Providencia alcalifaciens strains translocate from the gastrointestinal tract and are resistant to lytic activity of serum complement

Antonia B. R. Vieira,^{1,2} Ivan H. J. Koh³ and Beatriz E. C. Guth¹

Correspondence

Beatriz E. C. Guth

becguth@ecb.epm.br

¹Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo – Escola Paulista de Medicina, São Paulo, SP, Brazil 04023-062

²Departamento de Patologia, Universidade Federal do Pará, PA, Brazil

³Disciplina de Técnica Operatória, Universidade Federal de São Paulo – Escola Paulista de Medicina, São Paulo, SP, Brazil 04023-062

The ability of *Providencia alcalifaciens* strains, isolated from patients with diarrhoeal disease, to translocate from the gastrointestinal tract and their resistance to serum complement lytic activity were investigated and compared with previously characterized differential invasive capabilities in HeLa cells. Translocation ability to several extraintestinal sites and resistance to lysis by human serum complement were observed in both highly invasive and non-invasive strains. These characteristics have not been previously described in *P. alcalifaciens* and their potential role in causing disseminated infections should therefore be considered.

Received 11 June 2002

Accepted 7 March 2003

INTRODUCTION

Providencia alcalifaciens is a member of the *Enterobacteriaceae* and has been implicated as an agent of diarrhoeal disease in children and adults (Haynes & Hawkey, 1989; Albert *et al.*, 1992; Guth *et al.*, 1996). Recently, a large outbreak of food-borne infection was attributed to *P. alcalifaciens*, providing definitive evidence of the role of this organism in causing gastroenteritis (Murata *et al.*, 2001).

Several studies have shown that *P. alcalifaciens* is able to invade cultured epithelial cells (Albert *et al.*, 1992; Guth *et al.*, 1996; Janda *et al.*, 1998); invasion was also observed in intestinal tissues by using an adult rabbit ileal loop model (Albert *et al.*, 1992; Mathan *et al.*, 1993).

Previous data from our laboratory showed that invasive *P. alcalifaciens* strains, although frequently found, did not predominate among isolates from patients with diarrhoea in São Paulo, Brazil, and that non-invasive *P. alcalifaciens* strains were also isolated from diarrhoeal stools in pure culture from which no other bacterial enteropathogens were identified (Guth *et al.*, 1996). However, the presence of other virulence mechanisms and the role of non-invasive *P. alcalifaciens* strains as agents of disease have not been established.

Accumulating evidence has demonstrated that bacterial translocation, defined as the passage of viable indigenous bacteria from the gastrointestinal (GI) tract to the mesenteric

lymph node (MLN) complex and other extraintestinal sites, is an important event in the pathogenesis of opportunistic infections that originate in the human GI tract. Several bacterial species, including members of the family *Enterobacteriaceae*, are likely to translocate from the GI tract in animal models (Berg, 1999; Sakamoto *et al.*, 2000). *Enterobacteriaceae* are also the most common cause of infection in hospitalized patients (Sedman *et al.*, 1994; Tani *et al.*, 1997).

Although the mechanisms responsible for bacterial translocation have not been well-elucidated, several clinical conditions, as well as the presence of virulence-associated properties (including those that allow evasion of host defences), are all predisposing factors to bacterial translocation (Sedman *et al.*, 1994; Tani *et al.*, 1997).

The ability of *P. alcalifaciens* to translocate from the intestinal lumen to extraintestinal sites of the host, with possible aetiology for systemic infections, has not been studied. Thus, in the present study, we evaluate the capacity for translocation and resistance to lytic activity of serum complement of invasive and non-invasive *P. alcalifaciens* strains that were previously isolated from patients with diarrhoea in our community.

METHODS

Bacterial strains. In total, 30 *P. alcalifaciens* strains were studied (Guth *et al.*, 1996). Except for four strains that were isolated from asymptomatic patients, all other strains were isolated from diarrhoeal stools. Most strains were isolated in pure culture or from stool cultures in

Abbreviations: GI, gastrointestinal; MLN, mesenteric lymph node.

which no other enteropathogenic bacteria were identified. Fifteen strains were able to invade HeLa cells; the other strains did not show this characteristic. Invasion assays in HeLa cells were performed as described previously (Guth *et al.*, 1996), except that bacterial suspension (10^9 c.f.u. ml $^{-1}$) was prepared and diluted 1/10 in Eagle's Minimal Essential Medium (MEM; Sigma) supplemented with 10% fetal bovine serum (FBS) and 2% D-mannose. After 3 h incubation at 37 °C, the bacterial suspension was removed, the cell monolayer was washed and reincubated for 1 h at 37 °C in fresh MEM-FBS that contained 150 µg gentamicin ml $^{-1}$.

