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Genetic Heterogeneity and Antibiotic Susceptibility of *Vibrio alginolyticus* Strains Isolated from Horse-Mackerel (*Trachurus trachurus* L., 1758)

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Abstract: This study was performed to investigate the presence of halophilic *Vibrio* spp. in horse-mackerel (*Trachurus trachurus* L., 1758). In total, 80 fish samples were collected from fish markets and supermarkets between 04.04.2006 and 16.05.2006 in the city of Mersin. All fish samples were microbiologically tested for halophilic *Vibrio* spp. Nine (11.25%) *Vibrio* spp. strains were isolated and identified as *V. alginolyticus*. Molecular epidemiological discrimination and antimicrobial susceptibility patterns of *V. alginolyticus* strains were also analysed by arbitrarily primed polymerase chain reaction (AP-PCR) and Kirby-Bauer disc diffusion, respectively. Genotypic heterogeneity of these 9 isolates discriminated by AP-PCR determined that while only 3 isolates were from the same genotypic source the other 6 isolates were from different sources. All isolates were resistant to ampicillin, cephalothin, and streptomycin, and susceptible to levofloxacin, ofloxacin, cefotaxime, nalidixic acid, tetracycline, piperacillin-tazobactam, chloramphenicol, imipenem, and aztreonam. One isolate was resistant to cefepime, 1 to nitrofurantoin, and 1 to ciprofloxacin. One of the 9 isolates was susceptible to erythromycin and 1 to novobiocin, and 3 isolates were susceptible to sulbactam-cefoperazone.

Key Words: *Vibrio alginolyticus*, *Trachurus trachurus*, AP-PCR

İstavrit (*Trachurus trachurus* L., 1758) Balığından İzole Edilen *Vibrio alginolyticus* Suşlarının Genetik Heterojenitesi ve Antibiyotik Hassasiyeti

Özet: Bu çalışma istavrit cinsi (*Trachurus trachurus* L., 1758) balıklarda halofilik *Vibrio* türlerinin varlığının araştırılması amacıyla gerçekleştirilmiştir. Mersin ilindeki balık pazarları ve süpermarketlerden 04.04.2006 ve 16.05.2006 tarihleri arasında toplam 80 balık örneği toplanmıştır. Bütün balık örnekleri halofilik *Vibrio* türleri yönünden mikrobiyolojik olarak incelenmiştir. Dokuz adet (% 11,25) *Vibrio* türü izole edilmiştir ve bunların hepsi *V. alginolyticus* olarak tanımlanmıştır. *V. alginolyticus* suşlarının moleküler epidemiyolojik ayrımı arbitraril primed-polimeraz zincir reaksiyonu (AP-PZR) ve antibiyotik duyarlılıkları Kirby-Bauer disk difüzyon metotları ile incelenmiştir. AP-PZR ile genotipik heterojenite ayrımı gerçekleştirilen bu 9 izolattan, 3 tanesinin aynı genotipik kaynaktan, diğer 6 izolattan da farklı kaynaktan köken aldığı ortaya çıkarılmıştır. İzolatların tümü ampisilin, sefalotin ve streptomisine dirençli ve levofloksasin, ofloksasin, sefotaksim, nalidiksik asit, tetrasiklin, piperasilin-tazobaktam, kloramfenikol, imipenem ve aztreonam'a karşı duyarlı olarak bulunmuştur. İzolatlardan biri cefepime'e, biri nitrofurantoin ve biri de siprofloksasin'e dirençli bulunmuştur. Dokuz izolattan birinin eritromisin, birinin novobiosin ve üçünün sulbactam-sefaperozon'a duyarlı olduğu tespit edilmiştir.

Anahtar Sözcükler: *Vibrio alginolyticus*, *Trachurus trachurus*, AP-PZR

Introduction

The genus *Vibrio* includes more than 35 species, mostly in marine (1) and natural habitats of seawater, and this species is broadly distributed throughout the

world (2). There are halophilic marine bacteria of this genus known to cause diseases in humans. *Vibrio parahaemolyticus* has long been known to cause foodborne gastroenteritis associated with seafood (3).

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Among other halophilic vibrios, *V. alginolyticus*, *V. fluvialis*, and *V. metschnikovii* are also pathogenic for humans, while *V. anguillarum* is a pathogen for fish and other marine animals (1). A fairly less common organism, *Vibrio alginolyticus*, also causes wound infections after recent exposure to seawater, and is responsible for septicaemia and death (3).

