Isolation and identification of *Yersinia pestis* responsible for the recent plague outbreaks in India

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The district of Beed in Maharashtra state, India experienced a suspected bubonic plague outbreak in August, 1994. A month afterwards, Surat city, in Gujarat state was in the grip of an epidemic with a highly fatal illness resembling pneumonia. The present work describes the isolation and identification of *Yersinia pestis*, the causative agent of plague, from these outbreak areas. Eleven *Y. pestis* isolates were recovered from 82 pneumonic patients of Surat city. From Beed district, 6 *Y. pestis* isolates could be obtained from different batches of trapped rodents within a year of the outbreak. A single rodent isolate from Surat city was obtained from among 17 animals in August, 1995. Characterization of the *Y. pestis* isolates was done by biochemical tests, specific bacteriophage lysis, counter immunoelectrophoresis, fluorescent antibody test and SDS-PAGE analysis.

PLAGUE epidemics and pandemics have had devastating effects on human beings throughout the recorded history. It was a highly dreaded and fatal disease in the pre-antibiotic era and at times wiped out nearly half the human population in the affected countries. Worldwide, in the post-antibiotic period, plague has existed mainly as a sporadic disease with occasional epidemic outbreaks. According to WHO reports, between 1980 and 1986, many countries in Africa, Asia and America covering a substantial proportion of the globe had reported plague cases[1]. During the last decade, outbreaks of both bubonic and pneumonic forms have occurred in Myanamar, Vietnam, Tanzania, Zaire, Peru and Madagascar[2].

In India, plague along with cholera and small pox formed the 'trinity' of major epidemic infections until the 19th century and thereafter plague outbreaks continued up to 1968 with final disappearance in 1969. Though suspected outbreaks were reported in Maharashtra in 1975 and in Himachal Pradesh in 1984 (ref. 3), but conclusive evidence was lacking. Thus, after the disease was thought to have been conquered, suspected cases of bubonic plague were reported in village Mamla, District Beed of Maharashtra in August, 1994 following typical sequences of flea nuisance and rat falls. Within a month the industrial city of Surat in the neighbouring state of Gujarat reported an increasing number of patients with a highly fatal form of acute pneumonia. During the period 21 September – 20 October, 1994 a total of 1027 suspected cases were admitted to hospitals in Surat city, of which 146 were recorded as presumptive pneumonic plague based on seropositivity.

We report here the confirmatory evidence of *Yersinia pestis* isolation from human pneumonic patients of Surat city and from the rodents of both the outbreak areas.

Materials and methods

Human samples

Since during the suspected bubonic plague outbreak in district Beed no bubo aspirates or blood samples were available, *Y. pestis* isolation could not be attempted from human cases. From among the patients in Surat presenting a clinical picture of acute pneumonia with fever, cough, haemoptysis, dyspnoea affecting mainly the young adults, 82 sputum specimens could be processed for isolation work. Samples were collected by the Department of Microbiology, Surat Medical College, Surat.

Rodent samples

Tissue samples of spleen and liver from 30 rats (*Rattus rattus*) of Mamla village, district Beed and 4 rats (*Rattus rattus*) from Surat city were collected during August to November 1994. During the months of April and May, 1995, 11 rats (*Rattus rattus*) and in July and August, 53 rats (*Rattus rattus*) trapped from Mamla village and the adjoining Chinchoti village of district Beed were processed. At the same time 17 rodents belonging to *Tatera indica* trapped from the field areas around these villages were also processed for isolation work. Spleen and liver of these animals were kept in Cary Blair transport medium and processed in the laboratory. From the Surat city in the month of July and August, 1995, 17 *Rattus rattus* trapped from the household vicinity of the affected

four localities were also processed. Majority of rodent samples from outbreak areas were received through the dedicated efforts of Shyamal Biswas and his team of Plague Surveillance Unit, NICD, Bangalore.

