SEROLOGICAL STUDIES ON *VIBRIO FLUVIALIS*

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SUMMARY: The serology of 138 strains of *Vibrio fluvialis* was studied. Eighteen O-antigenic groups were defined among them and it was shown that the H antigens of all the strains were identical regardless of the biovar. The presence of mucoid antigen, which inhibits O agglutination, was found in some strains. As all O antisera for *V. fluvialis* contained some R antibody, all diagnostic O sera must be absorbed with R organisms before use. Some O antigens of *V. fluvialis* were identical with those of certain *Vibrio cholerae* serovars.

INTRODUCTION

Vibrio fluvialis, previously known as "group F vibrio" in England and "CDC group EF6" in the United States (Furniss et al., 1977; Huq et al., 1980), is a halophilic marine organism. It has been isolated from estuarine water and sea-fish (Furniss et al., 1977; Lee et al., 1978; Seidler et al., 1980; Sakazaki, 1982) and from numerous patients suffering from acute diarrhea (Spira et al., 1978; Blake et al., 1980; Furniss et al., 1977; Huq et al., 1980; McNicol et al., 1980; Seidler et al., 1980; Agarwal and Sanyal, 1981; Sakazaki, 1982; Tacket et al., 1982). Recently, several reports on the enteropathogenicity of the organism have appeared (Sanyal et al., 1980; Seidler et al., 1980; Lockwood et al., 1982; Kobayashi et al., 1983; Kudoh et al., 1983; Nishibuchi et al., 1983). However, no serological study of this organism has so far been described. The purpose of the present study, in which 138 strains of the organism isolated from various sources were examined, is to propose an antigenic schema for epidemiological and ecological use.

MATERIALS AND METHODS

Strains studied: A total of 138 strains of *V. fluvialis* was studied; of these 104 were isolated in Japan, 23 were received from India, eight from Bangladesh and three from Africa. Of the 138 strains, 112 were *V. fluvialis* biovar I, and the remaining 26 strains were biovar II. The majority of the strains were isolated either from the feces of patients with gastroenteritis or from seafish, and a few were derived from birds and seawater. An R antigen mutant derived

from strain GF25 was obtained after repeated colony selection by an oblique light method.

In addition, other 124 strains of related species including *Vibrio cholerae*, *V. parahaemolyticus* and *Plesiomonas shigelloides* were used to study their antigenic relationship to *V. fluvialis*.

O antisera: For preparation of the O antisera, strains were inoculated into infusion broth. After overnight incubation at 37 C with continuous shaking in a water bath, the cultures were heated at 100 C for 2 hr. The heated organisms were then washed twice and, after final centrifugation, the packed cells were resuspended in saline. The density of the suspension was adjusted to that of the original broth culture. Rabbits were each injected intravenously with 0.5-, 1-, 2- and 4-ml amounts of the suspension at intervals of 4 days. The animals were bled on the sixth day after completion of the course of injections and the serum separated.

H antisera: Crude flagellar preparations were used for production of the H antisera. Actively motile cultures, obtained by several passages of the organism through semisolid media, were inoculated into brain heart infusion (Difco). After incubation at 37 C for 18 hr, the growth was centrifuged at 15,000 rpm for 30 min and the sediment washed twice in saline. The packed cells were resuspended in saline and deflagellated in a blender at 8,000 rpm for 5 min. After deposition of the cells by centrifugation at 15,000 rpm for 30 min, the supernatant fluid was removed and then centrifuged at 70,000 $\times g$ for 60 min; the sediment was washed twice in saline. The presence of flagellar fragments in this sediment was confirmed by electron microscopy. The flagellar fragment obtained from a 1-liter culture was suspended finally in 20 ml of phosphate buffered saline (0.067 M, pH 7.2). Rabbits were each immunized with each of these suspensions in the same way as that for the preparation of O antisera. Since only crude flagellar preparations were used for immunization, O antibody in the resulting H antisera was removed by absorption with the homologous culture heated at 100 C for 2 hr.

