Antigenic Relationships Among Strains of Vibrio parahaemolyticus

R. M. TWEDT, P. L. SPAULDING, AND H. M. JOHNSON
Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio 45226

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The antigenic relationships of 79 strains of Vibrio parahaemolyticus representing 46 assigned K-types were studied by tube agglutination. Homologous titers of 46 anti-K sera ranged from 80 to 2,560. All but three sera exhibited from one to six heterologous reactions, the majority of which gave titers of \( \leq 20 \), but 19 sera showed cross-reactions whose titers exceeded 40. Nine reciprocal and 16 unilateral relationships were described. Some of the cross-reactions involved heat-extractable K-antigens, as determined by absorption with non-heated and heated heterologous antigens, whereas others did not involve K-antigens since absorption with the heterologous antigens had no effect on the homologous systems. On the basis of the reactions of selected antisera to the O-antigens of some of the K-strains, the cross-reactions could not be explained solely on the basis of O-specificities.

The enteropathogenic halophile, Vibrio parahaemolyticus, was first isolated by Fujino et al. (4) during an outbreak of food poisoning in Japan in 1950. The organism has subsequently been demonstrated responsible for summer epidemics of food poisoning accompanied by acute gastroenteritis (11). Morphological, cultural, and biochemical characteristics of the organism have been well established by various workers (12, 20, 21).

In early serological investigations (16–18, 22), vibrio isolates were divided into serotypes, but the vibrio antigens were not identified. Later, Sakazaki (11) described a heat-stable somatic O-antigen, a heat-labile capsular K-antigen, and a flagellar H-antigen. Omori et al. (9), in an immunochemical investigation of purified K-antigen, demonstrated that the material resisted heating to 100 C for 2 hr but was removed from the cell surface by the heat treatment. Sakazaki classified strains of V. parahaemolyticus into 10 O-antigen groups and 32 K-antigen types. He recommended that only K-antigen typing be carried out in routine procedure since O-antigen determination was tedious and K-antigen was specific for an individual O-group. However, a significant proportion, in many cases the majority, of recent isolates from fish and sea water (1, 2, 8, 13) as well as from clinical sources (5, 7, 14, 15) were inagglutinateable in known K-antisera. As a result, many new O- and K-antigens have been proposed (3, 5, 13) and several have been excluded (6). An antigenic schema incorporating

| Group | Strain designation | Source | Donor |
|-------|--------------------|--------|-------|
| KA    | K1, K4, T2194 (K6) | Patients' stools, sea fish, sea water; Japan | K. Aiso |
| JL    | K4, K15            | Patients' stools; Japan | J. Liston |
| SAK   | K1–K4, K13         | Sea water and sediment; Japan | J. Liston |
| LL    | K1, K3–K5, K18, K20, K22, K31–K33 | Sea fish; Germany | L. Leistner |
| YM    | K22, K33–K46       | Patients' stools, sea fish, sea water; Japan | Y. Miyamoto |
| RS    | K1, K3, K4, K6, K13, K18, K19, K23, K25, K29 | Patients' stools, sea fish, sea water; Japan | R. Sakazaki |
| HZ    | K1–K17, K20, K21, K23–K32, T2450(K5), HP939(K7), HP792(K29), T1965(K30), T2081(K30) | Patients' stools, sea fish, sea water; Japan | H. Zen-Yoji |
these changes was described by Zen-Yoji et al. (23) and includes 12 O-groups and 48 K-types. However, the inability of this antigenic schema to account for an increasing number of vibrio isolates suggested that further serological investigation was warranted. The present study was undertaken to clarify antigenic relationships among V. parahaemolyticus isolated from both marine and clinical sources.

MATERIALS AND METHODS

Bacterial cultures. Seventy-nine cultures of V. parahaemolyticus shown in Table 1 and described in our previous works (19-21) were used in the present studies. These strains, representing 46 assigned K-types, were isolated from feces of patients suffering from gastroenteritis, from food implicated in food poisoning outbreaks, and from sea fish or sea water.

Culture maintenance. Stock cultures were grown at 37 C on Trypticase soy agar (Difco) plus 2.5% NaCl for 18 to 24 hr in screw-cap tubes. They were maintained at room temperature, with monthly transfers.

