Survey of the *irp2* Gene Among *Yersinia pestis* Strains Isolated During Several Plague Outbreaks in Northeast Brazil

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The irp2 *gene codes for a 190 kDa protein (HMWP2) synthesized when highly pathogenic* Yersinia *are grown under conditions of iron starvation. In this work, the presence of* irp2 *in strains of* Y. pestis *isolated from different hosts during several plague outbreaks in the foci of Northeast Brazil was studied. For this purpose, 53 strains were spotted onto nylon filters and their DNA was hybridized with the A13 probe which is a 1 kb fragment of the* irp2 *coding sequence. All strains except two hybridized with the probe. However, when the initial stock culture of these two strains were analyzed, they both proved to be positive with the A13 probe, indicating that the locus was lost after subculture* in vitro *but was always present* in vivo. *To examine the degree of conservation of the chromosomal fragment carrying* irp2 *among Brazilian strains, the hybridization profiles of 15 strains from different outbreaks, different hosts and different foci were compared. The hybridization profiles of these strains were all identical when their DNA was digested with either* EcoR*I,* EcoR*V or* Ava*II, indicating that the restriction sites surrounding the* irp2 *locus are very well conserved among Northeast Brazilian strains of* Y. pestis. *Altogether, these results suggest that the* irp2 *chromosomal region should be of prime importance for the bacteria during their multiplication in the host.*

Key words: *irp2* – chromosome – deletion – *Yersinia pestis* – iron – plague – Brazil

Yersinia pestis, the etiologic agent of plague, is a gram-negative bacterial pathogen, transmitted from rodents to rodents by fleas. Although the plague reservoir is the rodent, the disease can also affect humans (Poland & Barnes 1979). The plague bacillus was introduced into Brazil during the last pandemic and persisted in rural areas, mainly in the Northeastern region, where several *Y. pestis* foci are still active nowadays (WHO 1965, Almeida et al. 1981, 1985, 1989). These foci share similar climatic conditions, rodent hosts and flea vectors. The field rodent *Bolomys lasiurus (Zygodontomys lasiurus pixuna)* and its fleas *Polygenis* spp., are the main animal reservoir and vector involved in the epizootics, but other species of rodents and fleas can also be responsible for plague outbreaks (Karimi et al. 1974a, b, c, Almeida et al. 1981, 1985, 1989). Cycles of plague activity have a periodicity of 5-10 years, with quiescent periods between each outbreak (WHO 1965, Almeida et al. 1981).

Many attempts have been made to find some differences among *Y. pestis* strains isolated from these foci but it has not been possible to link the geographical origin, the type of host or the period of isolation of the isolates to a peculiar phenotypic or genetic trait.

Virulence in *Y. pestis*, depends on the synthesis of products encoded by chromosomal and plasmid genes (Brubaker 1991). Carniel et al. (1987) demonstrated that highly pathogenic *Yersinia (Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* biotype 1B) produce high molecular weight proteins (HMWPs) under conditions of iron starvation. The synthesis of the 190 kDa protein (HMWP2) is encoded by the *irp2* chromosomal gene which is present in the highly pathogenic species only (Carniel et al. 1989, Almeida et al. 1993). This gene is absent from the non pathogenic or low pathogenicity strains and therefore could be used as a new genetic marker of high pathogenicity phenotype in *Yersiniae* (Carniel et al. 1991). The *irp2* locus is located on an unstable chromosomal fragment, which can delete spontaneously in *Y. pestis* and *Y. pseudotuberculosis*. Loss of the gene is obtained by repeated subcultures of *Y. pestis in vitro* (Almeida et al. 1993) and is accompanied by a marked reduction in the bacterial pathogenicity (Carniel et al. 1991).

In this work, the presence of the *irp2* gene among Brazilian *Y. pestis* strains was studied and the hybridization profiles of the chromosomal segments carrying this gene were com-

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pared. We demonstrate here for the first time that all the strains studied harbored the *irp2* locus upon primary isolation and, as found for strains of other geographical origins, that the gene could be lost after subcultures *in vitro*. The restriction profiles using three different enzymes were compared and were found to be all identical, indicating a high stability of the corresponding restriction sites in the *irp2* region.

