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Recent Emergence of New Variants of *Yersinia pestis* in Madagascar

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***Yersinia pestis*, the causative agent of plague, has been responsible for at least three pandemics. During the last pandemic, which started in Hong Kong in 1894, the microorganism colonized new, previously unscathed geographical areas where it has become well established. The aim of this longitudinal study was to investigate the genetic stability of *Y. pestis* strains introduced into a new environment just under a century ago and to follow the epidemiology of any new genetic variant detected. In the present study, 187 strains of *Y. pestis* isolated between 1939 and 1996 from different regions of Madagascar and responsible mainly for human cases of bubonic and pneumonic plague were studied. Our principal genotyping method was rRNA gene profiling (ribotyping), which has previously been shown to be an effective scheme for typing *Y. pestis* strains of different geographical origins. We report that all studied *Y. pestis* strains isolated in Madagascar before 1982 were of classical ribotype B, the ribotype attributed to the *Y. pestis* clone that spread around the world during the third pandemic. In 1982, 1983, and 1994, strains with new ribotypes, designated R, Q, and T, respectively, were isolated on the high-plateau region of the island. Analysis of other genotypic traits such as the *Not*I genomic restriction profiles and the *Eco*RV plasmid restriction profiles revealed that the new variants could also be distinguished by specific genomic and/or plasmid profiles. A follow-up of these new variants indicated that strains of ribotypes Q and R have become well established in their ecosystem and have a tendency to spread to new geographical areas and supplant the original classical strain.**

Plague has been one of the most devastating infectious diseases in human history. The typical signs of bubonic and pneumonic plague along with the epidemiological characteristics of the disease left reliable historical records that made possible the establishment of the occurrence of three pandemics during the Christian era. The first pandemic occurred during the 6th century and probably killed 100 million people (16). The second pandemic invaded Europe during the 14th century and persisted until the 18th century. The third pandemic started in Hong Kong in 1894, just over a century ago, and rapidly spread over five continents. Major advances in the knowledge of plague were made during the early years of the last pandemic. In 1894, Alexandre Yersin (23) identified the etiologic agent, a gram-negative rod now called *Yersinia pestis*, and showed that rats were the reservoir of the disease. Four years later, Paul-Louis Simond (20) demonstrated that plague was transmitted by fleas. The development of public health control measures, vaccines, and antibiotics considerably reduced the mortality, morbidity, and incidence of plague during the 20th century. However, plague persists in foci of endemicity in Africa, Asia, and North and South America. The recent increase in the number of cases of plague in humans (22) and the reappearance of epidemics in countries such as Malawi, Mozambique, and India led to its categorization as a reemerging disease (19).

Y. pestis can be subdivided into three biovars. On the basis of historical data and the bacteriological characteristics of the strains isolated from remnant foci of ancient plague, Devignat (5) hypothesized that biovars Antiqua, Medievalis, and Orientalis caused the first, second, and third pandemics, respectively. It is hardly possible to confirm the link between the first two pandemics and biovars Antiqua and Medievalis other than to argue for hypotheses on the basis of historical data. In contrast, the fact that plague colonized previously unscathed areas during the third pandemic could be used to correlate the characteristics of the strains with that pandemic. The observation that all strains isolated from the areas previously unaffected by plague were of biovar Orientalis, while isolates from ancient foci were of other biovars, argued for the clonality of a strain of biovar Orientalis as the causative agent of the third pandemic.

Recent genetic evidence has reinforced Devignat's hypothesis. Using pulsed-field gel electrophoresis techniques, Lucier and Brubaker (14) found that the *Spe*I DNA patterns of eight strains of *Y. pestis* were closely related to their respective biovars. Similar results were obtained by Rakin and Heesemann (17) by the same technique and use of the I-*Ceu*I restriction enzyme for nine *Y. pestis* strains. By restriction fragment length polymorphism analysis of rRNA genes, we were able to subdivide 70 strains of *Y. pestis* into 16 ribotypes (11), and we found a close association between biovar Orientalis and ribotype B. However, there were some striking exceptions to this association among the third-pandemic strains. For instance, five strains of biovar Orientalis isolated in Vietnam were of ribotype G or E (11). Similarly, we found that three *Y. pestis* strains of biovar Orientalis isolated during the 1994 outbreak in India harbored a new ribotype designated ribotype S (18). These results suggested that in less than one century (which, from an evolutionary point of view, represents a very short period of time), the original *Y. pestis* strain that spread over the world had undergone chromosomal rearrangements, leading to the local emergence of new ribotypes. To test this hypoth-

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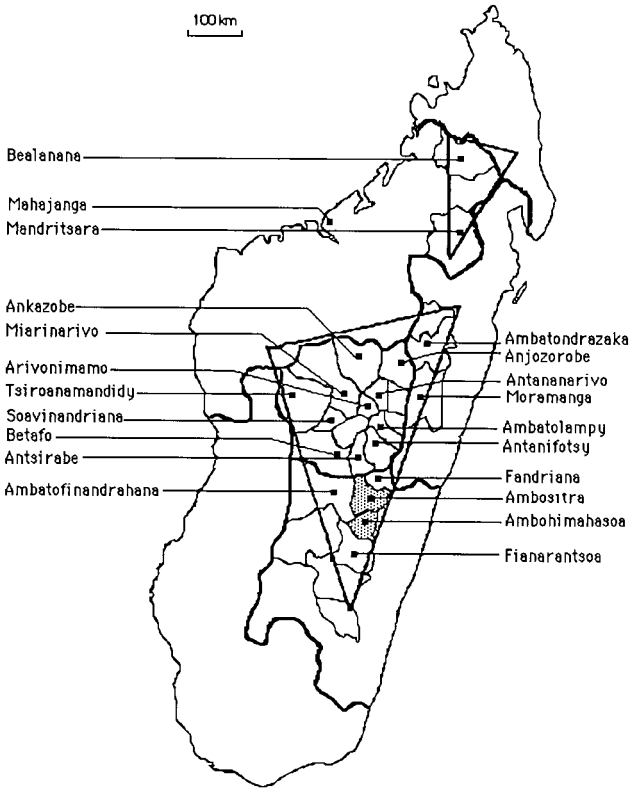

