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Vibrio anguillarum serovars associated with vibriosis in fish

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Abstract. A total of 517 *Vibrio anguillarum* strains isolated from diseased fish together with 14 *V. anguillarum* serogroup O2 and *V. ordalii* type strains were serotyped using the European serotyping system. Marked species differences were recorded. In isolates from salmonids serovar O1 (70·2%) and O2 (20·2%) were dominant, whilst a minor proportion belonged to other serogroups or were non-typeable. Figures for turbot were similar to those from salmonids. In 32 isolates from sea bass, sea bream and mullet, most strains belonged to serogroup O1, while one was O2a, one O7, and the rest non-typeable. In cod, serovar O2 was dominant while only a minor proportion belonged to other serogroups or were non-typeable. The eel isolates belonged equally to serovars O2 and O3. All O2 strains were subtyped with absorbed O2a and O2b antisera. O2a was dominant in all fish species, but in cod, the relative number of O2b isolates was considerably higher than in other fish species. The applicability of the European serotyping system is discussed and compared with other serotyping systems.

Introduction

Taxonomic investigations on strains of *Vibrio anguillarum* have shown significant biochemical variations in a number of characters (Lee, Shread, Furniss & Bryant 1981; Kaper, Lockman, Remmers, Kristensen & Colwell 1983; Larsen 1983; Bryant, Lee, West & Colwell 1986; West, Brayton, Bryant & Colwell 1986). However, it has been stated that there was not sufficient data for the establishment of specific biotypes on the basis of present information, and existing proposals were not robust (Kaper *et al.* 1983).

Wiik, Hoff, Andersen & Daae (1989) have demonstrated so much heterogeneity by phenotypical and genetic characterization that a thorough genetic characterization was recommended, and the existence of more than one species within *V. anguillarum* suggested.

Serotyping appears to be one of the most reliable ways of characterizing *V. anguillarum* isolates, especially from an epizootiological point of view. Pacha & Kiehn (1969) observed an apparent geographic distribution of serotypes which preferentially could be ascribed to the sources of the investigated strains. Later, it was found that three serotypes, I–III, were involved in vibriosis among North American salmonids in aquaculture (Gould, Antipa & Amend 1979; Johnson 1980; Chart & Trust 1984). Japanese publications have shown the presence of a variety of serotypes; however, the authors' nomenclature was quite different. Kitao, Aoki, Fukudome, Kawano, Wada & Mizuno (1983) dealt with serotypes A–C, Kusuda, Kawai & Sako (1975) with serotypes I–III, while J-O-1–J-O-3 were used by Ezura, Tajima, Yoshimizu & Kimura (1980). More recently, eight serotypes, J-O-1–J-O-8, have been described (Tajima, Ezura & Kimura 1986a, b, 1988).

Generally, only three of these Japanese serogroups appear to be important fish pathogens

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(Tajima *et al.* 1986a), and it should be emphasized that J-O-1 in Japan and serotype II in North America comprise the species *V. ordalii*, previously *V. anguillarum* biotype II (Schiewe & Crosa 1981). The present authors' main object in establishing a serotyping system for *V. anguillarum* was to further characterize isolates from epizootics among feral fish, and later in cultured fish, and to trace these organisms in the environment.

The purpose of this paper is to demonstrate the general applicability of serotyping and the distribution of serovars among various fish species to indicate which serovars could be relevant in the variety of vaccines that might emerge in the expanding aquaculture industry.

Materials and methods

Bacterial strains and culture conditions

Strains of *Vibrio anguillarum* were isolated in the present authors' own laboratories (Table 1–3) or kindly supplied by the donors as listed in Table 2. Additionally, 21 strains from sea bass, *Dicentrarchus labrax* (L.), 11 from sea bream, *Sparus aurata* (L)., and four from mullet (Mugilidae), isolated by Dr Giuseppe Ceschia, Italy, were included.

