Long-term persistence of virulent *Yersinia pestis* in soil

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Plague is characterized by geographical foci from which it re-emerges after decades of silence, a fact currently explained by enzootic and epizootic cycles between plague-susceptible and plague-resistant rodents. To assess the potential role of soil in plague epidemiology, we experimentally investigated whether *Yersinia pestis* could persist alive and virulent in soil. Sterilized soil inoculated with virulent *Y. pestis* biotype Orientalis was regularly sampled for 40 weeks in duplicate. Each sample was observed by acridine orange staining and immunofluorescence using an anti-*Y. pestis* polyclonal antibody, and DNA was extracted for PCR amplification and sequencing of the *Y. pestis* *ureD*, *caf1* and *pla* genes. All samples were inoculated onto selective agar, and samples from soil that had been incubated for 10, 60, 165, 210 and 280 days were also inoculated into each of two BALB/c female mice. The mouse experiment was performed in triplicate. Non-inoculated, sterilized soil samples were used as negative controls. Micro-organisms fluorescing orange and detected by immunofluorescence were identified as *Y. pestis* in all samples. They were recovered in pure agar cultures for up to 30 weeks but thereafter were contaminated with *Pseudomonas* spp. Soil that had been inoculated with *Y. pestis* proved to be fully virulent in mice, which died with *Y. pestis* septicaemia and multiple organ involvement. Negative control mice showed no signs of disease. These data indicate that *Y. pestis* biotype Orientalis can remain viable and fully virulent after 40 weeks in soil. This study is a first step on which to base further investigations of a potential telluric reservoir for *Y. pestis*, which could represent an alternative mechanism for the maintenance of plague foci.

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INTRODUCTION

Yersinia pestis, the causative organism of plague, has played an important role in shaping human history. Historical texts record two historical plague pandemics and a third pandemic is still on-going (Perry & Fetherston, 1997). Sporadic cases are also reported in several countries. In Algeria, plague re-emerged in 2003 in the Oran area after six decades of silence (Bertherat *et al.*, 2007; Bitam *et al.*, 2006). Indeed, long-term persistence in geographical areas of so-called plague foci, where plague re-emerges after decades of silence, is a characteristic feature of plague.

Plague is thought to exist indefinitely in rodent populations in so-called enzootic (maintenance) cycles that involve transmission between partially resistant rodents (enzootic or maintenance hosts) and their fleas. The disease can spread from enzootic to more susceptible animals (epizootic or amplifying hosts), causing rapidly

spreading die-offs (epizootics) (Gage *et al.*, 1995). Yersin (Yersin, 1894), Simond (Simond, 1898) and Raybaud (Gauthier & Raybaud, 1903) established that rat fleas transmit *Y. pestis* to humans. This scheme, later endorsed by the Indian National Advisory Committee on plague (Anonymous, 1906), was oversimplified and became the epidemiological dogma for plague (Drancourt *et al.*, 2004). This classical scheme, however, does not explain the particular epidemiological patterns observed during large historical pandemics or the existence of plague foci that appear to persist for many decades in the absence of human cases or noticeable die-offs among local rodent populations (Drancourt *et al.*, 2006).

The initial report by A. Yersin indicated that *Y. pestis* had been isolated from the soil of a house where the inhabitants had died of plague (Yersin, 1894). *Y. pestis* was later isolated from a burrow of plague-infected rodents (Karimi, 1963), and Mollaret (1963) reported the experimental persistence of *Y. pestis* in soil. Long-term persistence of virulent *Y. pestis* in soil could play a role in the particular epidemiology of plague. In this perspective, we assessed the

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Abbreviations: CIN, cefsulodin-irgasan-novobiocin; p.i., post-inoculation.

long-term preservation of live, virulent *Y. pestis* biotype Orientalis using a non-quantitative model of artificially inoculated soil and a mouse model of infection.

