

Biochemical and serological analysis of *Vibrio anguillarum* related organisms

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ABSTRACT: This work reports on the phenotypic and serological characterization of *Vibrio anguillarum* related (VAR) organisms isolated from diseased fish, shellfish, and from the environment in different geographical areas. Reference strains of *V. anguillarum* (serotypes O1 to O10) and *V. splendidus* (biotype I) were included for comparison. On the basis of numerical taxonomy analysis the strains were grouped in 4 phena, with similarity levels among them ranging from 77.6 to 86.8% and levels of internal similarity ranging from 84.1 to 86.8%. The serological analysis demonstrated that 22 of 45 (49%) VAR strains were typeable, being distributed into 7 O-serogroups (A, B, C, D, E, F, G). Six of these serogroups (A, B, C, D, F, and G) were recognized among 21 VAR strains isolated from diseased fish. Serotypes C and F were the predominant types among strains isolated from diseased turbot and cod, respectively. No dominating environmental serotype was found. Moreover, it was found that only serogroup F constituted a heterogeneous group with regard to phenotypic and serological characteristics.

KEY WORDS: *Vibrio anguillarum* related organisms · O-Serotyping · Fish · Shellfish · Environment

INTRODUCTION

Vibriosis is considered to be one of the most serious threats to the success of fish culture industry, occurring worldwide. The disease also affects farmed bivalve molluscs and crustaceans (Toranzo & Barja 1990). Although *Vibrio anguillarum* (serotypes O1, O2, and O3) is considered the main causative agent of vibriosis in marine fish, other *Vibrio* species taxonomically related to *V. anguillarum* have been associated with disease in larval and adult stages of marine fish (Masumura et al. 1989, Fouz et al. 1990, Toranzo et al. 1990, Myhr et al. 1991, Pazos et al. 1993) and shellfish (Lavilla-Pitogo et al. 1990, Paillard & Maes 1990, Castro et al. 1992, Riquelme et al. 1995) in different geographical areas. These vibrios correspond to different biotypes of *V. splendidus* and *V. pelagius* and can be grouped under the designation *V. anguillarum* like or *V. anguillarum* related (VAR) organisms (Bryant et al. 1986a, Fouz et al. 1990, Toranzo & Barja 1990, Myhr

et al. 1991, Pazos et al. 1993, Riquelme et al. 1995). At present, vaccines which contain antigen preparations of *V. anguillarum* of the serotypes O1 and O2 (subgroups α and β) have proved to be very effective for the control of vibriosis (Santos et al. 1991). However, VAR organisms have been isolated from vaccinated fishes (Myhr et al. 1991, Pazos et al. 1993). This fact emphasizes the need to carry out an extensive characterization of VAR isolates in order to determine if representative strains of this organism should be included in the vibriosis vaccines. In this sense, previous studies have demonstrated that VAR strains can be differentiated from *V. anguillarum* on the basis of biochemical characteristics (Bryant et al. 1986a, Fouz et al. 1990, Myhr et al. 1991, Pazos et al. 1993) and that the VAR strains do not share antigenic components with the serotypes O1 to O5 of *V. anguillarum* (Pazos et al. 1993). In the present paper we report the phenotypic and serological characterization of VAR organisms isolated from diseased fish, shellfish, and the environment in different countries (Chile, Denmark, Spain, United States).

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MATERIALS AND METHODS

Bacterial strains and phenotypic characterization.

In the present work we used 45 strains, isolated from diseased fish (34 strains), shellfish (2 strains), and the environment (9 strains) in our area (Spain) and other countries (Chile, Denmark, Norway, United States) (Table 1). Gram stain, oxidase and catalase tests, morphology and motility, growth on thiosulfate citrate bile sucrose (TCBS) agar (Oxoid, Basingstoke, England), fermentation of glucose, nitrate reduction, and sensitivity to the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (O/129) (Oxoid) were used to identify the isolates as members of the genus *Vibrio*. Reference strains of *V. anguillarum* (serotypes O1 to O10) and *V. splendidus* (biotype I) from the American Type Culture Collection (ATCC) were also included for comparative purposes (Table 1).