Resistance to lytic activity of serum complement. The turbidimetric assay described by Pelkonen & Finne (1987) was employed to test resistance of bacterial strains to serum bactericidal activity. Briefly, an overnight bacterial culture grown in Luria-Bertani (L)-broth was diluted 1/100 in fresh medium and grown until the exponential phase was reached. Bacterial cells were harvested and resuspended in L-broth to a concentration of $\sim 10^7$ cells ml $^{-1}$ and 75 µl bacterial suspension was distributed between the microplate wells. A volume of a pool of sera, prepared from blood from healthy human volunteers, was added to each well to achieve final concentrations of 36 and 50%. Each strain was

Fig. 1. Representative results on susceptibility to serum bactericidal activity of invasive and non-invasive *P. alcalifaciens* strains. (a) *P. alcalifaciens* 40448 (INV⁺); (b) *P. alcalifaciens* 2101-15 (INV⁻); (c) *P. alcalifaciens* 9938 (INV⁺); (d) *P. alcalifaciens* 0181-9 (INV⁻); (e) *Escherichia coli* K-12-711 (serum-sensitive); (f) *Enterobacter cloacae* 046 (serum-resistant). ■, 50% normal serum; ◆, 36% normal serum; ×, 50% inactivated serum; ▲, 36% inactivated serum; ×, bacterial suspension.

tested in triplicate with both normal and heat-inactivated (56 °C, 30 min) serum. The microplates were incubated at 37 °C and the growth ability of each strain was assessed by measurement of OD₆₂₀ at 30 min intervals for 3 h. *Escherichia coli* strain K-12-711 and *Enterobacter cloacae* strain 046 (Keller *et al.*, 1998) were used as negative and positive controls, respectively. *P. alcalifaciens* strains were considered to be resistant to the lytic activity of serum when they were able to grow equally in normal (complement-active) and heat-inactivated (complement-inactive) serum at both 36 and 50 %.

Bacterial translocation. Four *P. alcalifaciens* strains that presented different invasive abilities and different resistance profiles to the lytic activity of serum were selected for the test. Translocation assays were performed as described by Koh & Silva (1996); each *P. alcalifaciens* strain was tested six times on different days, with a total of 24 assays. Briefly, Wistar-EPM rats were fasted for 2 days and given water *ad libitum*. All surgical procedures were performed under aseptic conditions and the animals received ether-inhalation anaesthesia. Following oroduodenal catheterization, 10 ml bacterial suspension in PBS, which contained 10¹⁰ c.f.u. ml⁻¹, was inoculated into the intestinal lumen between the duodenum and ileum. MLNs, spleen, liver and blood were collected 2 h after inoculation. Aliquots of minced organs were cultured in MacConkey's agar at 37 °C for 24 h. Bacterial counts are expressed as log₁₀ c.f.u. g⁻¹. Colonies were confirmed as inoculated bacteria by morphological characteristics and biochemical tests (Ewing, 1986). *Escherichia coli* strain HB 101, which is unable to translocate from the GI tract, was used as the negative control (Koh & Silva, 1996).

RESULTS AND DISCUSSION

The role of *P. alcalifaciens* as an enteric pathogen has been observed by several authors (Haynes & Hawkey, 1989; Guth *et al.*, 1996; Murata *et al.*, 2001) and as it has been reported that these bacteria can invade eukaryotic cell lines (Albert *et al.*, 1992; Guth *et al.*, 1996), such invasion has been proposed as a virulence mechanism. Although invasive ability seems to be strain-dependent (Guth *et al.*, 1996; Janda *et al.*, 1998; Sobreira *et al.*, 2001) and, in our previous study, it was suggested to be clonally determined (Guth *et al.*, 1999), several non-invasive *P. alcalifaciens* strains have also been isolated from patients with diarrhoea (Guth *et al.*, 1996). However, the potential pathogenic role of these strains has not been described.