The present authors' own isolates were obtained by sampling from the pronephros of diseased fish with sterile swabs after sterilizing the skin with a burning iron, followed by an incision with a sterile knife. The swabs were plated onto blood agar (BA; Marine agar, Difco, supplemented with 5% citrated calf blood) and plates were incubated for 48 h at 20 °C. Principles for isolation and characterization are summarized in Larsen (1990). All *V. anguillarum* strains mentioned in this paper were tested using the characters listed by Larsen & Olsen (1991).

Preparation of antisera and O-antigen for agglutination test

Antisera against *V. anguillarum* serovars were raised in rabbits. Animals were injected intravenously with saline-washed suspensions (approximately 10^9 cells ml^{-1}) of formalin-killed cells. Injections were given twice weekly in consecutive doses of 0·2, 0·4, 0·8 and 1·0 ml. One week after the last injection, the rabbits were bled from the ear vein. Blood was allowed to clot for 3 h at room temperature. After removal of the coagulum, serum was collected by centrifugation. Two weeks later, this immunization and bleeding procedure was repeated, but with 1·0 ml doses throughout. The antisera were stored at −20 °C. For preparation of O-antigen for the slide agglutination test bacteria were grown on BA for 18–20 h at 20 °C and harvested with 3 ml of sodium acetate-buffered formalin-saline [0·05 M NaAc, 0·1 M NaCl, 1% (v/v) formalin, pH 7·5]. O-antigens, prepared by heating the bacterial suspension to 121 °C for 1 h, were used in the slide agglutination test. If needed, the antigens were concentrated by centrifugation. The principles for serotyping were described previously by Sørensen & Larsen (1986) using the serotyping system shown in Table 1.

Preparation of antisera for subgrouping of serovar O2 into subgroups O2a and O2b

Antisera prepared against the reference strains 1173/1 (O2a) and 820723–2/8 (O2b) (Rasmussen 1987) were used. Each strain was plated on five BA plates and incubated at 20 °C for 48 h. Cultures were harvested with 10 ml phosphate buffered saline (PBS) pH 7·3 and autoclaved at 121 °C for 1 h. Subsequently, suspensions were centrifuged at 4000 rpm for 20 min. The supernatant was removed and the cells washed once with PBS. When the cells were used for

absorption 3 ml of heterologous antiserum was mixed with the cell pellet, incubated at 37 °C for 1 h, stored at 5 °C overnight, and centrifuged at 4000 rpm for 20 min. Then the serum was tested for specificity with the homologous and heterologous strains. If necessary, the procedure was repeated.

Results

A comparison of the Danish serotyping system with those used in the USA and Japan is given in Table 1 together with the fish species from which the Danish reference strains were isolated and the ATCC numbers of the strains.

The results of serotyping 322 *V. anguillarum* strains isolated from dead or diseased salmonid

Table 1. Serotyping system for *Vibrio anguillarum* compared to other systems and with the Danish reference strains listed

Serovar					
Denmark	USA	Japan	Danish reference strain	ATCC no.	Isolated from:
01	I	C, J-O-3	6018/1	43305	*Oncorhynchus mykiss*
02	II	A, J-O-1	1173/1	43306	*Gadus morhua*
03		B, J-O-2	6062/A	43307	*Oncorhynchus mykiss*
04			1356/1	43308	*Gadus morhua*
05			1384/1	43309	*Gadus morhua*
06			1406/1	43310	*Gadus morhua*
07			6192/3	43311	*Anguilla anguilla*
08			1733/2	43312	*Gadus morhua*
09			1247/1	43313	*Gadus morhua*
010			1347/1	43314	*Gadus morhua*

Table 2. *Vibrio anguillarum* serovars isolated from salmonids

Country	Total number of strains	O1	O2	O3	O4	O5	Non-typeable
Denmark	163	111	30	5	3	4	10
Sweden	28	20	5			3	
Norway	57	46	11				
Finland	2	2					
UK	6	4	2				
Germany	23	12	9		1		1
Italy	22	18	1	1			2
France	4	2	1				1
USA	5	1	4				
Canada	12	10	2				
Total	322	226	65	6	4	7	14
Percentage		70·2	20·2	1·9	1·2	2·2	4·3