All the strains were examined using a total of 41 physiological and biochemical tests. The assays were performed following the criteria of Bryant et al. (1986a, b), Fouz et al. (1990), and Myhr et al. (1991). The results of the phenotypic tests of the 56 strains were subjected to numerical taxonomic analyses. Similarity among the strains was calculated by using the simple matching coefficient. Clustering was carried out using the unweighted pair group method with arithmetic averages (UPGMA) (Sneath 1972).

Source of antisera. Seven representative VAR strains (RM51, RC25.1, RPM30.2, RM36.1, 95-3-35, 95-3-72, 95-3-88) with distinct isolation sources and displaying phenotypical differences, and the strain of *Vibrio splendidus* ATCC 33125 (biotype I), were used to obtain the antisera. Sera were obtained in rabbits as described by Sørensen & Larsen (1986) using formalin-killed cells as antigen. Whole-cell antisera against reference strains of *V. anguillarum* (serotypes O1 to O10) were kindly provided by J. L. Larsen (Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Fredriksberg, Denmark).

Serological assays. The serological relationships between strains were determined by slide agglutination and quantitative agglutination tests following basically the procedures of Sørensen & Larsen (1986) and Santos et al. (1995). The assays were performed using the heat stable O-antigen and rabbit whole-cell antisera. The O-antigens were prepared by heating bacterial suspensions of each strain in phosphate-buffered saline (PBS) at 100°C for 1 h. Slide agglutination tests were conducted by mixing a drop of each O-antigen suspension (10^9 cells ml⁻¹, McFarland standard No. 3) with a drop of 1/10 diluted rabbit antiserum on a multiwell glass slide. A visible and immediate agglutination was registered as positive and no or only a weak agglutination occurring after 1 to 2 min as a negative

reaction. Quantitative agglutination tests were performed in microtiter plates using serial 2-fold dilutions of 50 µl aliquots of the antisera and O-antigen suspensions (10^9 cells ml⁻¹, McFarland standard No. 3). The agglutination titer was considered the reciprocal of the highest dilution of the antiserum giving a positive reaction after incubation with the antigen overnight at 15°C.

RESULTS

Phenotypical characterization and numerical taxonomy analysis

The results of the biochemical and physiological characterization (Table 2) indicated that all of the strains were rod shaped, motile, Gram-negative, oxidase and catalase positive, halophilic, and negative for lysine and ornithine decarboxylases. All the strains fermented D-glucose without gas production, reduced nitrate, and were sensitive to the vibriostatic agent O/129. None of the isolates grew at 44°C, and none produced acid from inositol or rhamnose. In the remaining biochemical tests, variation among the strains was detected (Table 2). The results of the assays for susceptibility to chemotherapeutic agents showed that the majority of the *Vibrio* strains were sensitive to chloramphenicol and tetracycline, resistant to streptomycin, and variable in their susceptibility to the other chemotherapeutic compounds assayed.

The results of numerical analysis of the strains are presented in Fig. 1 and Table 2. The isolates were distributed in 4 phenon, defined at similarity levels of 86.8% (phenon I), 84.6% (phenon II), 86.5% (phenon III), and 84.1% (phenon IV). Similarity levels among phenon ranged from 77.6 to 86.8%. Phenon I, which comprised 14 strains, included the majority of the *Vibrio anguillarum* reference strains (7 of 10 strains tested). All the isolates of this group were positive for Voges-Proskauer, indole, β-galactosidase, gelatinase, and caseinase. The majority of the strains produced acid from arabinose, mannitol, sorbitol, and amygdalin. Phenon II contained 23 strains (including the remaining reference strains of *V. anguillarum*) which were lecithinase positive and hemolytic (using turbot erythrocytes), and most of them produced acid from amygdalin and mannitol. Most of the strains of this group lacked elastase activity and did not produce acid from arabinose. The majority of the environmental strains (8 of 9 isolates tested) were included in this phenon. Phenon III was composed of 8 strains (including the reference strain of *V. splendidus* biotype I) which did not produce yellow colonies on TCBS or acid from sorbitol and arabinose, and lacked elastase activity.