FIG. 1. Map of the island of Madagascar. The two "plague triangles" located on the high plateau are indicated. Thin lines delineate the subprefectures located within these triangles. Dotted areas correspond to the areas where strains of ribotypes Q and R were isolated.

esis, the evolution of the ribotype of *Y. pestis* over time in a specific geographical area affected by the third pandemic has been followed. Madagascar was chosen for this study for several reasons: (i) plague arrived for the first time during the third pandemic, (ii) since it is an island, the risks of extraneous plague importation are limited, and (iii) a wide range of strains along with data on their epidemiological characteristics have been collected.

In the present study, we demonstrate that in Madagascar, all *Y. pestis* strains that were studied and that were collected before 1982 had the classical ribotype B. In 1982 and later, strains with new ribotypes, designated ribotypes R, Q, and T, emerged in a specific region of the island and are now well established in their ecosystem. We also demonstrate an association between the genomic profiles, the ribotypes, and the plasmid profiles of the different variants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 187 strains of *Y. pestis* isolated between 1939 and 1996 from different regions of Madagascar (Fig. 1) were analyzed in this study. They were taken from the collection of the Central Laboratory for Plague (Institut Pasteur, Antananarivo, Madagascar). All strains belonged to the biovar Orientalis (they did not ferment glycerol but did reduce nitrate to nitrite) (5). To obtain genomic DNA, bacterial suspensions were prepared from stock cultures and were streaked onto Trypticase soy agar plates. The plates were incubated for 24 to 48 h at 28°C, i.e., until confluent growth was visible. For plasmid preparation, bacteria were grown in peptone broth for 24 h at 28°C with shaking.

DNA extraction, restriction, and transfer to nylon membranes. Extraction of total DNA from each *Y. pestis* strain was performed as described previously (4, 11). Ten micrograms of each sample was digested overnight at 37°C with *Eco*RI or *Eco*RV restriction enzyme before being loaded onto a 0.8% agarose gel and subjected to an overnight electrophoresis as described previously (11). Alkaline denaturation, neutralization, and transfer of DNA onto nylon filters (Hybond-N; Amersham, Les Ulis, France) with a VacuGene apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) were performed as described previously (13).

For visualization of undigested plasmids, plasmid DNA was extracted by the procedure of Kado and Liu (12). For restriction endonuclease analysis of plasmids, plasmid extracts were obtained by the method of Birnboim and Doly (1) and were digested overnight at 37°C with the *Eco*RV restriction endonuclease. The DNA was loaded onto 0.8% agarose gels, subjected to overnight electrophoresis, and stained with ethidium bromide.

Hybridization with the rRNA probe. In the first part of this study, a nonradioactive acetylaminofluorene-labeled 16S plus 23S rRNA probe (10) was used. Hybridization and immunoenzymatic detection of hybridizing fragments were performed according to the manufacturer's recommendations (Eurogentec, Seraing, Belgium), with slight modifications (11). In the second part of this study, 16S plus 23S rRNA from *Escherichia coli* (Boehringer) was labeled with horseradish peroxidase by using the ECL gene detection system (Amersham) as described previously (13).

Antibiotic susceptibility testing. Susceptibilities to 14 antibiotics (ticarcillin, amoxicillin, amoxicillin-clavulanic acid, cefalothin, cefotaxime, tetracycline, minocycline, streptomycin, gentamicin, kanamycin, sulfonamide, trimethoprim, chloramphenicol, and nalidixic acid) was performed by the disk diffusion test (15) in Mueller-Hinton agar.

Pulsed-field gel electrophoresis. Genomic DNA was prepared in agarose plugs as described previously (3). Following digestion with *Not*I, macrorestriction fragments were resolved by contour-clamped homogeneous electric field electrophoresis with a CHEF-DRIII apparatus (Bio-Rad Laboratories), an electric field of 6 V/cm, and an angle of 120°. Migration of the DNA fragments was achieved in 0.5× Tris-borate-EDTA buffer and in 0.9% agarose gels maintained at 17°C. Pulse times were ramped from 1 to 10 s over 29 h.

Nomenclature. The hybridization profiles of the 16S plus 23S rRNA probe with the genomic DNA digested with *Eco*RI or *Eco*RV, named the *Eco*RI and *Eco*RV ribopatterns, respectively, are designated by the letters RI and RV, respectively, followed by a numeral (11). The ribotype of a strain (in capital letters) corresponds to the combination of the *Eco*RI and *Eco*RV ribopatterns. Pulsotype refers to the *Not*I genomic restriction pattern obtained after pulsed-field gel electrophoresis of total genomic DNA.

RESULTS

Identification of new ribotypes among strains from Madagascar. A representative sample of 59 *Y. pestis* strains was selected from the Madagascar culture collection (Table 1) on the basis of their year of isolation (from 1939 to 1996) and geographical origin (21 different regions) (Fig. 1).

