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Study of *Vibrio anguillarum* Strains from Different Sources with Emphasis on Ecological and Pathobiological Properties

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A total of 317 *Vibrio anguillarum* strains were isolated from water, sediment, and diseased as well as healthy rainbow trout at a Danish mariculture farm and from feral fish caught close to the farm. All strains were examined serologically. Ten sera permitted determination of the O group in 66.7% of the strains from diseased rainbow trout. Furthermore, the O group could be determined in 45.1 to 65.4% of the strains from mucus, gills, and intestinal contents of healthy rainbow trout, while only 22.2 to 28.8% of the isolates from water, sediment, and gills or mucus of feral fish were groupable. Serogroup O1 and to some extent O2 appeared to be associated with trout. Strains from these serogroups were selected for analyses of hemagglutinating activity and surface hydrophobicity. Serogroup O1 comprised hemagglutinating as well as nonhemagglutinating strains; from cases of vibriosis, all O1 strains were nonhemagglutinating. The strains belonging to serogroup O2 were generally hemagglutinating. Examinations of surface hydrophobicity by salt aggregation and hydrophobic interaction chromatography suggested that the O1 strains were more hydrophobic than the O2 strains. In pathogenicity tests, O1 strains isolated from gills and mucus of healthy rainbow trout killed all trout in the test groups. A strain from the intestinal contents of healthy rainbow trout did not produce significant mortality. This strain could, however, be frequently reisolated from the pronephros of fish in the test group concerned. After challenge with strains from eel mucus and seawater, mortality was not produced, and furthermore, these strains could not be reisolated from the pronephros.

Salmonids that are transferred from fresh water to salt water become exposed to another microbial flora and may become infected with *Vibrio anguillarum*. The origin of pathogenic *V. anguillarum* strains and the site(s) at the host where penetration takes place still need clarification (3, 8).

An important prerequisite for studies of these problems is a thorough characterization of isolated strains. Serological examinations of *V. anguillarum* have been made in many countries, especially dealing with isolates from diseased fish and, most often, from cultured species (10, 14, 19, 23, 24). A recent study presented a new serogrouping scheme and examined serological properties of strains from cultured as well as feral fish and from the environment (22). In cases of vibriosis among salmonids, serogroup O1 accounted for 75% of the strains, whereas serogroup O2 constituted 15%; the corresponding figures for feral fish presented an opposite distribution, namely, 2% and 75%, respectively. Host species preference of *V. anguillarum* strains has also been noticed by others (2, 23).

The introduction of a hemagglutination typing scheme also helps to characterize *V. anguillarum* and could provide important information toward understanding the pathobiological properties of this bacterium (17). In other bacterial species, hemagglutinating activity in addition to hydrophobic surface properties have been correlated with host colonization ability and sensitivity to phagocytosis (11, 21, 27, 28).

In this study, *V. anguillarum* strains from different sources were characterized by serogrouping, hemagglutinating activity, and surface hydrophobicity examinations. Furthermore, strains were tested for pathogenicity.

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MATERIALS AND METHODS

Sampling site. Samplings were made at a netcage farm situated at the southern coast of Sealand, Denmark. Water depth ranged from 2.5 to 3.5 m at the site, and salinity was approximately 1%. At the start of the season, the unit was stocked with 15,000 freshwater-reared rainbow trout (*Salmo gairdneri*) with a mean weight of 700 g. In Denmark, production of rainbow trout in marine localities is seasonally restricted, and this production usually only takes place from April to November.

At regular intervals, two samples of water from the surface and two samples of sediments were collected by procedures described previously (15). In addition, four healthy rainbow trout were taken from the netcages and killed. These samplings were repeated eight times during the season. In cases of increased mortality in the farm, swab samples were taken from the pronephros of moribund rainbow trout. The swab samples were placed in tubes containing Stuart transport medium (Oxoid, Ltd., London, England).

