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Transmission of Antimicrobial Resistant *Yersinia pestis* During a Pneumonic Plague Outbreak

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Background. Pneumonic plague (PP), caused by *Yersinia pestis*, is the most feared clinical form of plague due to its rapid lethality and potential to cause outbreaks. PP outbreaks are now rare due to antimicrobial therapy.

Methods. A PP outbreak in Madagascar involving transmission of a *Y. pestis* strain resistant to streptomycin, the current recommended first-line treatment in Madagascar, was retrospectively characterized using epidemiology, clinical diagnostics, molecular characterization, and animal studies.

Results. The outbreak occurred in February 2013 in the Faratsiho district of Madagascar and involved 22 cases, including 3 untreated fatalities. The 19 other cases participated in funeral practices for the fatal cases and fully recovered after combination antimicrobial therapy: intramuscular streptomycin followed by oral co-trimoxazole. The *Y. pestis* strain that circulated during this outbreak is resistant to streptomycin resulting from a spontaneous point mutation in the 30S ribosomal protein S12 (*rpsL*) gene. This same mutation causes streptomycin resistance in 2 unrelated *Y. pestis* strains, one isolated from a fatal PP case in a different region of Madagascar in 1987 and another isolated from a fatal PP case in China in 1996, documenting this mutation has occurred independently at least 3 times in *Y. pestis*. Laboratory experiments revealed this mutation has no detectable impact on fitness or virulence, and revertants to wild-type are rare in other species containing it, suggesting *Y. pestis* strains containing it could persist in the environment.

Conclusions. Unique antimicrobial resistant (AMR) strains of *Y. pestis* continue to arise in Madagascar and can be transmitted during PP outbreaks.

Keywords. antimicrobial resistance; outbreak; pneumonic plague; *Yersinia pestis*.

Plague, caused by *Yersinia pestis*, has 3 clinical forms. Bubonic plague (BP), the predominant form, results from a flea bite. If BP is untreated, *Y. pestis* can enter the bloodstream, causing septicemic plague, and/or spread to the lungs, causing secondary pneumonic plague (PP). Individuals with secondary PP can transmit *Y. pestis* to other individuals via respiratory droplets, resulting in primary PP [1, 2]; incubation period of primary PP is 1–4 days [2–6]. PP is usually 100% lethal if not quickly treated with antimicrobials and can spread within human populations [6, 7]. *Y. pestis* is susceptible to many antimicrobials [8–11] except 3 previously identified antimicrobial resistant (AMR) or multidrug-resistant (MDR) strains, all from Madagascar, wherein AMR/MDR was conferred by presence of 3 unrelated conjugative plasmids [8, 12, 13], and a recently described strain from China resistant to streptomycin due to a point mutation in the 30S ribosomal protein S12 encoding *rpsL* gene [14].

Plague is a reemerging [15] and neglected [16] disease, and Madagascar accounts for most global human plague cases [17], reporting hundreds of presumptive or confirmed cases every year [18], sometimes many more [19]. Current Madagascar treatment guidelines recommend streptomycin as first-line treatment [20]. We describe a PP outbreak in Madagascar involving transmission of a *Y. pestis* strain resistant to streptomycin; cases were exposed while participating in traditional funeral practices.

METHODS

Definitions

Cases were defined according to World Health Organization (WHO) recommendations for known endemic plague foci [17], adapted to tests used at the time of this study: confirmed if *Y. pestis* was isolated, presumptive if positive results were obtained from a rapid diagnostic test (RDT) targeting the *Y. pestis* fraction 1 (F1) antigen [21] and/or anti-F1 serology

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[22], and suspect if compatible clinical symptoms were present but tests were negative or no test samples were available (Supplementary Table 1). Onset date was defined as the first day plague symptoms were observed. Notification of cases to the Madagascar plague national surveillance system is mandatory, so no additional ethics approval was required for that information. For cases identified during a retrospective investigation, written informed consent was obtained before inclusion (Malagasy ethical committee authorization number 037/MSANP/CE-2013). All information has been anonymized.

