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# Analysis of *Vibrio parahaemolyticus* Soluble Antigens by Employing Passive Hemagglutination

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The passive hemagglutination assay was explored as a sensitive test of immunological reactivity of endotoxin and other antigens prepared from selected strains of *Vibrio parahaemolyticus*. The tannic acid procedure for passive hemagglutination, commonly used with protein antigens, was the only procedure yielding good results with *V. parahaemolyticus* protein extracts, endotoxins, and related preparations. These results were probably due to the presence of large amounts of protein in the *V. parahaemolyticus* endotoxins as determined by earlier work referenced in the text. Glucose and galactose as possible antigenic determinants in the endotoxin of a *Vibrio* strain were tested by inhibition tests. Cross-reactions were observed between endotoxin preparations, but were low in hemagglutination, suggesting reactions of common generic antigens. The ability of *V. parahaemolyticus* endotoxins to stimulate production of antibodies was determined.

*Vibrio parahaemolyticus*, a causative agent of food poisoning in Japan, has in recent years been implicated in seafood-borne disease in the United States (1, 9-11, 17, 20, 23) and in other countries (3, 4). The mode of pathogenesis of this microorganism remains incompletely understood, despite active investigation by a number of workers. Some interesting hypotheses include the following. A hemolysin that functions as an enterotoxin as well as a cardiotoxin has been described (12, 13, 16). A report of the isolation of a diarrheagenic factor prepared from a Kanagawa-negative strain (nonhemolytic on Wagatsuma medium) has been published (21). There is some evidence that *V. parahaemolyticus* may also be responsible for infections in organs other than the intestine (5, 7; B. K. Boutin, S. F. Townsend, P. V. Scarpino, and R. M. Twedt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B24, p. 19).

A comparative study of three serologically distinct strains of *V. parahaemolyticus* was undertaken to quantitate differences or similarities in antigenic properties. Antisera were prepared against each of the three strains, as well as against antigen extracts. For comparison of immunological cross-reactivity, the passive hemagglutination (HA) assay was selected as the most sensitive method for measuring reactivity of soluble bacterial antigens.

Passive HA procedures with antigens prepared from strains of *Enterobacteriaceae* are well documented. However, *Vibrio* antigens surveyed in the present study did not react effectively when such techniques were applied. Therefore, a passive HA method was developed that yielded reproducible results with endotoxin preparations and protein extracts. The method employs tannic acid for pretreatment of erythrocytes (RBC) and employs selected antigens for sensitization. The procedure also proved useful in determining the role of glucose and galactose as antigenic determinants in endotoxin preparations of *V. parahaemolyticus*. Thus, this paper reports on the passive HA assay applied to antigenic analysis of *V. parahaemolyticus*.

## MATERIALS AND METHODS

**Bacterial strains.** *V. parahaemolyticus* Sak-3 isolated from a patient and identified as serotype O2:K3 was obtained from R. Sakazaki, National Institutes of Health, Tokyo, Japan. Strain 11590, serotype O4:K11, isolated from a patient in Bainbridge, Md., in 1972 was provided by the late M. Fishbein, Food and Drug Administration, Washington, D.C. Strain FC 1011, O serotype unknown, K38, was isolated from a normal Chesapeake Bay blue crab by one of the authors.

**Media.** Brain heart infusion agar or broth (BHI, Difco Laboratories, Detroit, Mich.), with or without 3% NaCl added, was used to culture the organisms. BHI (BBL Microbiology Systems, Cockeysville, Md.) was also used in some experiments, as indicated below.

Medium containing 3% NaCl is toxic when injected into animals, but BHI prepared by BBL Microbiology Systems without NaCl added fails to support good growth of *V. parahaemolyticus*. However, BHI obtained from Difco Laboratories, Inc., supported growth of *V. parahaemolyticus*, with or without NaCl added. Hence, Difco BHI was used throughout the study.

**Experimental animals.** Male or female New Zea-

land white rabbits, 2 to 3 kg, were obtained from Camm Research Institute, Wayne, N. J.

