Hafnia alvei, a Probable Cause of Diarrhea in Humans

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Hafnia alvei, a member of the family Enterobacteriaceae, was the only species of bacteria cultured from the stool of a 9-month-old child who was admitted with a 3-day history of watery diarrhea. The isolated strain of H. alvei failed to produce heat-labile or heat-stable enterotoxins or Shiga-like toxin I or II and did not invade HeLa cells, nor did it cause keratoconjunctivitis (determined by the Sereny test) in a guinea pig’s eye. The strain, however, induced diarrhea in 8 of 12 adult rabbits with removable intestinal ties (removable intestinal tie-adult rabbit diarrhea [RITARD] assay) and in 1 of 2 orally fed animals. No diarrhea could be induced with Escherichia coli K-12 in eight RITARD assay rabbits and three orally fed rabbits, respectively. Microscopic examination of affected animals revealed moderate inflammatory cellular infiltration of the intestinal mucosa, in which bacterial attachment to the surface epithelium and loss of the microvillus border were evident in the ileum and colon. Electron microscopy demonstrated cellular modifications of the apical surface, with cupping or pedestal formation and increased terminal web density at sites of bacterial “attachment-effacement,” a well-known characteristic and mechanism of diarrhea of enteropathogenic E. coli. Identical lesions were also induced by H. alvei in rabbit ileal loops, which ruled out naturally occurring rabbit enteropathogenic E. coli strains, which are known to produce similar lesions. It is concluded that at least some strains of H. alvei have the potential to cause diarrhea and that attachment-effacement is a virulence characteristic shared by bacteria other than E. coli.

**MATERIALS AND METHODS**

**Case.** A 9-month-old girl with vomiting and diarrhea was brought to the Clinical Research Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) for treatment. The diarrhea, which lasted 3 days, was accompanied by vomiting, mild dehydration, fever, and distention of the abdomen. Stools appeared liquid, yellow, and without mucus or blood. Other signs and symptoms were unremarkable. She was treated with rice-based oral rehydration fluid and discharged 20 h after admission.

Diarrheal stool cultures for enteric bacterial pathogens did not yield any recognized bacterial pathogens, but H. alvei was isolated as a pure culture. Parasites and viruses were not sought in the diarrheal stool.

**Identification of H. alvei.** The identification of a non-lactose-fermenting colony on MacConkey agar was accomplished by a set of biochemical reactions outlined by Ewing (8) and by reaction in the biochemical strip API-20E for the family Enterobacteriaceae (API System, Montalieu, Vercieu, France).

**Enterotoxigenicity assay.** H. alvei was grown in Trypticase soy broth supplemented with 0.6% yeast extract (TSBY) (DIFCO) for 20 h with shaking. Cell-free culture filtrates and polymyxin B extracts of cell pellets (9) were tested in mouse adenral tumor Y-1 cells for heat-labile toxin (17), in suckling mice for heat-stable toxin (6), and in HeLa cells for Shiga-like toxins (3).

**Enteroinvasiveness assay.** The strain was tested for invasiveness in HeLa cells (7) and in a guinea pig’s eye by the Sereny test (18).

**Enteroadherence assay.** Adherence to HeLa cells was tested according to the method of Cravioto et al. (5). Briefly, HeLa cell monolayers were inoculated with overnight bacterial culture grown in Luria broth, and they were then incubated for 3 h in the presence of 0.5% d-mannose, after which they were fixed in 70% methanol and stained with Giemsa stain.

**Plasmid analysis.** Plasmid DNA extraction was carried out according to the method of Birnboim and Doly (2). After electrophoresis, the gel was stained with ethidium bromide and examined under UV light.

**RITARD assay.** All animals used in this assay were treated thrice, each time for 5 days, with metronidazole (125 mg per rabbit per day) and sulfaquinoxaline sodium (464 mg per rabbit per day) to clear the gut of giardias and coccidia, respectively; only giardia- and coccidium-free animals were used for experiments. The ability of H. alvei to cause diarrhea was tested in 12 rabbits with removable intestinal ties (RITARD model) (19). Abdominal surgery was performed after the rabbits had fasted for 24 h, and a permanent tie was introduced into the cecum. Ten-milliliter aliquots of an overnight culture of H. alvei in TSBY, each aliquot containing $10^{10}$ organisms, were injected into the intestine.
above the temporary small intestinal tie, and the tie was
removed 2 h later. Control experiments were performed on
eight rabbits with *Escherichia coli* K-12. Unless stated
otherwise, the animals were observed for 5 days for diarrhea
and death. Rectal swabs were taken daily and plated onto
MacConkey agar to monitor shedding of the challenge
organism. The animals were sacrificed at various periods
after challenge when they developed diarrhea. However, all
control animals which did not develop diarrhea (see results
below) were sacrificed at the end of the fifth day. At autopsy,
the intestines were examined for fluid accumulation and
other gross pathological changes. Intestinal sections were
taken from the upper and lower jejunum, ileum, cecum,
proximal and distal colon, appendix, and mesenteric lymph
nodes, and they were fixed in buffered formal saline (21).
Mucosal scrapings of the mid-jejunum and mid-ileum were
cultured quantitatively. Serial 10-fold dilutions of homoge-
nized tissue were prepared in sterile physiological saline,
plated on MacConkey agar, and incubated at 37°C for 24 h.

