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Identification of *eae* Sequences in Enteropathogenic *Escherichia coli* Strains from Rabbits

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DNA sequences coding for attachment and for verotoxin production were investigated in a collection of enteropathogenic *Escherichia coli* strains from rabbits. All of the strains produced diarrhea after experimental infection, attached to the brush borders of the intestinal lining, and possessed homology to the *eae* probe, whereas strains isolated from healthy rabbits did not. Sequences homologous to the AF/R1 fimbriae of strain RDEC-1 were not found. One strain reacted with the probe for the Shiga-like toxin type I gene.

During an epidemic of infant diarrhea in a nursery in Taunton, United Kingdom, an *Escherichia coli* strain, E2348/69, belonging to serotype O127:K63:H6 was isolated. The strain produced profuse watery diarrhea, intestinal cramping, vomiting, and nausea when administered orally to human volunteers (8), yet it did not produce enterotoxins or verotoxins and was not enteroinvasive. Histology showed that the strain attaches to the brush border of rabbit and porcine intestinal epithelial cells and causes effacement of microvilli (14). These features, i.e., inability to produce known enterotoxins and the lack of *Shigella*-like invasiveness (positive Sereny test and intracellular spread), and the attaching and effacing properties are typical of enteropathogenic *E. coli* (EPEC) strains stricto sensu according to the definition of Levine et al. (9). Therefore, strain E2348/69 is considered an EPEC strain.

In 1977, Cantey and Blake (4) isolated an EPEC strain called RDEC-1 and belonging to serotype O15:K—:H— from diarrheic rabbits. As is the case for E2348/69, RDEC-1 attaches to the microvilli of intestinal epithelial cells, causes effacement of the brush borders, does not produce known enterotoxins, and is not invasive and is therefore also classified as an EPEC strain stricto sensu. Attachment of RDEC-1 to enterocytes is mediated by fimbriae, which have been called AF/R1 by O'Hanley and Cantey (17). Later, other strains showing the same features but belonging to other serotypes, such as O26:K—:H11, O103:K—:H2, and O109:K—:H2, have been isolated from diarrheic rabbits (19).

Jerse et al. (6) cloned a sequence (*eae*) necessary for intimate attachment of strain E2348/69 to intestinal epithelial cells and constructed a DNA probe with it. This *eae* probe hybridizes with 99 other EPEC strains of human origin, enterohemorrhagic *E. coli* strains, and also the rabbit EPEC strain RDEC-1. Therefore we tested the possible presence of DNA sequences homologous to the *eae* sequences of strain E2348/69 in other rabbit EPEC strains. We also tested DNA sequences associated with virulence factors which are implicated in the physiopathology of human diarrhea and in enteritis of large animals, including DNA sequences coding for the verotoxins Shiga-like toxin I (SLT-I) and SLT-II and DNA sequences for effacing and attaching factor (EAF), diffuse adherence (DA), adhesin involved in diffuse adherence (AIDA-I), enteroaggregative *E. coli* (EAggEC), and attaching factor/rabbit 1 (AF/R1), coding for attachment structures other than *eae*. Some details of these probes are provided in Table 1.

The EAF probe recognizes DNA sequences responsible for a localized type of adhesion to HEp2 cells characteristic of numerous human EPEC strains. The DA and AIDA-I probes recognize DNA sequences coding for a diffuse type of adhesion to HEp2 cells, which is detected in some *E. coli* strains causing infant diarrhea. The EAggEC probe recognizes the DNA sequences responsible for the aggregative form of adhesion to HEp2 cells of other strains of *E. coli* associated with infant diarrhea. The AF/R1 probe codes for the fimbriae which are involved with the attachment of RDEC-1 to the epithelial cells of the rabbit intestinal mucosa. Finally, the SLT-I and -II probes hybridize with DNA sequences coding for the secretion of verotoxins by verotoxigenic *E. coli* (VTEC) strains. VTEC strains cause several intestinal and extraintestinal diseases in humans, pigs, and cattle. VTEC strains of human origin belong to serotypes O157:H7 and O26:H11 and react with both the *eae* and SLT-I or SLT-II probes (6).

A total of 41 EPEC strains were included in this study. Thirty-nine strains were isolated from Belgian and Dutch diarrheic rabbits. Routine microscopy confirmed the presence of *E. coli* attached to the brush borders of the small and cecal intestine lining. Strains were obtained from rabbits of different ages; 2 strains were isolated from reproduction stock, 35 strains originated from weaned rabbits, and 4 strains were isolated from suckling rabbits. Strain RDEC-1 (O15:K—:H—) was kindly provided by J. Cantey, University of South Carolina, Charleston, and strain V2700 (O103:K—:H2) was provided by L. Renault, C.R.C.B., Athis-Mons, France. Twelve more strains (healthy rabbit strains) were isolated by fecal swabs from healthy weaned rabbits in six Belgian rabbitries without demonstrable problems of colibacillosis. The O:K:H serotypes were determined by F. Ørskov and I. Ørskov (International *Escherichia* and *Klebsiella* Centre, Copenhagen, Denmark) or in our laboratory by standard methods (18).

