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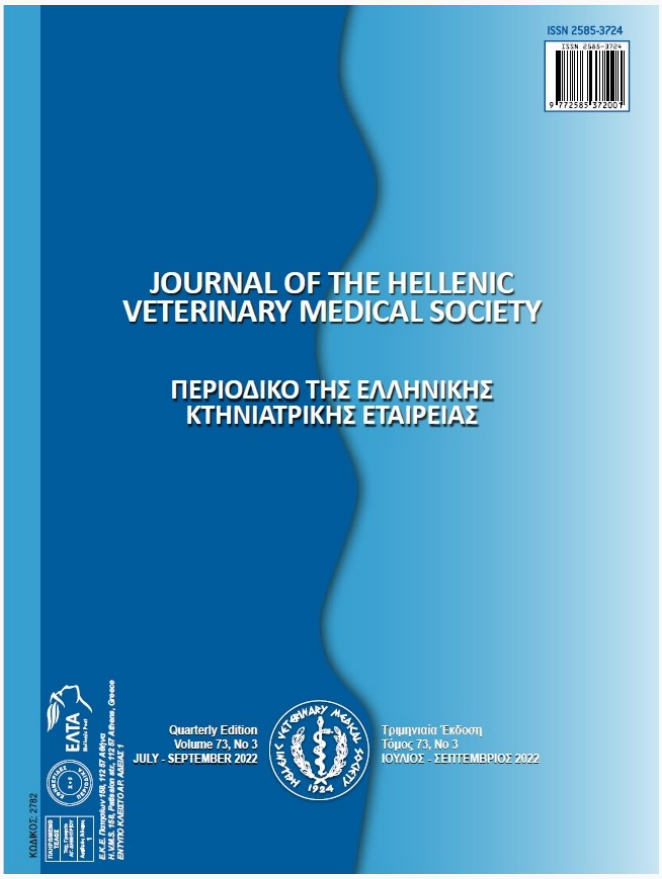

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Research article
Ερευνητικό άρθρο

First isolation of a *trh* positive *V. alginolyticus* from Atlantic bluefin tuna *(Thunnus thynnus)* farmed in Turkey

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ABSTRACT: Popularity of sushi-sashimi (raw-almost raw fish) has increased around the globe, and Atlantic bluefin tuna is among the most frequently used fish in these dishes. In this study, we used two adult Atlantic bluefin tunas sent to the laboratory from a farm in the Aegean region of Turkey in 2014. We isolated *trh* (Thermostable direct hemolysin-related hemolysin gene) positive *Vibrio alginolyticus* from the internal organs of these two tunas. Isolates were obtained by conventional microbiological methods and identified with Vitek 2 Compact fully automatic identification device. Confirmation of isolates and investigation of thermostable direct hemolysin (*tdh*) and tdh-related hemolysin (*trh*) genes were carried out by a Polymerase Chain Reaction (PCR) method. Two *V.alginolyticus* isolates from these two tuna samples were found to be *trh* positive and *tdh* negative. The aim of this study is to report first isolation of a *trh* positive *V. alginolyticus* from two bluefin tunas farmed in the Aegean sea, to draw attention to possible dangers in raw-almost raw fish consumption and supply information for further epidemiological studies. However, more samples are necessary for elucidating the prevalence of high virulence food pathogens in tuna. However, due to their economic value, multiple tuna samples are rarely sent to laboratories for diagnostic purposes.

Keywords: *Thunnus thynnus, V. alginolyticus, trh,* sushi, sashimi

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INTRODUCTION

Bluefin Tuna is of high economic value both around the world and in Turkey. There are seven species of tuna in Turkey: *Thunnus thynnus, Thunnus albacares, Euthynnus alleteratus, Thunnus alalunga, Thunnus obesus, Katsuwonus pelamis* and *Auxis rochei*. First tuna culture farm in Turkey was founded in 2002 and the number of facilities increases every year. These farms catch tuna from the wild during summer months and harvest occurs after animals are fed in net cages for 6-8 months (Captive Seawater Breeding business) (Karakulak, 2019). While Turkey's bluefin tuna export was 178,000 tonnes in 2018 (about 1 billion dollars), Ministry of Agriculture and Forestry targets $ 2 billion in 2023. Trajectory in the world is increasing rapidly (FAO, 2019).