Preparation of K-antigens. Strains representing each of the 46 K-types were grown on Brain Heart Infusion agar (Difco) plus 2.5% NaCl for 18 hr at 37 C, washed three times, and suspended in formalized physiological saline. The optical density (OD) of the suspension was adjusted to 0.25 at 620 nm in a Coleman Junior spectrophotometer.

Preparation of O-antigens. Strains prepared as above were suspended to an OD of 1.0. K-antigens were heat-extracted by the method of Miwatani et al. (7). The suspension was heated to 100 C for 1 hr, washed three times in formalinized saline, centrifuged at 15,000 x g, and resuspended to original volume in formalinized saline. The heat-extraction was repeated for a second hour, and again for 0.5 hr.

Final OD of suspension was 0.25.

Preparation of antisera. Antisera were produced by injecting rabbits intravenously with eight successive doses of antigen, increasing from 0.5 to 4.0 ml. After resting 1 week, animals were given booster doses and bled by cardiac puncture at weekly intervals. Sera were separated, heated to 56 C for 30 min, and preserved with 0.1% sodium azide. Samples with high agglutinin titers from a given animal were then pooled. Basal titers revealed no natural antibodies against V. parahaemolyticus.

Absorption of antisera. Antisera were absorbed with either heat-extracted or unheated cells. Approximately 1.5 ml of packed cells was incubated for 2 hr at 37 C and for 18 hr at 4 C with an equal volume of antisera. After centrifugation to separate absorbed sera, agglutinin titers were determined by tube agglutination.

Agglutination test. Tube agglutinations were performed by adding 0.5 ml of antigen to an equal volume of antisera serially diluted in buffered physiological saline. Tubes were incubated at 37 C for 2 hr and 4 C for 18 hr, centrifuged, and read.

RESULTS

The homologous and heterologous agglutination titers of the 46 K-antisera are presented in Table 2. Homologous titers ranged from 80 to 2,560. The majority (36 of 46) ranged between 160 and 640; the mode was 320. Except

| Table 2. K-antigen relationships |
|----------------------------------|
| K-antigen serum | Homologous titer | Heterologous titers |
|-----------------|------------------|---------------------|
|                 | ≤ 20             | 40-80               | ≥ 160               |
| 1 640*          | 3, 6, 8, 25,*    |                     |
| 2               | 160              | 8, 17, 19           |                     |
| 3 640           | 11, 16, 17, 32   |                     | 2, 27               |
| 4               | 320              | 3, 20, 24, 32       | 6, 7                |
| 5               | 80               | 11, 24, 27          |                     |
| 6               | 320              | 5, 24, 32, 36       | 4                   |
| 7               | 320              | 14, 24              |                     |
| 8               | 320              |                     |                     |
| 9               | 320              | 11, 21, 24, 27,     | 34, 42              |
| 10              | 320              | 8, 32, 34, 36       |                     |
| 11              | 160              | 3, 7, 32            |                     |
| 12 1,280        | 3, 8, 20         |                     |                     |
| 13               | 160              | 21, 22              | 24                  |
| 14 1,280        | 1, 3, 24, 27     |                     | 16                  |
| 15 320          | 24               |                     | 22                  |
| 16 640          | 1                |                     |                     |
| 17 1,280        | 1                |                     |                     |
| 18 160          | 45               |                     |                     |
| 19 320          | 8, 24, 25        |                     |                     |
| 20 1,280        | 11, 21, 24, 27   |                     |                     |
| 21 320          | 11, 22, 24       |                     |                     |
| 22 1,280        | 24, 25           |                     | 15                  |
| 23 160          | 24               |                     |                     |
| 24 2,560        | 3, 45, 46        |                     |                     |
| 25 1,280        | 1, 44            |                     |                     |
| 26 160          | 24, 36           |                     |                     |
| 27 1,280        | 17               | 2, 3, 40            |                     |
| 28 160          | 17, 24           |                     |                     |
| 29 160          | 24               |                     |                     |
| 30 320          | 24, 27           |                     | 14, 20              |
| 31 320          | 24               |                     |                     |
| 32 1,280        | 24, 27           |                     | 20                  |
| 33 640          | 24, 27, 42       |                     |                     |
| 34 320          | 27               |                     | 24                  |
| 35 160          | 27               |                     |                     |
| 36 640          | 24, 27           |                     |                     |
| 37 640          | 24, 27, 45       |                     |                     |
| 38 320          | 1                | 27                  | 39                  |
| 39 320          | 21, 24           | 27                  |                     |
| 40 160          | 16, 21, 24       | 27                  |                     |
| 41 160          | 1, 27, 36        |                     |                     |
| 42 640          | 24               | 3                   |                     |
| 43 320          | 3, 27, 40        |                     |                     |
| 44 640          | 3, 27            |                     |                     |
| 45 640          | 27               |                     |                     |
| 46 160          | 3, 24, 27, 36    |                     |                     |

