Yersinia pestis has been found in human body lice during plague outbreaks. To evaluate the role that the human body louse plays as a vector of plague, we allowed lice to feed on rabbits made bacteremic by intravenous inoculation of $10^9$ colony-forming units of 3 strains of Y. pestis. High mortality rates were observed in all lice 2 and 3 days after infection. The lice remained infected with the strains for their life span and excreted viable organisms in their feces from day 1, although they were unable to lay eggs. The lice infected with 2 virulent strains of Y. pestis transmitted the organisms during feeding to uninfected rabbits, which became septicemic and died of plague (with 1 exception) 1 day later. Infections were transmitted to naive lice that were fed on these rabbits, showing that lice can be vectors of Y. pestis in an experimental model.

Yersinia pestis is the agent of plague [1]. Three plague pandemics have been reported: the Justinian plague (fifth to seventh centuries), the Black Death (14th–18th centuries), and the modern plague (1870s to the present) [1]. The Black Death pandemic alone is estimated to have claimed the lives of one-third of the European population and is thought to have shaped the development of modern civilization. The recent emergence of multiresistant strains of Y. pestis [2] and the growing recognition of its potential as a biological weapon [3] mean that plague remains a significant threat to human health.

Y. pestis has a complex life cycle involving a mammalian reservoir (primarily rodents) and a flea vector [1]. Humans play no part in its long-term survival. Because of its role in previous pandemics, the Oriental rat flea (Xenopsylla cheopis) is considered to be the classic plague vector [4, 5]. Y. pestis has been isolated from other arthropods, mainly ticks [6–9] and lice [10]. Blanc and Baltazard transmitted Y. pestis to mammals and rats by inoculating them subcutaneously with infected human body lice or their feces [10].

To investigate the potential role that lice play in plague transmission, we tested the ability of Y. pestis (1 avirulent and 2 virulent strains) to infect and multiply in lice after ingestion of 1 meal of infected rabbit blood. We then evaluated the ability of lice to acquire Y. pestis and transmit plague to naive rabbits. For this, we used our experimental model of louse infection that was shown to simulate natural infection with Bartonella quintana [11], Rickettsia prowazekii [12], Borrelia recurrentis [13], and R. typhi [14].

MATERIALS AND METHODS

Study design. The general design of the study is presented figure 1.

Strains. Two virulent strains of Y. pestis were used in the present study—CEB 03-014 (Biovar Orientalis, origin Vietnam) and 6/69 (Biovar Orientalis, origin Madagascar)—as well as 1 avirulent strain (EV76). Iga Levet (Direction Générale de l’Armement) and Michel Simonet provided the CEB 03-014 and the 6/69 strains of Y. pestis, respectively. Numbers of colony-forming units per milliliter from stock cultures were determined 3 days after 100 μL of 10-fold serial dilutions (from undiluted to $10^{-12}$) in PBS (bioMérieux) were streaked onto both Columbia sheep blood (bioMérieux) and...
Yersinia-selective cervical intraepithelial neoplasia (CIN; Becton Dickinson) agars and incubated at 28°C. Counts were performed in duplicate experiments.

The Orlando reference strain of the human body louse [11] was used. Each group of lice was kept in a separate plastic container at 29°C with 70%–90% humidity. New Zealand White female specific-pathogen-free rabbits (Charles River) weighing 4.5–5 kg were used. Rabbits were kept in individual cages contained in a ventilated cabinet (Charles River). Rabbits were estimated to have 360–420 mL of blood.

Calculation of the size of blood meal ingested by a louse. To estimate how many bacteria 1 louse could ingest during a 1-h feeding on a bacteremic rabbit, 100 fifteen-day-old infected lice were weighed on an electronic balance (August Sauter) accurate to within 10⁻⁵ g. They were weighed again after feeding for 1 h on the shaved abdomen of an uninfected rabbit. The experiment was repeated 3 times. The amount of blood imbibed was calculated using the formula $B = \Sigma L_{\text{post}} - \Sigma L_{\text{pre}} / N - N_{nf}$, where $B$ is the weight of the ingested blood, $\Sigma L_{\text{post}}$ is the mass of the lice after feeding, $\Sigma L_{\text{pre}}$ is the mass of the lice before feeding, $N$ is the total number of lice, and $N_{nf}$ is the number of lice that were observed not to feed.