**Immunization procedures.** Rabbits were bled before immunization to determine base-line values for bacterial agglutinins and other antibodies. Bacteria were grown 18 h in BHI broth or on BHI agar, with or without 3% NaCl added. Broth cultures were treated with 0.5% Formalin and used as immunizing antigens without further treatment. Bacteria grown on agar were harvested in 0.9% saline, sedimented by centrifugation, and resuspended in 0.5% formalinized saline to a density equivalent of $10^9$ bacteria per ml. A total of four injections of agar-grown or broth-grown bacteria were administered to the rabbits via the marginal ear vein. The first injection consisted of 0.5 ml of bacterial suspension, and the second injection on day 3 was 1.0 ml, followed by 1.5 ml on day 5 and 2.0 ml on day 8. Animals were permitted to rest after the final injection and were trial bled on day 15.

**Determination of titers.** Pre- and post-immunization sera were tested in pairs. Standard procedures for bacterial agglutination were used to determine antibacterial titers of the sera (8).

**Passive HA. (i) Variations in treatment of antigens before sensitization of RBC.** The method for endotoxin sensitization of RBC, without prior treatment of antigen, has been documented (5). A variation of this technique, i.e., heating the endotoxin at 100°C in a water bath for 60 min, was also done (18), as was a variation involving NaOH treatment of sensitizing antigen (26).

**(ii) Treatments for sensitizing RBC.** Chromium chloride or tannic acid was used to pretreat RBC for sensitization with an antigen (2, 5).

For some experiments, outdated human type O, Rh+ or − RBC obtained from a blood bank were employed. Alternatively, 30 ml of blood of a similar type was drawn from volunteers. Sheep RBC were obtained from the University of Maryland animal farm. Fresh RBC were mixed with an equal volume of Alsever solution and allowed to equilibrate in the cold for 1 week before use. Phosphate-buffered saline was used except where indicated.

**Antigens employed for passive HA studies. (i) *V. parahaemolyticus* endotoxins.** Endotoxins were prepared from strains 11590, FC 1011, and Sak-3 with phenol water (Phe) and trichloroacetic acid, as previously described (22).

**(ii) Cell washes.** *V. parahaemolyticus* strains were cultured overnight at 37°C on BHI agar, and the cells were harvested in distilled water, dialyzed against distilled water in the cold for 4 days, and centrifuged at 3,000 × *g* in a Sorvall RC-2 centifuge, and the supernatant was stored in the cold until needed.

**(iii) Toxic fractions.** A culture filtrate, ranging from 50 to 80% protein, extracted from *V. parahaemolyticus* FC 1011, isolated from a normal Chesapeake Bay blue crab, was found to be toxic on forced feeding, producing diarrhea and death in BALB/c mice of 15 to 22 g. Intraperitoneal injection produced similar results (21). Briefly, this fraction was prepared as follows: *V. parahaemolyticus* FC 1011 was cultured overnight at 37°C in 2-liter volumes of seawater-yeast extract broth without agitation. The cultures were centrifuged at 7,000 rpm in a Sorvall RC-2 centrifuge (Ivan Sorvall Co., Norwalk, Conn.) for 30 min. The supernatant was decanted and filtered through two layers of no. 1 Whatman filter paper, followed by successive aseptic filtration through 1.2- and 0.45-μm Millipore filters. Merthiolate (Thimersol, Sigma Chemical Co., St. Louis, Mo.), at the final concentration of 1:10,000, was added to the filtrate to insure sterility. Sterility was confirmed by plating on BHI. Fractions ranging from 30 to 60% ammonium sulfate saturation were prepared with filtrates of the FC 1011 strain.

Fractions were dissolved in sterile distilled water and dialyzed against distilled water, with two changes per day over a 1-week period. All procedures were carried out in the cold. Filtrate fractions were concentrated to one-third volume, followed by lyophilization with a VirTis model 10-800 lyophilizer (VirTis Co., Gardiner, N.Y.). Ammonium sulfate fractions, 30 and 60%, prepared from sterile seawater-yeast extract broth, served as controls. Dried filtrate fractions were weighed and dissolved in 0.85% sterile saline before injection in experimental animals.

