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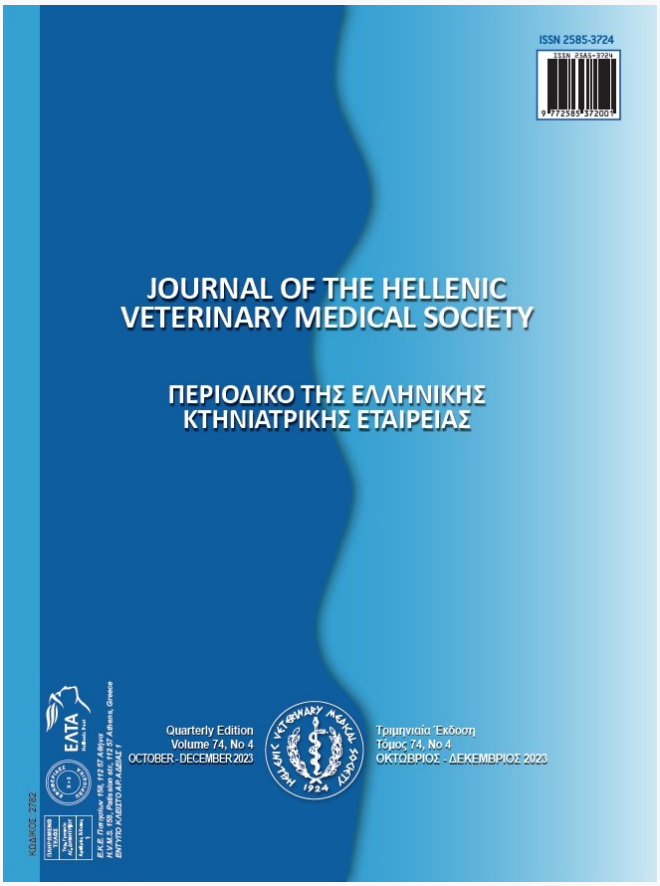

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ML Avsever, S Tunalıgil

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Research article
Ερευνητικό άρθρο

First isolation of *trh* positive *Vibrio alginolyticus* from *Engraulis encrasicolus* in Turkey

M.L. Avsever[1], S. Tunalıgil[2]

[1]*Manisa Celal Bayar University, Akhisar Vocational High School, Plant and Animal Production Department, Manisa-Turkey*

[2]*İzmir Bornova Veterinary Control Institute, Veterinary Biological Products Control Department, İzmir, Turkey*

ABSTRACT: *Engraulis encrasicolus* is the most popular fish species among consumers in Turkey especially during winter months. *E. encrasicolus*, which is generally found in public markets, is a small fish that takes a long time to clean and therefore is often marketed without cleaning. Although large fish are exclusively handled by sellers, more people come into contact with smaller fish such as *E. encrasicolus* during home cleaning. This means that a zoonosis in fish such as *E. encrasicolus* could pose a widespread public health concern. *Vibrio alginolyticus*, which has virulence genes, can particularly cause wound infection through wounds and abrasions on people's hands while cleaning fish. It can lead to severe food poisoning when it enters the body orally from hands contaminated with pathogens or from the environment. Thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) are the gene regions that significantly increase the pathogenicity of *V.alginolyticus.*In this study, a total of 200 (20 from each sale point) *E. encrasicolus* purchased from 10 different sale points in 4 different public markets were investigated bacteriologically. *V. alginolyticus* was isolated from the internal organs (spleen, kidney, liver) of 14 fish (7%) from 4 sale points in two public markets and was identified by biochemical methods. The isolates were then confirmed by PCR and *trh-tdh* virulence genes were investigated. While the *trh* was detected in 6 of 14 isolates (42.8 %), *tdh*was not found in any of them (0%). In this study, *trh* positive *V. alginolyticus* was isolated from *E. encrasicolus* for the first time in Turkey. It should be emphasized that zoonotic agents in *E. encrasicolus* may cause widespread public health problems. However, there is need for more comprehensive studies to be conducted especially in the Çanakkale province and Black Sea region where*E. encrasicolus* are extensively harvested.

Key Words: Aegean region; *Engraulis encrasicolus*; Thermostable direct hemolysin (*tdh*); *tdh* -related hemolysin (*trh*), *Vibrioalginolyticus*

Corresponding Author:
M.L. Avsever, Akhisar Vocational High School, Plant and Animal Production Department, Manisa-Turkey.
E-mail address: meric.avsever@cbu.edu.tr.

