Presence of *eaeA* sequences in pathogenic and non-pathogenic *Escherichia coli* strains isolated from weaned rabbits

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Summary. Seventy-one *Escherichia coli* strains isolated from diarrhoeic weaned rabbits from different areas of France were tested for the presence of DNA sequences specific for the EPEC, EHEC, DAEC and EAggEC strains and 16 of them were tested for pathogenicity in animal experiments. High pathogenicity was observed only with strains unable to ferment rhamnose. DNA from all 55 rhamnose-negative O103, O26 and rough strains hybridised with the *eaeA* probe. Similar hybridisation was obtained with six non-pathogenic rhamnose-positive strains belonging to serogroups O128 and O132. No hybridisation was observed with the other probes. This is the first report of the presence of *eaeA* sequences in genomic DNA of non-pathogenic strains.

Introduction

In industrial rabbit-fattening farms, severe enteric disease is associated with colonisation of the distal ileum and caecum by *Escherichia coli* strains. Different serogroups of diarrhoeagenic *E. coli* have been isolated from weaned rabbits.^{1,2} These strains produce profuse watery diarrhoea associated with a high mortality. In 1977, a highly pathogenic O15:H— strain, called RDEC-1, was described.³ This strain is now considered to be an enteropathogenic *E. coli* analogue and the reference model of *E. coli* diarrhoea in rabbits. In France, the disease is mainly associated with strains belonging to serogroup O103. These strains adhere to rabbit isolated intestinal villi and to HeLa cells in a diffuse pattern. A 32-kDa protein has been identified as being involved in these attachment mechanisms.⁴ One O103 strain has been shown to induce characteristic attaching and effacing (A/E) lesions as seen by electronmicroscopy.⁵ Identical A/E lesions are produced by human enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli* strains.^{6,7} A chromosomal locus designated *eae*,⁸ and more recently *eaeA*,⁹ was identified in human EPEC strain E2348/69 and was required for the production of A/E lesions in tissue-culture cells. The *eaeA* gene has been detected with a probe containing an internal 1-kb fragment of this gene in all the EPEC strains, in the rabbit pathogenic strain RDEC-1 and in the majority of EHEC strains tested. The *eaeA* gene is involved in

the intimate attachment stage leading to induction of the A/E lesions. The product of the *eaeA* gene is a 94-kDa outer-membrane protein termed intimin and its expression is enhanced by the presence of the EAF plasmid.¹⁰

In the present study, the *eaeA* probe was tested on strains of different serogroups, including O103, isolated from weaned diarrhoeic rabbits from different parts of France. All the isolates were also tested with other DNA probes specific for EPEC, EHEC, diffuse adhesion *E. coli* (DAEC) and enteroaggregative *E. coli* (EAggEC). The EAF probe, a DNA segment of a plasmid discovered in most EPEC strains, was used. This plasmid encodes the capacity for the strains to exhibit a localised adherence pattern to HEP-2 cells¹¹ and is required for the full expression of pathogenicity of EPEC strains.¹² EHEC strains are identified by the presence of structural genes for Shiga-like toxins SLTI and SLTII¹³ and of a plasmid pCVD419-derived fragment that hybridises with most EHEC strains.¹⁴ DAEC strains can be characterised by a fragment of a chromosomal gene coding for F1845, a fimbrial adhesin mediating diffuse adherence in tissue culture cells.¹⁵ A DNA fragment of a plasmid conferring an aggregative-adhesion pattern to HEP-2 cells is considered to be specific for EAggEC strains.¹⁶ All these sequences were used as probes in the present study.

Materials and methods

Strains

Seventy-one *E. coli* strains were studied. Their

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Table I. Properties and origins of the 71 *E. coli* strains studied

Strain nos.	O serogroup	Rhamnose fermentation	Reference no.
B10	103	—	2
C55, C70, D94, D139	103	—	2
E1, E13, E22, E31	103	—	2
BN9010–BN9051	103	—	This work
C102, C230, D145	26	—	4
C110	Rough	—	4
C124, C127	103	+	2
C6, C104	128	+	2
BN9080	128	+	This work
BN9081	132	+	This work
E40, E70	132	+	2
B76	102	+	4
A155	85	+	2
D28	15	+	2
C121	4	+	4
B72, C157, C178, D100	2	+	2

properties and origins are listed in table I. They were isolated between 1981 and 1990 from weaned diarrhoeic rabbits from different areas of France. They belonged to nine O serogroups and 53 of them were of the major serogroup O103; 51 strains of this serogroup were unable to ferment rhamnose, a property which correlates well with the expression of pathogenicity.² Most of these strains harboured several plasmids, including one of *c.* 120 kb.