Identification of cases and sample collection:

Suspected cases were identified when presenting to health centers. On day of presentation, physicians collected sputum and blood samples from some suspected cases, which were sent to the Central Laboratory for Plague at Institut Pasteur de Madagascar (IPM) for confirmation.

Laboratory Analyses

Presence of F1 antigen in sputum samples was tested using RDT [21], and presence of anti-F1 antibodies was detected in sera obtained from blood samples using an ELISA [22]. *Y. pestis* culture was attempted from sputum samples using cefsulodin-irgasan-novobiocin selective media; antimicrobial susceptibility testing was performed on isolates using disk diffusion and seven antimicrobials. Confirmatory susceptibility testing and minimal inhibitory concentrations (MIC) were determined using E-test strips for streptomycin and co-trimoxazole. Isolate growth was examined via culturing, and virulence via infection of laboratory mice (Supplementary materials).

Phylogenetic and Genetic Analyses

DNA was extracted from *Y. pestis* isolates and 5 sputum samples, and whole genomes sequences (WGSs) were generated for isolates. Single-nucleotide polymorphisms (SNPs) were identified from WGSs for outbreak and other *Y. pestis* isolates from Madagascar (Supplementary Table 2) and used to infer a maximum-likelihood phylogeny; WGSs of isolates with AMR phenotypes were screened for known AMR determinants (Supplementary materials). To examine specificity of SNPs identified in WGSs of outbreak isolates and/or closely related isolates, these SNPs were queried (Supplementary materials) in DNA extracts (Supplementary Table 3) and/or WGSs (Supplementary Table 4) obtained from other *Y. pestis* isolates from Madagascar and elsewhere. Assays targeting these SNPs (Supplementary Table 5) were used to genotype *Y. pestis* DNA present in sputum samples (Supplementary materials).

RESULTS

Outbreak Description

From 31 January to 26 February 2013, 22 plague cases (2 confirmed, 6 presumptive, and 14 suspected) were registered from Faratsiho commune. Detailed case information is provided in Supplementary materials and Supplementary Table 1; location and temporal information is provided in Figures 1 and 2, respectively. High proportion of suspect cases was due to lack of samples for bacteriology and available samples being sputum; sputum samples are commonly culture-negative but RDT-positive for *Y. pestis* F1 antigen, as isolating *Y. pestis* from sputum samples is complicated by sputum quality and contamination by commensal flora [19].

The first known case, case 1, was a 7 year-old male from Andavabiby who first exhibited symptoms 31 January. He was not diagnosed or treated and died at Andavabiby on 7 February. Because his symptoms (fever, headache, vomiting, diarrhea) were consistent with either BP or PP, and time between symptom onset and death was relatively long (7 days), he may have had BP that progressed to secondary PP, thereby representing the index case for this PP outbreak. His traditional funeral at Andavabiby (8–9 February) was attended by 6 secondary cases (2–7). Case 2, a 2 year-old female, first exhibited PP symptoms at her residence in Amparihivato on 14 February; she died there 16 February without being diagnosed or treated. Her traditional funeral at Amparihivato (16–17 February) was attended by 5 secondary cases (3, 8–9, 21–22). Case 3 was a 46 year-old female residing in Ankafotra and grandmother of case 2. She visited Amparihivato from 3 to 8 February and traveled from there to attend the wake for case 1, returning to Amparihivato from 10 to 13 February. After traveling to Antsirabe, she returned to Amparihivato to attend the wake for case 2; she first exhibited PP symptoms 15 February. On 20 February, her family attempted to take her to a health center in Andohariana, but she died on the way that same day in Fenoarivo without being diagnosed or treated. Her family traveled with her corpse to Ankafotra, stopping in Anafivato for ~1.5 hours. At Ankafotra, a wake was held 21–22 February followed by traditional burial. On 23 February, 17 persons (cases 4–20), including relatives of case 3 and community members that had direct or indirect contact with her and others by participating in death care and/or attending her wake, developed PP symptoms and were admitted to Faratsiho District Hospital. Two additional cases were reported 26 February, one at Faratsiho District Hospital (case 21) and one at Ambondrona Health Center (case 22). Cases 21 and 22, who attended wakes for cases 2 and 3, presented with chest pain and blood-stained sputum but without febrile signs. All 19 surviving cases were treated with intramuscular streptomycin starting on day of admission and, subsequently, with oral co-trimoxazole; all recovered. Among the 22 cases, number of males and females was the same, 15 were >15 years old, and most participated in patient care (n = 2) and/or funeral events (cared for corpses n = 5, attended wakes n = 18).