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INTRODUCTION

Engraulis encrasicolus, of the *Engraulidae* family, is partially found in temperate waters and is generally caught in the Black Sea and Çanakkale Straits in Turkey (Türksönmez and Diler, 2019). According to the fisheries statistics for 2020, 291.910 tons of *E. encrasicolus* wereharvested from Turkish waters and it is the pelagic sea fish species which is caught the most (TUIK, 2020). It has been reported that the total sum of *E. encrasicolus* catch in 2021 was 171.253 tons (TUIK, 2021). Again, in numerous scientific studies conducted in many cities in Turkey, *E. encrasicolus* ranks first among the most consumed marine fish (Selvi et al., 2022; Kuşat and Şahan, 2021; Deniz and Sarıözkan, 2020; Yüksel and Diler, 2019). The consumption of *E. encrasicolus* has spread from the Black Sea region to the rest of the country despite low consumption rate of other fish. *E. encrasicolus* may bethe only fish species consumed by the majority in Turkey.

Vibrioalginolyticus is the major pathogen of fish and bivalves; it is rare in humans and does not cause a serious infection except to immunosuppressed people. However, it can cause serious food poisoning to humans, when it has virulence genes such as *trh* and *tdh*. Therefore, *V. alginolyticus* is considered an emerging pathogen (Xie et al., 2005; Smolikova et al., 2001; W. Winn Jr., et al., 2006). In Turkey, large fish such as sea bass, sea bream, mullet, bonito, trout and carp are generally sold after they are cleaned by the sellers. However, small fish such as *E. encrasicolus* are often sold without cleaning,as they are cheaper and harder to clean. That's why only sellers come into contact with large fish, while smaller fish, such as *E. encrasicolus*, are handled by more people in their homes. *V. alginolyticus* can infect the eyes and ears of people who handle the fish through contact and wounds on the hands.It can cause food poisoning directly by taking the hand to the mouth, and indirectly by splatter of the fish parts to the greens (arugula, cress, parsley) served as a side dish to the fish (Rubin and Tilton , 1975; Pien et al., 1977; Lee et al., 2008; Reilly et al., 2011; Neill and Carpenter, 2010).

The first *trh* positive *V. alginolyticus* in the world was reported in Alaska and Tunisia (Narjol et al., 2006; Ben kahla-Nakbi et al., 2006). The first *trh* positive *V. alginolyticus* isolation in Turkey was reported by Avsever from bivalve molluscs in 2016 (Avsever, 2016). Both in Turkey and in the world, *trh* and *tdh* genes have mostly been studied in *Vibrio parahaemolyticus* (Terzi et al., 2009; Türk et al., 2011; Leoni et al., 2016; Raghunath, 2015;Terzi-Gulel and Martinez-Urtaza, 2023).There are fewer studies investigating *tdh* and *trh* genes in *V. alginolyticus* (Gonzales-Escolana et al., 2006; Avsever, 2016, 2022) as *tdh* and *trh* are important virulence factors in the pathogenesis of mainly *V. parahaemolyticus*. However, in recent studies, *V. alginolyticus* was also seen to rapidly increase its pathogenicity and was reported to transfer *V.parahaemolyticus* virulence genesto itself (Gonzales-Escolana et al., 2006).

Although *V. alginolyticus* infections have been reported from humans (Baran et al., 2016; Citil et al., 2015) and aquatic organisms (Demir, 2012; Dolgun, 2022) in Turkey, *trh*, *tdh* genes were often uninvestigated. This makes it difficult to understand the prevalence of *V.alginolyticus* positive for virulence genes in Turkey. The presence of *trh-tdh* genes of *V.parahaemolyticus* were investigated in *Sarda sarda, Merlangius merlangus* and frozen *E. encrasicolus* samples in Turkey (Doğruer et al., 2022). However, there are no reports of *trh* (+) *V. alginolyticus* in *E. encrasicolus* fromTurkey.

The aim of this study is to report *trh* positive *V. alginolyticus* isolation in *E. encrasicolus* for the first time in Turkey and to draw attention to the fact that home-cleaning *E. encrasicolus* may cause widespread public health problems.

