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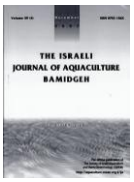

Identification and Genotyping of *Vibrio ordalii*: A Comparison of Different Methods

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Abstract

The phenotypical, serological, and molecular identification of *Vibrio ordalii* isolates recovered from diseased gilthead sea bream (*Sparus aurata*) in the Mediterranean area were investigated. Most isolates had similar phenotypic characteristics, but some showed small differentiation in biochemical analysis. Both slide agglutination and enzyme linked immunosorbent assay (ELISA) showed that the isolates were antigenically homogeneous and no different serotypes were detected. SDS-PAGE analysis showed that the structure of lipopolysaccharide (LPS) was heterogeneous, that outer membrane proteins (OMP) display homogeneous grouping protein banding patterns, and that the LPS profile of the isolates had different banding patterns. However, random amplified polymorphic DNA (RAPD) analysis revealed genetic variation (up to 90.4%) among isolates of the same species.

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Introduction

Vibrio pathogens can be characterized using biochemical identification and serological methods (Grisez and Ollevier, 1995). Serological methods have also been used to diagnose fish diseases (Toranzo et al., 1987; Austin and Austin, 1999).

Vibrio ordalii is an important pathogenic bacterium that causes vibriosis or hemorrhagic septicemia in salmonids (Schiewe et al., 1981; Chart and Trust, 1984; Austin and Austin, 1999). This bacterium was isolated from cultured sea bass (*Dicentrarchus labrax*) in the Mediterranean Sea (Grisez et al., 1997; Rodgers and Furones, 1998; Le Breton, 1999) and from gilthead sea bream in Turkey (Candan, 1993; Cagirgan, 1993; Akayli and Timur, 2004).

Molecular techniques have been used to analyze intraspecific genetic diversity among *V. ordalii* isolated from salmonids, for example, lipopolysaccharides typing (Chart and Trust, 1984; Grisez and Ollevier, 1995), total protein profiling (sodium dodecyl-sulfate-poly-acrylamide gel electrophoresis; Chart and Trust, 1984; Austin et al., 1997), and plasmid profiling (Austin et al., 1997). Recently, the molecular characterization of *V. ordalii* strains isolated from Atlantic salmon *(Salmo salar)* in Chile was determined by PCR based methods (Silva-Rubio et al., 2008). The aim of this study was to compare and evaluate the efficacy of different methods of identifying and typing *V. ordalii* isolates from gilthead sea bream (*Sparus aurata*).

Materials and Methods

Isolation and identification of bacteria. Diseased juvenile (2 g) and older (150-200 g) gilthead sea bream specimens were obtained from five farms located on the Aegean Sea coast of Turkey. Infected juveniles showed anorexia, darkening, small shallow skin ulcers, eye losses, and sudden death. Infected older fish showed anorexia, organized skin lesions namely large or small deep ulcerated skin lesions with scale losses, fin rot, jaw erosion, and hemorrhages on a considerably large area of the body surface.

Bacteria from infected visceral organs (liver, kidney, spleen), blood, and surface lesions of moribund fish were inoculated onto 1% NaCl (sodium chloride) supplemented with tryptic soy agar (TSA; Difco) and incubated at 22°C for 5-7 days.

Phenotypical, serological, and molecular characteristics of the bacteria isolates were examined and compared to representative reference strains (Table 1). Strains were identified taxonomically using conventional bacteriological methods according to standard morphological, physiological, and biochemical analyses (Schiewe et al., 1981; Larsen and Olsen, 1991; Holt et al., 1994), and API 20E, API 20NE, and API 50CH test kits.

Table 1. Reference bacteria used in this study and their source.