An important reason for the increase in tuna culture is the popularity of sushi-sashimi restaurants. One of the most preferred fish types for sushi-sashimi dish served in Japanese restaurants is Atlantic bluefin tuna (*Thunnus thynnus*). This situation rapidly reduces the number of Atlantic bluefin tuna in the world and threatens its existence. In the last 50 years, 80% of the population was reported to have disappeared. In addition, sushi-sashimi consumption creates food poisoning risks in humans as these dishes are made of thinly sliced raw fish served alone or with rice, sauce and various vegetables. This type of servicing is not enough to kill bacteria in raw-almost raw fish meat. For this, fish must be cooked for 20-25 minutes at 180-190 ^{o}C.

Many types of bacteria have been reported, such as *Aeromonas, Vibrio, Photobacterium* and *Mycobacterium*. They were isolated from the muscles, spleen, liver and kidneys of tuna (Chen et al., 2004; Munday et al., 2003, Sousa and Silva-Souza, 2001; Austin, 2002, Austin and Austin. 2016; Banja, 2002; Valdenegro-Vega et al., 2013; Kapetanović et al., 2017). In addition, bluefin tuna is one of the largest predators in the sea; it consumes many marine animals and plenty of water, therefore their microbiota colonizes the tuna (Ottolenghi, 2008; Austin, 2002). For these reasons, consumption of sushi/sashimi made out of tuna may be risky for human health (Ben-Gigirey et al., 1999; Emborg et al., 2005; Vardić Smrzlić et al., 2012; Van Spreekens, 1977; Banja, 2002; Barralet, 2004, Reich, 2008).

A microbiology surveillance (1997) carried out on sushi-sashimi dishes in Japan reported bacterial flora including important food pathogens such as *Vibrio parahaemolyticus, Staphylococcus aureus, Salmonella spp, Listeria monocytogenes* in 13.8% of 1020 sushi samples and in 11.1% of 906 sashimi samples. *V.alginolyticus* is also a common pathogen in frozen fish products. It was among the most isolated bacteria in a study on frozen mussels and fish in Sweden (Scharer et al.,2011). Cohen et al. (2007) found a 72% prevalence in a study about *V.alginolyticus* in frozen fish products in Casablanca.

Some strains of *V.alginolyticus* also have the *trh*, *tdh* gene regions which are detected more commonly in *Vibrio parahaemolyticus*. *V.alginolyticus* with these gene regions can cause severe food poisoning in humans (Gargouti 2015; Gonzalez-Escalona 2006; Vijayakumar, 2017, CDC, 2005; Kapetanović 2017, Scharer et al.,2011). *V.alginolyticus* also produces histamine by decarboxylase activity and may damage the meat quality by producing histamine in Atlantic bluefin tuna (Middlebroks, 1998).

Trh positive *V.alginolyticus* was not previously reported in the Atlantic bluefin tuna. This may be due to parasitological studies being more popular in bluefin tuna or due to lack of further investigations on the presence of *tdh-trh* genes in *V.alginolyticus* isolates from these products. (Kapetanović, 2017). *V.alginolyticus,* commonly found in seawater, causes primary and secondary diseases in many marine animals (Austin, 2002; Balcázar, 2010; Lee, 1996; Liu, 2004; Harriague et al., 2008).

The aim of this study is to report the first isolation of a *trh* positive *V.alginolyticus* from two tuna fish farmed in the Aegean sea, draw attention to possible dangers in raw-undercooked fish consumption and supply data for further epidemiological studies.

MATERIALS AND METHODS

Samples

In this study, two adult Atlantic bluefin tunas were sent to the laboratory from a farm in the Aegean Sea in 2014. Sea water temperature was 16 ^{0}C (+, - 2). Total number of adult tuna in the cage was around 500, 10% of animal population were symptomatic (Figure 1) but there was no mortality. 10 mg / kg dose of florfenicol was added to the feed upon disease notification. On the second day, 2 symptomatic tunas were sent to the laboratory. Florfenicol was continued for ten days and significant improvement was observed after treatment.

Bacterial isolation and identification

Inoculations from internal organ samples (liver, spleen, kidney) were cultivated in Alkaline Peptone Water, 22 °C and 37 °C for 24 hours. Subsequent subcultures were made on TCBS (Thiosulfate Citrate Bile Sucrose -Merck) agar and petri dishes were incubated at 22 °C and 37 °C for 48 hours. Yellow colored, Gram negative, motile, oxidase positive colonies, 2-3 mm in diameter were streaked onto Marine agar and were incubated at 22 ° C for 24 hours (TS/TS ISO 8914**,** 1998; Austin and Austin, 2016). Then, fresh culture suspensions were prepared with saline in an appropriate density and loaded in Vitec 2 Compact full automatic identification device. The following day, *V. alginolyticus* results were obtained from the device for both isolates (with a 99% probability).

