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Original Article

Investigation of vibriosis caused by *Vibrio anguillarum* in rainbow trout (*O. mykiss*)

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Abstract

Identifying pathogenic microorganisms causing disease is important for epidemiological research, antimicrobial therapy, and control. The current study was carried out to use different methods for the identification of *Vibrio anguillarum* from diseased rainbow trout (*Oncorhynchus mykiss*) obtained from Türkiye (Muğla-Fethiye), the damage caused by the pathogenic microorganism in the tissues and organs, and the determination of the antibiotic effective against the pathogen. Hemorrhagic and ulcerative skin lesions and diffuse petechial hemorrhage in the internal organs were clinically detected in diseased fish obtained from the rainbow trout farm. Bacteria isolated from diseased fish were subjected to analysis using conventional bacteriological methods, a commercial bacterial identification test kit (API), an automated bacteria identification system known as Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), BD Phoenix™, and 16S rRNA sequence analysis. All isolated bacteria were identified as *V. anguillarum* by API 20E and conventional bacteriological method. These results have been confirmed with 16S rRNA sequence analysis. However, the isolated bacteria were identified as *Grimontia hollisae* (syn. *Vibrio holisae*) with the BD Phoenix system. Histologically, tissue damage such as melano-macrophage centers and necrosis in the kidney and spleen, hyperemia and mononuclear cell infiltration in the liver, as well as mononuclear cell infiltration on muscles, talengectiasis in the gill tissue was observed. In addition, it has been determined that the most effective antibiotic against the pathogen was enrofloxacin. When comparing all identification methods used for this pathogen causing tissue damage, it was demonstrated that the MALDI-TOF MS provides better results than other methods in terms of cost and identification time, and it could be used as an alternative to the conventional method to the identification of *V. anguillarum*.

Keywords: Rainbow trout, 16S rRNA, API20E, BD Phoenix, Conventional method, *Vibrio anguillarum*, MALDI-TOF MS.

1. Introduction

Bacterial diseases are one of the most important problems identified in cultured freshwater and marine fish species worldwide with the increase in aquaculture production. It has been reported that bacterial infections leading to mass mortalities cause economic losses in trout production [1]. Pathogenic bacteria such as *Lactococcus garvieae*, *Vibrio anguillarum*, *Yersinia ruckerii*, *Aeromonas* spp, *Pseudomonas* spp, and *Flavobacterium* spp have been frequently reported to cause disease and mortality in rainbow trout [2].

Vibrio anguillarum, also known as *Listonella anguillarum*, is the causative agent of vibriosis, a deadly hemorrhagic septicemia disease affecting various marine and fresh/brackish water fish (over 50 teleost species), bivalves, and crustaceans [1, 2, 3]. In addition to water temperature and quality, stress factors are essential in disease outbreaks. The most prominent symptoms of the disease include darkening of the skin and ulcer formation on the skin. In the chronic form of the disease, intense hemorrhages are observed on the visceral organs and body muscles. Internally the spleen may be enlarged and the kidney liquefied [3, 4].

Türkiye has become one of the largest aquaculture producers in both Europe and the world. According to TUIK (Turkish Statistical Institute) data for 2021, rainbow trout produced in Türkiye constitutes 35.45% of the total aquaculture production with a production of 167241 tons [5].

Identification of bacteria on the species level is critical for epidemiological research, antimicrobial therapy, and control in aquaculture. The diagnosis of bacterial fish diseases has progressed from traditional culture-dependent methods involving the recovery of pathogens on agar-containing media and identification by conventional methods. Today, this method is frequently used in many laboratories [6]. However, the identification of bacterial fish pathogens using conventional methods is time-consu-

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ming, expensive, complicated, and requires expertise [7, 8]. Therefore, alternative identification methods are needed for bacterial fish pathogens. The present study aimed to identify *V. anguilarum*, which causes high mortality in cultured rainbow trout, using different identification methods, to detect tissue damage caused by pathogenic bacteria and determine antibiotics against the agent.

