

The 1994 plague epidemic of India: Molecular diagnosis and characterization of *Yersinia pestis* isolates from Surat and Beed

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PCR analysis of formalin-fixed human autopsy tissues, rodent tissues, fleas and bacterial isolates from pneumonic patients from the 1994 plague epidemic confirmed the presence of the *fl* and *pla* genes of *Yersinia pestis* in these samples. Several *Y. pestis* isolates from the epidemic areas were studied in respect of their plasmid profile, expression of F1 antigen and ribotype pattern. All the three plasmids known to be associated with virulence were present in the Surat isolates of *Y. pestis*. Presence of the F1 antigen, classically used for diagnosis of *Y. pestis* infection, was demonstrated by immunoblotting. All the Indian isolates from the 1994 epidemic showed an identical ribotype profile. This profile, however, was different from those of *Y. pestis* isolates tested from other regions of the world.

Upon digestion with *EcoRI* and *EcoRV*, and probing with *E. coli* 16S and 23S rRNA genes, DNA from these *Y. pestis* isolates gave two distinct profiles which, taken together, suggest that the present Indian isolates represent a new ribotype. The presence of *Y. pestis* signature genes in 5 out of 7 fleas collected from rodents in the affected areas, and the occurrence of the same ribotype in the *Y. pestis* isolates from domestic rodents, sylvatic rodents and the patients are strongly indicative of a clonal origin of this Indian strain and an epidemiological linkage among wild rodents, domestic rats and humans in the epidemic area.

IN August 1994 the village Mamla in Beed district of Maharashtra state experienced an unusually heavy flea nuisance and rat falls. Subsequently, a number of cases clinically resembling bubonic plague were reported from Mamla and nearby villages of Beed district. Based on the totality of ecological, clinical and serological

evidence, a presumptive diagnosis of bubonic plague was made and the health machinery of the State Government, with the active support of the Central Government, took prompt measures and the epidemic was successfully contained.

In September 1994, Government hospitals and private clinics in the city of Surat in the neighbouring state of Gujarat reported an increasing number of patients with an illness resembling acute pneumonia. Based on clinical, laboratory and radiological findings, a presumptive diagnosis of an outbreak of pneumonic plague was made.

Plague is an acute zoonotic bacterial disease caused by infection with *Yersinia pestis*. *Yersinia* belongs to a group of bacterial pathogens of the family Enterobacteriaceae. Three members of this group, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica* and *Yersinia pestis*, are human pathogens. These species carry one or more plasmids of molecular weights 110 kb, 70 kb and 9.5 kb which have proved to be of diagnostic value. Only *Yersinia pestis* carries all the three plasmids^{1,2}.

The pathogenicity in *Y. pestis* is multifactorial, with the genes responsible being present on the chromosome as well as on all the three plasmids. The 9.5 kb plasmid is unique to *Y. pestis* and is not present in the other two *Yersinia* species. This plasmid carries the *pla* gene which codes for a virulence factor called plasminogen activator. The complete sequence of this 1041 nucleotide-long gene is known³. Although *pla* has partial sequence homology to the 'omp'-T gene of *E. coli* and gene 'e' of *Salmonella typhimurium*, there are unique regions which can be used for amplification of the *pla* gene by PCR for identification of *Y. pestis* isolates⁴ and plague surveillance⁵. The two other plasmids, 110 kb and 70 kb, produce specific products like Fra I and

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Table 1. Source and place of origin of the *Y. pestis* isolates used in this study

| Isolate no. | Source | Place | Date of collection | Nature of specimen |
|-------------|--------------------------|---------------|--------------------|--------------------|
| 4 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 8 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 9 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 101 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 102 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 103 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 104 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 105 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 106 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 107 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 108 | Human (pneumonic plague) | Surat | Sept./Oct. 1994 | Sputum |
| 111 | <i>Rattus rattus</i> | Village Mamla | May 1995 | Liver and Spleen |
| 112 | <i>Tatera indica</i> | Village Mamla | Aug 1995 | Liver and Spleen |
| 113 | <i>Rattus rattus</i> | Surat | Aug 1995 | Liver and Spleen |
| 114 | <i>Rattus rattus</i> | Village Mamla | May 1995 | Liver and Spleen |
| 115 | Rodent (species ?) | Village Mamla | Nov 1994 | Liver and Spleen |
| 116 | <i>Rattus rattus</i> | Village Mamla | Nov 1994 | Liver and Spleen |
| 117 | <i>Rattus rattus</i> | Village Mamla | Aug 1995 | Liver and Spleen |