METHODS

Soil inoculation and sampling. All manipulations involving live *Y. pestis* were done in a P3 laboratory under biosafety cabinet level 3 systems (Fig. 1). *Y. pestis* strain 6/69M biotype Orientalis, a virulent strain (kindly provided by Professor Michel Simonet, Institut Pasteur, Lille, France), was cultured on 5% sheep blood agar (bioMérieux). Natural sand collected in the Marseille area was steam-sterilized and further humidified by addition of 20 ml sterile distilled water per 500 g sterilized sand. Sterility was assessed by inoculating 5 g sand onto 5% sheep blood agar and cefsulodin-irgasan-novobiocin agar (CIN, Becton Dickinson) and incubating at 30 °C under a 5% CO₂ atmosphere for 1 week. Soil was PCR-negative (see below). Sterilized soil was then thoroughly mixed with *Y. pestis* suspended in sterile

phosphate-buffered saline (PBS) in order to achieve a homogeneous inoculum of 10⁶ c.f.u. per g soil, and 500 g of this preparation was placed in an opaque plastic container incubated at room temperature for 40 weeks. Room temperature was monitored daily. Sterile distilled water (10 ml) was gently added twice a month to the container. The experiment was performed in duplicate. Before each sampling, soil was mixed evenly with a sterilized fork in order to ensure the homogeneity of the sample. An average 5 g aliquot of inoculated soil core was sampled 1 h post-inoculation (p.i.), and then every 24 h for 10 days, then every 2 weeks starting on day 15 for a total of 40 weeks. Aliquots of 1 g were immediately inoculated into 9 ml brain-heart broth (Difco) and gently agitated before 100 µl of the suspension was inoculated onto 5% sheep blood agar and CIN. A second aliquot was deposited on slides with a pen nib for acridine orange staining (Chapin & Murray, 2003) and indirect immunofluorescence detection was performed using an anti-*Y. pestis* rabbit polyclonal antibody and FITC-conjugated goat anti-rabbit IgG (Immunotech) diluted 1:400 in PBS containing 3% non-fat dry milk and 0.2% Evans blue (bioMérieux). Slides were washed, mounted with Fluoprep (bioMérieux) and examined under an Olympus BX-51 epifluores-

Fig. 1. Experimental protocol used in this study.

cence microscope at $\times 100$ magnification. The remaining sample was frozen at -80°C for further experiments.

Molecular analyses of soil. DNA was extracted from soil samples using the QIAamp DNA Mini kit with modifications of the manufacturer's protocol (Qiagen). Defrosted soil samples were vortexed, briefly centrifuged in order to sediment the soil particles and 100 μl supernatant was incubated at 56°C for 15 min with 180 μl ATL lysis buffer and 20 μl proteinase K. After centrifugation at 6000 g for 1 min, the pellet was incubated at 70°C for 10 min with 200 μl AL buffer, precipitated with 96 % ethanol, transferred into a QIAamp spin column and centrifuged at 6000 g for 1 min. Then 500 μl AW1 buffer was added and the tubes were centrifuged at 6000 g for 1 min. AW2 buffer (500 μl) was added and the tubes were centrifuged at 20000 g for 3 min. DNA was eluted by addition of 200 μl AE buffer and centrifugation at 6000 g for 1 min. Filtrate was collected into a sterile, DNase-free tube for PCR amplification of the plasminogen-activator gene *pla*, the *cafI* gene encoding F1 capsular antigen, and the urease gene *ureD* (Table 1). PCRs were carried out in an Applied Biosystems 2720 thermal cycler (MJ Research). The target genes were amplified in a 50 μl reaction mixture containing 10 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM dNTPs (Invitrogen), 0.1 mM primer (Eurogentec) and 1 U *Taq* DNA polymerase (Invitrogen). Amplification of the *pla* gene required an initial 5 min denaturation at 95°C and 39 cycles of 1 min denaturation at 94°C , 30 s annealing at 60°C , and a 1 min extension at 74°C followed by a final 15 min extension at 75°C ; *cafI* amplification used an initial 10 min denaturation at 95°C and 40 cycles of 1 min denaturation at 94°C , 40 s annealing at 55°C , and a 1 min extension at 74°C followed by 15 min final extension at 75°C ; *ureD* amplification used an initial 5 min denaturation at 95°C and 39 cycles of 1 min denaturation at 94°C , 30 s annealing at 54°C , and 1 min extension at 74°C followed by a 10 min final extension at 74°C . Sterile water was used as a negative control in each PCR assay and no positive control was used. PCR products were purified using a multiscreen PCR filter plate (Millipore). Sequencing reactions were done using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq polymerase FS (Perkin-Elmer), and sequencing products were resolved using an ABI 3100 automated sequencer (Perkin-Elmer). Sequence analysis was performed using the ABI prism DNA sequencing analysis software package version 3.0 (Perkin-Elmer) with a Power Macintosh 7200/120. GenBank databases were searched via Internet BLAST software from the National Center for Biotechnology Information homepage (<http://www.ncbi.nlm.nih.gov/BLAST/>), and the sequence results were compared.

