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Characterization of *Aeromonas trota* Strains That Cross-React with *Vibrio cholerae* O139 Bengal

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It has previously been shown that *Vibrio cholerae* O139 Bengal shares antigens with *V. cholerae* serogroups O22 and O155. We detected six surface water isolates of *Aeromonas trota* that agglutinated in polyclonal antisera to *V. cholerae* O139 and *V. cholerae* O22 but not in antiserum to *V. cholerae* O155. On the basis of agglutinin-absorption studies, the antigenic relationship between the cross-reacting bacteria were found to be in an *a,b-a,c* fashion, where *a* is the common antigenic epitope and *b* and *c* are unique epitopes. The antigen sharing between *A. trota* strains and *V. cholerae* O139 was confirmed in immunoblot studies. However, *A. trota* strains did not react with two monoclonal antibodies specific for *V. cholerae* O139 and, consequently, tested negative in the Bengal SMART rapid diagnostic test for *V. cholerae* O139 which uses one of the monoclonal antibodies. A polyclonal antiserum to a cross-reacting *A. trota* strain cross-protected infant mice against cholera on challenge with virulent *V. cholerae* O139. All *A. trota* strains were cytotoxic for HeLa cells, positive for adherence to HEp-2 cells, and weakly invasive for HEp-2 cells; one strain was heat-stable toxin positive in the suckling mouse assay; however, all strains were negative for cholera toxin-like enterotoxin. Studies on bacteria that share somatic antigen with *V. cholerae* O139 may shed further light on the genesis of *V. cholerae* O139.

Vibrio cholerae O139 Bengal has caused large epidemics of clinical cholera in the Indian subcontinent recently, and it is now considered to be the second etiologic agent of cholera. Since it has also spread to several other countries, *V. cholerae* O139 is also thought to be the etiologic agent of the eighth pandemic of cholera (1). *V. cholerae* O139 does not agglutinate with specific antisera to *V. cholerae* O1 or to 152 of the 154 non-O1 *V. cholerae* serogroups described to date. However, it agglutinates with specific antisera to *V. cholerae* O22 and *V. cholerae* O155 serogroups. The antigenic relationship of *V. cholerae* O139 to each of the cross-reacting serogroups mentioned above is described as an *a,b-a,c* type of relationship, where *a* is the common epitope and *b* and *c* are unique epitopes for the cross-reacting organisms. In addition, since the core or rough antigen is common for all *V. cholerae* isolates, *V. cholerae* O139 also shares this antigen with all other *V. cholerae* isolates. Therefore, for the production of specific polyclonal antiserum to *V. cholerae* O139, the antiserum must be absorbed with a rough strain of *V. cholerae* and then absorbed with strains belonging to *V. cholerae* O22 and O155 serogroups (20). The absorbed antiserum is then used for the confirmatory diagnosis of *V. cholerae* O139 in which the organisms from colonies are agglutinated with the antiserum.

We were interested in finding out whether other members of the family *Vibrionaceae* could cross-react with *V. cholerae* O139 Bengal. In the course of our screening, we detected six strains of *Aeromonas trota* that agglutinated with specific antiserum to *V. cholerae* O139 Bengal in a slide agglutination test. These strains were characterized with respect to their antigenic and virulence properties.

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MATERIALS AND METHODS

Bacteria. One hundred fifty strains of *Aeromonas* spp. isolated from surface water sources from Dhaka and surrounding areas in Bangladesh in 1989 were used for screening for a cross-reaction with *V. cholerae* O139. The *A. trota* type strain, ATCC 49657, was also used, but only to check for its reactivities with *V. cholerae* O139 antiserum and antiserum to *A. trota* that cross-reacts with *V. cholerae* O139. The strains were stored in T1N1 soft agar (1.0% Trypticase, 1.0% NaCl, 0.5% agar) at 25°C. *Aeromonas* spp. were identified by the method described previously (5).

