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Short Communication

Susceptibility to *Yersinia pestis* Experimental Infection in Wild *Rattus rattus*, Reservoir of Plague in Madagascar

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Abstract: In Madagascar, the black rat, *Rattus rattus*, is the main reservoir of plague (*Yersinia pestis* infection), a disease still responsible for hundreds of cases each year in this country. This study used experimental plague challenge to assess susceptibility in wild-caught rats to better understand how *R. rattus* can act as a plague reservoir. An important difference in plague resistance between rat populations from the plague focus (central highlands) and those from the plague-free zone (low altitude area) was confirmed to be a widespread phenomenon. In rats from the plague focus, we observed that sex influenced plague susceptibility, with males slightly more resistant than females. Other individual factors investigated (weight and habitat of sampling) did not affect plague resistance. When infected at high bacterial dose (more than 10^5 bacteria injected), rats from the plague focus died mainly within 3–5 days and produced specific antibodies, whereas after low-dose infection ($<$ 5,000 bacteria), delayed mortality was observed and surviving seronegative rats were not uncommon. These results concerning plague resistance level and the course of infection in the black rat would contribute to a better understanding of plague circulation in Madagascar.

Keywords: Experimental challenge, infectious disease resistance, Madagascar, pathogen-mediated selection, rodent-borne disease, *Yersinia pestis*

Rodent-borne diseases inflict a heavy toll on human health (Gratz, 1997; Meerburg et al., 2009). To increase our understanding of rodent reservoir dynamics, and consequently of disease risks, we need to consider evolutionary changes of the hosts in response to their pathogens (Woolhouse et al., 2002; Altizer et al., 2003). Plague is a rodent-borne disease caused by *Yersinia pestis* bacteria and transmitted by fleas (Prentice and Rahalison, 2007). In Madagascar, plague arrived in 1898 during the third pandemic and has persisted in the central highlands, at altitudes above 800 meters, since the 1930 s (Brygoo, 1966; Duplantier et al., 2005). This large region constitutes one of the main plague foci in the world (41% of the world's reported cases in 2000–2001, WHO, 2003). The black rat, *Rattus rattus*, is by far the most abundant small mammal and is the most probable plague reservoir in villages of the central highlands (Brygoo, 1966; Duplantier and Duchemin, 2003). However, this species is classically considered to be plague susceptible (Dennis et al., 1999), resulting in

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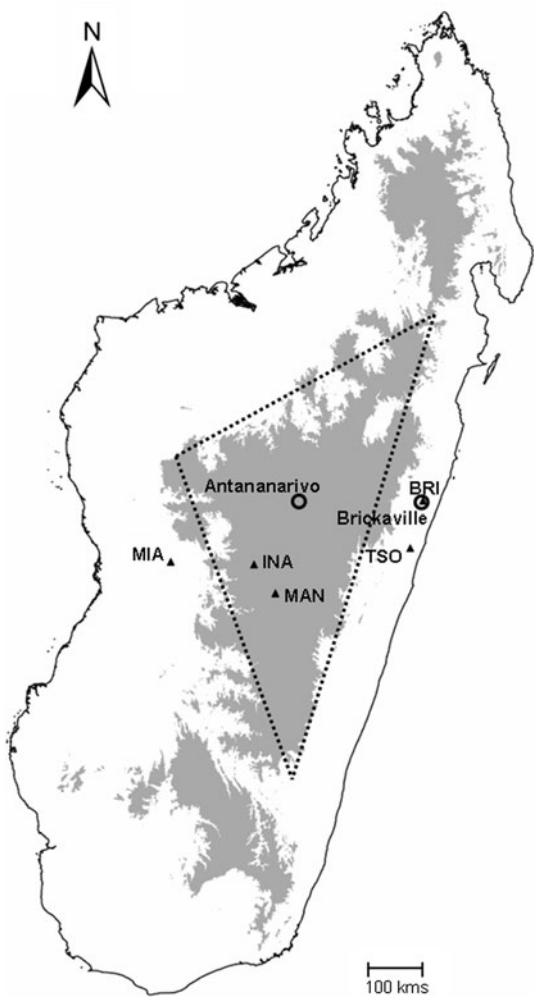

Figure 1. Location of study sites and plague focus in Madagascar. Dotted line: limits of the main plague focus. Grey area: zone where altitude is higher than 800 meters. Triangles: sampling sites [Brickaville (BRI), Inanantonana (INA), Mangarano (MAN), Miandrivazo (MIA), and Tsarasambo (TSO)]. Open circles: localities considered by Rahalison et al. (2003).

the paradox of attributing the role of reservoir to a highly susceptible host.