Oral feeding of rabbits. Five adult rabbits which had fasted
for 24 h were given 15 ml of a 5% sodium bicarbonate
solution via a gastric tube to neutralize gastric acidity. This
was followed by an oral challenge with 10 ml of a culture of
*H. alvei* containing 10^10^ bacteria for two rabbits and with *E.
coli* K-12 culture for the other three rabbits. The animals
were observed daily for development of diarrhea, and shed-
ding of the organisms was monitored as described above.

Rabbit ileal loop assay. Either 1 ml of a 20-h TSBY culture
containing 10^9^ bacteria or cell-free culture filtrates were
inoculated into the approximately 10-cm-long intestinal ileal
loops of four adult New Zealand White rabbits which had
previously fasted for 48 h. Not more than six loops were
made in each rabbit. After 20 h, rabbits were sacrificed and
loops were examined for fluid accumulation and other gross
pathological changes (12). Portions of loops were fixed in
buffered formal saline and processed for histopathology.

**RESULTS**

The organism was positive for the following tests: o-nitro-
phenyl-β-D-galactopyranoside, lysine decarboxylase, ornith-
ine decarboxylase, and the fermentation of glucose, man-
nitol, and arabinose. It was negative for the following tests:
oxidase; arginine dihydrolase; tryptophan deaminase; citrate
utilization; H₂S, urease, and indole production; methyl red;
Voges-Proskauer; gelatin liquefaction; and the fermentation
of inositol, sorbitol, rhamnose, saccharose, melibiose, and
amylose.

The organism was negative for the following virulence
properties: heat-labile and heat-stable enterotoxins, Shiga-
like toxins, and enteroinvasiveness in the HeLa cell assay
(survival of bacteria, 0%) and by the Serény test; it also
failed to adhere in the HeLa cell assay. In addition, no
plasmid could be identified by gel electrophoresis.

**RITARD assay.** All 12 rabbits inoculated with *H. alvei*
shed the organism in the feces either alone or mixed with *E.
coli*. Eight of them developed diarrhea between 1 and 4 days
after inoculation; the diarrhea lasted in all animals until they
were sacrificed (Table 1). Feces were soft, mucoid, and
without blood, which soiled the perianal region of the
affected rabbits. None of the eight rabbits inoculated with *E.
coli* K-12 developed diarrhea.

At autopsy, rabbits with diarrhea had moderate amounts
of greenish yellow, mucoid fluid in the lower part of the small
intestine and substantial amounts in the portion of the cecum
that remained patent.

Quantitative bacteriology was performed on the gut scrap-
lings from the jejunum and mid-ilea of five rabbits with diarrhea
(rabbit numbers 2, 6, 7, 9, and 11) and two rabbits without
diarrhea (numbers 10 and 12). Four rabbits with diarrhea had
pure growth of *H. alvei* in both the jejunum and ileum, and
the fifth had a mixture of *E. coli* and *H. alvei*. The number of
*H. alvei* in the jejunum ranged between 9 x 10^9^ and 6.5 x 10^9^
per g of mucosal scraping. In the two rabbits which did not
have diarrhea, only *E. coli* was cultured from the jejunum,
with only *H. alvei* cultured from the ileum of rabbit 10 and
*H. alvei* and *E. coli* cultured from the ileum of rabbit 12. The
number of *H. alvei* in the ilea of these rabbits ranged
between 9 x 10^9^ and 7 x 10^9^ per gm of mucosal scraping.

The histologies of rabbits 2, 9, and 11, which developed
diarrhea and contained only *H. alvei* in the mid-jejunum and
mid-ileum, showed bacterial attachment in the lower ilea of
all three (Fig. 1). All three rabbits had evidence of bacterial
attachment in the portions of the cecum that remained patent;
in addition, attachment of bacteria was seen in the
colon and rectum in rabbit 2 and the colon in rabbit 11.