V. alginolyticus is a curved, gram-negative bacillus, motile with a single polar flagellum and is a facultative anaerobic organism. *V. alginolyticus* is commonly found in coastal waters and sediments all over the world (4-6). This organism is considered a part of normal marine flora (7). With the exception of 2 reports from the United States, earlier cases came from Greece, Spain, Scandinavia, and the Black Sea, emphasising the widespread presence of *V. alginolyticus* in ocean waters (6).

Many papers describe isolation of *V. alginolyticus* from soft tissue infections (4,8,9). Wound infections and ear infections are usually mentioned. Eye infections are mentioned much less frequently. The data show the definite association of *V. alginolyticus* with infection at these sites; however, many authors list *V. alginolyticus* as a pathogenic *Vibrio* species, particularly associated with wound and ear infections. Antibiotic treatment has been used in most cases and surgical debridement has been used in some (8). Feingold and Kumar (6) and Ardiç and Özyurt (10) reported that *V. alginolyticus*, an organism typically found in seawater, is an unusual cause of otitis media.

More recently, attention has been focused on the halophilic vibrios, *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus*, and their relationship to human disease, because halophilic vibrios are increasingly recognised as important intestinal and extra-intestinal pathogens (11). Infections due to halophilic vibrios are generally acquired through ingestion of contaminated raw or undercooked shell-fish or seafood or by direct invasion through skin wounds (11,12). However, in contrast to the others, *V. alginolyticus* has infrequently been isolated from human infections despite its widespread saprophytic existence in coastal waters (11).

Disease due to this organism undergoes a significant increase during warmer months and mainly affects persons who have had direct contact with seawater or who have handled shellfish (7). *V. alginolyticus* is a species that is widely distributed in coastal waters of

temperate and tropical regions (13). Furthermore, as the growth speed of this organism is fairly high, if *Trachurus trachurus* is contaminated with *V. alginolyticus* and has not been appropriately stored, distributed, and processed, it may play an important role in food poisoning. For this reason, our study was performed in the warm season. The aim of this study was to investigate the prevalence, genotypic discrimination, and antibiotic susceptibility of potentially pathogenic halophilic *Vibrio* spp. for humans in *Trachurus trachurus* obtained from fish markets and supermarkets in Mersin province.

Materials and Methods

Sample collection and preparation for analysis

Marine fish samples were collected during 2 months (April and May) in 2006. In total 80 marine fish samples were included in this study and these samples were simultaneously collected as 10 units in fish markets and 10 units in supermarkets over 2 weeks (Table 1). Whole fish samples with internal organs were brought to the laboratory on ice and processed within 24 h. Samples were taken from muscular tissues and part of the intestines.

Table 1. Study groups.

Study groups	Date	Sample number	
		Supermarket	Fish market
Group 1	04.04.2006	10	10
Group 2	18.04.2006	10	10
Group 3	02.05.2006	10	10
Group 4	16.05.2006	10	10
Total		80 samples	

Isolation and identification of *Vibrio* species

Firstly muscle and intestine samples were homogenised in sterile porcelain dishes under sterile conditions. Homogenised tissue samples were inoculated in alkaline peptone water (pH 8.6) with 3% NaCl (w/v) and incubated at 35-37 °C for 8 h (14,15). Then the fluid cultures were transported and inoculated on thiosulphate citrate bile sucrose (TCBS) agar (Merck), in order to observe the separated colonies for each bacteria type. Petri dishes were incubated at 35-37 °C for 18 h. All

colony types (yellow, green, and blue) were evaluated on TCBS agar plate and growing colonies were subcultured on Tryptic Soy Agar (TSA) (Merck) supplemented with 3% NaCl (w/v) and incubated at 35-37 °C for 24 h. Gram negative, oxidase and catalase positive motile rods were used for identification. Growing on bile, lysine decarboxylase and ornithine decarboxylase, methyl red, Voges Proskauer, citrate and indol tests were used for conventional biochemical identification of *Vibrio* species. Biochemical characterisation was performed according to standardised methods (16-19). Furthermore, growth of isolates was examined in tryptone soya (TS) broth (Merck) supplemented with 0%, 6%, 8%, and 10% NaCl (20). We also used the ID 32 GN system (AUX medium; BioMerieux-France) for identification of bacterial colonies.