* Values represent reciprocal of serum dilution.

+ Reciprocal relationships are italicized.
for K8, K16, and K17, all antisera exhibited from one to six heterologous reactions. Most cross-reaction titers were ≤ 20. Some of these low-titered reactions resulted from the agglutination of a few K-types. However, 19 sera exhibited cross-reactions whose titers exceeded 40. Nine reciprocal relationships (italicized) were observed among the antisera. Some of these, i.e., K2 and K27, and K15 and K22, showed relatively high titers. Still other high-titered heterologous reactions, such as between antigens K7 and anti-K4 sera, and K14 and anti-K30 sera, were unilateral. Many of the reciprocal and nonreciprocal heterologous reactions occurred between K-types that are not currently regarded as members of the same O-group. We assumed from this that the cross-reactions noted among K-antisera that exhibited titers ≥ 40 were possibly attributable to their K-antigen content or to as yet unidentified antigens.

The low homologous titers of some antisera (K5) might indicate relatively low antibody content or binding capacity, or they could reflect the nature of K-antigen of a given bacterial strain. To shed light on this point, homologous agglutination reactions were conducted among 22 K-antisera and strains of several given types of V. parahaemolyticus (Table 3). Eight of the 38 antigens tested agglutinated with antisera to a titer more than eightfold less than that of the immunogen. These eight strains may be rough or possess other deficiencies in antigenic expression, or they may be incorrectly serotyped. The eight strains were distributed among five K-antisera (K4, K6, K15, K18, and K22) and should be studied further to clarify the unexplained antigenic relationships noted here. Cultural and morphological properties of these strains did not apparently differ from those of strains that were agglutinated by antisera.

Absorption properties of systems involving strong unilateral cross-reactions, as with K39 antigen and K38 antiserum, are illustrated in Table 4. Both homologous and heterologous titers were significantly reduced by absorption with unheated homologous or heterologous cells. Heated cells, however, did not absorb the antibody. The ability of the heterologous cells to absorb the antibody suggests that the K38 antigenic specificity may be included within that of K39. The inability of the heated cells to absorb the antibody suggests that the K-antigen involved is either heat-labile or heat-extractable.

### Table 3. Homologous titers with several sources of K-antigen

| K-antisera | Immunizing strain | Group, titers |
|------------|-------------------|---------------|
|            |                   | SJ | JL | KA | LL | RS | SAK | YM |
| 1          | SJ-K1             | 640 | 80 | 80 | 80 | 160 | 320 |
| 2          | SJ-K2             | 160 | 80 | 80 | 80 | 320 |      |
| 3          | SJ-K3             | 640 | 80 | 160| 160| 320 | 5   |
| 4          | SJ-K4             | 320 | 160| 320| 80 | 320 | 5   |
| 5          | SJ-T2450(K5)      | 40 | <5 | <5 | <5 | 5   | 320 |
| 6          | SJ-K6             | 320 | 160| 160| 160|     | 80  |
| 7          | SJ-K7             | 320 | 320| 320| 320|     |     |
| 12         | RS-K12            | 320 | 320| <5 | <5 | 1,280|     |
| 13         | SJ-K13            | 160 | 160| 160| <5 | 320 |     |
| 15         | RS-K15            | <5 | <5 | <5 | <5 | 320 |     |
| 18         | KA-K18            | 320 | 320| 320| 320|     |     |
| 19         | KA-K19            | 1,280| 1,280| 1,280| 1,280|     |     |
| 20         | RS-K20            |     |     |     |     |     |     |
| 22         | LL-K22            |     |     |     |     |     |     |
| 23         | SJ-K23            |     |     |     |     |     |     |
| 25         | SJ-K25            |     |     |     |     |     |     |
| 28         | SJ-K28            |     |     |     |     |     |     |
| 29         | SJ-K29            |     |     |     |     |     |     |
| 30         | SJ-K30            |     |     |     |     |     |     |
| 31         | SJ-K31            |     |     |     |     |     |     |
| 32         | SJ-K32            |     |     |     |     |     |     |
| 33         | YM-K33            |     |     |     |     |     |     |
TABLE 4. Agglutination absorptions of a unilateral cross-reaction