MATERIALS AND METHODS

Bacterial strains and growth conditions – The 53 Brazilian *Y. pestis* strains used in this study are listed in the Table. They were isolated from human patients, rodents, and fleas in the plague foci of Northeast Brazil between 1967 and 1986 (Karimi et al. 1974a, Almeida et al. 1981, 1985, 1989). The strains were named P. (for plague), followed by the name of the city (i.e. EXU or GRS) or the states (BA or PB), where the laboratories which isolated the strains were settled. Unless otherwise indicated, bacteria were routinely grown overnight at 28°C in peptone broth or on Blood Agar Base (Difco) for 48 hr. Each isolate was stabbed on duplicate Blood Agar Base (Difco) tubes and kept at +4°C. Care was taken to maintain one of the tubes as the original stock culture and to avoid subcultures. The *Y. pestis* vaccine strain EV76 was shown to be Irp2- (Carniel et al. 1987) and therefore was used in this study as a negative control. *Y. enterocolitica* strain Ye8081 (serotype O:8), *Y. pseudotuberculosis* strain IP32790 (serotype I) and *Y. pestis* strain 6/69 are all Irp2+ and the two formers have a different *Eco*RI hybridization profile with the Cla8 probe (Almeida et al. 1993). They were used as positive controls for Southern hybridizations.

Chemicals – Coomassie brilliant blue, agarose and ethidium bromide were purchased from Sigma chemical corp., St Louis, Mo. Nylon filters (Hybond N.), Random priming kit, [^{35}S] dATP were obtained from Amersham Corp. (Arlington Heights, III.). Restriction endonucleases *Eco*RI, *Eco*RV and *Ava*II were from Janssen Biochemica (Beerse, Belgium). Proteinase K was purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Lambda DNA-*Hind*III digests were obtained from New England Biolabs and were used as molecular weight markers. X-ray films XAR were purchased from Eastman Kodak Co., Rochester, N.Y.

DNA manipulations – Total DNA extractions and Southern Blots were performed as previously described (Carniel et al. 1989). For colony blotting, colonies grown on Blood Agar Base (BAB), were spotted onto nylon filters layered on BAB plates and incubated overnight at 28°C. Bacterial lysis, DNA denaturation and hybridization were carried out as described elsewhere (Almeida et al. 1993). Two probes were used for this study: a 1 kb fragment (A13) of the coding sequence of the *irp2* gene (Carniel et al. 1989), or an 8 kb DNA segment (Cla8), carrying the entire *irp2* gene and adjacent sequences (Carniel et al. 1992). Both probes were labelled by random priming using ^{35}S dATP. The A13 probe was used for colony hybridizations and the Cla8 probe for Southern hybridizations (Southern 1975). The filters were prehybridized, hybridized and washed at 65 °C as described elsewhere (Carniel et al. 1989). *Y. pestis* strain EV76 (Irp2-) was used as a negative control for colony hybridizations.

RESULTS

Study of the presence of irp2 *among 53* Y. pestis *Brazilian isolates* – In a previous study, we found that 44.7% of the *Y. pestis* strains isolated from different countries in the world had lost the *irp2* gene (Almeida et al. 1993). We wondered whether such loss occurred *in vitro* after repeated subcultures or whether *irp2* deleted strains could be found *in vivo*. Since the strains used in the above publication were subcultured several time, it was not possible to answer this question and we concluded that the study of a large number of fresh isolates would be of great interest. The existence of well documented plague foci in the Northeast of Brazil and of an important collection of *Y. pestis* strains isolated at different times from various hosts and places prompted us to examine these isolates for the presence of the *irp2* locus. 53 strains of *Y. pestis* were screened by colony blot with the A13 probe for the presence of *irp2*. A13 is an internal fragment of the *irp2* locus and was used as a probe because the absence of sequences adjacent to the *irp2* gene decreased the hybridization background. Only two strains (P.EXU 21 and P.EXU 424) did not harbor the *irp2* locus (Irp2). However, when the original stock cultures of these two Irp2- strains were taken, both strains were found to be originally Irp2+. Therefore, these results clearly demonstrate that all the freshly isolated *Y. pestis* strains studied harbored the *irp2* locus. These data also show that the Brazilian isolates, similarly to the strains isolated from other parts of the world (Almeida et al. 1993), can lose the *irp2* region after *in vitro* subcultures.

