



Title	Antigens of <i>Vibrio parahaemolyticus</i> . I. Preparation of Specific Antisera to Somatic (O) Antigen and Their Application in Antigen Analysis of <i>Vibrio parahaemolyticus</i>
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ANTIGENS OF *VIBRIO PARAHAEMOLYTICUS*. I. PREPARATION OF SPECIFIC ANTISERA TO SOMATIC (O) ANTIGEN AND THEIR APPLICATION IN ANTIGEN ANALYSIS OF *VIBRIO PARAHAEMOLYTICUS*¹

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SUMMARY The preparation of specific antisera to somatic (O) antigen of *Vibrio parahaemolyticus* is described. Strains isolated from clinical sources were analyzed for O-antigen and it was found that strains which could not be typed with K-antisera could be identified by specific antisera to ten known somatic (O) antigens (O-1 - O-10) and to 2 new O-antigens (O-N1 and O-N2) proposed here.

INTRODUCTION

Like many other gram negative rods, *Vibrio parahaemolyticus* has the following three antigenic components: 1) a heat stable somatic antigen (O-antigen) (Sakazaki, 1963; Okada, 1966; Torii et al., 1967; Miwatani, 1967), 2) a capsular or envelope antigen (K-antigen) (Omori et al., 1966; Hashimoto et al., 1966) and 3) a heat labile flagellar antigen (H-antigen) (Sakazaki, 1967; Miwatani and Nishimune, 1967). Sakazaki studied the antigenic structure of *Vibrio parahaemolyticus* and described 10 O-antigens and 32 K-antigens in this species (Sakazaki, 1965; Sakazaki, 1967). He also recommended K-antigen typing for antigen analysis of freshly isolated *Vibrio para-*

haemolyticus, as determination of O-antigen was impractical as a routine procedure for etiological investigations. Sakazaki pointed out 3 main reasons why O-antigen analysis was impractical and K-antigen analysis was suitable; 1) freshly isolated organisms have abundant K-antigen, and in the living state they are not agglutinated by homologous O-antiserum, 2) in some cases, when cultures are boiled at 100 C for 2 hours they lose their ability to be agglutinated by homologous K-antiserum, while still not being agglutinated by O-antiserum and 3) each K-antigen is specific for an individual O-antigen, for example K-2 and K-3 antigens were demonstrated only in the organisms with O-2 antigen (Sakazaki, 1967).

Further reasons why O-antigen analysis seems to be impractical are; 1) it is difficult to get an antiserum for somatic (O) antigen with

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a high agglutination titer, 2) cross reactions were observed, and specific antiserum has not been prepared, 3) it is complicated to prepare an agglutinating somatic (O) antigen.

Since 1965, however, many strains which cannot be typed with K-antigen have been isolated from clinical sources and new K-types have been reported every year.

During antigenic analyses of *Vibrio parahaemolyticus* isolated from clinical sources, we found that more than 40% of the organisms could not be typed with K-antigen when K-antigen analysis alone was available for determination of their antigenic structures. Accordingly, a specific antiserum to the somatic (O) antigen was necessary for antigen analysis of these organisms.

This paper reports the preparation of specific antisera to the somatic (O) antigen and their application to antigen analysis of *Vibrio parahaemolyticus* isolated from clinical sources. Two new somatic (O) antigens are also proposed.

MATERIALS AND METHODS

1. Strains

Ten pilot strains representing the O-antigens (one for each O-serotype) and two strains isolated from clinical sources (O-N1 and O-N2) were used for preparation of specific antisera to somatic (O) antigens. One hundred and forty-four strains of *Vibrio parahaemolyticus* isolated at the Public Health Research Institute of Kobe City, Tokyo-to Laboratories for Medical Sciences and Kanagawa Prefectural Public Health Laboratory, during outbreaks of food poisoning from 1965 through 1967, were analyzed for O-antigen employing the preparations of specific antisera.

2. Preparation of immunizing somatic (O) antigens

Organisms were grown on nutrient agar containing 3% sodium chloride, at 37 C for 18 hours, and washed off with 3% sodium chloride solution. The cell suspension was heated by passing steam (100 C) through it for one hour and then centrifuged at $5,000 \times g$ for 15 minutes. The resulting precipitate was suspended in the same volume of 3% sodium

chloride solution and reheated by steam (100 C) for one hour. Then the suspension was centrifuged, resuspended in the same volume of 3% sodium chloride solution and reheated by steam (100 C) for an additional 30 minutes. Finally, the suspension was adjusted to a concentration of 40 mg wet weight of cells per ml of 3% sodium chloride solution. This suspension was used as the preparation of immunizing antigens.