At the end of the season, feral fish (*Anguilla anguilla*, *Zoarces viviparus*, *Pleuronectes platessa*, *Pomatoschistus minutus*) were trapped close to the mariculture farm and killed.

During transport to the laboratory, fish as well as other samples were kept at 5°C.

Bacteriological examination. Water and sediment were processed by principles listed elsewhere (15). Gill tissue, skin mucus, and intestinal contents from healthy rainbow trout or feral fish were transferred to 0.9% saline containing 0.1% peptone. Subsequently, the specimens were homogenized and plated on TCBS (thiosulfate-citrate-bile salts-sucrose agar, (Difco Laboratories, Detroit, Mich.) as well as BA (Difco marine agar with 5% citrated calf blood). The

TABLE 1. Distribution of serogroups and groupable, nongroupable, and self-agglutinating *V. anguillarum* strains

Source of strains	No. of strains	*V. anguillarum* serogroups										No. groupable (%)	No. nongroupable	No. self-agglutinating
		O1	O2	O3	O4	O5	O6	O7	O8	O9	O10			
Environmental														
Water	49	2	2	2		3			2	2	1	14 (28.6)	33	2
Sediment	52		2	2	1	3		1	4	1	1	15 (28.8)	33	4
Rainbow trout														
Mucus[a]	71	25	2	1				1	2		1	32 (45.1)	29	10
Gills[a]	52	18	5	2	1			4	2	1	1	34 (65.4)	17	1
Intestines[a]	31	5	9	2								16 (51.6)	12	3
Pronephros	15	8			1		1					10 (66.7)	5[b]	
Feral fish														
Mucus	19	1	2						1	1		5 (26.3)	5	9
Gills	27		2	2				1	1			6 (22.2)	15	6
Intestines	1							1				1 (100.0)		

[a] Swab samples from pronephros of these fish were sterile.
[b] Three of these isolates occurred in mixed cultures with *A. salmonicida*.

plates were incubated for 24 h, and isolates were selected among colonies characteristic of *V. anguillarum*. Swab samples from the pronephros of healthy and diseased rainbow trout were streaked onto BA. After incubation for 24 and 48 h at 25°C, plates were inspected and representative colonies were chosen.

All isolates were identified by a series of examinations which comprised Gram stain, motility, catalase, oxidase, oxidation-fermentation test, arginine^{DH}, lysine^{DC}, ornithine^{DC}, 0/129 sensitivity (10 and 150 μg), growth on TCBS agar, and growth in 0, 6, and 7% NaCl (16).

Serological characterization. Serogrouping was done by slide agglutination as described previously (22). Only serogroup O1 and O2 isolates were subjected to the further examinations described below.

Hemagglutination test. The isolates were cultured on BA at 25°C. After 24 h, plates were harvested by suspending bacterial cells in phosphate-buffered saline (pH 7.1). The optical density of the suspensions was adjusted to 1.5 at 590 nm, corresponding to 4.0×10^{10} CFU/ml. These suspensions were mixed with equal amounts of 3% (vol/vol) erythrocyte suspensions. The tests were performed on a glass plate in the presence or absence of 0.5% D-mannose. Reactions were recorded after 4 min at 20 to 22°C. Erythrocytes harvested from humans (type O), poultry, guinea pigs, horses, pigs, and rainbow trout were used. Also, a 3% (vol/vol) yeast cell suspension was tested (17).

Hydrophobic interaction chromatography. For hydrophobic interaction chromatography (HIC), isolates were cultured on BA as described above. Plates were harvested with a solution of 1 M $(NH_4)_2SO_4$ in 0.01 M phosphate buffer (pH 6.8). Subsequently, the optical density at 590 nm was adjusted to 1.5 and the bacterial suspensions were applied onto Octyl Sepharose gels (Pharmacia, Uppsala, Sweden) in Pasteur pipettes stoppered with glass wool (18). The degree of adsorption was determined by absorbance measurements (12).