At the time of the outbreak, 228 individuals resided in the affected hamlets. Because the most recent previous outbreak in this area occurred in 2003 and recovered plague cases are rarely seropositive 10 years after exposure [23], it was assumed none

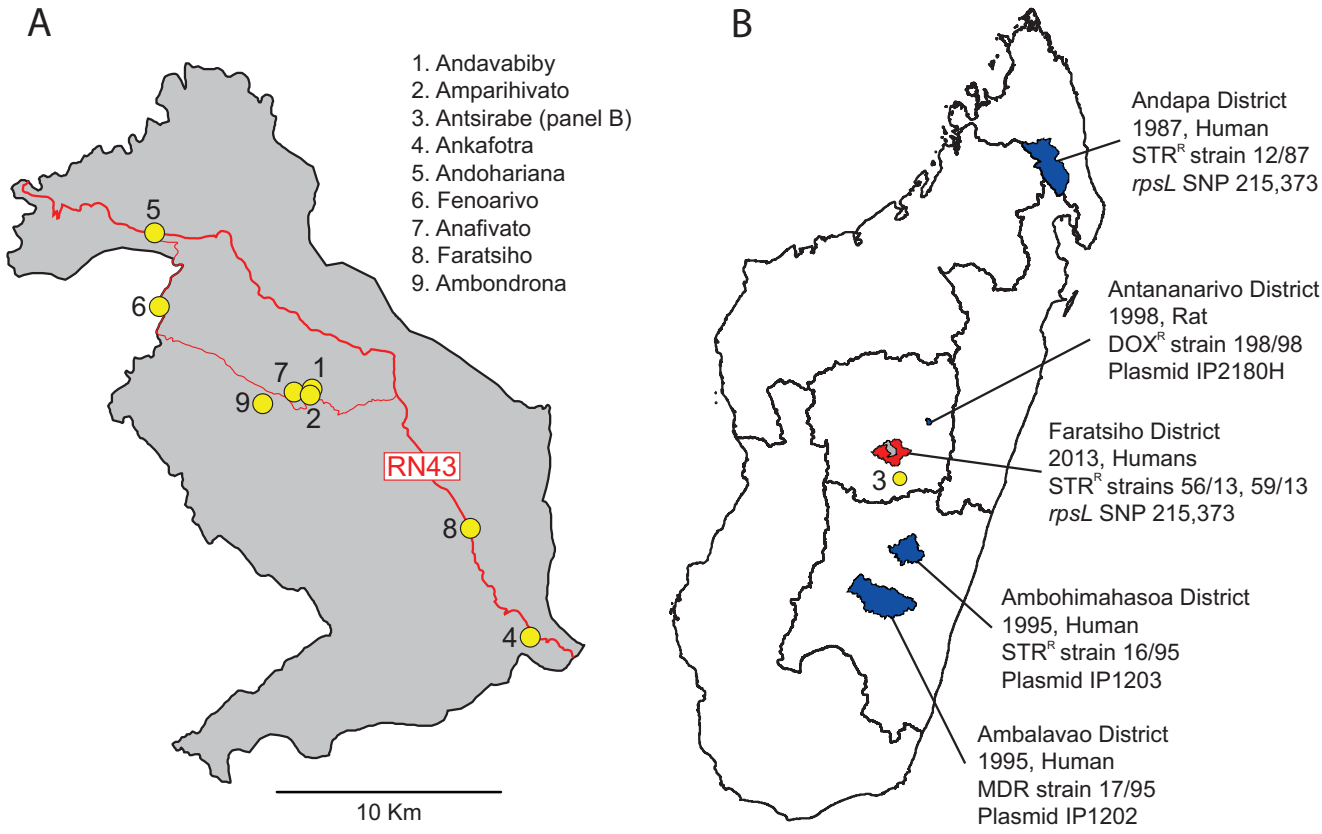

Figure 1. *A*, Map of commune of Faratsiho indicating locations associated with this PP outbreak; location 3 is shown on panel *B* as it is located outside of this commune. *B*, Map of Madagascar indicating the 5 different districts from which AMR/MDR strains of *Y. pestis* have been isolated, year and host of isolation, resistance phenotype (STR, streptomycin; DOX, doxycycline), and mechanism of resistance for each AMR/MDR strain. Faratsiho district is shaded red and, within it, the commune of Faratsiho is shaded gray; the latter corresponds to panel *A*. Abbreviations: AMR, antimicrobial resistant; MDR. multidrug-resistant; PP, pneumonic plague; SNP, single-nucleotide polymorphism.

of these 228 individuals were immune to *Y. pestis* due to previous exposure. Thus, the attack rate for this outbreak was calculated as 9.6% (22/228) and the case fatality rate as 14% (3/22; all three untreated).

Y. pestis Detected in Multiple Biological Samples

Six sputum and 18 serum samples were collected from surviving cases (Supplementary materials). Five sputum samples (cases 8, 12-14, 20) were F1 antigen positive with RDT; one sputum sample (Case 22) was negative with RDT but a serum sample from this case was anti-F1 immunoglobulin G (IgG) positive with ELISA. Sputum samples from cases 12 and 13 yielded *Y. pestis* isolates 59/13 and 