Yersinia Yops protein. The *Fra1* protein, encoded by *fl* on the 110 kb plasmid, is unique to *Y. pestis* and has been classically utilized for diagnosis of *Y. pestis* infection. This gene is highly conserved amongst various isolates of *Y. pestis* and thus provides the characteristic 'signature' for *Y. pestis*.

The present report describes results which demonstrate the presence of *Y. pestis* genes in human tissue samples collected at autopsies in Surat. The same signature sequences were demonstrated in fleas collected from rodents trapped during the epidemic in Beed. Molecular characterization of *Y. pestis* isolates from Beed and Surat is also described.

Materials and methods

Bacterial cultures. The bacterial isolates (Table 1) were grown from single colonies in nutrient broth for 24 hours. The cells were pelleted, resuspended in 70% ethanol and stored at -20°C until further use.

Formalin-fixed human tissues. Tissue samples from lungs and spleen were collected at autopsy from deceased persons and stored in 10% buffered formalin. All patients were from Surat with pre-mortem diagnosis of pneumonic plague.

Fleas. Fleas were collected from the epidemic areas in Beed district and stored in 70% ethanol.

Polymerase chain reaction

Primers. The primers used for PCR are listed in Table 2. These were synthesized on an automated oligonucleotide

Table 2. Sequences of oligonucleotide primers used in this study

For the plasminogen activator gene:

YP1-5' ATCTTACTTTCCGTGAGAAG 3'
 YP2-5' CTTGGATGTTGAGCTTCCTA 3'
 YP3-5' ATACTGTGACGGCGGGTCTG 3'

For the *fl* gene:

F1-1-5' ATACTGCAGATGAAAAAATCAGTTCC 3'
 F1-2-5' ATAAAGCTTTTATTGGTTAGATACGGT 3'
 F1-1405' GCCCGCATCACTCTTACATA 3'
 F1-3845' CATCCCCACAAGGTTCTCA 3'

synthesizer (Applied Biosystems, USA; Model 392) using phosphoramidite chemistry.

DNA preparation. The bacterial cells stored in 70% ethanol were centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded, the pellet was air dried and resuspended in 100 μl of sterile distilled water. The samples were boiled for 10 minutes and rapidly chilled on ice. The boiled material was centrifuged at 10,000 rpm for 5 minutes and the supernatant was used as template for PCR.

Approximately 10 mg of tissue was sliced from each of the formalin-stored samples and washed thrice with 0.05 M tris-HCl (pH 7.5). The material was ground in 50 mM tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl_2 , 0.45% Tween-20, 0.45% NP-40, using a glass rod. Proteinase K digestion was carried out overnight at 55°C in 500 μl of buffer (10 mM tris-HCl, 5 mM EDTA and 0.5% SDS) containing proteinase K (100 $\mu\text{g}/\text{ml}$). The samples were then extracted with hot phenol for 2 hours at 65°C . After chloroform extraction, the supernatant was precipitated at -70°C with 0.3 M sodium acetate and two volumes of ethanol. The extracted DNA was

pelleted and washed twice with 70% ethanol. The pellet was air-dried and reconstituted in 25 µl sterile distilled water. The samples were denatured for 5 min in boiling water and then chilled in ice.

Ethanol was drained from the flea samples which were then air dried. DNA was extracted by the hot phenol method as described.

Both positive and negative controls were included and all possible precautions were taken to avoid contamination.

