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Typing of *Yersinia pestis* isolates from the state of Ceará, Brazil

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Aims: To investigate whether modifications in *Yersinia pestis* isolates from three plague foci from the state of Ceará, Brazil, had occurred over the years as a consequence of genetic adaptation to the environment.

Methods and Results: The isolates were studied with respect to susceptibility to antimicrobial drugs, plasmid and protein profiling, pigmentation on Congo red-agar plates, and the presence of some pathogenicity genes using PCR. Most of the expected virulence markers were detected in the cultures examined. There was no evidence of any alteration that could be associated with their origin (patients, rodents and fleas) or period of isolation (1971–1997).

Conclusions, Significance and Impact of the Study: Phenotypic or genotypic changes were not detected in the cultures examined. However, the results obtained will serve as a reference to follow the evolution of *Y. pestis* in these foci.

INTRODUCTION

Yersinia pestis is the causative agent of plague, which has been responsible for three well-characterized pandemics during the Christian era. In spite of scientific and technological development, plague has never been eradicated and remains widespread around the world (Perry and Fetherston 1997; WHO 2000).

Plague arrived in Brazil by sea during the last pandemic which began in the 19th century in China. Although control measures were promptly undertaken, it's spread inland and establishment among wild rodents in most of the states of the north-east region could not be prevented (WHO 1965).

In the State of Ceará, plague was first registered in 1900 in Fortaleza, the capital city, from where it spread and became concentrated in three mountain areas: Ibiapaba Mountain, Baturité Mountain and Araripe Plateau. The incidence of human plague and the occurrence of epizootics declined in these areas. However, residual plague activity has been detected consistently among sentinel animals, suggesting that the infection could re-emerge at any moment if surveillance and control activities are neglected (Aragão *et al.* 2002).

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The epidemiological characterization of plague areas is highly significant for the development of efficient plague control strategies. Therefore, the typing of *Y. pestis* strains to determine their sensitivity to antimicrobial drugs, their plasmid content and pathogenic potential is very important. Furthermore, this knowledge would allow the detection of any emerging strains that might be introduced accidentally from another country, or by bioterrorism.

The purpose of this work was to perform a phenotypic and molecular characterization of *Y. pestis* isolates from patients, rodents and fleas from the three plague foci in the State of Ceará, to determine whether any modification of the plague bacillus had occurred over the years as a consequence of genetic adaptation to the environment.

MATERIALS AND METHODS

Bacterial samples and culture conditions

The study involved 53 *Y. pestis* isolates from the State of Ceará, originating from different hosts during different periods (Table 1). The isolates were grown in brain heart infusion broth (Difco) at 28°C from 24 to 72 h and plated on blood agar base (Difco). The pigmentation phenotypes of the isolates were determined on Congo red-agar (CRA) as recommended by Bahmanyar and Cavanaugh (1976). *Yersinia pestis* EV76 (the vaccinal Girard-Robic strain),

Table 1 Origin of the isolates, plasmid content, PCR amplification of plasmid and chromosomal genes, and percentage of pigmented colonies (Pgm +) on Congo red-agar

