MICROBIAL PATHOGENCITY

Molecular analysis of clinical isolates of *Providencia alcalifaciens*

MARISE SOBREIRA, NILMA C. LEAL*, M. MAGALHÃES, BEATRIZ E. C. GUTH† and ALZIRA M. P. ALMEIDA*

Laboratory of Immunopathology Keizo Asami, Universidade Federal de Pernambuco, Recife, *Department of Microbiology, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ/MS and †Disciplina de Microbiologia, Universidade Federal de São Paulo, Escola Paulista de Medicina, UNIFESP, São Paulo, SP, Brazil

In an attempt to elucidate the virulence factors and the pathogenic mechanisms of *Providencia alcalifaciens*, 36 isolates identified in 1994–1995 in Recife city, Brazil were analysed by PCR to investigate the presence of DNA sequences homologous to virulence genes described in other invasive enterobacteria, as well as their ability to invade HeLa cells, their plasmid profiles and antibiotic resistance patterns. The genetic diversity of the isolates was also analysed by RAPD-PCR. No homologous sequences of virulence genes were observed with any of the *P. alcalifaciens* isolates studied. Ten isolates had no plasmid and 26 harboured one-to-five plasmids of 147–<6.9 kb. Invasion of HeLa cells was observed in only 10 isolates. No correlation between the plasmid content of the strains, their invasion of HeLa cells or their resistance to antimicrobial drugs could be established. The isolates could be distributed into 10 genotypic groups by RAPD-PCR. Considering the genotypic profile and ability to invade HeLa cells, 7 of the 10 invasive isolates belonged to the same genotypic group. The presence of invasive isolates in the same or a related genotypic group suggests the existence of a clonal lineage responsible for the invasiveness.

Introduction

Providencia alcalifaciens is a gram-negative bacterium from the family Enterobacteriaceae and several studies have described its association with diarrhoea in travellers and children, although it can be found occasionally in non-diarrhoeic stools [1–5].

Although cell invasion has been proposed as the main virulence mechanism of this organism [4–8], studies by Guth and Perrella [4] showed that this characteristic was identified in only 42% of the isolates of *P. alcalifaciens* from diarrhoea in São Paulo city, Brazil. Subsequent clonal analysis studies showed that isolates with invasive properties represented specific clones, which differed from the non-invasive ones [9].

In 1994–1995, several *P. alcalifaciens* isolates were obtained from patients with diarrhoea in Recife city, Brazil. To determine the virulence properties of these isolates, the presence of DNA sequences homologous to virulence genes described in other invasive enter-

obacteria was investigated by PCR, as well as the ability of the isolates to invade HeLa cells, their plasmid profiles and drug resistance patterns. The genetic diversity of the isolates was also analysed by RAPD-PCR.

Materials and methods

Bacterial isolates and culture conditions

A total of 36 *P. alcalifaciens* isolates from diarrhoeic stools from unrelated people was analysed. The isolates were obtained in a private laboratory in the city of Recife, Brazil during 1994 and 1995. They were identified biochemically with API-20E strips (bioMérieux). The isolates were maintained as frozen stocks and, before testing, were grown in brain heart infusion (BHI) broth for 18 h at 37°C. Strains *Escherichia coli* ATCC 25922, *E. coli* K12 NCTC 39R861, *Yersinia pestis* P. PB 881 (Plague Laboratory, Centro de Pesquisas Aggeu Magalhães), *Y. pseudotuberculosis* 2952 (Institut Pasteur, Paris), *Shigella flexneri* 2 (strain 831), *Y. enterocolitica* 41 and *S. boydii* 1995 (Department of Bacteriology, Instituto Oswaldo Cruz/Fundação Oswaldo Cruz) served as controls in the different techniques.

PCR assays

PCR assays were performed to investigate the presence in *P. alcalifaciens* of gene sequences homologous with those of other invasive bacteria. Specific primers already described, such as those for *inv_{ent}* and *ail* of *Y. enterocolitica* [10], *inv_{pstb}* of *Y. pseudotuberculosis* [11], *senA* of *E. coli* and *Shigella* [12] and *psa* of *Y. pestis* and *Y. pseudotuberculosis* [13], were tried. Primer sequences used for detection of *aida* of *E. coli* (5'-ATGAATAAGGCCTACAGTATC-3' and 5'-CTCCGGAGTATATATTCTGAG-3') and *irp2* from *Y. enterocolitica* (5'-AAGGATTCGCTGTTACCGGAC-3' and 5'-TCGTCGGGCAGCGTTTCTTCT-3') were derived from published sequences of the genes [14, 15]. Amplification reactions were performed in a thermocycler (Perkin Elmer), programmed for 25 cycles, each cycle consisting of 1 min at 94°C for denaturation, 2 min at 50°C for annealing, 3 min at 72°C for extension, finishing with an extension step of 7 min at 72°C. Amplification with *inv_{ent}* and *irp2* primers was repeated with an annealing temperature of 55°C. The reaction mixtures, in a final volume of 25 µl, consisted of DNA 20 ng, *taq* DNA polymerase (CENBIOT/RS) 1 U, 160 µM of each deoxynucleoside triphosphate (dNTP-Pharmacia), 1.5 mM MgCl₂, 20 pmol of each primer, 50 mM KCl and 10 mM Tris-HCl, pH 8. Each amplification run included a negative control containing all the components of the mixture except DNA and a positive control, to which a sample of DNA was added. The products of amplification were electrophoresed in agarose 1% gels as described for plasmid analysis below. The reactions were repeated three times.