2. Materials and methods

2.1. Field sampling and bacteriological examination

10 fish (200-250 g) showing clinical signs of disease were obtained from the South-West Region of Türkiye (Muğla-Fethiye). Mortality rates varied from 20%. Bacteriological samples of kidney, liver, spleen, and blood were streaked onto Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA). Plates were incubated at 19-20 °C for 24-48 hours.

2.2. Conventional bacteriological method

Morphological, physiological and biochemical characterization of the isolates (n=10) was performed using tests such as Gram staining, motility, cytochrome oxidase and catalase activity, fermentative degradation of glucose (O/F), O/129 (2,4-diamino-6,7-diisopropyl pteridine) sensitivity,beta-galactosidase, Methyl Red (MR), Voges-Proskauer (VP), indole, citrate, urease, gelatin and amylase, H_2S (Hydrogen sulfide production), carbohydrate fermentations (glucose, sucrose, mannitol, melibiose, arabinose, rhamnose, inositol, trehalose and sorbitol), amino acid decarboxylation (ornithine, lysine and arginine) [2, 9].

2.3. Identification by commercial bacterial identification test kit (API 20E)

API 20E kit was used according to the manufacturer's instructions except for the incubation temperature, which was 19-20 °C [9].

2.4. Identification by automated bacteria identification system (MALDI-TOF MS Biotyper and BD Phoenix)

Each bacterial sample was added into Phoenix ID broth and the suspension matched a McFarland 0.5 standard. Each suspension was inoculated into the Phoenix system panel and was logged and loaded into the Phoenix system instrument [10]. Also, for MALDI-TOF MS, a single bacterial colony from each tested isolate was placed onto a 96-spot polished steel UV-absorbing MALDI-TOF plate. One drop of MALDI matrix (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) which absorbs laser energy was added to each bacterial colony and they were allowed to air dry. The prepared plate was then put on a fixed, pulsed laser beam (BrukerBiotyper MALDI Automation Control software) for analyzing the samples [11, 12]. All results were recorded electronically in a system database.

2.5. Identification by 16S rRNA sequence analysis of a representative strain

A representative strain (Va_5) of similar isolates was chosen for molecular analysis. One colony of Va_5 was transferred to a tube (2ml) containing Tryptic Soy Broth (TSB) and incubated during 24-48 h at 19-20 °C until the OD_{600} was 1. After incubation, 1.0 ml of the bacteria culture was centrifuged at 12,000 g for 1 min, the supernatant was discarded, and the pellet was frozen at -20°C until DNA extraction. Genomic DNA was extracted using the GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit, (E3580, Poland) according to the manufacturer's instructions. The extracted DNA from the Va_5 was subjected to PCR with the universal bacteria primer set 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT3') as reported by Weisburg et al. [13]. PCR products were sequenced bidirectional by BM Laboratuvar (Ankara, Türkiye).

2.6. Antimicrobial sensitivity

Antimicrobial susceptibility tests of the isolates were determined by using Kirby Bauer disc diffusion method on Mueller-Hinton Agar (MHA) (Himedia-M173) plate (Barry, 1985) by using the twelve antibiotics (ampicillin (10 µg), erythromycin (5 µg), flumequine (30 µg), chloramphenicol (30 µg), ciprofloxacin (1 µg), enrofloxacin (5 µg), florfenicol (30 µg), furazolidone (50 µg), kanamycin (30 µg), streptomycin (10 µg), oxytetracycline (30 µg), and sulfamethoxazole (25 µg)). The plates were incubated at 19 °C for 24 h. The antibiotic sensitivity test was carried out according to instructions of the Clinical and Laboratory Standard Institute [15] and performed in duplicates. The isolates were classified as sensitive (S), intermediary sensitive (I), or resistant (R).

2.7. Histopathological examination

Tissue samples from the visceral organs (liver, spleen, kidney), skin, and gill were immediately fixed in 10 % buffered formalin and processed for paraffin embedding. Paraffin blocks were sectioned (4-5 µm) on a microtome Leica RM2125 (Leica Microsystems GmbH, Austria), dewaxed and stained with haematoxylin (Sigma-Aldrich-HHS16), and eosin (Merck 109844, Germany) (H&E), according to the method described by Roberts et al. [16].