The same experiment was performed using 50 fifteen-day-old uninfected lice, which were fed on one of the investigators (L.H. or D.R.) or a technician (R. Morazzani). The volume of blood imbibed was calculated by dividing the weight of the blood ingested by the density of the rabbit (1.0416 g/mL) or human (1.058 g/mL) blood.

Infection of rabbits. Three rabbits were made bacteremic by the infusion of $10^9$ cfu of Y. pestis. Each strain of Y. pestis was diluted in 20 mL of PBS and injected into the ear vein of 1 rabbit over 15 min. Infected rabbits were examined daily for clinical signs of illness and development of pathological lesions. The duration of bacteremia was determined by polymerase
chain reaction (PCR) performed on 200 μL of EDTA-anticoagulated blood. Blood from Y. pestis (CEB 03-014)–infected rabbits was collected immediately and at 1, 2, 4, 7, 10, and 19 h after infection. Blood from Y. pestis (6/69)–infected rabbits was collected immediately and at 1, 3, 7, 10, 14, and 21 h after infection. Blood from Y. pestis (EV76)–infected rabbits was collected immediately and at 1, 3, 5, 23, and 28 h after infection. DNA was extracted using the QIAamp Tissue kit (Qiagen) in accordance with the manufacturer’s instructions. DNA was eluted in 100 μL of elution buffer, and PCR was performed using the Y. pestis–specific primers YP20D (5'-TATGGATGAC-TAGACTGG-3') and YP12R (5'-CAGCAGGATATCAGGAA-ACA-3'), which amplify the pla gene encoding for the plasminogen activator. PCR was performed with a Peltier model PTC-200 thermocycler (MJ Research); each cycle consisted of denaturation at 95°C for 60 s, annealing at 57°C for 30 s, and extension at 72°C for 90 s. After 44 cycles, an additional 7-min extension was performed at 72°C. DNA extracted from the 3 strains under study was used as positive control. PCR with β-globin–derived primers [15] was used to control for the efficiency of DNA extraction and PCR.

**Infection of lice.** Five minutes after the beginning of the intravenous infective injection, 3 groups of 300 fifteen-day-old lice were infected by allowing them to feed for 1 h on the shaved upper-right thigh of the bacteremic rabbits. The day of infection was referred to as day 0.

To determine whether Y. pestis multiplied in lice, cultures were performed on each of 5 lice collected 1 h after infection from each rabbit. Five lice were cultured every 3 h for the first 25 h and thereafter once every 24 h. Each louse was individually surface decontaminated as described elsewhere [16] and crushed in 500 μL of PBS, and 100 μL of 10-fold serial dilutions (from undiluted to 10^-4) in PBS were streaked onto blood agar. The generation time of each Y. pestis strain within the louse was calculated using standard formulas based on an exponential growth phase: generation time (minute/generation) = time elapsed to reach P/n; and n = 3.3 × log(P/p), where P is the final population number, p is the original population number, and n is the number of generations.

Lice were examined daily after they were collected from the bacteremic rabbits. Death rates for lice infected with each Y. pestis strain were compared using the χ² test (Epi Info; version 6.0; Centers for Disease Control and Prevention). On day 3, 2 infected lice from each group were fixed in absolute ethanol at 4°C for 2 weeks and embedded in paraffin. Three-micrometer sections were cut for an immunofluorescence assay (IFA) [13], which used 30 μL of an anti–Y. pestis (EV76) mouse polyclonal antibody (PAb) diluted to 1:1000 in PBS with 3% (wt/vol) nonfat dried milk. Uninfected lice were used as negative controls.

Twice daily, ~0.1 mg of feces was collected from each louse group for the IFA and for culture, as described elsewhere [13]. Uninfected lice feces were used as negative controls. For the IFA, the feces were examined with 30 μL of anti–Y. pestis PAb diluted to 1:1600. For culture, each sample was suspended in 500 μL of PBS, and 100-μL aliquots were streaked onto blood and CIN agars. Agar media were incubated at 28°C and read on day 3.

The numbers of eggs produced by each group of lice were recorded daily. Two eggs (starting from the laying of the first egg) and 2 larvae (starting from the hatching of the first egg) were sampled daily: 1 was processed for PCR, as described above, and 1 was cultured on blood agar after decontamination [16]. 18S rRNA PCR [17, 18] was used to determine the efficiency of DNA extraction and PCR.