Cla8 hybridization profiles of 10 Y. pestis *strains isolated during the same outbreak* – In order to determine the degree of conservation of the *Eco*RI hybridization profile of the chromosomal *irp2* region and to evaluate the potential

TABLE

Yersinia pestis strains isolated in Northeast Brazil and used in this study

Strains	Host (species)	Geographical origin	Year
P. EXU 21	Rodent (*Rattus r. alexandrinus*)	Exu	1967
P.EXU 25	Rodent (*Bolomys lasiurus*)	Exu	1967
P.EXU 41	Rodent (*Rattus r. frugivorus*)	Exu	1967
P.EXU 56	Rodent (*Bolomys lasiurus*)	Exu	1967
P.EXU 77	Flea (*Polygenis*)	Exu	1967
P.EXU 138	Flea (*Polygenis*)	Exu	1967
P.EXU 146	Flea (*Polygenis*)	Exu	1967
P.EXU 160	Rodent (*Rattus r. alexandrinus*)	Exu	1967
P.EXU 184	Human	Exu	1967
P.EXU 185	Human	Caruaru	1967
P.EXU 196	Human	Triunfo	1967
P.EXU 306	Flea (*Pulex irritans*)	Exu	1969
P.EXU 310	Flea (*Pulex irritans*)	Exu	1969
P.EXU 424	Rodent (*Gallea spixii wellsi*)	Exu	1970
P.EXU 559	Flea (*Polygenis*)	San Benedito	1972
P.EXU 662	Human	Exu	1974
P.EXU 795	Human	Aratuba	1978
P.EXU 796	Human	Aratuba	1978
P.EXU 800	Rodent (*Rattus r. alexandrinus*)	Triunfo	1978
P.EXU 801	Human	Ipu	1978
P.EXU 805	Human	Capistrano	1978
P.EXU 806	Rodent (*Calomys callosus*)	Aratuba	1978
P.EXU 810	Rodent (*Bolomys lasiurus*)	Natuba	1979
P.EXU 811	Rodent (*Bolomys lasiurus*)	Natuba	1979
P.EXU 817	Rodent (*Oryzomys subflavus*)	Natuba	1979
P.EXU 819	Flea (*Polygenis*)	Triunfo	1979
P.EXU 820	Rodent (*Bolomys lasiurus*)	Natuba	1979
P.EXU 834	Rodent (*Bolomys lasiurus*)	Guaraciaba Norte	1979
P.EXU 843	Rodent (*Bolomys lasiurus*)	Natuba	1980
P.EXU 851	Rodent (*Bolomys lasiurus*)	Natuba	1980
P.EXU 860	Rodent (*Bolomys lasiurus*)	Salgado S. Felix	1982
P.GRS 1	Rodent (*Bolomys lasiurus*)	Minas Gerais	1983
P.GRS 2	Rodent (*Bolomys lasiurus*)	Minas Gerais	1983
P.BA 2	Flea (*Xenopsylla cheopis*)	Bahia	1984
P.BA 3	Human	Bahia	1984
P.PB 862	Human	Solanea	1986
P.PB 863	Human	Barra Sta. Rosa	1986
P.PB 864	Rodent (*Rattus r. frugivorus*)	Solanea	1986
P.PB 865	Rodent (*Bolomys lasiurus*)	Solanea	1986
P.PB 866	Rodent (*Rattus r. frugivorus*)	Solanea	1986
P.PB 868	Rodent (*Bolomys lasiurus*)	Solanea	1986
P.PB 869	Rodent (*Holochilus sciureus*)	Solanea	1986
P.PB 870	Rodent (*Bolomys lasiurus*)	Solanea	1986
P.PB 871	Rodent (*Bolomys lasiurus*)	Solanea	1986
P.PB 872	Rodent (*Bolomys lasiurus*)	Solanea	1986
P.PB 873	Rodent (*Oryzomys subflavus*)	Solanea	1986
P.PB 874	Rodent (*Rattus r. frugivorus*)	Solanea	1986
P.PB 875	Rodent (*Rattus r. frugivorus*)	Solanea	1986
P.PB 876	Rodent (*Rattus r. frugivorus*)	Solanea	1986
P.PB 877	Rodent (*Rattus r. frugivorus*)	Solanea	1986
P.PB 878	Rodent (*Oryzomys subflavus*)	Solanea	1986
P.PB 879	Rodent (*Oryzomys subflavus*)	Solanea	1986
P.PB 881	Human	Remigio	1986

