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Determination of Their Antibiotic Susceptibility and Diagnosis of Using Convectional Culture and Molecular Methods Yersinia Ruckeri in the Rainbow Trout Farms in Kahramanmaraş, Turkey

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***Abstract*— This study is aimed at the isolation and identification of *Yersinia ruckeri* using bacteriological culture method as well as the amplification of DNA using polymerase chain reaction. (PCR) in the samples taken from the fish in the rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) farms in Kahramanmaraş (Turkey). For that purpose, samples were taken between April 2013 and September 2014 from the livers, spleens, kidneys, and intestines of rainbow trouts in 16 different trout farms. Brain Heart Infusion Agar (BHIA) and Tryptic Soy Agar (TSA) were used for isolating the *Yersinia ruckeri*. Pure strains obtained from fish samples were subjected to biochemical identification tests and Biolog System (The biolog GENIII micro plate) in order to be analyzed for their phenotypic characteristics. Specific primers were used in the confirmation via PCR technique of the 14 pieces of *Yersinia ruckeri* strains which were identified according to their phenotypic and biochemical properties. Consequently, all of these strains were confirmed to be molecular *Yersinia ruckeri*. Also, an antibiogram test was performed to identify their antibiotic susceptibility.**

***Keywords*— Rainbow trout, Yersiniozis, Yersinia ruckeri, BIOLOG GEN III, PCR, Antibiotic susceptibility testing.**

I. INTRODUCTION

One of the most critical problems faced by the continually growing and developing world is that of having a balanced, healthy and sufficient diet in proportion to the increasing population.

There is a growing deficit of animal protein in the world in parallel with the population growth. Fisheries, as a member of this group, is becoming more and more significant. Pollution of the inland waters and seas brought along by industrialization reduces the possibility of making use of water sources and fisheries. Therefore fish farming is gaining importance day by day both in our country and in the world. With an awareness of the importance of fisheries, some countries are looking for ways to obtain water products by making the best use of fishery sources in deriving animal proteins [43].

Fish farming has developed rapidly in the last 20 years. In many countries, fish farming has become an industry. Since fish farming is aimed at producing the highest number of fish in a unit area, overstocking of fish has led to increased fish diseases. Fighting successfully with the diseases has become very important for the future of fish farming [43].

In fish farming, any unfavorable changes in the physical, chemical, biotic and abiotic optimal living conditions of the living environment of the fish and the persistence of such changes without any improvement mainly lead to the emergence of many infectious diseases [4].

Enteric red mouth disease, known as ERM or Yersiniosis, was first reported in 1950's in the rainbow trout farms in the USA as a septicemic disease with high mortality rates in sub-acute and acute forms [8, 14, 21, 34, 38]. ERM disease is widespread in many parts of the world [11, 37, 33, 40].

In Turkey, Yersiniosis caused by *Yersinia ruckeri* was identified in rainbow trouts in 1991. As the trout farms increased in number, the disease became more widespread, starting to cause economic losses. This disease is currently frequently found in trout farms and has become the most important bacterial disease in our country [16; 29; 30; 44]. Its treatment is costly and requires labor. And since the treatment is based on the use of antibiotics, antibiotic-resistant bacteria growth begins in case of failure to use the proper and sufficient dose, making the treatment more difficult. The growth of antibiotic-resistant bacteria threatens human and animal health. Therefore, instead of fighting against the disease by way of medicines, the means of protection from the disease are sought and implemented. Medical intervention begins only when

the means of protection from *Yersinia ruckeri* prove fruitless [44].

Yersinia ruckeri is a gram negative; enteric bacteria generally sized 0.5 x 1.5 - 2 µm, slightly curled, rod-shaped, in motile, single or short-chain coccobacillus or bacillus forms with 7 – 8 peritrichous flagella [38, 14, 21, 4]. *Yersinia ruckeri* is actively motile at 15-27 ºC, inactive during the incubation at 9 ºC in spite of the presence of flagella and entirely immobile when incubated at 35 ºC due to the lack of flagella [35, 14, 18].