Hybridization of the *Eco*RI-digested genomic DNAs of these strains with the 16S plus 23S rRNA probe initially showed three different profiles. The most common profile was identical to the previously described ribopattern RI.1 (Fig. 2A, pattern 1) (11). This *Eco*RI pattern is found in ribotype B, the ribotype characteristic of most strains isolated during the third pandemic. A second *Eco*RI pattern was identified in two strains (strains 13/82 and 3/89). It differed from ribopattern RI.1 by the loss of one band at 7.9 kb and the appearance of a novel fragment of approximately 8.5 kb. Since this pattern differed from all 11 previously characterized *Eco*RI patterns (11), it received the designation RI.12 (Table 1 and Fig. 2A, pattern 12). A third *Eco*RI pattern, which differed from RI.1 by the presence of an additional band of 7.5 kb, was identified in two strains (strains 9/83 and 22/94). This *Eco*RI pattern was also different from all the known patterns and received the designation RI.13 (Table 1 and Fig. 2A, pattern 13).

When the *Eco*RV restriction enzyme was used, two different hybridization profiles were observed (Fig. 2B). The most frequent one corresponded to known pattern RV.2, which, combined with RI.1, forms ribotype B (11). A second *Eco*RV ribopattern was identified in two strains (strains 13/82 and 3/89). It differed from RV.2 by the existence of a thinner band at 7.5 kb (which corresponds to two fragments very close in size) and the presence of an additional band of 8.4 kb. Since this pattern was different from the already identified 11 *Eco*RV patterns (11), it received the designation RV.12 (Table 1 and Fig. 2B, pattern 12).

Interestingly, we showed in a previous work (11) that, by

TABLE 1. Characteristics of 59 strains of *Y. pestis* isolated at different times and from different regions of Madagascar[a]

Strain	Host	Site of isolation	Yr of isolation	Subprefecture	RI	RV	Ribotype
12/39	Human	Bubo	1939	Antananarivo	1	2	B
27/40	Human	Organ, sputum	1940	Ambositra	1	2	B
29/41	Lemur	Spleen	1941	UN	1	2	B
45/43	Human	Organ	1943	Antananarivo	1	2	B
62/46	Human	Lungs	1946	Miarinarivo	1	2	B
125/51	Human	Bubo	1951	Antsirabe	1	2	B
142/53	Guinea pig	Spleen, LN	1953	Antananarivo	1	2	B
4/54	Human	Bubo	1954	Miarinarivo	1	2	B
5/55	Human	Organ, bubo	1955	Ankazobe	1	2	B
4/56	Human	Bubo	1956	Ambatolampy	1	2	B
8/57	Human	Organ	1958	Soavinandriana	1	2	B
6/58	Human	Organ	1958	Antsirabe	1	2	B
12/59	Human	Bubo	1959	Ambositra	1	2	B
3/61	Human	Organ, bubo	1961	Arivonimamo	1	2	B
28/62	Rat	Spleen	1962	Moramanga	1	2	B
4/64	Human	Bubo	1964	Ambositra	1	2	B
22/68	Human	Organ, bubo	1968	Tsiroanomandidy	1	2	B
6/69	Human	Bubo	1969	Fandriana	1	2	B
13/70	Human	Bubo	1970	Tsiroanomandidy	1	2	B
9/71	Human	Organ	1971	Bealanana	1	2	B
15/71	Human	Bubo	1971	Bealanana	1	2	B
1/72	Human	Organ, bubo	1972	Bealanana	1	2	B
3/73	Human	Bubo	1973	Ambositra	1	2	B
4/74	Human	Bubo	1974	Soavinandriana	1	2	B
4/75	Human	Bubo	1975	Fianarantsoa II	1	2	B
6/75	Human	Bubo	1975	Betafo	1	2	B
3/76	Human	Bubo	1976	Bealanana	1	2	B
6/76	Human	Bubo	1976	Soavinandriana	1	2	B
5/77	Human	Bubo	1977	Antsirabe	1	2	B
6/77	Human	Bubo	1977	Antanifotsy	1	2	B
1/78	Human	Bubo	1978	Bealanana	1	2	B
2/79	Human	Bubo	1979	Antananarivo	1	2	B
10/81	Human	Bubo	1981	Tsiroanomandidy	1	2	B
16/81	Human	Bubo	1981	Ambatondrazaka	1	2	B
13/82	Human	Bubo	1982	Ambositra	12	12	R
9/83	Human	Bubo	1983	Ambohimahasoa	13	2	Q
10/84	Human	Bubo	1984	Ambatofinandrahana	1	2	B
16/85	Human	Organ	1985	Soavinandriana	1	2	B
14/86	Human	Organ	1986	Anjozorobe	1	2	B
4/88	Human	Bubo	1988	Antanifotsy	1	2	B
7/88	Human	Bubo	1988	Betafo	1	2	B
19/88	Human	Bubo	1988	Ambatofinandrahana	1	2	B
3/89	Human	Bubo	1989	Ambositra	12	12	R
88/90	Human	Bubo	1990	Betafo	1	2	B
3/91	Human	Bubo	1991	Ambatofinandrahana	1	2	B
72/91	Human	Bubo	1991	Mandritsara	1	2	B
10/92	Human	Organ	1992	Antananarivo	1	2	B
80/92	Human	Bubo	1992	Tsiroanomandidy	1	2	B
85/92	Human	Bubo	1992	Moramanga	1	2	B
31/93	Human	Bubo	1993	Ambositra	1	2	B
10/94	Human	Bubo	1994	Ambatofinandrahana	1	2	B
22/94	Human	Bubo	1994	Ambohimahasoa	13	2	Q
71/94	Rat	Organ	1994	Antananarivo	1	2	B
10/95	Human	Bubo	1995	Antananarivo	1	2	B
128/95	Human	Bubo	1995	Mahajanga	1	2	B
1/96	Human	Lungs	1996	Anjozorobe	1	2	B
6/96	Human	Bubo	1996	Miarinarivo	1	2	B
21/96	Human	Bubo	1996	Arivonimamo	1	2	B
24/96	Human	Bubo	1996	Antanifotsy	1	2	B