Antibiotic susceptibility patterns

Drug resistance patterns of bacterial isolates were determined by disc diffusion on Mueller-Hinton agar (Merck) supplemented with 3% NaCl. The diameters of the inhibition zones were measured and used for categorisation of the strains as susceptible, intermediate, or resistant as described by the National Committee for Clinical Laboratory Standards (21). Discs (OXOID) of levofloxacin (5 µg), ofloxacin (5 µg), cefotaxime (30 µg), nalidixic acid (30 µg), cefepime (30 µg), tetracycline (30 µg), erythromycin (15 µg), piperacillin-tazobactam (110 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), novobiocin (30 µg), imipenem (10 µg), ampicillin (10 µg), ciprofloxacin (5 µg), aztreonam (30 µg), cephalothin (30 µg), streptomycin (10 µg), and sulbactam-cefoperazone (30-75 µg) were used for antibiotic susceptibility testing.

Genotypic discrimination

Genotypic heterogeneity of *V. alginolyticus* isolates was determined by arbitrarily primed polymerase chain reaction (AP-PCR).

Bacterial DNA preparation

A rapid DNA extraction procedure was performed for *V. alginolyticus* colonies on Muller-Hinton plates. A loopful of organism was suspended in 1 ml of sterile distilled water. Then bacteria were lysed by boiling for 20 min at 80 °C. The cells were centrifuged (12,000 × g for 5 min) and supernatant was discharged. The pellet was mixed with 200 µl of chloroform and 200 µl of sterile water. Then the mixture was centrifuged at 12,000 × g

for 10 min. The supernatant was used as a template for amplification.

AP-PCR amplification

A universal primer (M13) was chosen from a method book by Durmaz and Ayan (22). Two microlitres of DNA solution were amplified in a 50 µl reaction containing 75 mM Tris-HCl pH 8.8, 0.2 mM dNTPs (Sigma, DNTP-100), 1.5 mM MgCl₂ (Promega, A3513), 0.5 mM of universal primer M13 (5'-GAGGGTGGCGTTCT-3'), and 1 U of *Taq* polymerase (Sigma, D1806). The PCR amplification was started with 2 cycles of 5 min at 94 °C and 5 min at 40 °C and 72 °C, followed by 40 cycles of 60 s at 94 °C, 60 s at 40 °C, and 60 s at 72 °C and also placed in a thermal cycler (Eppendorf Mastercycler, Hamburg, Germany). PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

Relatedness of bacterial isolates was determined according to Tenover et al. (23). The AP-PCR DNA band patterns of the isolates were compared with each other to determine their relatedness. In this technique, epidemiologically related isolates generate unique fragment patterns and epidemiologically unrelated isolates generate indistinguishable or, on occasion, closely related fragment patterns (23).

Results

Vibrio alginolyticus strains were detected in 9 of 80 (11.25%) fish samples from 2 different markets (Table 2). All isolates belong to group 1 and 4. Three strains, 1 of which was isolated in a supermarket in group 1, and 6 strains, 3 of which were isolated in a fish market in group 4.

Table 2. Isolated *V. alginolyticus* strains from different groups.

Study groups	No. of collected samples	No. of isolated <i>V. alginolyticus</i> strains
Group 1: A	10	1
B	10	2
Group 2: A	10	None
B	10	None
Group 3: A	10	None
B	10	None
Group 4: A	10	3
B	10	3
Total	80	9

A: Supermarket; B: Fish market

All isolates were resistant to ampicillin, cephalothin, and streptomycin, and susceptible to levofloxacin, ofloxacin, cefotaxime, nalidixic acid, tetracycline, piperacillin-tazobactam, chloramphenicol, imipenem, and aztreonam. One isolate (isolate 5) was resistant to cefepime, 1 (isolate 8) to nitrofurantoin, and 1 (isolate 2) to ciprofloxacin. One (isolate 6) of the 9 isolates was susceptible to erythromycin and 1 (isolate 5) to novobiocin, and 3 isolates (isolates 2, 3, and 5) were susceptible to sulbactam-cefoperazone.

These data are summarised in Table 3.