Pathological changes in the small intestine included erosion
of surface epithelium; dilatation of lacteals in the villi; and

**TABLE 1. Fecal shedding, diarrhea, and colonization of the gut in rabbits with removable intestinal tie inoculated with *H. alvei***

<table>
<thead>
<tr>
<th>No. of rabbit</th>
<th>Diarrhea* (day after inoculation)</th>
<th>Postinoculation shedding of organism in stool</th>
<th>No. of bacteria/g of mucosal scraping of:</th>
<th>Histological evidence of bacterial adherence in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mid-jejunum</td>
<td>Mid-ileum</td>
</tr>
<tr>
<td>1</td>
<td>+ (1)</td>
<td>H, E (5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>+ (3)</td>
<td>H, E (3)</td>
<td>1 x 10^9 (H)</td>
<td>3 x 10^9 (H)</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>H (4)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>+ (2)</td>
<td>H (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>+ (2)</td>
<td>H (10)</td>
<td>1 x 10^8 (H)</td>
<td>3 x 10^4 (E)</td>
</tr>
<tr>
<td>6</td>
<td>+ (4)</td>
<td>H (10)</td>
<td>1 x 10^7 (H)</td>
<td>6.5 x 10^4 (H)</td>
</tr>
<tr>
<td>7</td>
<td>+ (3)</td>
<td>H, E (4)</td>
<td>1 x 10^7 (H), 2 x 10^7 (E)</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>−</td>
<td>H, E (10)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>+ (2)</td>
<td>H, E (5)</td>
<td>1 x 10^7 (H)</td>
<td>4.3 x 10^9 (H)</td>
</tr>
<tr>
<td>10</td>
<td>−</td>
<td>H, E (4)</td>
<td>4 x 10^7 (E)</td>
<td>9 x 10^7 (H)</td>
</tr>
<tr>
<td>11</td>
<td>+ (2)</td>
<td>H (4)</td>
<td>8 x 10^7 (H)</td>
<td>4 x 10^6 (H)</td>
</tr>
<tr>
<td>12</td>
<td>−</td>
<td>H, E (5)</td>
<td>5 x 10^7 (E)</td>
<td>2.9 x 10^7 (E), 7 x 10^4 (H)</td>
</tr>
</tbody>
</table>

* +, Presence of diarrhea; −, absence of diarrhea.

* H, *H. alvei*; E, *E. coli*. Number in parentheses indicates day of sacrifice; shedding of indicated organism occurred until that day.

* ND, Not done.
HAFNIA ALVEI AND DIARRHEA

FIG. 1. Ideal sections (a, B, and C) from rabbit ileum 1 h after inoculation with H. alvei, showing successive stages of epithelial surface injury compared with a section taken from an animal inoculated with E. coli K-12 (D). (A) Attachment of bacteria to ileal intestinal epithelial cells (arrows), with several epithelial cells appearing at the basamembrane (g). (g) Bacteria invading deep or apical cells (arrowhead). Note inflammatory cellular infiltration and neutrophilic exudate (magnification, ×400, hematoxylin and eosin stain).
FIG. 2. Ultrathin ileal sections (A, B, C, and D) from rabbit 11 48 h after inoculation with *H. alvei*, showing various stages of cell surface-bacterium interface. (A) Bacteria are seen propagating (by bacterial cell division) (small arrow). Note that cells and microvillus border are still intact (magnification, ×8,000). (B) Affected cells with attached bacteria and disrupted cellular organelles can be seen on the left side of the micrograph, and unaffected cells with intact microvillus border can be seen on the right. Portion of a dead cell heavily invaded by bacteria (arrows) is seen in the lumen (magnification, ×6,470). (C) A villus surface is severely altered; attaching bacteria have effaced the microvillus border and caused the loss of most of the cytoplasm. Bacteria still attached to cellular debris can be seen in the lumen (magnification, ×6,470). (D) High magnification (×15,000) of apical plasma membrane showing cupping or pedestal formation beneath attached bacteria and increased density of the terminal web.
hypermia, edema, and an increased number of leukocytes in the lamina propria. The cecum and colon showed hyperplasia of epithelial cells; loss of goblet cells; and hyperemia, edema, and increased cellularity in the lamina propria, which were associated with bacterial attachment. The appendix and mesenteric lymph nodes appeared normal.

Of the three rabbits which did not develop diarrhea, rabbits 3 and 10 had bacterial attachment in the colon whereas in rabbit 12 no attachment was seen. In rabbits 10 and 12, there was also evidence of reactive hyperplasia in the mesenteric lymph nodes. Examination of the intestines of two rabbits infected with E. coli K-12 showed neither bacterial attachment nor any other abnormality.