Inoculation of mice. Protocols used for mice experiments were approved by Marseille's Medical School Animal Ethics Committee. BALB/c female mice weighing 17–18 g (Charles River) were kept under biosafety cabinet level 3 for 5 days before inoculation. Defrosted soil samples that had been incubated with *Y. pestis* for

10, 60, 165, 210 and 280 days were vortexed, centrifuged at 100 g for 30 s in order to sediment soil particles, and the supernatant was used for intraperitoneal inoculation in mice. Two mock-infected, negative control mice were inoculated with steam-sterilized soil held for the same length of time prior to inoculation as the *Y. pestis*-treated soil samples, suspended in sterile brain-heart broth in parallel. The experiment was performed in triplicate, so that six mice were inoculated for each sample, including the negative control. Negative control mice were sacrificed at the end of the 10 day observation period. Body weight and physical features were monitored daily after inoculation. After death, the kidneys, lungs, liver, spleen, heart, and brain were dissected, the lung fragment was inoculated onto blood agar, and all tissues were stored in 95 % ethanol for 20 days at room temperature before fixation with 4 % buffered formalin and paraffin embedding. Serial sections (3 μm) of lung specimens were obtained for routine haematoxylin-eosin and Giemsa staining. Blood samples collected by intracardiac puncture were inoculated onto blood agar, and also spread on slides and air-dried at room temperature. Non-infected mouse blood specimens were collected and examined in parallel. Slides were fixed in 100 % methanol, stained with freshly prepared acridine orange in the dark for 5 min, rinsed with tap water, air-dried at room temperature, overlaid with DAPI (4',6'-diamidino-2-phenylindole) ready-to-use solution (Molecular Probes) and examined under an FITC-rhodamine double-band filter using a Leica DM2500 Upright fluorescence microscope at $\times 100$ magnification. DNA was extracted from lung tissue using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's protocol (25 mg).

Identification and characterization of isolates. All isolates recovered after inoculation of agar plates and mice were identified as *Y. pestis* by Gram staining, oxidase test and API20E strip inoculation. Urease activity was tested in the tube (bioMérieux) after 24 h inoculation. Identification was further confirmed by PCR amplification and sequencing of the 16S rDNA (Drancourt *et al.*, 2004), *pla*, *ureD* and *cafI* genes present in single colonies.

RESULTS

Microscopic observation and axenic culture of *Y. pestis* from contaminated soil

During the 40-week observation, the room temperature varied from 18.7°C to 24°C , with a median of $20.8 \pm 1.8^{\circ}\text{C}$, in the P3 laboratory where inoculated soil samples were kept. Acridine orange staining of soil samples visualized *Yersinia* cells with morphology that was compatible with *Y. pestis*. Species identification was later confirmed by positivity of indirect immunofluorescence using polyclonal antibody in all samples (Fig. 2). Agar