Isolation procedures

The samples were either streaked directly or after 1 to 2 days incubation in peptone water/brain heart infusion broth at 28°C onto brain heart infusion agar (BHIA), desoxycholate citrate agar and blood agar (BA). Media used were from Diffco Laboratories. The agar plates were incubated at 28°C and 37°C for 24 to 48 h. All the samples yielded mixed growth of organisms. The suspected colonies were purified by repeated sub-cultures and tested for oxidase reaction, Gram staining and Wayson staining. The oxidase negative, Gram negative and Wayson bipolar safety pin positive colonies were processed for biochemical reactions. The biochemical tests performed were indole production; citrate utilization; hydrogen sulphide production; urea hydrolysis; fermentation of glucose, lactose, mannitol, sucrose, maltose, arabinose, xylose, rhamnose, adonitol, dulcitol, cellobiose and salicin; decarboxylation of lysine and ornithine; hydrolysis of arginine.

Bacteriophage lysis

Organisms exhibiting biochemical reactions characteristic of *Y. pestis* were subjected to bacteriophage lysis test. *Y. pestis* monospecific bacteriophage kindly supplied by May C. Chu (WHO Collaborating Center, CDC Laboratory, Fort Collins, USA) was utilized for the test. Phage lysis test was performed using bacteriophage reagent on streaked pure cultures on blood agar plates and incubated at 28°C. *Y. pseudotuberculosis* strain 1 A and *Y. pestis* strain A1122 kindly provided by May C. Chu through Technical Advisory Committee on Plague, Govt. of India, served as controls.

Fluorescent antibody test

A loopful of heat killed overnight broth cultures of four *Y. pestis* isolates, *Y. pseudotuberculosis* strain 1 A and *Y. pestis* strain A1122 fixed with precooled acetone were tested following standard protocol using rabbit anti F1 FITC conjugate received through the courtesy of WHO Reference Centre using Nikon Microphot Universal microscope.

Counter immunoelectrophoresis

Wells were punched off at a distance of 5 mm in agarose (0.8% in veronal buffer, pH 8.6) coated on greese-free clean glass slides. Wells, towards cathode were filled with crude antigens of *Y. pestis* isolates and other bacteria (*Y. pseuodotuberculosis*, *Y. enterocolitica*, *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhi*) prepared by sonication (5 watts, 20 cycles of 60 seconds each using Misonix ultrasonicator) of harvested growth from solid media. Hyper immune sera raised in rabbits against purified F1 antigen of *Y. pestis* was added in the well towards anode. Counter immunolectrophoresis (CIE) was carried out in an anodic current of 20 mA for 45 min using 0.075 M veronal buffer, pH 8.6 as per the method of Grabar and Williams[4]. After this the slides were incubated at room temperature overnight and washed with normal saline before staining with Coomassie brilliant blue.

Polyacrylamide gel electrophoresis

Bacterial cells were grown on blood agar overnight at 28°C from a single colony. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run as per the method of Laemmli[5]. Briefly, the cells were pelleted and washed in 0.5 M phosphate buffer saline. The pellets were resuspended in 50 µl distilled water and 50 µl SDS-PAGE loading dye. The samples were boiled for 4 to 5 minutes and run in a 10% acrylamide gel at 25 mA till the dye front was close to the bottom of the gel using electrophoresis unit of Atto Corporation. The protein bands were visualized by staining with Coomassie brilliant blue as per the standard protocol.

Results

The colony characteristics of suspected *Y. pestis* isolates on BA and BHIA were not clear following 24 h incubation as the colonies were barely visible. At 48 h, the size of the colonies increased to 0.5 mm in diameter and upon further incubation the size could reach up to 1–2 mm. The colonies on BA were greyish, non-mucoid, round and dome-shaped with a tendency of flattened irregular border on prolonged incubation. At times the appearance of the colonies typically resembled fried egg shapes (Figure 1).

The microscopic appearance of the organisms was coccobacilli to rods with rounded ends and slightly convex sides. Typical bipolar safety pin shapes were visible from young cultures in broth medium following Wayson staining (Figure 2). Organisms in the broth did not produce turbidity, instead floccular deposits at the bottom and rising along the walls of the tubes were very consistent and characteristic (Figure 3).

