

Hafnia alvei in Stool Specimens from Patients with Diarrhea and Healthy Controls

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We found an epidemiological association of *Hafnia alvei* with diarrhea, because the organism was isolated from 12 of 77 (16%) adult Finnish tourists to Morocco who developed diarrhea and from 0 of 321 tourists without diarrhea ($P < 0.001$). From another group of 112 adult Finnish diarrheal patients, only 2 (2%) yielded *H. alvei*. In contrast to some Bangladeshi strains of *H. alvei*, the Finnish strains were negative for the attachment-effacement lesion by an in vitro fluorescent actin staining test and also did not show homology to the *Escherichia coli* attachment-effacement gene (*eaeA*) by PCR. These results suggest that a mechanism or mechanisms other than the attachment-effacement lesion may also be involved in the association of *H. alvei* with diarrhea.

Recently, *Hafnia alvei* strains isolated from diarrheal feces of Bangladeshi children have been reported to cause attaching-effacing (AE) lesions typical of enteropathogenic *Escherichia coli* (EPEC) (2, 5). These strains also hybridized with the EPEC *eaeA* probe. The *eaeA* gene is the only virulence-associated factor described so far for *H. alvei*. Genes belonging to the *eaeA* gene cluster are reported to occur also in enterohemorrhagic *E. coli* and in one biotype of *Citrobacter freundii* (9). The aim of the present study was to clarify the potential role of *H. alvei* as an enteropathogen. We determined the isolation rate of *H. alvei* in subjects with and without diarrheal symptoms and screened the isolates for the ability to produce the AE lesion and for the possession of the *eaeA* gene.

Fecal samples were obtained from three groups of adult Finnish people (510 people in all). Diarrhea group 1 consisted of 77 travelers examined during or immediately after travel to Morocco between January and February 1989. Diarrhea group 2 consisted of 112 patients examined between March and June 1993 as a routine diagnostic measure when they consulted a physician because of diarrhea symptoms. Of these patients, 71 had recently traveled abroad, mostly to the Mediterranean or Far Eastern countries. The third group formed a control group of 321 nondiarrheal travelers examined immediately after their return from Morocco in 1989. All stool samples were cultured by standard methods and studied for *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Aeromonas*, and *Plesiomonas* species as described previously (6). The specimens of diarrhea group 1 and the control group were also processed for different types of diarrheagenic *E. coli*, rotaviruses and adenoviruses, and intestinal parasites (8). In diarrhea group 1 and the control group, one to three colonies were subcultured from primary nonselective Drigalski-Conradi agar for species identification with the API 20 E system (BioMerieux, Marcy l'Etoile, France). In diarrhea group 2, the cultures on nonselective cystine-lactose-electrolyte-deficient agar were screened for the presence of *H.*

alvei: 5 to 10 colonies were tested for the hydrolysis of L-proline-p-nitroanilide for detecting *H. alvei* (3). The final species identification was performed with the API 20 E system. Statistical analysis was done with the χ^2 test.

The study also included six *H. alvei* strains isolated from diarrheal patients in Bangladesh and previously reported to produce AE lesions (strains 10457, 10790, 19982, 38/90, 9194, and 12502 [2]). Strains 10790 and 19982 were also reported to hybridize with the EPEC E2348/69-specific *eaeA* probe. EPEC E2348/69 (127:H6) obtained from J. P. Nataro (Center for Vaccine Development, University of Maryland, School of Medicine, Baltimore) was used as a positive control when searching for the *eaeA* gene. In addition, *H. alvei* ATCC 13337 (type strain) and ATCC 29927 (DNA reference strain) were included. The ability to produce the AE lesion was screened by the fluorescent actin staining test with HEp-2 cells (2). Possession of the *eaeA* gene was tested by PCR. For this determination, bacterial strains were grown in brain heart infusion broth (Difco, Detroit, Mich.) overnight at 37°C. Each sample was prepared by boiling 100 μ l of a 10^{-3} dilution of bacterial culture (final bacterial concentration, 10^6 /ml) with 100 μ l of 2% Triton X-100 for 10 min. Primers (sense, 5'GATCTCTGAACGGCGATTACGCG3'; antisense, 5'CTTCGGTGCCATCCGCTTTAGCC3') were derived from the conserved region (residues 2182 to 3372) of the EPEC E2348/69 (GenBank accession numbers M58154 and M34051) *eaeA* gene. Amplifications were performed with a DNA thermal cycler (PTC-100; MJ Research, Watertown, Mass.) for 35 cycles of 30 s at 95°C, 30 s at 62°C, and 2 min at 72°C with 3 min of initial denaturation at 95°C and 5 min of final extension at 72°C in a 25- μ l volume containing 5 μ l of sample and 1 U of Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) in a buffer provided by the manufacturer and overlaid with 30 μ l of sterile paraffin oil. Negative (*Serratia liquefaciens*) and positive (EPEC E2348/69) controls were included with each experiment. The amplification product generated in the PCR assay with *H. alvei* reference strain 10457 was cloned into *Sma*I-digested Bluescript KS vector (Stratagene, La Jolla, Calif.). Two plasmids containing the insert were sequenced by the dideoxy method with the AutoRead kit (Pharmacia, Uppsala,