Table 1. Sequences of primers used for *Y. pestis* PCR amplifications

Target gene	Forward (F) and reverse (R) primers	Product size (bp)	Annealing temp. ($^{\circ}\text{C}$)	Reference
Plasminogen activator precursor	F 5'-ATCTTACTTTCCGTGAGAAG-3' R 5'-CTTGGATGTTGAGCTTCCTA-3'	480	60	Pouillot <i>et al.</i> (2005)
F1 capsular antigen	F 5'-CCCGCATCACTCTTACAT-3' R 5'-ACGGTTACGGTTACAGCA-3'	388	55	Kuske <i>et al.</i> (2006)
Urease	F 5'-ACCATGCTGACGAACGATTTCGG-3' R 5'-TCAGCGCCACAAAAATTGTTC-3'	402	54	Present study

Fig. 2. Acridine orange staining shows numerous *Y. pestis* cells in soil experimentally inoculated with *Y. pestis* 6/69M biotype Orientalis strain for 280 days (a), whereas mock-infected negative controls remained negative (b). *Y. pestis* cells were confirmed in soil using indirect immunofluorescence (c), whereas the negative control showed no fluorescence (d).

inoculation yielded >1000 colonies on blood agar over the 40-week observation period and >1000 colonies on CIN until week 25, followed by 100–1000 colonies from week 26 until week 40. Colonies were identified as *Y. pestis* until week 30, after which they were contaminated with *Pseudomonas* spp. No urease activity was observed in any *Y. pestis* colony. All *Y. pestis*-inoculated soil samples yielded positive PCR amplification of *pla*, *ureD* and *cafI* genes, whereas all negative controls remained negative. The sequence of the *pla* amplicons yielded 93–99 % similarity with the reference (GenBank CP000310.1), the sequence of *ureD* amplicons yielded 85–94 % sequence similarity with the reference (GenBank AL590842.1), and the sequence of *cafI* amplicons yielded 93–100 % similarity with the reference (GenBank CP000670.1). Variations in similarity values were due to variations in the sequence quality between various batches.

Mouse model

All mice inoculated with soil extracts after removal of the particle fraction died within 72 h p.i., whereas no death was observed in control mice over a 10 day period of observation. Before they died, inoculated mice exhibited hair loss, decreased appetite and decreasing weight, from 18.3 g at the time of inoculation to 16.7 g 24 h p.i., 15.2 g 48 h p.i. and 14.1 g 72 h p.i., whereas negative control mice demonstrated an increase in weight from 17.3 g at the time of inoculation to 21.1 g at the end of the 10 day observation period. The lungs had lost their recognizable architecture, exhibiting extensive haemorrhage, large foci

of necrosis and large numbers of neutrophils packed within what remained of the interstitium. The liver, red pulp of the spleen, and kidneys showed extensive necrosis and neutrophil infiltration. In contrast, the brain and heart showed no pathological changes. Specific *Y. pestis* were detected in the blood and lung tissue of infected mice, but not in negative controls; *pla* amplicons yielded 92–97 % similarity with the reference (GenBank CP000310.1), *cafI* amplicons yielded 94–99 % similarity with the reference (GenBank CP000670.1), and *ureD* amplicons yielded 98–99 % similarity with the reference (GenBank AL590842.1). *Yersinia* cells were easily detected by acridine orange staining in blood samples collected from dead mice inoculated with 10, 60, 165, 210 and 280 day soil extracts after removal of the soil particles. In contrast, no *Yersinia* cells were detected in negative control mice (Fig. 3). Moreover, *Yersinia* cells stained orange, suggesting that they were alive. Indeed, blood cultures yielded colonies identified as *Y. pestis* at 72 h. Likewise, cultures of lung tissues collected from dead mice yielded colonies identified as *Y. pestis* at 72 h, and specific *pla*, *ureD* and *cafI* sequences were detected in all lung tissue specimens collected in inoculated mice, whereas negative control lungs from mock-infected mice remained negative.

