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REVIEW

Yersinia pestis: the Natural History of Plague

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SUMMARY The Gram-negative bacterium *Yersinia pestis* is responsible for deadly plague, a zoonotic disease established in stable foci in the Americas, Africa, and Eurasia. Its persistence in the environment relies on the subtle balance between *Y. pestis*-contaminated soils, burrowing and nonburrowing mammals exhibiting variable degrees of plague susceptibility, and their associated fleas. Transmission from one host to another relies mainly on infected flea bites, inducing typical painful, enlarged lymph nodes referred to as buboes, followed by septicemic dissemination of

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the pathogen. In contrast, droplet inhalation after close contact with infected mammals induces primary pneumonic plague. Finally, the rarely reported consumption of contaminated raw meat causes pharyngeal and gastrointestinal plague. Point-of-care diagnosis, early antibiotic treatment, and confinement measures contribute to outbreak control despite residual mortality. Mandatory primary prevention relies on the active surveillance of established plague foci and ectoparasite control. Plague is acknowledged to have infected human populations for at least 5,000 years in Eurasia. *Y. pestis* genomes recovered from affected archaeological sites have suggested clonal evolution from a common ancestor shared with the closely related enteric pathogen *Yersinia pseudotuberculosis* and have indicated that *ymt* gene acquisition during the Bronze Age conferred *Y. pestis* with ectoparasite transmissibility while maintaining its enteric transmissibility. Three historic pandemics, starting in 541 AD and continuing until today, have been described. At present, the third pandemic has become largely quiescent, with hundreds of human cases being reported mainly in a few impoverished African countries, where zoonotic plague is mostly transmitted to people by rodent-associated flea bites.

KEYWORDS *Yersinia pestis*, epidemiology, lice, paleomicrobiology, plague

INTRODUCTION

Plague, caused by the bacterial pathogen *Yersinia pestis*, has been recognized by doctors and populations as a unique nosological entity for centuries because it is the sole disease characterized by swollen lymph nodes referred to as buboes to cause deadly epidemics. Historical sources in Europe have led to the delineation of three plague pandemics. The first pandemic, known as the Justinian Plague, devastated the Mediterranean Basin from 541 to 750/767 CE (1) and invaded northern Europe as far as Germany and England (2). The second pandemic, lasting from 1346 to the 18th century, including the so-called "Black Death" period of 1346 to 1353 (3), killed an estimated one-third of the European population (4). The third pandemic probably began in 1772 in the Chinese province of Yunnan (5, 6) and spread worldwide on the eve of the 20th century following human movement via steamship and railroad (7). *Y. pestis* infection may cause five principal forms of plague, including bubonic, septicemic, pneumonic, meningeal, and pharyngeal plague (8). In addition, *Y. pestis* may cause skin ulceration at its portal of entry, reported as carbuncles and ulcers, along with pustules, spots, petechiae, bruising, and gangrene (9). The plague etiology was resolved in 1894 in Hong Kong by the Swiss-French physician Alexandre Yersin, who was affiliated with the Pasteur Institute and who also contributed to resolving part of the cycle of transmission of *Y. pestis* involving the rat (*Rattus rattus*) and its ectoparasites (*Xenopsylla cheopis*) (10).

Obtaining a comprehensive overview of various sources of infection and various routes of transmission of *Y. pestis* in populations is critical for preventing and surveying plague. Cumulative field observations in plague foci combined with the critical review of data issuing from paleomicrobiological, anthropological, and historical studies continue to shed new light on questions related to the reservoirs, sources, transmission, and vectors of *Y. pestis* and to provide new avenues for addressing these questions.

Here, we critically review data reported in the English literature and some non-English publications to provide a comprehensive view of the various cycles of plague transmission as a basis for determining the appropriate medical management of plague in the countries in which it remains a zoonotic disease and a public health concern.

MODERN PLAGUE

Microbiology of Plague

Y. pestis is one of the three human-pathogenic *Yersinia* species, along with *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (8). *Y. pestis* is a nonmotile, nonsporulated, aerobic Gram-negative bacillus or coccobacillus exhibiting a hairpin morphology after Gram staining and growing within 24 to 72 h at a temperature range of 4 to 40°C (optimum, 28 to 30°C) at pH 7.4 (8). The sources and phenotypic characteristics of *Y. pestis* isolates allow their classification based upon the following: the region of isolation