3. Immunization procedure

The immunization procedure was essentially the same as that described by Burrows et al., (1946). Young rabbits, weighing about 2.5 to 3 kg, were immunized with a series of 5 inoculations at 4 day intervals: the first 2 intraperitoneal and the next 3 intravenous. The doses of cell suspension at each inoculation are summarized in Table 1. If the

TABLE 1. *A schedule for immunization of rabbits with heat-killed cell suspension of Vibrio parahaemolyticus*

Inoculation	Route of inoculation	Dose ^a (ml)
1	Intraperitoneal	0.5
2	"	1.5
3	Intravenous	0.5
4	"	1.0
5	"	2.0

a Rabbits weighed 2.5-3 kg.

rabbits lost more than 100 g weight, the next inoculation was the same as before, but if they gained more than 100 g, an additional 0.5 ml was inoculated the next time. A preliminary experiment on the rise of agglutinin titers in rabbits after inoculation indicated that this procedure produced an antiserum with sufficiently high titer for further experiments (Miwatani, 1967). After 5 inoculations, blood was drawn by 3 successive heart punctures at 3 day intervals and the serum was separated, pooled and kept at -20 C until use.

4. Preparation of agglutinating somatic (O) antigens

Test strains were grown on nutrient agar slants containing 3% sodium chloride at 37 C for 18 hours. The cells were washed off with 3% sodium chloride solution and centrifuged at $5,000 \times g$ for 15 minutes. They were then suspended in a small amount of 3% sodium chloride solution and heated with steam at

100 C for one hour. They were centrifuged, re-suspended in 3% sodium chloride solution and adjusted to an optical density of 0.3 at 550 m μ with a Coleman junior spectrophotometer.

5. Agglutinin absorption

To one volume of antiserum were added two volumes of 3% sodium chloride solution and two volumes of heat-killed cell suspension (100 mg wet weight per ml) prepared by the same method as the immunizing antigens. The mixture was incubated at 37 C for 2 hours and then stood overnight at 4 C. The cells were removed by centrifugation at 5,000 $\times g$ for 30 minutes, and this supernatant was used as the preparation of absorbed antiserum. In some cases, this absorption was repeated once more to reduce the agglutinin titers against heterologous antigens.

6. Agglutination test

The test tube method was employed for the agglutination test. Antiserum diluted 1:100 was used for the test and two fold serial dilutions were made 3% sodium chloride solution. To 0.5 ml of antiserum was added 0.5 ml of the agglutination antigens and the mixture was incubated at 55 C for 16 hours. The reciprocal of the highest dilution showing a

visible agglutination was recorded as the agglutination titer.

RESULTS

1. Agglutination test with unabsorbed antisera

Agglutination titers of unabsorbed antisera of ten pilot strains (O-1—O-10) were examined using heat-killed (100 C, 1 hour) cell suspensions as agglutinating antigens. The results are shown in Table 2. The agglutination titers of each unabsorbed antiserum against the homologous antigens were higher than 1,600. Although most agglutination titers against the heterologous antigens were lower than 200, higher titers (400 or 800) were observed in some cases; for example, the antiserum of O-1 gave agglutination titers of 400 against O-5, O-7, O-9 and O-10, and the antiserum of O-3 gave titers of 400 or 800 against all the heterologous antigens.

2. Agglutination test with absorbed antisera

The above results showed that unabsorbed

TABLE 2. *Agglutination tests with unabsorbed antisera of somatic (O) antigens of Vibrio parahaemolyticus*

Agglutinating antigen	Unabsorbed antiserum									
	O-1	O-2	O-3	O-4	O-5	O-6	O-7	O-8	O-9	O-10
O-1	1,600	200	800	200	200	200	400	200	—	200
O-2	200	1,600	800	200	200	200	800	200	400	200
O-3	—	—	3,200	—	400	—	400	—	200	—
O-4	200	—	400	3,200	200	200	800	200	200	200
O-5	400	200	400	200	3,200	200	800	200	400	400
O-6	200	200	300	200	200	3,200	800	200	400	200
O-7	400	200	400	200	200	200	3,200	200	800	400
O-8	200	200	400	200	200	200	800	6,400	800	200
O-9	400	200	400	200	200	400	800	400	6,400	400
O-10	400	—	400	200	200	200	800	200	400	1,600

Agglutination tests were carried out as described in the text.

Agglutination titers are expressed as the reciprocal of the highest dilution of antiserum showing visible agglutination.

The lowest dilution of antiserum tested was 1:200 and (—) indicates no detectable agglutination at the lowest dilution.