DISCUSSION

We herein demonstrate that *Y. pestis* 6/69M, a virulent Orientalis strain, remains viable and virulent after 40 weeks incubation in sterilized humidified sand. Our data do not

Fig. 3. Acridine orange staining shows a large number of bacterial colonies in the blood of mice inoculated with soil contaminated with *Y. pestis* for 280 days, after removal of the particle fraction (a), whereas the blood collected from mock-infected, control mice was negative (b).

merely result from initial contamination of the natural sand we collected in the Marseille area, since there have been no cases of plague in Marseilles for 97 years and the Marseille area has never been a long-term plague focus. Sand was autoclaved before inoculation and demonstrated to be sterile by the absence of any growth on blood agar under the appropriate incubation conditions. PCR tests were also negative for the presence of *Y. pestis* DNA. Moreover, all manipulations were done in a P3 laboratory with appropriate confinement measures, and no other experiment using *Y. pestis* was done in this laboratory during this period. Therefore, contamination during the course of the experiment is highly improbable.

Long-term persistence of *Y. pestis* in soil has remained a controversial issue, since several researchers failed to isolate *Y. pestis* from soil after Yersin's initial claim (Yersin, 1894) and most researchers believed that *Y. pestis* perishes quickly when outside its normal vector or hosts (Brubaker, 1991; Perry & Fetherston, 1997). Indeed, *Y. pestis* dies rapidly if exposed to a temperature exceeding 40 °C or when exposed to desiccation (Perry & Fetherston, 1997). Nevertheless, Mollaret (1963) reported survival of *Y. pestis* for 16 months in autoclaved soil and indicated that hydration of soil before experimental inoculation was necessary for *Y. pestis*

survival (Mollaret, 1965). Furthermore, *Y. pestis* was isolated from the chamber of a gerbil (*Meriones vinogradovi*) burrow in which an animal had died from plague, but in which no animal nor dead or living fleas had been observed for 7 months (Karimi, 1963). This field observation was reproduced twice, 10 and 11 months after the closure of a contaminated burrow (Karimi, 1963). However, poorly described methodologies in these previous reports and lack of an independent confirmation prevented definite acceptance of this paradigm. Nowadays, in Madagascar, there are observations of pneumonic plague cases among persons attending ritual excavation of plague corpses in the absence of direct contact with the dead (Harilanto Razafindrazaka, personal communication). It has been previously suggested that *Y. pestis* urease may play a role in environmental adaptation (Sebbane *et al.*, 2001). Indeed, unlike other *Yersinia* species, *Y. pestis* lacks urease activity due to a frame-shift mutation (Sebbane *et al.*, 2001). We did not observe recovery of urease activity during this experiment and we did not detect reversion of the frame-shift mutation in the *ureD* gene, indicating that such activity is not essential for soil survival, at least under our experimental conditions.

We further demonstrated that *Y. pestis* strain 6/69M remained fully virulent after a 40 week incubation in humidified soil. Mice inoculated with soil samples contaminated with *Y. pestis* for 10–280 days developed a fatal *Y. pestis* septicaemia within 3 days p.i., and *Y. pestis* was detected in most tissues examined. Importantly, we observed lung lesions similar to those reported in a mouse model of primary plague pneumonia (Lathem *et al.*, 2005). These data therefore indicate that *Y. pestis* 6/69 retained its virulence after long-term survival in soil.

Plague is characterized by decades of silence in fixed geographical foci where re-emergence of human plague has been linked to a continuous, low-level circulation of *Y. pestis* in rodent populations. Environmental changes leading to changes in the abundance of vector and rodent populations, reduction in rodent surveillance, and increased contact with rodents may explain the re-emergence of human plague (Duplantier *et al.*, 2005). Among factors leading to epizootics, climatic factors including variations in temperature and precipitation appear to be important in many, but perhaps not all, areas (Gage & Kosoy, 2005). Foci characterized by long inter-epizootic periods during which *Y. pestis* cells are not found in the host and vector populations are also a hallmark of plague, as recently illustrated in Oran, Algeria, where plague re-emerged in 1993 after more than half a century of silence (Lounici *et al.*, 2005). In this case, epidemiological and typing data suggested the existence of a natural focus of plague rather than recent importation of infected animals. Although such observations could simply result from a lack of adequate surveillance, this phenomenon could be explained by a chronic carrier state in rodents. The latter hypothesis relies on the co-existence of relatively resistant species that act as enzootic reservoir species and relatively susceptible animals that act as epizootic hosts.

