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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Diagnosis of bacterial fish diseases and classification of serotypes with slide agglutination method

Lam aglütinasyon metodu ile bakteriyel balık hastalıklarının teşhisi ve serotiplerin sınıflandırılması

Kaan Kumaş[1]* ● Tevfik Tansel Tanrıkul[2]

[1] Izmir Katip Çelebi University, Faculty of Fisheries, Department of Aquaculture, 35620, Izmir, Türkiye https://orcid.org/0000-0003-1583-8482
[2] Izmir Katip Çelebi University, Faculty of Fisheries, Department of Aquaculture, 35620, Izmir Türkiye https://orcid.org/0000-0002-1684-7965

*Corresponding author: kaan.kumas@ikcu.edu.tr
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Abstract: Bacterial fish pathogens cause significant losses in rainbow trout farms. In fish farms, bacterial pathogens cause threatening diseases which has made it necessary to develop rapid methods for disease diagnosis. Serological techniques which are applied with a small amount of antiserum and sample, are preferred for the rapid diagnosis of fish diseases. In this study, formalin-killed antigens prepared from reference strains of *Lactococcus garvieae*, *Yersinia ruckeri*, and *Vibrio (Listonella) anguillarum* were injected intravenously in consecutive doses to New Zealand rabbits. One week after the last injection, the sera separated to use in the slide agglutination tests. A total of 42 strains were studied, including *Y. ruckeri* (18 isolates), *V. anguillarum* (14 isolates), and *L. garvieae* (7 isolates) and 3 references (ATCC 43305, ATCC 29473, ATCC 49156) strains. Serotype O1 determined the predominant serotype (86%) in *V. anguillarum* and *Y. ruckeri* (84%) strains examined by the slide agglutination method. *L. garvieae* strains did not react against Japanese antisera but positively reacted against Turkish *L. garvieae* antisera.

Keywords: Bacterial fish pathogens, diagnosis, serological characterization

Öz: Bakteriyel balık patojenleri gökkuşağı alabalığı yetiştiriciliği yapan balık çiftliklerinde ciddi kayıplara sebep olmaktadır. Su ürünleri yetiştiriciliğinde hastalık etkeni bakteriyel patojenler, oluşan hastalıkların teşhisi için hızlı teşhis yöntemlerinin gelişmesine yol açmıştır. Hastalıklar yetiştiricilik tesislerinde görüldükten sonra balık patojenlerinin hızlı teşhis edilmesi, hastalıkların tedavi edilerek oluşacak ekonomik kayıpların önüne geçilmesi için önemlidir. Balık hastalıklarının hızlı teşhisinde az miktarda antiserum ve örnek ile uygulanabilen serolojik teknikler tercih edilmektedir. Bu çalışmada *Lactococcus garvieae*, *Vibrio (Listonella) anguillarum* ve *Yersinia ruckeri*'nin referans suşlarından antijenler kullanılmak üzere formalin ile inaktive edilerek, Yeni Zelanda tavşanlarına ardışık dozlarda intravenöz enjeksiyonlar gerçekleştirilmiştir. Son enjeksiyon yapıldıktan bir hafta sonra antiserum elde edilmiştir. 18 *Y. ruckeri*, 14 *V. anguillarum* ve 7 *L. garvieae* ve referans (ATCC 43305, ATCC 29473, ATCC 49156) suşlar dahil olmak üzere toplamda 42 suş ile çalışma yapılmıştır. Lam aglütinasyon metodu ile incelenen *V. anguillarum* suşlarında baskın olarak (%86) serotip O1, *Y. ruckeri* suşlarında (%84) serotip O1 tespit edilmiştir. *L. garvieae* suşlarının Japon KG- antiserumu ile aglütinasyon vermediği, ancak Türk KG- antiserumu ile pozitif reaksiyon oluşturduğu tespit edilmiştir.

