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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Preparation of O-antigen from *Yersinia ruckeri* Serotype O1 and Use in the Slide Agglutination Test [*]

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Abstract: In this study, it was aimed at early detection of yersiniosis caused by *Yersinia ruckeri* in rainbow trouts (*Oncorhynchus mykiss*) using an agglutination test. O-antigen of *Y. ruckeri* isolated from diseased fishes was prepared. This prepared O-antigen was injected into the ear vein of the rabbit. Serum was removed from blood samples taken on the 7th and 15th days after the last injection. Thus, the antibody of the *Yersinia ruckeri* was produced in the rabbit. Titers of the resulting antibodies were determined by microtitering in 96 well-based plates. It was used successfully in the slide an agglutination tests by making necessary dilutions according to the titer ratios of the antibodies. We determined that the motile and Gr (-) bacteria isolated from diseased rainbow trout with yersiniozis symptom were *Y. ruckeri* according to the results of slide agglutination test without any biochemical test. The isolated bacteria were confirmed to be *Y. ruckeri* with the results of API 20E test kit and PCR test. The antibodies were stored at -20 and -80°C for use subsequent studies.

Keywords: Antisera, O-antigen, slide agglutination test, *Yersinia ruckeri*.

Yersinia ruckeri Serotip O1'den O-antijenin Hazırlanması ve Lam Aglütinasyon Testinde Kullanımı

Öz: Bu çalışmada, aglütinasyon testi kullanılarak gökkuşağı alabalıklarında (*Oncorhynchus mykiss*) *Yersinia ruckeri*'nin sebep olduğu yersiniozisin erken teşhisi amaçlanmıştır. Hastalıklı balıklardan izole edilen *Y. ruckeri*'nin O-antijeni hazırlandı. Hazırlanan bu O-antijeni tavşanın kulak venasına enjekte edildi. Son enjeksiyondan sonraki 7. ve 15. günlerde alınan kan örneklerinden serum çıkarıldı. Böylece, *Y. ruckeri* antikoru tavşanda üretildi. Elde edilen antikorların titreleri, 96 kuyucuklu platelerde mikrotitrasyon ile belirlendi. Antikorların titre oranlarına göre gerekli dilüsyonlar yapılarak slayt aglütinasyon testlerinde başarıyla kullanılmıştır. Yersiniozis belirtisi gösteren hastalıklı gökkuşağı alabalıklarından izole edilen hareketli, oksidaz negatif ve Gr (-) bakterilerin biyokimyasal test yapılmadan lam aglütinasyon testi sonuçlarına göre *Y. ruckeri* olduğu tespit edildi. İzole edilen bakterilerin API 20E test kiti ve PCR testi sonuçları ile *Y. ruckeri* olduğu doğrulandı. Antikorlar, daha sonraki çalışmalarda kullanılmak üzere -20°C ve -80°C'de stoklandı.

Anahtar sözcükler: Antiserum, lam agglütinasyon testi, O-antijeni, *Yersinia ruckeri.*

[*]***This study was presented as a poster at the ITESDES 2018 symposium.***

INTRODUCTION

Enteric redmouth (ERM) disease or yersiniosis, caused by the enterobacterium *Yersinia ruckeri*, is an important disease which has led to significant economic losses of in salmonid and non-salmonid fish reared in both fresh and marine water aquacultures throughout the World (Austin & Austin, 1993). Infection may result in the development of chronic or acute septicemia with hemorrhages on the body surface and in the internal organs. Mortality, particularly in rainbow trout (*Oncorhynchus mykiss*) farms where ERM is present can reach up to 70% of the total population (Furones et al., 1993). (Walbaum) and other salmonids. *Y. ruckeri*, which was initially isolated from rainbow trout in the Hagerman Valley of Idaho, USA, in the 1950s 1966 (Austin & Austin, 1987), is now widely found in fish populations throughout North and South America, Australia, Africa and Europe (Austin & Austin, 2007). The disease has been reported from rainbow trout farms in the many regions of Turkey (Çagirgan & Yurekliturk, 1991; Timur & Timur, 1991; Çagırgan, 1996; Diler et al., 1998; Balta et al., 2005; 2010; 2016; Onuk et al., 2011). *Y. ruckeri* were identified by using conventional tests, API 20E test kit, serological test and PCR method (Austin & Auistin 1987; 1999; Balta et al., 2005; 2010; 2016; Altun et al., 2013, Onuk et al., 2011). Serotyping is particularly important for strain differentiation of *Y. ruckeri*, and the O-serotyping scheme of Davies is the most commonly used. *Y. ruckeri* has five O serotypes, O1, O2, O5, O6, and O7. İt was reported that *Y. ruckeri* is rapidly recognized by slide agglutination assay using defined O antigens (Davis, 1990). All the isolates from rainbow trout were represented almost exclusively by serotype O1 (Ormsby et al., 2016).