**Transmission from lice to rabbits.** Starting from day 1, each infected lice group was allowed to feed for 1 h on the shaved outer-thigh of a new previously unexposed rabbit. A group of 300 uninfected lice, used as a negative control, was allowed to feed daily on the outer-thigh of another uninfected rabbit. The entire experiment was performed in duplicate. To assess whether fewer lice could transmit Y. pestis, groups of 100 and 10 lice were infected with each virulent strain and allowed to feed for 1 h daily on the outer-thigh of new naive rabbits.

From day 1, all rabbits were examined daily for clinical signs of illness and the development of pathological lesions. Weekly, 4 drops of ear blood were collected onto blotting paper from each rabbit (Fisher Scientific) for the IFA, as described elsewhere [19]. EDTA-anticoagulated blood was collected twice daily from each rabbit for PCR (pla and β-globin) and culture (on both agars).

All rabbits used to feed Y. pestis (virulent strains)–infected lice were autopsied after they died of the infection or were killed after the detection of skin lesions. Various organs, including the skin from the thigh where lice had fed, spleen, liver, lungs, heart, kidneys, and lymph nodes, were collected aseptically and divided into 3 portions. Two portions were stored at ~80°C for pla PCR and culture (on both agars). The third portion was fixed in Bouin’s fixative (thigh skin, spleen, liver, and lymph nodes) or in 4% buffered formalin (lungs, heart, and kidneys), embedded in paraffin, sectioned (3 μm), and stained with hematoxylin-eosin or used for immunohistochemical analysis, as described elsewhere [20], by use of anti–Y. pestis PAb diluted to 1:2000 and an immunoperoxidase kit (Histostain Plus Kit; Zymed CliniSciences). Serum samples from healthy mice were used as negative controls.

**Transmission of Y. pestis from rabbits to naive lice.** Each of 4 naive groups of 100 lice was fed daily for 1 h on the previously shaved abdomen of each of the nurse rabbits 1 h after the feeding of the infected lice. Numbers of surviving lice...
were recorded daily, and dead lice were collected for culture and pla PCR.

RESULTS

The results show that rabbits were made bacteremic by our technique. We found that Y. pestis–bacteremic rabbits can infect lice and that infected lice can infect naive rabbits, which in turn, can infect a new set of lice. The number of colony-forming units of the 3 Y. pestis strains was 4 times higher on blood agar than on CIN agar. Colony aspect allows to differentiate contaminants on blood agar.

Y. pestis–infected rabbits died 19–24 h after infection (CEB 03-014), 26–28 h after infection (6/69), and 28 h after infection (EV76). All blood samples collected from the infected rabbits were positive by PCR (pla and β-globin). The lethal effect of EV76 may be related to the high inoculum used intravenously.

Infection of lice. We calculated (mean ± SD) that a louse ingested 0.63 ± 0.17 μL (0.66 ± 0.02 mg) of rabbit blood and 0.42 ± 0.01 μL (0.44 ± 0.01 mg) of human blood per meal. Furthermore, we calculated that, when fed on a bacteremic rabbit (2.27 × 10^6 to 2.63 × 10^6 bacteria/mL), a louse would ingest 1400–1600 bacteria.

Cultures performed with 360 lice fed on infected rabbits (120 lice for each Y. pestis strain) were positive on day 3 of culture. The louse disinfection protocol was effective: bacterial contaminants were present in only 12 cultures. After 1 h of feeding, and depending on the experiment, the number of Y. pestis cultured from infected lice varied from 1600 to 1295 (CEB 03-014), 1030 to 1100 (6/69), and 45 to 100 (EV76) (figure 2). This is in accordance with our prediction based on the ingested blood and the size of the inoculum injected into the rabbit. Y. pestis–generation time in lice was calculated to be 2.61 h (CEB 03-014), 2.74 h (6/69), or 1.96 h (EV76). The number of viable bacteria in lice fed on infected rabbits decreased on day 4 (figure 2).