TABLE 3. *Agglutination tests with absorbed antisera of somatic (O) antigens of Vibrio parahaemolyticus*

Agglutinating antigen	Absorbed antiserum									
	O-1	O-2	O-3	O-4	O-5	O-6	O-7	O-8	O-9	O-10
O-1	1,600	—	—	—	—	—	—	—	—	—
O-2	—	1,600	—	—	—	—	—	—	—	—
O-3	—	—	3,200	—	—	—	—	—	—	—
O-4	—	—	—	1,600	—	—	—	—	—	—
O-5	—	—	—	—	3,200	—	—	—	—	—
O-6	—	—	—	—	—	1,600	—	—	—	—
O-7	—	—	—	—	—	—	1,600	—	—	—
O-8	—	—	—	—	—	—	—	1,600	—	—
O-9	—	—	—	—	—	—	—	—	3,200	—
O-10	—	—	—	—	—	—	—	—	—	1,600

Explanation as in Table 2.

antiserum was not specific. To obtain a specific antiserum to the somatic (O) antigen, agglutinin absorption was carried out. Heat-killed cell suspensions of several strains were examined for absorption of agglutinins and it was found that the following combinations gave the best results: the absorption of the antisera of O-1, O-2, O-3, O-4 and O-5 by a heat-killed cell suspension of O-9, and the absorption of the antisera of O-6, O-7, O-8, O-9 and O-10 by a heat-killed cell suspension of O-2. The results of the agglutination test using absorbed antisera are shown in Table 3. The agglutination titers of each absorbed antiserum against the homologous antigens were higher than 1,600 and no agglutination was observed with the heterologous antigens when the absorbed antiserum was diluted more than 1:200. This indicated that specific antiserum to each somatic (O) antigen became available after agglutinin absorption.

3. Somatic (O) antigen analysis of strains from clinical sources

The specific antisera to somatic (O) antigens prepared by agglutinin absorption were applied in somatic (O) antigen analysis of 144 strains isolated from clinical sources (Table 4). Of

TABLE 4. *Antigen analysis of Vibrio parahaemolyticus isolated from clinical sources*

O antigen	Number of strains	
	K-typable	Not K-typable
O-1	9	4
O-2	12	3
O-3	20	8
O-4	17	4
O-5	17	12
O-6	1	0
O-7	0	0
O-8	1	9
O-9	1	0
O-10	0	0
O-7, O-10	0	21
Unknown	0	5
Total	78	66

O-antigen analysis was carried out by the agglutination test described in the text. K-antigen analysis was done by Drs. K. Maeshima, H. Zenyoji and T. Kodama before sending us the strains used in experiment.

144 strains examined, 78 could be typed, and 66 could not be typed with K-antigen.

The 78 strains with known K antigens were identified by 8 O-antisera and their antigen structures (O- and K-antigen) were in good accordance with the scheme proposed by Sakazaki (1965). Of the 66 strains which could not be typed by K-antisera, 40 were identified by 6 O-antisera, 5 were not agglutinated with any of the 10 antisera (O-1—O-10) prepared, and 21 showed agglutination with the antisera of O-7 and O-10. This indicated that most of these 66 strains could be identified by specific antisera to somatic (O) antigen.

4. Preparation of group specific antisera of new somatic (O) antigens

The results described in the previous section suggested that there was other somatic antigen(s) in *Vibrio parahaemolyticus*. One of the 5 strains, which were not agglutinated with any of the 10 antisera, was selected and designated as strain O-N1, and one of the 21 strains which showed agglutination with the

antisera of O-7 and O-10 was selected and designated as strain O-N2 (O-N1 and O-N2 were isolated from patients by Dr. K. Maeshima). The antisera of these 2 strains were prepared by the method described in Materials and Methods, and agglutination tests were carried out with these antisera (Table 5). Though the agglutination titer of each unabsorbed antiserum against the homologous strain was higher than 6,400, high agglutination titers were observed with some of the heterologous antigens. To obtain specific antiserum to each antigens, agglutinin absorption was applied as described in Materials and Methods. The agglutination titers of the absorbed antisera of O-N1 and O-N2 are shown in Table 6. When the antiserum of O-N1 was absorbed with a heat-killed cell suspension of either O-9, O-2 or O-5, the antiserum did not become specific. It was found that specific antiserum to O-N1 strain could be prepared by consecutive absorption of antiserum with heat-killed cell suspensions of both O-2 and O-5. The specific antiserum to O-N2 strain was also obtained by absorption of O-N2 antiserum with heat-killed cells suspensions of O-2, O-7, O-9 and O-10.

