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First isolation of *Vibrio furnissii* (emerging Vibrio) from mussels (Mediterranean mussel and bearded mussel) in Turkey

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Summary: *Vibrio furnissii* is an emerging pathogen that can cause acute gastroenteritis in humans. In this work, 652 bivalve mollusc samples (wedge shell-*Donax trunculus,* L.1758, oyster-*Ostrea edulis,* L.1758, cockle-*Venus verrucosa,* L.1758, clam-*Tapes decussatus,* L.1758, bearded mussel-*Modiolus barbatus,* L.1758, Mediterranean mussel-*Mytilus galloprovincialis* L.1819, striped venus-*Chamelea gallina* L.1753) sent to the laboratory from stations in Ayvalık and Balıkesir (Northern Aegean Region of Turkey) between 2007-2010 were researched with conventional microbiological methods and six isolates were identified as *Vibrio furnisii.* Isolates were later confirmed with PCR using primers specific to *toxR* gene. Isolation rate of *V. furnisii* from bivalve molluscs per years were 2007 (2%), 2008 (0.43%), 2009 (1.29%), 2010 (0%) and per shell fish species were mediterranean mussel (3.33%), bearded mussel (3.27%), oyster (3.27%), clam (0%), cockle (0%), wedge shell (0%), striped venus (0 %). In this work, *V. furnissi* was isolated from mussels in Turkey for the first time and attention was drawn zoonotic importance of the agent.

Keywords: Bearded mussel, emerging Vibrio, Mediterranean mussel, *Vibrio furnissii.*

Türkiye'deki midyelerden (Kara midye ve kıllı midye) ilk *Vibrio furnissii* (emerging Vibrio) izolasyonu

Özet: *Vibrio furnissii* insanlarda akut gastroenteritise sebep olan ve önemi gittikçe artan bir patojendir. Bu çalışmada, 2007-2010 yılları arasında, Ayvalık ve Balıkesir'deki (Türkiye'nin Kuzey Ege bölgesi) istasyonlardan laboratuvara gönderilmiş 652 takım çift kabuklu yumuşakça (kum şırlanı-*Donax trunculus,* L. 1758, yassı istiridye-*Ostrea edulis,* L. 1758, kidonya-*Venus verrucosa,* L. 1758, akivades-*Tapes decussatus,* L. 1758, kıllı midye -*Modiolus barbatus,* L. 1758, kara midye - *Mytilus galloprovincialis* L. 1819, kum midyesi, *Chamelea gallina* L.1753) konvansiyonel mikrobiyolojik yöntemlerle araştırıldı ve çalışmada izole edilen altı izolatın *V. furnissi* olduğu tespit edildi. İzolatlar daha sonra *toxR* genine spesifik primerler kullanılarak PCR ile konfirme edildi. Çalışmada kullanılan çift kabuklu yumuşakçalardan *V. furnissii* izolasyon oranı yıl ve tür bazında 2007 (% 2), 2008 (% 0.43), 2009 (% 1.29), 2010 (% 0); kara midye (% 3.33), kıllı midye (% 3.27), istiridye (% 3.27), akivades (% 0), kidonya (% 0), kum şırlanı (% 0), kum midyesi (% 0) olarak tespit edildi. Bu çalışmada Türkiye'deki midyelerden ilk kez *V. furnissi* izole edilmiş olup, etkenin zoonotik önemi vurgulanmıştır.

Anahtar sözcükler: Emerging Vibrio, kara midye, kıllı midye, *Vibrio furnissii.*

Introduction

Vibrio furnissii, which is a gram negative halophyllic bacterium, was originally thought to be an aerogenic (able to produce gas from glucose) strain of *Vibrio fluvialis*. *V. fluvialis* and *V. furnissii* were first described in 1977 after isolation from diarrheic patients and from environmental sources (14). In 1983, however, *V. furnissii* was shown to be a distinct species by genetic analysis (19, 20). It consists of two circular chromosomes (3.2 Mb, 1.6 Mb) and reveals novel genes likely to be involved in pathogenicity (7).

V. furnissii unlike *V. cholerae, V. parahaemolyticus* and *V. vulnificus* is not a lethal human pathogen (7). Still, *V. furnissii* is classified as an "emerging vibrio" (16, 17, 21), which can be pathogenic for humans and has been implicated in occasional outbreaks of acute gastroenteritis in which mortalities have been reported (9, 10, 25, 34). Recent studies have shown that *V. furnisii,* as a result of climate change, is a potential threat especially to people living in developing tropical coastal areas (21). *V. furnissii* is also capable of causing infections in bivalve molluscs and other aquatic organisms and posesses various virulence factors (1, 3, 24).

