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Subtyping Brazilian *Yersinia pestis* strains by pulsed-field gel electrophoresis

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ABSTRACT. We subtyped Brazilian *Yersinia pestis* strains by pulsed-field gel electrophoresis (PFGE). This was done with 22 Brazilian *Y. pestis* strains: 17 from an outbreak and 5 from endemic routine surveillance. The strains were divided into 2 groups (I and II), 8 subgroups (A-H) and 19 PFGE profiles or pulsotypes. PFGE did not separate outbreak from non-outbreak strains, as identical pulsotype patterns were found among outbreak strains and strains obtained from surveillance. However, it was able to detect intraspecific genetic diversity among Brazilian strains. This PFGE technique was able to differentiate a homogeneous group of Brazilian *Y. pestis* strains.

Key words: *Yersinia pestis*; Pulsed-field gel electrophoresis; Molecular epidemiology

INTRODUCTION

Yersinia pestis is the causative agent of plague, a zoonotic disease of rodents, mainly transmitted to humans by infected flea bites. After three major pandemics responsible for millions of deaths, the zoonosis still occurs in South and North America, Asia, and especially Africa. Currently, it is considered a reemerging disease and the agent is classified as class 3 biohazard and category A bioterrorism (Perry and Fetherston, 1997; Stenseth et al., 2008).

Y. pestis was introduced to Brazil by the sea route during the third pandemic, in 1899, and became established among the native rodents in rural areas in several ecological complexes (WHO, 1965; Baltazard, 2004). Apart from sporadic epidemics, from 20 to 100 cases were annually reported until the mid-1980s, when all the foci tended to quiescence (WHO, 2006). During a plague outbreak in September 1986 in the Chapada da Borborema focus (Paraíba State) in the northeastern region (Figure 1), 20 *Y. pestis* strains were isolated from humans (3) and rodents (17): *Rattus rattus* (6), *Necromys lasiurus* (6), *Oryzomys subflavus* (4), and *Holochillus sciurus* (1) (de Almeida et al., 1989). These cultures are deposited in the *Yersinia* spp Collection (Fiocruz - CYP) maintained by the National Service for Reference in Plague from the Centro de Pesquisas Aggeu Magalhães, FIOCRUZ-PE (SRP/FIOCRUZ-PE), Recife, PE, Brazil (Rocha et al., 2009). The strains appeared to be homogenous regardless of their origin concerning their plasmid content (Leal et al., 1989, 2000), outer membrane protein profile (Abath et al., 1989), and RAPD and PCR - ribotyping analysis (Leal NC and Sobreira MBS, unpublished results). Currently, studies on the molecular epidemiology of *Y. pestis* have been hampered due to the great similarity of isolates (Vogler et al., 2011). Pulsed-field gel electrophoresis (PFGE) is a highly discriminatory and versatile method and has been used in bacterial epidemiological and evolutionary studies and it proved useful for molecular subtyping of many bacteria whereas other molecular methods showed low discriminatory ability (Tenover et al., 1995).

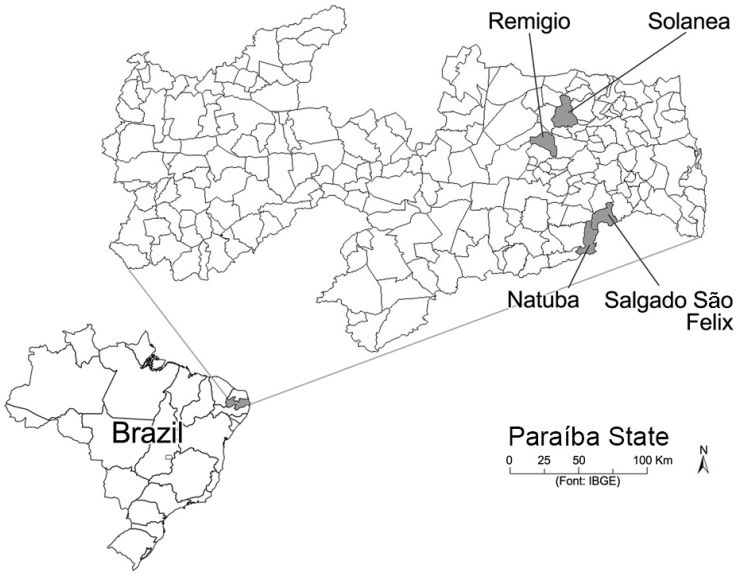

Figure 1. Map of Brazil and Paraíba State showing the counties from the *Yersinia pestis* strains originated.