Although all isolates were obtained from fish from the same geographical areas, genotypic similarity was not detected between group 1 and group 4 by AP-PCR (Figure). Although all group 1 isolates were represented by the same band patterns, all isolates in group 4 were represented by distinct band patterns.

Discussion

The halophylic vibrios reach important concentrations during July and August, as the water temperature rises in

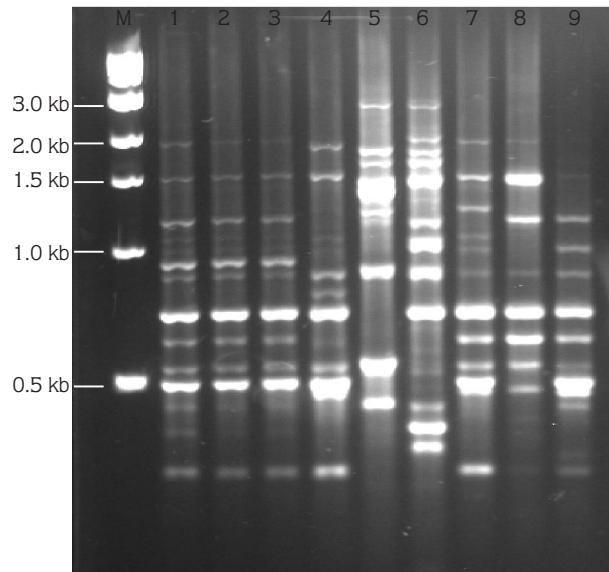


Figure. AP-PCR band patterns of *V. alginolyticus* strains isolated from fish. Lane M: 1 kb DNA ladder (Biolabs; #N3232S) lane 1: isolated strain (Isolate 1) in A in group 1, Lane 2-3: isolated strains (Isolate 2, 3) in B in group 1, lane 4-6: isolated strains (Isolate 4, 5, 6) in A in group 4 and lane 7-9: isolated strains (Isolate 7, 8, 9) in B in group 4.

Table 3. Antibiotic susceptibility patterns of *V. alginolyticus* strains.

Antibiotics	Measured zone diameter (mm) and susceptibility status of isolates									
	<i>V. alginolyticus</i> isolates									
	1	2	3	4	5	6	7	8	9	
Levofloxacin	19 (S)	20 (S)	20 (S)	21 (S)	17 (S)	18 (S)	18 (S)	18 (S)	18 (S)	
Ofloxacin	17 (S)	19 (S)	18 (S)	21 (S)	18 (S)	18 (S)	17 (S)	18 (S)	17 (S)	
Cefotaxime	22 (S)	22 (S)	21 (S)	22 (S)	19 (S)	25 (S)	18 (S)	22 (S)	25 (S)	
Nalidixic acid	18 (S)	20 (S)	20 (S)	21 (S)	20 (S)	22 (S)	20 (S)	21 (S)	22 (S)	
Cefepime	22 (S)	23 (S)	23 (S)	24 (S)	14 (R)	21(S)	17 (S)	17 (S)	22 (S)	
Tetracycline	20 (S)	21 (S)	21 (S)	24 (S)	22 (S)	24 (S)	21 (S)	17 (S)	20 (S)	
Erythromycin	11 (R)	10 (R)	11 (R)	14 (R)	11 (R)	16 (S)	11 (R)	0 (R)	11 (R)	
Piperacillin-Tazobactam	25 (S)	26 (S)	24 (S)	28 (S)	20 (S)	20 (S)	24 (S)	20 (S)	25 (S)	
Nitrofurantoin	20 (S)	20 (S)	20 (S)	22 (S)	20 (S)	17 (S)	19 (S)	11 (R)	22 (S)	
Chloramphenicol	24 (S)	25 (S)	21 (S)	30 (S)	25 (S)	27 (S)	24 (S)	23 (S)	25 (S)	
Novobiocin	10 (R)	9 (R)	14 (R)	11 (R)	20 (S)	9 (R)	10 (R)	15 (R)	7 (R)	
Imipenem	20 (S)	25 (S)	25 (S)	29 (S)	20 (S)	23 (S)	22 (S)	22 (S)	20 (S)	
Ampicillin	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	
Ciprofloxacin	19 (S)	12 (R)	20 (S)	20 (S)	17 (S)	20 (S)	17 (S)	20 (S)	17 (S)	
Aztreonam	22 (S)	20 (S)	21 (S)	21 (S)	20 (S)	21 (S)	19 (S)	19 (S)	18 (S)	
Cephalothin	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	
Streptomycin	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	
Sulbactam-Cefoperazone	0 (R)	16 (S)	16 (S)	14 (R)	19 (S)	14 (R)	12 (R)	14 (R)	12 (R)	

