Characterization of Aeromonas caviae Antigens Which Cross-React with Shigella boydii 5


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Received 23 September 1991/Accepted 20 February 1992

Live and boiled cells of 16 strains of Aeromonas caviae, isolated from patients with diarrhea, agglutinated with Shigella boydii 5 antiserum in a slide test. Further studies with seven selected strains showed agglutination with boiled cells in a tube test. Lipopolysaccharide antigen extracted from one of these strains cross-reacted with S. boydii 5 in enzyme-linked immunosorbent assay and immunoblot studies. Either all or the majority of the seven strains possessed properties deemed to be diarrheagenic.

The three well-recognized species of the genus Aeromonas are Aeromonas hydrophila, Aeromonas sobria, and Aeromonas caviae. Although these three species have been isolated from humans with diarrheal illnesses, their role as definitive agents of diarrhea remains to be established (3, 8). We have isolated several strains of A. caviae from stools of patients with diarrhea attending our treatment center. These strains cross-reacted with antiserum to Shigella boydii 5. We characterized the antigens responsible for the cross-reactions and also studied the virulence properties of these unique strains in an attempt to elucidate their diarrheagenic potential.

A. caviae was isolated from 16 patients (of both sexes, ranging in age from 2 months to 28 years) with diarrhea. The nature of the diarrhea was watery for all patients except one, who had bloody mucoid diarrhea. Shigella dysenteriae 2 was also isolated from this patient, and the invasive diarrhea could be attributed to this organism. Campylobacter jejuni was also isolated from another patient. No other recognized bacterial pathogens were isolated from the remaining 14 patients. The pathogens sought were Salmonella, Shigella, Vibrio, Campylobacter, Aeromonas, and Plesiomonas species, and they were identified by standard methods (17, 25). Isolates belonging to the genus Aeromonas were speciated according to the scheme of Popoff and Veron (15).

The isolates hydrolyzed esculin, grew in KCN medium, fermented salicin, and utilized L-arginine and L-arabinose but did not produce gas or acetoin from glucose. These reactions are characteristic of A. caviae. Isolates were further confirmed as A. caviae by N. Ryan, Fairfield Infectious Diseases Hospital, Fairfield, Victoria, Australia.

Live and boiled (for 1 h) suspensions of A. caviae from all 16 patients gave strong slide agglutinations with S. boydii 5 antiserum, but no agglutination was found against antiserum to other shigellae (Wellcome Diagnostics, Dartford, United Kingdom). Strains 11212, 6642, 2375, 13681, 14399, 1168, and 1183 were selected for further studies. For characterization of cross-reacting antigens, antiserum against A. caviae 11212 and an S. boydii 5 strain were produced in rabbits as described elsewhere (14).

Tube agglutination tests were performed with overnight growths of bacteria in Trypticase soy broth supplemented with 0.6% yeast extract (TSBY) (GIBCO, Paisley, Scotland, United Kingdom) at 37°C, washed in physiological saline, boiled for 1 h, and adjusted to a turbidity corresponding to that of McFarland standard no. 3. Doubling dilutions of antisera were tested, starting with 1:50 dilutions and moving to smaller increments when a more accurate estimate of the titer was required.

Lipopolysaccharide (LPS) antigens were extracted from A. caviae 11212 and S. boydii 5 by the hot phenol-water extraction procedure (24) for use in enzyme-linked immunosorbent assay (ELISA) and immunoblotting. ELISA was performed by using LPS (10 μg/ml) as the coating antigen for determination of the antibody titer in rabbit antisera by a previously described method (12). The antibody titer was calculated as the mean interpolated dilution giving an optical density of 0.2 above background (18).

For immunoblotting, LPS separated on 13.5% sodium dodecyl sulfate-polyacrylamide gels (11) was blotted onto a nitrocellulose membrane (21) and was reacted with rabbit anti-LPS antibody (1:200 dilution) and then with alkaline phosphatase-conjugated swine anti-rabbit immunoglobulin G (1:500 dilution), both 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).

