the water temperatures of 15–18°C (Busch, 1978). High mortality can be observed within 3-5 days under the stress factors if the chronic infection is present in the population. The incubation period lasts for 5-7 days at 15°C water temperature if the disease occurs for the first time in the population (Busch, 1982; Bullock and Cipriano, 1990). In the current study typical signs of Yersiniosis appeared on the 3rd day in experimentally infected fish, disease was observed in all the fish at the end of 8th day. The fact that infection occurred early may be due to high amount of the agent, excessive stress factors under aquarium conditions and optimal water temperature of 16 $^{\circ}$C which is needed for disease occurrence. In future research work, the use of larger controlled environments should be considered in order to provide clean water with abundant oxygen for the fish which will provide more reliable data.

Infection is usually transmitted through the discharges of diseased or infected but clinically healthy fish (Busch, 1982; Bullock and Cipriano, 1990). In this study, it was observed that healthy fish which were placed in the contaminated aquarium water at the temperature of 14-16^{o}C showed clinical signs of the disease within 11-15 days. It is therefore important to obtain background information about infectious diseases before introducing fish to the farm and to disinfect all the materials and equipments brought to the facilities in addition to taking control measures against stress factors such as oxygen deficiency, pollution and stocking. These precautions may decrease the risk of disease occurrence and can therefore minimize economic losses due to Yersiniosis.

Y. ruckeri identification was carried out by conventional bacteriological methods and PCR assay from blood samples collected from fish with clinical signs of disease. In diagnosing fish diseases, although clinical and pathological findings are important, definitive diagnosis is possible with *in vitro* isolation of the causative agent from infected tissues. *Y. ruckeri* can be isolated after cultivation in culture media such as TSA, BHIA, ROD and SW, by incubating for 48 hours at 22-25^{o}C. The comparison of SW with the other culture media suggested that there were no significant differences between them and SW medium can therefore be used for epidemiological studies owing to its advantages such as high sensitivity and shorter time requirement. (Austin and Austin, 1993). Characteristic colonies in blue-green color were obtained following the inoculations onto SW culture media after experimental infection. However, specific colonies were only distinctive in 96-120 hours, not in 24-48 hours as mentioned by the above researchers. For this reason,

evaluations of ERM suspected cases should be made after checking growths on SW selective medium for one week.

The delay in diagnosis of disease increases economic losses due to deaths and causes pessimism in fish producers which affect development of the sector negatively. Lack of appetite appears as the disease progresses which makes oral treatment with chemotherapeutics impossible In this study, it was possible to diagnose Yersiniosis infection within 12 hours by PCR examination of a small amount of blood sample taken directly from heart of live fish. Thus, early diagnosis can be made while fish are alive and diseased fish can be isolated and treated accordingly which will help decrease economic losses. The use of PCR assay in diagnosis of fish diseases should therefore become widespread because it is quick, sensitive and specific.

Table 1. Allocation of experimental groups and the doses of *Y. ruckeri* strain administered to each group.

Group	Number of Fish	Inoculate	Dose	Mode of Application
Control	20	aawPBS	___	i.m.
I	20	*Y. ruckeri*	$4x10^8$	i.m.
II	20	*Y. ruckeri*	$1x10^8$	i.m.
III	20	*Y. ruckeri*	$4x10^4$	i.m.
IV	20	*Y. ruckeri*	$1x10^4$	i.m.

Table 2. Distribution of rainbow trout fish which showed typical signs of Yersiniosis after injection of a reference *Y. ruckeri* strain on daily basis

Days	Experimental groups				
	I	**II**	**III**	**IV**	**Control**
1	—	—	—	-	—
2	—	—	—	—	—
3	8	—	—	—	—
4	2	3	—	—	—
5	3	5	1	—	—
6	4	4	2	—	—
7	3	7	2	—	—
8	—	1	6	2	—
9	—	—	4	5	—
10	—	—	5	9	—
11	—	—	—	1	—
12	—	—	—	3	—

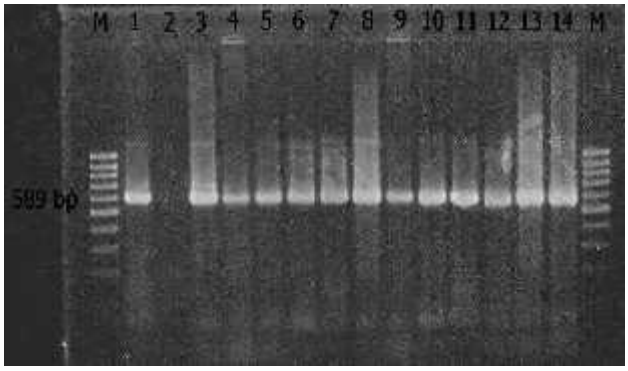

Figure 1. Agarose gel electrophoresis of *Y. ruckeri* specific PCR amplification products of DNAs obtained from blood samples taken from experimentally infected rainbow trout fish [1: Positive control (reference *Y. ruckeri* strain); 2: Negative control (*E. coli*); 3-14: DNAs of blood samples taken from rainbow trout infected with *Y. ruckeri*; M : 100 bp molecular marker].

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