Salt aggregation test. For the salt aggregation test (SAT), isolates were cultured on BA as described above. Bacterial suspensions were prepared with 0.002 M sodium phosphate (pH 6.8); the optical density at 590 nm corresponded to 1.5×10^{10} CFU/ml. On a glass plate, the bacterial suspensions were mixed with equal amounts of buffered ammonium sulfate solutions which ranged from 0.2 to 4.0 M. Bacterial suspensions were also tested by being mixed with the phosphate buffer without ammonium sulfate. Visual reading for aggregation was performed against a black background and concluded after 2 min at 20 to 22°C (18).

Pathogenicity testing. Eight *V. anguillarum* O1 isolates from the following sources were tested: pronephros of diseased rainbow trout; mucus, gill, and intestines of healthy rainbow trout; mucus of eel; and seawater (two isolates from trout mucus and trout intestines, respectively, and one isolate from each of the other sources were selected). Groups of rainbow trout of 10 individuals with a mean weight of 50 g were challenged by intramuscular injection of approximately 5×10^5 CFU per individual. The trout were held in fresh water at 10 to 12°C, and mortality was recorded at two or more daily inspections. Samples from the pronephros of recently dead fish were examined bacteriologically. All surviving trout were killed after 1 week; samples were collected and processed as above.

RESULTS AND DISCUSSION

A total of 317 *V. anguillarum* strains were isolated and examined serologically. The isolates were arranged according to origin and serogroup (Table 1). The ratios of isolates which could be serogrouped varied substantially depending on the origin. Serogroup O1 showed a tendency toward seasonal variation in prevalence; a high proportion of the O1 isolates derived from the start of the season.

The overall percentage of groupable strains from water and sediment was only 29%. These strains exhibited a broad serological spectrum without any predominant serogroup. This corresponds to a pattern described previously (22). The low groupability of strains from environmental sources should be associated with the fact that elaboration of the antigenic scheme was based on isolates from diseased fish (22). Among environmental strains, two were recorded as serogroup O1. Challenge with one of these strains did not produce disease (see below). The possibility that pathogenic strains originate from the environment has previously been discussed (8), and results from the present study do not exclude this possibility. Actually, the proportion of strains with a pathogenic potential in water and sediment could be very low owing to a poorer ability for survival and multiplication in the environment as compared with the nonpathogenic flora. A relatively high ratio of *V. anguillarum* strains from rainbow trout, diseased as well as healthy, could be serogrouped, and the predominance of O1 and to some extent O2 was apparent. The accumulation of O1 on the

TABLE 2. Agglutinating activity of O1 and O2 strains of *V. anguillarum*

Agglutination type[a]	Agglutination of:							No. of O1 strains	No. of O2 strains
	Erythrocytes						Yeast cells		
	Human	Poultry	Guinea pig	Trout	Horse	Pig			
A	+	+	+	+	+	+	+	19	21
C	−	+	+	+	+	+	+	0	1
D	−	+	+	−	+	−	+	2	0
G	−	−	−	−	−	−	−	38	2

[a] According to Larsen and Mellergaard (17).

surfaces of healthy rainbow trout provides evidence of a specific association in which chemotaxis could be involved as reported for other members of the family *Vibrionaceae* (5, 7). Most likely, the nongroupable *V. anguillarum* strains isolated from diseased rainbow trout, including the strains which occurred in mixed cultures with *Aeromonas salmonicida*, represented contaminants from the environment. The material from feral fish was sparse, and conclusions regarding their potential role as carriers of trout pathogenic strains on internal or external surfaces could not be made.