[a] Abbreviations: UN, unknown; LN, lymph nodes; RI, *Eco*RI ribopattern; RV, *Eco*RV ribopattern.

using the *Eco*RI enzyme, two hybridizing fragments of 18.2 and 7.6 kb were found in all *Y. pestis* strains of worldwide origin studied, and a third fragment of 4.7 kb was present in all but one of the strains examined. These three fragments were also conserved in the new ribopatterns identified in Madagascar (arrows in Fig. 2A). Similarly, with the *Eco*RV enzyme, the perfectly conserved fragments of 7.5 and 5.8 kb and the conserved fragment of 5.5 kb were present in the new ribopattern, ribopattern RV.12 (arrows in Fig. 2B).

Combination of the *Eco*RI and *Eco*RV ribopatterns indi-

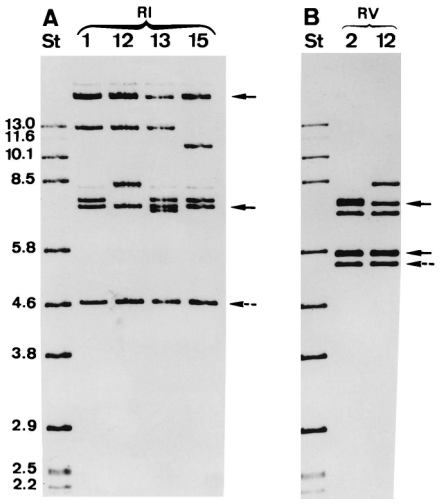

FIG. 2. Representative *Eco*RI (A) and *Eco*RV (B) ribopatterns of the strains of *Y. pestis* isolated from different plague foci in Madagascar. Ribotype B is the combination of RI.1 and RV.2, ribotype Q is the combination of RI.13 and RV.2, ribotype R is the combination of RI.12 and RV.12, and ribotype T is the combination of RI.15 and RV.2. Arrows on the right point to restriction fragments conserved in all strains (solid bar) or in all but one strain (dotted bar) of *Y. pestis* isolated from various parts of the world and ribotyped previously (11). Lanes St, *Eco*RI-cleaved DNA of *Xenorhabdus* sp. strain 278 used as a molecular mass standard; the sizes of the bands (in kilobases) are indicated on the left side of panel A.

cated that all strains that displayed the classical ribopattern RI.1 also displayed the classical ribopattern RV.2 and therefore belonged to ribotype B (Table 1). The two strains which exhibited *Eco*RI pattern RI.13 had classical *Eco*RV pattern RV.2. Following the alphabetical order, the combination RI.13 plus RV.2 was designated ribotype Q (Table 1). Interestingly, the two strains harboring new *Eco*RI pattern RI.12 were the same strains that also harbored new *Eco*RV pattern RV.12. The combination of these two patterns (RI.12 plus RV.12) was designated ribotype R (Table 1).

Analysis of the epidemiological characteristics of the strains harboring the two new ribotypes, ribotypes Q and R, showed that, like most of the classical strains, they were isolated from human bubo aspirates. More interestingly, a relationship was found between the geographical origins of the strains and their ribotypes: the two strains of ribotype R were isolated in the Ambositra subprefecture and those of ribotype Q were isolated in the Ambohimahasoa subprefecture (Table 1). These two neighboring regions are located on the high plateau, in the southern part of the Madagascar central "plague triangle" (Fig. 1).

Analysis of the ribotypes of the strains isolated in the Ambositra and Ambohimahasoa subprefectures. A more complete analysis of the *Y. pestis* strains isolated in the Ambositra and Ambohimahasoa subprefectures was undertaken. For this purpose, 131 isolates identified between 1940 and 1996 in the Ambositra (98 isolates) and Ambohimahasoa (33 isolates) subprefectures were analyzed. Three of these isolates came from rodents (*Rattus rattus*) and the 128 remaining strains came from humans.

Seventy-six of the isolates tested were of classical ribotype B. All were isolated from humans but came from different clinical specimens (buboes, lung, sputum, or other internal organs). It is noteworthy that all 13 strains isolated before 1982 were of classical ribotype B.

Twenty-four strains of ribotype R were identified. They all came from human patients and from different clinical samples. The first strain was isolated in 1982. Until 1994, the territory of this new variant was restricted to the northern part of the Ambositra region (Ilaka, Andina, and Tsarasoatra municipalities; Fig. 3). A field investigation of the precise locations of the villages where the patients were found to be infected with a strain of ribotype R revealed that they all lived in different small villages 5 km apart extending along a 20-km rocky hill. This *Y. pestis* variant was observed outside this area for the first