The suspected *Y. pestis* isolates from human and rodent samples showed positive biochemical reactions to

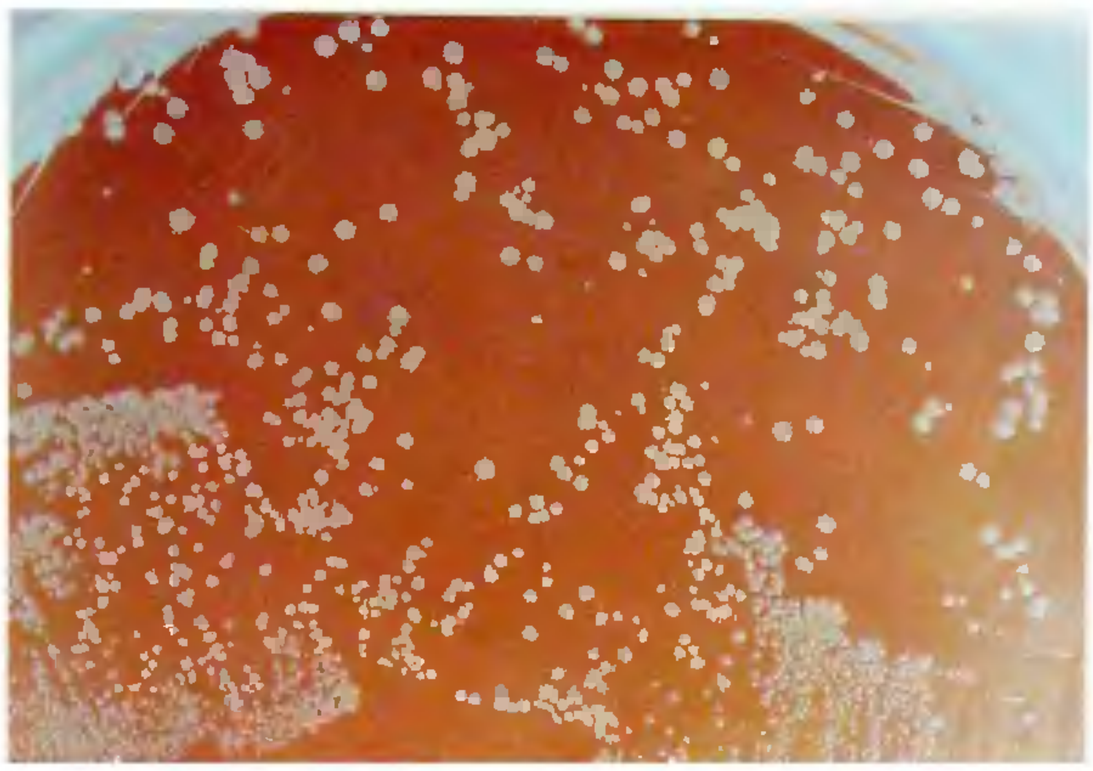

Figure 1. Colonies of a clinical *Y. pestis* isolate on blood agar plate following 72 h incubation at 28°C.