MATERIAL AND METHODS

Sampling

In this study, 200 fish (11 cm +-1) (20 from each sale point) of *E. encrasicolus* purchased from 4 different public markets and 10 different sale points in Izmir were used. The average air temperature in December, the month when sampling took place, was 12 (+- 2) ^{o}C. Each sample was placed in a separate small sterile bagand transported to the laboratory under cold chain.

Pathogen Isolation and Identification

Internal organs (liver, spleen and kidney) of each fish were cut into parts with sterile scissors and inoculated in Alkaline Peptone Water (APW). APW were incubated at 37 °C for 24 hours. Followed by subculture into TCBS (Thiosulfate Citrate Bile Sucrose -Merck) agar. Petri dishes were incubated at 37 ^{o}C for 48 hours. Gram-negative, oxidase-positive, motile colonies of 2-3 mm diameter were transferred to TSA (Triptic Soy Agar) and biochemical identification was

made from the colonies on TSAafter incubation for 24 hours at 37 °C. 14 isolates identified biochemically (TS/TS ISO 8914, 1998; Austin and Austin, 2016).

Molecular Identification of Pathogens

These isolates were confirmed with PCR(Polimerase Chain Reaction). DNA isolation was carried out from these isolates using a commercial DNA extraction kit (High Pure, Germany). Isolates were thereby confirmed as described by Luo and Hu (2008) with primers targeting the *gyrB* gene in *V. alginolyticus*. *V. alginolyticus* ATCC 17749 was used as a positive control. The primer sequence used in the study was 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; 0.4 µM AlgF1 (2 µl), 0.4 µM AlgR1 (2 µl), 5 µl genomic DNA, 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), and 11.22 µl nuclease free water. The PCR was performed as follows; an initial denaturation at 94 ° C for 4 min; 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 amplification product was loaded into a 1.0% agarose gel for electrophoresis. DNA size marker 100 DNA Ladder Plus (MBI Fermentas) was used. After electrophoresis, bands were visualized with designated equipment.

Investigation of *trh* and *tdh* genes

Primers used were the forward and reverse primers. (PCR) primers, targets and amplification sizes are shown in Table 1.

Vibrio parahaemolyticus DNA samples (Terzi et al.2009) were used as positive control, while negative control consisted of distilled sterile water. For PCR (*trh*), 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) and 11.22 µl nuclease free water (Total 25 µl) . For PCR (*tdh*), the master mix consisted of; 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) and 13.22 µl nuclease free water (Total 25 µl). PCR was performed as follows: initial denaturation at 94 ° C for 5 min., 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. Final extension was at 68 ° C for 7 min. PCR products were evaluated with electrophoresis on a 2% (w/v) agarose gel (1 hour, 75 volts). DNA size marker 100 DNA Ladder Plus (MBI Fermentas) was used. Visualisation of bands was carried out with designated equipment.

RESULTS

A total of 200 *E. encrasicolus* (20 from each sale point) purchased from 10 different sale points in 4 different public markets in Izmir province were investigated bacteriologically. *V. alginolyticus*was isolated from the internal organs (spleen, kidney, liver) of 14 fish (7%) from 4 sale points in two public markets. Hemorrhages on the head, operculum, abdomen (Fig. 1) and enlarged internal organs (liver/spleen) were overall observations. Enlargment of the liver-spleen was observed in all 14 fish from which *V. alginolyticus* was isolated. However, hemorrhages on the body surface and enlargement of the liver-spleen were also noted in some fish from which *V. alginolyticus* could not be isolated. Isolates were identified by biochemical methods. All isolates showed the same biochemical patterns. Biochemical identification results are provided in Table 1. After the isolates were confirmed by PCR (Fig.2), *trh* and *tdh* virulence genes were investigated (Fig. 3-4). While the *trh* gene region was detected in 6 of the 14 isolates (42.8 %), *tdh* gene was not found in any of them (0%). *Trh* positivity rate in *V. alginolyticus* isolates were33.33% , 33.33%, 0%, 80% and *trh* positive *V. alginolyticus* were found in 2.5%, 1.66%, 0%, 4% of the samples from four markets (A, B, C,D), respectively (Table 2).

