APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1986, p. 593–597
0099-2240/86/030593-05$02.00/0

Vol. 51, No. 3

Serotyping of *Vibrio anguillarum*

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Received 16 July 1985/Accepted 26 November 1985

A serotyping scheme based on the detection of O antigens by slide agglutination in fish-pathogenic strains of *Vibrio anguillarum* is presented. Over a period of 5 years 270 *Vibrio* strains from feral and cultured fish, 189 strains from the environment, and 36 strains from invertebrates were collected. The strains were divided into 10 distinct serotypes (O1 through O10). More than 90% of the fish-pathogenic strains, but only 40% of the environmental strains, were typable; 71% of the strains isolated from cultured rainbow trout were serotype O1, whereas 78% of the strains isolated from feral fish were serotype O2. No dominating environmental serotype was found. A serotyping system for *V. anguillarum* is proposed. A total of 90 strains received from culture collections and laboratories in different countries were typed according to the present system.

Vibrio anguillarum is an important infectious agent causing vibriosis among feral and farmed fish and shellfish (2, 6). It has been hypothesized that these aquatic animals acquire *V. anguillarum* infections from environmental sources (23, 24). Available biochemical data alone do not substantiate this because they do not permit a precise separation among strains of different origin (16, 20). Bergman (3) and Aaser (1) observed the occurrence of different "agglutination types" among fish-pathogenic vibrios. Serology was later used either in epizootological studies of vibriosis among feral fish (18) or in the characterization of strains from outbreaks of vibriosis among cultured fish (5, 9, 14, 25).

A pathobiological understanding of vibriosis must be based upon a detailed characterization of the causative strains to trace the sources of pathogenic isolates. This may be achieved by serological subdivision of the existing biochemical taxa (16, 21, 22), which provides a more solid basis for the investigation of the distribution and transmission of the strains in the aquatic environment and its fauna.

The importance of serology in epizootological and ecological studies of *V. anguillarum* was realized by Pacha and Kiehn (20), who in their work dealt with three serotypes: Northwest salmonid vibrios (serotype 1), European vibrios (serotype 2), and Pacific herring vibrios (serotype 3). At present three serotypes are recognized in Norway (12), two or three are recognized in the United States (25), and six are recognized in Japan (14).

Ezura and co-workers (7) have compared only some of these serotypes, and it seems pertinent to develop an unambiguous serotyping system of *V. anguillarum*.

In this paper we present a serological study of Danish *V. anguillarum* strains isolated from diseased feral and cultured fish, from invertebrates, and from the environment compared with a collection of international strains. On the basis of our results, we propose a serotyping system that is coordinated with the American (13, 25) and Japanese (7, 14) systems.

MATERIALS AND METHODS

Bacterial strains. The strains which were examined and their sources are listed in Table 1. Fish-pathogenic strains, strains from invertebrates, and environmental strains were isolated over a period of 5 years by the methods described below.

(i) Fish-pathogenic strains. Feral fish (cod, *Gadus morhua*; eel, *Anguilla anguilla*; and plaice, *Pleuronectes platessa*) were caught in pond nets or Danish seine at seven different geographical sites in internal Danish marine waters. Cultured rainbow trout (*Salmo gairdneri*) were collected from five Danish maricultures. Fish with lesions typical of vibriosis were selected for bacteriological examination and brought to the laboratory in plastic bags cooled with ice within 1 to 6 h after landing. Samples were taken from the pronephros with a sterile swab after sterilizing the skin with a burning iron followed by an incision with a sterile knife.

(ii) Strains from invertebrates. Crabs, starfish, and bivalves were collected from the seine (see above) and brought alive to the laboratory. Before the animals were opened with a sterile knife, the surfaces were sterilized with 70% (vol/vol) ethanol. Samples were taken from the internal organs with a sterile swab.

(iii) Environmental strains. Vibrio strains were isolated from seawater and sediment as described elsewhere (15).