Confirmation

DNA was extracted from isolates with a commercial DNA Extraction kit (High Pure, Germany) according to manufacturer's protocol for positive isolates in Vitec 2. For confirmation, *gyrB* gene was targeted and PCR protocol suggested by Luo and Hu (2008) was used. *V.alginolyticus* ATCC 17749 was used as positive control and *V. anguillarum* ATCC 19264 was used as negative control. The primer sequence was designed as 5'-TCA GAG AAA GTT GAG CTA ACG ATT-3 '(AlgF1, forward) and 5'-CAT CGT CGC CTG AAG TCG CTG T -3' (AlgR1, reverse). The total volume for the PCR reaction was 25 µl; 5 µl genomic DNA, 0.4 µM AlgF1 (2 µl), 0.4 µM AlgR1 (2 µl), Taq DNA polymerase (5 units / µl) (0.40 µl) (MBI, Fermantas), 10xPCR buffer (2.50 µl), 50 mM MgCl2 (1.25 µl), 10 mM dNTPs (dCTP, dATP, dTTP, dGTP) (0.63 µl), 11.22 µl nuclease free water. The amplification program consisted of an initial denaturation at 94 ° C for 4 min; then 32 cycles of denaturation at 94 ° C for 30 s, annealing at 64 ° C for 30 s, extension at 72 ° C for 1 min, and a final extension at 72 ° C for 8 min. After PCR amplification, 4 µl of each product was loaded into a 1.0% agarose gel for electrophoresis. DNA size marker 100 DNA Ladder Plus (MBI Fermentas) was used. Bands were visualized with designated equipment.

Investigation of *trh* and *tdh* genes in isolates by PCR (PCR 1, *trh*; PCR 2, *tdh*)

Trh and *tdh* genes in isolates were additionally confirmed by two seperate PCR assays (PCR 1, 2). PCR assays were performed using primer pairs GGCTCAAAATGGTTAAGCG and CATTTCCGCTCTCATATGC for *trh* (PCR 1), CCATCTGTCCCTTTTCCTGC and CCAAATACATTTTACTTGG for *tdh* (PCR 2) according to the procedure described by Cohen et al. *Trh* positive *V. parahaemolyticus* DNA extracts were used as positive control while distilled water was used as negative control.

Reaction volume consisted of, 5 µl genomic DNA, 5 µM *TRH*-L primer (2.0 µl), 5 µM *TRH*-R primer (2.0 µl), Taq DNA polymerase (5 units / µl) (0.40 µl) (MBI, Fermantas), 10xPCR buffer (2.50 µl), 50 mM MgCl2 (1.25 µl), 10 mM dNTPs (dCTP, dATP, dTTP, dGTP) (0.63 µl), 11.22 µl nuclease free water for PCR 1 (*trh*)

For PCR 2 (*tdh*) the master mix was; 5 µl genomic DNA, 5 µM *TDH*-L primer (1 µl), 5 µM *TDH*-R primer (1 µl), Taq DNA polymerase (5 units / µl) (0.40 µl) (MBI, Fermantas), 10xPCR buffer (2.50 µl), 50 mM MgCl2 (1.25 µl), 10 mM dNTPs (dCTP, dATP, dTTP, dGTP) (0.63 µl), 13.22 µl nuclease free water.

The reactions (PCR 1, 2) were performed with an automated thermocycler as follows: initial denaturation at 94 ° C for 5 min., followed by 40 cycles of denaturation at 94 ° C for 30 sec., annealing at 58 ° C for 45 sec., and primer extension at 68 ° C for 75 sec. A final extension was performed at 68 ° C for 7 min. PCR products were run by electrophoresis on 2% (w⁄v) agarose gel (1 hour, 75 volts). DNA size marker 100 DNA Ladder Plus (MBI Fermentas) was used. Bands were visualized with designated equipment.

RESULT AND DISCUSSION

Sample photo of first tuna with hemorrhages is supplied in Figure 1. Macroscopic and microscopic examination, isolation and identification results of both tuna samples are given in Table 1. Results of PCR studies are shown in Figures 2, 3 and 4.

Necropsy findings such as enlargement of internal organs (spleen, liver, kidney), hemorrhages in the head, gills, fins and intestines are consistent with the general clinical presentation of vibriosis (Austin and Austin, 2016). Only the first fish had hemorrhages in the intestines. Gram stain made from the internal organs of the second fish revealed weaker bacterial colonization. These may be due to the second fish responding better to antibiotic treatment or receiving antibiotic supplemented feed at a higher rate.