Amplification of DNA by PCR. For amplification of the *pla* gene, PCR was carried out in 100 µl reaction volumes with 50 pmol each of external primers (Table 2), 1X Taq buffer, 3 mM MgCl₂, 400 µM dNTPs, 2.5 units of Taq DNA polymerase (Stratagene) and 1/10th volume of template DNA. The reactions were carried out by programming the DNA thermocycler (Techne, UK) at 95°C for 5 min, 52°C for 1 min and 73°C for 1 min for 1 cycle followed by 2 cycles at 95°C for 2 min, 52°C for 1.3 min and 73°C for 1.3 min; 35 cycles at 95°C for 1 min, 51°C for 1 min and 73°C for 1.3 min. One tenth volume of the first PCR product was reamplified with internal primers using the same conditions. For amplification of *f1*, the reactions were set up as above except that 1.5 mM MgCl₂ was used. The reactions were carried out at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 51°C for 1 min and 72°C for 2 min.

Ribotyping

Bacterial cells were grown in nutrient peptone broth from single colonies for 48 h. The cells were pelleted and chromosomal DNA was extracted using SDS-Proteinase K digestion followed by phenol-chloroform extraction. DNA samples (2.5 µg each) were digested with *EcoRI* or *EcoRV* restriction enzymes (Promega, USA) for 4 h at 37°C according to manufacturer's instructions. Electrophoresis was carried out on 0.8% agarose gel in 1X TBE buffer (90 mM Tris, 9 mM boric acid, 2 mM EDTA, pH 8.0) overnight. Southern hybridization was performed using Hybond membranes (Amersham, England) essentially according to the manufacturer's instructions. Fragments carrying the 16S, 23S and 5S *E. coli* rRNA genes⁶, from plasmids pKK 3535/pL215 were used as probes. In addition, 16S and 23S ribosomal RNAs were purified from *E. coli* and used as probes. The probes were ³²P-labelled by nick translation or by 5' end labelling. PCR ribotyping was carried out using primers from the conserved regions of the 16S rRNA gene.

Plasmid analysis by pulsed field gel electrophoresis (PFGE)

The bacterial cultures were grown overnight in BHI broth from single colonies. Cells from 1.5 ml of culture

were pelleted and the plasmids isolated using commercially available kits (Wizard, Promega, USA). Purified plasmids were suspended in 100 µl of Tris-EDTA buffer (pH 8.0) and mixed with an equal volume of 2% low-melting agarose. The plugs were prepared in Biorad moulds and subjected to PFGE in 1% agarose in 0.5X TBE buffer for 21 hours at 18°C. Lambda oligomers were used as size markers. The plasmids were visualized after staining with ethidium bromide.

Protein profile studies using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Bacterial cells were grown overnight on BHI agar from single colonies. A loopful of cells was suspended in PBS, washed thrice in the same buffer and pelleted. The pellets were resuspended in 50 µl distilled water and 50 µl SDS-PAGE loading dye. The samples were boiled for 4 minutes at 100°C and run in a 10% polyacrylamide gel at 40 mA until the dye front was close to the bottom of the gel. The protein bands were visualized by staining with Coomassie blue using standard protocols.

Immunoblot analysis for F1 antigen expression

The bacterial cell lysates were subjected to electrophoresis in 10% SDS-PAGE as above and the proteins were transferred onto nitrocellulose membranes (S&S, Germany), at 100 mA for 1 hour. The membranes were blocked with 3% BSA overnight and incubated with rabbit anti-F1 antibodies at a dilution of 1:20,000 for 1 hour. The F1 antigen was detected with alkaline phosphatase-conjugated anti-rabbit IgG (1:6,000 dilution) for 1 hour. It was then developed with phosphatase substrate for 4–5 minutes.

All experiments reported here were performed independently in more than one laboratory.



Figure 1. PCR amplification of *pla* gene fragments (478 bp) from bacterial isolates. Lane 1 – positive control; 2 – negative control; 3 – *Y. pestis* isolate from human pneumonic patient, Surat; 4 – *Salmonella typhi*; 5 – *E. coli*; 6&7 – *Y. pestis* isolates from two different pneumonic plague patients, Surat; 8 – *Klebsiella pneumoniae*, 9&10 – *Y. pestis* isolates from two pneumonic plague patients, Surat; 11 – *Proteus* sp., 12 – *Y. pestis* isolate from plague patient, Surat; 13 – *Streptococcus pneumoniae*; 14 – *Y. pestis* isolate from plague patient, Surat; 15 – *H. influenzae*.