(iv) Strains from culture collections and other laboratories. A total of 90 *V. anguillarum* strains received from type culture collections and laboratories in different countries were included for comparison. Four strains of *V. anguillarum* biotype II (7448, 241-S, DF_3K, and MSC2-75) were also examined. These strains are listed in Table 1 as *Vibrio ordalii* (21). Strains belonging to this species have not been isolated in Denmark (16).

Bacterial identification. Swabs were inoculated on blood agar plates that were incubated for 48 h at 25°C. Typical bacterial colonies resembling *V. anguillarum*, i.e., low convex, semitranslucent, and hemolytic, were isolated for primary identification according to the following characteristics: gram-negative mobile rods which were sensitive to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine, 10 μg/disk); positive for catalase, oxidase, nitrate reduction, and arginine decarboxylase; negative for lysine and ornithine decarboxylase; and fermentative in the oxidation-fermentation test and able to grow in 6% NaCl but not in

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TABLE 1. Bacterial strains

Representative strain	No. of isolates	Isolated from:	Source[a]	Serotype	
				Stated by supplier	According to present system
V. anguillarum					
6018/1	157	*Salmo gairdneri*	J. L. Larsen	(Fig. 1A and Table 3)	
1173/1	113	Feral fish	J. L. Larsen	(Fig. 1B and Table 3)	
1300F/2	23	*Gadus morhua* (feces)	J. L. Larsen	(Fig. 2B)	
3-16 KR/1	36	Invertebrates	J. L. Larsen	(Fig. 2C)	
1.1.2.13/2	189	Environment	J. L. Larsen	(Fig. 2A)	
B.1913/80	20	*Salmo gairdneri*	O. Ljungberg	I[b]	O1
FT1578	8	*Salmo gairdneri*	T. Håstein	–[c]	O1
T.246	4	*Salmo gairdneri*	B. Austin	–	O1
408F	2	*Salmo gairdneri*	F. Baudin Laurencin	I?[d]	O1
4008	16	*Salmo gairdneri*	G. Giorgetti	–	O1
57/82/1	8	*Salmo gairdneri*	A. Saltzmann	–	O1
775	1	*Onchorhynchus kisutsch*	M. Schiewe	I	O1
53-507	2	*Onchorhynchus kisutsch*	K. A. Johnson	I	O1
R.73	1	*Scopthalmus maximus*	A. E. Toranzo	–	O1
PT213	1	*Plecoglossus altivelis*	T. Aoki	C	O1
B.2221/80	4	*Salmo gairdneri*	O. Ljungberg	II[b]	O2
FT1633	1	*Salmo gairdneri*	T. Håstein	–	O2
2887	1	*Salmo gairdneri*	G. Giorgetti	–	O2
63/82/1	5	*Salmo gairdneri*	A. Saltzmann	–	O2
T.268	2	*Salmo salar*	B. Austin	–	O2
ATCC 14181	1	*Salmo trutta*	ATCC	–	O2
ATCC 19264	1	*Gadus morhua*	ATCC	–	O2
NCMB 6	1	*Gadus morhua*	NCMB	–	O2
PT24	1	*Plecoglossus altivelis*	T. Aoki	A	O2
2337	1	*Salmo gairdneri*	G. Giorgetti	–	O3
PT493	1	*Plecoglossus altivelis*	T. Aoki	B	O3
74/82/1	1	*Salmo gairdneri*	A. Saltzmann	–	O4
B.2762/81	3	*Anguilla anguilla*	O. Ljungberg	–	O5
NCMB 407	1	*Pleuronectes platessa*	NCMB	–	O7
PB15	1	*Plecoglossus altivelis*	T. Aoki	D	N[e]
PB28	1	*Plecoglossus altivelis*	T. Aoki	E	N
ET1	1	*Anguilla japonicus*	T. Aoki	F	N
V. ordalii MSC 2-75	4	*Onchorhynchus kisutsch*	M. Schiewe	II	O2