BA: Bahia, PB: Paraíba, GRS: Garanhuns.

of host-induced variations, the genomic DNAs from 10 strains isolated during the same outbreak but from different hosts, were extracted, digested with *Eco*RI, transferred to nylon membranes and hybridized with the Cla8 probe. The Cla8 probe was used instead of the A13 probe because it is an 8 kb DNA segment which covers a larger DNA region surrounding the *irp2* locus. The same 16 kb *Eco*RI segment was recognized by the probe in all the strains tested (Fig. 1), indicating that the bacteria isolated during this outbreak were homogeneous regard-

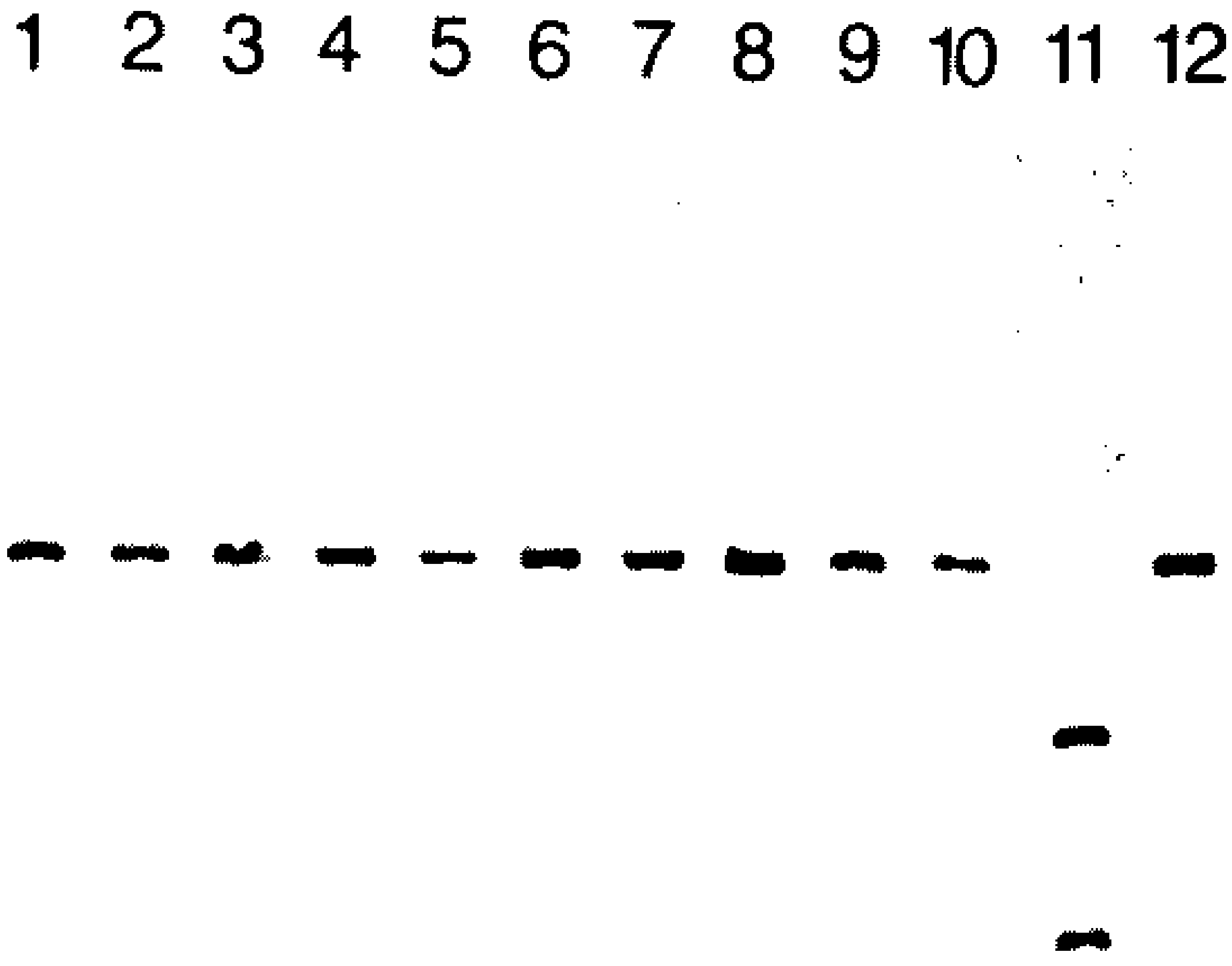

Fig. 1: southern hybridization with the Cla8 probe of *Eco*RI digested total DNA of 10 strains of *Yersinia pestis* isolated from different hosts during the same outbreak in Exu in 1967. Lines 1, P.EXU 21; 2, P. EXU 25; 3, P.EXU 41; 4, P.EXU 56; 5, P.EXU 77; 6, P.EXU 138; 7, P.EXU 146; 8, P.EXU 160; 9, P.EXU 184; 10, P.EXU 185; 11, *Y. enterocolitica* O:8 strain Ye8081; *Y. pseudotuberculosis* I strain IP32790.

ing the *irp2* DNA segment. The *Eco*RI hybridizing fragment had a size similar to that of *Y. pseudotuberculosis* and different from the two bands recognized by the Cla8 probe in *Y. enterocolitica* (Fig. 1). This result also shows that the type of host infected with *Y. pestis* (humans, rodents or fleas) did not affect the *irp2* profile. These data could be expected since flea vectors, rodent reservoirs and humans all belong to the same epidemiological plague cycle.

Cla8 hybridization profiles of 15 Y. pestis *strains isolated during different outbreaks* – To determine whether the *Eco*RI fragment carrying *irp2* was similar in all Brazilian *Y. pestis* strains isolated or whether some clonal diversity appeared with time or with the geographical area where the outbreak occurred, the genomic DNAs of 15 strains listed in the Table were digested with *Eco*RI and hybridized with the Cla8 probe. As shown on Fig. 2A, all strains had a 16 kb hybridizing fragment. This pattern was similar to that previously found in various strains isolated in European, African and Asian countries (Almeida et al. 1993).

To further investigate the conservation of the restriction map of the *irp2* region, the chromosomal DNAs of these strains were also digested with *Ava*II or *Eco*RV and hybridized with the Cla8 probe. As shown on Fig. 2B, the probe recognized three *Ava*II restriction fragments of approximately 12, 3.5 and 1.5 kb in all strains tested. When the DNAs were digested with *Eco*RV, two fragments of ca. 6 and 7 kb hybridized with Cla8 in all the strains (Fig. 2C). These results indicate that the *Eco*RI, *Ava*II and *Eco*RV restriction sites were perfectly conserved in the segment carrying the *irp2* locus among different Brazilian strains. The profile obtained with the