The disease has symptom such as darkening color, hemorrhage inside and outside the mouth, in the operculum, on the body surface and at the base of fins, abdominal swelling and water accumulation, and single or double sided exophthalmos. The final diagnosis must be based on laboratory work and not on these symptoms [3]. The symptoms also include erosion around the anus, on the fins and the skin as well as hemorrhage in the eyeballs and the iris. The autopsy examination may reveal bleeding in internal organs and a yellowish liquid in the abdominal cavity and the stomach [19].

The pathogenicity of *Yersinia ruckeri* changes depending on the water temperature and the fish size as well as factors including environmental conditions, nutrition, and stress conditions. The disease occurs when the water temperature is above 10 ºC, while mortality is very low under 10 ^{o}C [14, 21, 5, 4]. Mortality increases in parallel with overfeeding, stress conditions and in fish smaller than 7.5 cm. This infection is primarily found in young rainbow trouts [9, 25].

Yersiniosis agent involves the intestines of healthy fish first. Stress triggers the re infection in carrier fish and the infection process in healthy fish [13, 26]. The infection becomes acute in undesirable cases such as handling the carrier fish or the fish with intestines full of colonized *Yersinia ruckeri*, overstocking the fish, increased amounts of ammonia and other hazardous materials in water and reduced oxygen levels [14].

Both human and veterinary physicians diagnose bacterial diseases using culture method and biochemical analyses as well as agglutination, immunodiffusion, immunofluorescense, hemagglutination, radioimmune assay, enzyme-linked immunosorbent assay (ELISA), serological tests such as complement fixation, histopathological methods and PCR technique which has become highly significant in molecular biology [1, 7, 36, 41].

Sensitivity against antibacterial medicines can be identified by using many methods. Routine laboratory tests are generally used to assess the bacteriostatic and bactericide activity of medicines [6, 27, 24, 32].

II. MATERIAL AND METHODS

The 16 establishments engaged in trout farming as discussed in this study are located in Kahramanmaraş (Turkey). Between April 2013 and September 2014, 115 pieces of liver, spleen, kidney and intestine samples were obtained from these farms. These samples were planted in Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) agars using loops. The agars were left for incubation at 24 ^{o}C for 48 hours. Subculture was prepared using the colonies where growth was complete. Then these bacteria were examined with regard to biochemical properties. Colony morphology, gram staining, gram reaction test with potassium hydroxide (KOH), oxidase test, catalase test, voges proskauer (VP)-Methyl red (MR) tests, oxidation/fermentation (O/F) tests, indole test and motion test were performed for purposes of identification. Also, Biolog System (The biolog GENIII micro plate) was used to define the bacteria according to their metabolic activities. For Biolog System, a bacteria suspension was prepared from the BIOLOG IF-A solution. Bacteria concentration was set at 92-98% using turbidimeter. The bacteria samples with adjusted concentration were added into the microplates, in the amount of 100 µl for each well. These microplates were left for incubation for 24 hours at 26 ºC. And finally, they were measured by the microplate reader and compared with the system databank in order to diagnose the bacteria.

Molecular identification was based on routine biochemical tests and the bacteria identified using the Biolog System.

