BACTERIAL CHARACTERISATION

Characterisation of diarrhoeagenic *Escherichia coli* clones by ribotyping and ERIC-PCR

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The ability of ribotyping and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) to discriminate diarrhoeagenic *Escherichia coli* clones of 122 strains belonging to 26 distinct serotypes was evaluated. The 26 serotypes corresponded to 24 ribotypes and 25 ERIC-types. Correlation between multilocus enzyme electrophoresis, ERIC-PCR and ribotyping was *c.* 90% for the dominant ribotypes. Related clones such as O55:H7 and O157:H7 presented similar ribotypes and clustered together in a dendrogram, and the two divergent clonal groups of enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) were included in distinct branches. The results suggest the possibility of applying these two simpler techniques as tools to identify clones of diarrhoeagenic *E. coli*.

Introduction

Escherichia coli shows a striking amount of intraspecific variation and includes strains able to colonise several habitats. A number of strains live as commensals in the animal and human intestine, but become opportunist pathogens when predisposing conditions favour their dissemination to extra-intestinal sites. Other strains are frank pathogens that can be associated with intestinal diseases. Criteria for characterising pathogenic *E. coli* include adherence pattern in cultured cells, presence of specific virulence genes and serotyping. Six categories of diarrhoeagenic *E. coli* (DEC) are recognised [1]: (i) enteropathogenic *E. coli* (EPEC), that show localised adherence and produce the attaching and effacing (AE) lesion in HEp-2 or HeLa cells; (ii) enterotoxigenic *E. coli* (ETEC), that produce the ST or LT enterotoxins, or both, and the CFA and CS adhesins; (iii) enterohaemorrhagic *E. coli* (EHEC), that produce Stx cytotoxins and cause AE lesions; (iv) enteroinvasive *E. coli* (EIEC), that cause shigellosis-like dysentery and share virulence genes of *Shigella*; (v) enteroaggregative *E. coli* (EAaggEC), distinguished by their ability to express an aggregative adherence pattern; and (vi) diffusely adhering *E. coli* (DAEC), also identified by their characteristic pattern of attachment to cultured cells.

The study of clonal structure has contributed to

assessing the pathogenic diversity of *E. coli* because it provides a reference framework for understanding the relationship between serotypes, virulence properties and strains [2]. Clonal analysis has revealed that most classical EPEC O serogroups include strains displaying different virulence characteristics [3–7]. Most of this information has been derived from multilocus enzyme electrophoresis (MLEE) [8], frequently combined with other techniques such as those identifying outer-membrane protein and lipopolysaccharide profiles. Recently, analyses based on direct assessment of genomic DNA differences have been widely used for typing purposes. These include ribotyping [9], an analysis of fingerprint patterns of DNA digested with restriction endonucleases after a hybridisation reaction with the 16S+23S cDNA probe, and comparison of DNA products amplified with primers for repetitive sequences such as the enterobacterial repetitive intergenic consensus (ERIC) [10]. The purpose of this work was to evaluate the ability of these techniques to discriminate clones, as defined by MLEE, of diarrhoeagenic *E. coli* strains of different serotypes and distinct virulence properties.

Materials and methods

Bacterial strains

A collection of 122 *E. coli* strains, comprising 26 serotypes originally isolated in different geographic areas from diarrhoeic children, and previously characterised by MLEE and determination of specific virulence factors (Table 1) was studied.

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Table 1. Serotypes, virulence determinants and electrophoretic types of 122 diarrhoeagenic *E. coli* strains