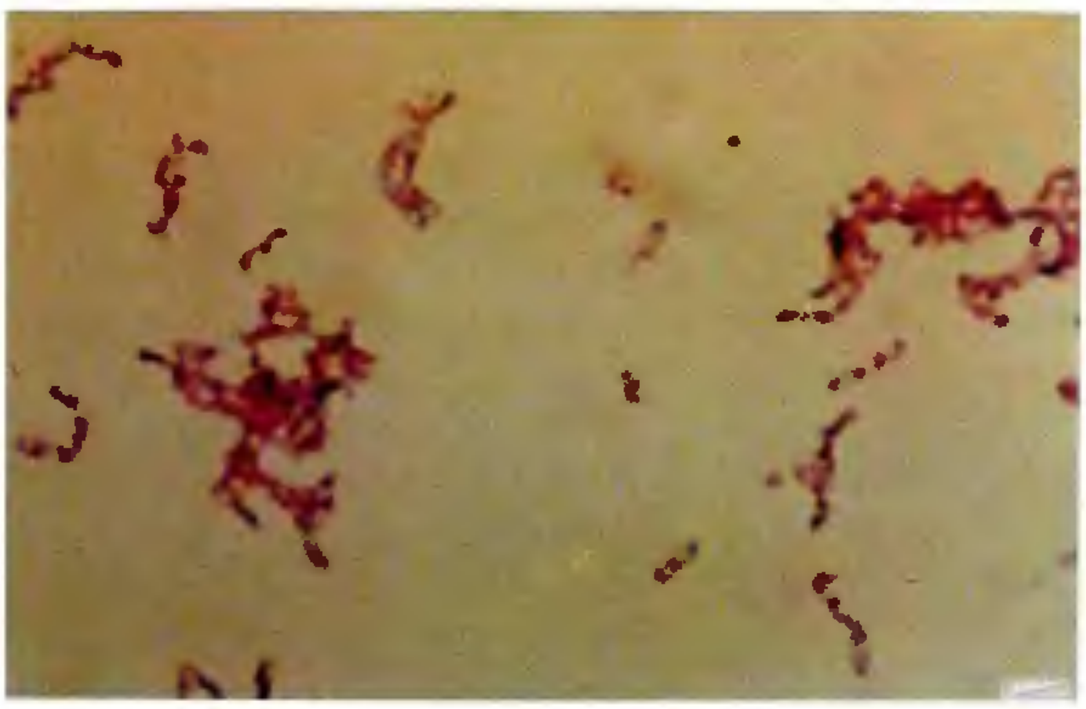

Figure 2. Wayson staining of a clinical isolate of *Y. pestis*.

glucose, mannitol, maltose, arabinose, xylose, salicin and scored negative in the remaining tests.

A total of 22 isolates exhibited these biochemical reactions. However, only 18 isolates could be lysed by the specific bacteriophage. SDS-PAGE revealed a characteristic uniform protein profile for all the 18 bacteriophage positive isolates (Figure 4 *a*, *b*); the other four biochemically resembling isolates had a very different pattern. Though the pattern of protein bands of the 18 isolates was nearly identical, a slight variation in the region of 34–35 kDa was observed for isolate numbers 9, 10 and 12, wherein a doublet could be seen that was not there in the rest of the samples.

Of the 18 *Y. pestis* isolates described above, 11 were recovered from the pneumonic patients and 7 from rodent specimens (Table 1). The age and sex of the patients yielding *Y. pestis* isolates are given in Table 2.

Figure 3. Growth of *Y. pestis* in broth tube.

