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Clonal structure of *Providencia alcalifaciens* strains isolated from diarrhoeal stools in São Paulo, Brazil

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Clonal analysis based on ribotyping demonstrated that *Providencia alcalifaciens* strains isolated mainly from diarrhoeal stools in São Paulo, Brazil, were clustered into two main groups. Eleven distinct ribotype patterns were identified with *Cla*I, *Eco*RV and *Mlu*I restriction endonucleases. *P. alcalifaciens* strains with invasive properties were of two ribotype patterns that differed from those identified among non-invasive strains. The ribotyping results confirmed that *P. alcalifaciens* strains associated with diarrhoeal disease in São Paulo represent distinct groups of strains. Although the invasive strains were isolated from different patients over an extended period they were clustered into two genetically related clones, which seemed to be distributed endemically in the population studied.

Introduction

Providencia alcalifaciens is a member of the Enterobacteriaceae and is thought to be a cause of diarrhoea [1]. Invasion has been proposed as the virulence mechanism of this organism [2] and this property was found commonly among *P. alcalifaciens* strains isolated in Bangladesh [3]. A previous study in São Paulo City, Brazil, [4] showed that although invasive *P. alcalifaciens* strains were encountered frequently, this virulence characteristic, in contrast with the data obtained in Bangladesh [3], did not predominate among isolates from patients with diarrhoea. These results led to the supposition that *P. alcalifaciens* strains associated with diarrhoea in São Paulo may comprise a diverse group of strains.

Several methods have been used to study genetic diversity among bacterial species. Analysis of DNA polymorphism in the chromosomal region containing the highly conserved rRNA genes, and referred to as ribotyping, was initially proposed as a general method for molecular identification of bacteria [5]. Since then, ribotyping has emerged as an important taxonomic tool, providing precise information about bacterial intra- and inter-species differentiation [6–8].

The purpose of this study was to analyse by ribotyping the genetic relationship of *P. alcalifaciens* strains described previously in association with diarrhoeal disease in São Paulo, Brazil.

Materials and methods

Bacterial strains

A total of 19 *P. alcalifaciens* strains was studied. Most of the strains were isolated as pure cultures or from stool cultures in which no other bacterial enteropathogen was identified. Nine strains were invasive; the other strains did not show this characteristic. Except for two strains isolated from asymptomatic patients all the other strains were from diarrhoeal stools [4]. Invasion assays of HeLa cells were performed as described previously [4]. The characteristics of the strains are presented in Table 1.

Ribotyping

The extraction of genomic DNA was performed according to Brenner *et al.* [9]. Initially, the DNA (2 µg) of one of the strains (9938) was incubated with 18 restriction endonucleases (*Sma*I, *Bam*HI, *Sal*I, *Hind*III, *Xba*I, *Pvu*I, *Kpn*I, *Hin*fI, *Bgl*I, *Eco*RI, *Cla*I, *Ava*I, *Acc*I, *Xho*I, *Mlu*I, *Eco*RV, *Bgl*II and *Hae*II) (1 U/µg of DNA) according to the manufacturer's instructions (New England Biolabs) to select for the more appropriate endonucleases. Based on the number and distribution of fragments *Cla*I, *Eco*RV and *Mlu*I

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restriction enzymes were selected for use. The DNA of all strains was then digested with these three enzymes, electrophoresed in agarose 0.8% gels and transferred to Hybond-N nylon membranes (Amersham International, San Jose, CA, USA) by Southern blotting [10]. The procedures for labelling the 16S + 23S rRNA and the detection and hybridisation conditions were as described previously [6]. The cDNA probe was prepared by reverse transcription of 16S + 23S rRNA from *Escherichia coli* with a DIG DNA labelling and detection kit (Boehringer Mannheim, Germany). *Haemophilus aegyptius* 3031 *EcoRI* DNA digest [11] was used as a size marker to estimate the fragment sizes of *P. alcalifaciens* DNA with a personal microcomputer and the DNA Star program. The genetic relationship and divergence between ribotypes were calculated from a genetic distance matrix, defined on the basis of similarity between the distinct fingerprints and represented in a dendrogram constructed with the Neighbor-Joining algorithm [12].

Results and discussion

In the present study ribotyping was used to genotypically characterise *P. alcalifaciens* strains associated with diarrhoea in São Paulo, Brazil.

Analysis of the *P. alcalifaciens* strains showed that different restriction endonuclease patterns were established after DNA digestion with *ClaI*, *EcoRV* and

MluI, electrophoresis and hybridising with the *E. coli* rRNA probe. rDNA profiles were considered distinct when there was a single band difference and were designated according to the restriction endonuclease used (C for *ClaI*, E for *EcoRV* and M for *MluI*). Side-by-side comparisons and precise size determinations were performed when similar patterns were observed among the strains studied. The ribotype patterns observed were reproducible, as several strains gave the same ribotype in different assays.

Nine *ClaI* rDNA profiles (C1–C9) were identified, and they had eight or nine rDNA bands ranging from 2.5 to 17.6 kb (Fig. 1). Ten *EcoRV* (E1–E10) and eight *MluI* (M1–M8) profiles were observed. Representative patterns obtained with *ClaI*, *EcoRV* and *MluI* are shown in Fig. 2. The *ClaI* and *MluI* restriction patterns produced better discrimination of *P. alcalifaciens* strains than the *EcoRV* pattern, although *EcoRV* discriminated more patterns among strains belonging to the same ribotype as determined by *ClaI* (Table 1).