The strains were also identified by biotyping as described before (19). After primary isolation, each strain was passaged twice on blood agar (tryptose blood agar base [Difco Laboratories, Detroit, Mich.] with 5% [vol/vol] sheep

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TABLE 1. Characteristics of the probes used

Probe	Recombinant plasmid	Restriction site(s)	Size (kb)	Strain of origin	Reference
eae	pCVD434	*SalI-KpnI*	1,000	E2348/69	6
EAF	pMAR22	*BamHI-SalI*	1,000	E2348/69	15
DA	pSLM852	*PstI*	500	F1845	3
AIDA-I	pIB6	*HindIII*	3,500	2787	2
EAggEC	pCVD432	*EcoRI-PstI*	700	17/2	1
AF/R1	pW9	*KpnI*	412	RDEC-1	22
SLT-I	pJN3719	*BamHI*	1,142	933	16
SLT-II	pNN111/19	*PstI*	842	933	16

blood). Two sterile tubes with nutrient broth were inoculated with one colony each and incubated aerobically for 4 h at 37°C. A standard volume (about 0.10 ml) of the resulting bacterial suspension was used to inoculate the medium. Fermentation of carbohydrates was tested on phenol red broth base (Difco Laboratories). The following carbohydrates were prepared as 10% (wt/vol) solutions in deionized water: dulcitol, D-raffinose, L-rhamnose, and sorbose. They were sterilized by filtration and added to sterile basal medium at a final concentration of 0.5% (wt/vol). The completed medium was dispensed in 2-ml volumes into sterile capped 10-ml tubes. Results were read after 48 h of incubation at 37°C. Motility and ornithine decarboxylase were tested on MIO medium (Difco Laboratories) and read after 24 h of incubation at 37°C.

The pathogenicity of all strains was tested by experimental infection of 4-week-old coccidium-free New Zealand White rabbits with strains lyophilized after primary isolation as described before (19). The inoculum was prepared from second-passage organisms grown on blood agar by inoculating colonies into Penassay broth and incubating them aerobically for 6 h at 37°C. For each strain, two rabbits were infected orally with 2 ml of inoculum containing approximately 2×10^6 CFU. Rabbits were checked for diarrhea on a daily basis. At 7 and 10 days postinfection, one rabbit was killed and necropsied. Segments of the terminal small intestine, cecum, and proximal colon were processed for histology. Coliform bacteria attached to the intestinal mucosa were traced in hematoxylin-eosin-stained sections at magnifications of ×500 and ×1,000 with a Leitz Laborlux 12 microscope. A bacterium was considered attached to an epithelial cell if it was immediately adjacent to the surface of the cell and if there was no mucus or other material between the bacterium and the cell surface. The presence of *E. coli* in the duodenum, jejunum, ileum, and cecum was evaluated after streaking plates of G2SN (Gassner agar [Merck], 77 g; yeast extract [GIBCO], 3 g; sodium thiosulfate · 5 H_2O, 5 g; ferric citrate, 0.5 g; distilled water, 1,000 ml [pH 7.2]; after autoclaving, 20 ml of 0.025% novobiocin was added) with intestinal contents and incubating them at 37°C for 18 h. Coliform colonies were identified by the method of MacKenzie et al. (10).

Hybridization was done with the *eae*, EAF, DA, AIDA-I, EAggEC, AF/R1, SLT-I, and SLT-II probes as described before (12) (Table 1). Briefly, cells of the different strains were transferred onto filter paper and subjected to cell lysis. The DNA was denatured and hybridized overnight with the DNA probes at 65°C in the absence of formamide. After being washed, the filters were autoradiographed for 1 to 4 days and read. The following strains were included in the study as positive controls: strain E2348/69 for the *eae* and EAF probes (6, 15); a localized adhesion (LA) positive strain for the EAF probe (21); a DA-positive strain 2787 for the AIDA-I probe (2); strain C1845 for the F1845 probe (3); AggA-positive strain 17-2 for the Agg probe (1); AF/R1 fimbria-producing strain RDEC-1 for the AF/R1 and *eae* probes (6, 22); and verocytotoxic strains 1625 and 211 for the SLT-I and SLT-II probes, respectively (11). These positive controls also served as negative controls for nonhomologous probes, and *E. coli* HS (5) served as a negative control for all probes. All positive control strains reacted only with the corresponding probes, whereas the negative control strain did not hybridize with the different DNA probes used.