Bacteria	*Source*
Vibrio ordalii	CECT 582[a]
Vibrio anguillarum (=*Listonella anguillarum*) serotype 01	IMR[b]
V. anguillarum (=*L. anguillarum*) serotype 02	IMR[b]

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Immunization of rabbits. Rabbit antisera were used to characterize the serological characteristics of the *V. ordalii* isolates. The antisera were raised against *V. ordalii* isolate 1, CECT 582, and *L. anguillarum* serotypes 01 and 02. Rabbits were injected intravenously with saline-washed suspensions (10^9 cells/ml) of formalin-killed bacteria. The suspensions were injected twice weekly in consecutive doses of 0.2, 0.4, 0.8, and 1 ml. One week after the last injection, the rabbits were bled by cardiac puncture and the sera were stored at -30°C until used (Sorensen and Larsen, 1986; Larsen et al., 1994).

Serotyping. Slide agglutination tests were conducted for serotyping the reference bacteria and *V. ordalii* isolates according to procedures of Sorensen and Larsen (1986) and Toranzo et al. (1987). Bacterial O-antigen suspension (containing a concentration of about 10^9 cells/ml) was mixed with one drop of 1/5 diluted rabbit anti-serum. Immediate and apparent agglutination was registered as positive.

Enzyme-linked immunosorbent assay (ELISA). An indirect ELISA was used to detect the antibody in the antirabbit serum samples against *Vibrio* species, as described by Arkoosh and Kaattari (1990) and Hanna et al. (1991). Each bacterial suspension was added to each well of a 96-well microtiter plate. Plates were blocked by the addition of 100 µl of 1% (w/v) bovine serum albumin (BSA). Plates were rinsed three times with a washing solution containing 0.05% Tween 20 (PBS-Tween) before the addition of 100 µl of the antiserum samples (1:50 dilution). After further incubation at 22°C for 1 h and additional washings, 100 µl substrate solution horse radish peroxidase (HRPO) enzyme conjugate (1:1000, Sigma P-6278) was added to the plates. Plates were incubated at 22°C for 1 h. The optical density (OD) was measured at 492 nm using a micro plate reader (Bio-Rad 3550).

Electrophoresis. Electrophoresis of lipopolysaccharides (LPS) and outer membrane proteins (OMP) was conducted on bacterial isolates cultured on brain heart infusion broth (BHIB; Difco), supplemented with 1.5% NaCl and incubated at 22°C for 24-36 h. Samples (10 µl) were subjected to sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The LPS samples were stained with silver nitrate and the OMP samples were stained with Coomassie brilliant blue.

RAPD. The fingerprints of four selected bacterial isolates (1, 3, 4, and 10) and CECT-582 were determined using twenty OPERON primers. These isolates, identified by conventional methods as *V. ordalii*, and one reference

strain were identified using RAPD analysis. Genomic DNA was extracted from bacteria grown in tryptone soy broth (TSB; Oxoid) supplemented with 1% NaCI, using a high pure PCR template preparation kit (Roche). Twenty OPERON 10-mer oligonucleotide primers from the OPA, OPC, OPD, and OPE series were screened by RAPD. Genomic DNA amplification was performed in 25 µl total volume, according to standard procedures (Welsh and McClelland, 1990). After initial denaturation, PCR was performed as follows: 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min, for a total of 40 cycles. Generated polymorphic RAPD markers were statistically evaluated. The Multi Variant Statistical Package (MVSP) 3.1 computer program was used to determine diversity. Pair-wise comparisons were carried out according to Nei-Li's coefficient (Nei and Li, 1979). A dendogram was constructed from the combined distance matrices of seven primers by an unweighted pair-group method using arithmetic averages (UPGMA).

Results

Phenotyping. All bacteria displayed the same morphological, biochemical, and physiological characteristics as the reference strain (CECT 582) and were classified as typical *V. ordalii* (Table 2). The main differential traits were between strains 1, 3, 4, and 10. Variations were detected in Simon's citrate reaction, pellicle formation in the broth, and arginine dihydrolase reaction in API 20E test.