Chromosomal DNA was prepared from the *Yersinia ruckeri* strain using DNA isolation kit in order to be used for reproducing the genes. The YER8 (5'-GCGAGGAGGAAGGGTTAAGTG-3') and YER10 (5' GAAGGCACCAAGGCATCTCTG-3') primary couple, derived from the 16S rRNA gene of *Yersinia ruckeri'*, was used [23]. For the reaction the total volume (40 µL) was completed with 1 µL (20 pikomol) forward primer, 1 µL (20 pikomol) reverse primer, 1 µL dNTP (1 mM), 4 µL buffer (NH4)2SO4 (10X), 1,6 µL MgCl2 DNA polymerase (5 u/µL) [25 mM Tris-HCl (pH 7,5), 0,1 mM EDTA, 1 mM DTT and %50 (v/v) glycerol], 1 µL template DNA (~400 ng/mL) and 29,4 µL dH2O. For PCR amplification, following the pre-denaturation phase at 94 ºC for 1 minute, a total of 35 PCR cycles were made including denaturation at 94 ºC for 1 minute, hybridization at 55 ºC for 1 minute, DNA synthesis at 72 ºC for 1 minute and the final extension at 72 ºC for 8 minutes. PCR products were run on agarose gel at a concentration changing between 0.6% and 2%. They were melted inside Agarose 1X buffer at proper concentrations and then discharged into tanks. Generally, 5 µL of the proper dilutions of the analysis samples were taken and mixed with 1 µL 6X loading. For DNA control, λ DNA 100 base farm or 1000 base farm DNA markers were used as the DNA reference with proper sizes. Agarose gel was stained with EtBr for 30 minutes, viewed at UV light and photographed.

III. RESULTS AND DISCUSSION

The samples taken from the rainbow trout farms in Kahramanmaraş are generally suspicious as carriers of disease. When the temperatures increased, and waters became warmer, the farms were searched for samples having symptoms of the disease. And samples were obtained also from some plants with no suspicion of disease. The number of samples was based on the production levels of the farms and the

situation of the farms and their owners. The reason why we waited for warmer temperatures is that in the previous researches this disease was not encountered at water temperatures below 10 ºC. In the farms; sampling was done by visual and manual examination of the fish, staying motionless at the sides or on the surface or swimming aimlessly, having darkened color, hemorrhage inside and outside the mouth, in the operculum, on the body surface and at the base of fins, abdominal swelling, exophthalmos in the eyes. The samples were from fish weighing 5-200 grams. The unhealthy fish exhibited symptoms such as darkening color and exophthalmos, bleeding and damage to internal organs.

Phenotypic and biochemical properties were identified using the pure cultures of the isolated gram-negative, rod-shaped 14 pieces of *Yersinia ruckeri* strains. Also, Biolog System device was used to verify the biochemical tests (Table 1) and to define the other phenotypic properties (Table 2).

Table 1. Morphological and biochemical characteristics of 14 *Yersinia ruckeri* isolated from Rainbow Trout

Phenotypic and Biochemical Features	***Yersinia ruckeri* (n:14)**
Colony color	White
Gram Coloring	-
Shape	rod
Oxidase	-
catalase	+
Movement	+
H_2S	-
Methyl Red	+
Voges Proskauer	-
indole	-
Urease Formation	-
Oxidation / Fermentation	F
MacConkey Agar	+
Mueller-Hinton Agar	+
Reproduction at 0°C	-
Reproduction at 5°C	-
Reproduction at 15°C	+
Reproduction at 20°C	+
Reproduction at 25°C	+
Reproduction at 30°C	+
Reproduction at 37°C	+
Reproduction in 0.0% NaCl	+
Reproduction in 0.5% NaCl	+
Reproduction in 1.0% NaCl	+
Reproduction in 2.0% NaCl	+
Reproduction in 6.5% NaCl	-

Table 2. Other phenotypic characteristics with Biolog System of 14 *Yersinia ruckeri* isolated from Rainbow Trout.