Table 1. (PCR) primers, targets and amplification sizes

PCR Primers	Targets	Amplification sizes	Literature
GGCTCAAAATGGTTAAGCG -CATTTCCGCTCTCATATGC	*Trh*gene	250 bp	Cohen et al. (2006)
CCATCTGTCCCTTTTCCTGC -CCAAATACATTTTACTTGG	*Tdh*gene	373 bp	Cohen et al. (2006)

Table 2. Biochemical properties of *V. alginolyticus* isolates

Gram	+	**Urease**	_	**LDC**	+	**Growth in 22°C**	+	**MR/VP**	+/+
Oxidase	+	**ONPG**	_	**ADH**	_	**Growth in 37°C**	+	**Growth in %0 NaCl**	–
Motility	+, Flagellar	**Indole**	+	**10µg O/129**	Resistant	**Mannitol**	+	**Growth in %3 NaCl**	+
Colony color in TCBS	Green	**Hemolysis in Blood Agar**	+	**150 µg O/129**	Sensitive	**ODC**	+	**Growth in %8 NaCl**	+
Glucose	+	**Lactose**	_	**Oxidation/ Fermentation**	+/+	**H_2 S/Gas**	_		

Table 3: Molecular analyzes by market place and sales points

Public Markets	A			B			C				D			
Sale Points	a	b		c	d	e	f	g	h	i			j	
Isolate No:	1	2	3	Not isolated	4, 5	6	Not isolated	7, 8, 9	Not isolated	10	11	12	13	14
gyrB	+	+	+		+	+		+		+	+	+	+	+
Trh	+	-	-		-	+		-		+	+	+	-	+
Tdh	-	-	-		-	-		-		-	-	-	-	-

Figure 1: External lesions such as haemorrhages in the head (A), operculum (B) and the abdominal area (C) of fish infected with *V. alginolyticus*

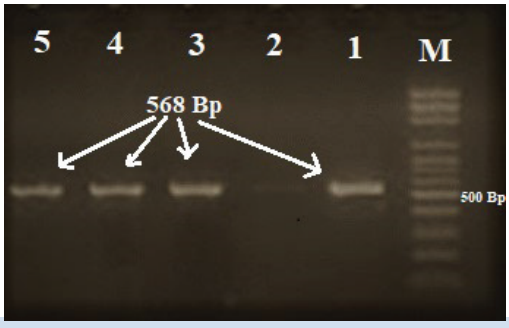

Figure 2: Results from *gyrB* PCR : Marker, 100 bp. Line 1: Positive control *V. alginolyticus* ATCC 17749, 568 bp. Line 2 Negative control distilled water. Line 3, 4, 5: Isolates (No 1,2,3), 568 bp (*gyrB*).

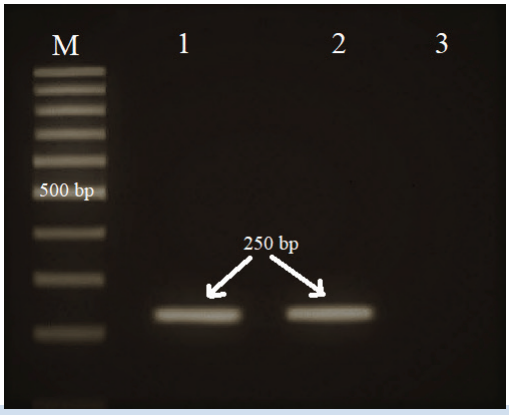

Figure 3: PCR results for virulence gene *trh*. M: Marker 100 bp. Line 1: *trh* positive control (Terzi et al, 2009), 250 bp. Line 2: *trh* positive isolate sample (No 1), 250 bp. Line 3: Negative control distilled water.