Electron microscopy was performed on ileal sections of rabbit 11, which developed diarrhea and from which H. alvei was the only bacterium cultured from the small intestine. The microvillus border and apical cytoplasm of infected enterocytes showed various degrees of degeneration. At sites of bacterial attachment, the microvilli were absent, with an increased density of the terminal web directly beneath attached bacteria. The plasma membrane was cupped or raised in the form of pedestals beneath attached bacteria. Affected cells showed evidence of loss of apical cytoplasm, and those severely affected showed extrusion. Dead cells were heavily invaded by bacteria (Fig. 2).

**Orally fed rabbits.** One of the two rabbits orally challenged with H. alvei developed diarrhea on the fourth day; the diarrhea lasted 3 days, during which time H. alvei was the only organism cultured from feces. Before diarrhea developed, after diarrhea stopped, and until the last day of observation (14th day), it shed a mixture of E. coli and H. alvei. In the other rabbit, which did not develop diarrhea, shed a mixture of E. coli and H. alvei throughout the 2 weeks of observation. None of three rabbits challenged with E. coli K-12 developed diarrhea.

**Rabbit ileal loop assay.** Inoculation of four loops in each of four rabbits did not cause fluid accumulation with either whole bacterial culture or with cell-free culture filtrate. Microscopic examination revealed areas of severely damaged hyperemic and edematous mucosa which also included broadening of villi, necrosis, and loss of epithelium at the villus tips. Bacteria were seen inside damaged villus tips and "streams" of inflammatory cells often extended from damaged mucosa into the lumen, forming a layer of mucopurulent exudate. Bacteria were seen attached to epithelial cell surfaces, with loss of microvillus border in a manner identical to that observed in the RITARD rabbits. Hyperemia, edema, and somewhat less inflammatory infiltration were also observed in loops inoculated with K-12, but there was no evidence of epithelial cell necrosis or bacterial adherence.

**DISCUSSION**

H. alvei was suspected as a possible cause of the watery diarrhea in this child because it was the only organism which was cultured and was in abundance in the stool. However, H. alvei is not known to cause diarrhea, and when it was tested, our isolate did not possess the conventional virulence properties of either toxin production or invasiveness.

The RITARD model has previously been used as a suitable model to investigate the diarrheagenic properties of a variety of organisms (15, 19). H. alvei produced diarrhea in 8 of 12 rabbits and in 1 of 2 orally inoculated rabbits. In both models, organisms colonized the gut, resulting in the excretion of bacteria in feces, sometimes with total displacement of normal flora.

Light and electron microscopy showed mucosal lesions which were indistinguishable from those which have been described for entero pathogenic E. coli (EPEC) and, to a lesser extent, enterohemorrhagic E. coli (EHEC) strains in either experimental animals (12, 20) or in intestinal biopsies obtained from patients (16, 22). These lesions include bacterial attachment to enterocytes, effacement of the microvillus border, and modification of the underlying apical cell surface, which often lead to loss of apical cytoplasm and disruption of cellular organelles. Colonization of the major part of the small intestine and often the large intestine with "attaching-effacing" bacteria is considered the major virulence characteristic by which EPEC strains induce diarrhea in experimental animals (20) and in humans (16, 22).

The strain appeared to lack a plasmid which in EPEC is associated with the expression of the EPEC adherence factor and which is identifiable by the characteristic localized adherence in HeLa or HEp-2 cells (1, 10, 14) and by the EPEC adherence factor probe (13). The H. alvei strain did not exhibit localized adherence in cell culture. It will be interesting to determine the degree of homology, if any, between the chromosomal genes encoding attachment-effacement of H. alvei and those encoding attachment-effacement of EPEC and EHEC.

It was important to establish that the rabbits used in these experiments were free from naturally occurring rabbit EPEC strains (4). This was clear, as diarrhea occurred in rabbits only when inoculated with H. alvei and not when inoculated with nonpathogenic E. coli K-12; characteristic lesions were seen in rabbits which had only H. alvei isolated from the gut and were also seen in the ileal loops of rabbits inoculated with H. alvei and not in those of rabbits inoculated with E. coli K-12.

As was demonstrated in the piglet model for EPEC and EHEC (20), the occurrence of diarrhea in rabbits was influenced by the degree and site of bacterial attachment in the gut. Of the three diarrheal rabbits in which histology and quantitative bacteriology were done, all had bacterial attachment in the lower part of the small intestine and in the colon and a high degree of colonization with H. alvei in the small intestine. In contrast, among rabbits which did not develop diarrhea, bacterial attachment was confined to the colon in two of three animals. Bacterial counts of H. alvei in the small intestines of these two rabbits were 10- to 100-fold less and were mixed with E. coli.

This study should now be followed by one establishing the prevalence of H. alvei in diarrheal and nondiarrheal populations to determine its contribution to disease.

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