	Origin			Presence of plasmids, extra DNA bands/ PCR amplification of the genes				
Samples	Source	Foci	Year	pYV *lcrV*	pPst/*pla*	pFra/*caf1*	> 90 kb	Pgm + * %
PExu 540	man	Ibiapaba	1971	+/+	+/+	+/+	–	100
PExu 554	man	Ibiapaba	1971	+/+	+/+	+/+	+	100
PExu 509	man	Ibiapaba	1971	+/+	+/+	+/+	–	75
PExu 527	man	Ibiapaba	1971	+/+	+/+	+/+	–	75
PExu 539	man	Ibiapaba	1971	+/+	+/+	+/+	–	100
PExu 541	man	Ibiapaba	1971	+/+	–/–	+/+	–	100
PExu 542	man	Ibiapaba	1971	+/+	+/+	–/+	–	100
PExu 547	man	Ibiapaba	1971	+/+	–/–	–/+	–	75
PExu 550	man	Ibiapaba	1971	+/+	+/+	+/+	–	100
PExu 553	man	Ibiapaba	1971	+/+	–/–	+/+	–	100
PExu 538	man	Ibiapaba	1971	+/+	+/+	+/+	–	75
PExu 555	man	Ibiapaba	1972	+/+	+/+	+/+	–	100
PExu 557	man	Ibiapaba	1972	+/+	–/–	+/+	–	100
PExu 558	man	Ibiapaba	1972	+/+	+/+	+/+	+	100
PExu 556	man	Ibiapaba	1972	+/+	+/+	+/+	–	100
PCE 01	man	Ibiapaba	1978	+/+	+/+	+/+	–	0
PExu 789	man	Ibiapaba	1978	+/+	+/+	+/+	–	100
PExu 804	man	Ibiapaba	1978	+/+	+/+	+/+	–	100
PExu 797	rr	Ibiapaba	1978	+/+	+/+	+/+	–	100
PExu 792	man	Ibiapaba	1978	+/+	+/+	+/+	–	25
PExu 801	man	Ibiapaba	1978	+/+	–/–	–/+	–	100
PExu 802	man	Ibiapaba	1978	+/+	+/+	+/+	–	100
PExu 807	man	Ibiapaba	1978	+/+	+/+	+/+	–	100
PCE 03	bl	Ibiapaba	1979	+/+	+/+	+/+	–	50
PCE 04	bl	Ibiapaba	1979	+/+	+/+	+/+	–	100
PCE 05	bl	Ibiapaba	1979	+/+	+/+	+/+	–	100
PCE 06	n/i	Ibiapaba	1979	+/+	+/+	+/+	–	50
PCE 07	bl	Ibiapaba	1979	+/+	+/+	+/+	–	100
PExu 835	bl	Ibiapaba	1979	+/+	+/+	+/+	–	100
PExu 833	bl	Ibiapaba	1979	+/+	+/+	+/+	+	75
PExu 842	bl	Ibiapaba	1979	+/+	+/+	+/+	+	75
PExu 809	man	Ibiapaba	1979	+/+	+/+	+/+	–	100
PExu 861	bl	Ibiapaba	1980	+/+	+/+	+/+	–	75
PCE 09	bl	Ibiapaba	1980	+/+	+/+	+/+	–	100
PCE 11	bl	Ibiapaba	1982	+/+	+/+	+/+	–	100
PCE 14	*pi*	Ibiapaba	1982	+/+	+/+	+/+	–	50
PCE 16	bl	Ibiapaba	1982	+/+	+/+	+/+	–	100
PCE 18	bl	Ibiapaba	1982	+/+	+/+	+/+	–	100
PCE 19	bl	Ibiapaba	1982	+/+	+/+	+/+	–	100
PCE 20	bl	Ibiapaba	1982	+/+	+/+	+/+	–	50
PCE 21	man	Ibiapaba	1982	+/+	+/+	+/+	–	100
PCE 22	bl	Ibiapaba	1982	+/+	–/–	–/+	–	50
PExu 559	pbj	Ibiapaba	1982	+/+	+/+	+/+	+	100
PCE 29	bl	Ibiapaba	1983	+/+	+/+	+/+	–	100
PCE 30	cc	Ibiapaba	1986	+/+	+/+	+/+	+	100
PCE 882	man	Ibiapaba	1997	+/+	+/+	+/+	–	100
PExu 795	man	Baturité	1978	+/+	+/+	+/+	–	100
PExu 796	man	Baturité	1978	+/+	+/+	+/+	–	75
PCE 17	bl	Baturité	1982	+/+	+/+	+/+	–	0
PCE 24	rr	Baturité	1982	+/+	+/+	+/+	–	50

Table 1 (*Continued*)

Samples	Origin			Presence of plasmids, extra DNA bands/ PCR amplification of the genes				
	Source	Foci	Year	pYV *lcrV*	pPst/*pla*	pFra/*caf1*	> 90 kb	Pgm + * %
PCE 13	bl	Baturité	1982	+/+	+/+	+/+	–	50
PCE 28	bl	Baturité	1983	+/+	+/+	+/+	–	50
PExu 683	rr	Araripe	1974	+/+	+/+	+/+	+	100

*All the cultures amplified the genes *psa*, *irp2*, *psn* (*fyuA*).
rr: *Rattus rattus*, bl: *Bolomys lasiurus* and cc: *Calomys callosus* are rodents; n/i: non-identified rodent; pi: *Pulex irritans* and pbj: *Polygenis bolhsi jordani* are fleas.