Table 1. Bacterial strains used in the present study. Data in parentheses indicate serotype or biotype

Strain	Species	Isolated from	Donor
ATCC 43305 (O1)	<i>V. anguillarum</i>	<i>Oncorhynchus mykiss</i> (Denmark)	ATCC
ATCC 43306 (O2)	<i>V. anguillarum</i>	<i>Gadus morhua</i> (Denmark)	ATCC
ATCC 43307 (O3)	<i>V. anguillarum</i>	<i>Oncorhynchus mykiss</i> (Denmark)	ATCC
ATCC 43308 (O4)	<i>V. anguillarum</i>	<i>Gadus morhua</i> (Denmark)	ATCC
ATCC 43309 (O5)	<i>V. anguillarum</i>	<i>Gadus morhua</i> (Denmark)	ATCC
ATCC 43310 (O6)	<i>V. anguillarum</i>	<i>Gadus morhua</i> (Denmark)	ATCC
ATCC 43311 (O7)	<i>V. anguillarum</i>	<i>Anguilla anguilla</i> (Denmark)	ATCC
ATCC 43312 (O8)	<i>V. anguillarum</i>	<i>Gadus morhua</i> (Denmark)	ATCC
ATCC 43313 (O9)	<i>V. anguillarum</i>	<i>Gadus morhua</i> (Denmark)	ATCC
ATCC 43314 (O10)	<i>V. anguillarum</i>	<i>Gadus morhua</i> (Denmark)	ATCC
ATCC 33125 (biotype I)	<i>V. splendidus</i>	Marine fish (United States)	ATCC
95-3-51	VAR	<i>Oncorhynchus mykiss</i> (Denmark)	J. L. Larsen
95-3-52	VAR	<i>Oncorhynchus mykiss</i> (Denmark)	J. L. Larsen
95-3-101	VAR	<i>Oncorhynchus mykiss</i> (Denmark)	J. L. Larsen
NF25.1	VAR	<i>Salmo salar</i> (Spain)	F. Pazos
SE2.1	VAR	<i>Oncorhynchus kisutch</i> (Spain)	F. Pazos
SE8.1	VAR	<i>Oncorhynchus kisutch</i> (Spain)	F. Pazos
RPM232.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RA60.2	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RI41	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RC25.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RPM167.2	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
AZ1.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
PC23.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RQ1.2	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RM51	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RM36.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RPM30.2	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RPM36.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RPM40.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
RPM41.1	VAR	<i>Scophthalmus maximus</i> (Spain)	F. Pazos
95-3-71	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-72	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-74	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-88	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-89	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-90	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-91	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-92	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-93	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-94	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
95-3-95	VAR	<i>Gadus morhua</i> (Denmark)	J. L. Larsen
SB	VAR	<i>Morone saxatilis</i> (United States)	A. Baya
88589-1	VAR	Tropical fish (United States)	A. Baya
88589-2	VAR	Tropical fish (United States)	A. Baya
OG	VAR	<i>Argopecten purpuratus</i> (Chile)	C. Riquelme
LL	VAR	<i>Concholepas concholepas</i> (Chile)	C. Riquelme
LV29	VAR	Seawater tank (Norway)	A. E. Toranzo
TKI	VAR	Seawater tank (Chile)	C. Riquelme
95-3-33	VAR	Seawater (Denmark)	J. L. Larsen
95-3-35	VAR	Seawater (Denmark)	J. L. Larsen
95-3-41	VAR	Seawater (Denmark)	J. L. Larsen
95-3-46	VAR	Seawater (Denmark)	J. L. Larsen
95-3-48	VAR	Seawater (Denmark)	J. L. Larsen
95-3-36	VAR	Sediment (Denmark)	J. L. Larsen
95-3-47	VAR	Sediment (Denmark)	J. L. Larsen

Table 2. Phenotypic characteristics of the phenon of the *Vibrio* strains used in this study. n: Number of strains in phenon. TCBS: thiosulfate citrate bile sucrose