Agglutination tests: For antigen determination, tube agglutination tests were carried out with heated cell suspension as used for preparation of O antisera. The results were read after incubation in a water bath at 50 C for 18 hr. The H agglutination tests were performed with suspensions of motile cultures. Antigens for the tube agglutination tests were prepared from 18-hr cultures on Trypticase Soy Agar. The cultures were harvested in saline containing 0.3% formalin. The density of the suspension was adjusted to that of an overnight broth culture. The H-agglutination tests were carried out by mixing equal volumes of antigen with appropriate dilutions of sera; the results were read after incubation in a water bath at 50 C for 18 hr. For R-agglutination tests, the R antigen culture was suspended in distilled water, and an equal volume of the suspension was added to diluted antisera with 0.85% saline to prevent autoagglutination.

Agglutinin-absorption tests: Agglutinin-absorption tests were performed

when reciprocal or unilateral reactions occurred with certain antisera. Diluted antiserum was mixed with packed cells derived from growth on plates of infusion agar for absorption of O antisera and on Nutrient Broth No. 2 (Oxoid) containing 1% agar (Difco) for H-antibody absorption.

TABLE I

Agglutinability of O antigen suspensions

Strain	Treatment	O titer to homologous antiserum
GF1	1	80
	2	80
	3	320
GF3	1	80
	2	80
	3	1,280
GF4	1	40
	2	40
	3	1,280
GF5	1	40
	2	40
	3	640
GF6	1	320
	2	320
	3	640
GF9	1	80
	2	80
	3	640
GF16	1	80
	2	80
	3	640
GF21	1	160
	2	160
	3	640
GF22	1	40
	2	160
	3	320
GF27	1	—
	2	20
	3	320
GF37	1	20
	2	160
	3	640

Treatment 1: Live state

2: Heated at 100 C for 1 hr

3: Heated at 100 C for 2 hr

The sign — indicates negative at a dilution of 1:20 and higher.

TABLE II
R agglutinin titers of diagnostic *O* antisera of *V. fluvialis*

Antiserum	Antigen (100 C, 2 hr)	
	GF25(R)*	Homologous <i>O</i> antigen
O1	160	320
O2	320	640
O3	40	1,280
O4	160	1,280
O5	320	640
O6	160	640
O7	160	640
O8	160	640
O9	160	640
O10	80	320
O11	160	320
O12	160	640
O13	160	1,280
O14	320	1,280
O15	160	320
O16	160	320
O17	320	2,560
O18	160	1,280

* GF25 was employed as a pilot strain for the R antigen of *V. fluvialis*.

TABLE III
O antigenic relationships

<i>O</i> antiserum*	Homologous titer	Titer with other <i>O</i> antigen
O1	320	
O2	320	
O3	320	
O4	640	
O5	320	
O6	640	O7 : 40
O7	320	
O8	320	
O9	320	
O10	320	
O11	320	
O12	640	
O13	1,280	
O14	640	O4 : 160
O15	320	
O16	320	
O17	1,280	
O18	1,280	

* R antibody in the antiserum was removed by absorption with R culture (GF25) heated at 100 C for 2 hr.

RESULTS

O Antigen

Since some live cultures of *V. fluvialis* did not agglutinate with homologous O antisera, 11 strains were first used to determine the best way of treating cell suspensions for optimal O agglutinability. Growth on infusion agar at 37 C for 18 hr was harvested in saline and the suspension divided into three parts. One part was retained; another heated at 100 C for 2 hr; and the other heated at 100 C for 1 hr. The organisms in each part were then washed in saline, centrifuged and the packed cells finally resuspended in saline. Subsequent agglutination tests showed that the antigens heated for 2 hr gave the highest titers with homologous O antisera; in contrast, no reaction or only weak agglutination occurred with live antigens (Table I). From these results, determinations of the O antigens of the 138 cultures were carried out with the cell suspensions heated at 100 C for 2 hr followed by washing.