The purpose of this study was to perform a molecular subtyping by PFGE of Brazilian *Y. pestis* strains: 17 from an outbreak and 5 from endemic routine surveillance. The results demonstrated intraspecific genetic diversity in the samples studied previously considered homogeneous. Therefore, PFGE allows large-scale analysis of strains and identify major genetic variation among the strains.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Two sets of *Y. pestis* strains from the FIOCRUZ - CYP collection (SRP/FIOCRUZ-PE), Recife, PE, Brazil): 17 from the 1986 outbreak in the Paraíba State, Brazil (de Almeida et al., 1989) and 5 from the endemic area routine plague program surveillance from 1978-1982 (de Almeida et al., 1985) (Table 1). They are maintained in peptone agar stab tubes at 4°C in a biosafety level 3 laboratory. Outbreak strains were identified as P (plague), PB (state of origin), and sequential number. Other strains were identified as P.Exu (laboratory location), and sequential number.

Table 1. Host source and spatial and temporal distribution of the *Yersinia pestis* strains studied.

Year	Locality		Source					Total
	Municipality	Sitio/Farm	Man	Rodents				
				Rr	Nl	Os	Hs	
1986	Solanea	Bacalhau	-	1	-	2	-	3
		Cinco Lagoas	-	2	1	-		3
		Pedra d'Agua	1	1	2	1	1	6
		Lagoa do Serrote	-	-	1	-	-	1
		Cinco Estrelas	-	-	1	-	-	1
		Sete Lagoas	-	-	-	1	-	1
		Valério	-	1	-	-	-	1
	Remigio	Serrinha	1	-	-	-	-	1
1979/1982	Natuba	Estivas	-	-	-	1	-	1
		Tauá	-	-	1	-	-	1
		Salgadinho	-	-	1	-	-	1
		Gito	-	-	1	-	-	1
	Salgado São Felix	Engenho Covão	-	-	1	-	-	1
Total			2	5	9	5	1	22

Rr = *Rattus rattus*; Nl = *Necromys lasiurus*; Os = *Oryzomys subflavus*; Hs = *Holochilus sciureus*.

Three unrelated strains (P.Exu 340, P.Peru 375 and P.CE 882) and 5 derived cultures obtained through multiple serial subcultures of the parental strains (Leal-Balbino et al., 2004) were included to assess the stability of PFGE profiles with subculture. The technique's reproducibility was evaluated by PFGE analysis of the strain P.PB 866 three times. All *Y. pestis* strains studied belong to biovar Orientalis (glycerol negative and nitrate positive) on the basis of their abilities to ferment glycerol and to reduce nitrate as primary determinants in assigning biotype (Perry and Fetherston, 1997).

The cultures were inoculated into brain heart infusion broth (Himedia, Vadhani Industrial Estate, Mumbai, India) and incubated at 28°C for up to 7 days. Culture growth was confirmed by the plague phage test (Karimi, 1978), plated on blood agar base (Himedia, Vadhani Industrial Estate) and incubated at 28°C for up to 5 days.

