Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*

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Summary. Seven strains of *Hafnia alvei* isolated from diarrhoeal stools of children resembled enteropathogenic *Escherichia coli* (EPEC) in that they produced attaching-effacing (AE) lesions in rabbit ileal loops and fluorescent actin staining in infected HEp-2 cells. In addition, a DNA probe from a chromosomal gene required by EPEC to produce AE lesions, hybridised to chromosomal DNA from all seven *H. alvei* strains. These findings indicate that there is a sharing of virulence-associated properties at the phenotypic and genetic levels by *H. alvei* and EPEC. *H. alvei* strains with these properties should be considered diarrhoeagenic.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) strains are an important cause of childhood diarrhoea and characteristically are unable to produce heat-labile enterotoxin (LT) or heat-stable enterotoxin (ST), or express *Shigella*-like invasiveness.¹ However, some strains produce a shiga-like cytotoxin.² The majority of EPEC possess a plasmid of c. 60 MDa associated with localised adherence (LA) to cultured HeLa or HEp-2 cells.³ An EPEC adherence factor (EAF) probe constructed from this plasmid shows good correlation with LA in tissue-culture assays.⁴ Furthermore, EPEC induce attaching-effacing (AE) lesions in the intestinal mucosa of affected animals.⁵ These lesions are characterised by intimate attachment of bacteria to the enterocyte with the resultant destruction of microvilli, cupping and pedestal formation of plasma membranes, and accumulation of electron-dense fibrillar material in the terminal web adjacent to attached bacteria. The fibrillar modification probably involves polymerisation of actin filaments, which can be detected in cultured cells by a fluorescent actin staining (FAS) assay.⁶

The genes required to produce the AE lesion are located on the bacterial chromosome.⁷ Although both LA (plasmid-borne) and AE (chromosomal) properties are required by EPEC for full expression of enteropathogenicity, EPEC unable to cause LA retain the ability to cause diarrhoea, albeit less effectively than strains which possess the LA phenotype.⁸ It appears that the plasmid-associated LA factor may facilitate initial contact of the bacteria with the

mucosa, after which the chromosomally encoded factor(s) produce the AE lesions.⁹

Recently, Jerse *et al.*¹⁰ used transposon mutagenesis to identify a chromosomal gene that is required by EPEC strain JPN15 (a plasmid-free derivative of EPEC strain E2348/69, serotype O127:H6) to produce AE lesions. This gene, termed *eae*, comprises a 2817-bp open-reading frame capable of encoding a 102-kDa protein. A 1-kb probe derived from this region hybridised to all *E. coli* strains of EPEC serogroups which demonstrate AE lesions, as well as to other pathogenic *E. coli* that produce these lesions, such as enterohaemorrhagic *E. coli* (EHEC) and *E. coli* RDEC-1 (an EPEC of weanling rabbits).

Recently, we described a strain of *Hafnia alvei* (referred to as 19982 in this report), isolated from the stool of a child with diarrhoea, that produced diarrhoea in RITARD rabbits and whole intact rabbits.¹¹ This strain did not have any recognised conventional virulence-associated properties, such as LT, ST, shiga-like toxins (SLTs) or shigella-like invasiveness of, or adherence to, cultured HeLa cells. However, histological and electronmicroscopic examinations of intestinal tissues from diarrhoeic rabbits and from inoculated small intestinal loops of adult rabbits showed typical AE lesions characteristic of EPEC.¹¹ We have since isolated six more strains of *H. alvei* from stools of children with diarrhoea. We characterised these strains and investigated the sharing of virulence-associated properties with EPEC at the phenotypic and genetic levels.

Fig. 1. Ileal loop section (A) from a rabbit inoculated with *H. alvei* strain 10790, compared with the ileal loop section inoculated with non-pathogenic *E. coli* K-12 (B). Note bacterial attachment and effacement of enterocytes in A, compared with no bacterial attachment to enterocytes in B. (Bar, 6 μ m; haematoxylin and eosin stain).