High mortality rates were observed in lice on days 2 and 3 in the groups of 300, 100, and 10 lice fed on infected rabbits (figure 3). When 300 lice were used, for instance, mortality rates were as follows: 50.36%–56.47% (CEB 03-014), 43.77%–45.8% (6/69), and 29.31%–28.13% (EV76) on day 2; and 16.42%–18.93% (CEB 03-014), 17.79%–17.55% (6/69), and 55.51%–53.13% (EV76) on day 3. The mortality of EV76–infected lice was delayed, and this may have been due to a lower initial inoculum in lice. At the same times, none or only 1 of the uninfected lice died. Compared with the mean survival of uninfected lice (40 and 39.5 days), the 6-day survival of lice fed on infected rabbits was significantly shorter (P < 10^-7) regardless of the Y. pestis strain (figure 3). Based on the results of the IFA (figure 4), lice infected with the 3 strains appeared to have a generalized septicemia with organisms distributed throughout the body. Lice fed on uninfected rabbits were IFA negative.

From day 1, Y. pestis was detected daily by IFA and culture in the feces of infected lice. Feces from uninfected lice were IFA and culture negative. No eggs were laid by infected lice, whereas uninfected lice began laying eggs on day 4 (6392–6583 eggs, depending on the experiment), and these hatched 11 days later. Culture and pla PCR performed on eggs and larvae from uninfected lice were negative. 18S rRNA PCR was positive, however, showing the efficiency of DNA extraction and PCR.

Transmission of Y. pestis from infected lice to naive rabbits. Rabbits hosting uninfected- and Y. pestis (EV76)–infected lice remained asymptomatic and seronegative for at least 180 days after infection. Blood cultures and pla PCRs were always negative. β-globin PCRs were positive, showing the efficacy of DNA extraction and PCR.

All the rabbits hosting Y. pestis virulent strain–infected lice died, except for one hosting 300 Y. pestis (6/69)–infected lice, which remained asymptomatic until killed on day 15. The survival time of the rabbits was directly correlated with the number of infected lice they hosted. When 300 lice were used, the host rabbits died 6 or 7 days (CEB 03-014) to 4 days (6/69) after infection. When 100 lice were used, rabbits died 8 days (CEB 03-014) to 9 days (6/69) after infection. When 10 lice were used, the rabbits died 13 days (CEB 03-014) to 17 days (6/69) after infection. At 2–4 days after infection, rabbits hosting Y. pestis (CEB 03-014)–infected lice developed a bluish-purple discoloration of the skin where the lice fed. Blood cultures and pla PCRs were negative until the day before the death of the rabbits hosting lice infected with the Y. pestis virulent strains. Cultures and PCRs were then positive for all rabbits except for the rabbit hosting 300 Y. pestis (CEB 03-014)–infected lice, which was killed on day 7 when it developed a bluish-purple discoloration of the skin at the site of the lice feeding.

Culture and pla PCR were positive for all the organs sampled...
Human Body Louse as Vector of Plague

Figure 3. Comparison of the survival rate over time of the uninfected control human body lice and the lice infected with each of the 2 virulent strains of *Yersinia pestis*: CEB 03-014 and 6/69. Day 0 is the day of infection. Values are given as percentages of lice surviving, excluding those killed for other tests.

**DISCUSSION**

To be an efficient vector, an arthropod must be able to acquire and maintain an infection that it transmits by feeding or by excreting viable bacteria in its feces. The ability of fleas to transmit plague (vector efficiency) was defined as the product of 3 potentials: infection potential (percentage of fleas that become infected after taking an infected blood meal), vector or infective potential (percentage of infected fleas that become capable of transmitting), and transmission potential (observed ability of each flea to transmit plague before its death) [17, 21].

Our experiments show that the human body louse, a strictly human ectoparasite, is a potential vector for plague. When lice fed on rabbits bacteremic with avirulent or virulent strains of *Y. pestis*, they all became infected and died of the infections, with the highest mortality rates between days 2 and 3 (figure 3). The death of the lice could be due to the multiplication of *Y. pestis* (figure 2) causing septicemia (figure 4). *X. cheopis* also died after its infection with *Y. pestis* [18], which formed a biofilm [22] blocking the foregut [22] and causing regurgitation of organisms into the host when the flea attempted to feed [21]. Infected lice excreted viable bacteria in their feces, as reported elsewhere with infected lice [10] and fleas [21]; their feces are a major source of human infection in louse-borne diseases [13, 23–25].