56/13, respectively. Just 5 of 18 tested sera samples yielded positive ELISA results (Supplementary materials), which is not unexpected given the acuteness of PP infections [22]; anti-F1 IgG typically occurs ~7 days after symptom onset and blood samples were collected on day of presentation.

Outbreak Isolates and Samples Ae Clonal

Phylogenetic analysis of WGSs from outbreak isolates 56/13 and 59/13 and 36 other *Y. pestis* isolates from Madagascar (Figure 3) revealed the outbreak isolates are identical at all examined SNPs. These 2 isolates share a SNP, position 215 373 in reference genome CO92 [24], not present in 762 previously examined [25] isolates from Madagascar (Supplementary Table 3). They share another SNP (CO92 position 3 504 440) with isolate 97/96, obtained from a human in Faratsiho district in 1996, and the outbreak isolates and 97/96 share a third SNP (CO92 position 4 404 925) with isolate 42/08, obtained from a human in Faratsiho district in 2008; these 3 SNPs are absent in 372 publicly available global *Y. pestis* WGSs (Supplementary Table 4). Two *Y. pestis*-specific targets [26] and derived states of SNPs 215 373 and 3 504 440 were detected in DNA extracted from sputum samples from Cases 8, 13–14, and 20; the derived state of SNP 4 404 925 was detected in samples from cases 13 and 20 (Supplementary materials). The clonal nature of the outbreak isolates/samples suggest a single transmission event from the environment with subsequent amplification and spread among the cases.

Resistance to Streptomycin

Isolates 56/13 and 59/13 are resistant to streptomycin (MIC > 1024 μg/mL with E-test, no inhibition zone)