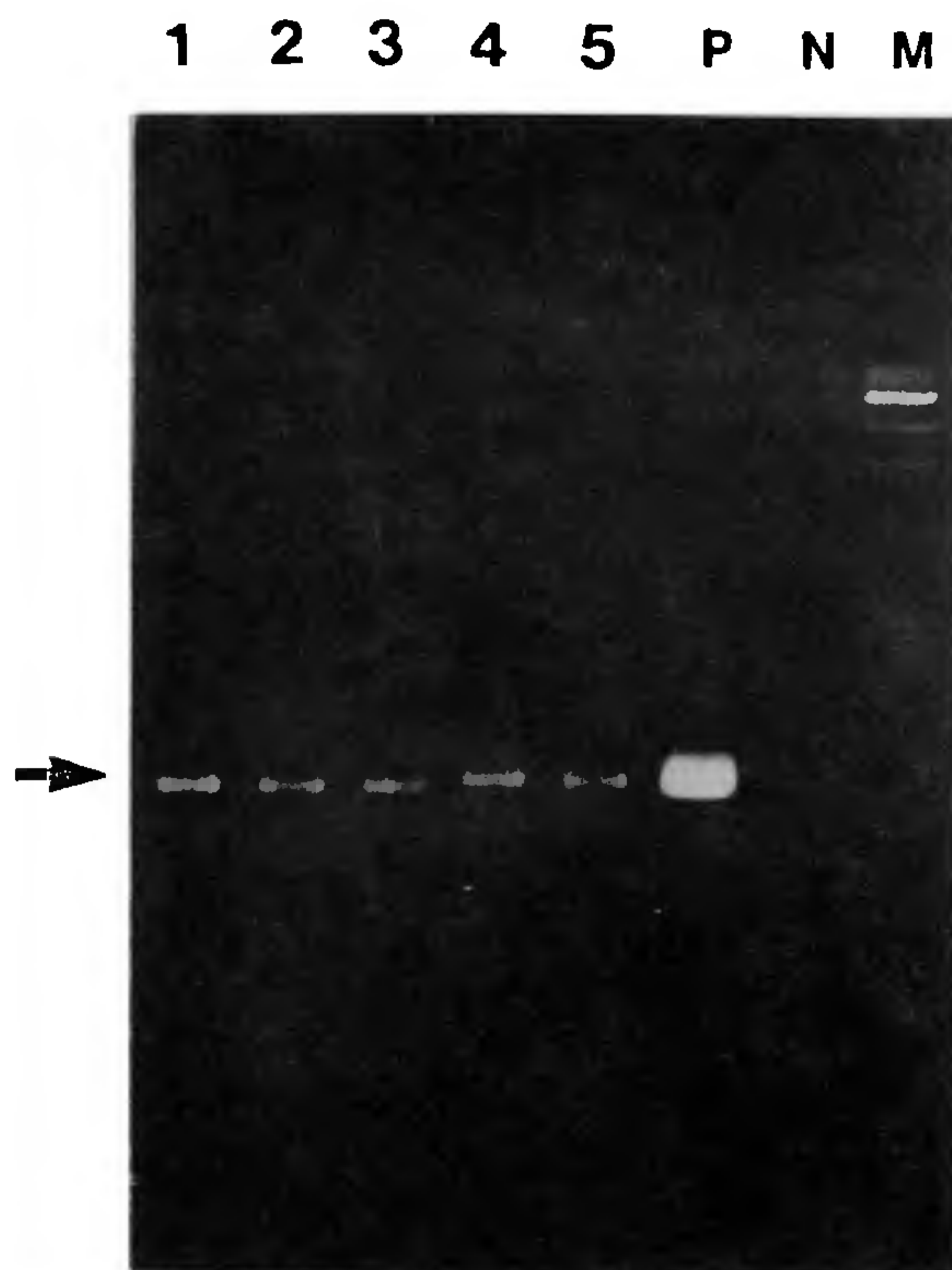


Figure 2. PCR amplification of *f1* gene fragments (274 bp) from autopsy samples. Lane 1 – A1/94, 2 – A6/94, 3 – A4/94, 4 – A10/94, 5 – A13/94. P, positive control A1122 *Y. pestis*; N, negative control; M, molecular weight marker.