Anahtar kelimeler: Bakteriyel balık hastalıkları, teşhis, serolojik karakterizasyon

INTRODUCTION

The risk of disease in fish increases as a result of adverse changes in the interaction between pathogen, host and environment (Toranzo, 2005). The rod-like or spherical cocci Gram-negative and Gram-positive bacterial species can cause disease outbreaks in aquaculture (Austin and Newaj-Fyzul, 2017). Infectious bacterial pathogens have been reported in the majority of the taxonomic groups. However, in the extensive production, only a few bacterial species are responsible for significant economic losses worldwide. (Toranzo et al., 2009). In addition, an extensive antigenic variation has been reported with bacterial pathogens associated with fish diseases (Leblanc et al. 1981; Nakai et al., 1981; Kitao et al., 1983; Stevenson and Airdrie, 1984; Nomura and Aoki, 1985; Sorensen and Larsen, 1986; Toranzo et al., 1987). In the rainbow trout farms, major bacterial pathogens that cause disease are *Pseudomonas fluorescens, Flavobacterium psychrophilum, Flavobacterium columnaris, Listonella anguillarum, Aeromonas hydrophila, Yersinia ruckeri* and *Lactococcus garvieae* (Toranzo, 2004).

The rapid diagnosis of diseases by serological methods has increased the accuracy in the diagnosis and reduced the time required for diagnosis from days to hours (Austin and Newaj-Fyzul, 2017). Since the identification of *Aeromonoas salmonicida* with a simple slide agglutination test by Rabb et al. (1964), the procedure was improved and applied to numerous bacterial fish pathogens. (Eurell et al., 1979, Toranzo et al., 1987; Romalde et al., 1995). Several

monoclonal and polyclonal antibodies against fish pathogens are available commercially, and the selected antibody used in the tests are critical for immunoserological diagnosis. Monoclonal antibodies (mAbs) detect only one epitope on a single target antigen and comprise a homogenous cloned immunoglobulin with high specificity, whereas polyclonal antibodies contain heterogeneous mixed immunoglobulin molecules that can recognize multiple epitopes on a single antigen are superior for the detection of pathogens (Austin and Newaj-Fyzul, 2017).

Vibriosis caused by *V. anguillarum* is probably one of the oldest recognized bacterial fish diseases and is pathogenic to many fish and shellfish (Larsen, 1990; Hickey and Lee, 2017; Hansen et al., 2020). So far, *V. anguillarum* has been divided into 23 O serogroups, (Pacha and Kiehn, 1969; Sorensen and Larsen, 1986; Kitao et al., 1983; Kitao et al., 1984 Grisez and Ollevier, 1995; Pedersen et al., 1999) however between these serotypes O1 and O2 associated with the most isolated and virulent serotypes (Toranzo et al., 2017). *Y. ruckeri,* the causative agent of Enteric Redmouth Diseases has two commonly used serological schemes for the classification. Davies divided *Y. ruckeri* into five serotypes named O1, O2, O5, O6, O7 (Davies, 1990) and Ormsby et al. (2016) extended this scheme with serotype O8. Romalde et al. (1993) described four serotypes subdivided into subgroups O1 (a, b), O2 (a, b, c), O3, and O4. In the serological tests, *L. garvieae* strains have been divided into two serotypes named KG^- and KG^+ that can be differentiated by an agglutination test (Kitao, 1982; Yoshida et al., 1997; Romalde and Toranzo, 2002). In addition, the KG^- strain produces a capsule on its cell surface, which is pathogenic to fish (Yoshida et al., 1997) however, isolates might have result with losing capsule due to subculturing (Morita et al., 2011)

In this study, the major bacterial fish pathogens (*V. anguillarum, Y. ruckeri, L. garvieae*) isolated from different rainbow trout farms between 2014-2021 in the South Aegean region of Turkey were tested for serological diagnosis and classification of serotypes. Proper and rapid diagnosis for the diseases leads to appropriate treatment and avoid indiscriminate use of chemotherapeutics in the fish farm. However, it is essential to study characteristics of bacterial strains and develop better control and treatment strategies in order to prevent economic losses besides the serological classification of the serotypes would contribute to vaccine studies.

MATERIAL AND METHODS

Bacterial strains

Total of 42 strains, including three reference strains (ATCC 43305, ATCC 29473, ATCC 49156) received from Izmir Katip Celebi University Fish Diseases and Biotechnology Laboratory for determination of serological characteristics. In the slide agglutination tests *V. anguillarum* (15 isolates), *Y. ruckeri* (19 isolates), and *L. garvieae* (8 isolates) were examined. *V. anguillarum, Y. ruckeri, L. garvieae* isolates (except ATCC 49156, ATCC 43305 and ATCC 29473) were isolated from rainbow trout in the cases of Vibriosis, Yersiniosis, and Lactococcosis occurred between 2014-2021 in the Southern Aegean Region of Turkey. *V. anguillarum* and *Y. ruckeri* isolates were subcultured on TSA (Tryptic Soy Agar) and incubated at 21ºC, *L. garvieae* strains were subcultured to TSA and incubated at 30ºC to check purity by morphological characteristics and biochemical analysis.