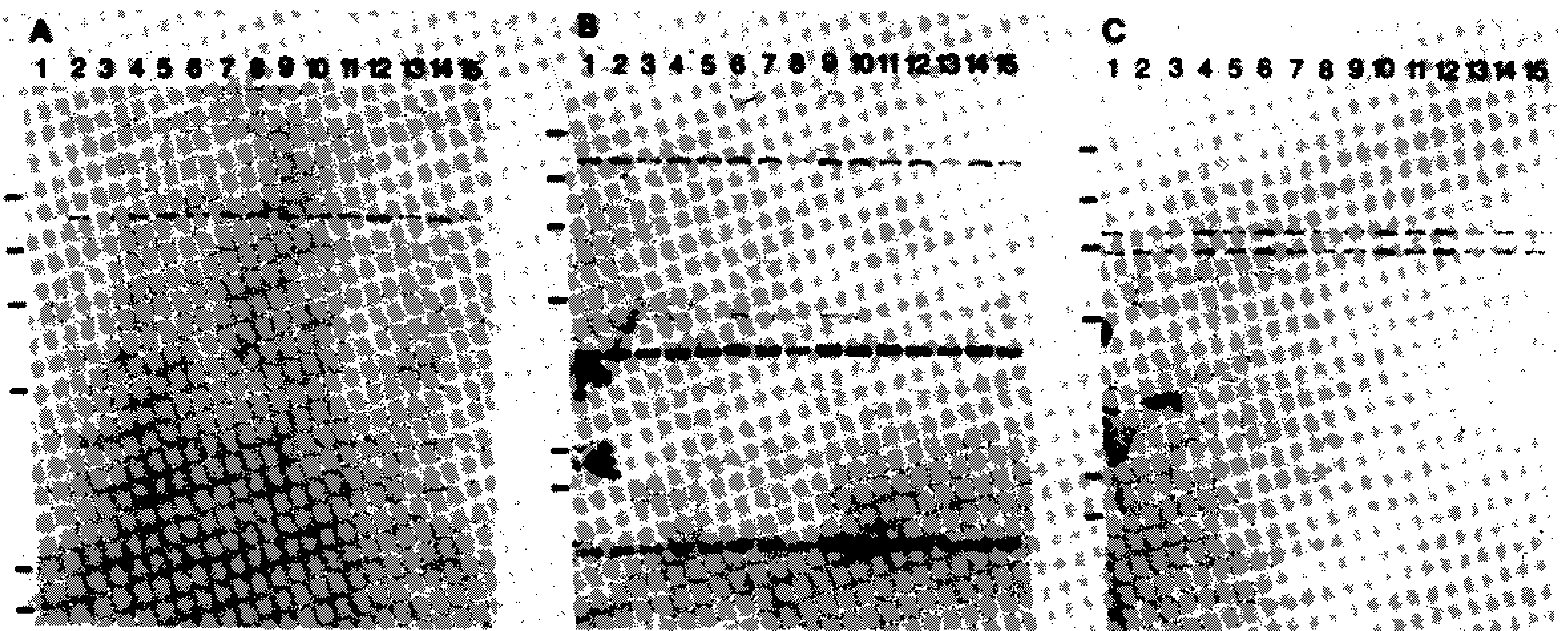

Fig 2: southern hybridization with the Cla8 probe of total DNA of 15 strains of *Yersinia pestis* isolated from different hosts during different outbreaks in Northeast Brazil. The DNAs were digested with either *Eco*RI (A), *Ava*II (B) or *Eco*RV (C). Line 1, P.EXU 819; 2, P.EXU 559; 3, P.EXU 834; 4, P.EXU 800; 5, P.EXU 795; 6, P.GRS 2; 7, P.BA 2; 8, P.BA 3; 9, P.EXU 196; 10, P.EXU 310; 11, P.EXU 662; 12, P.EXU 796; 13, P.EXU 801; 14, P.EXU 805; 15, P.GRS 1. Tick Marks on the left indicate the positions of the Lambda DNA-*Hind*III digests used as molecular weight markers: from top to bottom 23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 kb.

three different enzymes was also similar to that found in the *Y. pestis* 6/69 strain which was isolated in Madagascar (data not shown), suggesting that these profiles were not specific for the Brazilian strains but were conserved among *Y. pestis* strains of different geographical origins.