and circulation and the main hosts; the biochemical pattern, including the fermentation of rhamnose, melibiose, arabinose, glycerol, and melecytose, denitrification, fibrinolytic and coagulase activity, pesticin production and susceptibility, and dependence on amino acid sources; the frequency of mutation from a Pgm^+ to a Pgm^- phenotype in 10 generations; and virulence in guinea pigs (11). In particular, the biochemical pattern divides *Y. pestis* isolates into *Y. pestis* subsp. *pestis*, considered typically human (divided into the Intermedium, Antiqua, Medievalis, and Orientalis biovars), and *Y. pestis* subsp. *microtus*, considered typically zoonotic (11–13). *Y. pestis* bv. Orientalis isolates are unique in their capacity to stabilize the prophage YpfΦ (a filamentous phage) in their chromosome, which probably confers *Y. pestis* a selective advantage under natural conditions (14). *Y. pestis* is a gammaproteobacterium with a 4.60- to 4.65-Mb genome exhibiting numerous insertion sequences, intragenomic recombination and lateral gene transfer events, and remnants of an enteric life cycle (15, 16). The *Y. pestis* CO92 reference strain (biovar Orientalis) harbors three plasmids, a 70- to 75-kb plasmid common to the three human-pathogenic *Yersinia* species (designated pCD1, pCad, pVW, pYV, or pLcr) encoding the type III secretion system (T3SS) (including *Yersinia* outer proteins, or Yops) preventing the host's immune response, the V antigen (LcrV), which is also implicated in immunosuppressive activity, and the yersiniabactin siderophore system gene (*ybt*), allowing *Y. pestis* to acquire iron from blood (8, 17). A 100- to 110-kb plasmid (designated pFra/Tox, pFra, pTox, pMT1, or pYT) encodes the capsular F1 glycoprotein antigen and the *Yersinia* murine toxin Ymt, allowing the survival of *Y. pestis* in the flea gut (18), and a 9.5-kb plasmid (designated pPst, pPla, pPCP1, or pYP) encodes the plasminogen activator Pla and pesticin, a bacteriocin (8). However, the plasmid contents can be altered by successive subculturing (19). Additional plasmids have also been characterized, illustrating the plasticity and ongoing evolution of *Y. pestis* (12, 20–22). The pPCP1 plasmid encodes the plasminogen activator Pla, regarded as a major virulence factor promoting the systemic spread of *Y. pestis* from peripheral sites (23). The *pla* gene is an outer membrane omptin member, and omptins are detected in several Gram-negative bacteria, including animal and plant pathogens (24, 25). Ectoparasite bites may provoke discrete local inflammation (26) at the skin portal of entry of *Y. pestis*, which then spreads via the lymphatic route toward regional lymph nodes, in which pathogen growth results in the development of a bubo (27). *Y. pestis* further spreads via the lymph (28, 29) and blood vessels to the spleen and liver and causes rapidly fatal septicemia, with dissemination in the lungs (resulting in secondary pneumonic plague) and the meninges and cerebrospinal fluid (causing meningitis). The hematogenous dissemination of the bacteria may cause intravascular coagulation and endotoxic shock (30). During this process, *Y. pestis* rapidly multiplies in tissues, being protected from the immune system by serum resistance (31) and the evasion of innate immune functions, including the neutralization of immune cells mediated by the T3SS (encoded by the virulence plasmid pYV/pCD1) (32), the absence of detectable pathogen-associated molecular patterns (30), and the modulation of host innate immune cell interactions (33). Thus, *Y. pestis* is a facultative intracellular bacterium that multiplies in macrophages (34). A role of the mediator of inflammation MyD88 has been identified in the pathology of primary pneumonic plague, with MyD88 exhibiting a biphasic inflammatory response that ultimately limits systemic infection in a mouse model (35).

Plague in the 21st Century

The natural history of plague is shaped by time and geography (Table 1). The geographical extension of plague can be retrieved from World Health Organization (WHO) notifications, but plague has not been a WHO-notifiable disease since 2005, with the exception of pulmonary plague cases and cases documented in countries where the disease is nonendemic (36) (Fig. 1 and 2; Table 1). In the last few decades, 21 countries scattered across the Americas, Africa, and Asia have reported a total of 26,237 plague cases to the WHO under different epidemiology regimens (data from 2000 to 2018) (37–39). In the Americas, 371 plague cases have been reported over the last

TABLE 1 Currently available epidemiological information for the 19 countries that have reported plague cases over the last 19 years[a]