As shown in Table II, it was recognized that, although they had been

TABLE IV

O antigen determination and distribution of 138 strains of *V. fluvialis*

O group	Source				Total
	Human	Seafish	Bird	Seawater	
O1	2	3	—	—	5
O2	1	2	—	—	3
O3	1	4	—	—	5
O4	8	4	1	—	13
O5	1	1	—	—	2
O6	5 (3) *	1	—	—	6 (3)
O7	15 (4)	2	—	—	17 (4)
O8	1	—	—	—	1
O9	3	2	—	1	9
O10	7 (2)	3	—	—	10 (2)
O11	4 (2)	4 (3)	—	—	8 (5)
O12	1	—	—	—	1
O13	1	4	—	—	5
O14	4	5	—	—	9
O15	1	—	—	—	1
O16	2	5	—	—	7
O17	1	2	—	1	4
O18	1	1	—	—	2
UK	—	7 (5)	3	—	10 (5)
R	5 (2)	15 (5)	—	—	20 (7)
Total	64 (13)	68 (13)	4	2	138 (26)

* Numbers in parentheses indicate the number of strains of biovar II.

TABLE V

H agglutinin-absorption tests with *V. fluvialis*

Antiserum*	H-antigen								
	Type strain	GF3 (O3)	GF4 (O4)	GF5 (O5)	GF9 (O7)	GF37 (O12)	Refer. strain	GF6 (O6)	GF102 (O7)
<i>V. fluvialis</i> GF4-H (biovar I)									
Unabsorbed	2560	2560	2560	1280	2560	2560	2560	2560	1280
Absorbed with									
Type strain (biovar I, O7)	—	—	—	—	—	—	—	—	—
GF3 (biovar I, O3)	—	—	—	—	—	—	—	—	—
GF5 (biovar I, O5)	—	—	—	—	—	—	—	—	—
GF9 (biovar I, O7)	—	—	—	—	—	—	—	—	—
GF37 (biovar I, O12)	—	—	—	—	—	—	—	—	—
Reference strain (biovar II)	—	—	—	—	—	—	—	—	—
GF6 (biovar II, O6)	—	—	—	—	—	—	—	—	—
GF102 (biovar II, O7)	—	—	—	—	—	—	—	—	—
<i>V. fluvialis</i> Reference strain-H (biovar II)									
Unabsorbed	1280	1280	1280	640	1280	1280	1280	1280	640
Absorbed with									
Type strain (biovar I, O7)	—	—	—	—	—	—	—	—	—
GF3 (biovar I, O3)	—	—	—	—	—	—	—	—	—
GF4 (biovar I, O4)	—	—	—	—	—	—	—	—	—
GF5 (biovar I, O5)	—	—	—	—	—	—	—	—	—
GF9 (biovar I, O7)	—	—	—	—	—	—	—	—	—
GF37 (biovar I, O12)	—	—	—	—	—	—	—	—	—
GF6 (biovar II, O6)	—	—	—	—	—	—	—	—	—
GF102 (biovar II, O7)	—	—	—	—	—	—	—	—	—

* O antibody in the antiserum was removed by absorption with homologous cultures heated at 100C for 2hr.

The sign—indicates negative at a dilution of 1:20 and higher.

produced with S cultures, all O antisera for *V. fluvialis* contained some R agglutinin.

By cross-agglutination and agglutinin-absorption tests, these cultures except some R forms and unknown non-reacting strains, were divided into 18 O-antigenic groups. As shown in Table III, additional reactions occurred with the antisera of O groups 6 and 14, but no actual cross-reaction was observed among any combination of the 18 O-antigen groups.

The results of O-antigen determinations of the 138 strains of *V. fluvialis* from various sources are summarized in Table IV. Of these, 108 strains were grouped with the 18 O antisera; of the remainders, 20 were R-form cultures and 10 strains did not react with any of the antisera.