Serotype	Number of strains	Virulence determinants*	Electrophoretic types	Reference
O55:H6	9	<i>eaeA</i> , <i>bfpA</i> , EAF	ET 1	[6]
O55:H7	4	<i>eaeA</i>	ET 5	[6]
O55:H7	1	<i>eaeA</i>	ET 7	[6]
O111ac:H ⁻	1	None	ET 2	[4]
O111ab:H ⁻	1	None	ET 7	[4]
O111ac:H ⁻	1	<i>eaeA</i> , <i>stx1</i>	ET 8	[4]
O111ac:H ⁻	2	<i>eaeA</i> , <i>stx1</i> , EHEC plasmid	ET 8	[4]
O111ac:H ⁻	3	<i>eaeA</i>	ET 8	[4]
O111ab:H ⁻	3	<i>eaeA</i>	ET 9	[4]
O111ab:H ⁻	4	<i>eaeA</i> , EAF	ET 12	[4]
O111ab:H ⁻	5	<i>eaeA</i> , EAF, <i>bfpA</i>	ET 12	[4]
O111ab:H2	5	<i>eaeA</i> , EAF	ET 12	[4]
O111ab:H2	1	<i>eaeA</i> , EAF, <i>bfpA</i>	ET 12	[4]
O111ac:H4	1	None	ET 1	[4]
O111ac:H8	1	<i>eaeA</i> , <i>stx1</i> , EHEC plasmid	ET 8	[4]
O111ab:H9	1	<i>eaeA</i>	ET 16	[4]
O111ab:H9	2	<i>eaeA</i>	ET 9	[4]
O111ac:H9	3	<i>eaeA</i>	ET 9	[4]
O111ab:H10	3	AA	ET 1	[4]
O111ab:H10	1	None	ET 5	[4]
O111ab:H12	11	None	ET 1	[4]
O111ab:H25	1	<i>eaeA</i>	ET 3	[4]
O111ab:H25	1	<i>eaeA</i>	ET 4	[4]
O119:H ⁻	1	<i>eaeA</i>	ND	[5]
O119:H ⁻	1	<i>eaeA</i> , EAF, <i>bfpA</i>	ET 1	[5]
O119:H ⁻	2	<i>eaeA</i>	ET 1	[5]
O119:H ⁻	1	<i>eaeA</i> , <i>bfpA</i>	ET 1	[5]
O119:H2	4	<i>eaeA</i> , <i>bfpA</i>	ET 7	[5]
O119:H2	1	<i>eaeA</i> , <i>bfpA</i>	ET 8	[5]
O119:H2	1	<i>eaeA</i> , <i>bfpA</i>	ET 9	[5]
O119:H6	6	<i>eaeA</i> , EAF, <i>bfpA</i>	ND	[5]
O119:H6	2	<i>eaeA</i>	ND	[5]
O119:H6	5	<i>eaeA</i> , EAF, <i>bfpA</i>	ET 1	[5]
O119:H6	2	<i>eaeA</i>	ET 1	[5]
O119:H6	1	<i>eaeA</i> , EAF, <i>bfpA</i>	ET 5	[5]
O119:H6	2	<i>eaeA</i> , EAF, <i>bfpA</i>	ET 6	[5]
O119:H6	1	<i>eaeA</i> , EAF	ET 5	[5]
O119:H7	1	<i>eaeA</i> , <i>bfpA</i>	ET 8	[5]
O119:H18	1	None	ET 11	[5]
O128ab:H2	2	None	ND	—
O128ab:H2	1	<i>eaeA</i> , <i>bfpA</i>	ND	—
O128ab:H8	1	None	ND	—
O128ac:H8	1	None	ND	—
O128ab:H12	2	None	ND	—
O128ac:H27	2	None	ND	—
O128ab:H35	3	None	ND	—
O157:H ⁻	1	LT-I	DEC 7	[17]
O157:H7	3	<i>eaeA</i> , <i>stx1</i> , <i>stx2</i> , EHEC plasmid	DEC 3	[17]
O157:H7	1	<i>eaeA</i> , <i>stx2</i> , EHEC plasmid	DEC 3	[17]
O157:H7	4	<i>eaeA</i> , <i>stx2</i> , EHEC plasmid	DEC 4	[17]
O157:H7	1	<i>eaeA</i> , <i>stx1</i>	DEC 4	[17]
O157:H43	1	None	DEC 7	[17]
O157:H43	2	LT-I	DEC 7	[17]

ND, ET not determined.
*Virulence determinants: *eaeA*, *E. coli* attaching and effacing gene encoding intimin; EAF, EPEC adherence factor; AA, aggregative adherence factor; *stx1* and *stx2*, genes coding for Shiga cytotoxins 1 and 2, respectively; LT-I, heat-labile enterotoxin gene; *bfpA*, gene coding for bundlin, the structural subunit of the bundle forming pili (BFP).