Preliminary experimental infections (Rahalison et al., 2003) showed that rats from one locality within the Malagasy plague focus (Antananarivo) were approximately 1,000 times more resistant to plague infection than rats from one plague-free locality (Brickaville; Fig. 1). Within the plague focus, rats from different habitats carry different flea species (Brygoo, 1966) and exhibit contrasting levels of plague seroprevalence (higher outdoors than inside houses; Dromigny, 1997; Rahelinirina, 2009), so they may differ in their susceptibility to plague. Moreover, plague resistance heterogeneity within species could be modulated by individual factors, such as sex or age of animals (Twigg, 1978; Gage and Kosoy, 2005). This study was designed to further increase knowledge about plague resistance in natural populations of *R. rattus* in Madagascar, using experimental plague challenges. The main goals were (1) to confirm the difference in rat plague resistance between the plague focus and the plague-free zone, (2) to describe the kinetics of plague infection and the production of antibodies according to rat's origin and the bacterial dose injected, and (3) to examine whether resistance was associated with individual (sex or weight) or ecological (habitat) factors.

Rat Sampling

Wild-trapped rats were used in preference to laboratory-born animals to ensure that host factors would match closely those found in their natural environment (see also Isaacson et al., 1983). Sampling was conducted between November 2006 and May 2008 in two central highland zones, where human plague cases are reported each year, and three plague-free zones (Fig. 1). Rats were live-trapped within houses, in sisal hedges, and around irrigated rice fields (as described in Gilabert et al., 2007). For each sampled rat, a blood sample was collected to perform an anti-F1 (*Y. pestis* specific) antibodies ELISA assay (Dromigny, 1997; Rasoamanana et al., 1997). No seropositive rats were found within plague-free zones, whereas seroprevalence levels ranged between 0.4% and 14.6% within the plague focus. Seropositive rats were euthanized by cervical dislocation as recommended by Mills et al. (1995). Seronegative rats (supposed plague naïve) were housed in group boxes of five rats each, at ambient temperature, with food and water *ad libitum*. Plague challenge was performed at least ten days after trapping.

Plague Challenge and Follow-up of Animals

For all experiments, we used the same *Y. pestis* strain (named 23:07S), which was isolated in 2007 from a Malagasy patient during a routine diagnostic test (Institut Pasteur de Madagascar). The patient was severely affected and one fatal case occurred in the same village. The bubon punction was intensified by injection to mice (which died within 2 days) and stored at −20°C.

Table 1. Description of the plague challenge experiments conducted and results in term of mortality rate and antibody production

Experiment	i1	i2	i3	i4	i5	i6	i7
Date	15/02/07	27/09/07	26/05/08	26/05/08	04/06/07	16/10/07	23/05/08
Origin		Plague focus			Plague-free zone		
(locality[a])	(INA + MAN)	(INA)	(INA)	(INA)	(BRI + MIA)	(TSO + MIA)	(BRI + MIA + TSO)
Bacterial dose	10^5	125	1000	2.10^5	150	750	4150
No. of rats %[b]							
Infected	146	82	86	31	12	20	22
Dead	76	15	5	6	10	18	20
	52.1%	18.3%	5.8%	19.4%	83.3%	90%	90.9%
Surviving seronegative	4	16	30	0	N.A.	N.A.	N.A.
	5.7 %	23.9 %	37.0 %	0 %			

[a]See Fig. 1 for location

[b]Percentages were calculated according to the total number of infected rats, or according to the survivors for surviving seronegatives

N.A. = not analyzed

Because the bacterial dose injected during a flea bite can be highly variable (from zero to more than 4,000 bacteria; Lorange et al., 2005) and variable numbers of fleas can feed on one rat (up to 70 fleas on a single rat, J.M. Duplantier, unpublished observation), we conducted several experiments (Table 1) with various bacterial doses (between 125 to 2.10^5 bacteria injected). Before infection, the bacterial concentration of two days of culture was estimated by measuring the optical density of the solution. A better estimate of the injected dose was obtained a posteriori by deposing different dilutions on selective agar plates. Animals were weighed and 100 µL of bacterial solution was injected subcutaneously in the thigh. We then followed animals during 18 days, as preliminary experiments showed that subsequent mortality was not attributable to plague (L. Rahalison, unpublished observation). All rats were examined twice per day. An antigen F1 rapid diagnostic test (RDT, Chanteau et al., 2003) was used on the spleen of dead rats to confirm that the death was caused by plague. Dead rats with negative RDT were rare (mean 2% of infected rats) and removed from the analyses. At the end of the experiment (18^{th} day), blood was sampled for an ELISA assay (as described above), to estimate the proportion of surviving rats presenting anti-F1 antibodies.