3. Results

Water samples were collected 30 cm below the water surface in aseptic containers and were immediately measured. The water temperature, dissolved oxygen concentration, and pH values were measured as 14 °C, 8 mg/l, and 7.5, respectively.

Clinical findings such as external hemorrhagic and ulcerative lesions on the skin (Figure 1a, 1b) and bilateral exophthalmos with hemorrhage in the eyes of some individuals, were observed. Diseased fish samples exhibited

Fig. 1. Diseased rainbow trout. Hemorrhagic (arrowed) and ulcerative (arrowed) skin lesions on the lateral (a) and caudal (b) region of the body; hemorrhages in the gills (arrowed) (c), liver (arrowed), internal organs and muscles (*) and splenomegaly (arrowed) (d).

splenomegaly internally (Figure 1d), along with diffuse petechial hemorrhage in internal organs, especially in the liver, gas bladder, muscles, and gills (Figure 1c, 1d).

After the incubation of the bacteriological inoculations from the visceral organs, raised with round, convex, cream-colored colonies on TSA and BHIA were observed at 19-20 °C for 48 hours. They (n=10) were motile, Gram-negative, bacil, facultative fermentative, cytochrome oxidase and catalase positive, sensitive to O/129 (2,4-diamino-6,7-diisopropyl pteridine) as reported by West and Colwell [17]. Therefore, the isolates were identified as *Vibrio* sp. According to biochemical test the isolated bacteria were identified as *V. anguillarum* because arginine dihydrolase, beta-galactosidase, indole, Voges-Proskauer, citrate, amylase, and gelatinase had positive reactions; lysine, and ornithine dihydrolase, Methyl Red, urease, H_2S gave a negative reaction. The isolates produce acid from glucose, arabinose, mannitol, sorbitol, and sucrose, but not from inositol, melibiose, and rhamnose (Table 1).

According to API 20E results, it was determined that our isolates showed positive reactions in beta-galactosidase, arginine, indole, citrate, Voges-Proskauer, gelatinase, glucose, sorbitol, sucrose, mannose, and arabinose tests; lysine decarboxylase, ornithine decarboxylase, H_2S, urea, tryptophan deaminase, rhamnose, melibiose, and amygdalin tests gave negative reactions and also inositol fermentation test varied among the isolates. Therefore, all isolates identified as *V. anguillarum* with 324752656 API 20E profile number.

As a result of the biochemical analyses of the isolates in BD Phoenix ID microbiological system, it was found that the isolated bacteria were identified as *Vibrio holisae*.

In MALDI-TOF MS analysis, the criteria for a successful identification were within a confidence score of ≥2.0 for the species level and ≥1.7 for the genus level according to the manufacturer. The recorded MALDI-TOF MS Biotyper scores of the isolates were between 2.3-2.1 indicating highly probable to secure species identification in the present study (Table 2).

For confirmation of all identification results, gene sequencing with 16S rRNA revealed that the representative strain was *V. anguillarum*. The sequence obtained in this study is defined as GenBank accession number OR432444.

The results of antibiogram susceptibility tests indicated that all the *V. anguillarum* strains were susceptible to enrofloxacin, erythromycin, florfenicol, streptomycin, oxytetracycline, sulfamethoxazole and resistant to ampicillin,

Table 1. Morphological, physiological and biochemical characterization of the isolates.

Characteristics	Isolates (n=10)	Characteristics	Isolates (n=10)
Gram staining	-	**Carbohydrate fermentations**	
Motility	+	Glucose	+
Cytochrome oxidase	+	Sucrose	+
Catalase activity	+	Mannitol	+
O/F	F	Melibiose	-
O/129 -150	S	Arabinose	+
Beta-galactosidase	+	Rhamnose	-
Methyl Red	-	Inositol	-
Voges-Proskauer	+	Sorbitol	+
Indole	+	**Amino acid decarboxylation**	
Citrate	+	Ornithine	-
Urease	-	Lysine	-
Gelatin	+	Arginine	+
Amylase	+		
H_2S	-		