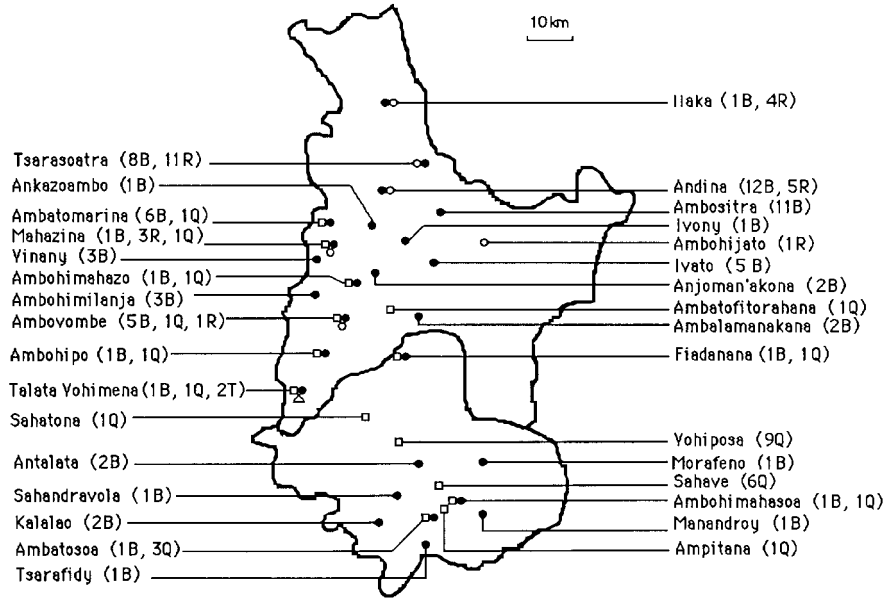

FIG. 3. Map of the Ambositra (upper area) and Ambohimahasoa (lower area) regions. The different names correspond to the municipalities where strains of ribotypes R (white circles), Q (white squares), T (white triangle), or B (black circles) were isolated. The number of strains isolated from each municipality and their ribotypes are indicated in parentheses.

time in 1994, in the Mahazina municipality. The same variant was again isolated from this municipality in 1995, suggesting local implantation. Expansion to more distant areas such as Ambohijato and Ambovombe was observed in 1996 (Fig. 3).

Twenty-eight strains of ribotype Q were found. Three of them were isolated from rodents, and the 23 other strains were isolated from human patients, essentially from bubo aspirates (22 strains). Ribotype Q strains were isolated for the first time in 1983. However, they appeared simultaneously in widely separated areas such as Ambovombe and Vohiposa (Fig. 3), suggesting that they in fact emerged before 1983. This variant is now found in a large area covering the western part of the Ambositra and Ambohimahasoa subprefectures.

The ribotype of one strain (strain 22/95) isolated in the Mahazina municipality (Fig. 3) in 1995 from a 40-year-old female who presented with clinical symptoms characteristic of bubonic plague (axillary bubo and fever at 40°C) was difficult to characterize. This strain displayed the *Eco*RI fragments of both 7.5 and 8.5 kb specific for the RI.13 and RI.12 ribopatterns, respectively, and, when the *Eco*RV enzyme was used, both the thick band of 7.5 kb and the novel 8.4-kb fragment found in RV.2 and RV.12 ribopatterns, respectively (data not shown). This result was suggestive of a double infection with two strains of different ribotypes. To confirm this hypothesis, strain 22/95 was streaked onto an agar plate and 10 different colonies were picked and analyzed. Eight of them displayed the RI.12 and RV.12 ribopatterns, and the other two strains displayed the RV.13 and RV.2 ribopatterns, indicating that the patient was simultaneously infected by two strains, one of ribotype R and one of ribotype Q.

Finally, two strains (strains 57/94 and 61/94) harboring a new *Eco*RI ribopattern were identified in the Ambohimahasoa subprefecture, in the Talata Vohimena municipality, in 1994 (Fig. 3). Their *Eco*RI ribopatterns differed from RI.1 by the loss of the 13.1-kb fragment and the appearance of a smaller band of 11.4 kb (Fig. 2A). This modification of the *Eco*RI ribopattern was not accompanied by a modification of the *Eco*RV pattern, which remained RV.2. Since the designation RI.14 was already attributed to the strains from the 1994 outbreak in India (3a), this new ribopattern received the designation RI.15. Similarly, because the designation ribotype S was already in use to describe the Indian strains, the new Malagasy ribotype, corresponding to the combination RI.15 plus RV.2, was designated T. As for the other variants, the three conserved restriction fragments were also conserved in the new RI.15 ribopattern (Fig. 2A). Only two other strains from the Talata Vohimena municipality were previously studied: one of ribotype B isolated in 1988 and one of ribotype Q isolated in 1989. No other *Y. pestis* strain was recovered from this municipality after 1994. Analysis of clinical and epidemiological records indicated that the two strains of ribotype T were isolated a week apart from two members of the same family, a 14-year-old female and an 18-year-old male, who both presented with bubonic plague. In fact, a total of five members of this family developed clinical symptoms of plague within a 3-week period. The first two patients were an 8-month-old baby and a 9-year-old boy, both of whom died of the infection. The third patient was a 4-year-old boy who received treatment and survived but from whom no bubo aspirate was taken. The last two cases of bubonic plague were in the two patients from whom the strains of ribotype T were isolated.

Evolution of the new variants over time in the Ambositra and Ambohimahasoa subprefectures. A longitudinal study (Fig. 4) of the ribotypes of the *Y. pestis* strains isolated in the Ambositra and Ambohimahasoa subprefectures indicated that (i) the total number of isolates has increased significantly since the 1980s (this recrudescence of plague cases could reflect both a natural evolution of the disease and the result of the disorganization of the surveillance system); (ii) ribotype B is still the major ribotype found among the strains from this region; and (iii) since their first isolation in 1982 and 1983, strains of ribotypes Q and R have been isolated more frequently over an increasingly wide area, suggesting not only stable implantation but also colonization of the local ecosystem. Together, the new variants represent 42% of the strains isolated in the Ambositra and Ambohimahasoa subprefectures since 1990.