Preparation of thermostable somatic "O" antigens

For agglutination tests, heat-stable somatic O antigens of *V. anguillarum* and *Y. ruckeri* were prepared as described by Davies (1990) and Toranzo et al. (1987). These suspensions are used in the slide agglutination tests as somatic antigens.

Antigens for immunization

Antigens were prepared as described by Toranzo et al. (1987). Reference strains of *V. anguillarum* O1 (ATCC 43305) were streaked on TCBS, *Y. ruckeri* O1 (ATCC 29473) on Waltman-shotts medium to incubated at 21ºC for 24-48h. *L. garvieae* KG- (ATCC 49156), biochemically and molecularly identified (GenBank: MT876413) *L. garvieae* (C3) steaked on TSA and incubated at 30ºC for 24-48 hours. Bacteria inoculated into TSB for grown overnight and killed by adding 2% (v/v) formalin into the culture. Formalin-killed cells were centrifugated and washed twice with 0.3% (v/v) formalin. Formalin-killed cells resuspended with 0.85% saline for centrifugation and density were adequate to 10^9 cells/ml, the density of a McFarland standard No.3.

Obtention of antisera

Antisera is produced from New Zealand rabbits according to Toranzo et al. (1987). Rabbits were injected intravenously with saline washed suspensions (the density of McFarland No.3) of formalin-killed cells. Injections were given to the rabbits on day 1 (0.25 ml), 2 (0.50 ml), 3 (1.0 ml), 4 (2.0 ml) and 11 (1.0 ml), respectively.

One week after the last injection, rabbits bled from the ear vein (Figure 1). Blood was allowed to clot at room temperature for one hour and left at 4ºC overnight. The serum is separated and stored at -20ºC until agglutination assays (Davies, 1990). In addition, blood was collected from non-immunized rabbits to obtain the serum and used in the slide agglutination tests for controls.

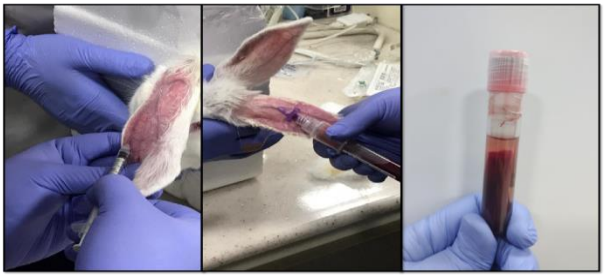

Figure 1. One week after the last injection, blood collection from rabbits and the obtained antiserum

Slide agglutination tests

Serological identification of bacterial strains performed on a black background by slide agglutination method using a loopful of whole-cell antigens against undiluted antisera. Serological classification of *V. anguillarum* and *Y. ruckeri* strains was tested by using heat-stable O antigens against representative antisera.

In the slide agglutination tests intensity of reactions (Figure 2) was determined as; no reaction (-), weak agglutination (+) after 5 minutes considered as a negative result, and a distinct and immediately occurring moderate (++), strong (+++) very strong (++++) agglutination considered as a positive result.

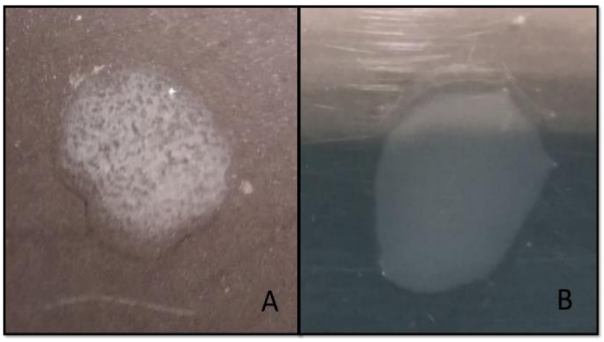

Figure 2. Formation of antigen-antibody clumps visible with naked eye in the slide agglutination test. A: Positive reaction, B: Negative reaction

RESULTS

Agglutination reactions

Whole-cell of 15 *V. anguillarum* isolates showed positive reaction against representative antisera in the tests, however strains V12 and V13 considered as negative when tests performed with somatic O antigens other strains (86%) belonged to serotype O1. Whole-cell and thermostable somatic antigens of *Y. ruckeri* strains did not show any difference against representative antisera and majority (%84) of the strains belonged to serotype O1. *L. garvieae* strains show serological differences based on the geographical origin of the isolate. In addition, in their groups, the biochemical properties of the strains (except ATCC 49156, ATCC 43305, ATCC 29483) were the same for *L. garvieae* isolates, *Y. ruckeri* isolates besides *V. anguillarum* where strain V12 and V13 did not ferment arabinose.