Table 3. Detection of *pla* and *f1* genes in autopsy tissues using the polymerase chain reaction

| Source | Autopsy ID no. | <i>pla</i> gene | <i>f1</i> gene |
|-----------------|----------------|-----------------|----------------|
| Bhula Bhai | A21/94 | -ve | NT |
| Fulmani Loknath | A20/94 | -ve | NT |
| Chandu Bhai | A1/94 | +ve | +ve |
| Guruji Bhai | A6/94 | +ve | +ve |
| Dinesh Bhai | A4/94 | -ve | +ve |
| Chirag | A10/94 | +ve | +ve |
| Vedi Ben | A13/94 | -ve | +ve |

NT – not tested.

Results

Conditions for PCR amplification of the *pla* and *f1* genes were standardized. DNA samples isolated from a number of bacteria pathogenic to humans, but known to lack the *pla* and *f1* genes, were used as controls in the PCR experiments. DNA samples from the 18 *Y. pestis* isolates from the epidemic areas (Table 1) showed the presence of both the *pla* and *f1* genes. Of the 7 autopsy tissue samples, 3 were positive for the *pla* gene (Table 3) and 5 were positive for the *f1* gene (Figure 2 and Table 3). Five of the 7 flea samples (C1, C2, C3, ANJ4

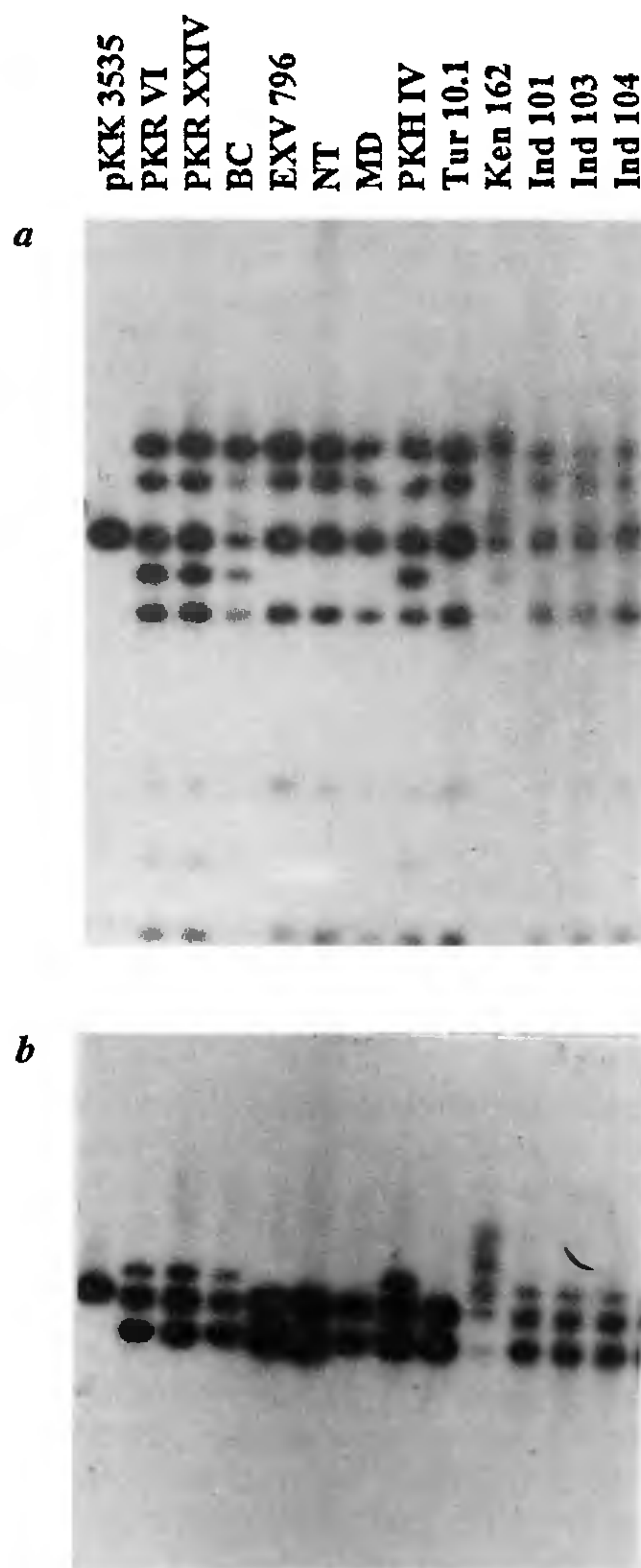


Figure 3. Southern hybridization of *EcoRI*- and *EcoRV*-digested *Y. pestis* chromosomal DNA. 32 P-labelled *E. coli* 16 S and 23 S rDNAs derived from pKK3535 were used as probes. Panel A indicates *EcoRI* digests and Panel B, *EcoRV* digests. Names of *Y. pestis* strains tested are indicated at the top. Patterns for only a few Indian isolates are shown. None of the Indian isolates (Ind 101, 103 and 104) has a profile similar to any other *Y. pestis* strains tested.

and ANJ6) collected from Beed during the epidemic period were positive for the *f1* gene (data not shown). The amplified products were cloned and sequenced and the identity of the genes confirmed. The *pla* and *f1* genes did not amplify in any of the control samples.

For ribotyping of the *Y. pestis* isolates from Surat and Beed, restriction endonucleases *EcoRI* and *EcoRV*, which are known to be the most discriminatory⁷, were used. A few strains isolated from different parts of the world and already ribotyped at the WHO *Yersinia*

