BACTERIAL PATHOGENICITY

Prevalence of invasive ability and other virulence-associated characteristics in *Providencia alcalifaciens* strains isolated in São Paulo, Brazil

BEATRIZ E. C. GUTH and E. PERRELLA

Department of Microbiology, Immunology and Parasitology, Universidade Federal de São Paulo, Escola Paulista de Medicina, São Paulo, SP, Brazil

Providencia alcalifaciens is an invasive enteric pathogen. The present study determined the prevalence of invasive ability in *P. alcalifaciens* strains isolated in São Paulo, Brazil, mainly from patients with diarrhoea. Invasion of HeLa cells was found in 17 (42%) of 41 strains studied. Most (88%) of the invasive strains were isolated from diarrhoeal stools. The invasive property was identified in 50% of *P. alcalifaciens* strains isolated as pure cultures or from stool samples where no other enteropathogen was identified. All the invasive strains caused actin condensation in infected cells. Plasmid profile analysis showed the presence of plasmids of 35.8–180 kb in 70% of the strains regardless of their invasive ability, suggesting that invasiveness in *P. alcalifaciens* is not plasmid related. No homology with a probe for gene sequences for invasion of enteroinvasive *Escherichia coli* and *Shigella* strains was identified in colony hybridisation assays. The invasive property of *P. alcalifaciens* was confirmed in the present study, but this characteristic did not predominate among strains isolated from patients with diarrhoea in São Paulo City. The presence of other virulence mechanisms and the role of non-invasive *P. alcalifaciens* strains as a cause of diarrhoea remain to be established.

Introduction

The possible role of *Providencia alcalifaciens* as an aetiological agent of diarrhoea in man was suggested several years ago [1–3]. *Providencia* spp. have been isolated – usually as the main organism or as pure cultures – from the diarrhoeal stools of patients, particularly children, in the absence of other recognised enteropathogens [4]. While studying travellers' diarrhoea, Haynes and Hawkey [5] found an increased frequency of *P. alcalifaciens* strains in patients who had travelled abroad, suggesting that they might be a major cause of diarrhoea among British travellers.

Invasion was first suggested as the virulence mechanism of *P. alcalifaciens* by Albert *et al.* [6], who showed that two of three *P. alcalifaciens* isolates from patients with diarrhoea were invasive to HEp-2 cells, producing actin condensation. Later, this observation was extended with another 14 strains from diarrhoeal

stools, suggesting that invasiveness is a property commonly found among *P. alcalifaciens* strains [7]. Invasion of cells by *P. alcalifaciens* has also been detected in rabbit intestinal tissues by electron microscopy [8].

The purpose of this study was to investigate the prevalence of invasive ability among *P. alcalifaciens* strains isolated mainly from patients with diarrhoea in São Paulo, Brazil. Data from the isolates on their ability to promote actin condensation, their plasmid profile, and on tests for their homology with invasion gene sequences of enteroinvasive *Escherichia coli* and *Shigella* spp. are also presented.

Materials and methods

Bacterial strains

Forty-one *P. alcalifaciens* strains isolated from 35 diarrhoeic and six non-diarrhoeal stools, between 1987 and 1990 in São Paulo were studied. Fifteen of the strains were isolated at a private laboratory from adults and children with diarrhoea and were kindly supplied

by Dr L. R. Trabulsi. The remaining strains were isolated at Escola Paulista de Medicina (EPM); 16 of them featured in a project on the aetiology of diarrhoea in children, conducted with the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA [9]. All the strains were identified biochemically [10] and confirmed as *P. alcalifaciens* as described by Farmer *et al.* [11]. Among the 41 strains studied, at least eight were isolated as pure cultures and 16 were isolated from stool samples in which no other recognised enteropathogen (such as *Shigella*, *Salmonella*, *Yersinia*, *Aeromonas*, *Campylobacter*, enteropathogenic *Escherichia coli*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, *E. coli* O157:H7 or rotavirus) was found; seven strains were associated with another known pathogenic bacterial species or rotavirus and no data were available for the remaining strains.