Experimental pathogenicity

Animal experiments were performed with 16 bacterial strains. The strains BN9010–BN9019, BN9080 and BN9081 from our laboratory (table I) were tested in experimental conditions as follows: specific-pathogen-free New Zealand White rabbits from the Station de Pathologie Aviaire et Parasitologie, INRA, Tours, France, were inoculated at 32 days of age with 10⁷ bacteria by stomach tubes. Each strain was tested on eight animals allotted to two cages which were maintained in controlled conditions, avoiding cross-contamination.

Strains from other sources (table I) had been tested already under experimental conditions described previously.^{2,4,17} To compare these animal experiments, strains B10, C6, C124 and E40 were included in the present study.

Diarrhoea and mortality were recorded daily. The animals were weighed three times per week over a period of 17 days. The samples for bacteriological study were taken immediately before inoculation and every 3 days until the twelfth day after inoculation.

DNA probes

The probes used are listed in table II. DNA probes were prepared from recombinant plasmids containing the probe fragments as inserts. Caesium chloride-purified plasmids from the different recipient strains

Table II. Properties of the probes used

Probe	Recombinant plasmid	Size (bp)	Restriction site	Reference no.
eaeA	pCVD434	1000	<i>SalI</i> - <i>KpnI</i>	8
EAF	pJPN18	1000	<i>Bam</i> HI- <i>SalI</i>	11
SLTI	pJN37-18	1142	<i>Bam</i> HI	13
SLT II	pNN111-19	842	<i>Pst</i> I	13
CVD419	pCVD419	3400	<i>Hind</i> III	14
F1845	pSLM852	370	<i>Pst</i> I	15
EAggEC	pCVD432	1000	<i>Eco</i> RI- <i>Pst</i> I	16

were digested with appropriate restriction endonucleases under the conditions recommended by the manufacturer (Boehringer Mannheim, Germany) and electrophoresed on a low-melting-point agarose 0.8% gel. Appropriate fragments were cut out of the gel and eluted by an agarase extraction procedure.¹⁸ Probes were labelled with α -³²P-CTP (Amersham International) by a random primed DNA labelling kit (Amersham).

Colony and Southern blot hybridisation

Bacterial colonies were transferred to several Hybond-N membranes (Amersham), grown for 4 h at 37°C, lysed and hybridised at 65°C to each labelled DNA probe in 6 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhart's solution (1 × Denhart's: polyvinylpyrrolidone 0.02%, Ficoll 0.02%, bovine serum albumin 0.02%), SDS 0.5%, denatured herring sperm DNA 250 µg/ml. Colony blots were washed successively at 65°C in 2 × SSC, SDS 0.1% and in 0.1 × SSC. The filters were autoradiographed for 5 h–2 days at –80°C with intensifying screens.

Plasmid DNA was prepared as described by Takahashi and Nagano.¹⁹ Total DNA was purified by the method of Wilson²⁰ with some modifications: bacterial cells were lysed with SDS; proteins and cellular debris were removed by digestion with proteinase K and precipitation with hexadecyltrimethyl ammonium bromide. DNA was obtained after two extractions with phenol-chloroform-isoamyl alcohol and precipitation with isopropanol. DNA samples were digested with *Hind*III and *Pst*I according to the manufacturer's instructions (Boehringer). Plasmids or total DNA fragments were separated by electrophoresis on an agarose 0.8% gel, transferred to a nylon membrane and incubated with the radiolabelled *eaeA* probe as described for colony blot hybridisation.

Results

Experimental pathogenicity

Strains BN9010–BN9019 were highly pathogenic for rabbits, with a mortality rate of 50–90% from day 4 to 12. Before death, all animals exhibited depressed growth and diarrhoea, generally of haemorrhagic type.

Fig. 1. Analysis of DNA by colony hybridisation. Total DNA was transferred to a nylon membrane and hybridised to the ^{32}P -radiolabelled *eaeA* probe. 1 and 59, HB101 as negative control; 2 and 60, E2348/69 as positive control; 3–6, B72, C157, C178, D100; 7, A155; 8–10, C102, C230, D145; 11, D28; 12, B10; 13–47, BN9010–BN9044; 48 and 49, C124, C127; 50, C55; 51–53, C6, C104, BN9080; 54–56, E40, E70, BN9081; 57, C110; 58, B76. Other strains from table I, data not shown.

Fig. 2. Southern blot analysis. Chromosomal DNA of strains indicated at the top was digested with *Hind*III, separated by gel electrophoresis, transferred to a nylon membrane and hybridised with the ^{32}P -radiolabelled *eaeA* probe. Raoul[®] was used as mol. wt marker after hybridisation with ^{32}P -radiolabelled pUC18 (Appligene, Illkirch, France).