Production of antisera. Antisera were produced against a clinical isolate of *V. cholerae* O139 (strain AI-1852), a *V. cholerae* serogroup O155 strain, and a cross-reacting *A. trota* strain (strain 1354). Bacteria were grown in brain heart infusion (BHI) broth (Difco, Detroit, Mich.) for 20 h with continuous shaking. The bacteria were pelleted by centrifugation, washed once in physiological saline, and reconstituted in physiological saline to 10^9 CFU/ml. Adult New Zealand White rabbits were immunized intravenously at 6-day intervals with 0.2-, 0.5-, and three 1.0-ml doses of bacterial suspension. A booster dose of 2 ml was administered 20 days after the last injection. The rabbits were exsanguinated 7 days after the last dose. The antisera to *A. trota* and *V. cholerae* O155 were made specific by absorbing the antisera with a rough strain of *V. cholerae* (strain CA385), and that to *V. cholerae* O139 was made specific by absorbing the antisera with strain CA385 and strains representing serogroups O22 and O155 of *V. cholerae* as described previously (20). Antiserum to *V. cholerae* O22 was that prepared previously at the National Institute of Health, Tokyo, Japan (20).

Slide agglutination test. The 150 stock cultures of *Aeromonas* spp. and the *A. trota* ATCC 49657 type strain were subcultured onto MacConkey agar (Difco) and were incubated at 37°C for 20 h. Each strain was agglutinated with specific antiserum to *V. cholerae* O139 in a slide agglutination test. Strains agglutinating in antiserum were further checked for agglutination in antisera to *V. cholerae* O22 and O155 and in two mouse monoclonal antibody preparations, ICL11 and ICL12, which are specific for *V. cholerae* O139, as described previously (14).

Tube agglutinin test. The antigens used in the tube agglutinin test consisted of bacteria grown in BHI broth (Difco) as shaker cultures at 37°C for 20 h, washed in physiological saline, boiled for 1 h, and adjusted to a turbidity corresponding to that of a McFarland turbidity standard number 3. Doubling dilutions of antisera were tested starting with 1:20 dilutions and moving to smaller increments when a more accurate estimate of the titer was required. Results were read after incubating the test tubes containing antigen-antibody mixtures in a water

bath at 42°C for 1 h and then at 4°C for 20 h. Agglutination was scored on a scale of 0 to 4+, and the endpoint titer was defined as the reciprocal of the highest dilution of antiserum yielding a 2+ agglutination of cells.

Agglutinin-absorption test. Bacteria were grown on BHI agar (Difco) at 37°C for 20 h and were harvested in physiological saline. The bacterial cells were packed by centrifugation and were added to antiserum (1 g of packed cells plus 1 ml of antiserum). The antigen-antibody mixture was incubated in a 42°C water bath for 1 h and then at 4°C for 20 h and centrifuged at 3,000 rpm at 25°C for 15 min. After filtration through a membrane filter (0.45-μm-diameter pore size; Sartorius, Gottingen, Germany), the supernatant was used for agglutination. If the antiserum was absorbed with more than one antigen, the filtration step was performed at the end of the last absorption step. *A. trota*, *V. cholerae* O139, and *V. cholerae* O22 and antisera to these strains were used in agglutinin-absorption tests.

Serogrouping. The *A. trota* strains were subjected to O serogrouping with antisera to the 44 serogroups (serogroups O1 to O44) (18) and to the other 50 serogroups recognized subsequently (serogroups O45 to O94) (19).

Studies with LPS antigens. Bacteria were grown on BHI agar (Difco) at 37°C for 20 h, and lipopolysaccharide (LPS) antigens were extracted by the hot phenol-water extraction method (24). Components of LPS were separated on sodium dodecyl sulfate (SDS)–13.5% polyacrylamide gels and were visualized by silver staining. For immunoblotting, LPS separated by SDS-polyacrylamide gel electrophoresis was blotted onto a nitrocellulose membrane (23) and was reacted with rabbit polyclonal antiserum (1:200 dilution) to the relevant bacteria and then with alkaline phosphatase-conjugated swine anti-rabbit immunoglobulin G (1:500 dilution), both of which were done in phosphate-buffered saline–Tween 20. Bound conjugate was detected with the substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, Mo.) as described previously (4).

Bengal SMART test. The Bengal SMART test is a rapid test for the detection of *V. cholerae* O139. It is a colorimetric immunoassay and uses the colloidal gold-labelled monoclonal antibody ICL12 to *V. cholerae* O139 (15). The ability of the SMART test to detect cross-reacting *A. trota* strains was tested according to the manufacturer's instructions.