**Inhibition of passive HA by glucose and galactose.** Endotoxin prepared from *V. parahaemolyticus* 11590 with phenol water was used initially. Samples of immune rabbit antiserum were incubated with 5 mg of glucose or galactose before doing the passive HA test. The samples were then serially diluted, and sensitized RBC were added (see Table 5). Positive and negative controls were included, and tests were read according to standard procedures for incubation that were determined experimentally. The positive controls consisted of sera giving titers of 5,120, 160, and 320. Thus, the control refers to antibody.

**Immunizing properties of endotoxins.** Endotoxins prepared from strains FC 1011 and 11590 by phenol-water extraction were diluted in 0.8% saline. Rabbits were injected via the marginal ear vein with increasing doses ranging from 0.1 to 0.4 mg, with seven injections being spaced over a 19-day interval. Animals were trial bled 1 week after completion of the injections. Both sera obtained before and after immunization were tested by passive HA.

**Controls used in HA experiments.** Control sera were obtained from animals before their immunization. Control antigens consisted of RBC sensitized with antigens described above and not exposed to serum. Control RBC were washed and not exposed to serum or antigens.

## RESULTS

Immunizations of rabbits with *V. parahaemolyticus* yielded results shown in Table 1. Titers, expressed as reciprocals of the final dilution of sera giving observable bacterial agglutination, ranged from a minimum of 320 to a maximum of 5,120 for strains 11590 and FC 1011, when washed, formalinized whole cells were employed as antigens. Strain Sak-3, although not producing as high a titer serum under similar conditions, produced titers of 2,560, when 6-h, for-

TABLE 1. *Agglutination reactions of V. parahaemolyticus strains 11590, FC 1011, and Sak-3*

| Rabbit | *V. parahaemolyticus* strain no. | Antigen preparation | Reciprocals of titer[a] | |
|---|---|---|---|---|
| | | | Preimmune serum | Immune serum |
| 24 | 11590 | Washed cells, Formalin treated | 0 | 5,120 |
| 25 | 11590 | Washed cells, Formalin treated | 0 | 5,120 |
| 20[a] | FC 1011 | Washed cells, Formalin treated | 0 | 5,120 |
| 00 | Sak-3 | Washed cells, Formalin treated | 0 | 640 |
| 02 | Sak-3 | Washed cells, Formalin treated | 0 | 320 |
| 01 | Sak-3 | 6-h broth culture, formalinized | 0 | 2,560 |
| 03 | Sak-3 | 6-h broth culture, formalinized | 0 | 2,560 |

[a] Only one rabbit was immunized with *V. parahaemolyticus* strain FC 1011.

malinized broth cultures were employed.

Results of passive HA, with untreated endotoxin as sensitizing antigen for human type O, Rh+ RBC, yielded reciprocal HA titers ranging from 0 to 160. Titers, generally expressed as reciprocals, are considered to be low if in the range of 1:0 to 1:160 or below (8) as compared with immune sera. Endotoxin prepared from *V. parahaemolyticus* 11590 produced very low HA titers with phenol water (Phe) and trichloroacetic acid preparations at reciprocals of 160. Cell washes yielded no reaction. Strain FC 1011 (Phe) provided very little reaction, as indicated by a titer of 80 at 1:5 dilution that was similar to that of the 11590 trichloroacetic acid preparation. As in the case of strain 11590, the wash showed no reaction. Sak-3 preparations consistently induced no reaction. However, all preparations gave positive precipitin reactions when undiluted samples were reacted with undiluted immune serum. Apparently these conditions were not appropriate for passive HA for *V. parahaemolyticus* soluble antigens.

Other passive HA tests included heat treatment of endotoxins prepared from strains 11590 (Phe) and FC 1011 (Phe) before use as sensitizing antigen for human type O, RH+ RBC. Also, experiments were done with strain 11590 (Phe), employing sheep RBC and $CrCl_3 \cdot 6H_2O$ (0.1%) during sensitization. In addition, tests were done with strain Sak-3 NaOH extract, employing human type O, Rh− RBC. Only strain 11590 (Phe) heat-treated endotoxin produced a response, but the reciprocal titer of 1.0 to 0.1 mg of antigens was 40 or less (not significant, data not shown).