HeLa cell invasion assay

Invasion assays were performed as described by Marques *et al.* [12] except that bacteria were grown overnight in Tryptic Soy Broth (TSB; Difco) and that a suspension of 3.0×10^8 cfu/ml was prepared and diluted 1 in 10 in Eagle's Minimal Essential Medium (MEM; Gibco) supplemented with fetal bovine serum (FBS) 10% and D-mannose 2%. After incubation for 3 h at 37°C, bacterial suspensions were removed, the cell monolayers were washed with phosphate-buffered saline (PBS, pH 7.3) and were then re-incubated for 3 h in fresh MEM-FBS containing gentamicin 100 mg/L. The monolayers were then washed with PBS, fixed with methanol, stained with May Grünwald followed by Giemsa, mounted on slides, and examined by light microscopy. Invasive *E. coli* strain 9-82::Tn5 of serogroup O28ac [13] and non-invasive *E. coli* strain C600K12 were used as controls. To determine the number of intracellular bacteria after gentamicin treatment, the monolayers were washed three times with PBS and then incubated with 0.4 ml of Triton-X100 1% for 5 min at room temperature [14]. Different dilutions of the lysed monolayers in saline (NaCl 0.85%) were plated on MacConkey agar and incubated at 37°C for 18 h. Experiments were run in duplicate and repeated twice for each isolate. The susceptibility of all 41 *P. alcalifaciens* strains studied to gentamicin was previously confirmed by standard methods [15] (data not shown).

Fluorescent actin staining (FAS) test

All invasive and 10 non-invasive strains were tested for actin condensation with HeLa cell monolayers in 6-h assays as described previously [16]. The control strains were those used in invasion assays.

Plasmid DNA analysis

Plasmid DNA was prepared by the method of Birnboim and Doly [17] and examined by electrophoresis in

agarose 0.8% slab gels. Plasmid sizes were determined by comparison of their relative migration rates with those of plasmids of known size (147, 63, 35.8 and 6.9 kb) present in *E. coli* K12 strain NCTC 39R861.

Colony hybridisation

The presence of genes associated with invasiveness of enteroinvasive *E. coli* (INV) and *Shigella* spp. was tested by colony hybridisation assays [18] with the 2.5-kb *HindIII* fragment from plasmid pSF55 as a probe [19]. The *E. coli* K12 strain harbouring pSF55 and the invasive *E. coli* O28ac strain [13] were used as positive controls, and *E. coli* strain K12 containing pBR322 [19] was used as a negative control.

Results

Invasion of HeLa cells was observed in 17 (42%) of the 41 *P. alcalifaciens* strains. However, of the 24 *P. alcalifaciens* strains found in faeces either as a pure culture or when no other enteropathogens were present, 12 (50%) were identified as invasive strains.

The origin and characteristics of the invasive strains studied are shown in Table 1. Most (15 of 17 strains) were isolated from diarrhoeal stools. In the invasion assay, the number of intracellular bacteria varied from 4.0×10^3 to 1.8×10^5 cfu/ml, while the invasive and negative controls used yielded 4.2×10^5 and 0 cfu/ml respectively. No colonies were recovered from the MacConkey plates of the non-invasive strains. All the invasive strains promoted actin condensation as shown by the FAS test, and none of the non-invasive strains caused actin condensation.

Plasmid profile analysis showed the presence of plasmids ranging from 180 to 35.8 kb in 29 of the 41 *P. alcalifaciens* strains. Of the 17 invasive strains, nine did not harbour a plasmid, seven harboured one plasmid, and one harboured three plasmids. The plasmids of the invasive strains varied in size, although some of them were of similar mol.wt (Table 1).

Under the conditions used, none of the 41 *P. alcalifaciens* strains studied showed hybridisation with the INV probe when compared to the results of the invasive positive control.

Discussion

Invasion of cells by *P. alcalifaciens* was demonstrated originally by Albert *et al.* [6, 7] in the HEP-2 cell assay. In the present study, the invasive ability of *P. alcalifaciens* strains was evaluated in HeLa cells. Forty-two percent of *P. alcalifaciens* strains were found to be invasive and no differences in invasive ability were

Table 1. Origin and characteristics of invasive *P. alcalifaciens* strains

Strain no.	Patient data			Intracellular bacteria [†] (cfu/ml)	FAS test	Presence of plasmids (size in kb)
	Age	Diarrhoea	Other pathogen isolated [†]			
0351-6	4 years	Yes	Rotavirus	4.0×10^3	+	—
681-8	1 year 2 months	Yes	ETEC	1.0×10^5	+	90,63,50
762-8	2 years 3 months	No	—	1.2×10^4	+	—
2401-2	4 years 11 months	Yes	EIEC	5.6×10^4	+	—
3761-8	2 years 2 months	Yes	<i>A. caviae</i>	1.8×10^5	+	50
4002-10	1 year 8 months	No	—	4.1×10^4	+	45
P-56	Child*	Yes	ND	2.1×10^4	+	35.8
40448	2 years	Yes	P	2.2×10^4	+	54
P-40	Child*	Yes	P	1.0×10^5	+	72
9045-3	Child*	Yes	P	1.4×10^5	+	180
47176	3 years 5 months	Yes	—	1.6×10^4	+	—
9400	11 months	Yes	—	2.0×10^4	+	—
4631-10	Child*	Yes	—	4.8×10^4	+	—
52713	1 year 8 months	Yes	—	3.1×10^4	+	—
65473	4 months	Yes	P	7.0×10^4	+	—
66878	8 months	Yes	—	3.4×10^4	+	—
9938	32 years	Yes	P	4.0×10^3	+	63