Country	Localization of plague foci	Characteristic(s) of plague foci	Current main source	Reference(s)
Democratic Republic of the Congo	Ituri, Haut-Uele districts, North Kivu province	Dry tropical, tropical mountain zones, elevation > 1,000 m	*Mastomys natalensis, Arvicanthis abysinnicus, Lemniscomys striatus, Mus minutoides, Rattus rattus*, fleas	48, 71, 192, 245
Madagascar	Center, North, and East of the country	Higher elevation than in coastal region rural zones	*Rattus rattus*, shrew, fleas	57–61, 183, 347
Uganda	West Nile regions	Plague risk increases during the dry season and with an elevation of >1,300 m	Rats, fleas	36, 49, 50, 51, 146, 184
Tanzania	Lushoto, Mbulu, Monduli district		Brush-furred mice, *Praomys* spp., African dormice, zebra mice, commensal rats, fleas	52–54, 56, 289
Zambia	Namwala, Sinda, Chama, Lundazi, Zambezi district	Semiarid zones	Gerbils, rats, fleas	46, 47, 55
Brazil	Pedra branca, Feira de Santana areas	Semiarid and xeric zones, plague risk increases with precipitation, elevation > 200 m	Grass mice, *Oryzomys* spp., vesper mice, *Bolomy* spp., Amazonian marsh rat, gray short-tailed opossum, Marsupialia, *Oligoryzomys, Cerradomys*, Spix's yellow-toothed cavy, Sao Lourenço punaré, fleas	45–47, 290, 360
Peru	Cajamarca, Otuzco, Ascope, Trujillo, Pacasmayo regions	Desert and arid zones, plague risk increases with an elevation of >1,300 m	*Askodon*, *Oryzomys*, cotton rat, *Phyllotis, Rattus rattus*, guinea pig, fleas	42, 45
United States	West of the country	Arid and saline zones, winter/spring rainfall, high elevation < 850 m	Prairie dog, squirrel, chipmunk, mountain lion, cat, dog, coyote, marmot, rabbit, hare, mice, vole, rat, fleas	41, 69, 70, 76, 93, 138, 353
China	North, Northeast, and South of the country; Junggar Basin	Plague risk increases with an elevation of >3,100 m; arid, desert, and subtropical zones; calcium- and iron-enriched environment	Marmot, Tibetan sheep, *Spermophilus dauricus*, dog, *Meriones*, rat, rabbit, mice, fleas	74, 271–276
Kazakhstan	Lake Balkhash areas, Aktyubinsk, Atyrau, Kyzylorda districts	Arid, saline, and desert zones	Camel, great gerbil, rodents, fleas	102, 277, 279
Mongolia	West of the country	High elevation > 650 m, desert and arid zones	Pallas's pika, Daurian pika, Siberian marmot, gray marmot, long-tailed suslik, Redcheeked suslik, Russian hamster, silver mountain vole, flat-headed vole, narrow-headed vole, Brandt's vole, Mongolian gerbil, midday gerbil, great gerbil, Siberian five-toed jerboa, corsac fox, Siberian polecat, mountain weasel, northern wheatear, fleas	64, 65
Vietnam	Central highlands region	High elevation zones > 850 m, plague risk increases during the dry season	*Rattus rattus*, Pacific rat, *Rattus norvegicus*, Asian house shrew, tree shrew, fleas	75
Algeria	Oran, Mascara, Laghouat areas	Arid and saline zones (Chott)	Camel, *Rattus norvegicus*, *Rattus rattus*, *Meriones shawii*, *Psammomys obsesus*, *Mus spretus*, *Apodemus sylvaticus*, *Cricidura russula*, fleas	62, 77, 268, 269
India	Andhra Pradesh, Karnataka, Tamil Nadu areas	Elevation > 300 m, tropical zones	*Rattus rattus*, *Mus musculus*, Indian gerbil, lesser bandicoot rat, fleas	99, 105
Libya	Nofilia area		Camel, rodents, fleas	63
Indonesia	Central and West Java regions	High elevation > 1,000 m, humid zones	*Rattus rattus*, fleas	436
Bolivia	Franz Tomayo, Andres Ibanez provinces	Tropical and subtropical dry zones, plague risk increases with an elevation of >1,300 m	*Akodon*, *Rattus rattus*, fleas	45
Russia	Mountain-Altai, Tuva (Mongun-Taigin) areas	High elevation, arid and desert zones	Camel, marmot, rodents, fleas	67, 68, 96, 154, 278, 280, 281, 283
Kyrgyzstan	Tien Shan, Alai, Talas mountains	High elevation > 2,700 m, continental climate	Marmot, mountain vole, rodents, fleas	66, 283, 284

[a]Including the locations of plague foci, characteristics of plague foci, and plague sources based on data published in the peer-reviewed literature.