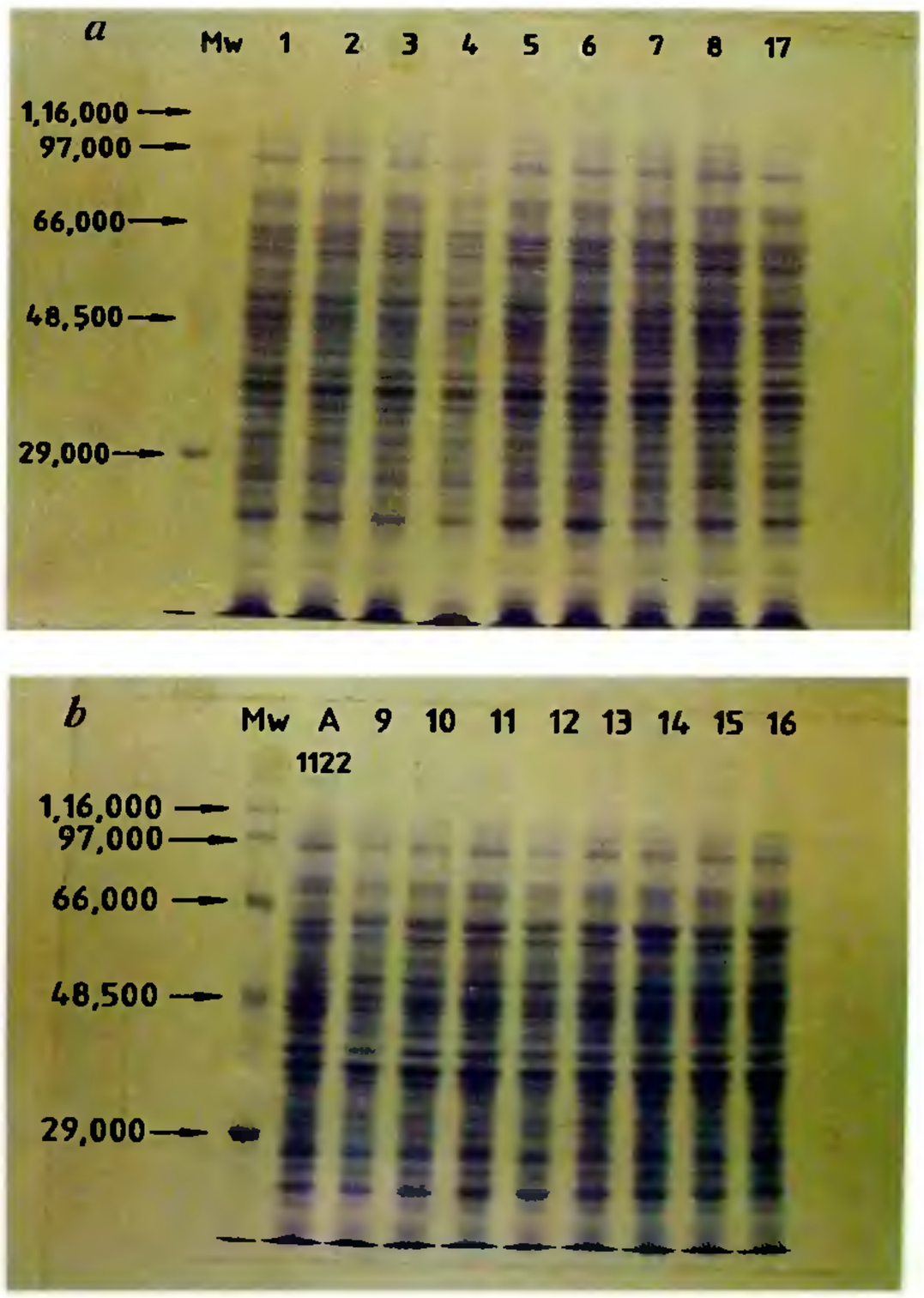

Figure 4 *a*, *b*. Protein profiles of *Y. pestis* isolates using 10% SDS-PAGE.

Due to the limited amount of anti F1-FITC conjugate available, only 4 of the *Y. pestis* isolates (nos. 1, 2, 3 and 4) could be tested. All the four showed the presence of F1 antigen by fluorescent antibody test (Figure 5).

Table 1. Summary of bacteriological (*Y. pestis*) isolation and identification experiments carried out on samples from Surat city and Beed district

Sample collection				Number positive			
Place	Period	Source	Number processed	Biochemical tests	Phage lysis	F1-FAT	F1-CIE
Surat city	Sept.–Oct. 1994	Human	82	14	11	4*	11
Surat city	Oct.–Nov. 1994	*Rattus rattus*	4	nil	nil	NT	nil
Surat city	Jul.–Aug. 1995	*Rattus rattus*	17	1	1	NT	1
Beed dist.	Aug.–Nov. 1994	*Rattus rattus*	30	4	3	NT	2
Beed dist.	Apr.–May 1995	*Rattus rattus*	11	1	1	NT	1
Beed dist.	Jul.–Aug. 1995	*Rattus rattus*	53	1	1	NT	1
		Tatera indica	17	1	1	NT	1

*Only four samples were tested.
NT = Not tested.