Figure 1: Sample photo of one of the tuna brought to the laboratory (No 1). Hemorrhage in the gill, fin and mouth area.\

Table 1: Examination results

Sample	Macroscopy	Necropsy	Gram Stain from internal organs	Bacterial isolation from internal organs	Isolate
No 1	Hemorrhage on gills, mouth, fins	Hemorrhage on intestines; enlarged liver, spleen, and kidney	Gram negative slightly curved rods in liver and spleen (Dense)	Spleen (No isolation from kidney and liver)	*Trh* Positive *V.alginolyticus*
No 2	Hemorrhage on gills and mouth	Enlarged liver, spleen and kidney	Gram negative slightly curved rods in liver and spleen (Less Dense)	Spleen (No isolation from kidney and liver)	*Trh* Positive *V.alginolyticus*

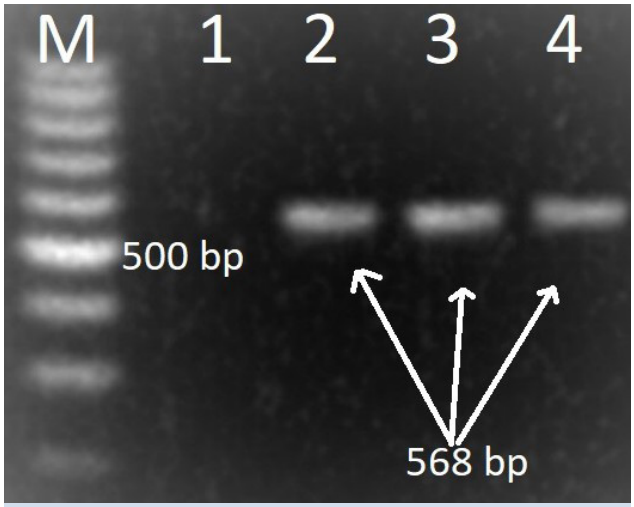

Figure 2. Confirmation of *V.alginolyticus* isolates with PCR. M: Marker, 100 bp. Line 1: Negative control *Vibrio anguillarum* ATCC 19264. Line 2: Positive control *V.alginolyticus* ATCC 17749, 568 bp. Line 3, 4: Isolates (No 1, 2), 568 bp(*gyrB*).

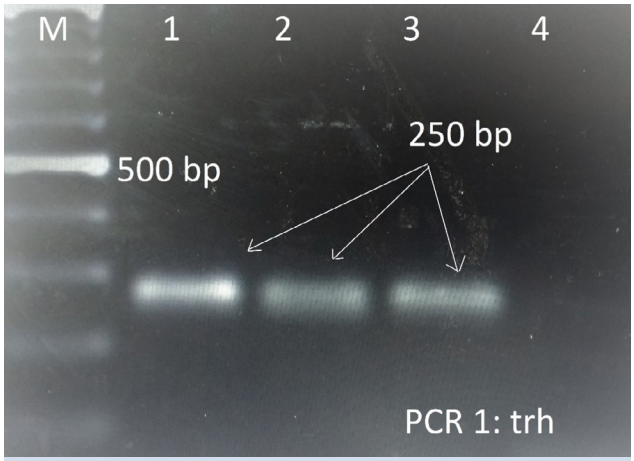

Figure 3: PCR 1, *trh*. M: Marker 100 bp. Line 1: *trh* positive control (Terzi et al, 2009), 250 bp. Line 2, 3: *trh* positive isolates (No 1, 2), 250 bp. Line 4: Negative control distilled water.

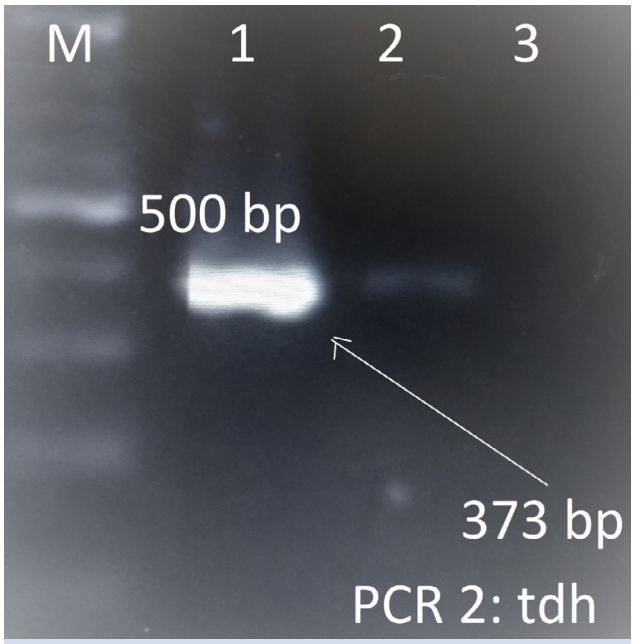

Figure 4. PCR 2, *tdh*. M: Marker 100 bp. Line 1: *tdh* positive control (Terzi et al, 2009), 373 bp. Line 2, 3*: tdh* negative isolates (No 1, 2)