Slide agglutination. Agglutination reactions between 0-antigens and the antisera varied, but some cross reactions were detected in the serological characterization by slide agglutination test. Serum from rabbits that were immunized with *V. ordalii* antigens reacted with *V. ordalii* and serotype 02 common antigens but not with serotype 01. In contrast, rabbit anti-*L. anguillarum* 02 serum did not interact with 0 antigens from *V. ordalii*.

ELISA. The antisera against *V. ordalii* had a strong positive reaction with the ten isolates and CECT 582 but a weak reaction with *L. anguillarum* serotype 01 and 02 antigens (Fig. 1). *Listonella anguillarum* 01 and 02 rabbit antisera did not produce a cross reaction with any *V. ordalii* isolate, similar to results of the slide agglutination reaction test.

SDS-PAGE. OMP structures were homogeneous in all *V. ordalii* and reference strains. The most prominent band was about 37 kDa, but heterogeneity was observed in LPS profiles (20-34 kDa).

RAPD. Seven primers (OPC02, OPC05, OPC08, OPC19, OPD03, OPD07, OPE03) were selected for further data analysis since they amplified polymorphic and repeatable RAPD markers (Fig. 2). The markers ranged 100-1500 bp. Similarity values varied (Table 3). The similarity between isolates 1 and 4 was only 13.7%, even though they were isolated from individuals living in the same region. The ultra metric tree (UPGMA dendogram) was divided into two main clusters (Fig. 3).

Table 2. Physiological and biochemical characteristics of ten *Vibrio ordalii* isolates from gilthead sea bream cultured in Turkey (1-10), CECT 582 *(*11), and *Listonella anguillarum* serotypes 01 (12) and 02 (13).

Characteristic	*Isolate*										*Reference bacteria*		
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
B-Galactosidase	-	-	-	-	-	-	-	-	-	-	-	+	+
Arginine dihydrolase	-	w	-	-	-	-	w	-	-	-	-	+	+
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-
Simon's citrate	w	+	+	-	-	w	-	-	-	w	+	+	w
Indole	-	-	-	-	-	-	-	-	-	-	-	+	-
Voges proskauer	-	-	-	-	-	-	-	-	-	-	-	+	+
Acid from:													
Mannitol	w	+	w	w	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	+	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	+
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	+	-
Galactose	-	-	-	-	-	-	-	-	-	-	-	+	-
Sucrose	+	w	+	+	+	w	+	+	+	w	+	+	+
Growth at 37°C	-	-	-	-	-	-	-	-	-	-	-	+	+
Pellicle formation	w	-	-	-	-	w	-	-	-	w	-	-	+

+ positive, - negative, w = weak positive

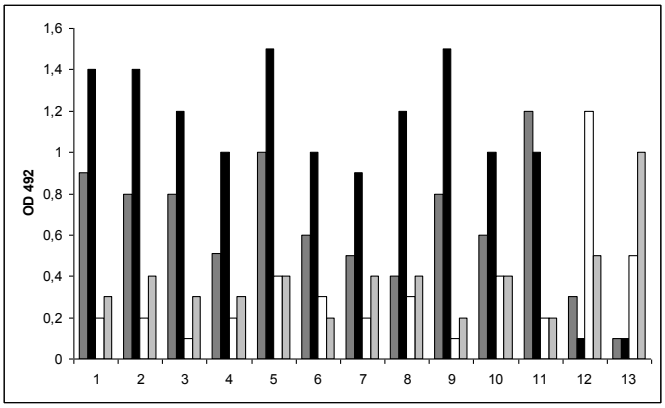

Fig. 1. ELISA testing rabbit antisera antigens from ten bacterial isolates (columns 1-10), CECT 582 (column 11), and *Listonella anguillarum* serotypes 01 (column 12) and 02 (column 13) against CECT 582 (■), *Vibrio ordalii* isolate 1 (■), and *L. anguillarum* serotypes 01 (□) and 02 (■).