Resistance to human serum and bacterial translocation from the GI tract have been considered to be important virulence characteristics for several species, including members of the family *Enterobacteriaceae* (Taylor, 1983; Sedman *et al.*, 1994; Sakamoto *et al.*, 2000). In the present study, we report for the first time that invasive and non-invasive *P. alcalifaciens* strains demonstrated resistance to human serum bactericidal activity and the ability to translocate from the GI tract.

Resistance to lytic activity of serum complement was observed in nine of 30 (30 %) *P. alcalifaciens* strains studied; these included five invasive and four non-invasive strains. Complement-resistant invasive and non-invasive *P. alcalifaciens* strains were able to survive and multiply in 36 and 50 % human serum, showing a growth rate 8–20 % higher than the initial inoculum (Fig. 1). All other *P. alcalifaciens* strains were susceptible to serum bactericidal activity.

By using a small-animal model to study bacterial translocation, we observed that *P. alcalifaciens* strains demonstrated the ability to translocate from the intestinal lumen to extraintestinal sites: they were detected in MLNs, liver and spleen (Table 1). Moreover, the results showed that invasion of eukaryotic cells and resistance to human serum bactericidal activity do not appear to be determining factors in the occurrence of *P. alcalifaciens* translocation.

Although a strong correlation between serum resistance and the ability of a variety of Gram-negative bacteria to invade and survive in the human bloodstream has been demonstrated in several studies (Roantree & Rantz, 1960; Taylor, 1983; Keller *et al.*, 1998), the survival of apparently serum-susceptible bacteria in the blood of bacteraemic patients has also been reported; this may be related to serum ineffectiveness at killing the infectious agent or bacterial circumvention of the serum bactericidal system by adoption of an intracellular location in the blood or reticuloendothelial system (Roantree & Rantz, 1960; Taylor, 1983).

Providencia spp. have been implicated as agents of bacteraemia and other extraintestinal infections (Cornaglia *et al.*, 1995; Pinto *et al.*, 1999), although *P. alcalifaciens* strains have

Table 1. Translocation of *P. alcalifaciens* to extraintestinal sites, invasion of HeLa cells and susceptibility to serum bactericidal activity

Strain	Invasion of HeLa cells*	Susceptibility to serum bactericidal activity†	Bacterial count (c.f.u. g ⁻¹)			
			MLN (n = 6)	Spleen (n = 2)	Liver (n = 2)	Blood (n = 2)
40448	+	R	8 × 10 ⁴	9 × 10 ³	8 × 10 ³	0
9938	+	S	2.8 × 10 ⁴	3 × 10 ³	8.9 × 10 ³	0
0181-9	—	S	4.8 × 10 ⁴	2 × 10 ³	1.8 × 10 ³	0
2101-15	—	R	2.2 × 10 ⁴	4.6 × 10 ³	1.1 × 10 ³	0

*+, Invasive; —, non-invasive.

†R, Resistant; S, susceptible.

been related mainly to diarrhoeal diseases (Guth *et al.*, 1996; Janda *et al.*, 1998; Murata *et al.*, 2001). Despite this knowledge, *P. alcalifaciens* is not recognized as a potential pathogen in most clinical laboratories and can therefore pass easily through routine screening, although a selective medium for *P. alcalifaciens* has been reported (Senior, 1997). The results obtained in the present study reinforce the importance of *P. alcalifaciens* as a pathogen by showing that some strains were able to translocate from the GI tract and survive the killing action of complement, giving support to the suggestion that these bacteria may have the potential to cause more disseminated infection.

ACKNOWLEDGEMENTS

This work was supported by Financiadora de Estudos e Projetos/Ministério da Ciência e Tecnologia/Programa de Apoio a Núcleos de Excelência (FINEP/MCT/PRONEX). A. B. R. V. received a research fellowship from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-PICDT).