Products generated with the primer directed to *irp2* gene were transferred from the gels to a nylon membrane by the technique of Southern [16] and hybridised with an *irp2* probe. This probe was obtained by PCR with the *irp2* primers described above and the DNA of *Y. pestis* P. PB 881. The probe was labelled by random-priming with a Dig DNA labelling and detection kit (Boehringer Mannheim), according to the manufacturer's instructions.

Invasion test in HeLa cells

Invasion assays of HeLa cells were performed as described previously [4].

Antimicrobial susceptibility

The antibiotic sensitivity of the isolates was tested by the diffusion method described by Bauer *et al.* [17] on Mueller-Hinton agar with commercial disks of ampicillin, cephalosporin, chloramphenicol, co-trimoxazole, gentamicin, neomycin, norfloxacin, sulphamethoxazole and tetracycline. *E. coli* ATCC 25922 was used as the control.

Extraction of plasmid DNA

Plasmid DNA was extracted by the alkaline lysis method of Birnboim and Doly [18] and electrophoresed in agarose 0.6% gels in Tris-borate buffer at a constant voltage of 100 V, followed by staining with ethidium bromide and visualisation under UV light. The plasmid sizes were determined by comparison of their migration with plasmids of known size (147, 63, 35.8 and 6.9 kb) present in *E. coli* K12 NCTC 39R861.

Extraction of total DNA

Total DNA was extracted according to Maniatis *et al.* [19] and quantified after electrophoresis in agarose 1% gel by comparison with a known amount of DNA from phage λ cleaved by *Hind*III.

RAPD typing

Preliminary assays were performed with one strain (Pa16) and 10 primers of different sizes (9-, 15- and 20-mer), to test their ability to generate clear and reproducible bands. The amplifications were done in a thermocycler (Perkin Elmer) programmed for 30 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, finishing with 7 min at 72°C.

The reactions were prepared in a final volume of 25 µl that contained DNA template 20 ng, *Taq* DNA polymerase (CENBIOT/RS) 1 U, 10 mM Tris-HCl, pH 8, 3 mM MgCl₂, 50 mM KCl, gelatin 100 µg, 200 µM of each dNTP (Pharmacia) and primer 20 pmol (Primer 785: 5'-CCGCAGCCAA-3' was selected for this work). Products of amplification were electrophoresed in agarose 1.5% gels as described above, and for analysis the stained gels were photographed with a Polaroid camera.

A negative control containing all the components of the mixture except DNA was included in each amplification run. Reproducibility of the results was evaluated by at least three repetitions of the PCR reactions.

The clearest and most reproducible bands were considered for analysis of their presence or absence in each isolate. They were analysed by the divergence index proposed by Nei and Li [20] and a dendrogram was generated with the MEGA-UPGMA program (Molecular Evolutionary Genetics Analysis).

Results

PCR assays

No amplification with the primers directed to the sequences of the genes *ail*, *inv_{pstb}*, *psa*, *aida* and *senA* was observed in the *P. alcalifaciens* isolates studied.

Surprisingly, instead of amplification of one fragment

of 352 bp, as expected, there was amplification of two or three larger fragments in nine isolates of *P. alcalifaciens* with the primer targeted to *inv_{ent}*. There was also amplification of two-to-five fragments larger than the expected 276-bp fragment in 20 strains with the primer targeted to the *irp2* gene. Amplification reactions with primers directed to *inv_{ent}* and *irp2* were then repeated at a higher stringency temperature (55°C) of annealing, and no amplification was observed, suggesting that the previously amplified fragments were not specific. DNA from *Y. enterocolitica* and *Y. pestis* strains used as positive controls resulted in amplification of only one fragment of the expected sizes. The fragments generated with *irp2* primer and the nine *P. alcalifaciens* isolates were transferred from the gels to nylon membranes and hybridised with a probe directed to the *irp2* gene. The *Irp2* probe did not hybridise with any of the amplified fragments.