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TABLE 1. Isolation of *H. alvei* from stool samples of diarrheal and nondiarrheal subjects

Group	No. (%) of <i>H. alvei</i> -positive stool samples from:		
	Diarrheal patients		Nondiarrheal subjects ^a (n = 321)
	Group 1 ^a (n = 77)	Group 2 ^b (n = 112)	
All subjects with <i>H. alvei</i>	12 (16) ^c	2 (2)	0
Subjects with <i>H. alvei</i> alone	4 (5)	2 (2)	0
Subjects with <i>H. alvei</i> together with other enteropathogen(s)	8 (10) ^d	0	0
Subjects with known enteropathogens	48 (62) ^e	12 (11) ^f	34 (11) ^e

^a Finnish travelers to Morocco in 1989 with or without diarrhea (partly described previously [6]).

^b Finnish patients examined in 1993 by a general practitioner because of diarrheal symptoms.

^c $P < 0.001$ versus diarrheal group 2 and nondiarrheal subjects.

^d *C. jejuni*, five patients; *Aeromonas* spp., three patients.

^e See reference 6.

^f *Salmonella enterica*, six patients; *C. jejuni*, four patients; *Aeromonas* spp., two patients; *Shigella sonnei*, one patient. In one sample, two pathogens were isolated.

Sweden) and analyzed with an automated laser fluorescent DNA sequencer (Pharmacia, Uppsala, Sweden). The sequences were aligned by the Bestfit program (Genetics Computer Group package, University of Wisconsin, Madison).

Among 398 travelers to Morocco, 12 of 77 (16%) with diarrhea had *H. alvei* in their stool samples in contrast to 0 of 321 without diarrhea ($P < 0.001$) (Table 1). The total number of *H. alvei* isolates recovered was 24 (1 to 3 from each of 12 patients). In diarrheal group 2, only 2 of 112 subjects (2%) had *H. alvei* in their stool samples; in these 2 specimens, a total of 15 isolates studied were *H. alvei*. One subject had recently visited Sweden, and the other did not travel abroad. *H. alvei* was significantly more common ($P < 0.001$) in diarrheal group 1 than in group 2. Other enteropathogens together with *H. alvei* were isolated from stool specimens of eight patients in diarrheal group 1 (tourists who visited Morocco); these included five patients with *Campylobacter jejuni* and three with *Aeromonas* spp. Thus, *H. alvei* was the only putative pathogen in four (5%) of the tourists with diarrhea (Table 1). The recognized pathogens isolated from stool samples of diarrheal group 1 and the control group have been published (6). In diarrheal group 2, stool specimens of 12 patients (11%) contained known enteropathogens (Table 1). In this group, neither of the two specimens with *H. alvei* contained recognized enteropathogens. All 47 *H. alvei* isolates (39 from Finnish patients, 6 from Bangladesh, and 2 American Type Culture Collection strains) were tested for AE lesions by the fluorescent actin staining test and for the *eaeA* gene by PCR assay with EPEC-specific (E2348/69) primers. All isolates from Finnish patients and the American Type Culture Collection strains were negative in both tests, whereas all Bangladeshi strains reacted positively in the fluorescent actin staining test and generated a product of the predicted size (Fig. 1). The 1,191-bp fragment generated in the PCR assay with strain 10457 was sequenced. The sequence obtained showed about 90% homology with other published *eaeA* gene sequences (result not shown).