19 years, corresponding mainly to sporadic cases in the United States and clustered cases in South America (9, 40–45). Africa has experienced the largest number of plague cases worldwide (97%) (46–56), including 25,409 cases reported mainly by the Democratic Republic of Congo and Madagascar. In Madagascar, plague foci are located in the center and north of the island, especially in the Ambalavao and Tsiroanomandidy districts. In endemic highland foci, rats were found to be 1,000 times more resistant to plague than rats collected along the coast (57). *Xenopsylla* fleas feeding on these rats

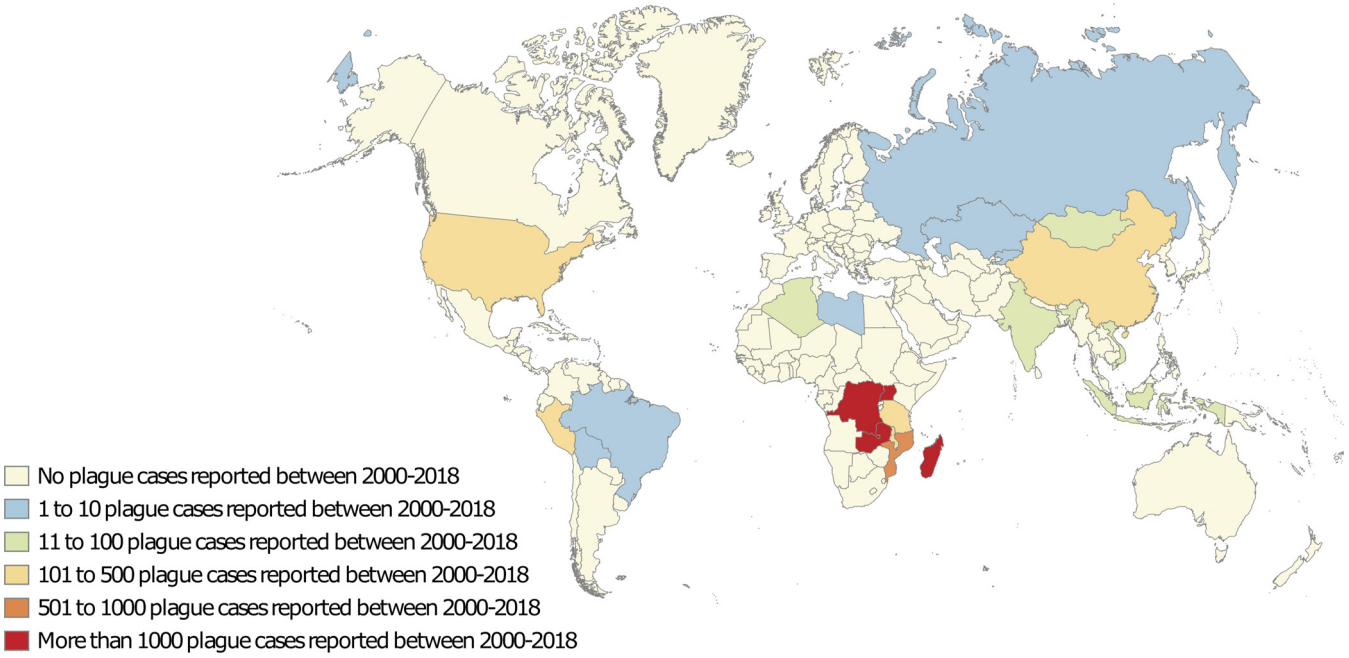

FIG 1 World map of plague cases reported to the World Health Organization in the 21st century (39).

and spreading plague among susceptible rodents are considered essential key factors responsible for dissemination of plague in Madagascar (58). Accordingly, most cases are bubonic (transmitted by fleas), with possible secondary pneumonic forms (58–61). In 2017, a large outbreak accounted for approximately 2,400 cases, mostly of the pneumonic plague form (78%); however, only 32 cases were microbiologically confirmed (59). In northern Africa, Algeria reported 15 cases in 2003 (62), and 13 cases were

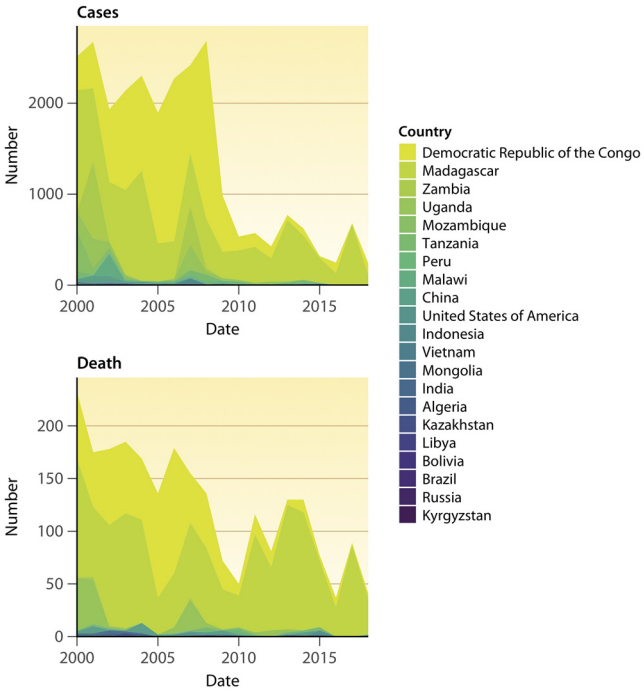

FIG 2 All plague cases and plague deaths by year and country reported by the World Health Organization over the last 19 years (39).