Invasion of HeLa cells

Ten (27.8%) of the 36 *P. alcalifaciens* isolates analysed were able to invade HeLa cell monolayers.

Antimicrobial susceptibility

All the *P. alcalifaciens* isolates were sensitive to chloramphenicol, gentamicin, neomycin and norfloxacin; all except one were also sensitive to cephalosporin and four isolates were sensitive to all the antibiotics tested. Resistance to tetracycline alone was identified in four isolates, and multiple resistance to ampicillin, cotrimoxazole, sulphamethoxazole and tetracycline was observed in 11 isolates. In some isolates, resistance to two, three, four or five antibiotics simultaneously was observed (Table 1).

Plasmid profile

The plasmid content of the isolates analysed is shown in Table 1 and a representative profile of the isolates is

Table 1. The plasmid content, drug resistance and ability to invade HeLa cells of the *P. alcalifaciens* isolates from Recife city

Isolate no.	Plasmids (kb)	Drug resistance	Invasion
Pa 1	49	Te, Co, Su	+
Pa 2	147, 49	Am, Te, Co, Su	+
Pa 3	147, 6.9	Am, Te, Co, Su	—
Pa 4	—	Am, Te	—
Pa 5	147, 63, 49, (2x) <6.9*	Am, Te	—
Pa 6	147, 49	Te	—
Pa 7	147	Am, Te, Co, Su	—
Pa 8	147	Am, Te, Co, Su	—
Pa 9	125, 55	Te	—
Pa 10	147	—	—
Pa 11	147, 55	Am, Te	+
Pa 12	147	Am, Te, Co	+
Pa 13	147	Am, Te	+
Pa 14	147	Am, Te, Co, Su	—
Pa 15	147	Am, Te, Co, Su	—
Pa 16	—	Am, Ce, Te, Co, Su	—
Pa 17	49	Am, Su	+
Pa 18	130, 49, (2x) <6.9*	Am, Te, Co, Su	—
Pa 19	147	Am, Te, Co, Su	—
Pa 20	—	Am, Te	—
Pa 21	—	Am, Te, Su	—
Pa 22	147, 49	Am, Te, Co, Su	+
Pa 23	—	Am, Te, Su	—
Pa 24	—	Am, Te, Co, Su	—
Pa 25	77, 8.9, <6.9	Am, Te, Co	—
Pa 26	147, 55	Am, Te	+
Pa 27	41, 7.5, (2x) <6.9*	Te	—
Pa 28	—	Am, Te	—
Pa 29	—	Te	+
Pa 30	—	—	—
Pa 31	147, 6.9	Am, Te	—
Pa 32	147	Am, Te, Co, Su	—
Pa 33	—	Am, Te	—
Pa 34	147	Am, Te	—
Pa 35	147, 63	—	—
Pa 36	147, 49	—	+

Te, tetracycline; Am, ampicillin; Su, sulphamethoxazole; Co, cotrimoxazole; Ce, cephalosporin.

*Two plasmids <6.9 kb.

presented in Fig. 1. A single 147-kb plasmid was identified in 10 isolates, and different plasmids of 147–<6.9 kb were observed in several other isolates. Ten isolates did not contain any plasmid. One of the 10

Fig. 1. Representative plasmid profiles on agarose 0.6% gel of the *P. alcalifaciens* isolates. Lane 1, Pa 13; 2, Pa 17; 3, Pa 6; 4, Pa 26; 5, Pa 35; 6, Pa 31; 7, Pa 3; 8, Pa 25; 9, Pa 18; 10, Pa 27; 11, Pa 5; 12, *E. coli* 39R861.

invasive isolates had no plasmid whereas the others harboured one or two plasmids of 147, 55 or 49 kb.

RAPD profile analysis

Primer 785 and the DNA of the 36 *P. alcalifaciens* isolates generated a polymorphic pattern, three-to-seven bands 300–2400 bp in size were produced (Fig. 2). Based upon their respective amplification patterns, the 36 isolates of *P. alcalifaciens* were grouped into 10 genotypic groups. Fig. 3 shows the dendrogram derived from analysis of the RAPD profiles and the distribution of the 36 isolates in their respective genotypic group. These 10 genotypic groups were reduced to seven (G1, 2, 5, 6, 7, 9 and 10) when only the isolates without plasmids were analysed.

Discussion

The virulence factors and the pathogenic mechanisms of *P. alcalifaciens* are still unclear. In the present study, the presence of sequences homologous to virulence genes from several other invasive enterobacteria was investigated by PCR in an attempt to elucidate the pathogenicity of *P. alcalifaciens* isolates identified in Recife city.