Results of passive HA, employing tannic acid-treated sheep RBC sensitized with different concentrations of *V. parahaemolyticus* endotoxins, are provided in Table 2. Length of time for sensitization and incubation for optimum results for passive HA were determined. As can be seen from data given in Table 2, maximum titers were obtained when sensitization of tannic acid-treated sheep RBC with endotoxin preparations proceeded for 60 min at 37°C, followed by incubation in the presence of serial serum dilutions for 2 h at room temperature and overnight incubation in the cold. Results given in Tables 2 and 3 show that: (i) tannic acid pretreatment of RBC was required for successful passive HA; (ii) human RBC or sheep cells can be used; and (iii) soluble antigens extracted by NaOH extraction and cell washings can be employed.

The passive HA procedure was further tested with protein antigens prepared from culture filtrates of the three strains of *V. parahaemolyticus* (Table 4). HA titer against *V. parahaemolyticus* FC 1011 (1.0 mg) showed no reaction, most likely representing inhibition by excess antigen, although other possibilities exist. This antigen was found to possess diarrhea-provoking properties in mice when force-fed to the mice or injected intraperitoneally (21). Antigen preparations of strain 11590 and Sak-3 revealed little or no toxic effect in animals, perhaps accounting for the relatively weak response in the HA test.

Endotoxin prepared from strain 11590 (Phe) was used in an inhibition test to determine whether glucose or galactose would inhibit a standard HA test. Table 5 shows that the standard titer of the 11590 (Phe) system was 5,120. Galactose inhibited HA, resulting in a greater than fourfold drop in titer, whereas glucose inhibited HA, with only a fourfold drop in titer. Thus, it appears that glucose and galactose are involved as antigenic determinants in *V. parahaemolyticus* 11590, since a fourfold drop in HA titer is considered significant (18). However, for absolute certainty, more sophisticated methodology would be required, including analysis of carbohydrate moieties of endotoxin, for example.

The passive HA test developed for *V. parahaemolyticus* was used to determine whether cross-reactions could be demonstrated between endotoxin preparations for the three *V. parahaemolyticus* strains. As can be seen from the data provided in Table 6, weak reactions were

TABLE 2. *Passive HA: tannic acid-treated sheep RBC sensitized with V. parahaemolyticus endotoxin versus immune serum*[a]

| Untreated endotoxin preparation | Endotoxin concn (mg) | RBC sensitization time and temp | HA test incubation time and temp | HA titer |
|---|---|---|---|---|
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | 15 min, 37°C | 2 h at room temperature | 0 |
| | 0.5 | 15 min, 37°C | As above | 0 |
| | 0.25 | 15 min, 37°C | As above | 0 |
| | 0.125 | 15 min, 37°C | As above | 0 |
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | 60 min, 37°C | As above | 20,480 |
| | 0.5 | 60 min, 37°C | As above | 20,480 |
| | 0.25 | 60 min, 37°C | As above | 10,240 |
| | 0.125 | 60 min, 37°C | As above | 10,240 |
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | 120 min, 37°C | As above | 0 |
| | 0.5 | 120 min, 37°C | As above | 0 |
| | 0.25 | 120 min, 37°C | As above | 0 |
| | 0.125 | 120 min, 37°C | As above | 0 |
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | 15 min, 37°C | 2 h at room temp followed by overnight at 5°C | 0 |
| | 0.5 | 15 min, 37°C | As above | 0 |
| | 0.25 | 15 min, 37°C | As above | 0 |
| | 0.125 | 15 min, 37°C | As above | 0 |
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | 60 min, 37°C | As above | 20,480 |
| | 0.5 | 60 min, 37°C | As above | 40,960 |
| | 0.25 | 60 min, 37°C | As above | 40,960 |
| | 0.125 | 60 min, 37°C | As above | 20,480 |
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | 120 min, 37°C | As above | 160 |
| | 0.5 | 120 min, 37°C | As above | 320 |
| | 0.25 | 120 min, 37°C | As above | 10 |
| | 0.125 | 120 min, 37°C | As above | 10 |

[a] HA titers are reciprocals of the highest serum dilution producing HA discernible to the unaided eye. Control sera were pre-immunization sera tested the same way as the immune sera. All controls were negative in this passive HA assay.