reported in Libya near Tobruk in 2009 (63). Molecular analyses revealed two different strains, *Y. pestis* IP1860-64 bv. Orientalis in Algeria and *Y. pestis* IP1973-75 bv. Medievalis in Libya, suggesting that several independent plague foci exist in these two countries (63). In Mongolia in 2010, most recorded bubonic plague cases were traced to marmots (75.2%) (64, 65), similar to the situation in Kyrgyzstan and Russia, where two cases were contracted by marmot hunters (37, 66–68). These data indicate the diversity of current plague sources, including mammals such as dogs, cats, camels, and rodents and one species of bird (65). Some characteristics common to most plague foci include (i) locations in low-density rural zones (this characteristic may explain the low number of cases, because contacts between human populations and vectors are infrequent) (6), (ii) higher altitudes relative to the rest of the country (36, 40, 48, 65, 69–75), (iii) locations in arid or semiarid zones (low precipitation) (6), (iv) locations in or around saline soil (76, 77), (v) the presence of at least two hosts/vectors and one species of flea, and (vi) enzootic epidemiology seemingly regulated by subtle interplay between resistant hosts (arising from pressure selection) and susceptible hosts.

Natural Sources of *Y. pestis*

***Y. pestis* in soil.** The hypothesis that soil might be a source of plague was proposed prior to the 1894 discovery of *Y. pestis*. In 1882, in a report to the British authorities, General Osbert Chadwik raised concerns regarding the wastewater disposal systems in the Taipingshan District of Hong Kong, leading to the idea that "the soil of Taipingshan was typically soaked with sewage discharged from dysfunctional drains and through the broken floors of the buildings above" (78). Equally widespread was the fear that the soil under inappropriately constructed houses in Taipingshan might become a receptacle of plague-infected bacteria, objects, or infected bodies falling through the porous nonintact floors of homes. An outbreak in Hong Kong in 1894 subsequently confirmed these fears, and the health commission proposed the burning of infected houses to "purify" the soil, which was believed to be the source of the infection (79). In an 1894 publication, Alexandre Yersin wrote that he had isolated one attenuated *Y. pestis* strain from the soil 4 to 5 cm below the surface of one house that was the home of plague victims in Hong Kong (80), and Kitasato reported the isolation of plague bacilli from "soil dust." Alexandre Yersin verified his observations by collecting soil specimens from other houses where plague victims lived and from negative-control houses; he isolated the plague agent from 4 of 10 test houses and from none of the negative controls. Alexandre Yersin further confirmed his observations in Canton, where in contrast to Hong Kong, houses had not been disinfected. He found plague bacilli 20 to 30 cm below the surface but not at a depth of 1 m (79). At this time, the plague was considered by plague experts to be caused by a telluric bacterium from soil contaminated by feces from rats or other infected animals; even the direct inhalation of *Y. pestis*-contaminated soil was reported to cause primary pulmonary plague (81, 82). These preliminary data were not confirmed until 70 years later, when Karimi reported the isolation of *Y. pestis* from a burrow where plague-infected mammals had died approximately 11 months previously in Iran (83). More recently, *Y. pestis* was isolated under natural conditions in Arizona from soil under the corpse of a mountain lion 3 weeks after the death of the animal (84). These natural observations were completed by isolating the Algeria3 strain (biotype Orientalis) from saline soil (40 g/liter NaCl) collected at the edge of a saltwater lake in Algeria (77). It was recently found that such observations could be extended to the United States, where the location of significant plague foci is correlated with the aridity and salinity of the soil (76). These field observations were correlated with experimental observations recorded during the 16-month persistence of *Y. pestis* in soil by Mollaret (85). These experimental observations have been refined and have demonstrated the persistence of a virulent *Y. pestis* Orientalis biovar in soil for up to 280 days (86). The persistence of plague in soil contrasts with the limited survival of *Y. pestis* on steel or glass, where it survives for less than 72 h (87). Altogether, at least four *Y. pestis* strains have been isolated from soil (77, 80, 83, 84). Only the *Y. pestis* strain isolated in Hong Kong appears to be attenuated, as

reported by Alexandre Yersin, which is potentially explained by the so-called dormancy phenomenon (80). Indeed, *Y. pestis* has the capacity to pass from a coccobacillus to an L-form in response to environmental stresses, such as extreme salinity, low temperatures, or the conditions of the flea host (77, 88, 89). The L-form might be a persistent form of *Y. pestis* outside mammalian hosts under extreme environmental conditions, as suggested by the conservation of the *pst* and *pim* genes despite their lack of function during mammalian infection (90). Moreover, under nonextreme conditions, *Y. pestis* remains alive and virulent in soil for long periods of time (85, 86) and in soil amoeba trophozoites, which may serve as temporary reservoirs (91, 92).