| K-antiserum | Absorbing antigen | Agglutinating antigen | K-type | Treatment* | K-type | Titer |
|-------------|------------------|-----------------------|--------|------------|--------|------|
| 38          |                  |                       | 38     | 38         | 39     | 320  |
| 38          |                  |                       | 38     | 38         | 39     | 160  |
| 38          | H                |                       | 38     | <5         | 39     | 10   |
| 38          | H                |                       | 38     | 38         | 39     | 80   |
| 38          | H                |                       | 39     | 39         | 39     | 5    |
| 39          | H                |                       | 38     | 38         | 39     | 160  |
| 39          | H                |                       | 39     | 39         | 39     | 80   |

* No designation indicates untreated cells. H indicates cells extracted by heat.

TABLE 5. Agglutination absorptions of reciprocal cross-reactions

| K-antiserum | Absorbing antigen | Agglutinating antigen | K-type | Treatment* | K-type | Titer |
|-------------|------------------|-----------------------|--------|------------|--------|------|
| 15          |                  |                       | 15     | 15         | 15     | 1,280|
| 15          |                  |                       | 15     | 22         | 160    |
| 15          | H                |                       | 15     | 15         | 20    |
| 15          | H                |                       | 15     | 22         | 40    |
| 15          | H                |                       | 15     | 22         | 2,560 |
| 15          | H                |                       | 15     | 22         | 80    |
| 15          | H                |                       | 22     | 15         | 2,560 |
| 15          | H                |                       | 22     | 22         | 20    |
| 15          | H                |                       | 22     | 15         | 2,560 |
| 22          |                  |                       | 22     | 22         | 10    |
| 22          |                  |                       | 22     | 22         | 2,560 |
| 22          | H                |                       | 22     | 22         | 2,560 |
| 22          | H                |                       | 22     | 22         | 150   |
| 22          | H                |                       | 22     | 22         | 40    |
| 22          | H                |                       | 22     | 15         | 10    |
| 22          | H                |                       | 22     | 22         | 640   |
| 22          | H                |                       | 15     | 22         | 80    |
| 15          | H                |                       | 15     | 22         | <5    |
| 15          | H                |                       | 22     | 15         | 2,560 |
| 15          | H                |                       | 15     | 15         | 10    |

* No designation indicates untreated cells. H indicates cells extracted by heat.

Results of absorptions involving a system with fairly strong reciprocal cross-reactions are presented in Table 5. The K15 and K22 antigens gave homologous reactions that were 8- to 16-fold greater than that of the heterologous antigen. The cross-reactions were, however, substantial. Absorption with unheated homologous antigens significantly reduced the homologous and heterologous reactions, whereas heated antigens were generally without effect on the homologous systems. Heterologous antigens, unheated or heated, did not affect the homologous reactions. The cross-reactions observed here, then, appear to be due to minor relationships between the K15 and K22 antigens. In general, the cross-reactions were susceptible to absorptions by heated antigens, although K15 and K22 belong to different O-groups (23).

Five other reciprocal cross-reactions behaved as described for K15 and K22 in absorptions, whereas three reciprocal cross-reacting systems (K2-K27, K3-K27, and K27-K40) were shown to share substantial antigenic relationships by absorption. Three unilateral cross-reactions with titers > 40 (K8, anti-K11; K16, anti-K14; K3, anti-K45) gave absorption patterns similar to those of K39 and anti-K38. The remainder of the unilateral cross-reactions showed that the homologous reaction was absorbable only with specific antigens.