Extraction of genomic DNA

DNA extraction was performed according to Brenner *et al.* [11].

DNA cleavage and reaction with the 16S+23S cDNA probe

DNA (2 µg) was digested with *Bgl*I and *Hind*III (New England Biolabs Inc., Beverly, MA, USA), electro-

phoresed in agarose 0.8% gel, and transferred to nylon membranes (Schleicher and Schuell Inc., Keene, USA) by Southern blotting [12]. The procedures for labelling the 16S+23S rRNA, and the detection and hybridisation conditions were performed as described by Popovic *et al.* [13]. The 16S+23S cDNA probe was prepared by reverse transcription of the 16S+23S rRNA with a DIG DNA labelling and detection kit (Boehringer Mannheim, Germany).

ERIC-PCR conditions and primers

PCR was performed with the following primer sequences [10]: ERIC1R, 5'ATGTAAGCTCCTGGGG-ATTCAC3' and ERIC2, 5'AAGTAAGTGACTGGGGT-GAGCG3'. Each 20- μ l PCR reaction mixture contained: 100 pmol of forward and reverse primers; 1 μ l of template DNA (*c.* 0.10 μ g); 0.8 μ l each of 1.5 mM dATP, dCTP, dGTP and dTTP; 2 μ l of Mg-free 10 $\times$ amplification buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0; 1% triton X-100); 1.5 μ l of 25 mM MgCl₂; 2.5 U of *Taq* DNA polymerase (Perking-Elmer/Cetus) and dimethyl sulphoxide (Sigma) 10%. The mixture was overlaid with one drop of sterile mineral oil and placed in an automated thermocycler (DNA MiniCycler, MJ Research, Watertown, MA, USA).

The DNA in the reaction mixture was denatured at 95°C for 5 min. PCR amplification was done as follows: denaturation at 92°C for 45 s, annealing at 52°C for 1 min, and extension at 70°C for 10 min, with a final extension at 70°C for 20 min at the end of 35 cycles. The samples were then held at 4°C. The PCR products were separated by agarose gel electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA).

Fingerprint analysis

The sizes of DNA fragments that hybridised with the 16S+23S cDNA probe and those of the bands generated by ERIC-PCR were calculated with the DNA-STAR software (DNASTAR Computer Systems for Molecular Biology and Genetics, London) with *Haemophilus aegyptius* 3031 DNA digested with *Eco*R1 and a 1-kb DNA ladder (BRL-Gibco) as reference markers for ribotyping and ERIC-PCR, respectively. Groups of strains showing identical fingerprints (FPs) were designed as ribotypes (RTs) or ERIC-types.

Genetic relationships and divergence between RTs were calculated from a genetic distance matrix, defined on the basis of similarity between the distinct FPs and represented in a dendrogram constructed with the Neighbour-Joining algorithm [14].

Results

Ribotypes and ERIC-types

RFLP analysis of DEC strains revealed 24 distinct ribotypes (RTs), defined by fingerprints (FPs) with band sizes ranging from *c.* 1.7–15.8 kb (Fig. 1). Twelve of the 24 RTs were represented by multiple strains and 74% of these were included in six RTs (D, H, K, O, U and V). In the ERIC-PCR analysis, the strains presented 25 different FPs (ERIC-types) with band sizes of 0.8–4.5 kb (data not shown). Multiple strains were observed in 12 of the 25 ERIC-types.

Because there was a good association between RT and ERIC-types (as shown below), the data on ERIC-PCR

are presented only where divergence occurred between the two techniques.

Serotypes, virulence markers and genetic relationship among RTs

A dendrogram based on a matrix of similarity of the different FPs was constructed that illustrates the degree of divergence among the RTs, clustered in three groups (I, II, III) (Fig. 2).