This study was carried out in 2003 as an undergraduate student thesis. The aim of this study was too rapid diagnoses of *Y. ruckeri* by using slide agglutination. *Y. ruckeri*'s body antigens have been prepared. These antigens were given intravenously to an adult rabbit to obtain antibodies. These antibodies were used in the rapid diagnosis of *Y. ruckeri* in the slide agglutination test.

MATERIAL and METHODS

Bacterial isolation: Diseased fish were showed typical symptoms such as dark skin, bilateral exophthalmos, ascites of the ventral body, eyes hemorrhage, redness in the throat and mouth, bleeding at the base of pelvic fins, prolapsed anus, pale liver, petechial hemorrhage in the pyloric caeca and redness in the last part of the intestines (Figure 1). *Y. ruckeri* was isolated from ten sickly rainbow trout in a fish farm in the Eastern Black Sea Region of Turkey. Fish kidneys were cultured aseptically by streaking a loop onto tryptic soy agar (TSA) plates and incubated at 20°C for 48 h. The bacteria were purified by passing Shotts Waltman (SW) medium (Figure 2) as previously described (Waltman & Shotts, 1984)

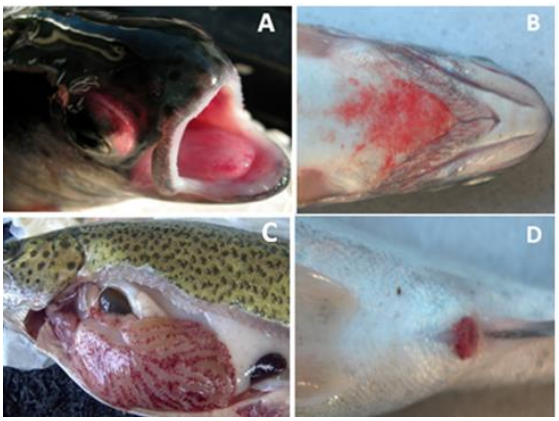

Figure 1 Darkening on the skin, exophthalmos, and bleeding in the eye, bleeding in the mouth (A), bleeding in the throat (B), petechial hemorrhage in the pyloric caeca (C), prolapsed anus (D) (Original).

Figure 2. Colonies and surrounded by a zone of hydrolysis of Y. *ruckeri* in SWA (Original).

Bacterial identification: *Y. ruckeri* was identified by using conventional biochemical tests and API 20E strips (BioMerieux, France) (Austin & Austin, 2007; Balta et al., 2010; 2016). API 20E test results were shown in Figure 3. Also, *Y. ruckeri* was confirmed by using PCR the Y. ruckeri-specific 16S rRNA primers YER3 and YER4 (Gibello et al., 1999) and checked by a slide agglutination test using a rabbit anti-serum of *Y. ruckeri* serotype O1 (Balta et al., 2010; 2016). PCR and slide agglutination tests were shown in Figure 4 and 5.

Figure 3. Biochemical test results of *Y. ruckeri* strain with API 20 E test kit (Original).

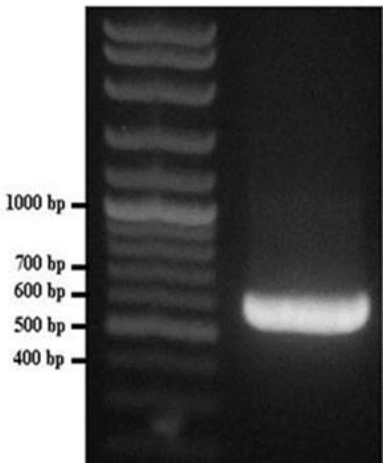

Figure 4. Coding gene amplification for 16S rRNA of *Y. ruckeri* (573bp), lambda DNA 100-bp molecular size marker (Promega, USA).

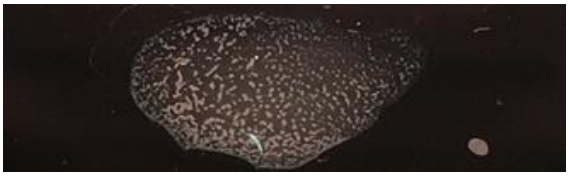

Figure 5. *Y. ruckeri* strains of type I antibody-positive result using the slide agglutination test (Original).