F: Fermentative, S: Sensitive, +: Positive reaction, -: Negative reaction

Table 2. MALDI-TOF MS results of the isolates**.**

Analytic name	Organisms Best Match	Score value	Organisms (Best Match II)	Score value
+++ A	*V. anguillarum*	2.313	*V. anguillarum*	2.282
+++A	*V. anguillarum*	2.398	*V. anguillarum*	2.387
++A	*V. anguillarum*	2.133	*V. anguillarum*	2.133
++A	*V. anguillarum*	2.131	*V. anguillarum*	2.109
++A	*V. anguillarum*	2.115	*V. anguillarum*	2.037
++A	*V. anguillarum*	2.128	*V. anguillarum*	2.116
++A	*V. anguillarum*	2.285	*V. anguillarum*	2.218
++A	*V. anguillarum*	2.289	*V. anguillarum*	2.257
++A	*V. anguillarum*	2.349	*V. anguillarum*	2.253
++A	*V. anguillarum*	2.273	*V. anguillarum*	2.205

+++: highly probable species identification, ++: secure genus identification, probable species identification, A: species consistency

Table 3. Results of the antibiotic antibiogram susceptibility test of *V. anguillarum* strains.

Antibiotics	Resistance	Inhibition Zone Diameter (mm)
Ampicillin (10 µg)	R	12<13
Erythromycin (5 µg)	R	10<11
Flumequine (30 µg)	I	15<16<25
Chloramphenicol(30 µg)	R	0<15
Ciprofloxacin (1 µg)	I	15<20<25
Enrofloxacin (5 µg)	S	29>21
Florfenicol (30 µg)	S	23>19
Furazolidone (50 µg)	R	0<15
Kanamycin (30 µg)	R	12<15
Streptomycin (10 µg)	S	16 >15
Oxytetracycline (30 µg)	S	20>13
Sulfamethoxazole (25 µg)	S	22>17

(**R**: resistant; **I**: intermediate; **S:** sensitive)

chloramphenicol, furazolidone (Table 3).

Histopathologically, melano-macrophage centers, necrosis and peritubular edema in the kidney (Figure 2a); vacuolar degeneration and necrosis of hepatocytes, hyperemia and mononuclear cells infiltration in the liver (Figure 2b) melano-macrophage foci and focal necrosis in the spleen were observed. Severe mononuclear cell infiltration on muscles (Figure 3a) and talenjectiasis in the gill tissue (Figure 3b) were noted.

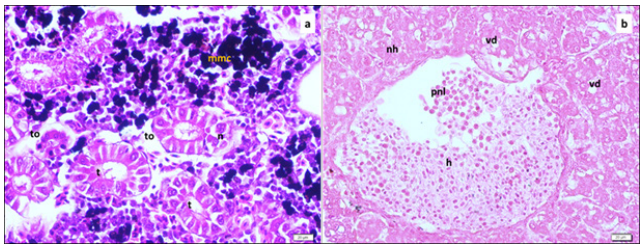

Fig. 2. Melano-macrophage centers (mmc), necrosis (n) and peritubular edema (to) in the kidney (a), vacuolar degeneration (vd) and necrosis of hepatocytes (nh), hyperemia (h) and polynuclear leukocyte (pnl).

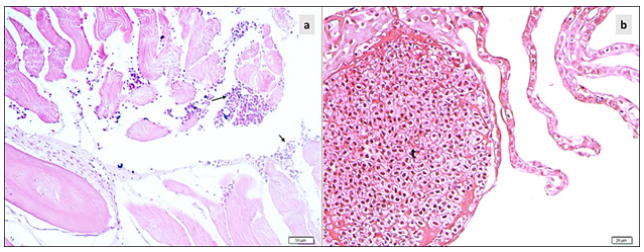

Fig. 3. Mononuclear cell infiltration (arrowed) on muscle (a),talenjectiasis (t) in the gill tissue (b).

4. Discussion

Vibrio anguillarum is the causative agent of vibriosis, a lethal haemorrhagic septicaemic disease affecting various marine and fresh/brackish water fish, bivalves, and also crustaceans. This pathogenic bacterium is responsible for serious economic losses in the aquaculture sector. There are reports that *V. anguillarum*, the causative agent of vibriosis, was isolated from many marine and freshwater fish such as salmon, rainbow trout, turbot, sea bass, and sea bream [2, 3].