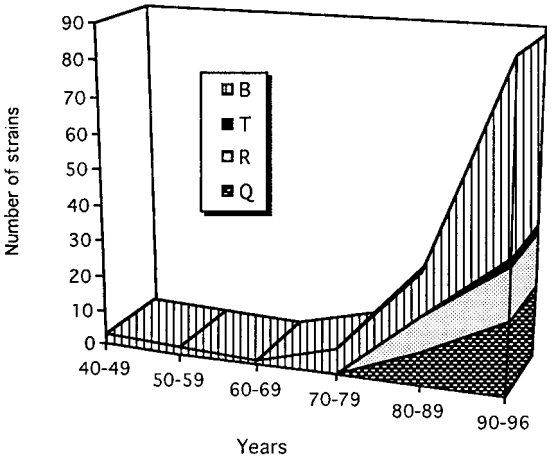

FIG. 4. Evolution of the strains of ribotypes B, Q, R, and T over time in the Ambositra and Ambohimahasoa subprefectures.

Genomic restriction profiles of *Y. pestis* strains of ribotypes B, Q, and R isolated in 1996 from the Ambositra and Ambohimahasoa subprefectures. In order to determine whether alterations of the rRNA gene profiles were accompanied by other genomic modifications, the *Not*I genomic restriction patterns (pulsotypes) of all 28 strains of *Y. pestis* isolated in 1996 in the Ambositra-Ambohimahasoa subprefectures (5 strains of ribotype Q, 6 strains of ribotype R, and 17 strains of ribotype B) were analyzed by pulsed-field gel electrophoresis. Only strains isolated in 1996 and kept at −20°C were studied by this method in order to avoid chromosomal rearrangements that may be generated during prolonged in vitro storage of the microorganisms.

The overall *Not*I genomic patterns were not identical but displayed a high degree of conservation (Dice similarity coefficient [6], close to 0.9) in the strains studied. Subtle differences involving a few restriction fragments could nonetheless be observed. Taking into account any change, even in only one restriction fragment, the 17 strains of ribotype B could be separated into eight pulsotypes (Fig. 5). Pulsotypes 4, 5, 7, and 8 were found in one strain each; pulsotypes 1 and 2 were found in two strains each; pulsotype 6 was found in four strains; and pulsotype 3 was found in five strains. These results suggest that spontaneous genomic rearrangements occur in *Y. pestis* in its natural environment. This could represent a simple point mutation in a restriction site or a more complex chromosomal rearrangement (21). No direct relationship could be established between the pulsotype and the site of isolation of the ribotype B strains.

Of the six strains of ribotype R studied, five displayed pulsotype 10 and one displayed pulsotype 11. None of these pulsotypes were identified in strains of ribotypes B and Q (Fig. 5). Furthermore, comparison of the geographical origins of the strains with their pulsotypes indicated that the only pulsotype

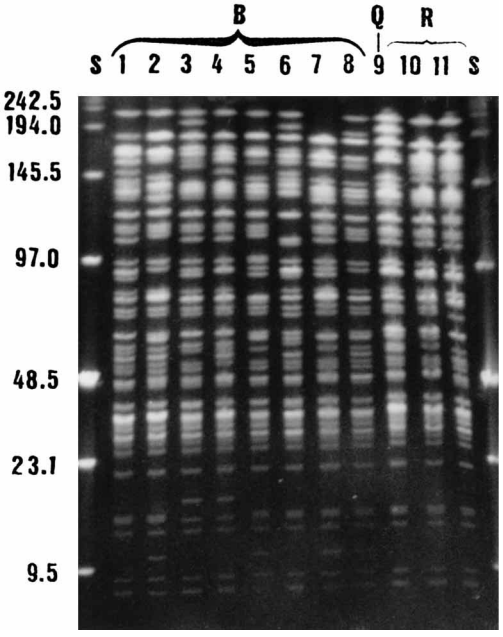

FIG. 5. Representatives of the 11 *Not*I genomic profiles found in the 28 strains of *Y. pestis* isolated in the Ambositra and Ambohimahasoa subprefectures in 1996. Lanes S, standard markers whose sizes (in kilobases) are indicated on the left side of the gel.

11 strain was isolated in Ilaka, i.e., in the most northern part of the primary territory of the strains of ribotype R (Fig. 3). The five pulsotype 10 strains were isolated in Andina (the south of the ribotype R territory) and in the newly colonized municipalities of Ambohijato and Ambovombe (Fig. 3). These results confirm the ribotyping data, again implying the geographical spread of this new variant from the northern territory to the southern regions.

All five strains of ribotype Q studied shared the same pulsotype (9), which differed from the eight pulsotypes observed in strains of ribotype B and the two pulsotypes of strains of ribotype R (Fig. 5). These strains were isolated in two different municipalities, Vohiposa (three strains) and Ambatosoa (two strains), from both humans and rats, demonstrating the epidemiological link between human plague cases due to *Y. pestis* of ribotype Q and the animal reservoir.

Whatever may be the exact genetic basis for the banding patterns observed, the consistent association of pulsotype and ribotype for strains of ribotypes Q and R suggests that these two variants are clonal in origin.