DISCUSSION

Between 1966 and 1986, 881 *Y. pestis* strains have been isolated from: plague patients (155 strains), several species of rodents (490 strains) and fleas (236 strains), in Northeast Brazil (Karimi et al. 1974a, Almeida et al. 1981, 1985, 1989). We wondered whether the host exerted some selective pressure which would result in some genetic and/or phenotypic divergence among strains isolated from diverse species of rodents (*Cavidae, Muridae* and *Cricetidae*) or fleas.

Previous studies on Brazilian *Y. pestis* isolates concentrated on biochemical tests. Most of the isolates belonged to the *Y. pestis Orientalis* biovar (glycerol-negative, nitrate-positive), that was spread through the modern world during the last pandemic (Devignat 1951, WHO 1965, Karimi et al. 1974a). A few strains were glycerol-negative and nitrate-negative (an atypical, undescribed variant) (Karimi et al. 1974a) but no correlation could be established between the biovar of the strain and a geographical or host specificity.

Hudson et al. (1973) analyzed biochemical properties and total protein electrophoretic patterns of 17 *Y. pestis* strains isolated during a short period (1966 to 1967) from two plague foci in Northeast Brazil. They identified two groups of strains, based on the quantitative content of a protein which was latter recognized as the murine toxin (Tengerdy & Hillam 1973). However, no correlation could be established between these two groups and the epidemiological characteristics of the strains.

More recently, Abath et al. (1989) carried out a study on the outer membrane protein profiles of 38 strains isolated during almost 25 years from several sources in various foci of the northeast. No major difference was found in the outer membrane protein profile of these strains.

Searching for other distinctive traits, the plasmid contents of 19 strains of *Y. pestis* collected during a plague outbreak in 1986, was investigated and compared to those of other strains previously isolated. All strains analyzed had a similar plasmid profile (Leal et al. 1989).

A very good correlation between the presence of the *irp2* gene and the virulence of *Y. pestis* has been established (Carniel et al. 1991, Almeida et al. 1993). Therefore, the presence of the *irp2* gene could be regarded as a marker of high virulence in *Y. pestis.* In a recent screening of *Y. pestis* strains isolated in several countries (but not in American plague foci) for the presence of *irp2,* it was observed that the gene was absent from approximately 45% of the strains tested. However, it was not clear whether the gene was lost *in vivo* or after repeated subcultures *in vitro.*

In the present study, the *irp2* gene was found in all but two strains tested (P. EXU 21 and P. EXU 424). However, original cultures of these strains were Irp2+, further illustrating that *irp2* is easily lost *in vitro* upon repeated subcultures. This *datum* also indicates that all the bacteria harbored the *irp2* locus *in vivo* and therefore strengthens the hypothesis that the presence of this gene should be of prime importance for the bacteria in its host, either as a growth factor or as a virulence determinant. Furthermore, the presence of the *irp2* locus in all the strains tested indicate that they are all potentially highly pathogenic for humans. The attenuated vaccine strain EV76 which is non-pigmented and had lost the *irp2* locus is not pathogenic upon subcutaneous injection but can persist for some time in the host. One can wonder whether the attenuated clinical forms or plague (*pestis minor*) may correspond to strains which have lost *irp2.* A study of the *Y. pestis* strains isolated from these patients would be of great interest.

Usually, regions of genomic DNA which do not carry important functions are subjected to frequent point mutations which lead to the loss or the acquisition of new restriction sites. The finding that the *Eco*RI, *Ava*II and *Eco*RV restriction sites on the *irp2* chromosomal segment were perfectly conserved among bacteria isolated from different geographical areas and different hosts over a period of 20 years further suggests that the *irp2* DNA segment is of prime importance for *Y. pestis,* either for its survival or for the expression of virulence functions in its hosts.

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