Materials and methods

Isolation of *H. alvei*

H. alvei strains were isolated from the stools of six children with diarrhoea. The stools were cultured for enteric bacterial pathogens by standard methods¹² and *H. alvei* was identified as described previously.¹¹ The *H. alvei* strains were designated as 38/90, 9194, 12502, 10790, 924/91 and 10457. One patient also yielded *Aeromonas sobria* on stool culture, but the other patients did not yield any recognised bacterial pathogens.

Clinical histories were available for five of the children, all of whom were < 5 years old. Three children had watery diarrhoea with mild dehydration which was corrected by oral fluid; the other two had mucoid stools, but no dehydration, and were treated with antibiotics. Of the children with mucoid stools, one was diagnosed as having post-measles diarrhoea, the other was infected with *A. sobria*.

Enterotoxigenicity assay

Bacteria were grown in Trypticase Soy Broth supplemented with yeast extract 0.6% (TSB; Gibco) at 37°C for 20 h with shaking. Cell-free culture filtrates and polymyxin B extracts of cell pellets¹³ were tested in

mouse adrenal tumour Y1 cells for LT,¹⁴ in suckling mice for ST,¹⁵ and in HeLa cells for SLTs.¹⁶

Enteroadherence assay

Adherence to HeLa cells was tested by the method of Cravioto *et al.*¹⁷ Briefly, HeLa cell monolayers were inoculated with an overnight bacterial culture grown in Luria broth at 37°C, then incubated for 3 h in the presence of D-mannose 0.5%, after which they were fixed in methanol 70% and stained with Giemsa stain.

Enteroinvasiveness assay

Bacteria were tested for invasiveness in HeLa cells¹⁸ and in a guinea-pig's eye by the Sereny test.¹⁹

Plasmid analysis

Plasmid DNA was extracted according to the method of Birnboim and Doly.²⁰ After gel electrophoresis, it was stained with ethidium bromide and examined under UV light.

FAS assay

This test was performed as described by Knutton *et al.*⁶ Each isolate was examined by incubating bacteria for 3 and 6 h with HEP-2 cell monolayers. Quantitation of LA was performed in the 6 h assays.²¹

Fig. 2. Fluorescein actin staining of control HEP-2 cells (A) and *H. alvei* 10790 infected HEP-2 cells (B) after 6-h assay. Note the fluorescent bacterial forms in panel B which are absent in panel A. (Bar, 6 μ m).

Fig. 3. Southern blot analysis of *H. alvei* 10790, *H. alvei* 19982, and EPEC strains 4084 C3 and E2348/69 (lanes 1–4 respectively). Chromosomal DNA was digested with *Eco*RI, separated by gel electrophoresis, transblotted to a nylon membrane and probed with a 1-kb region of the *eae* gene of *E. coli* strain E2348/69.

Rabbit ileal loop assay

One ml of each 20 h TSB culture containing 10^9 bacteria was inoculated into *c.* 10-cm-long intestinal loops of an adult New Zealand White rabbit that had previously been fasted for 48 h. Six loops were made in each rabbit. After 20 h, the rabbit was killed and loops were examined for fluid accumulation and other gross

pathological changes.⁵ Portions of loops were fixed in buffered formal saline and processed for histopathological examination. All the isolates were tested in two rabbits and a non-pathogenic *E. coli* K-12 strain was included as a negative control.

EAF and *eae* probe tests

The EAF probe was described by Nataro *et al.* (pJPN16)⁴ and consisted of a 1-kb *Bam*HI-*Sa*II restriction enzyme fragment isolated from plasmid pMAR22, that was cloned from plasmid pMAR2. The *eae* probe was described by Jerse *et al.* (pCVD434)¹⁰ and consisted of a 1-kb *Sa*II-*Kpn*I restriction enzyme fragment isolated from the *eae* gene of EPEC strain E2348/69. The probes were labelled with deoxyadenosine- α -(³²P)-triphosphate (3000 mCi/mmol; Amersham) by the random primer method.²² Hybridisation was performed as described by Echeverria *et al.*²³

Southern blot hybridisation

Bacterial chromosomal DNA was purified by the method of Stull *et al.*²⁴ and digested with *Eco*RI. Fragments were separated by electrophoresis on an agarose 1% gel, transferred to a nylon membrane²⁵ and treated with the *eae* probe.