REFERENCES

- Albert, M. J., Alam, K., Ansaruzzaman, M. & 7 other authors (1992). Pathogenesis of *Providencia alcalifaciens*-induced diarrhea. *Infect Immun* **60**, 5017–5024.
- Berg, R. D. (1999). Bacterial translocation from the gastrointestinal tract. In *Mechanisms in the Pathogenesis of Enteric Diseases*, vol. 2, pp. 11–30. Edited by P. S. Paul & D. H. Francis. New York: Plenum.
- Cornaglia, G., Frugoni, S., Mazzariol, A., Piacentini, E., Berlusconi, A. & Fontana, R. (1995). Activities of oral antibiotics on *Providencia* strains isolated from institutionalized elderly patients with urinary tract infections. *Antimicrob Agents Chemother* **39**, 2819–2821.
- Ewing, W. H. (1986). *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th edn. New York: Elsevier.
- Guth, B. E. C. & Perrella, E. (1996). Prevalence of invasive ability and other virulence-associated characteristics in *Providencia alcalifaciens* strains isolated in São Paulo, Brazil. *J Med Microbiol* **45**, 459–462.
- Guth, B. E. C., Irino, K. & Trabulsi, L. R. (1999). Clonal structure of *Providencia alcalifaciens* strains isolated from diarrhoeal stools in São Paulo, Brazil. *J Med Microbiol* **48**, 205–209.
- Haynes, J. & Hawkey, P. M. (1989). *Providencia alcalifaciens* and travellers' diarrhoea. *BMJ* **299**, 94–95.
- Janda, J. M., Abbott, S. L., Woodward, D. & Khashe, S. (1998). Invasion of HEp-2 and other eukaryotic cell lines by *Providenciae*: further evidence supporting the role of *Providencia alcalifaciens* in bacterial gastroenteritis. *Curr Microbiol* **37**, 159–165.
- Keller, R., Pedroso, M. Z., Ritchmann, R. & Silva, R. M. (1998). Occurrence of virulence-associated properties in *Enterobacter cloacae*. *Infect Immun* **66**, 645–649.
- Koh, I. H. J. & Silva, R. M. (1996). Novel *in vitro* small intestinal graft model for study of bacterial translocation in the rat. *Transplant Proc* **28**, 2667–2668.
- Mathan, M. M., Mathan, V. I. & Albert, M. J. (1993). Electron microscopic study of the attachment and penetration of rabbit intestinal epithelium by *Providencia alcalifaciens*. *J Pathol* **171**, 67–71.
- Murata, T., Iida, T., Shiomi, Y., Tagomori, K., Akeda, Y., Yanagihara, I., Mushiake, S., Ishiguro, F. & Honda, T. (2001). A large outbreak of foodborne infection attributed to *Providencia alcalifaciens*. *J Infect Dis* **184**, 1050–1055.
- Pelkonen, S. & Finne, J. (1987). A rapid turbidimetric assay for the study of serum sensitivity of *Escherichia coli*. *FEMS Microbiol Lett* **42**, 53–57.
- Pinto, V., Telenti, M., Bernaldo de Quiros, J. F. & Palomo, C. (1999). Two cases of fatal transfusion-associated bacterial sepsis provoked by *Providencia rettgeri*. *Haematologica* **84**, 1051–1052.
- Roantree, R. J. & Rantz, L. A. (1960). A study of the relationship of the normal bactericidal activity of human serum to bacterial infection. *J Clin Invest* **39**, 72–81.
- Sakamoto, K., Hirose, H., Ezaki, T., Kawamura, Y., Onizuka, A., Hayashi, M., Yamada, T. & Sago, T. (2000). Translocation of *Salmonella typhimurium* in rats; effect of enteral and parenteral nutrition. *Eur J Surg* **166**, 814–817.
- Sedman, P. C., Macfie, J., Sagar, P., Mitchell, C. J., May, J., Mancey-Jones, B. & Johnstone D. (1994). The prevalence of gut translocation in humans. *Gastroenterology* **107**, 643–649.
- Senior, B. W. (1997). Media for the detection and recognition of the enteropathogen *Providencia alcalifaciens* in faeces. *J Med Microbiol* **46**, 524–527.
- Sobreira, M., Leal, N. C., Magalhães, M., Guth, B. E. C. & Almeida, A. M. P. (2001). Molecular analysis of clinical isolates of *Providencia alcalifaciens*. *J Med Microbiol* **50**, 29–34.
- Tani, T., Hanasawa, K., Endo, Y., Kurumi, Y., Shiomi, H., Kodama, M., Kushima, R. & Hattori, T. (1997). Bacterial translocation as a cause of shock in humans: a report of two cases. *Surg Today* **27**, 447–449.
- Taylor, P. W. (1983). Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. *Microbiol Rev* **47**, 46–83.