Unabsorbed antibodies to the O-antigens (O1 through O9) of _V. parahaemolyticus_ K32, K2, K30, K12, K13, K18, K19, K20, and K44, respectively, gave agglutination patterns similar to the _K-anti-K_ reactions (Table 6), including cross-reactions, that showed no relationship to the _K-anti-K_ reactions of these organisms (Table 7; data extracted from Table 2). The patterns of the _K-anti-K_ reactions, then, do not appear to be due to a predominance of O-specificity in the sera.

**DISCUSSION**

The data presented here broadly substantiate the antigenic specificity of the _K-anti_K antigens reported by the Japanese (23). Considerable cross-reactivity, however, most of which is weak (titers of ≤ 20), occurs between the different _K-anti_K antigens (Table 2). Still, some cross-reactions of a moderate and strong degree were observed. Some were reciprocal, whereas others were unilateral. Further, absorptions

**TABLE 6. Agglutination by unabsorbed anti-O-sera**

| Antigen | O-1  | O-2  | O-3  | O-4  | O-5  | O-6  | O-7  | O-8  | O-9  |
|---------|------|------|------|------|------|------|------|------|------|
| O-1     | 160a | 40   | 20   | 40   | —    | 5    | 20   | 20   | —    |
| O-2     | 80   | 320  | 40   | 80   | 40   | 5    | 80   | 80   | 20   |
| O-3     | —    | 04   | 640  | 40   | 5    | 5    | 80   | 80   | 80   |
| O-4     | 10   | 80   | 20   | 320  | 5    | 20   | 5    | 80   | —    |
| O-5     | —    | 04   | 40   | 80   | 640  | —    | 10   | 10   | 80   |
| O-6     | —    | 04   | 40   | 80   | 40   | 10   | 320  | 10   | 80   |
| O-7     | 40   | 80   | 80   | 80   | 40   | 80   | 40   | 80   | 80   |
| O-8     | 5    | 04   | 40   | 80   | 20   | 40   | —    | 80   | 80   |
| O-9     | 20   | 40   | 40   | 40   | 40   | 20   | 20   | 5    | 80   |

* Reciprocal relationships are italicized.
* — Indicates a titer < 5.
showed that some strong cross-reactions indicated substantial antigenic relationships among the systems involved, whereas others showed that the homologous systems were specific. Sakazaki et al. (13) described reciprocal antigenic relationships between several pairs of K-antigens, including K1-K25, K4-K5, K8-K11, and K20-K21. Further, he stated that K14 was identical to K30 and that K2, K27, and K28 were partial antigens of K3. We confirmed the relationship between K2, K3, and K27, but could find no significant heterologous reactions linking the other antigens. We detected other heterologous reciprocal relationships: K4-K6, K15-K22, K27-K40. Our absorptions with some systems indicate that some specificities are included within others (Table 4).

The K-antigens of *V. parahaemolyticus* were originally thought to be destroyed by heating (11); however, a recent study of the immunochemistry of a K-antigen has indicated that the K-antigen is removed from cells by boiling but is not destroyed (9). This is the basis of the term heat-extraction as used in this paper. More studies are necessary, however, to determine if this property is shared by K-antigens of all *V. parahaemolyticus* strains.

Agglutination studies with homologous K-antigen organisms from different sources revealed some variations in reactivity with homologous antisera (Table 3). In five instances, the homologous types gave no reactions with the antisera, whereas two gave a homologous titer of 1:5, and one, a titer of 1:20. There could be several reasons for these observations. The organisms may have been mistyped originally or mislabeled. Also, the organisms may have lost their K-antigens; this, however, was not possible to confirm morphologically and culturally. In spite of the above exceptions, most of the strains listed in Table 3 gave the expected reaction with the homologous K-antisera.

Recently, the K-antigens of *Escherichia coli* were re-examined by Ørskov and Ørskov (10). They concluded that K-antigens were quite heterogenous serologically, which reflected chemical and physical heterogeneity. Also, they cautioned against the use of bacterial agglutination as the sole criterion for investigation of K-antigens.

The data presented here certainly indicate that more work is needed before the serotyping of *V. parahaemolyticus* according to their K-antigens can become a routine procedure.

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