(i) *Y. pestis* and protozoa. In the environment, *Y. pestis* may reside in protozoa, including amoebae (93). In 1999, Domaradsky hypothesized that the plague was a protonosis of the soil living in protozoan vegetative and cyst forms (94). Based on studies of soil ecology in Russia, it was suspected that *Y. pestis* could engage in intracellular interactions with protozoan cysts and blue-green algae (formerly cyanobacteria), enabling long-term preservation in the soil (95). Accordingly, several amoebae, belonging mainly to the *Acanthamoeba* genus (including *A. castellani*), were isolated from the soil in a natural plague focus in the Caspian region at a concentration of 300,000 cells/g of soil (96). Further experimental studies involving the coculture of soil free-living amoebae and *Y. pestis* demonstrated the survival and replication of *Y. pestis* for more than 48 h in *Dictyostelium discoideum* trophozoites (92) and for a minimum of 5 days after phagocytosis by *A. castellani* trophozoites (91). Similar to macrophages, amoeba infection is dependent on *Y. pestis phoP*, which encodes a transcriptional regulator (17, 97), and the *Y. pestis* type III secretion system (T3SS) (98). Similarly, protozoa present in rodent and lagomorph digestive tracts might host *Y. pestis* and act as temporary reservoirs (94).

***Y. pestis* in animals.** More than 200 species of mammals (rodents principally) can be infected with *Y. pestis*, and plague-resistant species are regarded as a source of *Y. pestis* (8, 9, 93, 99). However, not all mammalian species are plague susceptible, and species such as grasshopper mice (100), marmots (101), great gerbils (102), four-striped mice (103), deer mice (104), rats (105), California voles (106), kangaroo rats (93), and dogs (107–109) are suspected to be selectively plague resistant, resulting from selection pressure during relatively long periods of coexistence with *Y. pestis* (105). Although Gunnison's and black-tailed prairie dogs are highly plague susceptible (showing almost 100% mortality) (93), some research hypotheses suggest that a few resistant animals might exist among these populations (69, 110). The subtle balance between plague-susceptible and plague-resistant species has been advocated for use in the modeling of plague epizootics (93). Nevertheless, resistant hosts appear to harbor very low quantities of bacteria in their blood, too low to infect fleas, for which the minimum required concentration is 10 million CFU/ml (111). Carnivores can be infected after the ingestion of contaminated rodents (100). Additionally, camels in the Maghreb, the Middle East, and Asia are highly susceptible to plague following contact with dead rodent-contaminated carcasses or excrement (6, 112). Camels were reported to develop plague after experimental subcutaneous inoculation with *Y. pestis* (2/4 died) and *Y. pestis* inhalation (6/6 died, with primary pneumonic symptoms), whereas fodder ingestion induced bubonic plague (3/3 recovered after exhibiting bubonic plague symptoms with submaxillary buboes) (113). A second set of experiments with experimental parameters closer to natural infection conditions was performed in 1954 to 1956. In total, 28 Bactrian camels were infected by natural camel ectoparasites, such as blocked *Xenopsylla* and *Coptopsylla* flea species and ticks (*Hyalomma asiaticum* and *Ornithodoros tartakovskyi*) that had previously fed on infected guinea pigs. The blocked fleas successfully transmitted plague to the eight camels they bit. The ticks transmitted plague to the camels only in rare cases; when they did so, it was within 1 to 2 days after infection, and the authors suggested mechanical transmission via the infection of their buccal parts (112). These experiments indicated that the susceptibility of camels to plague varied based on the route of contamination. Concerning domesticated animals, canids are relatively resistant to plague (9). Indeed, among 10 dogs experimentally

infected with the *Y. pestis* 195/P strain via oral and parenteral routes, all 10 showed signs of infection; however, none of them died from plague, and after 7 days, the dogs recovered. All the dogs developed antiplague antibodies, which were present up to 300 days postinfection (107). Accordingly, it was recently demonstrated that the FRP1 receptor in mammals promotes the translocation of bacterial effectors; the absence of this receptor in canids confers enhanced resistance to plague (109). Nevertheless, dogs can present primary pneumonic plague and act as a source of contamination in humans (41). Additionally, domesticated cats can be infected following the hunting and consumption of infected wild prey, such as rodents, or by flea bites. Wild animals, livestock, and domesticated animals are therefore sources of plague for other animals and humans living in contact with them.

