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RESEARCH ARTICLE

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The *eaeA* gene is not found in *Hafnia alvei* from patients with diarrhea in Aragón, Spain

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Abstract A total of 102 *Hafnia alvei* clinical strains isolated from different patients with diarrhea has been tested, using polymerase chain reaction and dot-blot hybridization, for the enteropathogenic *Escherichia coli* attaching and effacing A (*eaeA*) gene to establish their role as a causative agent of diarrhea in our environment. None of them was positive for the *eaeA* gene. We cannot consider the *eaeA* gene as the virulence-associated factor implicated in the *H. alvei* strains isolated from diarrheal feces in our region.

Keywords *Hafnia alvei* · *eaeA* gene · Diarrhea · Pathogenic enterobacteria · Virulence factor

Introduction

The enteropathogenic *Escherichia coli* (EPEC) attaching and effacing A gene (*eaeA*) is a virulence-associated factor described in enterohemorrhagic *E. coli* and EPEC in one biotype of *Citrobacter freundii* and in *Hafnia alvei* [3, 4, 7, 11, 13]. The *eaeA* gene is necessary for the intimate adherence of the pathogen to epithelial cells and for the formation of the characteristic attaching–effacing (AE) lesions in the intestinal brush border [7].

Recently, *H. alvei* and unusual biotypes of *E. coli* have been reported as etiological agents of diarrhea able to produce AE lesions and having the *eaeA* gene [2, 3, 6, 9, 10, 11]. Their prevalence in clinical isolates shows noticeable geographical differences; and they are more frequent in developing countries than in developed ones: *eaeA*-positive *H. alvei* strains have frequently been isolated from diarrheal feces in Bangladesh [11, 12] and no *eaeA*-positive strains have been detected in *H. alvei* isolated in Finland and Canada [5, 12].

Detecting *eaeA*-positive *H. alvei* strains could be important, but it is not routinely done in clinical laboratories. The aim of our study was to determine the isolation rate of *H. alvei* in patients with diarrhea and investigate the presence of the *eaeA* gene in these strains, to establish their role as a causative agent of diarrhea in our environment.

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Materials and methods

The study included 4,024 fecal samples from to 2,200 different patients received in our laboratory from November 1998 to November 1999. The samples were cultured by standard methods for *Aeromonas*, *Campylobacter*, *Plesiomonas*, *Salmonella*, *Shigella*, and *Yersinia* species. The presumptive identification of lactose non-fermenting colonies on MacConkey agar was accomplished by a set of biochemical reactions: (1) D-glucose acid, D-glucose gas, and lactose fermentation in Kliger iron agar (Difco Laboratories, Detroit, Mich., USA) (2) motility, sulfide, and indole production with SIM medium (Oxoid, Basingstoke, Hampshire, UK) (3) lysine decarboxylase with lysine iron agar (Oxoid, Basingstoke, Hampshire, UK), and (4) citrate utilization with Simmons citrate agar (Difco Laboratories, Detroit, Mich., USA). Strains testing D-glucose acid-positive, D-glucose gas-negative, lactose fermentation-negative, motile, indole production-negative, lysine decarboxylase-positive, and citrate-negative were further submitted to the tests included in the API 20E identification system (API System, Biomerieux, Marcy l'Étoile, France).

The 102 strains identified as *H. alvei* from 102 different patients were tested for the *eaeA* gene by PCR and dot-blot hybridization. DNA was extracted by standard methods for enterobacteria. Primers C1E (5′-TCG TCA CAG TTG CAG GCC TGG T-3′) and C2E (5′-CGA AGT CTT ATC CGC CGT AAA GT-3′), delimiting a central region (residues 2,182–3,372) of the *eaeA* gene (GenBank accession numbers M58154, M34051), were used. Amplification was performed as described previously [5]. Negative (*E. coli*) and positive EPEC E2348/69 controls were included with each experiment.

Dot-blot hybridization was performed by standard techniques. A 1.1-kb *eaeA* probe, C1-C2, was made by PCR amplification with the primers described above and EPEC E2348/69 as a template. The PCR product was purified from a gel band after electrophoresis through 1% agarose gels. Appropriate fragments were bound to a glass fiber matrix, using a chaotropic agent, and were eluted in a concentrated form with water (Amersham, Les Ulis, France).

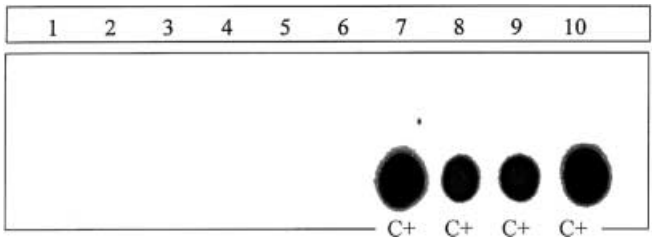

Fig. 1 Dot-blot hybridization performed with a 1.1-kb *eaeA* probe. None of the *Hafnia alvei* isolates were positive for the *eaeA* gene (*lanes 1–6*). Enteropathogenic *Escherichia coli* E2348/69 was the positive control (*lanes 7–10*)

The probes were labeled with (α^{32}P)-dCTP as recommended by the manufacturer (Amersham, Les Ulis, France). Hybridization was carried out at 60 °C overnight. Filters were exposed to Hyperfilm ECL (Hyperfilm-ECL RPN2103, Amersham International, Buckinghamshire, UK) for 18–20 h. The presence of DNA on the membranes was assessed with the *eaeA* probe following rehybridization.