Conventional microbiological methods are frequently used in the identification of *V. furnissii* (8, 16). Molecular methods such as PCR are reported to be faster with lower costs and are also less laborious. Still, during identification with conventional and molecular methods *V. furnisii* has to be discriminated from *V. fluvialis* (19, 33). Primers developed by Schirmeister et al. (28) targeting a specific region in the *toxR* gene successfully discriminates between *V. furnisii* and *V. fluvialis* as well as 41 members

of the *Vibrio* genus. *ToxR* gene is involved in the regulation of virulence-associated genes in several *Vibrio* species (23, 27).

Incidence of *V. furnissi* in bivalve molluscs is less than that of *V. parahaemolyticus* and *V. alginolyticus.* Matte et al. (24), reported the incidence of various *Vibrio* spp. isolated from oysters to be *V. alginolyticus* (81%), *V. parahaemolyticus* (77%), *V. cholerae* non-01 (31%), *V. fluvialis* (27%), *V. furnissii* (19%), *V. mimicus* (12%), and *V. vulnificus* (12%), respectively. However, *V. furnissii* was often isolated from human feces (10, 15, 21). Demir (11), has been reported of *V. furnissi* isolated from oyster in Turkey but there have yet been no reports of *V. furnisii* isolated from musels in Turkey. The aim of this work is to investigate the presence of *V. furnissii* from bivalve molluscs and to draw attention to the zoonotic importance of this pathogen.

Materials and Methods

In this work, 652 bivalve mollusc sample groups sent from production stations to the laboratory between 2007-2010 (wedgeshell-*Donax trunculus*,L.1758, oyster-*Ostrea edulis*, L.1758, cockle-*Venus verrucosa*, L.1758, clam-*Tapes decussatus*, L.1758, bearded mussel-*Modiolus barbatus*, L.1758, Mediterranean mussel-*Mytilus galloprovincialis* L.1819, striped venus-*Chamelea gallina*, L.,1753) were used. Sampling was done throughout the year with the exception of summer (May 1st-August 31st) when shellfish collection was prohibited. Average water temperature was 20ºC (± 2 °C) during sampling seasons. Samples were sent to the lab after being seperated and grouped according to species.

Isolation and identification: For pre-enrichment bivalve mollusc tissue homogenates were inoculated into Saline Water (ASP) and were incubated at 37°C for 18-24 hours. Inoculations were made on Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) (Sigma-Aldrich) agar from this enrichment. After 24 hour incubation at 37°C, the colonies oxidase positive and yellowish color in TCBS were investigated further with conventional methods (4, 16, 18). Accordingly, isolates positive for methyl red (1% NaCI), arginine (1% NaCI), citrate (Simmons) test, glucose, mannose and sucrose fermentation, gas production in glucose, growth at 25 and 37ºC and negative for Voges-Proskauer (1% NaCI), indole (1% NaCI), H_2S on Triple Sugar Iron (TSI) (1% NaCI), urea (1% NaCI) (Sigma-Aldrich), lysine (1% NaCI), ornithine (1% NaCI) were identified as *V. furnissii*.

Confirmation with PCR: DNA extraction was carried out from six isolates with a commercial kit (High Pure PCR Template Preparation Kit- Rosche Life Science, Germany; Lot: 11054300) previously identified as *V. furnissii* with conventional microbiology and grown on Tryptic Soy Agar (TSA) (Sigma-Aldrich) according to the manufacturer's instructions. *toxR* was determined to be the target gene for confirmation and the PCR protocol reported by Schirmeister et al. (27) was used. *V. furnissii* ATCC 35016 was used as a positive control, *V. anguillarum* ATCC 19264 was the negative control. The primer sequences were Vfurn-toxR2-fo AGACGCTGATCTCGATCCAC and Vfurn-toxR2-re TTGTCAAAGACCGCCAGAC. PCR amplification was performed in a 25 µl volume with 1 × PCR buffer (2 mM $MgCl_2$), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.2 µM of each primer, 1.5 U Taq DNA polymerase (MBI, Fermantas) and 2 ng of template DNA. The *toxR* PCR assay specific for *V. furnissii* was performed for 30 cycles at 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 20 s preceded by an initial incubation at 94°C for 5 min and followed by a final extension step at 72°C for 7 min (Techne, TC-412). After PCR amplification, 4 µl of each product was added into a 1.0 % agarose gel, electrophoresed (Thermo, Primo^{TM}). DNA size marker 100 DNA Ladder (MBI Fermentas) was used. Bands were visualised with designated equipment (Vilber Lourmant, E-BOX VX5).

