

## RESEARCH NOTE

## Invasion of HeLa Cells by *Providencia alcalifaciens* Presumably is Plasmid-encoded

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*Providencia alcalifaciens*, a well-characterized species in the tribe *Proteeae*, is the most recent organism included among invasive enterobacteria. In fact, it enters HeLa cell monolayers mobilizing actin filaments and invades the intestinal mucosal epithelium inducing diarrhea in adult rabbits with removable intestinal ties (MJ Albert et al. 1992 *Infect Immun* 60: 5017-5024).

In this animal model, the bacterium penetrates epithelium directly by endocytosis or disrupting cell junctions with entry into and proliferation in intercellular spaces (MM Mathan et al. 1993 *Pathol* 171: 67-71). Thus, *P. alcalifaciens* shares potential virulence properties with *Shigella* spp., the prototype of enteroinvasive bacteria.

Now we report that the capacity of *P. alcalifaciens* to invade HeLa cells is probably associated with the carriage of a 45- to 50- Kb plasmid.

We selected for the study eleven strains of *P. alcalifaciens*, isolated from equal number of children with symptoms of enteritis. The cultures were obtained in 1993, during a survey carried out in Recife, State of Pernambuco, Brazil, to evaluate the role of *P. alcalifaciens* in the etiology of gastroenteritis (manuscript in preparation). *Providenciae* selected for the study were present in the fecal samples in the absence of any other recog-

nized enteropathogenic organisms. After recovering, strains were maintained at room temperature in brain heart infusion broth (Difco Laboratories, Detroit, Michigan, USA), containing 0.4% of agar, for no more than three months before starting the tests.

The gentamicin-HeLa cell invasion assays were conducted and analyzed essentially as reported for the system *Edwardsiella* spp. and HEp-2 cell monolayers (MJ Janda et al. 1991 *Infect Immun* 59: 154-161). But, when the strain PA-5 was tested, we shortened the post-infection period from 3 to 1 hr to avoid detaching HeLa cells from coverslips. All *P. alcalifaciens* strains submitted to invasion trials were susceptible to gentamicin. An invasive strain of *Shigella flexneri* was the positive control.

Tests for production of conjunctivitis in rabbits, for detecting plaque formation, and contact hemolytic activity were done as previously described (B Sereny 1955 *Acta Microbiol Sci Hung* 2: 293-296, V Oaks et al. 1985 *Infect Immun* 48: 124-129, PJ Sansonetti et al. 1986 *Infect Immun* 51: 61-69).

Plasmid DNA was extracted by the method of HC Birboim and J Doly (1979 *Nucleic Acids Res* 7: 1513-1537) and examined by electrophoresis of cell lysates through 0.6% agarose gels. Plasmid bands were stained with ethidium bromide and visualized with UV illumination. Their molecular lengths were determined by comparing their electrophoretic mobilities with those of reference plasmids.

Of the eleven strains of *P. alcalifaciens* studied, seven were invasive for HeLa cell monolayers. As first noticed (Albert et al. *loc. cit.*), some strains are more invasive than others. The strain PA-5, for instance, was highly invasive, destroying the cell monolayer if the replication phase exceeds 2 hr (Fig. 1). All invasive strains, independently of the degree of invasiveness, carried a plasmid of 45-to 50-Kb (Fig. 2). In contrast, the four strains plasmidless were unable to invade HeLa cells. These findings show a clear correlation between *inv*-plasmid carriage and invasiveness.

Despite the strains now studied were recent clinical isolates, four of them did not display the *inv*-plasmid. Whether such strains lost the *inv*-plasmid immediately after culturing, or this plasmid is not essential for invasiveness, remains to be determined. In this vein, another study, analyzing the virulence factors of *P. alcalifaciens*, from São Paulo, State of São Paulo, Brazil, did not find any connection between plasmid profile and invasiveness (BEC Guth & A Perella 1994 *Abstr Annu Meet Am Soc Microbiol* B-375 p. 95). Thus, new investigations should be done to clarify this discrepancy.

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Fig.1: surviving cells of a monolayer detached after 3 hr of postinfection with the strain PA-5 of *Providencia alcalifaciens* in the gentamicin-HeLa cell invasion test. Giemsa stain. Magnification: X900.