Plague-resistant species of animals may develop long-term subclinical bacteraemia and, therefore, be a durable reservoir of infection (Baltazard & Mofidi, 1950). However, field observations and experiments led the authors to conclude that resistant species are not able to sustain plague in plague foci (Baltazard *et al.*, 1963).

The experimental data reported here provide a basis on which to examine the role of soil in the epidemiology of plague (Fig. 4). *Y. pestis* may exist between a short and unstable parasitic phase associated with rodents and fleas and a more stable soil phase, the so-called saprophytic phase, that would allow for survival between epizootics (Baltazard, 1964; Levi, 1997). Some animals could acquire *Y. pestis* by burrowing in contaminated soil, and thus initiate a new rodent/rodent flea cycle of transmission. Initial experiments by the Indian Plague Commission showed that guinea pigs could be contaminated by running freely over freshly (<24 h) contaminated soil (Anonymous, 1906). Further experiments showed that rodents, including 'plague-resistant' *Meriones* species, could acquire plague by burrowing in soils experimentally infected several days earlier. A rapid course of plague and death were hallmarks of these experiments. Of 110 dead animals, 63 had lung lesions, 31 intestinal lesions, and 20 solely spleen/lymph node lesions (Anonymous, 1906). These data indicate that either inhalation or ingestion of contaminated soil, or both, could be the routes of contamination for rodents. Other animal models have indicated that susceptible animals (e.g. mice and cats) can acquire lethal plague by either inhalation or ingestion of *Y. pestis* (Butler *et al.*, 1982; Welkos *et al.*, 2002), as reported for patients after consuming raw camel meat (Bin Saeed *et al.*, 2005).

Our experimental data are reminiscent of those for its closely related ancestor *Y. pseudotuberculosis*, which is a

soil- and water-borne enteropathogen (Tan *et al.*, 2002). The precise environmental factors contributing to its survival in soil remain to be identified. *Y. pestis* is highly susceptible to variations in environmental temperature and desiccation (Mollaret, 1965; Perry & Fetherston, 1997). According to Tan and co-workers, the distribution of plague foci could be closely related to calcium- and iron-enriched soils, in agreement with the known role of calcium in the regulation of *Y. pestis* virulence factors (Perry & Fetherston, 1997; Tan *et al.*, 2002). The determination of these factors may be of interest from the perspective of plague control. The experimental telluric persistence reported herein should prompt field investigations in plague foci in order to better assess the role of telluric persistence in the epidemiological chain of plague. If long-term survival of *Y. pestis* in natural soils that have not been sterilized can be demonstrated, along with the routine recovery of plague bacteria from soils in plague-endemic areas, *Y. pestis* biotype *Orientalis* should be added to the growing list of pathogens that persist fully virulent in soil and may be responsible for zoonoses such as *Mycobacterium bovis* (Young *et al.*, 2005), *Bacillus anthracis* (Logan *et al.*, 2007) and *Coxiella burnetii* (Evstigneeva *et al.*, 2007). A striking parallel could be made with *Vibrio cholerae*, a soil- and water-borne pathogen existing in well-defined cholera foci and responsible for pandemics (Zuckerman *et al.*, 2007). *Y. pestis* is therefore an additional candidate for the 'sit-and-wait' strategy previously described for human respiratory pathogens (Walther & Ewald, 2004). This strategy should favour evolution of pathogens towards high levels of virulence. If confirmed, the hypothesis of the telluric persistence of *Y. pestis*, along with the recently demonstrated transmission of *Y. pestis* by the body louse (Houhamdi *et al.*, 2006), should be incorporated into revised plague epidemiology schemes.

Fig. 4. Soil as an epidemiological link in the epidemiology of plague.

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