Our experiments demonstrated for the first time, to our knowledge, that as few as 10 lice can transmit plague to a host while feeding. Only *Y. pestis* virulent strains were transmitted by lice, as is the case with fleas that were found to be unable to transmit the avirulent strains EV51, TRU, and A1122 [26, 27]. Bluish-purple discoloration of the thigh skin where lice fed (CEB 03-014) was seen at 2–4 days after infection, which is consistent with a report that most animals (but not all) develop bubonic plague [1]. Moreover, we were able to establish a cycle of infection by using infected lice to transmit *Y. pestis* to rabbits, which then served as a source of infection for naive lice.

Our inocula (2.27 × 10⁶ to 2.63 × 10⁷ bacteria/mL) was in the range of that reported to occur during bacteremia in pa-
patients with plague (≥10^2 to <10^8 bacteria/mL) [28]. In a mouse model, blood meals containing 4 × 10^6 to 4.2 × 10^7 bacteria have been used to infect X. cheopis [29]. Average ± SD volumes of blood ingested were reported to be 0.12 ± 0.07 µL (female X. cheopis) [29], 0.03 µL (X. cheopis, nonspecified sex) [21], 0.0579 µL (female human head louse), 0.0657 µL (male human head louse), and 0.0387 µL (human head louse nymph) [30]. We found that the human body louse ingests an average ± SD of 0.63 ± 0.17 µL of rabbit blood and 0.42 ± 0.01 µL of human blood.

Several ectoparasites were found to be naturally infected with Y. pestis; at least 80 flea species are involved in maintaining plague cycles, and ≥31 are proven vectors [1]. Several ticks were incriminated as vectors and reservoirs of plague in China and the former Soviet Union [1]. Y. pestis was isolated from Ornithodoros pools collected in Brazil [31] and several ixodid and argasid species in Russia [7, 32, 33]. Infected human body lice and human fleas (Pulex irritans) were infected from patients with septicemic plague [10, 34–37]. Transmission of plague was demonstrated when human body lice or their feces were inoculated subcutaneously into guinea pigs or rats [10].

A widely held notion is that ancient plague pandemics resulted from the concentration of unusually high numbers of people with anthropophilic rodent fleas, mainly X. cheopis, responsible for transmission [34, 35, 38]. However, data reported during and after various outbreaks has challenged the universality of a rat-flea-human cycle in human plague outbreaks [10]. Bubonic plague pandemics in Iranian Kurdistan occurred in the absence of domestic rats [10]. X. cheopis, which was not encountered in Europe in the Middle Ages, is not adapted to the European climate [36], and the European rat flea (Nosopsyllus fasciatus) very rarely feeds on humans [37, 39]. Moreover, X. cheopis is not readily infected by Y. pestis, and subsequent transmission efficiency is low [29]. This made the etiology of

Figure 4. Demonstration of Yersinia pestis in an infected louse 3 days after infection by use of immunofluorescence staining and confocal microscopy; original magnification, ×100. Because of the louse dimensions, several photographs were taken and reconstituted by digital overlay.
the Black Death (the medieval plague pandemic) a matter of intense speculation [40, 41] until we and others found Y. pestis DNA in the remains of victims of the Black Death and the Justinian plague [42–45]. Although direct airborne transmission of plague from patients with pneumonic plague is possible [46–48], it occurs rarely [49] at louse range, and historical sources report the majority of ancient epidemics involved bubonic plague, most likely transmitted by ectoparasites [10].

Therefore, it is possible that vector-host cycles with different transmission dynamics occurred with plague in ancient times, when people commonly harbored ectoparasites. Diemerbroeck in 1665 [10] reported transmission of plague by clothes in which human body lice live. Our experiments show for the first time, to our knowledge, that human body lice transmit plague while feeding and support the hypothesis that they might be vectors in plague pandemics. This is particularly important in light of the reemergence of the human body louse in underdeveloped countries [50] and also in homeless people in developed and industrialized countries.

In conclusion, we demonstrated that the human body louse is a vector of plague in an experimental model. One infected blood meal was sufficient to infect lice, in which Y. pestis multiplied and produced a generalized infection; viable Y. pestis were excreted in feces. We believe that the louse, in addition to X. cheopis, might have played a role in past plague pandemics that occurred when human ectoparasites were common.

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