Local-Scale Genotype Diversity of *Yersinia Pestis*: A Case Study from Ambohitromby, Ankazobe District (2003-2016), Madagascar

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Abstract

Background

Plague is still a major public health concern in Madagascar despite the effort to reduce human cases and understand its epidemiology. In several localities known as plague foci, human cases are reported but the origin of the infection is most on the time unknown. In the present study, we report the presence of different genotypes of *Yersinia pestis* co-occurring in the same locality.

Methods

Human case was sampled in October 2016 and sent to the Central Laboratory for Plague for confirmation. Further, we undertook small mammal sampling to identify the circulation of plague in reservoirs. Isolated strains from human case, rodents as well as some archived strains from the same locality were combined with previously published strains to document the genotype of circulating strains. Further, blood sample from rodents were collected for seroprevalence analysis.

Results

In 2016, two different strains of *Y. pestis* from a human case and a reservoir circulated concurrently in the Ambohitromby commune (Ankazobe District) based on plague investigation. One type had been persisting there for more than 10 years but at least one other type may have been recently introduced. Seroprevalence of plague in rodents indicates that portion of the local murine population may resist to plague. These findings have implications for plague public health investigations and surveillance in Madagascar. Multiple distinct types of *Y. pestis* were circulating concurrently in the Ambohitromby commune (Ankazobe District) in Madagascar. Three strains genotype are now documented in Ambohitromby with the strain isolated in rats being a new genotype which is probably new to this locality or unobserved in previous years.

Background

Plague, caused by a bacteria *Yersinia pestis*, is widespread zoonotic diseases that is associated with animal reservoirs and fleas [1]. However, human cases generally occur leading to an outbreak when the case is reported with delay. In African region, plague is still a public health problem [2]. In Madagascar, human plague cases are notified annually from endemic foci that are mainly in the central highland (altitude over 800 m) as well as in Mahajanga (coastal area) [3]. Despite the effort to reduce human cases, Ankazobe District is still a highly active plague focus in Madagascar and needs continuous surveillance in order to reduce plague cases. Further, country-wide phylogeographic analyses have revealed that distinct subpopulations of *Y. pestis* occur and persist in different locations in Madagascar [4] but local-scale patterns of *Y. pestis* diversity remain poorly understood. Here, we report on diversity of *Y. pestis* strains circulating and persisting in the Ambohitromby commune in Ankazobe.

Methods

Human case investigation

In Madagascar, reporting of suspected human plague cases by health centers is mandatory. In October 2016, a human bubonic plague case was reported to the Central Laboratory for Plague (CLP; Institut Pasteur de Madagascar, IPM) from Ambohitromby commune, District of Ankazobe, an active plague focus in the Central Highlands region of Madagascar. A bubo aspirate collected from the case tested positive with a rapid diagnostic test (RDT) for detection of the *Y. pestis* F1 antigen [5] and bacteriological culture using selective media [6].

Small mammal trapping

In December 2016, one night of small mammal capture was conducted in the vicinity of the victim's home using 57 live-traps (39 National traps outdoors, 9 Sherman and 9 National traps indoors) and 20 rodents were captured: 19 *R. rattus* and one *M. musculus*. A second three-night session of capture, as part of plague surveillance among rodent population, was conducted in April 2017 using the same trapping system as in December (Table S1). All animals were euthanized and spleen samples were collected and stored in Cary Blair media for transportation to IPM prior to analysis. Sera or blood spot samples were also collected from each animal. Sera samples were stored at 4 °C whereas blood spots were stored at ambient temperature in the field. These activities were conducted in accordance with directive 2010/63/EU of the European Parliament (<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF>) and the guidelines accepted by the scientific community for the handling of wild mammals [7].

Laboratory analyses

Spleen samples were crushed with PBS and assessed with the RDT [5]. RDT positive samples were confirmed with molecular biology using three genes (*caf1*, *pla* and *lpx*) [8] and bacteriological culture using selective media.

Blood samples (Serum or blood spot) from each animal were tested for the presence of antibodies against the *Y. pestis* F1 antigen using an enzyme-linked immunosorbent assay (ELISA) [9].