Twenty-six strains, antigenic structures of which were not determined using the ten (O-1—O-10) antisera, were examined with the specific antisera to O-N1 and O-N2. It was found that the 21 strains, which were agglutinated with the antisera of O-7 and O-10, were all homologous each other and were agglutinated with the specific antiserum to O-N2. Among the 5 strains which were not agglutinated with any of the 10 antisera, strain O-N1 and another strain (T 1890, isolated from a patient by Dr. H. Zenyoji) were homologous and were agglutinated with the specific antiserum to O-N1, but the remaining 3 strains were not agglutinated with any group specific antiserum tested, indicating they had still other somatic (O) antigen(s).

TABLE 5. Agglutination tests with unabsorbed antisera of the somatic (O) antigens of O-N1 and O-N2

Agglutinating antigen	Unabsorbed antiserum	
	O-N1	O-N2
O-1	400	800
O-2	800	400
O-3	400	400
O-4	200	400
O-5	3,200	400
O-6	—	400
O-7	400	1,600
O-8	400	400
O-9	400	1,600
O-10	400	3,200
O-N1	6,400	400
O-N2	400	> 6,400

Explanation as in Table 2.

TABLE 6. *Agglutination tests with absorbed antisera of somatic (O) antigens of O-N1 and O-N2*

Agglutinating antigen	Absorbed antiserum									
	O-N1					O-N2				
	Absorbed with					Absorbed with				
	O-9	O-2	O-5	O-2, O-5		O-2	O-7	O-9	O-10	O-2, O-7 O-9, O-10
O-1	—	—	—	—		—	—	—	—	—
O-2	800	—	400	—		—	—	—	—	—
O-3	—	—	—	—		—	—	—	—	—
O-4	—	—	—	—		—	—	—	—	—
O-5	1,600	1,600	—	—		—	—	—	—	—
O-6	—	—	—	—		—	—	—	—	—
O-7	—	—	—	—		800	—	400	200	—
O-8	—	—	—	—		—	—	—	—	—
O-9	—	—	—	—		1,600	1,600	—	—	—
O-10	—	—	—	—		> 1,600	800	1,600	—	—
O-N1	> 1,600	> 1,600	> 1,600	> 1,600		—	—	—	—	—
O-N2	—	—	—	—		> 1,600	> 1,600	> 1,600	> 1,600	800

Explanation as in Table. 2.

DISCUSSION

Freshly isolated living cells of *Vibrio parahaemolyticus*, are not agglutinated by homologous O-antiserum. This can be explained by supposing that freshly isolated organisms have abundant K-antigen around the cell surface and this interferes with the reaction of O-antiserum with the homologous somatic antigen. As in the case with the B-antigen of *Escherichia coli*, the inagglutinability of living cells of *Vibrio parahaemolyticus* with its homologous O-antiserum does not occur even after the cells are heated at 100 C for 1 hour. Sakazaki (1967) reported that in some cases when cells are boiled at 100 C for 2 hours they are still not agglutinated by O-antiserum and this was one reason why he considered that O-antigen analysis of this organism was impractical and so recommended K-antigen analysis.

Since the recommendation of Sakazaki (1967), K-antigen analysis has been widely employed as a routine procedure in etiological

investigations. However, many strains which cannot be typed with K-antigen have been isolated from clinical sources and many new K-antigens have been proposed (Hashimoto et al., 1966; Shimouchi, 1968a; Shimouchi, 1968 b; Zenyoji, personal communication). Thus it may not be possible to determine the antigenic structure of many strains of *Vibrio parahaemolyticus* using only K-antigen analysis. We have prepared specific antisera to ten known somatic (O) antigens and two new O-antigens (O-N1 and O-N2). Thus antisera have high agglutination titers and do not show any cross reaction, so they are useful for O-antigen analysis of strains isolated from clinical sources. We employed O-antigen analysis on more than 150 strains of *Vibrio parahaemolyticus* and found that most of these strains were agglutinated with any of these 12 specific antisera. Only 3 strains were not agglutinated with any of these antisera, indicating that they have another new somatic

(O) antigen. Although these 3 strains were isolated during an outbreak of food poisoning, there has been no other case of food poisoning caused by the organisms with the same antigens. Further studies on the somatic (O) antigens and on the etiological significance of these strains are now in progress.

The two new O-antigens proposed in this paper will be established by further etiological investigation of their distribution. Although both O-N1 and O-N2 strains were agglutinated with at least two antisera for K-antigens, their K-antigenic structure requires further study.

NOTE ADDED IN PROOF

Recently, Sakazaki et al (Japan. J. Med. Sci.

Biol., 21; 313-324, 1968) reported the serological characteristics of *Vibrio parahemolyticus* and proposed 11 O-antigens and 41 K-antigens in this organism.

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