All the RTs of group I, except RT E, had the O111 antigen and included strains possessing some virulence markers of DEC. The dominant RT in this group (D) included strains having the aggregative adherence phenotype or genotype, or both. In addition to H12 (the most common H type in this group), one H4 and three H10 strains were also included in this RT. Furthermore, the single H4 strain had the O111lac antigen, in contrast to the remaining ones which were typed as O111ab (Fig. 2).

RTs of group II were marked by serotypes of four distinct serogroups: O55, O119, O128 and O157. RTs F-I clustered in a subgroup of O55:H7, O157:H7, O157:H43 and O157:H⁻ strains. RT H marked the O157:H7, Stx-producing strains, referred to as EHEC 1 by Whittam and McGraw [15]. Group II also included RTs with the H6 antigen and harbouring a complete EAF plasmid (with the *bfpA* gene and the EAF region, see Table 1). Strains of these RTs are considered as typical EPEC [16], and as belonging to the EPEC 1 clonal group [15].

Group III was a second divergent group of O111, but also included strains of serogroups O119 and O128. The dominant RT (U) has all these three serogroups and shows features of EPEC 2 [15]: namely, the presence of the H2 antigen and of some O119 and O128 strains with an incomplete EAF plasmid (EAF⁻, *bfpA*⁺) [5] (Table 1). The O111/Stx-producing strains, which belong to the EHEC 2 clonal group [15], were marked by RT V. Strains of RTs Q, R and T had no known virulence genes and that of RT S represented the O119:H7 strain with an incomplete EAF plasmid. Strains of remaining RTs (W and X) had only the *eaeA* gene (Fig. 2).

ERIC-types and ETs within DEC ribotypes

Twenty-one of the 24 RTs included strains of a single motile serotype and RTs of non-motile serotypes. Fig. 3 illustrates the relationship between ERIC-types, ETs and RTs for 10 representative RTs with more than three strains. There was 100% agreement in the discriminatory power of ERIC-PCR, ribotyping, serotyping and MLEE for five of the 10 RTs. For MLEE and ribotyping the absolute association was observed in eight ribotypes and for ERIC-PCR and ribotyping, the association was seen in seven ribotypes (Fig. 3). For

Fig. 1. Fingerprints that define DEC clones. (a) RTs (left) and ERIC-types (right) of dominant RTs in each of the three divergent clonal groups. (b) Schematic representation of fingerprints of each RT obtained by DNA digestion with the enzyme *Bgl*II.

Fig. 2. Genetic relationship between RTs of DEC and common virulence traits. Serotypes are indicated on the right. Box and ellipses mark the two main clonal groups of EPEC and EHEC, respectively. Empty symbols, EPEC 1 and EHEC 1; shaded, EPEC 2 and EHEC 2. #, indicates absence of known virulence markers of diarrhoeagenic *E. coli*; (), number of strains; [], includes nonmotile strains.

RT D, which included more than one serotype, the association between ET, ERIC type and RT was > 90%. RT H with nine O157:H7 strains, was resolved in a single ERIC type, but corresponded to two distinct ETs each of four and five strains.

Discussion

One hundred and twenty-two DEC strains were characterised by ribotyping and ERIC-PCR. With the exception of strains of serogroup O128 and some of

O119, all the strains had been analysed previously by MLEE and investigated for characteristic virulence properties. All strains, including 23 ETs in 26 serotypes and displaying virulence markers of EPEC, EHEC, EAggEC and ETEC, were grouped in 24 distinct RTs and 25 ERIC-types. Generally, strains of a single ET were included in the same RT and ERIC-type and most RTs had motile and non-motile strains of the same O serotype. However, RT D contained O111 strains of three different H types (H4, H10 and H12) and RT U contained strains of serogroups O111, O119 and O128. The O111:H4, O111:H10 and O111:H12

Fig. 3. Percentage of strains of major RTs that correspond to single ETs (■) or ERIC-types (□). *RTs D and U include more than one serotype; [], include non-motile strains.