Whole genome sequencing, SNP discovery, and phylogenetic analyses

Draft Illumina whole genome sequences were generated in this study for ten *Y. pestis* isolates; accession numbers for these new sequences are provided in Supplementary Table 2. DNA library construction for whole-genome sequencing was performed using KAPA Hyper Prep Kits (Roche, Pleasanton, CA) for Illumina NGS platforms per manufacturer's protocol, with double-sided size-selection performed after sonication. Non-combinatorial dual indexing was used with adapters and 8 bp index oligos from IDT (Integrated DNA Technologies, San Diego, CA) used in place of those supplied in the KAPA kit. The final

libraries were quantified on an Applied Biosystems QuantStudio 7 Flex Real-Time PCR System (Invitrogen, ThermoFisher) using the KAPA SYBR FAST ROX Low qPCR Master Mix (Roche, Pleasanton, CA) for Illumina platforms. The libraries were then pooled together at equimolar concentrations and quality was assessed with a Bioanalyzer High Sensitivity (Agilent Technologies, Santa Clara, CA). Final quantitation by qPCR preceded sequencing of the final library. Final pools were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA) with the 600-cycle v3 kit for 500 cycles.

Sequence data for 34 previously sequenced *Y. pestis* strains (Table S2) were downloaded from public databases. Among these strains was the North American strain CO92, which was used as a reference. Raw sequence reads for the 43 other *Y. pestis* strains were aligned against CO92 with bwa-mem v0.7.17-r1188 [10] and single nucleotide polymorphisms (SNPs) were called with the UnifiedGenotyper methods in GATK v3.3.0 [11, 12]. SNPs calling within duplicated regions, based on nucmer reference self-alignment, were filtered from downstream analyses; these methods were all wrapped with NASP v1.1.2. [13]. SNPs associated with known errors (n = 12) in CO92 [14], as well as those falling in short tandem repeats as identified by Tandem Repeats Finder v4.09 [15], were also filtered from the analysis. A maximum likelihood phylogeny was inferred on a concatenation of 270 SNPs with IQ-TREE 1.6.10 [16] using the TVMe + ASC model based on the integrated ModelFinder method [17]. Strains were assigned to previously identified lineages and subgroups within *Y. pestis* in Madagascar using a set of 249 previously-characterized SNPs [4] extracted from the NASP SNP matrix.

Results

Human case confirmation

Laboratory confirmation of the human plague case revealed that the sample was RDT positive with *Y. pestis* isolated by bacteriological culture (strain 25/16). The case had no known recent travel history outside the commune before his disease.

Plague in rodents

A total of 64 small mammals were tested for RDT of which 13 sample were RDT positive. In addition, four samples were molecularly confirmed and four of samples positive via *Y. pestis*-specific PCR. One sample tested positive with both RDT and PCR and yielded *Y. pestis* isolate (65/16). This strain was isolated from one individual *R. rattus* collected in December 2016 (Supplementary Table 1). Further, serological analysis revealed that 28 of 60 *R. rattus* were seropositive for *Y. pestis*: 12/19 from the December 2016 trapping session (with 3 positive with both PCR and ELISA) and 16/41 from the April 2017 trapping session (all negative on PCR); all four *M. musculus* individuals were negative with RDT, PCR and ELISA (Table S1).

Whole genome sequencing and analysis

Whole genome sequences (WGSs) were generated for isolates 25/16, 65/16, and eight additional archived isolates obtained from humans and rats in Ambohitromby in previous years (2004–2014). Single nucleotide polymorphisms (SNPs) identified from these 10 WGSs, and 33 publicly-available WGSs from other strains representing known diversity of *Y. pestis* in Madagascar [4], were used to construct a phylogeny (Table S2, Fig. 1), which revealed several interesting patterns. Isolated strains were assigned to the s group and composed of three subgroups (S03, S05 and S13) and the human and rat isolates from 2016 are quite distinct and assigned to different subgroups S05 and S13, respectively and these types co-occur locally in the environment. Even though the rat isolate was obtained ~ 10 m from the home of the human case.