Enterotoxin production. Bacteria were grown in Casamino Acids–yeast extract broth (6) in a water bath at 37°C for 20 h with continuous shaking (150 oscillations per minute). Membrane (0.45-μm-diameter pore size; Sartorius)-filtered supernatant was tested in a Y1 adrenal tumor cell assay for cholera toxin-like enterotoxin (17) and in a suckling mouse assay for heat-stable enterotoxin (8). The positive control used for the Y1 cell assay was a toxigenic *V. cholerae* O1 El Tor strain, the positive control used for the suckling mouse assay was a heat-stable toxin-producing *Escherichia coli* strain, and the negative control for both assays was the nonpathogenic laboratory strain *E. coli* K-12. The criterion for a positive test result in the Y1 cell assay was rounding of ≥50% of the cell monolayer, and that for the suckling mouse assay was a ratio of ≥0.085 for intestinal body weight to remaining body weight from five mice per test.

The bacteria were also tested in a PCR assay for the cholera toxin gene (*ctx*) by using two primers corresponding, respectively, to nucleotide bases 712 to 735 and 990 to 1013 of the *ctx* operon of *V. cholerae* O1 as described previously (21). The amplified product was separated by gel electrophoresis.

Cytotoxin production. Bacteria were grown in BHI broth (Difco) as shaker cultures as described above, and filtrates prepared as described above were tested for their cytotoxicities in HeLa cell monolayers (9). Undiluted and serially diluted (1:10 to 1:160) filtrates in phosphate-buffered saline (pH 7.2) were tested. Filtrates from a clinical isolate of *Shigella dysenteriae* 1 and *E. coli* K-12 were tested as positive and negative controls, respectively. Cytotoxicity (rounding, granularity, or detachment) of ≥50% of the monolayer was considered a positive result.

HEp-2 cell adherence assay. Adherence to the HEp-2 cell monolayer was tested by the method of Cravioto et al. (7). Briefly, HEp-2 cell monolayers were inoculated with bacteria grown at 37°C for 4 h in BHI broth (Difco) as shaker cultures and were then incubated for 3 h in the presence of 1% D-mannose. After washing to remove nonadherent bacteria, cells on coverslips were fixed in 70% methanol and stained with Giemsa. A localized adherence-positive strain of enteropathogenic *E. coli* serotype O127:H6 and the *E. coli* K-12 strain were used as positive and negative controls, respectively.

HEP-2 cell invasion assay. The HEp-2 cell invasion assay was performed as described previously (22). Briefly, bacteria grown at 37°C for 4 h in BHI broth (Difco) as shaker cultures were inoculated into a HEp-2 cell monolayer (multiplicity of infection, 100). After a 2-h infection period, the monolayer was washed to remove the nonadherent bacteria, and the remaining extracellular bacteria were killed by a further 1-h incubation at 37°C in medium containing 100 μg of gentamicin per ml. The progeny surviving the incubation in gentamicin were quantified by plating different dilutions of a Triton X-100-treated monolayer. The result was expressed as the ratio of the number of gentamicin-resistant cells to the number of cells in the input inoculum. A clinical isolate of *Shigella flexneri* 2a was used as a positive control, and *E. coli* K-12 was used as a negative control. All isolates were also incubated in gentamicin-containing medium without HEp-2 cells to ensure that they did not survive gentamicin treatment.

Passive protection in infant mouse cholera model. The 50% lethal dose (LD_{50}) of the clinical strain of *V. cholerae* O139 (strain AI-1852) was determined in an infant mouse cholera model as described previously (2). Bacteria from a 4-h BHI broth (Difco) shaker culture grown at 37°C were washed once in physiological saline and were resuspended in physiological saline. One-tenth of 1-ml volumes of 10-fold serial dilutions corresponding to 10^8 to 10^3 CFU were inoculated into 4-day-old suckling mice. Each dilution was inoculated into a group of six mice. The mice were watched for death for up to 48 h. The LD_{50} was calculated according to the formula of Reed and Muench (16).

To study the protective efficacy of polyclonal antiserum to cross-reacting *A. trota*, 0.1 ml of a 1:10-diluted antiserum in physiological saline was mixed with an equal volume of *V. cholerae* O139 culture (strain AI-1852) containing 100 LD_{50}s, and the mixture was incubated for 30 min at 25°C and inoculated into a group of six 4-day-old mice. The controls included mice inoculated with *V. cholerae* O139 mixed with 1:10-diluted *V. cholerae* O139 rabbit polyclonal antiserum, *V. cholerae* O139 mixed with 1:10-diluted rabbit preimmune serum, and mice inoculated only with 100 LD_{50}s of *V. cholerae* O139. The animals were watched for death as described above.