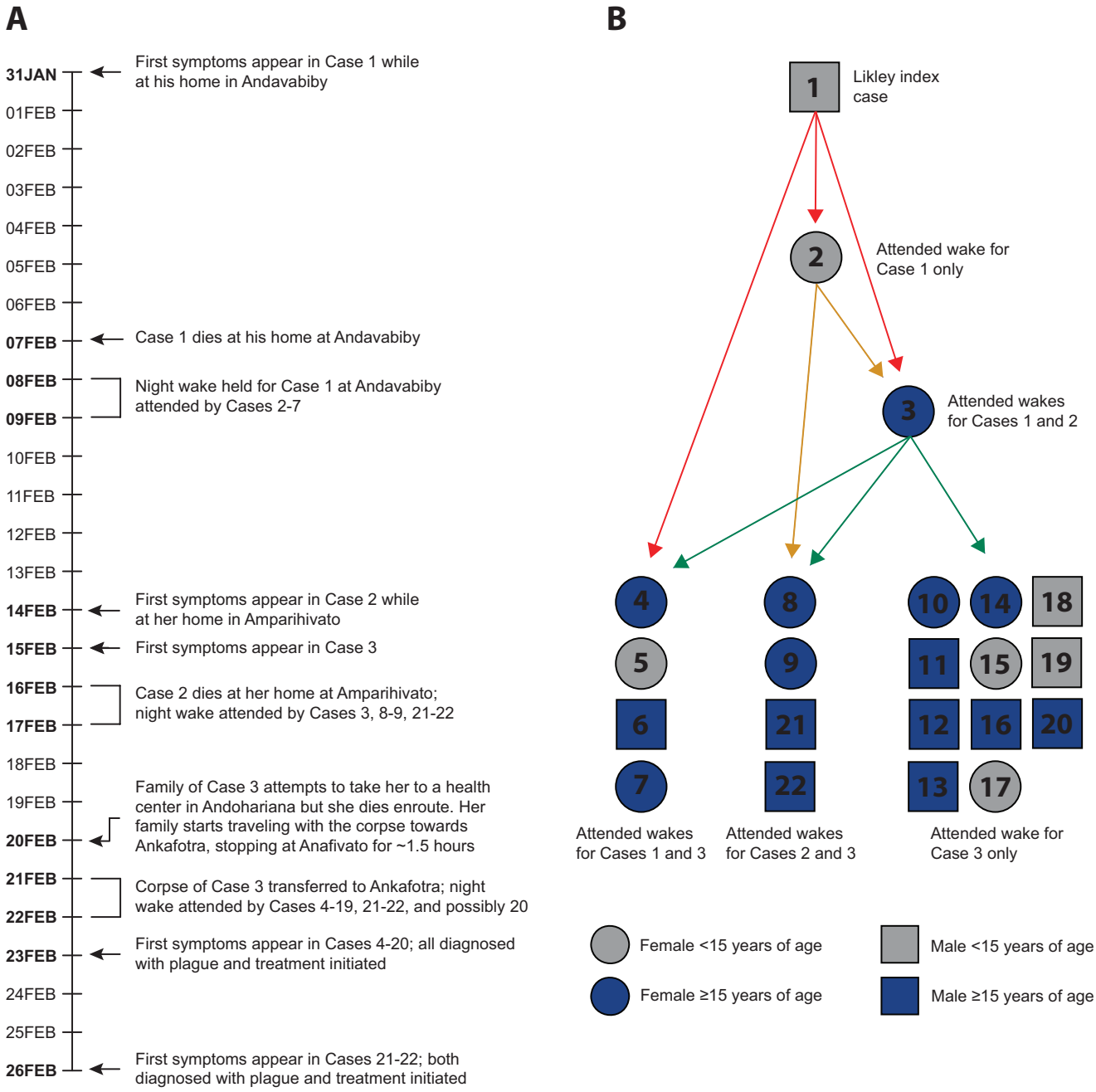

Figure 2. Outbreak transmission. *A*, Timeline of events associated with this PP outbreak. *B*, Case interactions during wakes as well as information on case-characteristics (age and gender). Abbreviation: PP, pneumonic plague.

but susceptible to other tested antimicrobials, including co-trimoxazole; closely related isolates 42/08 and 97/96 are susceptible to all tested antimicrobials. SNP 215 373 (adenine to guanine), shared by 56/13 and 59/13, occurs at CO92 nucleotide 128 of the *rpsL* gene causing the K43R (lysine to arginine) amino acid substitution common in other bacterial species exhibiting spontaneously acquired high-level streptomycin resistance [27–29]. AMR phenotype and genotype were maintained following repeated laboratory passaging (>500 generations) of 56/13 on non-selective media; no other AMR determinants were identified in 56/13 or 59/13. Introducing SNP 215 373 into attenuated streptomycin susceptible (MIC = 2 μg/mL) *Y. pestis* strain A1122 caused it to become streptomycin resistant (MIC >1,024 μg/mL; Supplementary materials).