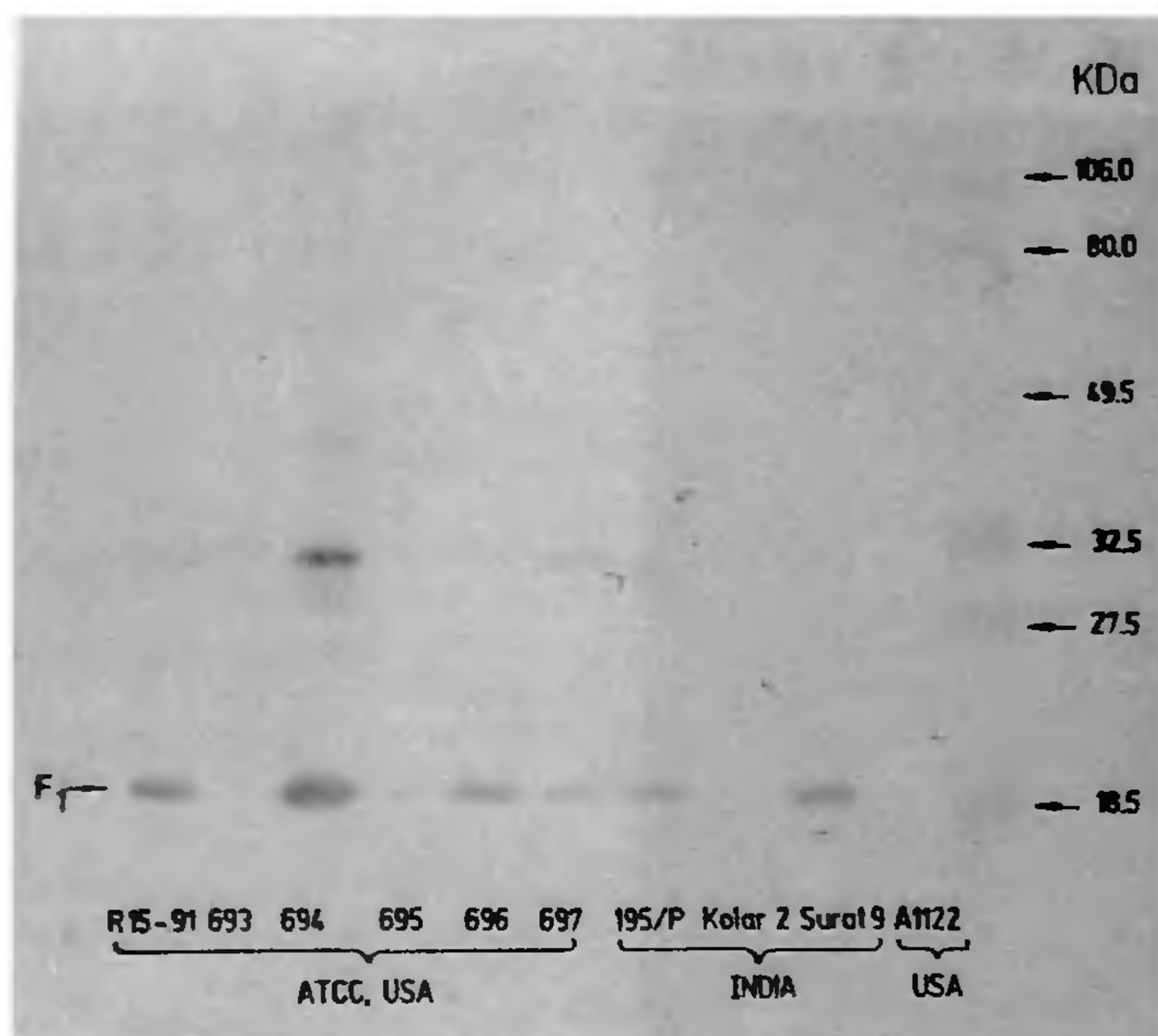


Figure 4. Western blot analysis of *Y. pestis* isolate no. India-9 (from Surat) in comparison with the other older Indian isolates and controls showing expression of F1 antigen of ~ 19 kd.

Reference Centre, Pasteur Institute, Paris, were included in this study.

EcoRI ribotype profiles displayed by the Indian isolates were identical to one another and were similar to those displayed by strains EXV 796, Nhatrang, Madagascar and Turkey 10.1 but differed from those displayed by strains PKR VI, PKR XXIV, Belgian Congo, PKH IV and Kenya 162 (Figure 3). When an identical set of experiments was performed with the restriction endonuclease *EcoRV*, it was observed that, again, all Indian isolates had the same ribotype profile (Figure 3). Unlike the *EcoRI* case, however, this profile differed markedly from the profiles displayed by EXV 796, Nhatrang, Madagascar and Turkey 10.1 but were similar to those displayed by PKR VI, PKR XXIV, Belgian Congo and PKH IV (Figure 3). Of particular interest is the fact that when both *EcoRI* and *EcoRV* ribotype profiles are considered together, the Indian isolates had profiles distinct from those of the foreign isolates, (Figure 3). This combination of *EcoRI* and *EcoRV* patterns seen in the present Indian isolates has not been seen in any of the *Y. pestis* strains examined earlier (Guiyoule *et al.*⁷ and personal communication⁸). Thus, the ribotype observed in this study constitutes a new ribotype, named 'S' for Surat by the WHO *Yersinia* Reference Centre, Pasteur Institute, Paris. All the present Indian isolates were found to belong to this ribotype. Similar ribotype patterns were obtained irrespective of the type of probe used. Upon PCR amplification of the 16S rRNA gene, products of identical size were obtained from all isolates originating from Beed and Surat.