S: Susceptible; R: Resistant

the summer and the occurrence of human infections also increases during the late summer months (3). Therefore, we collected fish samples in April and May; this period is a fairly warm season in this region. *Vibrio alginolyticus* strains were detected in 11.25% fish samples from 2 different markets (Table 2). All of the 4 isolates (44.4%) were detected in the supermarket and the others (55.6%) were detected in the fish market. Although hygienic conditions were poor in the fish markets, there were no significant differences between the supermarkets and fish markets.

Human diseases caused by *V. alginolyticus* include ear, soft tissue, and wound infections (4). Wound infections account for 71% of *V. alginolyticus* infections. Gastroenteritis was thought to be a rare presentation of *V. alginolyticus* infection, but it accounted for 12% of infections in one series (24). Antibiotic resistance in *V. alginolyticus* is a significant problem for wound infections related to the aquatic environment. Recent reports stated that 40% of *V. alginolyticus* isolated from the environment produce β -lactamases conferring resistance to ampicillin (4). In this respect, all *V. alginolyticus* isolates in our study were resistant to ampicillin (Table 3).

Other clinical syndromes reported in association with *V. alginolyticus* infection include chronic diarrhoea in a patient with AIDS, conjunctivitis, and post-traumatic intracranial infection. Resistance to tetracycline and chloramphenicol has been reported in a few isolates of *V. alginolyticus*, but all strains appear to be sensitive to ciprofloxacin (24). In contrast, all isolates were susceptible to tetracycline and chloramphenicol, but only one isolate was resistant to ciprofloxacin in our study (Table 3). Thus, antimicrobial resistance of this natural isolate could be an important problem for therapy of these serious infections caused by *V. alginolyticus*.

V. alginolyticus was reported to be the most prevalent species within *Vibrio* spp. in mussels and seawater samples (12). According to another study, toxigenic *V. alginolyticus* strains were isolated as a predominant species from the Adriatic coastal waters and seafood has been produced and consumed by baiting in this area. Therefore, researchers have stated that these potentially pathogenic isolates may significantly contribute to the

onset of sporadic and epidemic outbreaks of diarrhoeal disease in humans (25). The clinical significance of these potentially pathogenic vibrio strains are in association with gastroenteritis and/or invasive septicaemia and usually spread to humans via consumption of raw or undercooked shellfish or wound infections acquired by contact with fish, shellfish, or seawater. Furthermore, in French coastal waters the presence of predominant *V. alginolyticus* strains has also been demonstrated and sporadic vibriosis cases have been reported in France (12).

Although techniques like ribotyping, RAPD PCR, PCR fingerprinting, PFGE, AFLP, and AP-PCR have been used for detecting bacterial strain variability (23,26-28). We used AP-PCR because of its simplicity and appropriateness in our laboratory conditions. This technique is a DNA polymorphism assay, based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. Primer annealing temperature is fairly low (40 °C) and the primers used are not sequence specific. Whole bacterial genomic DNA has been randomly amplified by a 8-9 base sequence of primer (29). Isolated bacterial strains exhibited 7 different band patterns in our study (Figure). There are *V. alginolyticus* isolates, supposed to have the same band pattern of amplification (Figure; lane, 1-2-3), but fairly distinct isolates (Figure; lane, 5-6) were also determined. However, we observed closely related isolates (Figure, lane, 1-2-3-4-7-8-9). While 3 isolates were from the same genotypical source, 6 isolates (Figure, lane, 4-5-6-7-8-9) were from different sources.

In conclusion, there are no reports about the presence of *V. alginolyticus* in coastal waters or seafood samples in Turkey. In this respect, the presence of *V. alginolyticus* isolates in seafood collected in the Mediterranean coastal region indicates a public health problem. Nevertheless, these organisms were also detected in several coastal regions of the world, in both temperate and tropical areas, and there should be an awareness of the potential for zoonotic disease when handling these animals or their tissues.

Furthermore, AP-PCR is quite useful for genotypical discrimination of this organism. It is easily applicable for many laboratories working in this field.

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