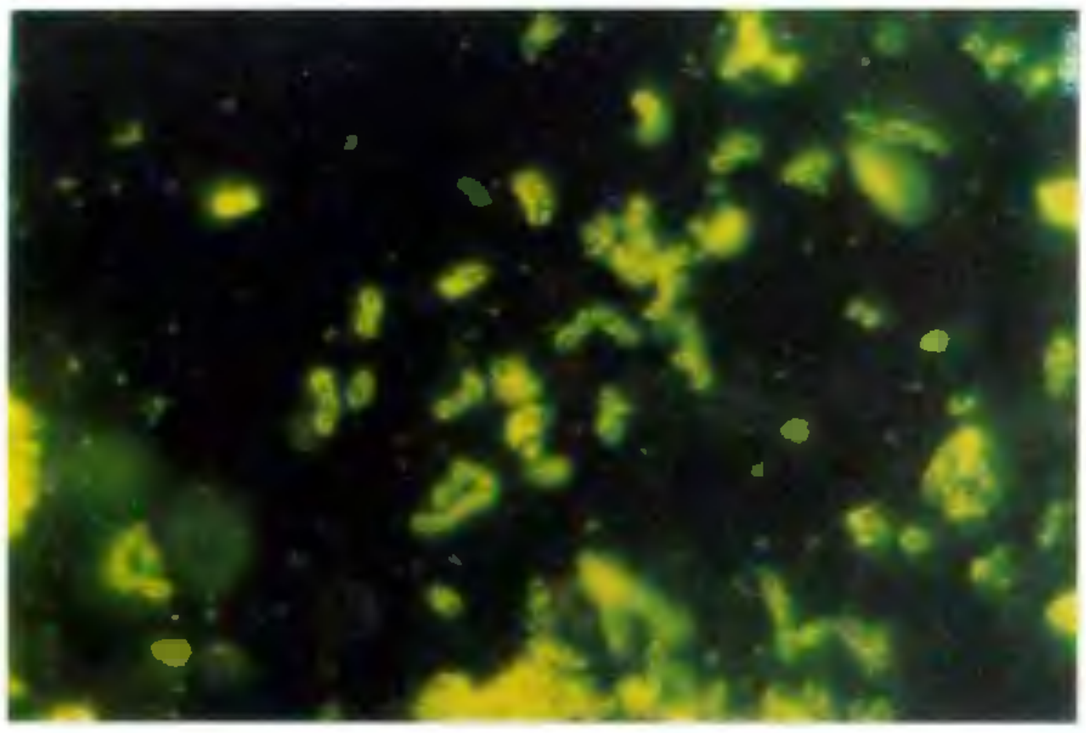

Figure 5. Presence of F1 antigen in a clinical isolate of *Y. pestis* tested by FAT.

Table 2. Age and sex of pneumonic patients yielding *Y. pestis* isolates

S. no.	Date of admission	Age (years)	Sex
1	01.10.94	2	F
2	22.09.94	25	M
3	29.09.94	9	M
4	22.09.94	11	M
5	22.09.94	19	M
6	22.09.94	24	M
7	22.09.94	34	M
8	22.09.94	18	M
9	22.09.94	60	F
10	22.09.94	33	M
11	22.09.94	18	M

17 of the 18 isolates exhibited clear precipitin bands against rabbit anti F1-antibodies. Isolate no. 12 did not show the presence of F1 antigen by this test. Similarly other non *Y. pestis* organisms including the *Y. pseudotuberculosis* and *Y. enterocolitica* were negative.