The previous studies of the role of *H. alvei* in causing diarrhea were concerned only with case reports (1, 7, 8, 11). Ours is the first controlled study which suggests an epidemio-

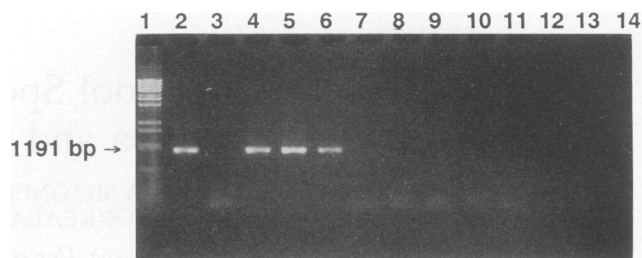


FIG. 1. PCR assay to detect the *eaeA* gene in *H. alvei* strains. Lanes: 1, molecular weight marker (Lambda DNA *Bst*II digest); 2, EPEC E2348/69 (positive control); 3, *S. liquefaciens* (negative control); 4 to 6, *H. alvei* 10457, 10790, and 1982, respectively; 7 and 8, *H. alvei* ATCC 13337 and 29927, respectively; 9 to 12, *H. alvei* IH 50978, IH 51199, IH 51030, and IH 51323, respectively, isolated from study group 1; 13 and 14, *H. alvei* IH 53200 and IH 53201, respectively, isolated from study group 2. Note that a PCR product of 1,191 bp is generated by isolates from Bangladesh only.

logical association with diarrhea for this organism. It can be argued that *H. alvei* cells are transient organisms; if so, they should also have been isolated from controls without diarrhea. Since controls did not yield this organisms, this possibility is unlikely. In positive specimens, we have found 2 to 10 colonies of *H. alvei*, and this organism was found significantly more frequently in patients with diarrhea acquired in Morocco than in diarrheal patients in Finland. It may be that *H. alvei* is more prevalent in Morocco than in Finland.

The present study confirmed that some Bangladeshi *H. alvei* strains previously reported to be positive for gene sequences homologous to *eaeA* were indeed so (2). However, strains isolated from Finnish subjects did not contain such a gene sequence. The absence of the *eaeA* gene sequence was further confirmed by a negative fluorescent actin staining test. It is possible that these strains may possess other virulence factors for production of diarrhea. The demonstration of such virulence factors and other case-control studies are needed to further argue for a role for *H. alvei* in causing diarrhea. In addition, the taxonomy of this species needs to be studied more closely. According to DNA-relatedness studies, biochemically defined species of *H. alvei* may consist of several genospecies (4, 10).

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REFERENCES

1. Albert, M. J., K. Alam, M. Islam, J. Montanaro, A. S. M. H. Rahman, K. Haider, M. A. Hossain, A. K. M. G. Kibriya, and S. Tzipori. 1991. *Hafnia alvei*, a probable cause of diarrhea in humans. *Infect. Immun.* 59:1507-1513.
2. Albert, M. J., S. Faruque, M. Ansaruzzaman, M. Islam, K. Haider, K. Alam, I. Kabir, and R. Robins-Browne. 1992. Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. *J. Med. Microbiol.* 37:310-314.
3. Fanghänel, S., R. Reissbrodt, and H. Giesecke. 1991. L-Prolineaminopeptidase activity as a tool for identification and differentiation of *Serratia marcescens*, *Serratia liquefaciens* and *Hafnia alvei* strains. *Zentralbl. Bakteriol.* 275:11-15.
4. Fanning, G. R., F. W. Hickman, E. Cadet, G. Vaughn, and D. J. Brenner. 1976. Genotypic and phenotypic similarity in *Hafnia alvei* (*Enterobacter hafniae*), abstr. D-5, p. 52. *Abstr. Annu. Meet. Am.*

- Soc. Microbiol. 1976. American Society for Microbiology, Washington, D.C.
5. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc. Natl. Acad. Sci. USA* **87**:7839–7843.
 6. **Mattila, L., A. Siitonen, H. Kyrönseppä, I. Simula, P. Oksanen, M. Stenvik, P. Salo, H. Peltola, and the Finnish-Moroccan Study Group.** 1992. Seasonal variation in etiology of travellers' diarrhea. *J. Infect. Dis.* **165**:385–388.
 7. **Ratnam, S.** 1991. Etiologic role of *Hafnia alvei* in human diarrheal illness. *Infect. Immun.* **59**:4744–4745. (Letter.)
 8. **Reina, J.** 1993. Acute gastroenteritis caused by *Hafnia alvei* in children. *Clin. Infect. Dis.* **16**:443. (Letter.)
 9. **Schauer, D. B., and S. Falkow.** 1993. The *eae* gene of *Citrobacter freundii* biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia. *Infect. Immun.* **61**:4654–4661.
 10. **Steigerwalt, A. G., G. R. Fanning, M. A. Fife-Asbury, and D. J. Brenner.** 1976. DNA relatedness among species of *Enterobacter* and *Serratia*. *Can. J. Microbiol.* **22**:121–137.
 11. **Westblom, T. U., and T. W. Milligan.** 1992. Acute bacterial gastroenteritis caused by *Hafnia alvei*. *Clin. Infect. Dis.* **14**:1271–1272. (Letter.)