Plague Transmission

Transmission of *Y. pestis* to animal populations. *Y. pestis*-contaminated soil can be a source of infection in mammals. This finding was demonstrated in a series of experiments performed by H. Mollaret et al., who exposed various species of *Meriones* to soil contaminated with *Y. pestis* (114). These experiments led to the conclusions that humid soil is a better contaminant than dry soil, that burrowing *Meriones* species are more likely to die from plague than nonburrowing *Meriones* species, and that some burrowing *Meriones* species are more susceptible to plague than others. However, it was not possible to determine the exact route of contamination (i.e., whether *Meriones* individuals inhaled or ingested contaminated soil or both) when a hemorrhagic digestive tract and hemorrhagic pneumonia were present (114). Further experiments performed 50 years later involve susceptible Swiss-Webster mice in which a scarified paw was left in contact with soil that had been contaminated for 10 days with blood containing *Y. pestis* ($>10^8$ CFU/ml). In this experiment, only 1 of 104 animals became infected, and none of the other mice seroconverted, suggesting that this route of contamination is unlikely to sustain epizootics (115). Beyond studies involving soil, the potential of plants to serve as a plague source in animals has been poorly investigated, except in the Russian literature (95, 116, 117). The colonization of *Impatiens walleriana* plants by the *Y. pestis* EV strain was observed after scarifying and immersing stems of this plant in an infected solution (117). This observation was met with skepticism and was poorly cited. Field observations incorporating a large number of plants, including negative-control plants, should clearly be performed to determine precisely whether plants play any role in the epidemiology of plague. Similarly, the ability of fleas to become infected through contact with soil remains questionable: one study showed greater vector efficiency in mice (50% infection against 23% in the control group) when *Oropsylla montana* was in contact with soil infected with wild flea feces (118). In parallel, it was shown that *Y. pestis*-contaminated flea feces could contaminate soil and burrows (89, 90, 118–120). The inhalation or ingestion of contaminated soil material associated with burrowing activities remains to be investigated in the field using appropriate experiments. Indeed, very few experiments have been aimed at observing the contamination of mammals via the oral route. Paul-Louis Simond failed to orally infect rats, monkeys, and squirrels by feeding them an infected culture or infected spleen, liver, blood, feces, and urine samples (27). Furthermore, tests in the rodents *Mus musculus*, *Zygodontomys pixuna*, and *R. rattus* in which doses of 5.46 to 9.62 $\log_{10}$ viable *Y. pestis* bacteria were administered intragastrically yielded 100% mortality, similar to the observations made following the addition of 10^8 *Y. pestis* bacteria/ml of drinking water to infect mice, which resulted in death within 3 days. Notably, *Y. pestis* was not found in any rodent fecal samples, which is related to the fact that the *Y. pestis* strain used for challenge does not survive at pH less than 3 (121). A confirmatory study showed that 3/20 *Onychomys leucogaster* individuals from a parental population exposed to plague and 7/20 *O. leucogaster* individuals from a plague-naive parental population died after they were fed plague-infected mice, whereas 4/20 and 13/20 individuals of these species, respectively, survived and developed positive serology (100). These laboratory

experiments demonstrate the capacity of rodents to be infected via the oral route either by eating contaminated food or by burrowing into contaminated soil.