PFGE protocol

Genomic DNA of the *Y. pestis* strains was prepared in agarose plugs following a CDC Pulsenet protocol (www.cdc.gov/pulsenet/protocols.htm), with some modifications. *Y. pestis* cells grown on blood agar base plates for 48 h at 28ºC were suspended in cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0) and adjusted to an absorbance of 1.0 at 600 nm in a spectrophotometer (Biotech Photometer, WPA, Cambridge, UK). Pulsed-field certified agarose (2%; Bio-Rad, Hercules, CA, USA) was used for genomic DNA plug preparation. Cell lysis within agarose plugs was carried out in lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0, 1% sarcosyl) and proteinase K at 54°C for 2 h under constant and vigorous shaking. Digestion was carried out at 37°C with 20 U/µL *Asc*I enzyme at 37°C for 6 h in the enzyme buffer supplied by the manufacturer. Restricted plugs were washed at 50°C with 2X sterile ultrapure water and 4X TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), 10 min each. Digested fragments were separated using CHEF-DR III, Bio-Rad system (Countour-Clamped Homogeneous Electric Fields/Bio-Rad) on 1% Seakem Gold agarose (Lonza, Rockland, ME, USA) gels and running buffer of 0.5% TBE at 14°C with a ramping time of 4.5 V/cm for 22 h.

Lambda PFGE marker (New England BioLabs, Country RD Ipswich, MA, USA) was the molecular weight standard. Ethidium bromide (1 µg/mL)-stained bands were visualized under UV and the images captured by the 1-D Image Analysis Software, version 3.5 (Kodak Digital Science, New Haven, CT, USA).

PFGE pattern analysis

The BioNumerics program (Applied Maths, Sint Martens Latem, Belgium) was used to determine the presence or absence of bands and the similarity between samples was inferred using the BioNumerics program version 6.1 (Applied Maths). A dendrogram based on PFGE data was constructed with the Dice coefficient (Dice, 1945), using UPGMA at 1.5% tolerance, and the PFGE protocol discriminatory ability was determined by the Simpson diversity index (discrimination index; Hunter and Gaston, 1988).

RESULTS

PFGE protocol optimization

Protocol optimization that provided consistent and reproducible results comprised the following modifications (Table 2).

Table 2. Comparison between the standard and optimized protocols for pulsed-field gel electrophoresis typing of *Yersinia pestis*.

Procedure	Standard protocol (CDC)	Optimized protocol
Bacterial concentration	610 nm wavelength, absorbance of 1.35	610 nm wavelength, absorbance of 1.0
Agarose plugs preparation	1% Agarose SeaKem Gold	2% Pulsed-Field Certified Agarose Bio-Rad
Restriction digestion	*Asc*I: 40 U; 4 h	*Asc*I: 20 U; 6 h
Running conditions	6 V/cm	4.5 V/cm

PFGE protocol performance

Discriminatory ability

The PFGE protocol appeared to be highly efficient in discriminating between *Y. pestis* strains (Simpson diversity index, 0.93). *Asc*I restriction generated neat and clear bands. DNA fragments ranging from 50 to 300 kb were considered for analysis (Figure 2). The dendrogram based on PFGE divided the strains into 2 clusters or groups (I and II) at a 70% similarity cutoff, and 8 subgroups (A-H) at an 80% similarity cutoff and allowed the distinction of 19 PFGE profiles or pulsotypes (Figure 2). Cluster I grouped together 7 samples (1 human and 6 outbreak and non-outbreak rodent strains) subdivided into 2 subgroups (A and B) and 6 PFGE profiles or pulsotypes. Cluster II grouped together 15 isolates (1 human and 14 outbreak and non-outbreak rodent strains) subdivided into 6 subgroups (C-H) and 13 PFGE profiles or pulsotypes. All (A-G subgroups) but subgroup H included 2 to 4 isolates (19 PFGE profiles or pulsotypes) (Figure 2).