Yersinia enterocolitica Ye02 and *Escherichia coli* 25922 (ATCC) served as controls.

Antimicrobial susceptibility

Susceptibility to antimicrobial drugs was determined by the diffusion method described by Bauer *et al*. (1966), using Mueller-Hinton agar plates with commercial discs of penicillin (10 μg), ampicillin (10 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), sulphametrin (25 μg) and kanamycin (30 μg).

DNA manipulations

Total and plasmid DNA were extracted as previously described (Leal *et al*. 1997; Leal and Almeida 1999). The presence of chromosomal pathogenicity markers was determined by PCR under the conditions described by Leal, Leal and Almeida (1997) and Leal and Almeida (1999), using DNA extracted from the cultures and primers targeted to the genes *psaA*, *irp2* and *pns* (*fyuA*). The presence of the plasmidial genes *caf1*, *pla* and *lcrV* was also investigated. The molecular weight standard used to determine the sizes of the amplified fragments was the 100 bp DNA marker. A negative control was included in each PCR run with no target DNA.

Analysis of the proteins by SDS-PAGE

Total proteins were extracted from cells collected by centrifugation at 20 000 *g* from 1 ml of the broth culture following incubation at 28°C overnight. The pellet was suspended in 100 μl Laemmli buffer (Laemmli 1970), boiled for 10 min, and electrophoresed in homogeneous 12·5% SDS-polyacrylamide gels followed by staining with Coomassie blue R using Phast System equipment (Amersham Biosciences do Brasil Ltda, São Paulo, Brasil). Molecular weight markers (LMW) were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20·1 kDa) and lactalbumin (14·4 kDa).

RESULTS

Antimicrobial susceptibility

All the strains of *Y. pestis* were sensitive to all the antimicrobial drugs tested, except penicillin.

Pigmentation on CRA plates

Among the 53 *Y. pestis* cultures analysed, 34 developed 100% red colonies (Pgm^+) on CRA plates, eight developed 75% red and 25% white colonies (Pgm^-), eight showed the same proportion of Pgm^+ and Pgm^- colonies, one culture had 25% Pgm^+ and 75% Pgm^- colonies, and two developed 100% Pgm^- colonies (Table 1).

Plasmid content

Agarose gel electrophoresis of the plasmidial DNAs extracted from the 53 *Y. pestis* cultures showed variation in plasmid content. The three prototypical plasmids (pYV, pPst and pFra) were visualized in 39 samples. Among the remaining cultures, the three plasmids plus an extra DNA band greater than 90 kb were found in seven cultures, while seven others lacked any plasmids; three exhibited only pYV, three exhibited pYV and pFra, and one exhibited pYV and pPst (Table 1, Fig. 1).

Amplification of virulence genes by PCR

There was amplification of the segments of expected size with the primers directed to the genes *caf1* (506 bp), *lcrV* (800 bp), *irp2* (300 bp) and *psaA* (476 bp) in all *Y. pestis* samples examined. The gene *pla* was amplified only in the 47 cultures harbouring the pPst plasmid where this gene is located; it was not amplified in the six cultures lacking pPst. In contrast, the *caf1* gene was also amplified in the four cultures lacking the plasmid pFra, and the gene *irp2*, located in the *pgm* locus, was even amplified in two non-pigmented cultures (Table 1). The primer directed to the *psn* (*fyuA*) gene generated a DNA segment of approximately 1500 bp