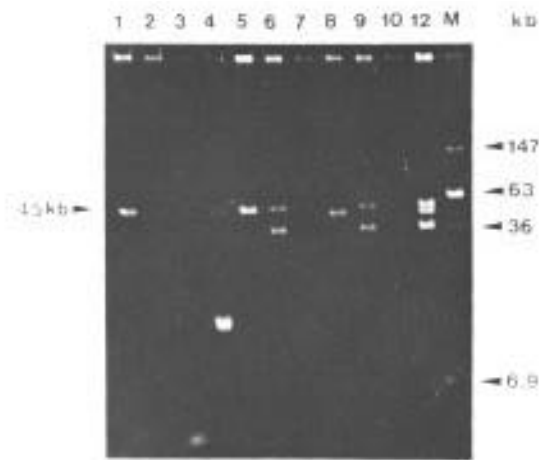


Fig.2: agarose gel electrophoresis of plasmids from eleven clinical isolates of *Providencia alcalifaciens*. Lanes 1, 4, 5, 6, 8, 9, and 11 symbolize invasive strains; all displaying the 45- to 50-Kb invasion plasmid. Lanes: 2, 3, 7, and 10 represent noninvasive strains. Eight different plasmid profiles are discerned. Lane M contained molecular size markers. Chr = chromosomal DNA.

In some invasive assays, two strains nonharboring the *inv*-plasmid showed occasional intracellular bacteria (in less than 1% of the cells) on the Giemsa stained coverslips, or few colonies (less than 10/ml) on the agar plates seeded with the triton X-100 cell lysate. This, apparently was due to

heterogeneity of the bacterial inoculum used for infecting HeLa cells, that is, occasional organisms still carried the *inv*-plasmid, but the DNA burden was insufficient to allow its visualization on the agarose gels. Since the *inv*-plasmid of *P. alcalifaciens* does not bear selective marks, refined genetic procedures are required before we can handle uniform plasmid-harboring bacterial populations.

Besides the *inv*-plasmid, we noticed other extrachromosomal DNA bands on the agarose gels. One of them, characterized as a large plasmid of 147-Kb, was displayed by ten of the eleven cultures examined. The only strain, which did not carry this plasmid, was invasive, pointing out that the 147-Kb plasmid is not implicated in HeLa cell invasion. Probably this plasmid occurs as a large unicopy, since it was well evident on the gels but appeared as a hazy band on contrasted photos (Fig. 2).

Many plasmids with different molecular sizes and arranged in distinctive patterns were exhibited by some strains. This diversity of plasmid outline might be useful as an epidemiologic marker for tracing relevant strains of *P. alcalifaciens*. Indeed, among the eleven strains examined, eight different plasmid profiles were observed (Fig. 2). Curiously, of the four noninvasive strains, three had the same plasmid fingerprint. In contrast to the *inv*-plasmid, all the others were considered cryptic since they could not be related to drug-resistance or bacteriocin production.

Our strains did not produce plaques in HeLa cell monolayers, did not show contact hemolytic activity, neither induced keratoconjunctivitis in rabbits, as did the *Shigella* control strain. In reality, unlike shigellae and similar to salmonellae or yersiniae (BB Finlay & S Falkow 1989 *Bact Rev* 53: 210-230), the ingested *Providencia* remains enveloped within endocytic vacuoles (Albert et al. *loc. cit.*). *Providencia* multiplies actively within the vacuoles producing large bacterial cluters and ultimately the cell lysis (Fig. 1). Concerning this, the intracellular behavior of *P. alcalifaciens* parallels that observed with *Edwardsiella tarda* (Janda et al. *loc. cit.*), although this bacterium appears does not carry large invasive plasmids (LRM Marques et al. 1984 *Curr Microbiol* 10: 129-132), neither *P. alcalifaciens* produce hemolysin.

Present data supplement the earliest work of Albert et al. (*loc. cit.*), who, documenting that *P. alcalifaciens* invades cultured cells and the rabbit intestinal epithelium, encouraged the acceptance of this organism as a bona fide potential enteropathogen. Yet, genetic studies for identifying the *inv*-gene are need before we can admit with confidence that the 45-50-Kb plasmid is actually responsible for the invasive phenotype displayed by many clinical isolates of *P. alcalifaciens*, as our findings insinuated.