No PCR amplification was observed with any of the isolates of *P. alcalifaciens* and the primers targeted to *psa*, the structural gene of a fimbrial adhesin of *Y. pseudotuberculosis* and *Y. pestis* [13], and *aida* and *ail* coding for adhesins in *E. coli* [14] and *Y. enterocolitica* [21], respectively. Furthermore, there was no amplification homologous to *inv_{pstb}* coding for the invasins of *Y.*

Fig. 2. Representative RAPD patterns generated on agarose 1.5% gel of *P. alcalifaciens* isolates and primer 785. Lane 1, Pa 2; 2, Pa 7; 3, Pa 14; 4, Pa 10; 5, Pa 6; 6, Pa 5; 7, Pa 23; 8, Pa 31; 9, Pa 34; 10, Pa 3; 11, negative control; 12, 100-bp DNA ladder.

Fig. 3. Dendrogram derived from analysis of the RAPD-PCR patterns of *P. alcalifaciens* isolates. Invasive isolates are shown in **bold** type.

pseudotuberculosis [22], nor *senA* coding for an enterotoxin of enteroinvasive *Shigella* and *E. coli* strains [12]. However, there was amplification of several DNA segments whose sizes were different from those expected with the primers directed to *inv_{ent}* [10, 21] from *Y. enterocolitica* and *irp2* found in pathogenic strains of *Yersinia* [23] and some strains of *E. coli*, *Klebsiella* and *Citrobacter* [24]. Subsequent tests at higher stringency and failure of hybridisation with an *irp2* gene probe confirmed these PCR products to be non-specific. No sequence homology to invasion genes of enteroinvasive *E. coli* and *Shigella* was also reported by Guth and Perrella [4] in *P. alcalifaciens* strains isolated in São Paulo.

Several studies have demonstrated the invasive ability of *P. alcalifaciens* strains isolated from diarrhoeic stools [4–8]. Magalhães *et al.* [5] observed that all the invasive strains, independently of the degree of invasiveness, harboured a 45–50-kb plasmid. In contrast, the non-invasive strains harboured no plasmid. This suggested a correlation between the presence of that plasmid and invasiveness.

In the present study, only five of the 10 invasive isolates harboured a plasmid of 49 kb and one had no plasmid. These data call into question the role of plasmids in the pathogenicity of *P. alcalifaciens*. Others [4] studying *P. alcalifaciens* strains isolated from diarrhoeic and non-diarrhoeic stools in São Paulo city also found no correlation between the presence of a 45–50-kb plasmid and invasiveness.

In accordance with previous observations [25, 26], most of the *P. alcalifaciens* isolates studied were sensitive to several antimicrobial drugs. Although multiple resistance to ampicillin, co-trimoxazole, sulphamethoxazole and tetracycline was observed in some isolates, no correlation between antibiotic resistance and the presence of a particular plasmid could be demonstrated.

Based on their RAPD pattern, the 36 *P. alcalifaciens* isolates were distributed into 10 genotypic groups. This multiplicity of groups could be attributed to amplifications of the plasmid DNA in addition to chromosomal DNA, as most of the isolates harboured between one and five plasmids. In order to homogenise the DNA template, the isolates carrying plasmids were excluded and only isolates without plasmids were considered for grouping. Even so, seven groups could still be identified. This diversity of genotypic groups is in agreement with the numerous serotypes described for *P. alcalifaciens* [27].

Considering the genotypic profile and invasiveness for HeLa cells, seven of the 10 invasive isolates belonged to the same genotypic group (G1) and two isolates belonged to two different but closely related groups, G5 and G6. On the other hand, one invasive

isolate belonged to an unrelated genotypic group (G10).

These results showed that most of the invasive isolates were clustered in genotypic groups that are related, suggesting the existence of a clonal lineage responsible for the invasive potential. However, there were also non-invasive isolates in those groups. In a recent study, Guth *et al.* [9] showed that invasive and non-invasive isolates had different ribotypes. *P. alcalifaciens* isolates with invasive properties belonged to two ribotype patterns that differed from those identified among non-invasive isolates, suggesting that the invasive and the non-invasive isolates were derived from different clones.

Although, in the present study, the heterogeneity of *P. alcalifaciens* was also demonstrated by RAPD, studies are still necessary to understand the mechanisms of pathogenicity of this organism.

We acknowledge the skilful technical assistance of Silvana Santos, Yara Nakasawa, Raimundo Pimentel, George Lins and Ulisses Montarroyos. M.S. had a scholarship from the Brazilian Research Council (CNPq).

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