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PLASMID COMPOSITION AND VIRULENCE-ASSOCIATED FACTORS OF Yersinia pestis ISOLATES FROM A PLAGUE OUTBREAK AT THE PARAIBA STATE, BRAZIL (1)

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SUMMARY

Pathogenic **Yersinia pestis** isolates were collected during a plague outbreak at the Paraiba State in 1986. The **Y. pestis** isolates were investigated for the presence of virulence-associated factors and plasmid content. All strains analysed were proficient in the expression of the VW and fraction 1 antigens, pigment adsorption and pesticin-fibronolysin-coagulase production. A similar plasmid profile composed by four plasmid with molecular weight of 60, 44, 14.9, and 6.4 Megadaltons (MD) was found in all strains. DNA cleavage with EcoRI restriction enzyme further demonstrated the uniform plasmid content of the **Y. pestis** isolates. Seven additional **Y. pestis** strains, previously isolated in the same region but in an endemic state, showed the same plasmid fingerprint. The lack of any detectable difference between epidemic and endemic isolates as well as the value of plasmid fingerprints in epidemiology of **Y. pestis** is discussed.

KEY WORDS: Yersinia pestis, plasmids, virulence-associated factors.

INTRODUCTION

When expressing its full virulence, **Yersinia pestis**, the causative agent of plague, is one of the most pathogenic organisms to the human being. The pathogenicity of **Y. pestis** is determined by the expression of at least six different virulence factors: production of the V and W antigens (VWa +) and fraction 1 antigen (Fra +), synthesis of pesticin-fibrinolysin-coagulase (Pfc +), ability to adsorb exogenous pigments (Pgm +), requirement for free calcium in growth medium at 37°C; and autoagglutination (BEESLEY et al., 1967; BRUBAKER, 1979; BRUBAKER, 1972; LAIRD & CAVANAUGH, 1980).

Several independent groups reported the involvement of plasmids in the expression of the virulence factors of **Y. pestis** (BEN-GURION & SHAFFERMAN, 1981; FERBER & BRUBAKER, 1981; PORTNOY & FALKOW, 1981; PORTNOY et al., 1983; PORTNOY & MARTINEZ, 1985; TSUKANO et al., 1986). Production of the V and W antigens and Ca^{2+} requirement at 37°C are coded by a 42-48 Mdal plasmid (BEN-GURION & SHAFFERMANN, 1981; FERBER & BRUBAKER, 1981; PORTNOY & FALKOW, 1981; PORTNOY et al., 1983). Production of pesticin and fraction 1 antigen are associated with

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a 6-7 Mdal and a 12-20 Mdal plasmids, respectively (FERBER & BRUBAKER, 1981; TSUKANO et al., 1986). Genetic evidences demonstrated that the Pgm + character is also coded by a plasmid even though it could never be isolated, probably due to its high molecular weight (TSUKANO et al., 1986).

MATERIALS AND METHODS

Bacterial strains

Y. pestis strains used in this work are listed in Table 1. **Escherichia coli** 39R861 was obtained at the National Collection Institute of Type Culture, Public Health Laboratory, Collin Dale, London.

Growth conditions and media

Strains were normally grown in liquid cultures with agitation in YT medium (2% tryptone, 1% yeast extract, 1% sodium chloride) at 28°C during 24 hours. Media used for detection of virulence factors were described elsewhere (BAHMANYAR & CAVANAUGH, 1976).

Isolation of Y. pestis from infected rodents or humans and maintenance of bacterial stocks

Samples of **Y. pestis** were recovered from spleen or blood (rodents) and from swollen limphonodes or wounds (humans) as described by ALMEIDA et al. (1989) according to BALTAZARD et al. (1956). Cultures were maintained at 4°C in agar stabs.

Detection of virulence-associated factors

Virulence-associated factors were determined essentially as described by BAHMANYAR & CAVANAUGH (1976). Pgm^+ cells grow as dark brown colonies on Congo-red agar at 28°C after 3 days. Vwa^+ cells were able to grow on magnesium oxalate agar at 28°C but not at 37°C. Production of fraction 1 antigen (Fra^+) was assayed by immunodiffusion on glass slides with polyclonal rabbit-anti F1 serum. The Pcf^+ character was evaluated by the coagulation of rabbit plasma at 26°C after mixing with **Y. pestis** cells.