[a] ATCC, American Type Culture Collection, Rockville, Md.; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland; T. Aoki, Miyazaki University, Miyazaki, Japan; B. Austin, Ministry of Agriculture, Fisheries and Food, Dorset, England; G. Giorgetti, Laboratorio di Ittiopatologia, Udine, Italy; T. Håstein, Veterinærinstituttet, Oslo, Norway; K. A. Johnson, Connaught Labs, Ltd., Ontario, Canada; J. L. Larsen, Royal Veterinary and Agricultural University, Copenhagen, Denmark; F. Baudin Laurencin, Laboratoire National de Pathologie des Animaux Aquatiques, Brest Cedex, France; O. Ljungberg, Statens Veterinärmedicinska Anstalt, Uppsala, Sweden; A. Saltzmann, Universität Kiel, Kiel, Federal Republic of Germany; M. Schiewe, National Oceanic and Atmospheric Administration, Seattle, Wash.; and A. E. Toranzo, Universidad de Santiago, Santiago, Spain.

[b] For 10 of the 24 strains supplied by O. Ljungberg, the serotypes were determined by D. H. McCarthy, Tavolek Inc., Washington, D.C.

[c] –, Type not stated.

[d] I?, Cross-reactive with serotype O1.

[e] N, Nontypable.

7% NaCl. A detailed biochemical characterization of *V. anguillarum* strains has been given elsewhere (16).

Serological analysis. Antisera, antigens, and serotyping were basically as described by Pacha and Kiehn (20).

(i) Antigens for immunizations. The strains used for immunization were grown for 18 to 24 h in broth culture containing 1% NaCl. The bacteria were killed with 2% (vol/vol) Formalin (40%). Only strains that did not exhibit autoagglutination after heating for 1 h at 120°C were used for immunization.

(ii) Antisera. Rabbits were injected intravenously with saline-washed suspensions (~10^9 cells per ml) 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. Two weeks later, this immunization procedure was repeated, now with 1.0-ml doses throughout. The antisera were stored at −20°C.

(iii) O antigen for agglutination tests. The bacteria were grown on blood agar plates (9-cm diameter) for 18 to 20 h at 25°C and harvested in 3 ml of sodium acetate-buffered Formalin-saline (0.05 M $NaCOOCH_3$, 0.1 M NaCl, 1% [vol/vol] Formalin [40%], pH 7.5). O antigens, prepared by heating the bacterial suspension to 100°C for 1 h, were used in the slide agglutination test. If needed, the antigens were concentrated by centrifugation.

(iv) Slide agglutination tests. A test was performed by mixing a loopful of O-antigen suspension on a slide with a loopful of undiluted antiserum. Agglutination was observed by inspecting the slide in sidelight against a dark background. A distinct and immediately occurring agglutination was registered as positive, and no or only a weak agglutination occurring after several minutes was registered as negative. One O-antigen preparation was examined simultaneously on one slide with 10 antisera. Controls were made with saline and serum from nonimmunized rabbits. Autoagglutinating strains were excluded.

RESULTS

To begin with antisera were raised against a few strains of *V. anguillarum* isolated from diseased fish, and two serotypes were thus established. With these two antisera a number of fish-pathogenic strains were tested. Some were nontypable, and they were then used for preparation of new antisera with which all previously nontypable strains were reexamined. In this way new serotypes were gradually established, and we ended up having 10 different antisera (Table 2) that reacted with more than one strain. There were no cross-reactions between types when agglutination was carried out and interpreted as described above.

The 90 strains received from collections and other laboratories were typed with our 10 antisera (Table 1). Six out of the 10 serotypes were found among these strains, and only three strains belonging to Japanese serotypes D, E, and F were nontypable with our antisera. These types have not been detected since 1973 (14). The four *V. ordalii* strains tested were all type O2. Serotypes O1, O2, and O3 are found worldwide (Table 1).