TABLE 3. *Passive HA: tannic acid-treated human type O, Rh+ RBC*[a]

| Endotoxin preparation | Endotoxin concn (mg) | HA titer |
|---|---|---|
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | 40,960 |
| *V. parahaemolyticus* FC 1011 (Phe) | 1.0 | 40,960 |
| | 0.5 | 20,480 |
| | 0.25 | 10,240 |
| | 0.125 | 5,120 |
| *V. parahaemolyticus* Sak-3 (Phe) | 1.0 | 1,280 |
| | 0.5 | 640 |
| | 0.25 | 640 |
| *V. parahaemolyticus* Sak-3 NaOH | 1.0 | 2,560 |
| *V. parahaemolyticus* 11590 cell wash | 1.0 | 1,280 |

[a]Sensitized with endotoxin, cell wash, or NaOH extracts from *V. parahaemolyticus* versus immune antiserum. Criteria were as described in Table 2. Control sera were preimmune sera tested the same way as immune sera. All control sera were negative in the HA test.

noted between 11590 (Phe) and FC 1011 and Sak-3 (Phe) versus FC 1011 or 11590. The latter, at an HA titer of 160, can be interpreted as indicating significant antigenic material held in common, even though strains 11590 and Sak-3 are not serologically similar.

Immunizing properties of *V. parahaemolyticus* endotoxins were evaluated by passive HA, results of which are given in Table 7. The FC 1011 preparation proved to be more antigenic than 11590.

## DISCUSSION

Antisera can easily be prepared against the moderately halophilic vibrio, *V. parahaemolyticus*, and immunizing antigens can be obtained from cells grown on BHI (Difco) without added NaCl. The tannic acid method was the only procedure that yielded consistently good passive HA for *V. parahaemolyticus* antigens when sheep RBC or human type O, Rh+ RBC were employed.

Passive HA applied to soluble antigens for

TABLE 4. *Passive HA: tannic acid-treated sheep RBC*[a]

| Antigen preparation | Concn | Antiserum | HA titer |
|---|---|---|---|
| *V. parahaemolyticus* FC 1011 60% (toxin) fraction[b] | 1.0 | Anti-FC 1011 | 0 |
| | 0.5 | Anti-FC 1011 | 5,120 |
| | 0.25 | Anti-FC 1011 | 2,560 |
| Control | 1.0 | NRS[c] | 0 |
| *V. parahaemolyticus* 11590 60% fraction[b] | 1.0 | Anti-11590 | 160 |
| | 0.05 | Anti-11590 | 80 |
| Control | 0.1 | NRS[c] | 0 |
| *V. parahaemolyticus* Sak-3 60% fraction[b] | 0.1 | Anti-Sak-3 | 80 |
| | 0.05 | Anti-Sak-3 | 80 |
| Control | 0.1 | NRS[c] | 0 |

[a] Sensitized with protein fractions prepared from culture filtrates of *V. parahaemolyticus* strains FC 1011, 11590, and Sak-3 versus homologous antiserum.
[b] Ammonium sulfate-precipitated fractions of culture filtrates.
[c] Normal rabbit serum (pre-immunization).

TABLE 5. *Inhibition of passive HA*[a]

| Endotoxin preparation | Endotoxin concn (mg) | Inhibiting sugar | Inhibiting sugar concn (mg) | HA titer |
|---|---|---|---|---|
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | None added | 0 | 5,120 |
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | Galactose | 5.0 | 160 |
| *V. parahaemolyticus* 11590 (Phe) | 1.0 | Glucose | 5.0 | 320 |

[a] Endotoxin antigens of *V. parahaemolyticus* strain 11590 pretreated with glucose and galactose.