Donors: Denmark, present authors; Sweden, O. Ljungberg; Norway, T. Håstein; Finland, D. Williams; UK, B. Austin; Germany, A. Saltzmann; Italy, G. Georgetti & G. Ceschia; France, F.B. Laurencin; USA, J.L. Fryer & M.H. Schiewe; Canada, P.A. Greer.

fish showed that the most important serotypes were O1 (70·2%) and O2 (20·2%) (Table 2). Only a small number were non-typeable (4·3%) or belonged to serovars O3–O5, while O6–O10 were not registered in salmonid fish.

Table 3 shows the results for strains isolated in Denmark from other fish species. In cod, *Gadus morhua* L., the dominant serogroup was O2 while the remaining groups only occurred in a few cases. The O1 serogroup was insignificant in cod, and this was also true in the eel *Anguilla anguilla* (L.). The strains from eel included in this study exclusively belonged to serogroups O2 and O3. The figures for strains from turbot, *Scophthalmus maximus* (L.), were similar to those obtained for salmonid isolates.

Eighteen out of 21 strains isolated from sea bass were O1, whilst one was O2a, one was O7 and one was non-typeable. Six of 11 strains from sea bream were O1 while the remaining five were non-typeable. The four isolates from mullet were all O1. These 36 strains were all Italian or Greek.

Results of subtyping of O2 strains into O2a and O2b are shown in Tables 4 and 5. The O2a subgroup was dominant in all fish species. However, marked differences between fish species were noticed. In salmonids and eel, about 85% of the strains were O2a, while in cod, the proportion was less than 60%. Some strains only reacted with unabsorbed O2 antiserum, and not with O2a or O2b antisera. Therefore, these strains were not subtypeable.

In addition, a number of type culture strains of *V. anguillarum* O2 were tested with absorbed O2a and O2b sera. The majority of these strains belonged to subgroup O2a. Only two strains, both isolated from coalfish, *Pollachius virens* (L.), in Norway, belonged to subgroup O2b (Table 6).

Table 3. Serovars of *Vibrio anguillarum* in non-salmonid fish species from Denmark

Fish species	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10
Cod, *Gadus morhua* L.	1	51	1	3	2	1	0	1	1	1
Turbot, *Scophthalmus maximus* (L.)	55	9	0	0	0	0	0	0	0	0
Eel, *Anguilla anguilla* (L.)	0	17	19	0	0	0	1	0	0	0

Table 4. Subtyping of the O2 serogroup of *Vibrio anguillarum* isolates from salmonids into O2a and O2b

Origin	O2	O2a	O2b	O2*
Denmark	30	25	2	3
Sweden	5	3	2	
Norway	11	9		2
England	2	2		
Germany	9	7	2	
Italy	1	1		
France	1	1		
USA**	4	4		
Canada**	2	2		
Total	65	54	6	5

*Reacting with the unabsorbed antiserum only.
**Selected strains including *V. ordalii*.

Table 5. Subtyping of *Vibrio anguillarum* serovar O2 isolates from non-salmonid fish species

Isolated from	Number	O2a	O2b	O2*
Cod	51	30	18	3
Turbot, Denmark	9	4	2	3
Turbot, Norway	29	29	0	0
Eel	17	15	0	2

* Reacting with unabsorbed sera only.

Table 6. Subtyping of selected type strains of *Vibrio anguillarum* serovar O2 and *Vibrio ordalii* with O2a and O2b antisera