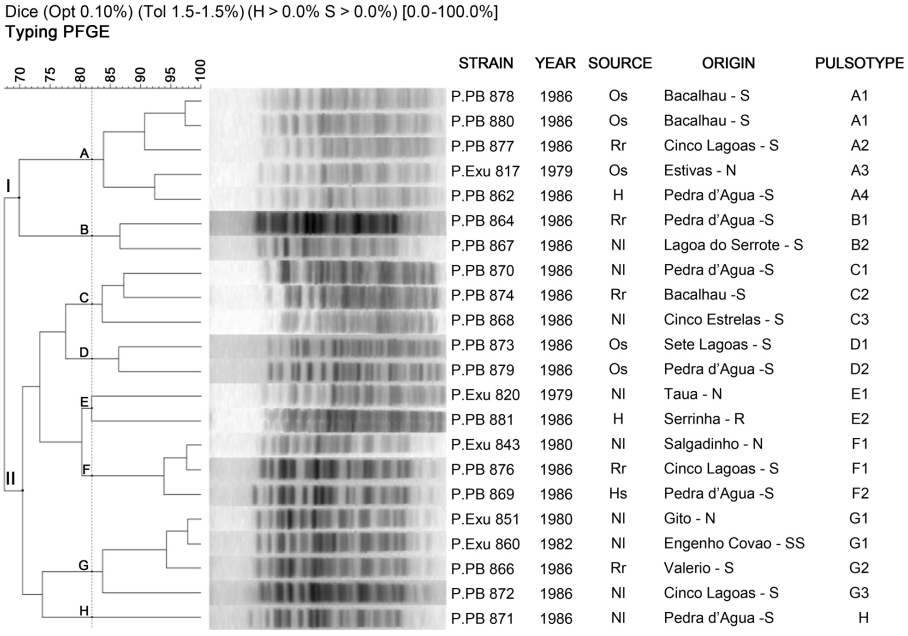

Figure 2. Dendrogram generated by the BioNumerics software from pulsed-field gel electrophoresis (PFGE) cuts by *Asc*I on 22 Brazilian *Yersinia pestis* strains. N = Natuba; R = Remigio; S = Solanea; SS = Salgado de São Felix municipalities. Os = *Oryzomys subflavus*; Nl = *Necromys lasiurus*; Rr = *Rattus rattus* rodent species; Hs = *Holochilus sciureus*; H = humans.

PFGE profile stability and technique reproducibility

The stability of the PFGE profiles was investigated by the analysis of serial subcultures of the strains P.Exu 340, P.Peru 375 and P.CE 882. Each strain exhibited a unique PFGE

profile, and derived cultures revealed the same parental profile (data not shown). The technique's reproducibility was evaluated by repeating the PFGE analysis of strain P. PB 866 three times, where an identical profile was generated in the three assays (data not shown).

Host distribution

Different pulsotypes were found in strains from identical hosts: the two human strains displayed one pulsotype each (A4, E2), and with a few exceptions, rodent strains also displayed one pulsotype each, e.g., 2 *R. rattus* from Sitio Cinco Lagoas/Solanea (A2, F1) and 2 *N. lasiurus* from Sitio Pedra d'Agua/Solanea (C1, H).

Identical pulsotypes occurred only twice among identical rodent sources: pulsotype A1 among two *O. subflavus* and G1 among two *N. lasiurus* but from different counties and year (Figure 2).

Spatial distribution

Different pulsotypes were found in strains from the same geographic area. Outbreak strains came from 8 rural sites (called sitios or farms) from 2 municipalities or counties (Figure 1) and non-outbreak strains from 5 sites from 2 municipalities. Most of the strains analyzed (16) were from 7 sites from Solanea (Figure 1) and distributed among 7 subgroups (A-D; F-H) and 15 pulsotypes (Figure 2).

Human and rodent outbreak strains from the same place did not match, e.g., 1 human and 5 rodent strains from Sitio Pedra d'Agua/Solanea displayed 6 pulsotypes (A4, B1, C1, D2, F2, H).

Identical pulsotypes only occurred among 2 rodent outbreak strains (A1) from the same place (Sítio Bacalhau/Solanea) and 2 non-outbreak rodent strains but from 2 different places (Sitio Gito/Natuba and Eng. Covão/Salgado de São Felix) and years (G1).

Temporal distribution

Identical pulsotypes were found in different years; outbreak strains were not separated from non-outbreak strains by their PFGE patterns: pulsotype F1 occurred in 1980 and 1986 (P.Exu 843 and P.PB 876) in different rodent hosts and counties, and pulsotype G1 in 1980 and 1982 (P.Exu 851 and P.Exu 860) from the same host but different counties (Figure 2).

DISCUSSION

Among the several typing molecular methods, PFGE macrorestriction analysis has been shown to be highly valuable for grouping and differentiating many pathogenic bacteria. Although PFGE sensitivity and discriminatory ability depend on the restriction enzyme and organism studied, its high performance has become valuable for typing many bacteria and discriminating *Y. pestis* strains (Guiyoule et al., 1994; Huang et al., 2002; Revazishvili et al., 2008; Zhang et al., 2009).