Analysis of the plasmid restriction profiles of strains of the four ribotypes. Wild-type *Y. pestis* strains usually contain three plasmids: (i) pFra/Tox (90 to 100 kb), which codes for fraction 1 antigen and a murine toxin; (ii) pYV (70 kb), the virulence plasmid common to the three pathogenic species of *Yersinia*; and (iii) pPla (9.5 kb), which encodes the bacteriocin pesticin and a plasminogen activator (7, 8). To determine whether DNA rearrangements also occurred in these plasmids, the plasmids of 76 *Y. pestis* strains were extracted and subjected to *Eco*RV restriction enzyme digestion. The *Eco*RV enzyme was chosen because, in our hands, it gave the best separation of the plasmid fragments. As indicated in Table 2, the 76 isolates studied included 20 strains of ribotype B (isolated from different regions of Madagascar and from the Ambositra-Ambohimahasoa subprefectures) and all strains of ribotypes Q, R, and T identified.

TABLE 2. *Eco*RV plasmid restriction profiles of various strains of *Y. pestis* of the four ribotypes

Ribotype (no. of strains)	REAP[a]	No. of strains
B (20)	P1	12
	$P1^-$	2
	$P1^+$	6
R (25)	P1	16
	$P1^-$	3
	$P1^+$	6
Q (29)	P2	28
	$P2^-$	0
	$P2^+$	1
T (2)	P3	2
Total		76

[a] REAP, *Eco*RV plasmid restriction profile; P^-, loss of one or two of the classical plasmids harbored by *Y. pestis*; P^+, acquisition of an extraneous plasmid.

After digestion, the bands visualized on a gel correspond to the restriction fragments of the three plasmids as a whole. The plasmid profile of our *Y. pestis* reference strain, strain 6/69, was used as the reference plasmid profile and was called P1 (Fig. 6). The loss of one or more plasmids, characterized by the disappearance of several restriction fragments but identical remaining fragments, was observed in only five of the strains studied (Table 2). In 13 other strains, one or more bands were seen in addition to the normal profile, suggesting the presence of an extra plasmid. By the method of Kado and Liu (12), which better differentiates undigested plasmids, one additional

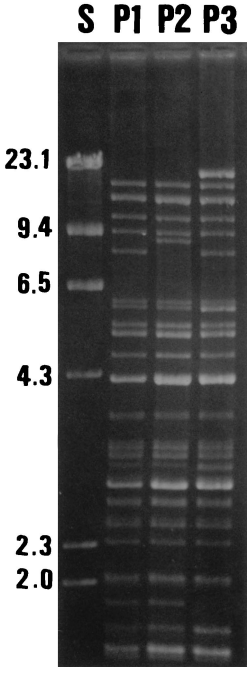

FIG. 6. Representative *Eco*RV plasmid profiles found in Madagascar. P1, the classical restriction profile found in strains of ribotypes B and R; P2 and P3, specific for strains of ribotypes Q and T, respectively; S, *Hin*dIII-digested bacteriophage lambda DNA as molecular size markers; the sizes of the bands (in kilobases) are indicated on the left side of the gel.

band was visible in most of these strains (data not shown), further suggesting the presence of a fourth plasmid in some isolates. Comparison of the additional *Eco*RV fragments indicated that the 13 strains harboring a fourth putative plasmid could be separated into four groups: the first group included three strains of ribotype B and five strains of ribotype R, the second group included three strains of ribotype B, the third group included one strain of ribotype R, and the fourth group included one strain of ribotype Q. Therefore, it seems that at least four different types of cryptic "extra" plasmids are circulating among *Y. pestis* strains in Madagascar and that their presence is not strictly linked to the ribotype of the strains. The presence of extra plasmids in addition to the three plasmids classically found in *Y. pestis* has already been reported for strains of various geographical origins (9). We did not further analyze these plasmids.

If the *Eco*RV plasmid profiles were compared without taking into account the missing bands due to plasmid loss or the additional bands due to plasmid acquisition, it appeared that all strains of ribotype B isolated at different times from different regions of Madagascar, including the Ambositra and Ambohimahasoa subprefectures, displayed the same *Eco*RV plasmid restriction profile, called P1 (Fig. 6 and Table 2). Similarly, all strains of ribotype R harbored the P1 plasmid profile found in strains of ribotype B (Table 2). These results suggest that the plasmid profile may be more stable than the ribotype.

All strains of ribotype Q displayed identical *Eco*RV restriction profiles. This profile, designated P2 (Table 2), differed from the P1 profile by the presence of an additional band of approximately 9 kb and the loss of an 8.5-kb fragment (Fig. 6). Therefore, strains of ribotype Q underwent not only chromosomal modifications (as evidenced by changes in their rRNA genes and genomic profiles) but also plasmid rearrangements. However, the fact that the 29 strains identified displayed a unique ribotype, pulsotype, and plasmid profile demonstrates the clonality of the strain that spread in the Ambositra-Ambohimahasoa region.

Similarly, the two strains of ribotype T displayed the same *Eco*RV plasmid profile which differed from both the P1 and the P2 profiles and, therefore, was designated profile P3 (Table 2). This *Eco*RV restriction profile was characterized by the presence of an extra band of approximately 22 kb and the loss of a small fragment of 1.8 kb (Fig. 6). Electrophoresis of undigested plasmid extracts clearly showed the presence of an additional plasmid in these two strains. Therefore, the plasmid profile of the two strains of ribotype T differed from the classical profile both by the loss of an *Eco*RV fragment normally found in *Y. pestis* and by the acquisition of a fourth plasmid.

Altogether, these results indicate that the *Eco*RV plasmid profiles of strains of ribotypes B and R are identical and perfectly conserved, whatever the date of isolation or the geographical origin of the strains in Madagascar. In contrast, strains of ribotypes Q and T each have a specific and conserved plasmid profile, which reinforces the hypothesis of a geographical expansion of specific clones.