Pulsed field gel electrophoresis carried out on three isolates (4, 8, 9) demonstrated the presence of all the three plasmids.

In SDS-PAGE, the overall migration pattern of the proteins was similar in all the Indian isolates. A protein band of approximately 25 kd was observed in the Surat isolates 4, 8 and 9 at the WHO Reference Centre, CDC, Fort Collins, USA. However, our own attempts to reproduce this result were unsuccessful (see Discussion below).

The F1 antigen was detected in all the isolates by immunoblot assay, indicating that the isolates were pathogenic (Figure 4 shows results for isolate-9).

Discussion

After the outbreaks in Beed and Surat, it was obviously necessary to identify and characterize the causative organism. This was achieved by PCR analysis of autopsy tissues from 7 individuals using primers for the *Y. pestis*-specific *pla* and *f1* genes. Five out of the 7 individuals whose autopsy tissues were analysed were positive for the *f1* gene and three were also positive for *pla*, providing unequivocal evidence of the involvement of *Y. pestis* in these deaths. It is worth mentioning that this is perhaps the first time that such *in situ* demonstration of *Y. pestis* in historical material had been attempted. By PCR analysis, eighteen bacteriologically characterized *Y. pestis* isolates were shown to be positive for the *Y. pestis* *pla* and *f1* genes (Table 1). Eleven of these isolates were from pneumonic plague patients from Surat city and seven were from rodents collected from the

epidemic areas during the period August 1994 to August 1995.