Clinically, characteristics, especially in severe vibriosis, in addition to skin lesions, include swelling and abscesses in the muscles. In the terminal stage of the disease, these abscesses may burst and cause deep wounds [18]. Similar to previous reports, this study identified externally hemorrhagic ulcerative skin lesions and muscle hemorrhage as the main clinical findings in all diseased fish, a condition commonly referred to as red-pest. A less common symptom of infection is a hyperemic and protruding anus [19, 20]. Hyperemic and protruding anus, which is an indicator indicative of intestinal inflammation, was not detected in the diseased fish in this study. In the present study, diseased fish exhibited internally splenomegaly, along with diffuse petechial hemorrhage in internal organs, mainly in the liver, gas bladder, muscles, and also gills. These internal clinical findings align with the main clinical symptoms observed in rainbow trout infected with *V. anguillarum*, as reported by other investigators [2, 16, 18-20].

It has been reported in other studies that gills absorb pathogenic fish bacteria [21, 22], and that gills rich in blood vessels are identified as the primary site of *V. anguillarum* colonization [22]. *V. anguillarum* leads to acute mortality once it enters the fish's body due to its short incubation time and rapid systemic spread. The pathogen characterized by hemorrhagic septicemia easily reaches other organs through blood vessels. Internally, it causes widespread hemorrhages in internal organs [3, 20]. Similar morphological signs of hemorrhage and hyperemia in the internal organs (liver and gas bladder, muscles, and gills) (Figure 1d; Figure 3b) show that *V. anguillarum* reaches the other organs by blood vessels.

In the present study, the bacteria isolated from diseased rainbow trout were identified as *V. anguillarum*, exhibited a fermentative characteristic, and were sensitive to O/129 vibriostat test. Moreover, their physiological and biochemical characteristics were similar to the characteristics reported by other researchers [23-25],

Many commercially available diagnostic tests (API, BD Phoenix, Biolog MicroPlates, BBL Crystal E/NF, Bionor Aqua) kits form part of routine laboratory diagnostics [26]. Among these systems, API kits are the most used kits for the identification of fish pathogenic bacteria [27, 28].

In previous studies, it was reported that the use of citrate, gelatinase, sorbitol, rhamnose and amygdalin fer-

mentation gave false positive or negative reactions in the identification of *V. anguillarum* with the API 20E [29]. It has been reported that some strains may be incorrectly identified as the API system is not included in the database [27, 30]. However, similar to the results of studies conducted in recent years [9, 19, 31], it was determined that our isolates showed positive reactions in beta-galactosidase, arginine, indole, citrate, Voges-Proskauer, gelatinase, glucose, sorbitol, sucrose, mannose, inositol tests; lysine decarboxylase, ornithine decarboxylase, H_2S, urea tryptophan deaminase, rhamnose, melibiose, amygdalin and arabinose tests gave negative reactions. When the API 20E results was compared with conventional methods, it was determined that there were no differences between *V. anguillarum* strains. It was determined that API 20 E was variable in the inositol test and gave a negative reaction in the manual method. These results were similar to Balta and Balta, [23], Onuk et al [24].

The isolated bacteria were identified by the BD Phoenix system as *Vibrio holisae*. Saticioglu et al [32] reported that BD Phoenix panels were used in the identification of *Shewanella* sp., which causes lens atrophy in rainbow trout trout eyes. In addition, the BD Phonex system has been successfully used to identify pathogenic Gram-positive fish bacteria such as *Lactococcus garviaea*, *Staphylococcus aureus* [33] and vibriosis agents such as *Vibrio alginolyticus, Vibrio parahaemolyticus* isolated from diseased European sea bass (*Dicentrarchus labrax*) [34]. With this system, not only the identification of bacteria but also the antibiotic test can be performed. For this reason, it is often used in human and veterinary medicine [10]. However, this system was insufficient to identify the pathogenic bacteria isolated in this study.