The results obtained from whole-cell and thermostable somatic O antigens of *V. anguillarum* isolates in the slide agglutination tests are presented in Table 1.

Table 2 shows the agglutination reactions of whole-cell and thermostable somatic antigens of *Y. ruckeri* strains against representative antisera.

Table 1. Slide agglutination test results of *V. anguillarum* strains

Whole-cell antigens	*V. anguillarum* O1 (ATCC 43305) antisera	O antigens	*V. anguillarum* O1 (ATCC 43305) antisera
ATCC 43305	++++	ATCC 43305	++++
V3	+++	V3	+++
V12	++	V12	+
V13	++	V13	+
V17	+++	V17	+++
V20	++++	V20	++++
V22	+++	V22	+++
V24	++++	V24	++++
V29	++++	V29	++++
V31	+++	V31	+++
V32	++++	V32	++++
V34	++++	V34	++++
V35	++++	V35	++++
V37	++++	V37	++++
SVA	++++	SVA	++++

Intensity of the reaction: No reaction; -, weak; +, moderate; ++, strong; +++, very strong; ++++

Table 2. Slide agglutination test results of *Y. ruckeri* strains

Whole-cell antigens	*Y. ruckeri* O1 (ATCC 29473) antisera	O antigens	*Y. ruckeri* O1 (ATCC 29473) antisera
ATCC 29473	++++	ATCC 29473	++++
YR5	++++	YR5	++++
YR241118	++++	YR241118	++++
Y1	++++	Y1	++++
Y3	++++	Y3	++++
Y6	++++	Y6	++++
Y12	+++	Y12	+++
Y31	+++	Y31	+++
Y32	-	Y32	-
Y33	++++	Y33	++++
Y34	-	Y34	-
Y35	-	Y35	-
Y36	+++	Y36	+++
Y37	+++	Y37	+++
C26	++++	C26	++++
C27	++++	C27	++++
C29	++++	C39	++++
S31	++++	S31	++++
KYB	++++	KYB	++++

Intensity of the reaction: No reaction; -, weak; +, moderate; ++, strong; +++, very strong; ++++

Table 3 shows the agglutination reactions of *L. garvieae* strains against Japanese KG$^-$ (ATCC 49156) and Turkish KG$^-$ (C3) antisera. In the slide agglutination tests, 7 strains isolated from rainbow trout in the Southern Aegean Region of Turkey did not react with Japanese KG$^-$ *L. garvieae* antisera. However, in the tests against Turkish KG$^-$ *L. garvieae* antisera, 6 strains show very strong agglutination (++++), and 1 strain reacted as strong agglutination (+++).

ATCC 49156 showed no reaction (-) with Turkish KG- *L. garvieae* antisera but gave very strong agglutination (++++) with its own antisera (Japanese KG- antisera).

Table 3. Slide agglutination results of *L. garvieae* strains

Antigen	*L. garvieae* ATCC 49156 (Japanese KG-) antisera	*L. garvieae* C3 (Turkish KG-) antisera
ATCC 49156	++++	-
C3	-	++++
C12	-	+++
ELG	-	++++
SLG	-	++++
BLG	-	++++
LGSO1	-	++++
LGSO4	-	++++

Intensity of the reaction: No reaction; -, weak; +, moderate; ++, strong; +++, very strong; ++++

DISCUSSION

It is critical to apply appropriate control strategies against the causative agents of fish diseases. Different serological procedures have been used to diagnose fish pathogens. It is known that long-term intensive vaccination can cause a consistent selective pressure resulting with the appearance of a distinct serotype (Bachrach et al., 2001). Antigenic variations have been reported for bacterial fish pathogens associated with fish diseases. Bacterial fish pathogens from different sources of isolation or origins can be identified in slide agglutination tests (Toranzo et al., 1987; Kang et al., 2004; Balta et al., 2010; Ürkü and Timur, 2014; Balta et al., 2016). Rapid and preliminary screening of the majority for bacterial pathogens is applicable with whole-cell antigens against representative antisera. However, it is necessary to use thermostable somatic O antigens for serogroups. (Toranzo et al., 1987; Davies, 1990; Romalde et al., 2003; Ormsby et al., 2016).