Test	% of strains positive in phenon:			
	I (n = 14)	II (n = 23)	III (n = 8)	IV (n = 11)
Motility	100	100	100	100
Gram-negative stain	100	100	100	100
Oxidase	100	100	100	100
Catalase	100	100	100	100
β -Galactosidase	100	100	62.5	0
Arginine dihydrolase	100	100	100	45.4
Lysine decarboxylase	0	0	0	0
Ornithine decarboxylase	0	0	0	0
Yellow colonies on TCBS	100	100	0	100
Citrate utilization	85.7	91.3	75	63.6
Urease	0	0	0	0
Indole	100	100	25	0
Nitrate reduction	100	100	100	100
Gas from glucose	0	0	0	0
Voges-Proskauer	100	78.2	25	0
Salt tolerance (% NaCl)				
0	0	0	0	0
3	100	100	100	100
5	100	82.6	100	100
8	0	13	0	0
10	0	0	0	0
Growth at:				
15	100	100	100	100
25	100	100	100	100
37	100	82.6	50	18.2
44	0	0	0	0
Acid from:				
Glucose	100	100	100	100
Mannitol	92.8	95.6	37.5	27.3
Inositol	0	0	0	0
Sorbitol	92.8	34.8	0	0
Rhamnose	0	0	0	0
Sucrose	100	100	100	91
Melibiose	0	0	0	0
Amygdalin	71.4	60.9	12.5	18.2
Arabinose	100	8.7	0	9
Hemolysis of erythrocytes:				
Sheep	57.1	34.8	50	0
Turbot	92.8	100	100	0
Production of:				
Amilase	85.7	65.2	100	9
Gelatinase	100	95.6	100	9
Caseinase	100	87	100	18.2
Elastase	71.4	13	0	0
Lipase	71.4	87	50	0
Lecithinase	57.1	100	87.5	0
Sensitivity to:				
Vibriostatic agent (O/129)	100	100	100	100
Chloramphenicol	100	100	87.5	100
Ampicillin	0	21.7	12.5	0
Oxytetracycline	100	100	75	100
Tetracycline	92.8	100	87.5	91
Oxolinic acid	100	91.3	50	100
Streptomycin	7	4.3	0	0
Trimethoprim-sulphamethoxazole	71.4	87	50	72.7

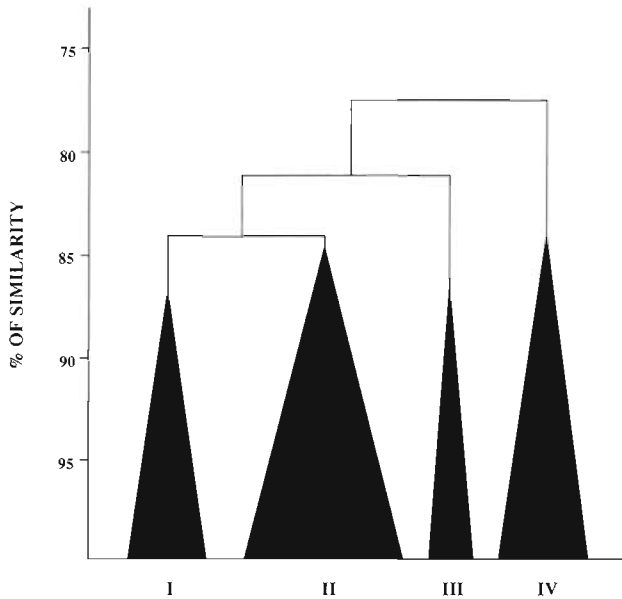


Fig. 1. Simplified dendrogram showing relationships among *Vibrio* stains determined by simple matching coefficients and the unweighted pair group method with arithmetic averages

Phenon IV included 11 isolates which lacked lecithinase, lipase, elastase, and hemolytic activities.

Serological testing

The slide agglutination test demonstrated that 22 of 45 VAR strains tested (49%) were typeable, being distributed into 7 serogroups (A, B, C, D, E, F, G) (Table 3). There were no cross reactions between groups. The majority of the typeable VAR strains were isolated from cod *Gadus morhua* (11 isolates) and turbot *Scophthalmus maximus* (9 isolates). While strains from cod exclusively belonged to serogroups F (10 of 11 strains) and G (1 of 11 strains), more heterogeneity was observed among turbot isolates (Table 3). Among strains isolated from the environment (seawater and sediment), no type predominated. None of the VAR strains agglutinated with the *Vibrio anguillarum* (serotypes O1 to O10) or *V. splendidus* (biotype I) antisera.