*Child: age data not available.

[†]—, no pathogen identified; P, pure culture; ND, data not available; ETEC, enterotoxigenic *E. coli*; EIEC, enteroinvasive *E. coli*.

[‡]Values are averages of duplicate assays; the invasive control strain showed 4.2×10^5 cfu/ml and non-invasive K12 *E. coli* showed 0 cfu/ml.

found when HEP-2 cells were used instead of HeLa cells (data not shown). Except for two strains, all the invasive *P. alcalifaciens* strains identified were isolated from patients with diarrhoea. A higher frequency of invasive ability (from 42% to 50%) was observed if only the *P. alcalifaciens* strains isolated from stools as pure culture or when no other recognised enteropathogen was identified were considered.

Although a high frequency of invasiveness was found among these *P. alcalifaciens* strains isolated in São Paulo City, almost half of them did not present this virulence trait. These results contrast with those of other studies [6, 7], in which all *P. alcalifaciens* isolates from diarrhoeal stools in Bangladesh were invasive. However, in support of Albert *et al.* [6], no other virulence factor such as the production of enterotoxins and cytotoxins was detected in any of the *P. alcalifaciens* strains studied (data not shown).

On the other hand, it is possible that the invasive and non-invasive *P. alcalifaciens* strains isolated in São Paulo may belong to different subgroups or biotypes. Preliminary results show that the invasive and non-invasive strains are of different ribotypes, thus suggesting the presence of distinct clones among the strains studied (unpublished observation).

Cell invasiveness is plasmid related in some classical invasive enterobacteria such as *Shigella* spp. [20], enteroinvasive *E. coli* [21, 22] and *Y. enterocolitica* [23]. Therefore, the loss of these elements could be responsible for lack of expression of such property. However, this seems not to be the case for the *P. alcalifaciens* strains studied, because plasmids were not found in nine of the 17 invasive strains. These results suggest that the ability of *P. alcalifaciens*

strains to invade HeLa cells is not plasmid related. Similar observations have been described for invasive strains of *Edwardsiella tarda* [12].

All the invasive *P. alcalifaciens* strains studied caused polymerisation of actin filaments demonstrated by the FAS test, confirming previous work [6, 7].

Some analogies between invasion of *P. alcalifaciens* and *Shigella* spp. have been suggested. These bacteria presented similar patterns of actin condensation [6] and their invasion processes were inhibited by cytochalasin D, a microfilament inhibitor [7]. Moreover, both organisms required prior growth at 37°C for optimal expression of the invasive phenotype.

Colony hybridisation assays with the INV probe pSF55 [19] were performed to determine if the gene sequences for invasion of enteroinvasive *E. coli* and *Shigella* spp. could be used to identify invasive *P. alcalifaciens* strains. Although the specific genes present on this probe are not known, Wood *et al.* [24] and Gomes *et al.* [25] showed that this sequence presented 100% sensitivity in detecting both *Shigella* and enteroinvasive *E. coli* Serény-test positive strains, and was able to identify some Serény-negative *E. coli* strains that retained their ability to invade HeLa cells. No hybridisation with the INV probe was observed with any of the strains in the present study, suggesting that the genome of *P. alcalifaciens* shares no homology with the invasion gene sequences of those bacteria. In this respect, it is like that of invasive *Salmonella* spp. [24].

Although the invasive property of *P. alcalifaciens* was confirmed, this virulence characteristic did not predominate among the strains isolated from patients with diarrhoea in São Paulo City, Brazil. The possible

presence of other virulence determinants and the role of non-invasive *P. alcalifaciens* strains as the aetiological agents of diarrhoea remain to be established. Animal models should be developed to assess the pathogenic potential of the non-invasive strains isolated in this study. Moreover, further epidemiological case-control studies in different geographical areas are required to help elucidate the role *P. alcalifaciens* strains play in diarrhoeal disease.

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