In Table 2 we have combined our serotyping results with those obtained by others into a new serotyping system, based on O antigens, designated by Arabic numerals 1 through 10 with the letter "O" as a prefix. A total of 270 *V. anguillarum* strains were isolated from diseased fish (Table 1) and serotyped; 157 isolates were from rainbow trout, 64 were from cod, 40 were from eels, and 9 were from plaices (less than 5% of the strains were autoagglutinating; these strains were excluded).

About 10% of the strains were nontypable (Fig. 1). A marked difference was found in the distribution of serotypes among the bacterial strains isolated from cultured rainbow trout and those isolated from feral fish. O1 is the dominant serotype (70%), causing vibriosis in rainbow trout; infections with O2 are not uncommon (15%), whereas the other serotypes only occur sporadically (Fig. 1A). O2 is the serotype most commonly isolated from feral fish with vibriosis (75%). Because no difference in the serotype distribution of strains isolated from cod, eel, and flatfish was observed, these results were compiled (Fig. 1B).

Of the 189 strains isolated from the environment (Table 1), only 40% were typable, and no type predominated. A few strains of serotype O1 or O2 were isolated. Data from strains from seawater and sediment were combined because they were alike (Fig. 2A).

The serotype distribution of the strains isolated from feces of cod (Fig.2B) and from invertebrates (Fig. 2C) resembles the distribution found among the environmental strains, i.e., many nontypable strains and no predominant serotype. Interestingly, eight of nine strains isolated from skin mucus of healthy cod belonged to serotype O2.

TABLE 2. Serotyping system for *V. anguillarum* based on O antigens

Serotype			Danish reference strain	Isolated from:
Denmark	United States[a]	Japan[b]		
O1	I	C, J-0-3	6018/1	*Salmo gairdneri*
O2	II	A, J-0-1	1173/1	*Gadus morhua*
O3		B, J-0-2	6062/A	*Salmo gairdneri*
O4			1356/1	*Gadus morhua*
O5			1384/1	*Gadus morhua*
O6			1406/1	*Gadus morhua*
O7			6192/3	*Anguilla anguilla*
O8			1733/2	*Gadus morhua*
O9			1247/1	*Gadus morhua*
O10			1347/1	*Gadus morhua*

[a] See references 4, 9, and 26.
[b] See references 7 and 14.

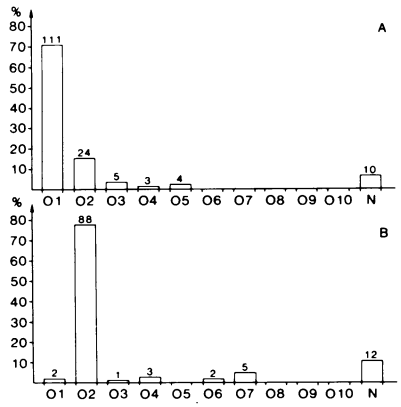

FIG. 1. Serotype distribution among fish-pathogenic *V. anguillarum* strains. A, 157 strains isolated from rainbow trout; B, 113 strains isolated from seawater fish. Serotypes O1 through O10 and nontypable strains (N) are indicated on the abscissa. The ordinate indicates the percentage, and figures above the columns indicate numbers of the tested strains belonging to each serotype.

DISCUSSION

Many reports about the serology of *V. anguillarum* have been published; most have been concerned with vibriosis in cultured fish, whereas the feral fish population has been studied only more sporadically (1, 3, 5, 7, 9, 10, 12, 14, 20, 25).

As a result of our study we have found new serotypes of

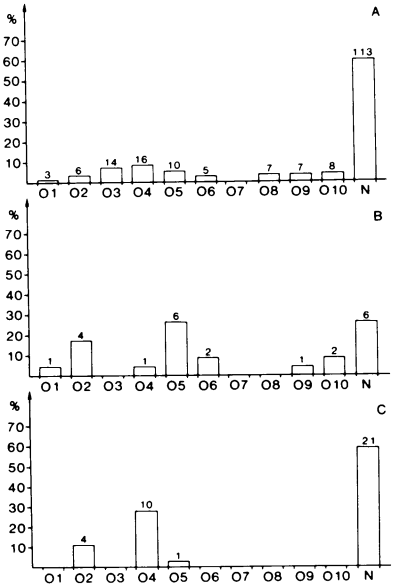

FIG. 2. Serotype distribution among *V. anguillarum* strains. A, 189 environmental strains; B, 23 strains isolated from feces from normal cod; C, 36 strains isolated from invertebrates. See legend to Fig. 1 for further explanation.