The quantitative agglutination assays confirmed the existence of 7 serogroups and indicated the existence of 2 serological subgroups within serogroup F (Table 3).

Phenon I grouped the majority of the reference strains of *Vibrio anguillarum* (serotypes O1, O2, O3, O4, O6, O7, and O10), 3 strains isolated from cod and belonging to serogroup F, 2 strains of serogroup G (isolated from cod and rainbow trout), and 2 non-typeable

strains (RPM 232.1 and LV 29). Phenon II included the *V. anguillarum* strains of serotypes O5, O8, and O9, the remaining strains of serogroup F isolated from cod, 1 strain isolated from seawater and belonging to serogroup E, and 12 non-typeable strains from different sources. Only 3 of the strains included in Phenon III were typeable, displaying a positive reaction with the antisera against the strain of *V. splendidus* ATCC 33125 (only the homologous strain) and against strain RC25.1 (2 isolates, serogroup B). Phenon IV included the strains isolated from turbot belonging to serogroups A, C, and D, as well as 4 non-typeable strains.

DISCUSSION

Vibriosis due to *Vibrio anguillarum* is one of the most important bacterial diseases of fish throughout the world. Recently, other *Vibrio* species taxonomically related to *V. anguillarum*, and commonly considered environmental strains without pathogenic importance, have been associated with the disease in marine fish and shellfish. Identification of *Vibrio* species that possess similar morphological, physiological and biochemical characteristics is not easy because of the large number of tests that are involved and the existence of variable reactions within each species (Bryant et al. 1986a, b, Fouz et al. 1990, Pazos et al. 1993). Alternatively, serological methods are a fast and reliable way of identifying of *Vibrio* species as well as for use in epidemiological and ecological studies (Sørensen & Larsen 1986, Fouz et al. 1990, Chen et al. 1992, Pazos et al. 1993, Santos et al. 1995). At present, few studies (Fouz et al. 1990) on the serological relationships among VAR strains have been conducted. This paper reports the phenotypic and serological characterization of VAR organisms isolated from diseased fish and shellfish and from the environment.

The results of the biochemical studies showed heterogeneity within *Vibrio anguillarum* and VAR organisms. Examination of numerical taxonomy showed that *Vibrio* strains were distributed in 4 phenons with values of internal similarity ranging from 84.1 to 86.8%. Moreover, Phenon II to IV, which are composed of related vibrios, differed from *V. anguillarum* in the Indol and Voges-Proskauer reaction, the hydrolysis of elastin, fermentation of arabinose and mannitol. These findings are similar to those obtained by Bryant et al. (1986a), Fouz et al. (1990) and Romalde et al. (1990). Therefore, these phenons can be considered to consist of the same species of vibrios described by these authors (*V. pelagius*, *V. splendidus*, and *Vibrio* spp.). In keeping with results previously reported by other authors (Fouz et al. 1990, Myhr et al. 1991), no taxonomical differences were observed between *Vibrio* strains iso-

Table 3. Slide agglutination test and quantitative agglutination assay with O-antigen from 45 VAR strains and rabbit whole-cell antisera against 7 VAR strains. VAR organisms were not typeable with the sera raised against *Vibrio anguillarum* of serotypes O1 to O10 or with serum against *V. splendidus* (biotype I). Sero.: serogroup. Values in parentheses indicate titers obtained on quantitative agglutination test. Titers lower than 20 were scored as negative