RESULTS

Identification of cross-reacting *A. trota*. Of the 150 isolates of *Aeromonas* spp. tested, 6 isolates agglutinated in *V. cholerae* O139 diagnostic polyclonal antiserum and in polyclonal antiserum to *V. cholerae* O22. However, these six isolates did not agglutinate with polyclonal antiserum to *V. cholerae* O155 or with the two monoclonal antibodies specific for *V. cholerae* O139. The six isolates were identified as *A. trota* according to previously published criteria (5). They gave negative reactions for esculin hydrolysis, arabinose fermentation, and the Voges-Proskauer test and positive reactions for cellobiose fermentation and lysine decarboxylation and for susceptibility to ampicillin. The six cross-reacting *A. trota* isolates were given strain numbers of 1354, 1203, 1330, 1191, 1220, and 1211, respectively. The *A. trota* type strain did not agglutinate with *V. cholerae* O139 antiserum.

Tube agglutination and agglutinin-absorption tests. The results of the agglutination and agglutinin-absorption tests are presented in Table 1. Cross-absorption of antisera to *V. cholerae* O139 and *A. trota* with heterologous bacteria of *A. trota* and *V. cholerae* O139 absorbed out most of the antibody. However, significant amounts of antibody to homologous antigens were retained in both antisera. This suggested that the antigenic relationship between the two bacteria constituted an *a*,*b*-*a*,*c* type of relationship, where *a* is the common epitope and *b* and *c* are unique epitopes. Cross-absorption of antiserum to *A. trota* with *V. cholerae* O22 caused a reduction in the homologous titer, but the titer against *V. cholerae* O139 was unaffected. Combined absorption of the antiserum with both *V. cholerae* O139 and *V. cholerae* O22 still left a significant amount of homologous antibody, as was the case when the antiserum was absorbed with *V. cholerae* O139 alone. Cross-absorption of antiserum to *V. cholerae* O22 with *A. trota* caused a reduction in the homologous antibody titer, yet a significant amount of homologous antibody was retained. This suggested that, as with *V. cholerae* O139, the relationship of *A. trota* with *V. cholerae* O22 is that of the *a*,*b*-*a*,*c* type.

The remaining five strains of cross-reacting *A. trota* gave a titer of 2,560 in antiserum to *A. trota* 1354, which was the same as the homologous titer. The antigenic relationships of these five strains of *A. trota* with *V. cholerae* O139 and *V. cholerae* O22 were studied in a qualitative manner in a slide agglutination test with relevant unabsorbed and absorbed antisera. In the slide agglutination test, all five strains showed antigenic relationships to *V. cholerae* O139 and *V. cholerae* O22 similar to that shown by strain 1354. Also, the *A. trota* type strain did not agglutinate with unabsorbed antiserum to cross-reacting *A. trota* 1354 in a slide agglutination test.

O serogrouping. The six cross-reacting *A. trota* strains could not be O serogrouped with antisera to the 94 O serogroups of *Aeromonas* spp. The *A. trota* type strain belonged to serogroup O11.

LPS antigens. Of the *Aeromonas* sp. strains, only the LPS of

TABLE 1. Agglutination and agglutinin-absorption tests with antisera to *A. trota* 1354, *V. cholerae* O139 strain AI-1852, and *V. cholerae* O22

Antiserum[a]	Absorbing organism[b]	Reciprocal titer of antisera to O antigens[c] from:			
		A. trota 1354	*V. cholerae* O139 strain AI-1852	*V. cholerae* O22	*V. cholerae* O155
A. trota	None	2,560	1,280	160	—[d]
	V. cholerae O139	640	—	160	—
	V. cholerae O22	1,600	1,280	—	—
	V. cholerae O139+ *V. cholerae* O22	640	—	—	—
V. cholerae O139	None	1,280	2,560	160	80
	A. trota	—	320	160	80
V. cholerae O22	None	320	40	320	—
	A. trota	—	—	160	—

[a] Rough antibody in these antisera was removed by absorption with culture of a rough strain, strain CA385.
[b] Absorbed with live organisms.
[c] Boiled (100°C for 1 h) cultures were used.
[d] —, no agglutination at a dilution of 1:20 or higher.