Comparison of Plague Susceptibility between Plague Focus and Plague-free Zone

We assessed whether the issue of plague challenge was related to the origin of the rats, the dose injected, and the interaction between both factors, using a generalized linear model (GLM, PROC LOGISTIC: binomial error distribution, logit link function, backward selection) in SAS for Windows 9.1. (SAS Institute, Cary, NC, USA 2002). Plague survival was higher in rats from the plague focus than for the low-altitude zone ($\chi^2(1) = 56.2$; $P < 0.0001$) and decreased with the injected dose ($\chi^2(1) = 21.7$; $P < 0.0001$; Table 1; Fig. 2). The large difference in plague susceptibility between plague focus and plague-free zone populations,

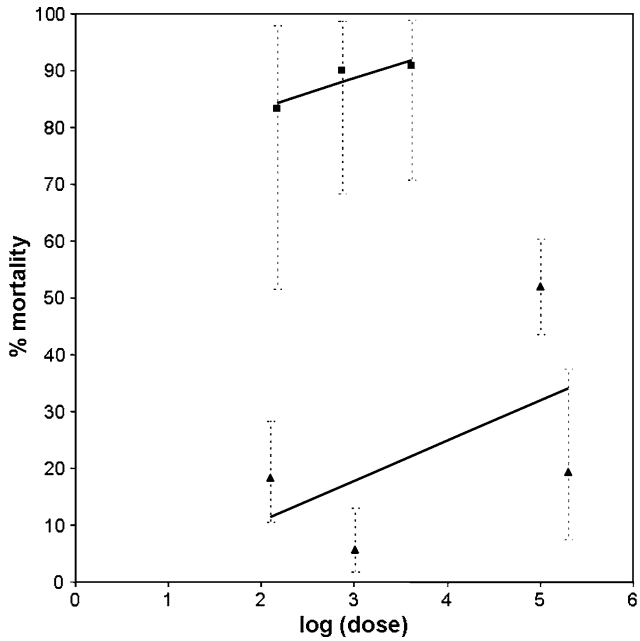

Figure 2. Dose-response curves in term of mortality rate for the rats originating from the plague focus (central highlands, triangles) and rats originating from the plague-free zone (low altitude zone, squares). Mortality rates are indicated for each infection experiment, along with their binomial confidence interval (Clopper and Pearson, 1934).

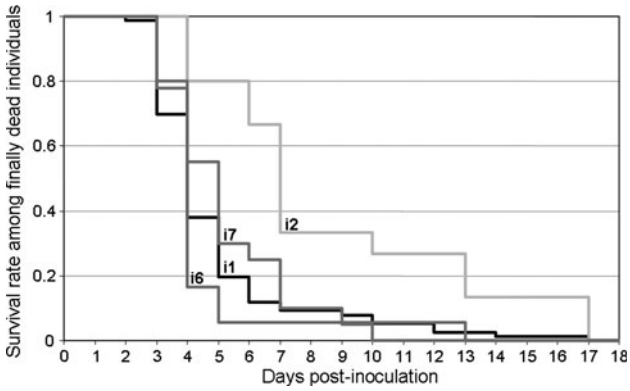

Figure 3. Survivorship functions for experiments resulting with more than 14 dead individuals. The proportion of surviving individuals among finally (after 18 days) dead individuals is represented following Kaplan-Meier representation. i1 (black) was a high-dose infection, whereas i2, i6, and i7 (grey) were low- or moderate-dose infections. i1 and i2 used rats from the plague focus, whereas i6 and i7 used rats from the plague-free zone. The mean survival time was higher in i2 (8.60 ± 1.14 days) compared with i1 (4.84 ± 0.30 days), i6 (4.39 ± 0.52 days), or i7 (5.15 ± 0.44 days).