DNA manipulation techniques

Plasmid DNA were isolated according the method of CASSE **et al.** (1979) as modified by R. R. BRUBAKER (personal communication). Briefly, cells grown in liquid YT medium were centrifuged (12.000 G, 4°C, 10 min) and washed once with 50 mM Tris-HCl, 10 mM EDTA (pH 8.0) buffer. Cell lysis was obtained after addition of the lysing mixture (1% SDS, 50 mM, 20 mM EDTA-Na, pH 12.45) to the cell suspension. To improve cell lysis the mixture was incubated at 37°C for 25 min and, then, 2M Tris HCl (pH 7.0) was added for neutralization followed by 5M NaCl. After extraction with 3% NaCl-saturated phenol, the DNA was ethanol precipitated from the supernatant. An additional purification step upon cesium chloride gradient centrifugation was necessary for cleavage with restriction enzymes.

Restriction endonuclease digestions were carried out under conditions described by MANIATIS et al. (1982). Cleaved and uncut plasmid DNA were subjected to electrophoresis in 5 mm thick horizontal 1% or 0.8% agarose gels, respectively, in Tris-acetate or Tris-borate (restriction fragments) buffers at 60 V. Gels were stained with ethidium bromide and photographed under UV light illumination.

RESULTS

Isolation of Y. pestis strains and detection of virulence-associated factors

Y. pestis is endemic at the Paraiba State since its introduction into the continent at the end of the nineteenth century. Since that time sporadic small epidemic plague outbreaks are registered in this region roughly at every ten years. During the spring of 1986, after a quiescent period of more than 10 years, a plague outbreak bursted at the Paraiba State spreading over several counties and causing five deaths and 48 serological confirmed infected persons (ALMEIDA et al., 1989).

A total of 19 **Y. pestis** samples were obtained from rodents (16 strains) and humans (3 strains) during the epidemic outbreak. These strains were investigated in relation to the expression

of the virulence-associated factors: production of fraction 1 antigen and coagulase, pigmentation and calcium dependence were assayed in each of the **Y. pestis** samples isolated. All strains proved to be proficient in all virulence-associated factors tested (Table 1).

Seven **Y. pestis** strains previously isolated from rodents in the same area, but in endemic conditions, were analysed for comparison purposes. Table 1 shows that these strains exhibit the same pattern of virulence-associated factors displayed by the epidemic isolates.

Plasmid content of the Y. pestis **strains**

Plasmid DNA of all 26 **Y. pestis** strains were isolated and compared after electrophoresis on agarose gels. All strains harbored a similar four plasmid set with molecular weights of 60, 44, 14.9 and 6.4 Mdal in spite of differences in the isolation periods, mammalian host and infection status, i. e., endemic or epidemic conditions (Figure 1).

To further evaluate the homology among the plasmid DNA of the several **Y. pestis** isolates,

TABLE 1

Yersinia pestis strains analysed and detection of virulence associated factors.

Strain No.	Locality (county)	Isolation date	Source	Virulence-associated factors[a]				Plasmids[b]
				Fra^+	Vwa^+	Pcf^+	Pgm^+	
810	Natuba	1979	Rodent	+	+	+	+	+
811	Natuba	1979	Rodent	+	+	+	+	+
817	Natuba	1979	Rodent	+	+	+	+	+
820	Natuba	1979	Rodent	+	+	+	+	+
843	Natuba	1980	Rodent	+	+	+	+	+
851	Natuba	1980	Rodent	+	+	+	+	+
860	S. S. Félix	1982	Rodent	+	+	+	+	+
862	Solânea	1986	Human	+	+	+	+	+
863	B. S. Rosa	1986	Human	+	+	+	+	+
864	Solânea	1986	Rodent	+	+	+	+	+
865	Solânea	1986	Rodent	+	+	+	+	+
866	Solânea	1986	Rodent	+	+	+	+	+
867	Solânea	1986	Rodent	+	+	+	+	+
868	Solânea	1986	Rodent	+	+	+	+	+
869	Solânea	1986	Rodent	+	+	+	+	+
870	Solânea	1986	Rodent	+	+	+	+	+
871	Solânea	1986	Rodent	+	+	+	+	+
872	Solânea	1986	Rodent	+	+	+	+	+
873	Solânea	1986	Rodent	+	+	+	+	+
875	Solânea	1986	Rodent	+	+	+	+	+
876	Solânea	1986	Rodent	+	+	+	+	+
877	Solânea	1986	Rodent	+	+	+	+	+
878	Solânea	1986	Rodent	+	+	+	+	+
879	Solânea	1986	Rodent	+	+	+	+	+
880	Solânea	1986	Rodent	+	+	+	+	+
881	Remígio	1986	Human	+	+	+	+	+