Our study did not separate outbreak from non-outbreak strains. Identical pulsotype patterns were found among outbreak strains as well as in those obtained during surveillance activi-

ties (Figure 2). Outbreak strains were isolated within a short period (September to December 1986) and the endemic strains were isolated 10 years earlier from July 1979 to August 1982. These strains came from a small area (~145 km) located in the ecological complex Chapada da Borborema, the largest but not the most active Brazilian plague focus (Baltazard, 2004).

Analysis of multiple-locus variable-number of tandem repeats has demonstrated intraspecific genetic diversity among Brazilian plague strains. This analysis establishes a relative correlation among genetic groups, which is related to the temporal and geographic origin of the isolates (Oliveira et al., 2012).

PFGE also revealed genetic heterogeneity among the 22 strains analyzed. Most strains from the same spatial, temporal and host origin displayed different pulsotypes. Surprisingly, strains from the same rodent species during the outbreak displayed different profiles; the same profile would be expected in strains closely related epidemiologically. These different *Y. pestis* populations may reflect microevolution of the invading strain due to environmental pressure experienced while spreading inland from the coast. In fact they are all descendant from the strain disseminated worldwide during the third pandemic, biovar Orientalis (glycerol negative and nitrate positive) (Perry and Fetherston, 1997).

On the other hand, identical patterns for strains from different localities and time periods confirm their common origin, in line with a single introduction of *Y. pestis* in Brazil (WHO, 1965; Baltazard, 2004).

PFGE pattern alterations associated with serial subculturing of *Y. pestis* isolates have been reported (Lucier and Brubaker, 1992; Guiyoule et al., 1994). Leal-Balbino et al. (2004, 2006) and Leal et al. (2000) observed spontaneous alterations in the genome (plasmid and chromosomal) of Brazilian *Y. pestis* as a result of prolonged storage and handling in the laboratory. The stability of the PFGE profiles was investigated by the analysis of serial subcultures of three strains. Each strain exhibited a unique PFGE profile and derived cultures revealed the same parental profile.

The generally accepted explanation for hypervariability in PFGE types of *Y. pestis* strains is intragenomic recombination between insertion sequence elements, usually leading to inversion of genome segments rather than deletion, although deletion can obviously occur (Lucier and Brubaker, 1992). Pigmentation segment deletion and plasmid deletions in Brazilian strains have been reported (Almeida et al., 1994; Leal et al., 2000; Leal-Balbino et al., 2006). Moreover, Guiyoule et al. (1994) found a pigmentation deletion *in vitro*, which gave a new PFGE type, although they did not suggest that all new PFGE types were due to this deletion.

In Brazil, since 2005, there have been no laboratory-confirmed human cases and the last *Y. pestis* isolation occurred in 1997 in the Ibiapaba focus (Ceará State); however, serological surveys continuously detect plague antibodies in sentinel animals in plague areas, suggesting that they remain active (Leal and Almeida, 1999; Aragão et al., 2009; WHO, 2006, 2009). Quiescent periods characterized by the absence or occurrence of rare human cases, may be misleading, since plague may reappear after many apparent control decades and its spreading potential by international trade or bioterrorism must be considered (Duplantier et al., 2005; Gage and Kosoy, 2005; WHO, 2006, 2009). Therefore, the knowledge of the characteristics of local strains and the availability of effective and rapid typing methods are essential to elucidate the origin of new strains, whether by natural focal emergence, reemergence, coming from other countries, or bioterrorism action, as well as to allow health surveillance and effective

control measures (Stenseth et al., 2008).

In conclusion, in the present study, we demonstrated the usefulness of PFGE for discriminating Brazilian *Y. pestis* isolates. The results obtained by the analysis of two sets of strains (17 from an outbreak and 5 during the endemic period) revealed intraspecific genetic diversity in the samples studied, which will provide insight into the homogeneity of the group of Brazilian *Y. pestis* strains.

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