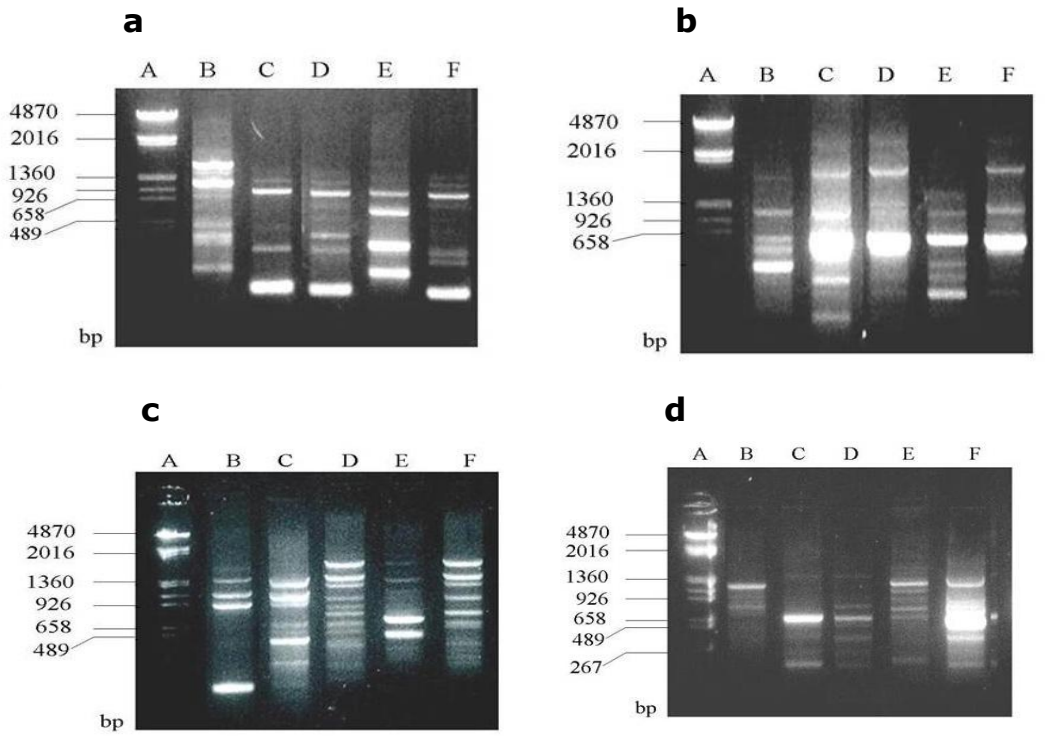

Fig. 2. RAPD markers amplified by using (a) OPC02, (b) OPC05, (c) OPC08, (d) OPC19, primers. Lane A = 500 bp DNA ladder (TAKARA), lane B = *Vibrio ordalii* (CECT-582), lane C = isolate 1, lane D = isolate 3, lane E = isolate 4, lane F = isolate 10.

Table 3. Similarity (genetic distance) between two reference strains and three bacteria isolates.

	Reference strains		*Isolate*		
	Listonella anguillarum serotype O1	*Vibrio ordalii (CECT-582)*	*1*	*4*	*10*
L. anguillarum serotype O1	1.000	-	-	-	-
V. ordalii (CECT-582)	0.547	1.000	-	-	-
Isolate 1	0.415	0.539	1.000	-	-
Isolate 4	0.380	0.302	0.137	1.000	-
Isolate 10	0.348	0.566	0.605	0.096	1.000