a — Fra^+ — production of the F1 antigen; Vwa^+ — production of the V and W antigens; Pcf^+ — synthesis of pesticin, fibrinolysin, coagulase; Pgm^+ — ability to adsorb exogenous pigments.
b — plasmids found: 60, 44, 14.9, and 6.4 MD.

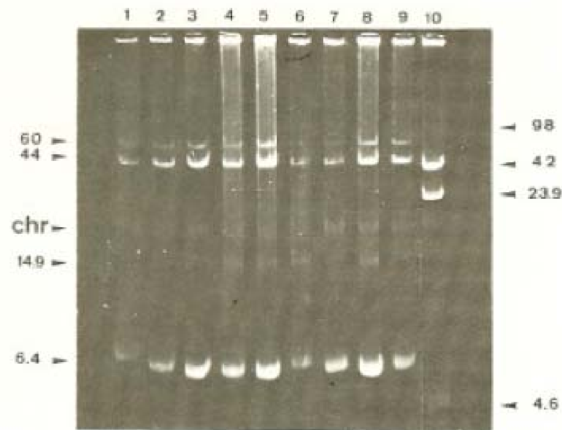

Fig. 1 — Agarose gel electrophoresis of plasmid DNA from **Y. pestis** isolates. 1 — strain 810; 2 — strain 811; 3 — strain 817; 4 — strain 862; 5 — strain 863; 6 — strain 881; 7 — strain 864; 8 — strain 871; 9 — strain 874; 10 — **E. coli** 39R861. Molecular weight marker plasmids of **E. coli** 39R861 are indicated on the right.

restriction endonuclease reactions of the total plasmid DNA content were carried out with all samples. Figure 2 shows the result of the cleavage reaction with plasmid DNA of some representative strains. EcoRI cleaved plasmid DNA of the **Y. pestis** strains resulted in a common fragment profile. These results suggest that the **Y. pestis** isolates from the plague outbreak at the Paraiba State were quite uniform in terms of their plasmid DNA content.

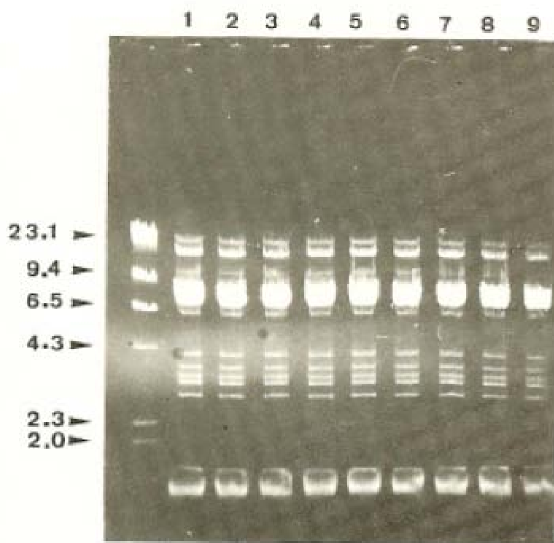

Fig. 2 — EcoRI restriction endonuclease digestion pattern of plasmid DNA isolated from **Y. pestis** strains. 1 — 810; 2 — 811; 3 — 817; 4 — 862; 5 — 863; 6 — 881; 7 — 864; 8 — 871; 9 — 874. Phage lambda DNA digested with Hind III was used as molecular weight marker and is indicated on the left.