sensitizing RBC is a relatively simple procedure and is used with soluble protein or lipopolysaccharide (or endotoxin) antigens (18, 19). However, endotoxins and related antigens of *V. parahaemolyticus* strains, from results of this study, require a modified approach for passive HA. Although tannic acid is known to be useful when protein antigens are involved, tannic acid treatment of RBC promoted sensitization of *V. parahaemolyticus* cells when endotoxin, cell washes, or NaOH extracts were employed. In the latter, NaOH extraction provides a detoxification of endotoxin preparations without inactivating immunological reactivity (18). The unusual reactivity of endotoxin antigens of *V. parahaemolyticus* compared with reports of other endotoxins (18) may arise from the array of proteins distributed in the endotoxin, with the net effect being demonstration of behavior similar to that of protein antigens. Also, endotoxins may contain more protein than the endotoxins of *Enterobacteriaceae*, but these hypotheses reported further study for verification. In our studies, however, the total Lowry nitrogen of *V. parahaemolyticus* endotoxins was greater than that reported for the *Enterobacteriaceae* (22). Aside from being less potent in lethal dosage than *Salmonella typhi* endotoxin, the *V. parahaemolyticus* endotoxins were found to be remarkably similar in biological properties (22).

Based on the inhibition demonstrated by preparations of strain 11590, the results with glucose and galactose show good evidence that these carbohydrates are involved in serological specificity. Galactose, but not glucose, is known to be a component of the cell structure of *Vibrio*

TABLE 6. *Cross-reactions between endotoxins prepared from strains of V. parahaemolyticus measured by passive HA*[a]

| Endotoxin preparation | Antiserum titers: Anti-*V. parahaemolyticus* 11590 serum | Anti-*V. parahaemolyticus* FC 1011 serum | Anti-*V. parahaemolyticus* Sak-3 serum |
|---|---|---|---|
| *V. parahaemolyticus* 11590 (Phe) | 40,960 | 40 | 80 |
| *V. parahaemolyticus* FC 1011 (Phe) | 80 | 40,960 | 40 |
| *V. parahaemolyticus* Sak-3 (Phe) | 160 | 40 | 1,280 |

[a] Endotoxin-sensitized human type O, Rh+ RBC in reaction against immune sera.

TABLE 7. *Immunizing properties of phenol-water endotoxin extracts prepared from V. parahaemolyticus strains*[a]

| Endotoxin preparation | Rabbit serum no. | Rabbit treatment | HA titer |
|---|---|---|---|
| *V. parahaemolyticus* FC 1011 (Phe) | 6 | Pre-immunization | 0 |
| | 6 | Post-immunization | 40,960 |
| *V. parahaemolyticus* FC 1011 (Phe) | 7 | Pre-immunization | 0 |
| | 7 | Post-immunization | 20,480 |
| *V. parahaemolyticus* 11590 (Phe) | 27 | Pre-immunization | 0 |
| | 27 | Post-immunization | 640 |
| *V. parahaemolyticus* 11590 (Phe) | 29 | Pre-immunization | 0 |
| | 29 | Post-immunization | 320 |

[a] Passive HA in serum of rabbits injected intravenously with phenol-water extracts.

(24, 25). The function of these and other carbohydrates in *V. parahaemolyticus* endotoxin is not known, despite the fact that the structure of endotoxins of other gram-negative, fermentative rods, including members of the *Enterobacteriaceae*, are well characterized (6, 15).

Results of cross-reaction tests suggest that endotoxins prepared from the three strains of *V. parahaemolyticus* share antigenic components, despite the relatively low titers of cross-reacting antigens (Table 6). Unimmunized, i.e., normal, rabbits did not demonstrate an antibody reaction against *V. parahaemolyticus* by passive HA, bacterial agglutination, agar gel double diffusion (21), or immunoelectrophoresis (21). Therefore, a titer of 40, although low, can be considered to be significant, clearly not the case for antigens from *Enterobacteriaceae*, *Staphylococcus*, or *Streptococcus*, where the nonimmunized, normal animal often possesses antibody reactivity to a titer of 40 or even greater (14). This extraneous antibody reactivity could, of course, arise from antibody stimulation by related bacteria or to antigens ingested or inhaled.

Endotoxins prepared from strains FC 1011 and 11590 were tested for ability to act as antigens. Low passive HA titers were obtained after immunization with strain 11590, whereas good titers were obtained with strain FC 1011, an unusual phenomenon since FC 1011 endotoxin was found to be less biologically potent than the 11590 preparation (21, 22). However, the antisera so obtained were not tested for ability to protect experimental animals.

The results reported here suggest that the passive HA test, employing the relatively simple and inexpensive tannic acid procedure, is useful for analysis of soluble antigens of *V. parahaemolyticus*.

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