V. anguillarum and have proposed a typing system based on serologically distinct, heat-stable somatic O antigens, i.e., lipopolysaccharides.

The designations of the types are in agreement with the American type system (4, 8, 13), but we suggest the use of Arabic rather than Roman numerals and the letter "O" as a prefix, because preliminary investigations in our laboratories have demonstrated that many strains of *V. anguillarum*, like other gram-negative bacteria (19), possess a polysaccharide capsule, a K antigen. These K antigens may explain the heterogenous antigenic patterns found when using immunodiffusion methods for the study of *V. anguillarum* antigens (9, 12, 25). By heating, most of the K antigens are removed from the bacterial cell walls, exposing the thermostable O antigens.

In the present study more than 500 *V. anguillarum* strains isolated from feral or farmed fish, from invertebrates, and from the environment were examined. Ten distinct serotypes were established (Table 2). Six of these types were recognized among the 90 strains received from culture collections and other laboratories (Table 1). Only three of these strains (PB15, PB28, and ET1 from Japan; Table 1) were nontypable. However, these types have not been detected since 1973 (14).

Serotype O1 was found to be the type most commonly isolated form infections in cultured salmonids (Fig. 1A). This type might be the same as serotype 1, which we did not manage to obtain, described as the Northwest salmonid vibrios (20). Serotype O1 has previously been described as type 775A (25), type I (8), type 408 (17), type J-0-3 (7), and type C (14) (Tables 1 and 2).

Serotype O2 has been described as the European vibrios, or as serotype 2 (20), and by others as type 569 (25), type II (8), type J-0-1 (7), and type A (14). This serotype is the predominant type isolated from diseased feral seawater fish (Fig. 1B). However, it also infects salmonids (Fig. 1A). In Japan, 91% of the *V. anguillarum* strains isolated from ayu (*Plecoglossus altivelis*) were found to belong to this type (14). Four *V. ordalii* strains tested carried the same O antigen. Serotype O3 has only rarely been isolated from diseased fish in Denmark (Fig. 1), but has been described as a fish pathogen by others (14).

All 10 serotypes were isolated from diseased fish. However, serotypes O8, O9, and O10 were only reisolated from the environment (Fig. 1 and 2). These serotypes seem to be true environmental types of very low pathogenicity.

The serology of environmental *V. anguillarum* strains has not been examined before, despite the fact that it has been hypothesized that aquatic animals acquire their infections from this source (23, 24) and despite the fact that *V. anguillarum* occurs abundantly in the marine environment (16).

Serotypes O3, O4, O5, and O6 are more common in the environment and may therefore cause a higher infectious pressure than the rarer types. Serotype O7 was isolated from diseased seawater fish and not from the environment. Notably, strain NCMB 407 isolated from plaice also belongs to this serotype.

Strains of serotype O1 or O2, which cause the majority of the infections in fish (see above), were also isolated in low numbers from the environment (Fig. 2A). These strains could be released into the environment from a specific reservoir, e.g., from diseased feral fish, or they may belong to the indigenous environmental microflora and possess an exceptionally high potential for colonizing fish and causing diseases (11).

Finally, we suggest that a coordinated serotyping system may become the basis for an international harmonization of diagnostics, development of vaccines, and environmental studies important for environmental authorities.

ACKNOWLEDGMENTS

The technical assistance of Bente Sølvig Jensen and Maj Britt Højgaard is greatly appreciated.

The work was supported by Agricultural and Veterinary Research Council grant no. 13-3412.

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