DISCUSSION

The analysis of plasmid content as a tool in the epidemiology of bacteria has received increasing attention from researches in recent years (MAYER, 1988). In several cases it was possible to elucidate the origin of specific outbreaks or to trace the dispersal routes of certain strains by comparing plasmids or restriction fragment profiles as, for example, in **Salmonella, Escherichia coli,** and **Neisseria gonorrhoea** (BRUNER et al., 1983; TIETZE & TSCHAPE, 1983; YEUNG et al., 1986).

Analysis of plasmid fingerprints as an approach to the epidemiology of **Yersinia** was mainly restricted to the virulence plasmid of **Y. enterocolitica** and **Y. pseudotuberculosis** (ISHIGURO et al., 1985; WARTENBERG et al., 1988). Even though a clear molecular relatedness exists among the calcium-dependence coding plasmids of **Yersinia,** nothing is known about the potential use of the **Y. pestis** specific plasmids as epidemiological markers (PORTNOY & FALKOW, 1981; PORTNOY & MARTINEZ, 1985). The small differences in the reported molecular weights of plasmids coding for virulence-associated genes in **Y. pestis** could suggest a possible diversity among isolates from different places or periods (PORTNOY et al., 1983; FEBER & BRUBAKER, 1981; TSUKANO et al., 1986).

Our results demonstrated a uniform expression of virulence-associated factors and similar plasmids in **Y. pestis** samples obtained at different places, periods and mammalian hosts. The identical EcoRI restriction pattern confirmed the probable identity of the plasmidial content in these isolates. Such results would indicate a probable common origin of the **Y. pestis** strains established at the Paraiba State, which is part of a broader plague focus represented by the Borborema plateau (ALMEIDA et al., 1985; ALMEIDA et al., 1981).

The identical plamid profile and virulence-associated factors in the **Y. pestis** samples isolated in endemic or epidemic conditions would

suggest that epidemic isolates have no plasmid — associated special feature providing them with an increased virulence.

Thus, it is conceivable that the rise of a plague outbreak is probably more related to deterioration of sanitation conditions or to ecological alterations, which would result in unusual increases in the flea or rat populations, rather than to the selection of strain expressing higher virulence.

Plague is a serious public health concern in Brazil. Several active endemic focuses are still present in the Northeast region of the country where health conditions and sanitation are worsening due to uncontrolled population growth and low incomes. The molecular characterization of **Y. pestis** strains in this region could give significant contributions to the identification of specially pathogenic strains or to elucidate dispersal routes of future epidemic outbreaks. The analysis of **Y. pestis** isolates from different plague focuses in Brazil is under investigation in our laboratory.

RESUMO

Composição plasmidial e fatores associados à virulência em cepas de Yersinia pestis **de um surto de peste no Estado da Paraíba, Brasil.**

Cepas patogênicas de **Yersinia pestis** foram coletadas durante um surto de peste no Estado da Paraíba em 1986. Os isolados de **Y. pestis** foram analisados quanto a presença de fatores associados à virulência e conteúdo plasmidial. Todas as linhagens analisadas foram proficientes na expressão dos antígenos VW e fração 1, além de possuírem capacidade de adsorção de pigmentos e produção de pesticina fibrinolisina-coagulase. Um perfil plasmidial semelhante composto por quatro plasmídeos com peso molecular de 60, 44, 14.9, e 6.4 MD foi encontrado em todas as linhagens. A clivagem do DNA plasmidial com a enzima de restrição EcoRI demonstrou o conteúdo plasmidial uniforme dos isolados de **Y. pestis.** Sete outras linhagens de **Y. pestis,** isoladas previamente no mesmo local mas em condição endêmica, mostraram o mesmo perfil plasmidial. A falta de diferenças entre os isolados epidêmicos e endêmicos assim como o uso do perfil plasmidial na epidemiologia de **Y. pestis** é discutida.

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