Biochemical criteria	**Isolate reaction (n: 14)**	**Biochemical Criteria**	**Isolate reaction (n: 14)**
pH 5	-	%8 NaCl	-
pH 6	+	%4 NaCl	-
Positif Kontrol	+	%1 NaCl	+
Stachyose	-	N-Acetyl NeuraminicAcid	+
D- Turanose	+/-	N-Acetyl-D-Galactosamine	+
Sucrose	-	N-Acetyl- β-D-Mannosa-mine	+
Gentiobiose	-	N-Acetyl-D-Glucosamine	+
D-Cellobiose	-	D-Salicin	-
D-Trehalose	+	β- Methyl-D-Glucoside	+/-
D-Maltose	+	D-Melibiose	-
Dextrin	+	α-D-Lactose	-
Negatif Kontrol	-	D-Raffinose	-
D-Serine	-	Minocycline	-
Fusidic Acid	-	Rifamycin SV	-
%1 Sodium Lactate	+	Troleando-mycin	+
I Nosine	+	D-Serine	-
L-Rhamnose	-	D-Aspartic Acid	-
L-Fucose	-	D-Fructose-6-Phosphate	+
D-Fucose	+/-	D-Glucose-6-Phosphate	+
3-Methyl Glucose	+/-	Glycerol	+
D-Galactose	+	myo-İnositol	-
D-Fructose	+	D-Arabitol	+/-
D-Mannose	+	D-Mannitol	+
α-D-Glucose	+	D-Sorbitol	+/-
Niaproof 4	-	Tetrazolium Blue	+-
Guanidine HCl	+/-	Tetrazolium Violet	+/-
Lincomycin	+	Vanco-mycin	+
L-Serine	+	D-Saccharic Acid	-
L-Pyroglutamic Acid	-	Quinic Acid	-
L-Histidine	-	Mucic Acid	-

L-Glutamic Acid	+	Glucoronamide	+
L-Aspartic Acid	+	D-Glucoronic Acid	+
L-Arginine	-	D-Gluconic Acid	+
L-Alanine	+	L-Galactonic Acid Lactone	+
Glycyl-L-Proline	+	D-Galacturonic Acid	+
Gelatin	-	Pectin	-
Potassium Tellurite	-	Sodium Bromate	-
Lithium Chloride	+/-	Sodium Butyrate	+
Nalidixic Acid	+	Aztreonam	-
Bromo-Succinic Acid	+	Formic Acid	-
L-Malic Acid	+	Acetic Acid	+
D-Malic Acid	-	Propionic Acid	-
α-Keto-Glutaric Acid	+/-	Acetoacetic Acid	+/-
Citric Acid	+/-	α-Keto- Butyric Acid	-
L-Lactic Acid	+	β- Hydroxy-D,L-Butyric Acid	-
D-Lactic Acid Methyl Ester	+/-	α-Hydroxybutyric Acid	+/-
Methyl Pyruvate	+	γ-Amino-Butryric Acid	-
p-Hydroxy-Phenylacetic Acid	-	Tween 40	+/-

After isolating *Yersinia ruckeri* antibiogram sensitivity test was applied to measure sensitivity against antibiotics and 12 different antibiogram disks (Table 3) were used for that purpose.

Table 3. Antibiogram test result of 14 *Yersinia ruckeri* isolated from Rainbow Trout.

Antibiotics	**Sensitivity (mm)**
Erythromycin (15 µg)	R (0)
Streptomycin (10 µg)	R (0)
Enrofloxacin (5 µg)	S (22)
Florfenicol (30 µg)	S (30)
Amoxicillin (ax) (25 µg)	R (6)
Ampicillin (amp) (10 µg)	R (10)
Bacitracin (b) (30 µg)	R (7)
Gentamicin (cn) (10 µg)	S (16)
Nalidixic acid (na) (30µg)	S (21)
Oxytetracycline (ot) (30 µg)	R (13)
Neomycin (n) (30 µg)	R (10)
Batrim-trimetoprim+sulfamethoxazole (bc) (1,25µg+23,7 µg)	R (18)

S: Sensitive (Duyarlı)
R: Resistant (Dirençli)

PCR pictures taken with the use of the pure cultures of 14 isolated *Yersinia ruckeri* strains are presented in Figure 1.