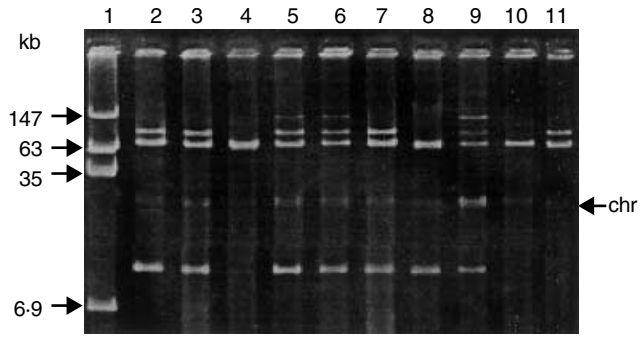

Fig. 1 Representative plasmid profiles of *Yersinia pestis* strains from the state of Ceará on 0·6% agarose gel. Lane 1: known plasmids from *Escherichia coli* 39R861; lane 2: plasmids from *Y. pestis* EV76 (reference strain); lane 3: P. EXU 882; lane 4: P. EXU 547; lane 5: P. EXU 842; lane 6: P. EXU 559; lane 7: P. EXU 861; lane 8: P. EXU 542; lane 9: P. EXU 833; lane 10: P. EXU 801; lane 11: P. EXU 553

with all *Y. pestis* cultures and a larger fragment (approximately 1800 bp) with the *Y. enterocolitica* Ye 02 used as positive control.

Protein profile

All the *Y. pestis* cultures examined exhibited an overall similarity in their protein profiles. A protein band of approximately 17 kDa, corresponding to the F1 antigen encoded by the gene *caf1*, was visualized in all 53 *Y. pestis* cultures (Fig. 2).

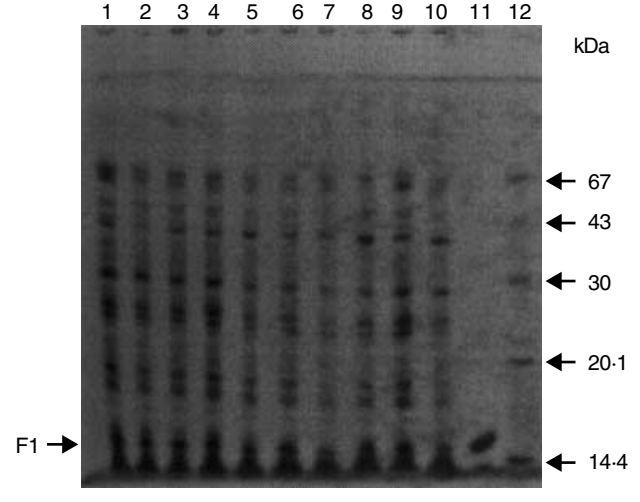

Fig. 2 SDS-PAGE with homogeneous 12·5% PhastGel and Coomassie blue R staining. Lane 1: P. EXU 683; lane 2: P. EXU 833; lane 3: P CE 22; lane 4: P. EXU 557; lane 5: P. EXU 801; lane 6: P. EXU 547; lane 7: P. EXU 553; lane 8: P. EXU 541; lane 9: P. EXU 542; lane 10: P. EXU 842; lane 11: *Y. pestis* F1 protein; lane 12: molecular weight markers

DISCUSSION

There is evidence that *Y. pestis* is a recently-emerged clone of *Yersinia pseudotuberculosis* (Achtman *et al*. 1999). *Yersinia pestis* has adapted to different mammalian hosts (rodents, rabbits, man) and several species of fleas in different climates and geographical regions. Therefore, it is surprising that it is a very homogeneous species. There is only one serotype, one phagetype and one biotype with three biovars defined based on glycerol fermentation and nitrate reduction (Perry and Fetherston 1997; Parkhill *et al*. 2001). However, strains with new characteristics have recently been identified in Indian and Madagascar plague foci, suggesting adaptation of the plague bacillus in its natural environment (Guiyoule *et al*. 1997).

Multi-drug resistance in *Y. pestis* had not been recognized (except for penicillin) until recently. However, during the actual plague epidemic in Madagascar, *Y. pestis* strains resistant to the drugs usually employed for plague treatment were detected. This resistance is mediated by a transferable plasmid, which means that acquisition of the resistance plasmid is a dynamic process taking place in *Y. pestis* (Galimand *et al*. 1997).