Discussion

Plague is still a major public health concern in Madagascar with about 75% of the human cases worldwide [2]. Although different studies have been done to understand human plague, the island still report cases from known plague foci or new localities. Thus, it is important to know strains diversity and identify the source of infection in humans. Our findings document that diverse subtypes of *Y. pestis* can co-occur locally in the environment in Madagascar which is possibly due to importation of novel subtype from other regions. Public health investigations to identify environmental sources of human plague infections, such as trapping and testing suspected rodent hosts as well as flea vectors, should be extensive and involve genotyping of resulting isolates to increase the likelihood of sampling and identifying all types of locally circulating *Y. pestis* that may be potential sources. Detailed spatio-temporal studies of local rodent plague hosts in Madagascar would better elucidate how multiple diverse types of *Y. pestis* are concurrently maintained in the environment. Our findings are consistent with this idea as almost half of the *R. rattus* we examined were seropositive, suggesting many rats in Ambohitromby can survive *Y. pestis* infection and serve as local reservoirs for plague. *Y. pestis* subgroup s13 may have been recently transported to Ambohitromby as no other isolates from Ambohitromby or from any other locations within Ankazobe District examined in this or previous studies [4, 18] were assigned to subgroup s13. Movement of *Y. pestis*, likely via inadvertent human-mediated movement of infected rats and/or flea vectors, has been documented in Madagascar [4], and isolation of this subgroup from a rat captured in Ambohitromby suggest it is now ecologically-established in this area. First, strains isolated in Ambohitromby are diverse. Although all were assigned to major lineage “s”, this is the most widely distributed *Y. pestis* lineage in Madagascar and Ambohitromby isolates were assigned to three distinct subgroups within this lineage (s03, s05, and s13) that are all also widely distributed in Madagascar [4]. Third, the human infection was likely obtained locally in Ambohitromby [18] from a subgroup (s05) that had been persisting in this region for more than a decade, as the human isolate is closely-related to seven other human and rat isolates from this commune obtained from 2003–2014 that are also assigned to this same subgroup; previous studies also have documented this subgroup in an adjacent commune within Ankazobe District [4, 18]. Local, long-term persistence of *Y. pestis* in Madagascar likely occurs in *R. rattus* populations [4, 18–20].

Subgroup 03 also is represented in Ambohitromby by a single isolate described here (Fig. 1) and was not found in Ambohitromby or elsewhere in Ankazobe District in previous studies [4, 18], suggesting this subgroup likewise may have been recently transported to this area. However, as the sole subgroup s03 isolate was obtained from a human with unknown travel history, acquisition of infection in another region of Madagascar where this subgroup is common cannot be ruled out.

Conclusion

The present study showed strains diversity at local scale in an endemic plague focus. Seroprevalence analysis on rodents highlighted that portion of local murine population resists to the diseases. Further research should focus on the phylogeography of strains circulating in this District to have an exhaustive overview of strains diversity and origins.

Declarations

Funding

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Designed the experiments: BR, FR, MP, MR and DW; Performed experiment: BR, FR, MP, SR MeR, SR, and DW; Analyzed the data: BR, FR, MP, VA, VAJ, SJ, DW; Contributed to reagents/materials: BR, MR, DW; Wrote the paper: BR, MP, FR, MR, SR, VA and DW. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was carried out in accordance with activities were conducted in accordance with directive 2010/63/EU of the European Parliament (<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF>) and the guidelines accepted by the scientific community for the handling of wild mammals [7]

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Figures

Figure 1

Maximum likelihood phylogeny for 43 *Y. pestis* isolates from Madagascar created using 270 core genome SNPs discovered from WGSs; the phylogeny is rooted using North American strain C092 (branch not shown). Letters on branches indicate previously-identified major *Y. pestis* lineages within Madagascar [4], and labels for each isolate indicate identification number, year of isolation, host, District-Commune-Fokontany (Village) of isolation, and assigned phylogenetic subgroup. Isolates from Ambohitromby commune are indicated with bold text.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.doc](#)
- [TableS2.doc](#)