The *rpsL* Mutation Has Occurred Independently Three Times in *Y. pestis*

Streptomycin resistant *Y. pestis* isolate 12/87 was obtained from a fatal PP case in the Andapa district of Madagascar in 1987 (Figure 1B). A WGS for 12/87 revealed it contains the *rpsL* mutation present in 56/13 and 59/13 but not any other

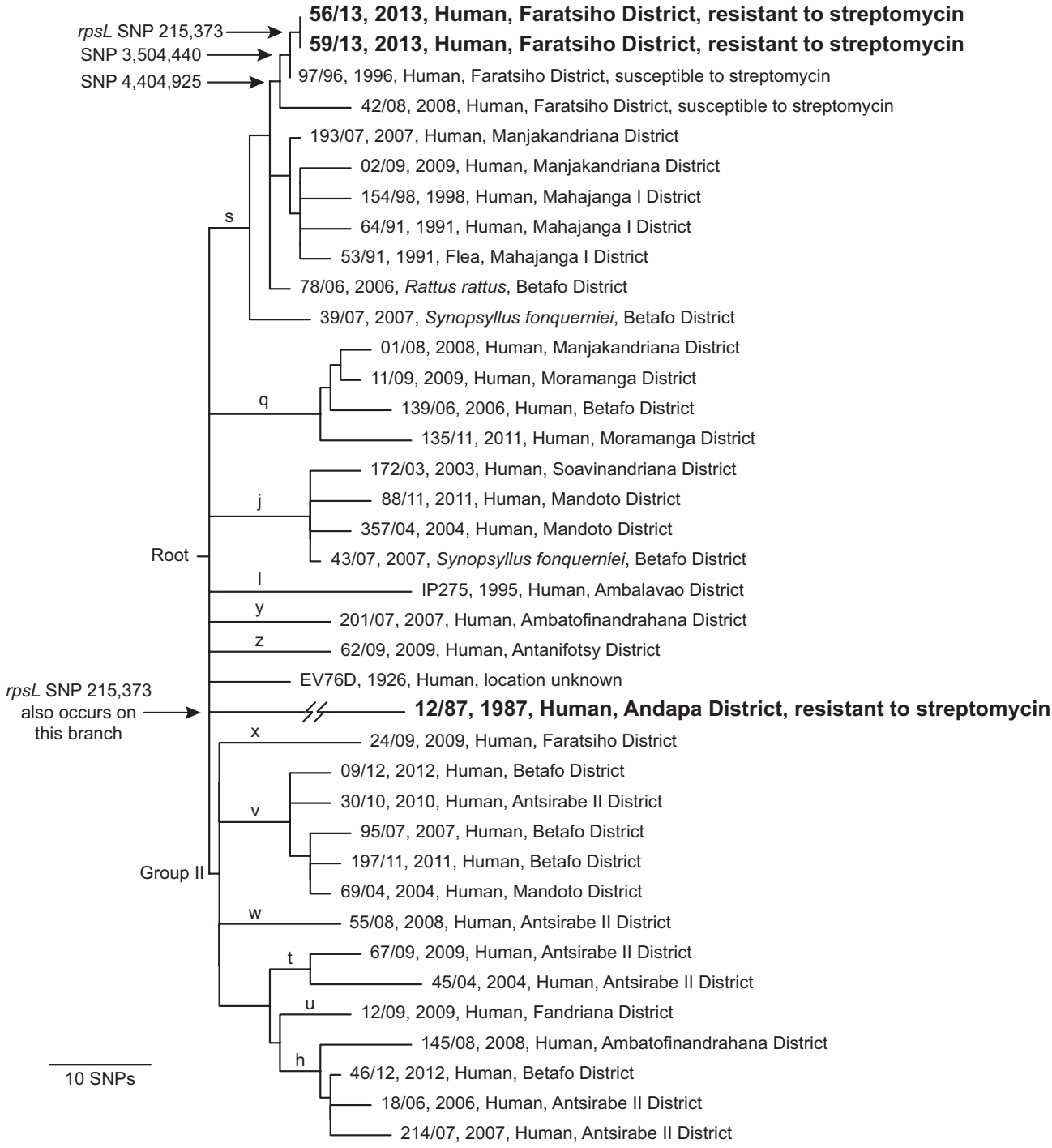

Figure 3. Maximum likelihood phylogeny for 38 *Y. pestis* isolates from Madagascar created using 387 core genome SNPs discovered from WGSs and rooted using North American strain CO92 (CO92 branch not shown). Labels for each isolate indicate identification number, year of isolation, host, and district of isolation; letters on branches indicate previously identified lineages [25]. Two isolates obtained from this PP outbreak in Faratsiho district, 56/13 and 59/13, are identical at all examined SNPs and share the *rpsL* SNP 215 373, which confers resistance to streptomycin. The *rpsL* SNP 215 373 is additionally found on the branch leading to isolate 12/87, which is also resistant to streptomycin; SNP 215 373 is the only homoplastic SNP in this phylogeny. Abbreviations: PP, pneumonic plague; SNP, single-nucleotide polymorphism; WGS, whole genomes sequence.

known AMR determinants. Also, 12/87 is highly distinct from 56/13 and 59/13 (Figure 3), indicating the *rpsL* mutation arose independently in this strain. Recently this same SNP was identified as the source of streptomycin resistance in a *Y. pestis* strain isolated from a fatal PP case in China in 1996; laboratory growth characteristics, virulence in animal models, and susceptibility to other antimicrobials were not evaluated for that strain [14].

Isolates Containing SNP 215 373 Exhibit Typical Growth and Virulence

When cultured, colonies from AMR isolates 56/13, 59/13, and 12/87, and AMS isolate 42/08 all appeared at ~48–72 hours. There were no *in vitro* growth differences between wild-type A1122 and A1122 mutants containing SNP 215 373. Mice infected with isolates 56/13, 59/13, and 42/08 died within 5 days of inoculation, and survival curves were similar among isolates (Supplementary materials).

DISCUSSION

PP outbreaks occasionally still occur, including a recent large outbreak in Madagascar [19] but are typically limited due to rapid identification of cases followed by antimicrobial treatment. We describe a PP outbreak in Madagascar involving 22 cases. A *Y. pestis* strain resistant to streptomycin was transmitted during this outbreak.