Cross-reactions were found in tube agglutination tests and ELISA (Table 1). In general, homologous titers were higher than heterologous titers, and the differences were more pronounced in ELISA than in tube tests. Cross-reactions were also seen in the immunoblot test (Fig. 1). In this test, both the homologous and heterologous antisera recognized the core antigens (thick bands at the bottom end of the gel) and the O-antigenic repeating polysaccharides (ladderlike pattern in the top portion of the gel). The homologous reactions (lanes 1 and 3) were stronger than the heterologous reactions (lanes 2 and 4) (Fig. 1), reflecting homologous and heterologous titers (Table 1). Since cross-reactions were found with boiled cells and purified LPSs, it appears that the cross-reacting antigens are LPSs.

To find out whether the cross-reactions observed between A. caviae and S. boydii 5 were due to a common antigen(s) shared by gram-negative aerobic rods, we performed slide and tube agglutination tests with live and boiled cells of two strains each of E. coli, Klebsiella pneumoniae, Enterobacter aerogenes, Salmonella typhi, A. hydrophila, and A. sobria.
TABLE 1. Antigenic cross-reactivities between *A. caviae* and *S. boydii* 5 in agglutination tests and ELISA

<table>
<thead>
<tr>
<th>Type of test and antigen used</th>
<th>Reciprocal titer of antiserum to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. caviae</em> 11212</td>
</tr>
<tr>
<td>Tube agglutination test with live cells from:</td>
<td></td>
</tr>
<tr>
<td><em>A. caviae</em> 11212</td>
<td>3,200</td>
</tr>
<tr>
<td>2375</td>
<td>3,000</td>
</tr>
<tr>
<td>13681</td>
<td>3,200</td>
</tr>
<tr>
<td>14399</td>
<td>3,200</td>
</tr>
<tr>
<td>1183</td>
<td>3,200</td>
</tr>
<tr>
<td>1168</td>
<td>3,200</td>
</tr>
<tr>
<td>6642</td>
<td>3,200</td>
</tr>
<tr>
<td><em>S. boydii</em> 5</td>
<td>3,000</td>
</tr>
<tr>
<td>Tube agglutination test with boiled cells from:</td>
<td></td>
</tr>
<tr>
<td><em>A. caviae</em> 11212</td>
<td>74,240</td>
</tr>
<tr>
<td><em>S. boydii</em> 5</td>
<td>5,400</td>
</tr>
<tr>
<td>ELISA with LPS antigens from:</td>
<td></td>
</tr>
<tr>
<td><em>A. caviae</em> 11212</td>
<td></td>
</tr>
<tr>
<td><em>S. boydii</em> 5</td>
<td></td>
</tr>
</tbody>
</table>

all isolated from feces, against *A. caviae* 11212 antiserum. No agglutination was seen in the slide test (with neat antiserum) or the tube test (with a 1:50 dilution of antiserum) with any bacteria, which ruled out the involvement of a common antigen.

Culture filtrates of the seven strains, after overnight growth of the organisms in brain heart infusion broth (Difco, Detroit, Mich.) at 37°C in a shaker-incubator, were tested for enterotoxin in 18-h adult rabbit ileal loop assays (7), heat-labile enterotoxin in Y1 adrenal tumor cell assays (19), heat-stable enterotoxin in suckling-mouse assays (6), and cytotoxin in HeLa cell assays (10). The organisms were negative for all of these properties. However, when the Y1 cell assay, the HeLa cell assay, and the suckling-mouse assay were repeated with culture filtrates obtained after growth in double-strength Trypticase soy broth without glucose (Difco), all isolates, except strain 6642, exhibited cytotoxic effects on Y1 cells. These consisted of rounding, granulation, and detachment of cells. No changes were seen in the HeLa cell assay and the suckling-mouse assay. These results confirmed the earlier observation that toxin production in *A. caviae* is medium dependent (13). Cell culture adherence and invasion assays (5, 23) were done by using HEP-2 cells with overnight bacterial culture grown at 37°C in brain heart infusion broth. However, all except strain 1168 invaded HEP-2 cells, with the intracellular survival of bacteria ranging from 100 to 1,000 CFU/ml. Again, all except strain 1168 adhered to HEP-2 cells, with bacteria seen around the cells. All strains were negative for *Shigella*-like invasiveness in Sereny test (20) when tested with overnight bacterial growth in TSBY at 37°C.