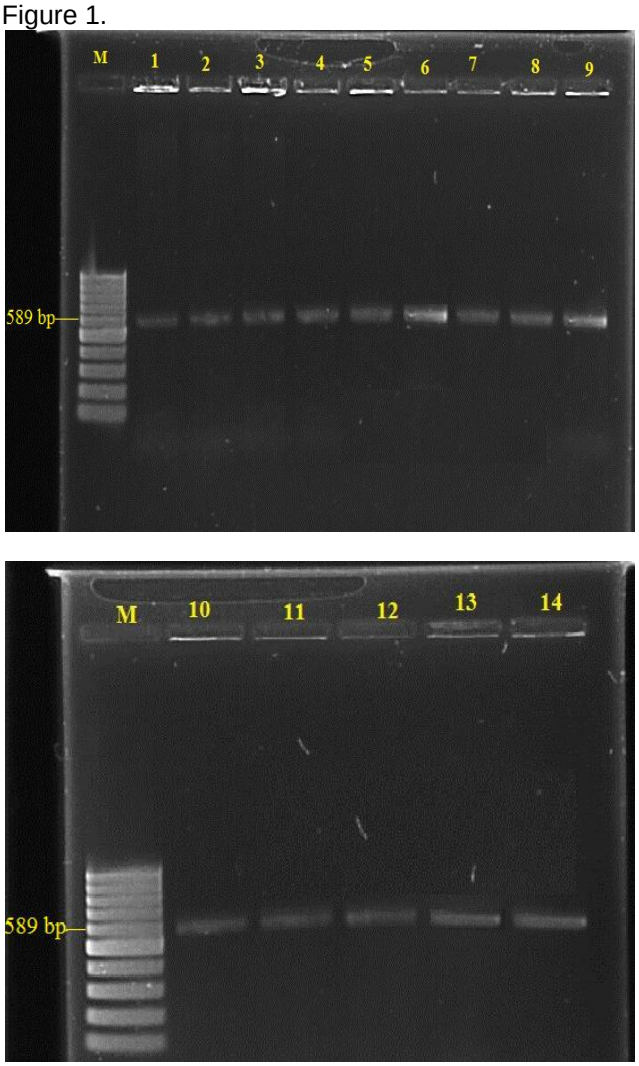

Figure 1. Image of PCR samples 14.

In this study aimed at identifying the current status of the presence of *Yersinia ruckeri* in the region of Kahramanmaraş, disease symptoms were observed in the carrier fish as the water temperatures increased and it became easier to make a morphological distinction between the unhealthy fish and the healthy fish. Symptoms increased in the carrier fish in parallel with increasing water temperatures. Also in the laboratory work, warmer water temperatures brought along a higher number of *Yersinia ruckeri* identified in the analyses. The isolation was at higher levels especially during June and July where waters become warmer. This situation supports the relation between increased temperatures and the incidence of the disease. In his work, [39] reported that *Yersinia ruckeri* epidemics are seen more frequently during the period with higher water temperatures.

In many of the samples, redness and hemorrhage were found around the fins, mouth, eyes, and operculum and the analyses resulted in the identification of *Yersinia ruckeri*. This condition is in support of the explanation which says that Yersiniosis is a disease characterized by the formation of hemorrhagic zones in various tissues and organs,

progressing with general hemorrhagic septicemic findings and subcutaneous hemorrhages are found particularly around the chin, mouth, bottom of fins and anus [10, 22]. These clinical findings are in parallel with the findings obtained in studies conducted in other countries.

Samples were obtained randomly from some farms because of failing to find a sample with morphological symptoms, but the analysis still resulted in the identification of *Yersinia ruckeri*. This condition is in support of the research where [20] isolated *Yersinia ruckeri* from the internal organs of some clinically asymptomatic fish in various trout farms in Fethiye region and where [28] isolated bacteria from fish with no symptoms indicating the presence of yersiniosis.

Yersiniosis, caused by *Yersinia ruckeri*, is a high mortality disease found in rainbow trout farms, resulting in severe economic losses [5]. Particularly among the young fish, the mortality rate may rise up to 75% [42]. Within a period of 1 week, the disease exhibited a mortality rate of 60%. This rate was found to be less than those reported in previous studies. This difference is related with environmental conditions, stress factors and intense stocking.

Factors such as organic pollution of the stressful environment, heat, overstocking, manual handling and oxygen insufficiency play a role in the occurrence of the disease [10]. In our research, the rate of isolated *Yersinia ruckeri* was higher in fish farms with extremely cloudy water, warm water, and overstocked fish.