It has been reported that MALDI-TOF MS is used in the identification of *Aeromonas salmonicida*, *Flavobacteriump sychrophilum*, *Yersinia ruckeri*, *Streptococcus agalactiae*, *Streptococcus iniae*, *Aeromonas hydrophila*, and *Aeromonas veronii*, which are bacterial fish pathogens that cause disease in rainbow trout [11, 35, 36]. Similarly, in this study, the identification of *V. anguillarum* was accomplished successfully with the MALDI-TOF MS. MALDI-TOF MS Biotyper identification score criteria are those recommended by the manufacturer: score ≥ 2,000 indicates species-level identification; score ≥1,700 and <2,000 indicate a genus-level description, and a score of <1,700 indicates the absence of a reliable description [37]. Therefore, a score of ≥ 2.00 was obtained in the identification of the isolated bacteria, and the species-level identification was successfully performed for *V. anguillarum* isolates.

Molecular methods are frequently used in the identification of pathogenic bacteria because conventional methods require a long time. The confirmation of the bacteria isolated in this study was made by 16S rRNA analysis. Conventional bacteriological methods including morphological, physiological and biochemical tests and also molecular techniques based on 16S rRNA sequencing have been reported to be the gold standard for identification of bacterial species [28, 38]. Nowadays, in the identification of pathogen fish bacteria, molecular biology methods such as 16S ribosomal RNA (rRNA) gene, sequencing polymerase chain reaction, and other related PCR-based methods are very popular. Gene sequencing with 16S rRNA revealed that the isolate was *V. anguillarum*. The sequence obtained in this study is defined as GenBank accession number OR432444.

The misuse of antibiotics in aquaculture leads to the development of resistance in bacteria and limits the treatment of the disease. Early detection and rapid administration of antibiotics with effective oral therapy are essential to reduce the effect of vibriosis [40]. It has been reported that *V. anguillarum* strains carry R-factor and develop resistance, especially to streptomycin, sulfonamides, and tetracycline via plasmids [3]. However, in the current study, it was determined that *V. anguillarum* strains were sensitive to oxytetracycline, streptomycin and sulfamethoxazole.

Histopathologically, although melano-macrophage centers in the kidney, vacuolar degeneration and necrosis of hepatocytes, hyperemia and mononuclear cells infiltration in the liver, melano-macrophage foci and focal necrosis in the spleen, severe mononuclear cell infiltration on muscles and talenjectiasis in the gill tissue were noted. These histopathological findings are similar to previous studies [4, 41]. However, mononuclear cell infiltration eosinophilic hyaline drops and hemosiderin deposits in proximal tubules epithelium generally reported in the kidney tissue in vibriosis [20, 42, 43] were not detected in this study as reported by Akaylı et al [25]. Ceylan et al. [20] reported severe mononuclear cell infiltration on primary lamella and desquamations on secondary lamellae in gill tissue; however, talenjectiasis was commonly observed in the gill tissue due to vascular changes in this study.

5. Conclusion

Identifying pathogenic bacteria causing disease is important for epidemiological research, antimicrobial therapy, and control. Although it is known that the traditional bacteriological method, which includes morphological, physiological, and biochemical tests, is the gold standard for the identification of bacterial species, the steps from sample collection to interpretation of biochemical results for culturing and identifying a bacterium usually take up to five days or 96 hours. Currently, automatic and semi-automatic systems are the most commonly used biochemical methods for identifying microorganisms in terms of time, cost, and workload. For this reason, it is necessary to expand the use of identification systems, which can provide fast, reliable, and cost-effective results in a short time, in the aquaculture sector. In this study, it was found that the MALDI-TOF MS system gave results in one hour in the identification of the pathogen *V. anguillarum*, which causes clinical and histopathological tissue damage; compared to other systems, it is more advantageous in terms of time, laboratory experience, and cost. In addition, the BD Phonex system was found to be inadequate for the identification of pathogenic bacteria.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the Van YüzüncüYıl Univer-

sity Animal Experiments Local Ethics Committee (2019).

Informed consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Cigdem Urku: Project administration, research design, conceptualization, investigation, writing – original draft, writing – review & editing; Faik Sertel Secer: Conceptualization, investigation, writing – review & editing; Sukru Onalan: Investigation, writing – review & editing; Tulay Akayli: Investigation, writing – review & editing

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