Results

As was found with the original strain of *H. alvei* 19982, the current six strains gave negative results in tests for the production of enterotoxins and SLTs, invasiveness and LA to HeLa cells. The original isolate and three of the current isolates (10457, 12502 and 924/91) did not possess any plasmids. Isolate 9194 had a plasmid of 80 MDa, isolate 38/90 had four plasmids of 80, 5.8, 3.8 and 1.0 MDa and isolate 10790 had four plasmids of 130, 4.3, 4.0 and 1.5 MDa respectively. Like the original isolate, all the current isolates

produced AE lesions in rabbit ileal loops (fig. 1). All isolates, including the original, also produced AE lesions in the FAS assay *in vitro*, giving a pattern of poor localised adherence (LA/P) (fig. 2).

None of the seven strains hybridised with the EAF probe. All strains hybridised with the *eae* probe, although the intensities of the signals obtained were weaker than those obtained with two EPEC strains, E2348/69 and 4084C3, a strain of serogroup O114.

Two *H. alvei* strains (19982 and 10790) were analysed further for homology with the *eae* probe by Southern blot hybridisation. The probe hybridised to fragments of different mol.wt in *H. alvei* and EPEC (fig. 3).

Discussion

H. alvei strains are considered to be members of the normal faecal flora. The first definitive proof of their enteropathogenicity was our demonstration of AE lesions in experimental models with a strain of *H. alvei* isolated from a child with diarrhoea.¹¹ The demonstration of the same lesion with six further strains of *H. alvei* from children with diarrhoea suggests that our original observation was not fortuitous and that the prevalence of AE *H. alvei* strains could be more common. Therefore, the ability of *H. alvei* to produce diarrhoea should be considered when investigating the aetiologies of diarrhoeal diseases.

Although the AE property is shared by EPEC and EHEC,⁶ EHEC is differentiated from EPEC by production of copious amounts of SLTs.²⁶ Since our

H. alvei isolates did not produce SLTs, they resembled EPEC more closely. However, unlike most EPEC, they did not show LA with cultured cells or hybridise with the EAF probe.

All the seven *H. alvei* strains also produced AE lesions in the FAS test. This again suggested a correlation between in-vivo and in-vitro assays for AE lesion with *H. alvei* as found with EPEC.⁶ Similarity with EPEC extended to the genetic level. The *eae* probe constructed from a strain of EPEC hybridised with all *H. alvei* strains, although the intensities of signals were weaker in colony hybridisation tests, perhaps suggesting a weaker homology between the sequences. In Southern blot hybridisations, the probe hybridised with fragments of differing size suggesting restriction length polymorphisms in the sequences. As carriage of this sequence corresponded with the ability of *H. alvei* to produce AE lesions in rabbit intestine and a positive FAS assay in HEP-2 cells, it appears that AE genes in *H. alvei* and EPEC may be homologous. The demonstration of homology between AE genes of EPEC and *H. alvei* points to either horizontal transmission of AE gene or the evolutionary relatedness of these two genera of bacteria.

In conclusion, the results suggest a sharing of virulence-associated properties at the phenotypic and genetic levels between *H. alvei* and EPEC, and the diarrhoeagenic potential of *H. alvei*.

This research was supported by the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), through the support of many countries and international agencies. We thank J. B. Kaper (Center for Vaccine Development, University of Maryland, Baltimore, MD, USA), for provision of EPEC attachment-effacement probe pCVD434 and M. Haque of ICDDR,B for secretarial assistance.

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