H Antigen

H antisera were prepared with two cultures, *V. fluvialis* GF4 (biovar I, O

TABLE VI

O antigenic relationships between *Vibrio fluvialis* and *V. cholerae* serovars

Antiserum	Antigen (100C, 2hr)	
	<i>V. fluvialis</i> -O4	<i>V. cholerae</i> -O41
<i>V. fluvialis</i> -O4		
Unabsorbed	640	640
Absorbed with <i>V. cholerae</i> -O41	—	—
<i>V. cholerae</i> -O41		
Unabsorbed	640	640
Absorbed with <i>V. fluvialis</i> -O4	—	—
	<i>V. fluvialis</i> -O5	<i>V. cholerae</i> -O39
<i>V. fluvialis</i> -O5		
Unabsorbed	320	320
Absorbed with <i>V. cholerae</i> -O39	—	—
<i>V. cholerae</i> -O39		
Unabsorbed	640	640
Absorbed with <i>V. fluvialis</i> -O5	—	—
	<i>V. fluvialis</i> -O10	<i>V. cholerae</i> -O6
<i>V. fluvialis</i> -O10		
Unabsorbed	320	320
Absorbed with <i>V. cholerae</i> -O6	—	—
<i>V. cholerae</i> -O6		
Unabsorbed	640	640
Absorbed with <i>V. fluvialis</i> -O10	—	—

The sign—indicates negative at a dilution of 1:20 and higher.

group 4) and NCTC 11328 (biovar II reference strain). By cross agglutination and agglutinin-absorption tests with these two H antisera and nine strains of *V. fluvialis* chosen at random, it was demonstrated that the H antigens of all nine cultures were identical (Table V).

H-agglutination tests were also carried out with all the strains of *V. fluvialis* received. Of the 138 strains of the organism, 135 were agglutinated with both H antisera at homologous titers. The remaining three non-motile cultures were not agglutinated. The H agglutination of *V. fluvialis* cultures was flocculant in nature and it appeared only after incubation at 50 C for 8 hr or longer.

From these results, it is concluded that all the strains of *V. fluvialis* examined in the present study possessed an identical H antigen.

Extragenetic Relationships

Antigenic relationships between *V. fluvialis* and other biochemically related organisms including *V. cholerae*, *V. parahaemolyticus* and *Plesiomonas shigelloides* were investigated. Heated antigen suspensions were prepared with representative cultures of the 18 O groups of *V. fluvialis*, 83 serovars of *V. cholerae*, 11 O groups of *V. parahaemolyticus* and 30 O groups of plesiomonads for cross-

TABLE VII

Representative strains for serovars of V. fluvialis

Serovar	Strain
1	12-68
2	26-68
3	209-73
4	210-73
5	212-73
6	1997-80
7	305-77
8	99-77
9	910-77
10	1184-77
11	96-79
12	391-79
13	1562-80
14	1988-80
15	1561-80
16	881-81
17	950-82
18	1030-82

agglutination and agglutinin-absorption tests. Although no significant reciprocal relationships were recognized between the O antigens of *V. fluvialis* and *V. parahaemolyticus* or plesiomonads, some close relationships were demonstrated between those of *V. fluvialis* and *V. cholerae*. As shown in Table VI, O antigens 4, 5 and 10 of *V. fluvialis* were apparently identical with those of *V. cholerae* serovars 41, 39 and 6, respectively.

DISCUSSION

In the present study, 18 O-antigen groups were defined among 138 strains of *V. fluvialis*. Since all strains of *V. fluvialis* possess identical H antigens, O-antigen groups of the species should therefore be referred to as serovars. The representative strain for each serovar is shown in Table VII; these strains should be used for preparation of the diagnostic antisera.

The R form is indistinguishable in colonial morphology from the parent S form, but may be distinguished by agglutination with anti-R serum. All O antisera against *V. fluvialis* contain some R antibody which may cause cross reactions in determination of the O groups. It should be emphasized, therefore, that for practical use all diagnostic O antisera must be absorbed with R culture of *V. fluvialis* strain GF25.

Although the mucoid antigens of *V. fluvialis* have not been considered in this study, the majority of strains were O inagglutinable in the living state. For identification of O antigens, the cultures to be tested should therefore be heated at 100 C for 2 hr and washed twice before examination.

There was no significant difference in the distribution of serovar strains in man or fish (Table IV).

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