Species	Strain no.	Fish	O2a	O2b	Country
Vibrio anguillarum	VA 42/86	Atlantic salmon, *Salmo salar* L.	1		Canada
	T264	Sea trout, *Salmo trutta* L.	1		England
	T268	Atlantic salmon, *Salmo salar* L.	1		England
	8506171/1	Pike, *Esox lucius* L.	1		Finland
	2887	Rainbow trout, *Oncorhynchus mykiss* (Walbaum)	1		Italy
Type cultures	ATCC 14181	Brown trout, *Salmo trutta* L.	1		Scotland
	ATCC 2-19264	Cod, *Gadus morhua* L.	1		Denmark
	NCMB 6	Cod, *Gadus morhua* L.	1		Denmark
	NCMB 2130	Coalfish, *Pollachius virens* (L.)		1	Norway
	NCMB 2133	Coalfish, *Pollachius virens* (L.)		1	Norway
Vibrio ordalii	241-S, MSC275, DF_3K	Salmonids	3		USA
	VA-58-7448	Sockeye salmon, *Oncorhynchus nerka* (Walbaum)	1		Canada

Discussion

The serotyping system proposed by Sørensen & Larsen (1986) began by preparing two antisera and eventually described 10 serovars. However, it was found appropriate to harmonize existing serotyping systems, which was done by requesting reference strains from colleagues. The simplest way to name the O-serovars was to follow common rules used in serotyping of many other Gram-negatives. It was decided to use Pacha & Kiehn's (1969) proposal for the first two serovars in our serotyping scheme (Sørensen & Larsen 1986), which appear to be adequate, when comparing the sources of these isolates. It has been shown that serovar O2 may be subdivided into two serologically distinct subgroups, O2a and O2b (Rasmussen 1987). This subdivision has been confirmed by Bolinches, Lemos, Fouz, Cambra, Larsen & Toranzo (1990).

Besides being an important epizootiological tool, the O-antigen residing in LPS is considered to be the most important antigen in vaccines against vibriosis (Evelyn 1984), although some proteinaceous moieties may be involved (Smith 1988).

With the serotyping system for *V. anguillarum* used in this investigation, 308 out of 322 strains from salmonids from various geographic regions were typeable. Isolates from salmonids mainly belonged to serogroups O1 and O2, irrespective of geographic origin. Seventy per cent of salmonid strains belonged to serogroup O1 while 20·3% were O2. The remaining strains were non-typeable or belonged to serogroups O3-O10. These results are in accordance with those of Toranzo, Santos, Lemos, Ledo & Bolinches (1987).

Marked differences were noticed between fish species. While serogroup O1 was dominant in salmonids, sea bream, sea bass, turbot and mullet, O2 was the most abundant serotype in cod, and O2, together with O3, the most frequent in eel.

Strains from serogroup O2 have been shown to constitute a very heterogeneous group with respect to parameters such as biochemical characters and plasmid content (Larsen 1990), and O2 strains can be divided into subgroups O2a and O2b (Rasmussen 1987; Bolinches *et al.* 1990). The biochemical mechanisms behind this, and its ecological significance, are not fully understood at present. Research is presently in progress in the authors' laboratory to elucidate these problems. Subgrouping of the O2 isolates in the present investigation together with a number of type cultures showed that O2a was the most abundant in all fish species. However, the O2b strains constituted a considerable part of the O2 strains isolated from cod, and both cultures from coalfish belonged to this subgroup. These results are in accordance with those obtained by Fouz, Conchas, Lemos & Toranzo (1989). All *V. ordalii* strains included in this study reacted with O2a antiserum.