Results of the bacterial counts performed after inoculation showed that rabbits excreted these strains rapidly at a concentration of 10^8 – 10^9 cfu/g of faeces. Strain B10 was confirmed as highly pathogenic by the same criteria, as already described.²

No diarrhoea and no mortality were observed after inoculation with strains BN9080 or BN9081. The mean daily weight gain of the rabbits was approxi-

mately 30 g. The bacterial counts were *c.* 10^5 cfu/g of faeces. As expected,^{2,17} strains C6, C124 and E40 were non-pathogenic in our experimental conditions.

Colony and Southern hybridisation

The 55 rhamnose-negative O103, O26 or rough strains hybridised with the *eaeA* probe. The *eaeA*

sequence was also detected in six rhamnose-positive strains. Three of them belonged to serogroup O128 and three were of serogroup O132 (fig. 1). Total DNA of these *eaeA* positive strains was analysed further by Southern blot hybridisation for homology with the *eaeA* probe. The probe hybridised with fragments of similar mol. wt in all these strains: an 8-kb fragment generated by digestion with *Hind*III (fig. 2) and a 6-kb *Pst*I-generated fragment (data not shown). The *eaeA* sequence was never detected on plasmids in any of these strains.

The 10 other rhamnose-positive strains belonging to serogroups O2, O4, O15, O85, O102 and O103 did not hybridise with the *eaeA* probe.

None of the 71 strains were observed to hybridise with the EAF, SLTI, SLTII, CVD419, F1845 and EAggEC probes.

Discussion

The results of animal experiments in this study were in good agreement with those previously obtained by Milon *et al.*^{2,17} Four strains were used to allow a comparison between these independent experiments. These strains exhibited non-variable and reproducible results and confirmed the validity of such experimental work. The 10 rhamnose-negative O103 strains tested in this study were highly pathogenic for rabbits. From these results and those mentioned above, it appears that there is a good correlation between the inability to ferment rhamnose and the pathogenicity of the O103 strains isolated in France. In France, the strains isolated most frequently in cases of diarrhoea in weaned rabbits belong to serogroup O103 and are unable to ferment rhamnose. Until now, only a few O103 strains able to ferment rhamnose have been detected.

The two strains BN9080 and BN9081, belonging to serogroups O128 and O132, respectively, were non-pathogenic. Similar results were obtained with strains C6 and E40, belonging to the same serogroups, as already described.^{2,17}

A total of 71 *E. coli* strains isolated in France from weaned diarrhoeic rabbits were studied with different DNA probes specific for EPEC, EHEC, DAEC and EAggEC of human origin. Positive results were observed only with the *eaeA* probe for all rhamnose-

negative and some rhamnose-positive rabbit strains. All these *eaeA* positive strains showed chromosomal hybridisation without noticeable restriction length polymorphisms in the sequences.

Sequences homologous to the *eaeA* gene were found in all the rhamnose-negative highly pathogenic strains. Therefore, the *eaeA* gene was not limited to the RDEC-1 strain⁸ but occurred in other rabbit pathogenic strains of different origins belonging to various serogroups.

The *eaeA* probe also hybridised with the O128 and O132 strains which were non-pathogenic under experimental conditions. Thus, the presence of the *eaeA* sequences in the rabbit *E. coli* strains was not strictly correlated with the full expression of pathogenicity in animal experiments. To our knowledge, this is the first report of the presence of the *eaeA* sequences in non-pathogenic strains.

The expression of the A/E factors in the EPEC strains of human origin is positively regulated by the EAF plasmid.¹² In the EHEC and RDEC-1 strains lacking this plasmid, other non-homologous plasmids encoding fimbriae could have a similar function.⁹ At the present time, the role of the initial attachment in the development of A/E lesions is not clearly defined. The highly pathogenic O103 strains and the non-pathogenic O128 strains showed the same characteristics of adhesion to 6-week-old rabbit intestinal villi and HeLa cells, with a diffuse pattern. These properties were associated with the presence of a 32-kDa adhesin in the O103 and O128 strains.⁴ The non-pathogenic O132 strains also exhibited a diffuse adherence pattern to HeLa cells.⁴ Thus, the difference of expression of pathogenicity in these *eaeA*-positive strains was not due to an alteration in their abilities to adhere *in vitro*.

This preliminary study has provided the basis for future research which could determine the conditions of expression of the *eaeA* gene in highly pathogenic rabbit *E. coli* strains and the involvement of the gene product in the expression of pathogenicity. Understanding of the significance of such sequences in non-pathogenic strains is also necessary.

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