Except case 1, putative index case and first known fatality, all other cases were likely exposed to infection via participation in traditional funeral practices for one or more of the three fatal cases (Figure 2). Traditional funeral practices have been associated with previous PP outbreaks in Madagascar [5, 15, 30] and increase risk of *Y. pestis* transmission. Immediately after death, the family cleans and dresses the corpse, and a traditional wake is held for 2–3 nights involving close relatives (some of whom may have been infected during care of the deceased) and friends, resulting in close interactions among people in crowded and poorly ventilated rooms, factors associated with PP transmission [31]. First symptoms of primary PP occur within 1–6 days postexposure [2, 4-6], slightly longer than the typical PP incubation period [3]; cases typically refer to the beginning of symptoms as the day when they feel very sick. Attack rate (9.6%) was similar to those from other PP outbreaks associated with funerals/death care in Madagascar and Uganda (8%) [1, 3, 5].

Novel AMR *Y. pestis* strains continue to arise in Madagascar, which is concerning given there is no available vaccine. Morbidity/mortality prevention in Madagascar and elsewhere is based upon rapid identification of cases followed by antimicrobial treatment. To date, 5 AMR/MDR strains of *Y. pestis* exhibiting 4 mechanisms of resistance have been isolated from 5 different locations in Madagascar (Figure 1B), documenting that novel AMR/MDR strains have independently emerged throughout plague-endemic regions of Madagascar.

This study documents that AMR *Y. pestis* strains can be transmitted among humans via droplet transmission during a PP outbreak. The *rpsL* mutation was present in *Y. pestis* isolates from cases 12–13 and sputa from cases 8, 14, and 20. Cases 8, 12, 13, and 14 (and possibly 20) attended the third wake associated with this outbreak, the only known interaction among some of them, suggesting transmission of AMR *Y. pestis* during that event. There possibly was transmission of the AMR strain earlier in the outbreak, during the second wake, as case 8 also attended that event and may have been exposed to infection there. However, this is impossible to determine as no isolates or sputa were available from other cases that attended that wake.