Although bacterial colonization of the spleen, kidney and liver was observed in both fish, isolation could only be made from the spleen. This may be due to the fact that antibiotics are more concentrated in the liver and kidney and suppress bacterial growth in these organs. However, despite the application of antibiotics in both fish, bacteria were isolated. This may be because both fish have had a limited 2 day exposure to antibiotics or insufficient uptake of antibiotic supplemented feed.

Florfenicol was administered to the fish at a dose of 10 mg / kg. as an antibiotic feed additive. Difficulties in isolating bacteria from tuna at the end of the second day suggests bacterial susceptibility to florfenicol. As the case was followed up, after the completion of 10 day florfenicol treatment, other fish were seen to have recovered. Bacterial fish pathogens are known to have developed resistance to antimicrobials in recent years. Despite this, florfenicol is still applied successfully in many bacterial infections. However, inappropriate flofenicol treatments may trigger resistance development (Santos and Ramos, 2018).

Although vibriosis is caused by many species, *V.alginolyticus* is one of the most common disease agents in marine animals (Balcázar, 2010; Lee, 1996; Liu, 2004; Harriague et al., 2008). However, parasitic diseases of tuna hold a more significant place than bacterial diseases and have been researched more thoroughly (Munday et al., 2003; Mldineo, 2006). This may be the reason for lack of data on *V.alginolyticus* isolations from tuna. In this study, existence of symptoms and isolation of *V.alginolyticus* from both tuna are consistent with the prevalence of *V.alginolyticus* infections in marine animals.

Tdh, trh pathogenicity genes are rarely investigated in *V.alginolyticus* isolates. This may be because these genes are mostly asssociated with *V. parahaemolyticus*. However, pathogenicity genes can be horizontally transferred between different *Vibrio* spp. (Deng et al., 2019). On the other hand, *V.alginolyticus* is not considered to be as dangerous as a food pathogen as *V. parahaemolyticus* or *V. cholerae*. Therefore, *V.alginolyticus* used to receive less attention from researchers. However, *V.alginolyticus* has recently started to stand out as an "emerging pathogen" in food poisoning cases (Ravikumar and Vijayakumar, 2017).

In this study, *trh* positive *V.alginolyticus* was isolated from Atlantic bluefin tuna that were cultivated and harvested for human consumption. If *V.alginolyticus* positive tuna are consumed without or little heat treatment (sushi-sashimi techniques), they pose a serious risk of food poisoning. Additional treatments such as salt addition or saucing are not enough to kill bacteria in raw fish. Safe consumption of all kinds of meat products is possible by cooking them at a sufficient temperature and time. On the other hand, any food pathogen in the internal organs of fish can also contaminate other fish during cleaning. In our study, initial isolation of *V.alginolyticus* was carried out at 22 ºC. Although the incubator in Vitek device was 37 oC, this presented no obstacles in bacterial identification as the growth range of *V.alginolyticus* is quite wide. It can also grow in storage conditions and in the human body (Austin and Austin, 2016; Gargouti 2015, Vijayakumar, 2017). In addition, as a single tuna is often shared by many people, even a few fish can make a large population sick .

Trh positive *V. alginolyticus* was mostly detected and reported in bivalve molluscs. There were isolates in bluefin tuna kept in storage conditions but it was not previously reported as an infectious agent in this species. Our study provides new data in this aspect. However, more samples need to be studied to verify the prevalence of high virulence food pathogens in bluefin tuna. However, tuna sample size submitted for diagnostic purposes to laboratories is generally limited to 1-2 fish because of its economic value. There-

fore, having 2 tunas in our study does not limit the importance of our findings.

As a result, in 2014, *trh* positive *V. alginolyticus* was isolated for the first time from two Atlantic bluefin tunas sent to the laboratory from a farm in Turkey's Aegean region (Izmir). We also aimed to draw attention to public health risks posed by sushi-sashimi prepared from raw/ undercooked tuna and to contribute to existing scientific literature.

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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