A. trota 1354 was studied. The separation profile of LPSs from *A. trota* 1354 and *V. cholerae* O139 strain AI-1852 is shown in Fig. 1. The profile of *A. trota* resembled that of smooth bacteria revealing a core antigen (thick bands at the bottom of the gel) and an O-antigenic repeating polysaccharide (ladder-like pattern in the top portion of the gel). However, the LPS pattern of *V. cholerae* O139 resembled that of semirough-type bacteria, as reported earlier (11), revealing a modified core structure and no high-molecular-weight O-antigen-specific side chain. Immunoblot studies carried out with antisera to *V. cholerae* O139 and *A. trota* recognized homologous and heterologous LPS antigens equally well (Fig. 2; immunoblot shown with antiserum to *A. trota* only).

Bengal SMART test. As expected, since the *A. trota* strains did not agglutinate with monoclonal antibody ICL12, they also tested negative in the Bengal SMART test, which was based on the same monoclonal antibody.

Enterotoxin and cytotoxin production. None of the six *A. trota* isolates produced a cholera toxin-like enterotoxin when culture filtrates were tested in Y1 cells (rounding of cells, 0 to 5%), but one isolate (isolate 1354) produced a heat-stable enterotoxin in the suckling mouse assay (gut weight to remaining body weight ratios, 0.09 for isolate 1354 and <0.07 for the other five isolates). All of the isolates were also negative in the PCR assay for the *ctx* gene; no PCR product was generated, but a control strain of toxigenic *V. cholerae* O1 El Tor produced a 320-bp amplicon corresponding to *ctx* (21). All six isolates were cytotoxic for HeLa cells (50 to 100% of the cells were affected), and the cytotoxin titers varied between 1:10 and 1:100. The control cultures behaved as expected.

HEp-2 cell adherence. All six strains of *A. trota* adhered to the HEp-2 cell monolayer; the majority of cells had numerous adherent bacteria. The control organisms behaved as expected.

HEp-2 cell invasion. In the quantitative invasion assay, with an input inoculum of 10^7 CFU/ml, the average number of bacteria recovered in the presence of gentamicin was 1×10^3 to 3×10^3 CFU/ml for all six strains. The corresponding value for the positive control, *S. flexneri* 2a, was 10^5 CFU/ml, and that for the negative control, *E. coli* K-12, was 0 CFU/ml. Thus, it appeared that *A. trota* strains were weakly invasive for cell culture. All test and control isolates were killed in gentamicin-containing medium without HEp-2 cells.

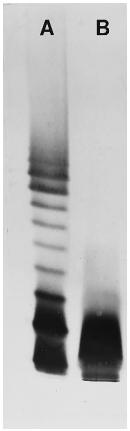

FIG. 1. LPS profile of *A. trota* 1354 (lane A) and *V. cholerae* O139 strain AI-1852 (lane B). The LPS preparations were electrophoresed on SDS–13.5% polyacrylamide gels and were silver stained.

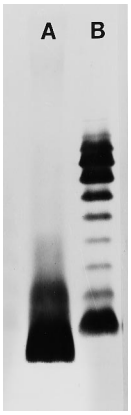

FIG. 2. Immunoblot analysis of LPSs from *V. cholerae* O139 strain AI-1852 (lane A) and *A. trota* 1354 (lane B) separated on SDS–13.5% polyacrylamide gels by electrophoresis, blotted onto a nitrocellulose membrane, and probed with rabbit antiserum to *A. trota* 1354.

Passive protection in infant mouse cholera model. The LD_{50} of *V. cholerae* O139 strain AI-1852 for infant mice was 10^4 CFU in 0.1 ml of inoculum. A 1:10 dilution of polyclonal antiserum to *V. cholerae* O139 or *A. trota* completely protected mice against a challenge dose of *V. cholerae* O139 of 100 LD_{50}s. On the other hand, a 1:10 dilution of preimmune rabbit serum did not protect mice; all mice developed diarrhea and died.