Preparation of somatic "O" antigen of Y. ruckeri: *Y. ruckeri* was grown as a pure culture on the TSA plate for 48 h. Colonies were harvested from the TSA plate with sterile phosphate-buffered saline (PBS) onto the plate. To dislodge colonies in plates were gently rubbed the surface of the agar with a bent glass rod. The bacterial slurry was placed into a 25 ml centrifuge tube and gently mixed with a magnetic stirrer for 10 minutes. Pour suspension into a centrifuge tube was centrifuged 1600 X g for 25 minutes. The supernatant was thrown. The bacteria cells were washed by centrifugation until clear and resuspended in PBS ten times volume. The bacterial suspension was heated at 100°C for 2 hours and centrifuged at the same speed and time. The supernatant was discarded. Bacterial cells were resuspended in 95% ethyl alcohol (cell pack, ten times fluid) and incubated at 37°C for hours. The bacterial suspension was centrifuged and resuspended with sterile PBS. This suspension was stored at 4°C until used (Ewing, 1972). This suspension (heat-stable O-antigen) was used as positive control in the slide agglutination assays.

RESULTS

Obtaining of antisera: The O- antigen of heat-killed bacterial was intravenously injected into the ear vein of the rabbit. The bacterial suspension has adjusted the density of a McFarland standard No 3. İnjections were given in consecutive doses on days 1 (0.25 ml), 2 (0.5 ml), 3 (1.0 ml) 4 (2.0 ml), and 11 (1.0 ml). One week after the last injection, blood was taken from vena jugularis of the rabbit. Blood was allowed to clot at 4°C overnight. The coagulated blood was centrifuged at 3000 rpm for 10 minutes. Thus, serum was removed. An equal amount of antibody (0.5 ml) was divided into Eppendorf tubes. It was stocked at -20°C and -80°C for use in subsequent studies.

Determination of antibody titer: The serum agglutinating titer of immunized rabbit with *Y. ruckeri* was detected in a 96-well microtiter plate with round bottom wells. The antibody titer determination assay was initiated with a dilution of 1:1 (50 µl of phosphate buffer: 50 µl of serum). And consequently, two-fold serial serum dilutions were carried out by adding 50 µl of diluted serum into the remaining wells with 50 µl of PBS. As a result, the serum dilutions were adjusted as 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048. Thereafter, 50 µl of inactive *Y. ruckeri* (1x109 CFU) suspension was added to each well and then microplates were covered with plastic parafilm and incubated at 20°C in cooled incubator for 18 h. The agglutination endpoint was determined as the last serum dilution where agglutination was visible (1/64 for one week and 1/16 for two weeks). Agglutination antibody titers were expressed as the highest serum dilution showing visible agglutination as compared to the positive control. The last well was used as a negative control, where there was only 50 µl PBS buffer.

Slide agglutination test: *Y. ruckeri* was grown on TSA plates for 24 at 20°C. A sterile loop was used to take *Y. ruckeri* colonies from the TSA were resuspended in PBS to obtain a concentration of about 10^9 cells/ml. This cell suspension was used as an antigen in the slide agglutination assay. The slide agglutination test was performed by mixing a drop of *Y. ruckeri* suspension with a drop of undiluted or 1/5 diluted rabbit antiserum of the *Y. ruckeri*. The immediately occurring agglutination within 1-2 minutes was determined as positive, and a weak agglutination after 5 min was accepted as a negative test. Controls were made with saline and serum from no immunized rabbits.

CONCLUSION

In this study, the antiserum against *Y. ruckeri* serotype O1 on the rabbit was produced. it was determined that the antibody titer in the received blood after one week was higher than that after two weeks. The obtained antibody in the rabbit against *Y. ruckeri* serotype O1 was used to determine the serotype of the *Y. ruckeri* strain isolated from diseased rainbow trout. Rabbit antisera were used to determine the strain of *Y. ruckeri* isolated from diseased rainbow trout. The antibody obtained from the rabbit was successfully used in dilution of 1/5 in the rapid diagnosis of *Y. ruckeri*. In this study, it is determined that *Y. ruckeri* serotype O1 was caused the majority of the infections in rainbow trout.In this study, it is determinated that *Y. ruckeri* serotype O1 was caused the majority of the infections in rainbow trout.

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