Numerous reports have shown that ribotyping is an extremely useful tool for the molecular characterization of bacterial species. To differentiate strains belonging to the same species by ribotyping, it is essential to choose restriction endonucleases which are most discriminatory. Guiyoule *et al.*⁷ have demonstrated that for the analysis of *Y. pestis* strains, restriction endonucleases *EcoRI* and *EcoRV* are particularly useful. *Y. pestis* strains isolated from Surat and Beed (including those isolated from trapped rodents) showed the same ribotype profile. Further, this profile (i.e. *EcoRI* and *EcoRV* patterns considered together) differed from those obtained with all other isolates examined so far (Guiyoule *et al.*⁷ and personal communication⁸), justifying the conclusion that these Indian strains are clonal in origin. Among the rodent isolates studied, one was from a *Tatera indica* specimen, a sylvatic species, and six were from *Rattus rattus*, a domestic species. The ribotype profile observed in the bacterial isolates from these domestic and sylvatic rodents was the same as that observed in the isolates from the patients. This is strongly supportive of an epidemiological linkage among wild rodents, domestic rats and humans in the epidemic area. The observation that five out of the seven flea samples collected from trapped rodents in the affected area also showed the presence of *Y. pestis* signature genes is in support of such an epidemiological connection.

The 85 *Y. pestis* strains examined by Guiyoule *et al.*⁷ have been classified into 16 ribotypes⁷. Even though the ribotype of the Surat strain is distinct from the 16 previously known ribotypes, it does not necessarily mean that it defines a strain of recent origin. This is because, as mentioned above, ribotyping has been done on only 85 out of the approximately 6000 strains known to exist in *Y. pestis* collections worldwide. Therefore, it is entirely possible that among the remaining 5900 or so strains, the Surat ribotype could be present in one or more strains. This can be only ascertained by further investigation.

Even though one of the worst pandemics in history occurred in India, and about 12 million people died between 1896 and 1930, only a few strains from these years have survived in various culture collections, mostly outside India. Considering that the only isolate ribotyped from India was collected in 1908 and that very few other Indian isolates are available over the last 100 years, it is not possible to say whether the Surat strain is of recent origin. This is because of our ignorance of the ribotype characteristics of the large number of strains that must have existed in the country during the past 90 or so years.

Studies carried out by the Pasteur Institute, Paris on *Y. pestis* isolates from Madagascar indicate the presence of more than one ribotype in this small island country (E. Carniel, personal communication). Moreover, there is a specific geographical distribution of these variants on

the island. Drug-resistant strains of *Y. pestis* have reportedly arisen recently in this endemic area.

The presence of all the three plasmids characteristic of *Y. pestis* in the Surat isolates was confirmed by pulsed field gel electrophoresis. This observation suggests that the Surat strain is different from the Russian vaccine strain EV because the latter lacks one of the three plasmids.

Upon single dimension SDS-PAGE, the protein profiles of the different *Y. pestis* strains studied by us showed an overall similarity. However, in Surat strains 4, 8 and 9, an additional 25 kd protein band was observed in experiments done at the WHO Reference Center, CDC, Fort Collins, USA. This band was not detected in subsequent studies done by us on the same isolates. This is perhaps not surprising in view of the fact that protein profiles obtained by single dimension SDS-PAGE can produce non-identical patterns depending upon the culture conditions, degradation of high molecular weight proteins and other variables. Even in case of *Y. pestis* it has been observed that though there is an overall similarity in the protein profiles produced by different strains, several display one or more unique bands.

The presence of the F1 antigen, which is routinely used for serodiagnosis of *Y. pestis*, was demonstrated in Surat isolates by immunoblotting, confirming that these isolates were indeed *Y. pestis*.

In conclusion, these results (i) confirm the association of *Y. pestis* with the epidemic at Surat and Beed; (ii) demonstrate that the *Y. pestis* isolates obtained from these regions were identical, indicating that the pathogen was most likely clonal in origin; and (iii) suggest that the pathogen has had an enzootic existence in the region. Further epidemiological studies, as well as collection and characterization of *Y. pestis* isolates from different parts of the Indian subcontinent, are necessary for a better understanding of the dynamics of this important pathogen.

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