Hemagglutinating activities were arranged according to a typing scheme presented by Larsen and Mellergaard (17) and supplemented with reactions toward erythrocytes from horses and pigs (Table 2). All agglutinations were mannose sensitive. Among O1, most strains were nonhemagglutinating and none of the eight O1 strains isolated from diseased fish hemagglutinated. Considering O1 strains obtained from healthy rainbow trout, 8 of 25 strains from gills and 5 of 18 strains from mucus hemagglutinated, whereas all 5 strains from intestinal contents exhibited hemagglutinating activity. These observations suggest the existence of separate populations of O1 strains differing in hemagglutinating activity or an ability of these strains to shift from a hemagglutinating to a nonhemagglutinating phase during the infection process (6). In this connection, the phagocytic sensitivity of hemagglutinating *V. anguillarum* strains versus nonhemagglutinating variants deserves attention. In other bacterial species, mannose-sensitive hemagglutination has been associated with increased phagocytic sensitivity (21). In contrast to the O1 strains, most of the O2 strains agglutinated a broad spectrum of erythrocytes (hemagglutination types A and C). These O2 strains were all obtained from surfaces of healthy fish. Among strains belonging to serogroup O2 and derived from outbreaks of vibriosis, hemagglutinating activity also predominates (J. L. Larsen, unpublished data). Often hemagglutination testing of *V. anguillarum* strains is reported without the serogroup being stated (17, 25, 26). Results from this study indicate that the hemagglutination pattern should be considered with reference to serogroup.

Comparisons of surface hydrophobicity of the strains were facilitated by the establishment of HIC and SAT groups that were based on principles used elsewhere (4, 9, 13). In the HIC test, the O1 strains showed a high degree of retention and were assigned to groups 2 and 3; the O2 strains were placed in HIC group 1. Approximately 98% of the O1 strains were recorded as SAT group 2, while the O2 strains mainly (96%) were incorporated into SAT group 4 (Table 3). Apparently, the O1 strains were more hydrophobic than the O2 strains. This observation in combination with results from the hemagglutination tests suggest differences between O1 and O2 strains as regards host interaction.

In pathogenicity tests, strains from cases of vibriosis, mucus, and gills of rainbow trout killed all the trout. With these strains, deaths generally occurred 3 to 4 days after injection. A strain from the intestines of rainbow trout was recovered from seven fish, but only one of these fish succumbed to the infection. Mortality was not produced by the strains from eel mucus and from seawater (Table 4). Although additional studies are needed, the present results suggest that gills and skin mucus of healthy saltwater-reared rainbow trout are in particular colonized with potentially pathogenic *V. anguillarum* strains. Actually, gills and integument could represent sites where the vibrios penetrate and invade the host.

Analyses of the O1 strains have shown that the water and intestinal strains did not harbor plasmids, while the pJM1 plasmid (1) was frequently present in strains from mucus and gills of rainbow trout (J. L. Larsen and J. E. Olsen, unpublished data). Also, we have initiated K antigen analyses of the mentioned strains. Previously, a K antigen was suggested to be present in O1 strains isolated from cases of vibriosis in various countries (20).

Obviously, future studies on the ecology of *V. anguillarum* should focus on virulence markers. Such studies would

TABLE 3. HIC and SAT groups of *V. anguillarum* O1 and O2

Group	Value	O1 (%) (n = 59)	O2 (%) (n = 24)
HIC			
1	<50%	0	24 (100)
2	50–75%	13 (22)	0
3	>75%	46 (78)	0
SAT			
1	<0.1 M	0	0
2	0.1–0.9 M	58 (98)	0
3	1.0–1.5 M	1 (2)	1 (4)
4	>1.5 M	0	23 (96)

TABLE 4. Pathogenicity of *V. anguillarum* O1 to rainbow trout

Source of strain	Mortality[a]	No. of fish from which *V. anguillarum* was isolated
Rainbow trout		
Vibriosis	10/10	10
Mucus	20/20	20
Gill	10/10	10
Intestines	1/20	7
Eel (mucus)	0/10	0
Water	0/10	0
Control	0/10	0

[a] Number of fish which died within 1 week after injection/number at start of experiment.

benefit from a more detailed understanding of the pathogenicity mechanisms of the bacterium, including factors associated with adhesion and colonization.

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