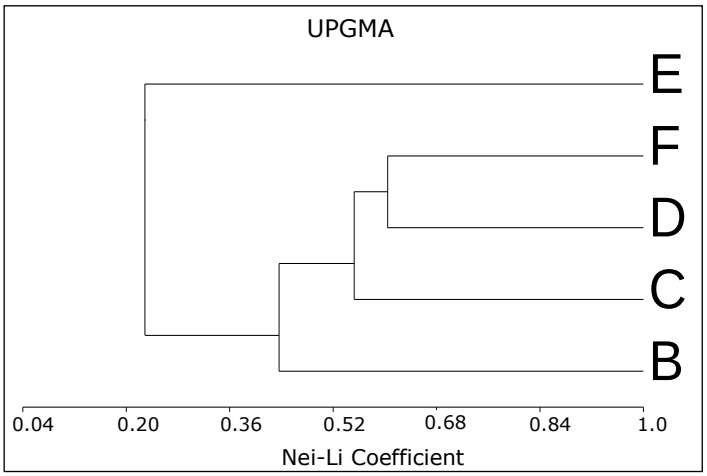

Fig. 3. RAPD dendrogram for UPGMA clustering of *Vibrio* species based on Nei-Li similarity coefficients. E = isolate 4, F = isolate 10, D = isolate 1, C = *Vibrio ordalii* (CECT-582), B = *Listonella anguillarum* serotype 01.

Discussion

Vibrio ordalii has been isolated from salmonids mainly in North America, New Zealand, Japan, Australia, and Chile (Austin and Austin, 1999; Toranzo et al., 2005). In this study, *V. ordalii* isolates from diseased gilthead sea bream cultured in the coastal region of the Aegean Sea, Turkey, were characterized by phenotypical, serological, antigenical, and genetic methods.

The ten isolates displayed mostly similar biochemical reactions. All were identified as *V. ordalii.* Therefore, there is a close correlation between our isolates and those reported by other researchers (Schiewe et al., 1981; Grisez and Ollevier, 1995, Austin and Austin, 1999). Five isolates showed variable positive Simon's citrate reactions while five strains showed negative Simon's citrate reactions. Three showed weak pellicle formation in broth. Pellicle formation is a specific characteristic of *L. anguillarum* serovar 02 (Larsen and Olsen, 1991). The isolates were more similar to *V. ordalii* than *L. anguillarum* serovar 02 (Schiewe et al., 1981).

Serological assays indicated that the *V. ordalii* isolates were highly homogeneous because whole cells and 0 antigens produced strong slide agglutination and ELISA with antisera raised against isolate 1 and CECT-582. These two assays showed that the ten *V. ordalii* strains were antigenically homogeneous and no serotypes were detected, as described by other researchers (Toranzo et al., 1987; Hanna et al., 1991; Chen et al., 1992; Grisez and Ollevier, 1995; Austin et al., 1997).

LPS and OMP are major antigenic determinants of marine vibrios that are pathogenic for fish (Chart and Trust, 1984; Austin et al., 1997). In this study, SDS-PAGE showed that the OMP composition was similar to earlier reports as

they share common protein antigens (Chart and Trust, 1984; Grisez and Ollevier, 1995). The LPS profiles, however, were heterogeneous, as described by other researchers (Grisez and Ollevier 1995; Austin et al., 1997).

SDS-PAGE showed intraspecific variation of LPS among the *V. ordalii* isolates but this variation is not as distinct as that of genotyping. Therefore, further molecular characterization of the *V. ordalii* isolates present in gilthead sea bream culture systems in Turkey is necessary. Within the molecular markers, RAPD is a useful method of analyzing genetic diversity among strains of *Vibrio* species (Najiah et al., 2003; George et al., 2005; Silva-Rubio et al., 2008). In the present study, this method was used to determine the relationships between the *V. ordalii* isolates. Although Silva-Rubio et al. (2008) did not observe any differences in RAPD profiles, the results of the present study show that genetic profiles of *V. ordalii* isolates vary and that the *V. ordalii* isolates from Turkey belong to different clonal lineages.

In conclusion, our *V. ordalii* isolates were heterogeneous in terms of LPS and RAPD, and homogeneous in terms of phenotypical, serological, and OMP profiling. RAPD was the most discriminative typing method, although phenotype and LPS profiling were useful complementary methods. Their combined use may be a feasible approach for epidemiological analysis of *V. ordalii*.

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