Results

Within the 1-year period of study, a total of 625 diarrheagenic bacterial strains were isolated. They belonged to the following species: *Campylobacter* spp (274), *Salmonella* spp (271), *Aeromonas* spp (33), *Y. enterocolitica* (32), *P. shigelloides* (12), and *Shigella* spp (3). *H. alvei* was isolated from 117 samples collected from 102 different patients. It was found in pure culture in 6 samples. Organisms cultured concomitantly with *H. alvei* were: *Campylobacter* spp (2), *S. enterica* (2), and *Y. enterocolitica* (2). None of them was more frequently associated with *H. alvei*.

PCR and dot-blot hybridization were performed to investigate the presence of the *eaeA* gene in the 102 *H. alvei* isolates. None of them was positive for the *eaeA* gene (Fig. 1).

Discussion

Agents of gastroenteritis previously thought to be mere commensals of the gastrointestinal tract have been described [1]. In recent years, several cases of diarrhea due to *H. alvei* have been reported [2, 3, 9, 10], but few data have been published about the rate of isolation of *H. alvei* from stool specimens in routine practice and its clinical significance remains to be defined [9]. From diarrheic patients in Bangladesh, *eaeA*-positive *H. alvei* have been isolated [11, 12]. In Finland and Canada, in contrast, this gene has not been found in *H. alvei* strains isolated from diarrheal patients, which suggests that there may be other virulence-associated factors [5, 11, 12]. All our *H. alvei* strains have also been *eaeA*-negative.

Nonetheless, there is another factor to be considered: *eaeA*-positive "*H. alvei*" isolates could actually be atypical *E. coli*. Ridell et al. [12] found a high homology in the sequence of the 16S rRNA between these strains and the EPEC strain E2348/69. Janda et al. [6] thoroughly studied five *eaeA*-positive strains identified by Albert et al. [3] as *H. alvei* with API 20E. Some biochemical reactions (negative Voges–Proskauer reaction and the inability to ferment l-rhamnose) suggested that these strains might not be hafniae; and further tests supported their identification as *Escherichia* spp. Our strains did not show atypical reactions for *Hafnia*, so we can consider them *H. alvei*. It would be interesting to investigate the *eaeA* gene in unusual biotypes of *E. coli*.

In conclusion, we did not find any *eaeA*-positive *H. alvei* strains; and so, we cannot consider our *H. alvei* strains as diarrheagenic pathogens unless other virulence-associated factors were implicated. These strains were isolated from stools of patients suffering diarrhea, but their etiological relationship to enteritis is unclear. It is possible that they were transient organisms and a part of the commensal flora of the intestine [8]. More studies are needed to define the role of both *H. alvei* and atypical *E. coli* as agents of diarrhea.

References

1. Abbott SL, Janda JM (1992) Bacterial gastroenteritis. I. Incidence and etiological agents. Clin Microbiol Newsl 14: 17–21
2. Albert MJ, Alam K, Islam M, Montanaro J, Rahaman AS, Haider K et al (1991) *Hafnia alvei*, a probable cause of diarrhea in humans. Infect Immun 59:1507–1513
3. Albert MJ, Faruque SM, Ansaruzzaman M, Islam MM, Haider K, Alam K et al (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
4. Frankel G, Candy DCA, Everest P, Dougan G (1994) Characterization of the C- terminal domains of intimin-like proteins of enterohemorrhagic *Escherichia coli*, *Citrobacter freundii*, and *Hafnia alvei*. Infect Immun 62:1835–1842
5. Ismaili A, Bourke B, Azavedo JC de, Ratnam S, Karmali MA, Sherman PM (1996) Heterogeneity in phenotypic and genotypic characteristics among strains of *Hafnia alvei*. J Clin Microbiol 34:2973–2979
6. Janda JM, Abbott SL, Albert MJ (1999) Prototypal diarrheagenic strains of *Hafnia alvei* are actually members of the genus *Escherichia*. J Clin Microbiol 37:2399–2401
7. Law D (1994) Adhesion and its role in the virulence of enteropathogenic *Escherichia coli*. Clin Microbiol Rev 7:152–173
8. Matsumoto H (1963) Studies on the *Hafnia* isolated from normal human. Jpn J Microbiol 7:105–114
9. Ratnam S (1991) Etiologic role of *Hafnia alvei* in human diarrheal illness. Infect Immun 59:4744–4745
10. Reina J (1993) Acute gastroenteritis caused by *Hafnia alvei* in children. Clin Infect Dis 16:443
11. Ridell J, Siitonen A, Paulin L, Mattila L, Korkeala H, Albert MJ (1994) *Hafnia alvei* in stool specimens from patients with diarrhea and healthy controls. J Clin Microbiol 32:2335–2337
12. Ridell J, Siitonen A, Paulin L, Lindroos O, Korkeala H, Albert MJ (1995) Characterization of *Hafnia alvei* by biochemical tests, random amplified polymorphic DNA PCR, and partial sequencing of 16S rRNA gene. J Clin Microbiol 33:2372–2376
13. Schauer DB, Falkow S (1993) The *eae* gene of *Citrobacter freundii* biotype 4280 is necessary for colonization in transmissible murine colonic hyperplasia. Infect Inmmun 61: 4654–4661