Most strains isolated from diarrheic rabbits produced various levels of diarrhea, all attached to the epithelial cells of the distal small intestine and cecum, and all produced lesions characteristic of attaching EPEC strains, whereas strains isolated from healthy rabbits did not. The attaching strains belonged to one of the pathogenic serobiotypes described before for rabbits (19); most strains belonged to the widespread, highly pathogenic weanling rabbit sero- and biotypes O15:K—H—/3— (10 strains), O26:K—:H11/4+ (3 strains), and O103:K—:H2/8+ (5 strains) or to the highly pathogenic suckling rabbit sero/biotype O109:K—:H2/1+ (6 strains) (Table 2). Three of the latter strains did not induce diarrhea in 4-week-old rabbits, which confirms previous evidence that O109:K—:H2/1+ strains are mainly pathogenic for unweaned suckling rabbits. Besides these highly pathogenic serobiotypes, three of the six strains belonged to the moderately pathogenic weaning rabbit serobiotypes O128:K—:H2/2+ and O132:K—:H2/2+. Although the exact serotype was determined for only 6 of the 12 healthy rabbit strains, none of them belonged to the pathogenic rabbit serobiotypes described before.

Hybridization trials with the *eae* probe showed that not only the RDEC-1 strain but also the complete collection of strains isolated from diarrheic rabbits possessed DNA sequences homologous to the *eae* sequence of the human EPEC strain E2348/69 (Table 2). Serotyping showed that these rabbit strains differed greatly from the classical human EPEC strains; indeed, they do not belong to serogroup O55, O86, O111, O119, O125 to O128, or O142 and do not react with the EAF probe (7). The healthy rabbit strains, on the contrary, did not react with the *eae* probe or with the other probes tested.

Although all the rabbit EPEC strains reacted with the *eae* probe, most of them did not react with the other probes. Only the RDEC-1 strain reacted with the AF/R1 probe, whereas one of three O26 strains reacted with the SLT-I probe (Table 2). As the AF/R1 sequence codes for specific fimbriae involved in attachment to enterocytes (22), this finding suggests that other adhesins may be associated with the virulence of EPEC strains for rabbits. This hypothesis is sustained by the data of Milon et al. (13), who observed six

TABLE 2. Characteristics of *E. coli* strains isolated from diarrheic and healthy rabbits

Origin and serotype[a]	Biotype	No. of strains	No. of strains showing a positive reaction[b] for:				
			Diarrhea after infection	Attachment	*eae* probe	AF/R1 probe	SLT-I probe
Strains from diarrheic rabbits							
O15:K—:H—	3	10	10	10	10	1	0
O20:K—:H7	1	1	1	1	1	0	0
O26:K—:H11	4	3	3	3	3	0	1
O103:K—:H2	8	5	5	5	5	0	0
O109:K—:H2	1	6	3	6	6	0	0
O128:K—:H2	2	3	3	3	3	0	0
O128:K—:H?	2	1	1	1	1	0	0
O132:K—:H2	2	6	6	6	6	0	0
O153:K—:H7	1	1	1	1	1	0	0
Rough:H2	1	1	1	1	1	0	0
Rough:H2	2	2	2	2	2	0	0
Rough:H2	8	2	2	2	2	0	0
Strains from healthy rabbits							
O109:K?:H+	3	5	0	0	0	0	0
O109:K?:H+	7	1	0	0	0	0	0
NT:H+	7	2	0	0	0	0	0
NT:H+	2	2	0	0	0	0	0
NT:H+	3	1	0	0	0	0	0
Rough:H−		1	0	0	0	0	0

[a] NT, no agglutination with antisera against O15, O20, O26, O103, O109, O128, O132, and O153.
[b] None of the strains reacted with the EAF, DA, AIDA-I, EAggEC and SLT-II probes.

different adhesion phenotypes among EPEC strains isolated from diarrheic rabbits. Although these colonization factors still have to be identified, our data suggest that they are different from the EAF, DA, AIDA-I, and EAggEC factors found in EPEC strains responsible for human diarrhea. Further evidence that rabbit EPEC strains show a complex pathogenicity pattern is furnished by the fact that one rabbit EPEC strain belonging to serogroup O26 reacted with the SLT-I probe. It is possible that this strain is in fact a VTEC strain, as observed in humans and cattle (7, 20). This should be investigated further.

In conclusion, human and rabbit EPEC strains are responsible for intestinal disorders. Both are able to attach themselves to the intestinal lining. Although they belong to different serotypes and do not possess the same adhesion properties, both human and rabbit EPEC strains possess homologous *eae* sequences.

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