Ribotyping has been used widely for typing several bacterial pathogens, but its discriminatory ability varies from species to species. For *Serratia marcescens* strains it was more discriminating than biotyping [13] and for *Yersinia enterocolitica* it has been viewed as an alternative to bioserotyping [14]. However, it was not a reference method to discriminate between *Salmonella* strains [15]. Owen *et al.* [16] ribotyped

Fig. 1. *ClaI* restriction fragments of 18 *P. alcalifaciens* strains hybridised with *E. coli* rRNA probe labelled with digoxigenin-11-dUTP. Lanes: 1–10, non-invasive strains (39133, 8894, 5271-0, 565, 6634, 4521-8, 4201-7, 0301-9, 2101-15, 0181-9, respectively); 11–18, invasive strains (681-8, 4002-10, 3761-8, 40448, 762-8, 9045-3, 2401-2, 47176, respectively); 19, *H. aegyptius* 3031 *EcoRI* digest. Fragment sizes (kb) are shown on the right. Letters at the bottom correspond to the ribotypes listed in Table 1.

Fig. 2. Representative ribotype patterns of *P. alcalifaciens* strains obtained after digestion with *EcoRV*, *MluI* and *ClaI*. Panel A, ribotypes of non-invasive strains; panel B, ribotypes of invasive strains. Letters correspond to the different ribotypes listed in Table 1; P, *H. aegyptius* 3031 DNA digested with *EcoRI*. Fragment sizes (kb) are shown on the right.

Table 1. Ribotypes and characteristics of *P. alcalifaciens* strains isolated in São Paulo, Brazil

Strain No.	Diarrhoea	Year of isolation	Invasive phenotype	Ribotypes		
				<i>ClaI</i>	<i>EcoRV</i>	<i>MluI</i>
9938	Yes	1990	+	C1	E1	M1
47176	Yes	1990	+	C1	E1	M1
40448	Yes	1990	+	C1	E1	M1
3761-8	Yes	1989	+	C1	E1	M1
4002-10	No	1989	+	C1	E1	M1
681-8	Yes	1989	+	C1	E1	M1
2401-2	Yes	1989	+	C2	E2	M2
9045-3	Yes	1988	+	C2	E2	M2
762-8	No	1989	+	C2	E2	M2
0181-9	Yes	1989	-	C3	E3	M3
2101-15	Yes	1989	-	C4	E4	M5
0301-9	Yes	1989	-	C5	E5	M6
4201-7	Yes	1989	-	C3	E6	M3
4521-8	Yes	1989	-	C5	E7	M6
5271-0	Yes	1989	-	C5	E7	M6
6634	Yes	1990	-	C7	E8	M7
565	Yes	1986	-	C8	E9	M4
8894	Yes	1988	-	C6	E10	M8
39133	Yes	1990	-	C9	E2	M4

P. stuartii strains isolated from several hospitals in the UK and showed that the method distinguished between strains and also separated them from allied *Providencia* and *Proteus* spp.

In the present study, a combination of the ribotype patterns obtained with three selected enzymes produced 11 distinct ribotypes among 19 *P. alcalifaciens* strains isolated from patients with diarrhoea in São

Paulo City (Table 1). These strains could not be distinguished by biochemical tests, because all had the same biotype (data not shown). Moreover, it should be noted that the invasive *P. alcalifaciens* strains were found in only two of the 11 ribotype patterns (1 and 2) irrespective of the restriction endonuclease used, and most of them (67%) were in pattern 1 (Table 1; Fig. 1).

Recently, Dalla-Costa *et al.* [17] showed that ribotyping was able to discriminate clones among diarrhoeagenic *E. coli* strains displaying different virulence markers. Related clones such as O55:H7 and O157:H7 had similar ribotypes and were clustered together, and the two divergent clonal groups of enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) were distributed in distinct branches.

The genetic relationship of the *P. alcalifaciens* strains studied is presented in a dendrogram (Fig. 3). Strains were clustered into two main groups (A and B). The invasive *P. alcalifaciens* strains represented two genetically related clones in group A, whereas the

other strains not harbouring this virulence property comprised a more diverse group distributed either in clusters A or B. These results confirmed an earlier suggestion [4] that *P. alcalifaciens* strains isolated from diarrhoeal stools in São Paulo comprise different subgroups of strains. *P. alcalifaciens* strains harbouring invasive properties represent specific clones that seem to be distributed endemically in the population studied.

Despite the differences observed among the non-invasive *P. alcalifaciens* strains, it should be remembered that they too were isolated from diarrhoeal stools in pure culture or from faeces where no other bacterial enteropathogen was identified, and some may be implicated as agents of diarrhoea by mechanisms not yet identified.

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Fig. 3. Dendrogram showing the relationships among *P. alcalifaciens* strains isolated from diarrhoea in São Paulo City. The tree was constructed by applying UPGMA to a matrix of similarity established from the ribotype data obtained with *Cla*I cleavage.

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