previously described by Rahalison et al. (2003), is here confirmed and demonstrated in other localities. Because this pattern was also observed in laboratory-born animals (Rahalison et al., 2003), it suggests the evolution of plague resistance restricted to central highland populations where plague has been endemic for 90 years. Plague resistance in these *R. rattus* populations would therefore be an example of rapid evolution (Altizer et al., 2003), which may be explained by the high virulence of plague bacteria (Stenseth et al., 2008) and the short generation time of the black rat (approximately 0.5 years; J.-M. Duplantier, unpublished data). Evolution of plague resistance may not be restricted to this system, as variability in resistance related to plague occurrence has been described in other recent plague foci: *Onychomys leucogaster* (Thomas et al., 1988) and *Microtus californicus* (Quan and Kartman, 1962) in North America; *Mastomys natalensis* in South Africa (Shepherd et al., 1986). Unlike these native species, the black rat was introduced to Madagascar a few thousand years ago, and consequently displays relatively low levels of neutral genetic variability, especially in the central highlands (Tollenaere et al., 2010), but this does not appear to have prevented rapid evolution (see also Koskinen et al., 2002; Dlugosch and Parker, 2008).

Kinetics of Infection

We conducted Kaplan-Meier survival analyses to compare experiments resulting in at least 15 dead individuals (i1, i2, i6, i7; Fig. 3) using the LIFETEST procedure of SAS. Log-rank tests indicated significant differences among survival curves ($\chi^2(3) = 17.06$; $P = 0.0007$). Most plague death occurred between the third and fifth days after infection (more than 90% before the seventh day) for experiments involving rats from the plague-free zone (i6 and i7) and for high-dose infections of rats from the plague focus (i1). Mean survival time was higher for the low-dose experiment involving rats from the plague focus (i2) with some plague death still occurring after 17 days (Fig. 3).

These results mostly confirmed the rapidity of plague disease in *R. rattus*, as observed in humans (Perry and Fetherston, 1997) and laboratory rats (Sebbane et al., 2005). However, the prolonged lifespan of infected rats within plague foci could have a significant influence on flea infection rates and, therefore, plague transmission, but information on bacteraemia levels in the blood through the course of infection would be necessary to evaluate this hypothesis.

Antibody Production in Surviving Animals

Most rats surviving to 18 days produced antibodies. However, the proportion differed according to the dose injected (Table 1), with 24–37% of rats surviving a low-dose infection (i2, i3) testing seronegative compared with only 0–6% at high dose (i1, i4). Individuals resistant to infection with no antibody production were rare in high-dose but fairly common in low-dose infections. Because the natural dose injected by fleas may be highly variable (Lorange et al., 2005), this result indicates that seroprevalence levels could underestimate plague circulation in rodent populations.

Individual Factors Affecting Susceptibility within the Plague Focus

The effect of sex, weight (at infection time), habitat (houses, sisal fences, and rice fields), and their interactions on plague survival was tested using GLMs (see above). Only experiment i1 was considered due to insufficient number of dead rats in other experiments. Sex was the only variable included in the final model ($\chi^2(1) = 4.43$; $P = 0.035$), revealing that males (66 individuals) were more resistant than females (80 individuals). More experiments are needed to confirm this finding. In humans, plague incidence

often differs between males and females, although the direction of the sex-bias differs between foci (for example Migliani et al., 2006 and Davis et al., 2006). This pattern is classically attributed to variation in risk due to differential behavior, rather than differences in susceptibility (but see Boisier et al., 2002).

Animal weight and habitat did not influence susceptibility. Other individual factors—transitory (physiological or immune status) or permanent (genetics)—also may influence resistance levels (Gage and Kosoy, 2005) and should be investigated in Malagasy *R. rattus*.

Conclusions

Epidemiological Consequences of Resistance Level within the Plague Focus

The proportion of plague-resistant or susceptible individuals in a population characterizes the role that population can play in transmission and maintenance of plague (Biggins and Kosoy, 2001). Indeed, highly resistant individuals allow the maintenance of rat populations during outbreaks, whereas highly susceptible rats allow plague transmission (Eisen and Gage, 2009), because fleas can only be infected by feeding on animals with terminal septicemia (Lorange et al., 2005). Resistance levels found in *R. rattus* populations from the Malagasy plague focus (50–94%) were close to those found in classical enzootic hosts in Asia (40–80%; Biggins and Kosoy, 2001). Moreover, epidemiological models show that an introduction of bubonic plague can lead to a highly persistent enzootic focus when the initial level of resistance in rodent population is 50–75% (Keeling and Gilligan, 2000). Developing specific epidemiological models would allow a more detailed investigation of the role of *R. rattus* resistance for plague persistence in Madagascar. The results of this study provide crucial insight for developing such models.

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