Typical strains of *Y. pestis* harbour three well-characterized plasmids (pPst, pFra, pYV) coding key virulence determinants. Atypical strains lacking some plasmids or containing additional cryptic plasmids have been found in several foci around the world. *In vitro*, the *Y. pestis* genome is very plastic; the prototypical plasmids may be eliminated spontaneously at a high frequency during storage in the laboratory, or through successive subcultures (Perry and Fetherston 1997). Other changes have also been described, including the emergence of additional DNA bands, the increase of plasmid molecular mass, and the integration of plasmids into the bacterial chromosome with or without loss of functions (Protsenko *et al*. 1991; Perry and Fetherston 1997). These changes are probably deletion, multimeric or recombination products of the three prototypical plasmids (Perry and Fetherston 1997).

The plasmid profile of the *Y. pestis* isolates from three independent plague areas from the State of Ceará fit into three plasmidotypes previously described by Leal *et al*. (1997): complete, harbouring the three prototypical plasmids; complete, with extra DNA-bands; and incomplete, missing some plasmids. The pYV, which is essential for virulence revealed to be the most conserved and was found in all the isolates in this study. Four cultures did not exhibit the plasmid pFra on agarose gels. However, all of them amplified the gene *caf1* by PCR. This gene encodes the structural subunits of the F1 antigen, a capsular protein of *Y. pestis* which is highly antigenic for man and animals. Most of the serological tests for plague diagnosis are based on the detection of a response against F1. Therefore, an

infection by a strain unable to produce F1 would lead to a false-negative result in a test based on F1 detection. The presence of a 17 kDa band (F1 antigen) was observed by SDS-PAGE in all the cultures examined, including the cultures lacking pFra. These results suggest that this plasmid is integrated into the bacterial chromosome and is still functional.

The *Y. pestis* chromosome harbours an unstable DNA segment (*pgm* locus) composed of two, physically and functionally distinct regions: a pigmentation segment, associated with the pigmentation phenotype of the colonies on CRA plates and required for transmission by flea bites; and an iron acquisition segment which carries several genes, *irp*, *ybt and psn* (*fyuA*), required for the disease (Buchrieser *et al*. 1998). The study of the pigmentation phenotype and the distribution of *irp2* gene in *Y. pestis* cultures from several foci around the world have identified three phenotypes: Pgm+ Irp2+, fully virulent cultures; Pgm– Irp2–, avirulent cultures; and Pgm– Irp2+, potentially virulent cultures. The Pgm– Irp2+ phenotype was shown to retain some degree of virulence for mice by the intravenous route of inoculation but not by the subcutaneous route, indicating that the iron acquisition segment is essential for the peripheral route of infection (Iteman *et al*. 1993).

The analysis of the *Y. pestis* strains from Ceará State showed that most of them, independent of origin, produced only pigmented colonies on Congo red-agar plates; some cultures produced red and white colonies in varied proportions, and two cultures produced only white colonies. There was amplification of the genes *irp2* and *psn* (*fyuA*) in all the cultures, even in two non-pigmented cultures, suggesting that they are potentially pathogenic.

The antigen pH6 is a putative adhesin which has been identified in both *Y. pestis* and *Y. pseudotuberculosis* (Perry and Fetherston 1997). Studies relating to its biological role and distribution among these species are still scarce. In the present work, the presence of the gene *psaA* (encoding the structural subunits of the pH6 antigen) was observed, through PCR with specific primers, in all the cultures analysed.

In summary, no phenotypic or genotypic alteration was detected in the isolates of *Y. pestis* examined that could be associated with origin or epidemiological period. The extra cryptic DNA bands found in some cultures could not be related to drug resistance as none of them showed antimicrobial resistance (except for penicillin). It is likely that these extra DNA bands, as well as the elimination of some plasmids, resulted from DNA rearrangements during storage. It is also likely that the loss of pigmentation in some cultures occurred *in vitro* after isolation. In spite of the instability of the *Y. pestis* genome *in vitro*, modifications that could reflect adaptation of this bacterium under environmental pressure do not seem to have occurred in nature.

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