strains of RT D belong to a single ET (ET1 [4]). The O119:H2 strains of RT U belonged to the related ETs 7, 8 and 9 (cluster B, Fig. 2 of reference [5]). The MLEE genotype of ET 7 [5] matches that of DEC 11 (O128:H2) [17] and differs from the O111:H2/ET 12 [4] for only the PGD enzyme. The O128:H27 and O128:H35 strains, in turn, cluster with O128:H2 [17]. These ETs mark a clone complex representing a subgroup of EPEC, referred to as EPEC 2, and characterised by a high frequency of H2 strains [15]. In addition, some O119:H2 [5] and O128:H2 strains of this group harbour an incomplete EAF plasmid (data not shown). Interestingly, these groups of strains, although belonging to different ETs and O:H types, were included in a single RT.

RTs K and O of serogroups O119 and O55, respectively, represent EPEC 1, marked by EPEC serogroups having the H6 and H34 flagellar antigen [15], whose divergence from EPEC 2 had as a consequence the accumulation of differences in the intimin gene. The present study demonstrated that divergence also occurred in the 16S+23S rRNA conserved sequence and in the distribution of ERIC. The same was true for the two groups of EHEC (EHEC 1 and EHEC 2), which were included in separate branches of the dendrograms. EHEC 1 comprised the *stx*+ strains of the serotype O157:H7, and EHEC 2 the *stx*+ strains of serogroups O26 and O111 [15]. The close relationship between the O157:H7 and the O55:H7 clones (RTs F, G and H) was also evident from the ribotyping and ERIC-PCR data; this association was supported by a statistical significance level (bootstrapping value) of 68% (data not shown).

The analysis of clonal structure of bacteria has been performed by MLEE, a technique that detects polymorphism in a set of conserved metabolic enzymes, which is assumed to reflect genetic variation of the whole genome [8]. Recently, several other molecular typing methods have been applied with the same purpose. Since the initial proposal of Grimont and Grimont [9], ribotyping has emerged as an important taxonomic tool for bacterial pathogens. Yet the discriminatory ability varies from species to species. For strains of *Yersinia enterocolitica*, it has been viewed as an alternative for bioserotyping [18], but it proved not to be a reference method to discriminate *Salmonella* [19]. In 1991, Tarkka *et al.* [20] observed an association between O:H:K serotypes and ribotyping for clinical isolates of *E. coli*. In the present study, the lower association of ribotyping with MLEE was observed for the O157:H7 strains, marked by RT H. Four of the nine strains in this RT belonged to a distinct ET. This result supports that of Martin *et al.* [21] who showed that ribotyping was not the best technique to discriminate strains of O157:H7. However, the MLEE genotype of DEC3 and DEC4 differs in a single enzyme [17].

Analyses based on the distribution of ERIC sequences were done for environmental strains of several bacteria [22–23]. In their study of the genetic structure of two biovars of soil *Rhizobium leguminosarum*, Strain *et al.* [23] observed a very close association between MLEE and ERIC-PCR data. Although the latter was more discriminatory, strains of a single ET were included in closely related ERIC-types. In 1995, Rivera *et al.* [24] found that toxigenic strains of *Vibrio cholerae* O1 and O139 from different locations showed identical FPs for the ERIC sequences and were only distantly related to the non-toxigenic strains. For *Shigella sonnei*, the ability of ERIC-PCR to discriminate clones was equivalent to that of pulsed-field gel electrophoresis [25], which is increasingly becoming a reference molecular technique for typing bacteria [18, 19, 21]. More recently, ERIC-PCR was also evaluated in a comparative test with several PCR-based techniques to discriminate strains of the *Acinetobacter calcoaceticus*-*A. baumannii* complex [26]. However, for these bacteria REP-PCR presented a more discriminatory power than ERIC-PCR. Thus, ERIC-PCR seems more fitted for the identification of enteric bacteria.

Ribotyping and ERIC-PCR were used to identify 23 DEC clones, previously defined by MLEE, which has been the main reference technique for the study of genetic variation in natural populations of bacteria. Both ribotyping and ERIC-PCR could discriminate the clones well, and there was a good correlation between them. The results of the present study suggest the suitability of these easier and faster techniques for the study of genetic variation within DEC strains.

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