Results

From 652 shellfish sample groups used in this research *V. furnissii* was isolated from six (0.92%). Information on bivalve molluscs from which the isolates were obtained are supplied in Table 1. Phenotypic properties of isolates are in Table 2 and gel electrophoresis of PCR confirmation is supplied in Figure 1. *V. furnissii* isolation rates according to year and species are as follows; 2007 (2%), 2008 (0.43%), 2009 (1.29%), 2010 (0%); mediterranean mussel (3.33%), bearded mussel (3.27%), oyster (3.27%), clam (0%), cockle (0%), wedge shell (0%), striped venus (0%).

As a result of this work, 652 bivalve mollusc samples from production stations in Ayvalık and Balıkesir provinces of Turkey between 2007-2010 were investigated with conventional and molecular methods and six isolates (2 Bearded mussels, 2 Mediterranean mussels, 2 oysters) were identified as *V. furnisii.*

Discussion and Conclusion

Food-borne infections and toxications due to the consumption of raw or under-cooked shellfish are common. The symptoms seen in these cases are mainly, abdominal cramps, sicknes, vomiting, fever and severe headache (12). Within lethal human pathogens isolated from shellfish and which cause food infection. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* comes first (7). *V. furnissii* on the other hand is an "emerging vibrio" and its pathogenicity in humans is open to debate.

Table 1. Information on bi-valve mollusc samples from which *V. furnisii* were isolated and number of isolates.
Tablo 1. *V. furnissii* izole edilen çift kabuklu yumuşakçalara ait bilgiler ve izolasyon sayıları

	2007			2008			2009			2010		
	T*	P**	N***	T	P	N	T	P	N	T	P	N
Clam	44	-	-	48	-	-	44	-	-	37	-	-
Mediterranean mussel	43	2	MR (3, 4)	14	-	-	-	-	-	3	-	-
Oyster	50	1	MR 5	50	1	MR 6	61	-	-	-	-	-
Bearded mussel	23	1	MR 1	24	-	-	14	1	MR 2	-	-	-
Cockle	22	-	-	24	-	-	22	-	-	20	-	-
Wedge Shell	-	-	-	13	-	-	13	-	-	7	-	-
Striped venus	18	-	-	58	-	-	-	-	-	-	-	-
Total	200	4		231	1		154	1		67		
Distrubition of isolation (%)	2 %			0.43 %			1.29 %			0 %		

T* Bivalve mollusc samples P**:*V. furnissii* positive bivalve mollusc samples N*** Isolate names.

Table 2. Phenotypic properties of *V. furnissii* isolates.
Tablo 2. *V. furnissii* izolatlarının fenotipik özellikleri.

Strains	MR 1	MR 2	MR 3	MR 4	MR 5	MR 6
Source of isolation	Berded mussel	Berded Mussel	Mediterraneanmussel	Mediterraneanmussel	Oyster	Oyster
Gram stain	-	-	-	-	-	-
Oxidase production	+	+	+	+	+	+
Motilite	+	+	+	+	+	+
Growth on TCBS media	+, yellow	+, yellow	+, yellow	+, yellow	+, yellow	+, yellow
Utilization of citrate	+	+	+	+	+	+
Utilization of glucose (1% NaCI)	+	+	+	+	+	+
Utilization of sucrose (1% NaCI)	+	+	+	+	+	+
Utilization of arabinose (1% NaCI)	+	+	+	+	+	+
Utilization of lactose (1% NaCI)	-	-	-	-	-	-
Gas production from glucose	+	+	+	+	+	+
Voges-Proskauer reaction	-	-	-	-	-	-
Methyl red (1% NaCI)	+	+	+	+	+	+
Arginine (1% NaCI)	+	+	+	+	+	+
Ornithine (1% NaCI)	-	-	-	-	-	-
Lysine (1% NaCI)	-	-	-	-	-	-
Indole production	-	-	-	-	-	-
Urea	-	-	-	-	-	-
Growth on 0 % NaCl	-	-	-	-	-	-
Growth on 1 % NaCl	+	+	+	+	+	+
Growth on 3 % NaCl	+	+	+	+	+	+
Growth on 6 % NaCl	+	+	+	+	+	+
Growth on 10 % NaCl	-	-	-	-	-	-
Growth on 12 % NaCl	-	-	-	-	-	-
Growth 4 °C	-	-	-	-	-	-
Growth 22 °C	-	-	-	-	-	-
Growth 37 °C	+	+	+	+	+	+
ONPG test	+	-	-	+	+	-