The sources of the 'pathogenic serovars' O1 and O2, are still questionable. In the very interesting work of Kanno, Nakai & Muroga (1989), it was suggested that *V. anguillarum* was transmitted as a water-borne infection or by contact, and furthermore, certain areas of the skin were found to be more vulnerable for penetration than others, with some being even more sensitive than the gills. After such experiments, certain questions must be answered. For example, is the environment the real source of the pathogenic vibrios or are these organisms associated with hosts/carriers? In a Danish study of the *V. anguillarum* flora of mariculture-produced rainbow trout, *Oncorhynchus mykiss* (Walbaum), it was found that approximately 29% of the *V. anguillarum* isolates from the water and sediment could be typed, but only 6% belonged to serovars O1 and O2. Besides, the only two O1 strains isolated did not possess the pJM1 plasmid. However, the *V. anguillarum* isolates colonizing external and internal surfaces of the fish were typeable to a greater extent (45–65%) and 42% of these belonged to serovars O1 and O2 (Larsen, Rasmussen & Dalsgaard 1988). In a comprehensive investigation of fresh- and seawater environments, Muroga, Iida, Matsumoto & Nakai (1986) studied 89 *V. anguillarum* isolates, and among these 14.6% could be serotyped by the Japanese typing system (A-I) but only one belonged to known 'pathogenic serovars' (O2). The presence of *V. anguillarum* in feral ayu, *Plecoglossus altivelis* (Temminck & Schlegel), fingerlings was also examined by Muroga, Yamanoi, Hironaka, Yamamoto, Tatani, Jo & Takahashi (1984). They detected *V. anguillarum* in only 168 (1·7%) out of 9574 fish, but only isolates belonging to J-O-1 (equivalent to O2) and J-O-3 (equivalent to O1) were virulent. Another Japanese investigation (Tajima *et al.* 1988) dealing with the occurrence of *V. anguillarum* in coho salmon, *Oncorhynchus kisutch* (Walbaum), and their culture environment, showed that among a total of 5337 *Vibrio* isolates only 58 *V. anguillarum* isolates were detected. Among these, 33 J-O-3 (O1) were associated with fish, while two were found in mud and one in sea water. One J-O-1 (O2) was

isolated from sea water while the remaining isolates did not belong to known pathogenic serotypes. The low densities of pathogenic serotypes in the environment are supported by the fact that enrichment methods might be necessary to demonstrate their presence (Muroga *et al.* 1986; Toranzo & Barja 1990).

On the basis of such data, and considering the apparently close association with fish which was also demonstrated in vibriosis of ayu (Muroga & Egusa 1988), it is tempting to suggest that the pathogenic *V. anguillarum* serotypes are associated with carrier fish and transported by these to a fish farm, for example, where closer contact transfers the organisms to the cultured fish. The fate of the organism depends on the host–pathogen–environment relationships that could result in a stress condition which triggers outbreaks of vibriosis; subsequently, both contact and water-borne infection spread the organism in the aquaculture facilities.

It has been stressed that some host preferences exist within the 'pathogenic serovars' and that they interact with the host in different ways (Wiik *et al.* 1989). These may be conferred by specific characters of the organism which are not reflected in LD_{50} determinations where O1 and O2 strains have shown the same level of virulence (Toranzo *et al.* 1987).

As far as the remaining serovars in the serotyping system are concerned, Pazos, Santos, Magaronos & Toranzo (1992) proved serovars O3, O4 and O5 to be virulent to fish. Recently O4, O6 and O8 were isolated from yolksac larvae of the Baltic cod (Buchmann, Larsen & Dalsgaard 1993), and recently, a serious outbreak of vibriosis in artificially reared cod occurred on the west coast of Denmark where untreated fish suffered a 100% mortality due to a serovar O4 infection.

In a Taiwanese study of 119 *V. anguillarum* (100 from milkfish, *Chanos chanos* (Forskäl), 10 from ayu, one from tiger shrimp, *Penaeus monodon* (Fabricius), and eight from the environment), 75·6% could be regarded as O1—all isolated from fish—while O2 and O3 contained 3·4% of the isolates (Song, Chen & Kon 1988). Again it should be emphasized that a harmonization of the typing system is urgently needed for proper comparison between studies.

Environmental or non-typeable strains also require consideration as they have been shown to possess pathogenic properties (Muroga *et al.* 1986; Lemos, Mazoy, Conchas & Toranzo 1991). As many fish in aquaculture are vaccinated with commercial vaccines containing serovars O1 and O2, such fish when debilitated by different means may be affected by organisms recruited from the environment or other vibrios, as discussed by Myhr, Larsen, Lillehung, Gudding, Heum & Håstein (1991). Possibilities for exchange of genetic properties may also result in new pathogenic organisms whose serotype will be determined and added to the serotyping system whenever they cause an epizootiological problem.

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