Discussion

Confirmation of a plague outbreak is achieved chiefly by isolation of *Y. pestis* or by demonstrating the serological responses compatible with the infection. In the present suspected outbreaks in Maharastra and Gujarat states, the isolation work described here was undertaken after the Technical Advisory Committee was established and after the outbreaks had abated. The samples were collected by our collaborating institutions from patients in Surat at the time of the outbreak. There was a gap of over 45 days from collection to processing of samples from Surat patients. This could have been the main reason for finding mixed growth of organisms in all the 82 samples that were processed and for colonies resembling *Y. pestis* that could be purified only from 11 of these samples. Tissue specimens from 7 out of 132 rodents collected during and after the outbreaks and tested 10 to 30 days after field collection yielded *Y. pestis* organisms following purification from the mixed growth. Although *Y. pestis* grows easily on most of the bacteriological media, the growth is relatively slow. Problems are encountered in differentiating *Y. pestis* when other organisms are also present in the samples. Contaminating organisms are nearly always present in sputum samples and in most of the animal and flea specimens submitted from the field. Cultures showing mixed flora (including *Y. pestis*) on solid or broth media require the microbiologist to proceed promptly within 2–4 days to separate and identify *Y. pestis*. Otherwise, most contaminating organisms by virtue of more rapid growth become so predominant that it becomes difficult to obtain separate colonies of *Y. pestis*. Eventually the contaminants outgrow *Y. pestis* in the culture, making it impossible to confirm the latter's presence[6]. The long gap in processing of specimens from the time of collection is probably responsible for the smaller number of isolates from the Surat patients.

A striking observation was that 9 of the 11 patients from whom the *Y. pestis* was isolated were admitted to

the hospital on 22 September 1994. In accordance with the epidemiological information available, the outbreak in Surat affected mainly young adults and started on 19 September 1994. The clinical diagnosis of suspected pneumonic plague was made on 21 September 1994. Since at that stage therapy with antibiotics appropriate for plague had just been initiated, this could have contributed to a certain extent in the recovery of more isolates from these early cases. The maximum number of isolations (9) were from male patients in the young age group of 9 to 34 years.

It has been stated that positive results obtained by Gram, Giemsa or Wayson staining of smears to identify safety-pin appearance of *Y. pestis* and the F1-FAT test are not confirmatory for plague but are vital preliminary steps in making the diagnosis[7,8]. In our study, Gram and Wayson staining for safety-pin appearance was quite confusing even to arrive at a presumptive diagnosis. The F1-FAT was not done directly on clinical samples from the present outbreaks because of a scarcity of the necessary reagents.

Biochemical characterization using the above-mentioned biochemical tests was not conclusive to establish a firm identity. Four of 22 isolates which biochemically resembled *Y. pestis* turned out to be different organisms when SDS-PAGE technique for protein profiles was undertaken. These were also not lysed by specific bacteriophage.

Among the rodent isolates, 3 were from affected areas in district Beed from specimens collected within a period of 3 months after the outbreak. The remaining 3 from the same areas were recovered within a period of 9 months to one year after the outbreak. One among these was from *Tatera indica*, the pericommensal rodent, trapped within one kilometer radius of Mamla village with a flea index of 5.5. The lone Surat rodent isolate was from Ved Road locality, the most affected area during the outbreak in 1994. These rodent isolations are a pointer to the fact that the plague foci are still active in the two affected areas.

Earlier studies[9] have shown that *Rattus rattus* and *Rattus exulans* are susceptible commensal rodent species. In the present instance, 6 of the 7 rodent isolates were from trapped *Rattus rattus* specimens. Probably the *Y. pestis* infection in these animals did not reach the stage of being fatal or the infecting organisms are less virulent or may be the *Rattus rattus* in these areas are becoming relatively resistant. The presence of F1 capsular antigen, a virulent marker, was, however, demonstrated in 6 of the 7 isolates by CIE and the existence of 70 kb plasmid was confirmed in all the isolates.

It is well known that once plague becomes endemic in foci in a country, it is very difficult to eliminate it because the rodent reservoirs of these organisms cannot be eliminated. Regular monitoring of rodent populations and routine testing of suspected patients should form important components in plague surveillance systems. Awareness of the fact that apart from bubonic or pneumonic appearances, gastrointestinal symptoms have been frequently recorded as primary clinical manifestations of confirmed *Y. pestis* infection[10] requires the clinicians to be extra cautious. Abdominal pain may be attributed to intra-abdominal buboes, which can occur without inguinal lymph node involvement[7]. In a recent case study report, confirmed plague presented itself as upper respiratory tract infection, nonspecific febrile syndrome, gastrointenstinal or urinary tract infections and meningitis[11]. Therefore, for the differential diagnosis of such syndromes, a reliable diagnosis of plague is essential.

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