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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.

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.
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 Ankafoatra 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 Ankafoatra, stopping in Anaifavo for ~1.5 hours. At Ankafoatra, 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
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 Are 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)
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 \textit{rpsL} gene causing the K43R (lysine to arginine) amino acid substitution common in other bacterial species exhibiting spontaneously acquired high-level streptomycin resistance \cite{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) \textit{Y. pestis} strain A1122 caused it to become streptomycin resistant (MIC >1,024 µg/mL; Supplementary materials).

\textbf{The \textit{rpsL} Mutation Has Occurred Independently Three Times in \textit{Y. pestis}}

Streptomycin resistant \textit{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 \textit{rpsL} mutation present in 56/13 and 59/13 but not any other
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 intra-muscularly, 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 copyrighted and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes
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