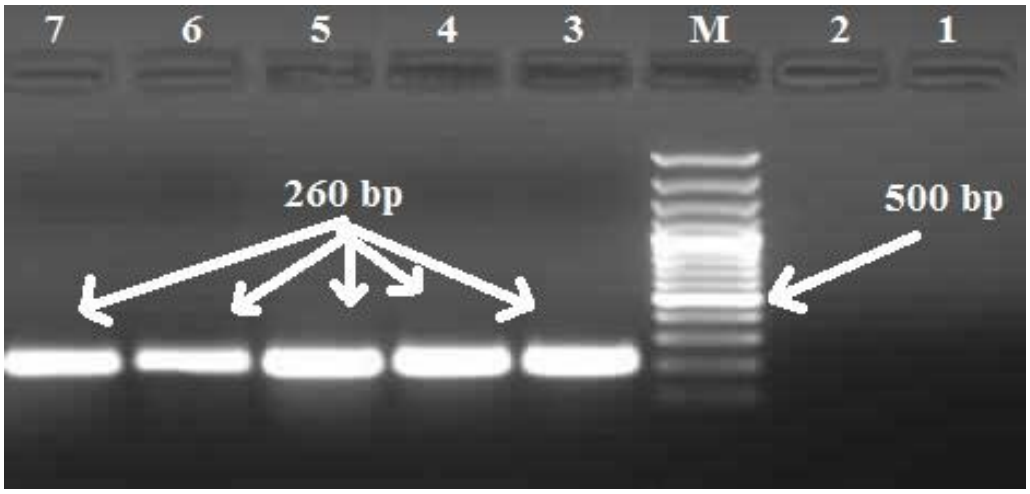

Figure 1. PCR confirmation of *V. furnisii* isolates. M: Marker, 100bp. Line 1: Negative control *Vibrio anguillarum* ATCC 19264. Line 2: Negative control, distilled water. Line 3: Positive control *V. furnissii* ATCC 35016, 260bp. Line 4-7: Isolates (MR1, 2, 3, 4), 260bp.
Şekil 1. *V. furnissii* izolatlarının PCR ile doğrulanması. M: Marker, 100bp. Hat 1: Negatif kontrol *Vibrio anguillarum* ATCC 19264. Hat 2: Negatif kontrol, distile su. Hat 3: Pozitif kontrol *V. furnissii* ATCC 35016, 260 bp. Hat 4-7: İzolatlar (MR 1, 2, 3, 4), 260 bp.

Still, recent reports have shown a higher potential for *V. furnisii* to be a food pathogen (10, 21). Therefore, isolation of *V. furnisii* from bivalve molluscs has increasing importance in terms of human health.

Amin et al. (2), in a work with 225 shellfish, 20 channel water and fecal swab samples from diarrhei patients have been isolated *V. parahaemolyticus* (2.6%), *V. vulnificus* (6.6 %), *V. fluvialis* (12%), *V. hollisae* (2.6%), *V. furnissii* (6.6%), *V. mimicus* (6.6%), *V. alginolyticus* (10.6%) and *V. damsella* (9.3%). In this work, *V. furnissii* isolation rate was 0.92% (6/652). This isolation rate was lower than Amin et al. (2). The reason for this, only shellfish were investigate in this work. *V. furnissii* may be isolated from human feces at a high rate (2, 10, 22). *V. furnissii* isolation from bivalve molluscs may also indicate fecal contamination in the sea. Still, more important parameters such as coliform count should be addressed before reaching a conclusion.

V. furnissii isolation rates were found by Amin et al. (2) to be 6.6% in shrimps and 6% in oysters whereas Sung et al. (29) found *V. furnissii* isolation rate to be 15% in shrimps in Taiwan. *V. furnisii* isolation rates in this work were found to be lower than both; mediterranean mussel (3.33%), bearded mussel (3.27%), oyster (3.27%), clam (0%), cockle (0%) , wedge shell (0%), striped venus (0%). This might be due to the other members of *Vibrio* genus being more common in shellfish in Turkey. Previous research has revealed *V. alginolyticus* (5) and *V. parahaemolyticus* (30, 32) to be the dominant species.

V. furnisii may also cause infections in shellfish (3, 24). Still, research has shown that bacterial agents affect bivalve molluscs mostly during the larval stage. Among pathogenic *Vibrio* that affect shellfish; *V. alginolyticus, V. anguillarum, V. splendidus, V. tapetis, V. tubiashi* are foremost (26). Tubiash (31) found that *V. anguillarum* (strain ATCC 1909, concentrations between 10^6 and 10^7 cells in 1 ml) is highly pathogenic to experimentally infected bivalve larvae, causing 90% mortalities after 48 hours. Important bacterial agents that affect mature shellfish are within *Rickettsia, Chlamydia* and *Mycoplasma* genus (6, 15, 26). In this work, no outbreaks were reported from sampling stations. This might be an indication of stocks being healthy or due to the difficulty larval diseases of shellfish.

On the other hand, phenotypic characteristics of *V. furnissii* isolates obtained in this work are compatible with other reports (8, 13, 16). Although isolates were mostly homologous in terms of phenotypic characteristics, when ONPG results are taken into account, at least two may be considered to be of different phenotypes. All isolates were positive for gas production from glucose, therefore easily differentiated from *V. fluvialis*.

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