Search for phenotypic differences associated with the ribotypes. We wondered whether the three ribotypes could be distinguished by phenotypic markers. For this purpose, the capacity to ferment the 50 sugars present in the API 50CH system was examined for the 187 strains examined in this study. No significant difference in the ability to ferment sugars was noted.

Similarly, the possibility of an association between a given ribotype and resistance to some antibacterial agents was investigated. The antibiotic susceptibilities of five strains each of ribotypes B, Q, and R and of the two strains of ribotype T were evaluated. All strains were susceptible to the 14 antibiotics tested, whatever their ribotypes.

Therefore, the different variants of *Y. pestis* could not be differentiated from each other on the basis of phenotypic markers such as fermentation of sugars or antibiotic susceptibility.

DISCUSSION

Plague was first introduced into Madagascar in November 1898, during the third pandemic, by a ship coming from India. In the following years, small outbreaks occurred in the major ports (16). Plague did not reach the high plateau of the island until 1921, when it caused a pneumonic outbreak in Antananarivo. A few months later, foci of bubonic plague and signs of rat infection became manifest in this area. The disease rapidly spread over the high plateau and became endemic in regions with altitudes of $\geq$800 m, subsequently disappearing from the seaports (2).

In a previous study (11), we hypothesized that the *Y. pestis* strain that spread over the world during the third pandemic was not only of biovar Orientalis, as suggested by Devignat (5), but also of ribotype B. The fact that all studied strains from Madagascar isolated before 1982 were of ribotype B argues for our hypothesis. However, we were puzzled by our previous observation that a few strains of biovar Orientalis isolated from different countries whose populations became infected during the third pandemic, such as Vietnam or India, harbored a ribotype other than ribotype B (11). The most likely explanation seemed that, once established in a new geographical area, the clone from the third pandemic underwent chromosomal rearrangements leading to the appearance of new ribotypes. This hypothesis has been tested in Madagascar. Our observation of the emergence of strains harboring new ribotypes such as ribotype R in 1982, ribotype Q in 1983, and ribotype T in 1994 in the Ambohimahasoa and Ambositra subprefectures strongly argues for this hypothesis.

The possibility that these strains do not correspond to new variants that emerged locally but rather to strains of *Y. pestis* introduced from abroad cannot be definitively ruled out but is highly unlikely for several of the following reasons. (i) None of the *Y. pestis* strains studied so far from anywhere in the world display these ribotypes, (ii) the new variants were isolated not in a seaport but on the high plateau, in an area that has very few exchanges with the outside world, (iii) they appeared recently, many years after effective plague surveillance was established in Madagascar, (iv) introduction of three different strains at three different times in the same remote area is extremely unlikely, and (v) there is as little divergence in the pulsotypes of strains of ribotypes B, Q, and R as within the strains of ribotype B isolated in Madagascar.

The fact that the three new variants were isolated from the same region of the island may be surprising. However, since the Ambohimahasoa and the Ambositra subprefectures are among the most active foci of plague on Madagascar, the detection of new variants may reflect intensive multiplication and circulation of the microorganism.

Strains of ribotype R were confined for many years to a specific ecological niche corresponding to a rocky hill located in the northern part of the Ambositra subprefecture. It is possible that some characteristics of this ecosystem favored the emergence of this variant. Its recent spread to the southern part of the Ambositra subprefecture indicates that this strain is now colonizing new geographical areas. In contrast, the territory of strains of ribotype Q is already quite large. The spread of this variant outside the Ambositra-Ambohimahasoa subpre-

fectures is likely to occur (or may already have occurred) and would be worth checking. Strains of ribotype T have only recently been recognized and at present seem to be limited to a small area. Whether this variant will be eliminated or whether its range will expand remains an open question.

The three new variants differed from the original strains not only in their ribotypes but also in their pulsotypes (strains of ribotype Q and R) and plasmid profiles (strains of ribotype Q and T). Whether there is a direct link between modifications of the rRNA genes, total genomic DNA, and plasmid DNA is unlikely. One more likely explanation is that the new variants were subjected to peculiar selective pressures in their specific ecosystems. These pressures may have been temporarily decreased, allowing genetic modifications which secondarily had no effect on the survival of the bacteria. On the other hand, the selective pressures may have been important, selecting for bacteria that acquired some specific traits that may have helped them survive in their specific environment. This second hypothesis could account for the progressive colonization of the Ambositra-Ambohimahasoa region by these new variants. An analysis of the clinical records of patients infected with strains of ribotype R suggested that they had more serious forms of the disease, with a higher lethality rate, than patients infected with regular strains of ribotype B. However, since the villages where these patients lived were distant from any health care center, the possibility that the higher mortality resulted from more limited access to health care cannot be excluded. An attempt to compare the levels of virulence of strains of ribotype B, Q, and R in the mouse model was not conclusive (10a) because the 50% lethal dose of wild *Y. pestis* is already so low (fewer than 10 bacteria by the subcutaneous route and approximately 1 bacterium by the intravenous route) that it makes this assay relatively insensitive to any increase in pathogenicity.

In conclusion, the results of this study confirm our initial hypothesis that distinct rRNA gene profiles of *Y. pestis* may evolve in a short period of time (i.e., in less than a century) in a specific geographical area. The isolation of strains of biovar Orientalis having a ribotype other than ribotype B in countries such as Madagascar, Vietnam, or India may thus be explained by a local modification of the original *Y. pestis* strain that spread over the entire world during the third pandemic. Whether these new variants have acquired selective advantages in a particular local environment and what the nature of these advantages could be will be challenging questions to be addressed in the future.

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