DISCUSSION

It is important that immunodiagnostic reagents for *V. cholerae* O139 be highly specific so that cross-reacting organisms are not misdiagnosed as true *V. cholerae* O139 strains. In this regard, Shimada et al. (20) have detected two *V. cholerae* strains belonging to serogroups O22 and O155 that cross-reacted with *V. cholerae* O139. However, those strains were not serologically identical to *V. cholerae* O139 but shared antigens in an *a*,*b*-*a*,*c* type of relationship. This finding has necessitated the cross-absorption of antiserum to *V. cholerae* O139 with *V. cholerae* O22 and O155 serogroups, in addition to a rough strain, to make the antiserum more specific. Our screening of bacteria has identified six strains of *A. trota* that share antigens with *V. cholerae* O139. However, the *A. trota* strains were not serologically identical to *V. cholerae* O139, but the relationship was the *a*,*b*-*a*,*c* type, as shown previously between *V. cholerae* O139 and *V. cholerae* O22 and O155. Again, it is interesting that *A. trota* strains did not cross-react with *V. cholerae* O155 but did react with *V. cholerae* O22, with which they also exhibited an *a*,*b*-*a*,*c* type of relationship. Shimada et al. (20) found that *V. cholerae* O22 and *V. cholerae* O155 did not share antigens. From the serological data presented above, it appears that *V. cholerae* O139, *V. cholerae* O22, and *A. trota* have a common antigen, while all of these bacteria also possess unique antigens. Again, *V. cholerae* O139 and *V. cholerae* O155 share a common antigen which seems to be absent from *V. cholerae* O22 and *A. trota.*

Provided that clinical and environmental specimens containing cross-reacting *A. trota* strains are cultured and colonies are biochemically identified as *A. trota*, there is no danger of misdiagnosing them as true *V. cholerae* O139. Therefore, it appears that there is no need to cross-absorb diagnostic antiserum to *V. cholerae* O139 with cross-reacting *A. trota* strains. Again, we have found that two monoclonal antibodies specific for *V. cholerae* O139 did not agglutinate with *A. trota* strains, and as expected, the Bengal SMART test designed with one of the monoclonal antibodies did not result in a diagnosis of *A. trota* as *V. cholerae* O139. This suggested that the antigenic epitope(s) to which the monoclonal antibodies are directed is absent from *A. trota*. Again, it is comforting that cross-reacting *A. trota* strains will not be misdiagnosed by the Bengal SMART test.

The cross-reacting *A. trota* strains did not belong to the 94 recognized O serogroups of *Aeromonas* spp., and therefore, a new O-serogroup number needs to be assigned to these strains. Also, even though these strains shared antigens with *V. cholerae* O139, they did not have an LPS characteristic of semirough-type bacteria; in fact they possessed an LPS characteristic of smooth bacteria. Of importance is the finding that polyclonal antiserum to a cross-reacting *A. trota* strain cross-protected infant mice against cholera upon challenge with a virulent *V. cholerae* O139 strain. This suggested that the antigenic epitope(s) shared between *V. cholerae* O139 and *A. trota* is immunodominant and protective.

A variety of virulence factors such as enterotoxin production, cytotoxin production, and tissue culture adherence and invasion have been demonstrated in strains of *Aeromonas* spp. (10, 12). Even so, the role of *Aeromonas* spp. in the etiology of diarrhea remains controversial (13). The cross-reacting *A. trota* strains appeared to be heterogeneous in the possession of virulence properties. Even though all strains were cytotoxic, tissue culture adherent, and weakly invasive (many common enteric bacteria exhibit a weak invasive ability), only one strain was enterotoxic. However, all strains were negative for cholera toxin-like enterotoxin production, which suggested that they may not cause cholera-like secretory diarrhea.

V. cholerae O139 shares striking similarities with the *V. cholerae* O1 El Tor biotype (1), and the available evidence suggests that *V. cholerae* O139 might have originated from an El Tor strain which acquired novel genes for LPS and capsule synthesis (3). Interestingly, unique gene sequences present in *V. cholerae* O139 have been demonstrated to be homologous to sequences in *V. cholerae* serogroups O69 and O141 (3), and the similarity of somatic antigens has been demonstrated with *V. cholerae* serogroups O22 and O155 (20) and now with *A. trota*. Further studies on antigen-sharing bacteria will shed more light on the genesis of *V. cholerae* O139 Bengal.

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