Although depending on the stressful conditions, the disease generally progresses in per-acute and acute forms in spring months with warmer waters and in young fish and in the chronic form in the autumn with cooler waters and in fish at age 1 [17, 45]. This study is supported by the fact that in our study the rate of fish death due to *Yersinia ruckeri* increases, especially in the spring period. [39] reported that bacterial epidemics are caused both by increased water temperature and by the fact that the fish is stressed because of the sudden changes in water temperatures.

[5] and [15] reported that the isolated bacterial agents may be present in the usual water microflora but that the incidence of disease increases in stressful cases for the fish such as sudden changes in water temperatures particularly due to seasons, hygienic defects, overstocking of fish in the production area and the lack of clean barriers.

Bacterial infections generally occur in situations where the fish are under physiological stress and in periods with poor hygienic conditions. The best way of protection from infection is to avoid overstocking, low oxygen levels and poor labor [2].

It is believed that any diseases or hazards suffered by one of a series of farms set up at short distances on a single water source may soon spread to all farms on the same water source and that the spread of disease may be considerably facilitated by the continuous transfer of eggs, larvae, and fish among the farms located in the region.

Although the disease is observed in some farms in this region, the rate of fish death is lower than the expectedly high levels, and this can be accounted for by the fact that the average altitude of the region is high, water temperatures do not exhibit a significant variation during the year and follow a very low trend and that the fish is not overstocked.

It was seen in the discussions made during the sampling that all farmers in the region are more or less informed about the disease. The farmers said that they did not experience any yersiniosis-related large-scale fish death and economic losses in the farms so far nor have they heard any complaints from the other farms that they had massive losses caused by this disease.

As a result of the interviews made in the area in relation to the vaccines against the disease, it was found that some farmers applied regular vaccination. However, the producers stated that because of the seasonal changes in recent years and the climate getting relatively warmer by virtue of the many lakes and ponds built in the region, the rate of the disease has been continually increasing and the farmers having minor businesses started to show interest in the subject. It was understood that the drugs were administered based on the recommendations and practices of other producers and not on the suggestions of aquaculture engineers or veterinaries. In general, it was found that there is an unconscious use of antibiotics in the farms.

It is emphasized that proper maintenance is a cornerstone of treating and keeping under control the bacterial diseases in fish farming and that vaccinations and immune system stimulants may also be used. However, for an effective fight against diseases that emerge for reasons such as stress, water pollution, and malnutrition, administrating a limited amount of antibacterial drugs may not be disregarded [12, 31].

Antibacterial drugs are commonly used for controlling and preventing infections in humans, animals, and plants. But many studies have demonstrated that overuse of these bacterial drugs has resulted in the emergence of resistant bacteria [15].

It was found that farm personnel generally ignore sterilization and the farms, in general, do not use control pools.

IV. CONCLUSIONS

In research, fish samples between 5-200 grams with suspicion of *Yersinia ruckeri* were taken from 16 different rainbow trout farms in Kahramanmaraş. Among the 16 farms from which various amounts of samples were taken at various times, *Yersinia ruckeri* was isolated from a total of 7 farms, namely the 2nd farm, the 4th farm, the 8th farm, the 9th farm, the 12th farm, the 14th farm and the 15th farm. A total of 115 samples were analyzed.Work was conducted in order to isolate and identify the *Yesinia ruckeri* using classical culture methods. And then Biolog device was used to verify the biochemical values and to identify the other phenotypic properties. 94 phenotypic tests

were carried out successfully. DNA work was done in PCR for the final diagnosis. Molecular *Yersinia ruckeri* was isolated in 14 samples. And finally; an antibiogram test was carried out to measure the antibiotics susceptibility of the *Yersinia ruckeri* found in the region. At the end of all this work, the phenotypical and genotypical properties of *Yersinia ruckeri* were identified.

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ETHICAL APPROVAL

All animal studies were approved by the Animal Ethics Committee of Kahramanmaraş Sütçü Imam University, Faculty of Agriculture (KSÜZİRHADYEK) and Research Institute (Protocol number: 2013/5-1).

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