V. anguillarum affects salmonid and non-salmonid fish worldwide and, this pathogen has been divided into 23 O serotypes however, serotypes that cause mortalities in fish reported for only serotype O1, serotype O2 and less extent serotype O3 (Toranzo et al., 2017). In the present study, antisera was raised from rabbit against the reference strain of *V. anguillarum* O1. In this study when whole cells were utilized agglutination was observed for all strains against O1 antisera, however, V12 and V13 coded strains show moderate agglutination. When heat stable O-antigens were used in the agglutination experiments, strains V12 and V13 were considered negative and were untypable. The biochemical properties of *V. anguillarum* strains were the same except fermentation of arabinose. All the *V. anguillarum* O1 isolates fermented arabinose, whereas untypable isolates (V12 and V13) were unable to ferment. In their review, Toranzo and Barja (1990) reported that strains of *V. anguillarum* serotype O1 fermented arabinose, but strains of serotype O2 could not. Likewise, Larsen and Olsen (1991) stated that *V. anguillarum* strains of serotype O1 were arabinose-positive (97%), whereas strains of serotype O2 were arabinose variable (37%).

Sorensen and Larsen (1986) reported 270 *V. anguillarum* strains were isolated from diseased fish (157 from rainbow trout; 64 from cod; 40 from eels; 9 from plaice). Agglutination assays against representative antisera revealed serotype O1 was the dominant serotype isolated from cultured fish and serotype O2 from wild fish. Tanrıkul (2007) isolated *V. anguillarum* from diseased fish in eight different rainbow trout farms in the South Aegean region and reported the *V. anguillarum* isolates belonged to serotype O1. Avsever and Un (2015) observed serological characterization of 51 *V. anguillarum* strains isolated from 6 different fish farms located in the Aegean Region of Turkey. In the slide agglutination tests against serotype O1, O2, and O3 antisera, the authors stated that 42 strains belonged to serotype O1 and 9 strains to serotype O2. Balta and Dengiz Balta (2017) observed diseased rainbow trout farms between 1999-2014 and isolated 32 *V. anguillarum* strains from 12 different farms located in the Black Sea Region of Turkey. To understand the diversity of the strains, the authors performed slide agglutination tests and reported *V. anguillarum* strains belonged to serotype O1. In accordance with the previous studies, the present study has demonstrated similar results within the diversity of *V. anguillarum* strains.

Wide diversity has been reported in *Y. ruckeri* isolates able to cause infection in rainbow trout and Atlantic salmon. *Y. ruckeri* outbreaks are associated with rainbow trout dominantly represented by serotype O1, whereas the predominant serotype associated with Atlantic salmon is associated with serotype O2, O5, and O8 worldwide. (Ormsby and Davies, 2021). The findings of this study indicate that serotype O1 was responsible for the majority of the ERM cases, except Y32, Y34, and Y35 did not agglutinate with serotype O1 antisera and were untypable. Davies (1990) observed serological characterization of 131 *Y. ruckeri* strains including 127 *Y. ruckeri* and 4 reference strains. Heat-stable O antigens of each 131 isolates were reacted against five antisera (O1, O2, O5, O6, O7) in the slide agglutination tests. Serotypes of the strains were 105 serotype O1, 11 serotype O2, 5 serotype O5, 4 serotype O6, 5 serotype O7, and 1 isolate was untypable. Romalde et al. (1993) demonstrated serological characterization of 53 *Y. ruckeri* strains isolated in Spain. Slide agglutination tests were performed against antisera raised for each serotype (O1 [a, b], O2 [a, b, c], O3, O4) from rabbits, and all the Spain strains positively reacted with serotype O1 antisera. Wheeler et al. (2009) stated serological diversity of 160 *Y. ruckeri* strain isolated from different countries. The serological tests revealed the serotypes; 128 strain determined serotype O1, 17 serotype O2, 11 serotype O5, 2 serotype O6, and 2 serotype O7. Bastardo et al. (2011) pointed out

serological characteristics of 11 *Y. ruckeri* strains isolated from diseased *Salmo salar* in Chile. Serological examinations of the isolates show the majority of the isolates were serotype O1 (9 strain O1b, one strain O1a) and 1 isolate belonged to serotype O2b. Altun et al. (2013) demonstrated the serological characterization of 15 *Y. ruckeri* strains isolated from diseased rainbow trout. Serological assays revealed the majority (11) of the isolates belonged to serotype O1 and, 4 strains were serotype O2. Our findings are consistent with the other researchers, in which serotype O1 is the predominant serotype.