DISCUSSION

Avsever (2022) had previously reported the isolation of *trh* positive *V. alginolyticus* from black mussels in Izmir and Çanakkale regions. In this study, *trh* positive *V. alginolyticus* was isolated from anchovies caught from the Çanakkale province and Black Sea region. According to these studies, *trh*-positive *V. alginolyticus* appears to be present in mussels from the Aegean Sea, the Dardanelles and the Black Sea. On the other hand, Terzi and Martinez (2016) detected *trh* positive *V.parahaemolyticus* in *Merlangius merlangus* and *Sarda sarda* samples from the Black Sea between 2006 and 2010. Thus, the *trh* gene may have been transferred from *V.parahaemolyticus* to *V. alginolyticus*. The findings of another report by Türk et al., (2011) also supports this possibility in which the *trh* or *tdh* gene was not detected in *V. parahaemolyticus* isolates from bivalve molluscs in the Aegean region in 2011. In conclusion, these studies show that the *trh* gene may have originated from the Black Sea. Since *E. encrasicolus* is mostly caught from the Black Sea, the presence of*trh* gene may be common in the *Vibrio*s of this fish species. Similarly, in an unpublished study investigating the mucilage problem in the Marmara Sea (Anonymous, 2021), it was stated that the dominant pathogen genus that decomposes mucilage was *Vibrio* and with *V. alginolyticus* being the most common species within the genus. However, all this is an assumption and should be investigated in more regions and more fish species in order to be proven.

It should also be noted that some fish species such as anchovy and sardines (*Sardina pilchardus*) are sold in Turkish markets without being cleaned (Anonymous, 2021). This practice causes more people to come into contact with it during cleaning at home. This study as well as other similar ones may lead to the requirement of anchovy and sardines to be precleaned in the market. A legislative action is considered by the authors to be beneficial in terms of public health. There are no reports found on *trh* positive *V. alginolyticus* isolates in sardines as in anchovies. Dogruer et al., (2022) detected *V. parahaemolyticus* in frozen sardine products, but did not investigate the presence of *trh-tdh* gene.

The biochemical structure of the isolates were similar to other studies (Ardıç and Özyurt 2002; Demir, 2012; Baran et al., 2022). However, there were also differences in the types of biochemical tests performed in these studies. However, biochemical properties do not affect the pathogenicity of pathogens as much as virulence gene regions. Serological tests, which are more deterministic on pathogenicity than the biochemical properties were not performed in this study. This was due to the imcreased cost of serological studies and the recent prominence of molecular studies. On the other hand, PCR detects the pathogen instead of a serological history of the infection and is usually able to detect its presence much earlier in the course of an infection.

We isolated *V. alginolyticus* from all four of the four public markets (A,B,C,D) (100%), while there was no isolation of *trh* positive *V. alginolyticus* in one (C). On the other hand, in one market, our *trh* positive *V. alginolyticus* isolation rate was 80% among *V. alginolyticus* isolates. There was no consistent response from the fishermen in the markets C and D when asked if they obtained their fish from different regional sources (Western Black Sea, East Black Sea etc.) However, we were informed that the fish were purchased from wholesale fish sales points in Izmir. This difference in the two markets is thought to be coincidental. To clarify this, it is necessary to repeat the study at least a few times and the origin of the fish in wholesale markets should be investigated through new research projects or by other researchers. Thus, *E. encrasicolus* health status in different section of the Black Sea can be elucidated.

Clinical symptoms seen in fish were overall compatible with infections caused by *Vibrio* spp. However, the fact that we detected hemorrhaging fins, skin and enlarged internal organs (liver/spleen) in fish negative for*V. alginolyticus* shows that there may be other pathogens in the collected samples. On the other hand, since the aim was to search for *Vibrio* species carrying the *trh-tdh* gene in these samples, the isolates that were not found to be *Vibrio* by TCBS and O 129 tests were not included in the evaluation. Therefore, different pathogens may have played a role in the lesions in fish. *Vibrio* spp. other than *V. alginolyticus* were not isolated in the samples used in the study. This may be due to the fact that *V.parahaemolyticus* is rare and fish infected with more pathogenic pathogens agents such as *Vibrioanguillarum* may have been eliminated because of the high mortality rate.

In Turkey, *V. alginolyticus* isolation from humans (Ardıç and Özyurt, 2002; Citil et al., 2015; Baran et al., 2022) was also reported. In order to determine whether the cause of these human cases is related to handling of fish such as *E. encrasicolus* at home, a full investigation should be carried out with evalu-

ating patient history, including possible contact with sea food followed by confirmatory phylogenetic analysis. This can be accomplished through joint multidisciplinary studies of both veterinarians and medical doctors.

CONCLUSION

As a result, in this study, *trh* positive *V. alginolyticus* was isolated for the first time in *E. encrasicolus* in Turkey and potential public health problems which may arise after handling and cleaning *E. encrasicolus* at home are emphasized.

CONFLICT OF INTEREST

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

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