Bacterial strain	Phenon	Antisera against VAR strain:						
		RM51 (Sero. A)	RC25.1 (Sero. B)	RPM30.2 (Sero. C)	RM36.1 (Sero. D)	95-3-35 (Sero. E)	95-3-72 (Sero. F)	95-3-88 (Sero. G)
Rainbow trout								
95-3-51	IV	-	-	-	-	-	-	-
95-3-101	II	-	-	-	-	-	-	-
95-3-52	I	-	-	-	-	-	-	+ (640)
Atlantic salmon								
NF25.1	II	-	-	-	-	-	-	-
Pacific salmon								
SE2.1	III	-	-	-	-	-	-	-
SE8.1	IV	-	-	-	-	-	-	-
Turbot								
RPM232.1	I	-	-	-	-	-	-	-
RA60.2	III	-	-	-	-	-	-	-
RI41	III	-	-	-	-	-	-	-
AZ1.1	IV	-	-	-	-	-	-	-
PC23.1	IV	-	-	-	-	-	-	-
RC25.1	III	-	+ (320)	-	-	-	-	-
RPM167.2	III	-	+ (320)	-	-	-	-	-
RM51	IV	+ (320)	-	-	-	-	-	-
RQ1.2	IV	+ (320)	-	-	-	-	-	-
RM36.1	IV	-	-	-	+ (320)	-	-	-
RPM30.2	IV	-	-	+ (2560)	-	-	-	-
RPM36.1	IV	-	-	+ (1280)	-	-	-	-
RPM40.1	IV	-	-	+ (2560)	-	-	-	-
RPM41.1	IV	-	-	+ (2560)	-	-	-	-
Cod								
95-3-71	II	-	-	-	-	-	+ (640)	-
95-3-72	II	-	-	-	-	-	+ (1280)	-
95-3-74	II	-	-	-	-	-	+ (1280)	-
95-3-89	II	-	-	-	-	-	+ (80)	-
95-3-90	II	-	-	-	-	-	+ (40)	-
95-3-91	I	-	-	-	-	-	+ (640)	-
95-3-92	II	-	-	-	-	-	+ (40)	-
95-3-93	II	-	-	-	-	-	+ (40)	-
95-3-94	I	-	-	-	-	-	+ (1280)	-
95-3-95	I	-	-	-	-	-	+ (1280)	-
95-3-88	I	-	-	-	-	-	-	+ (1280)
Striped bass								
SB	II	-	-	-	-	-	-	-
Tropical fish								
88589-1	III	-	-	-	-	-	-	-
88589-2	III	-	-	-	-	-	-	-
Scallop								
OG	II	-	-	-	-	-	-	-
'Loco'								
LL	II	-	-	-	-	-	-	-
Seawater								
95-3-35	II	-	-	-	-	+ (1280)	-	-
95-3-33	II	-	-	-	-	-	-	-
95-3-41	II	-	-	-	-	-	-	-
95-3-46	II	-	-	-	-	-	-	-
95-3-48	II	-	-	-	-	-	-	-
TKI	II	-	-	-	-	-	-	-
LV 29	I	-	-	-	-	-	-	-
Sediment								
95-3-36	II	-	-	-	-	-	-	-
95-3-47	II	-	-	-	-	-	-	-

lated from diseased fish and from the environment on the basis of classical biochemical tests used in this study.

The serological analysis showed the existence of serological heterogeneity among VAR strains, with 7 serogroups (A, B, C, D, E, F, G) (Table 3) being established. Six of these serogroups (A, B, C, D, F, and G) were recognized among 21 VAR strains isolated from diseased fish. Serotypes C and F were found to be the predominant types among strains isolated from diseased turbot and cod, respectively. Only 11% of strains isolated from the environment were typeable and none belonged to the serogroups detected among strains isolated from diseased fish. This raises a question about the possible source of pathogenic serogroups. Further studies, including a higher number of environmental strains and their respective antisera, are needed in order to clarify the origin of the pathogenic strains.

On the other hand, it was found that VAR strains of serogroups A, B, C, D, E, and G constitute homogeneous groups, while serogroup F constitutes a heterogeneous group with regard to phenotypic and serological characteristics. The existence of variability with respect to biochemical and serological characteristics has also been reported for strains belonging to serotypes O2 and O3 of *Vibrio anguillarum* (Toranzo et al. 1987, Larsen et al. 1994, Santos et al. 1995).

In conclusion, we have found that VAR strains can be typed on the basis of the serologically distinct heat-stable O-antigen. Attempts are currently being made to expand the serotyping system to enable it to characterize a higher proportion of VAR strains. In addition, the present results suggest that the formulation of present vibriosis vaccines may be improved if VAR strains representative of the antigenic groups which predominate in a particular area are tested in trial vaccines.

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