In the in vitro susceptibility test done by the disk diffusion method (1), three strains (11212, 6642, and 2375) were resistant to tetracycline, ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, and nalidixic acid but were susceptible to furazone and pirmecillin. The remaining four strains (13681, 14399, 1168, and 1183) were susceptible to all antibiotics except ampicillin and trimethoprim-sulfamethoxazole. Congo red binding studies (16) showed that all strains produced pigmented colonies on Congo red agar at 37°C but not at 30°C. All strains were relatively hydrophobic, since they agglutinated at 1.5 M ammonium sulfate (16). Plasmid studies (2) showed that only three strains possessed plasmids, which suggested that plasmids are probably not involved in encoding antigens responsible for cross-reaction in *A. caviae*. The three strains that possessed plasmids are 2375, 6642, and 11212. The first had four plasmids (2.2, 2.7, 3.0, and 3.2 Mdal), the second had two plasmids (<2 and 20 Mdal), and third had one plasmid (40 Mdal). Hemolytic activity of the isolates was studied according to the method of Furniss et al. (9) but with 5% human erythrocytes instead of sheep erythrocytes. All strains produced a strong (3+) alpha-hemolysis. A negative control strain, *E. coli* K-12, did not produce hemolysis, and a positive control strain, *A. hydrophila*, produced a strong beta-hemolysis.

In conclusion, the majority of the seven strains of *A. caviae* studied in detail had several virulence-related properties, such as multiple antibiotic resistances, Congo red binding, relative hydrophobicity, hemolysin production, cytotoxin production, and adherence to and penetration of HEP-2 cells. Most of the properties are characteristic of diarrheagenic bacteria, and it is possible that these organisms would have caused diarrhea in individuals from whom they were isolated. For reasons of economy, studies were confined to pathogens requested by physicians, and other diarrheal pathogens, such as giardiae, rotavirus, and *E. coli*, were not sought in these patients, so the etiological role of the *A. caviae* remains uncertain. The properties of hemolysis of human erythrocytes and invasion of HEP-2 cells by *A. caviae* contradict the previous reports (5, 22, 23). It is possible that there could be strain variations in these properties or that these twin properties are characteristic of strains sharing antigens with *S. boydii* 5.

It is likely that infection with either bacterium could offer cross protection against the other. Structural and genetic

FIG. 1. Immunoblot analysis of LPSs separated on 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and probed with rabbit antiseras. Lanes: 1, *S. boydii* 5 LPS × antiserum to *S. boydii* 5; 2, *S. boydii* 5 LPS × antiserum to *A. caviae* 11212; 3, *A. caviae* 11212 LPS × antiserum to *A. caviae* 11212; 4, *A. caviae* 11212 LPS × antiserum to *S. boydii* 5.
studies will shed more light on the antigenic cross-reactivities between the two genera of bacteria.

This research was supported by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). ICDDR,B is supported by countries and international agencies which share its concern for the health problems of developing countries. Current donors include the aid agencies of the governments of Australia, Bangladesh, Belgium, Canada, Denmark, France, Japan, the Netherlands, Norway, Sweden, Switzerland, the United Kingdom, and the United States; international organizations, including the United Nations Capital Development Fund, the United Nations Development Program, the United Nations Children’s Fund, and the World Health Organization; and private foundations, including the Ford Foundation and the Sasakawa Foundation.

We thank Manzurul Haque of ICDDR,B for secretarial assistance in preparing the manuscript.

REFERENCES