The *rpsL* mutation conferring resistance to streptomycin, which has occurred independently at least 3 times in *Y. pestis*, has no detectable impact on fitness or virulence, as indicated by normal laboratory growth by strains containing this mutation and their ability to still cause animal and human disease. All growth rates examined here were similar among strains with and without this mutation and it remained fixed despite multiple laboratory passages without streptomycin, suggesting no impact on fitness, at least under tested conditions; revertants to wild type are rare in other species containing this same or other spontaneous *rpsL* mutations [32, 33]. The 5 human cases documented to be infected with *Y. pestis* containing this mutation all rapidly progressed to acute disease and exhibited multiple symptoms upon presentation, demonstrating this AMR strain remains highly virulent in humans. The PP case infected with unrelated *Y. pestis* strain 12/87 containing this mutation was only treated with streptomycin and died after 5 days of treatment, and the PP case from China that yielded a *Y. pestis* isolate containing this same mutation also died [14]. Mortality in mice infected with closely related strains with and without this mutation was similar, revealing these AMR strains remain highly virulent in mice. These findings suggest AMR *Y. pestis* strains containing the *rpsL* mutation remain fully virulent and can potentially persist in the environment via the natural rodent-flea transmission cycle. A previous AMR *Y. pestis* strain from Madagascar was obtained from a rat [8], suggesting AMR *Y. pestis* strains naturally occur in the environment in Madagascar. However, this 2013 outbreak through the end of 2020, IPM obtained >700 additional *Y. pestis* isolates and none were streptomycin resistant.

It seems highly unlikely that the *rpsL* mutation present in the AMR *Y. pestis* strain transmitted during this PP outbreak was selected for by treatment, as suggested for the *Y. pestis* strain from China containing this same mutation [14]. The *rpsL* mutation was present in isolates obtained from sputum samples from two cases (12–13) and in *Y. pestis* positive DNA extracts generated from sputum samples obtained from 3 other cases (8, 14, 20) from this outbreak (Supplementary Table 1). Collection of sputum samples that yielded the isolates and DNA extracts containing the *rpsL* mutation and initiation of streptomycin therapy for these 5 cases were both initiated on the day that these cases presented to health centers. Thus, for the *rpsL* mutation to be selected for by treatment it would have needed to arise immediately and independently five different times. Similarly, the sputum sample from the fatal PP case that yielded isolate 12/87, which also contains the same *rpsL* mutation conferring resistance to streptomycin, was collected on the same day that treatment was initiated. Together these findings suggest that this particular mutation of the chromosomal *rpsL* gene

may spontaneously arise in *Y. pestis* in the absence of antibiotic selective pressure.

Beyond 24–36 hours PP often cannot be effectively treated and is almost invariably fatal but recovery rates are high if treated within 24 hours of symptom onset [7]. Plague is common throughout the central highlands of Madagascar, and physicians in endemic regions are trained to recognize symptoms. When 19 individuals presented with PP symptoms physicians immediately initiated therapy with intramuscular streptomycin, consistent with the established protocol for treating PP in Madagascar. However, given the large number of cases to be treated and limited streptomycin, they followed the established BP protocol instead of the established PP protocol, which included combination therapy with oral co-trimoxazole and streptomycin injections only every eight hours, instead of streptomycin every four hours in monotherapy for the PP protocol; all treated patients recovered. Importantly, laboratory results described here were only available to attending physicians after treatment was completed as antimicrobial susceptibility testing is not available in remote regions where most plague cases occur and are treated [18] and, where available, does not yield results quick enough to inform treatment of rapidly progressing PP. Thus, they did not know that at least some patients were infected with *Y. pestis* resistant to streptomycin. If these cases had received only streptomycin and not also co-trimoxazole it seems possible some would have died. Given that AMR/MDR *Y. pestis* strains continue to emerge in Madagascar, all but one of the 5 AMR/MDR strains identified to date in Madagascar have been resistant to streptomycin, the potential toxicity of this class of antimicrobial, and the need for it to be applied intramuscularly, streptomycin monotherapy is contraindicated and an alternative treatment regimen is warranted. To this end, a plague randomized clinical trial is ongoing for the effectiveness of ciprofloxacin monotherapy in the treatment of plague compared to streptomycin followed by ciprofloxacin [34].

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. The authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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