Serotypes of *L.garvieae* have been reported with absence (serotype KG+) or existence (KG-) of capsular material (Romalde and Toranzo, 2002). In addition, it has been reported that the biochemical and genetic characteristics of these two serotypes are very similar to each other. (Eldar et al., 1996). In the fish farms, capsulated isolates of *L. garvieae* are stated as highly virulent, while non-capsulated isolates are hardly able to establish an infection to rainbow trout (Barnes et al., 2002). *L. garvieae* KG+ antigens were detected around the cell surface and not in the cell capsules, whereas KG- antigens were over the capsule material (Oyama et al., 2002). In this study, antisera were raised from rabbits for Japanese and Turkish *L. garvieae* KG^- strains to use in the slide agglutination tests. The present results indicate that Turkish *L. garvieae* strains did not agglutinate with Japanese *L. garvieae* antisera and, the Japanese strain did not agglutinate with Turkish *L. garvieae* antisera. In contrast, antisera raised against Turkish *L. garvieae* agglutinated with *L. garvieae* strains isolated from diseased rainbow trout in the Aegean Region of Turkey. Likewise, in the serological tests against representative KG^- and KG^+ antisera, *Enterococcus seriolicida* strains isolated from diseased yellowtail in Japanese by Yoshida et al. (1996) showed that only KG^- strains agglutinated with KG^- antisera, whereas KG^+ strains reacted positively with both KG^+ and KG^- antisera. Barnes and Ellis (2004) compared serological characteristics of *L. garvieae* KG^-, KG^+ strains isolated from Europe (Italy, United Kingdom, Spain) and Japanese. In the agglutination tests, authors reported European KG- strains did not agglutinate with Japanese KG^- and KG^+ antisera, and Japanese KG- strains did not agglutinate with European KG^- antiserum and KG^+ antiserum. In addition, both European and Japanese KG^+ strains positively reacted to Japanese and European KG^+ antisera. Correlatively, Çağırgan (2004) stated 20 different *L. garvieae* strains were isolated from diseased rainbow trout in Turkey. Serological tests performed with KG^- antisera (Spain strain) and reported *L. garvieae* strains isolated from rainbow trout in Turkey reacted positively to representative antisera. Oinaka et al. (2015) reported an *L. garvieae* strain isolated from yellowtail in Japan between 2012-2013. In the slide agglutination tests, these strains did not agglutinate with reference KG^- and KG^+ antisera. In the current study, our findings revealed *L. garvieae* strains isolated from diseased rainbow trout in Southern Aegean Region showed no-reaction against Japanese *L. garvieae* antisera in the slide agglutination tests however, the strains showed a positive reaction against KG^- antisera produced from the Turkish strain. In accordance with the results of Çağırgan (2004) and Barnes and Ellis (2004) and this study, we suggest Turkish *L. garvieae* strains can be included European KG- serotype.

CONCLUSION

Diseases caused by *V. anguillarum, Y. ruckeri*, and *L. garvieae* is responsible for significant economic losses with a high mortality rate in rainbow trout. In this study, bacterial fish pathogens were diagnosed with the slide agglutination method, and serotypes of the strains were determined. The slide agglutination method allows the rapid diagnosis of pathogens causing fish diseases and the prevention of economic losses within the appropriate treatment.

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AUTHORSHIP CONTRIBUTIONS

Tevfik Tansel Tanrıkul, Kaan Kumaş: Fiction, literature, methodology, data analysis, manuscript writing. Tevfik Tansel Tanrıkul: Performing the experiment with rabbits, supervision. Kaan Kumaş: Preparation of antigens. All authors approved the final draft.

CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS APPROVAL

This study was conducted with the approval of Animal Experiments Local Ethics Committee of Ege University (Date: 24.03.2021, No: 2021-027).

DATA AVAILABILITY

Data supporting the findings of the present study are available from the corresponding author upon reasonable request.

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