(i) Flea-borne transmission in animal populations. Interanimal transmission can be achieved by animal ectoparasites, and approximately 80 flea species are listed as common vectors of *Y. pestis* among rodents (12, 122, 123). In 1897, one of Kitasato's mentors, Masanori Ogata, suspected fleas of playing a role in the transmission of plague (124) and successfully infected mice with a suspension of ground fleas that had fed on an infected rat (93). Simultaneously, Paul-Louis Simond et al. discovered plague bacilli in rat flea intestines in Cutch-Mandvi, India (125). Shortly thereafter, Simond caught an infected rat covered with fleas in the home of a plague victim in Karachi and harvested fleas from a stray cat lurking near his hotel. The experiment consisted of trapping the infected rat in a glass bottle, preventing it from moving, and placing the cat's fleas on the dying rat. Finally, a naive plague rat was placed in a suspended cage close to the dying rat. Simond found that the healthy rat died 5 days after the death of the infected rat without experiencing any direct contact with the infected rat. Paul-Louis Simond concluded that plague transmission from rat to rat, rat to human, and human to human could be mediated by fleas of any kind and that flea feces are contagious when used to inoculate rats (27). Currently, two major routes of plague transmission by fleas have been identified. First, early-phase or mass transmission was discovered between 1904 and 1947 (119). It has been shown that from 3 h to 7 days after infection and before blocking, the flea has the ability to infect a healthy animal during its next blood meal (104, 126). In this model, the fleas immediately become infectious (104) (*Y. pestis* can survive for only 3 h on a flea's mouthparts) (127) after their first plague blood meal because of the partial, early, ephemeral blockage of the proventriculus. Indeed, the ingested bacteria accumulate and form a small conglomerate in the proventriculus during a blood meal, and only a blood pulse clears this conglomerate and provokes blood reflux, carrying a few bacteria to the biting point (119). The efficiency of transmission varies greatly among flea species; for example, a transmission efficiency (TE) of 6.4% has been reported for *X. cheopis* (128), 17.88% for *Oropsylla tuberculata cynomuris*, 4.54% for *Oropsylla hirsuta* (129), and 7.7% to 10% for *Oropsylla montana* (130). *Nosopsyllus fasciatus* (0.213 ± 0.157 expected transmission per flea [ETF]) and *Orchopeas sexdentus sexdentus* (0.170 ± 0.138 ETF) (126) are quite effective vectors, while *Aetheca wagneri* (TE = 1.03%), *Ctenocephalides felis* (TE = 0.57%) (104, 131), *Opisodasys nesiotus*, *Megabothris abantis*, *Malaraeus telchinum*, and *Diamanus montanus* (126) appear to be very inefficient vectors. Two major limitations of early-phase/mass transmission implications were proposed by Hinnebusch et al. (119): first, an inoculum concentration higher than 10^8 bacteria/ml, which is required for effective transmission, is naturally achieved in mice but not in most other mammals except very shortly before death. Second, the number of bacteria transmitted individually by each flea is extremely low (less than 10 individual bacteria are sufficient to infect susceptible mammals) (132), and early-phase transmission (EPT) is effective only in a host that is highly sensitive to and has been bitten by a large number of fleas (133). EPT is ineffective for *X. cheopis* at low temperatures of $\sim$10°C (134). It is very difficult to draw conclusions from these studies, because the conditions under which the experiments were conducted differ greatly, leading to heterogeneous results. Indeed, the strains, the bacterial concentrations, the incubation times, and the hosts of the fleas differ. Sometimes, fleas can be blocked rapidly, and EPT can be confused with later-stage transmission. The results are also host dependent, as shown in a study conducted by Bland et al., who observed that 10% to 28% of fleas that fed on bacteremic rats or guinea pig blood showed the reflux of bacteria into their esophagus within the first 24 h postinfection and exhibited an increased vectorial capacity during EPT (135). Therefore, new experiments using standardized methods (as described above) to evaluate and compare transmission efficiencies are needed to understand the relative importance of EPT. The following second mechanism, referred to as "biofilm-dependent transmission," was described in articles in 1914 and 1915 by Bacot and Martin demonstrating the vectorial capacity of blocking *X. cheopis* and *Ceratophyllus fasciatus* in rats (136, 137): fleas can

develop a bacterial biofilm in the proventricular valve of the midgut from 1 to 3 days after ingestion (119, 126, 138). The biofilm partially or completely blocks the midgut such that the fleas can no longer feed and ultimately starve. The behavior of the fleas changes drastically; during the last days of their life, they relentlessly attempt to feed, considerably increasing the number of bites they inflict and the opportunities for *Y. pestis* transmission. Once blocked, aspirated blood comes into contact with the bacterial biofilm, mixing *Y. pestis* into noninfected blood. The fleas do not swallow the blood and thus release *Y. pestis*-infected blood at the biting point, sometimes infecting the host. The vectorial capacity of blocked fleas is much higher than that experienced during EPT. For example, blocked *X. cheopis* bites result in 25 to 50% transmission compared with the 0 to 10% probability of transmission during EPT. Flea proventriculus blockade allowing *Y. pestis* transmission is believed to involve approximately 25 genes (119). The factors that reportedly influence blocking and transmission efficiency include low temperature (139–142), seasonality (143, 144), the flea species involved and their proventricular morphology (93), and feeding frequency (131). These data indicate that the blocking mechanism is more efficient than EPT for the transmission of *Y. pestis* and probably contributes to the cycle of plague transmission (119). The interzoonotic persistence of *Y. pestis* may rely on the tissue sequestration of *Y. pestis* in plague-resistant hosts (depending on the plague focus, geography, and climate) (8, 145); the subtle balance among host susceptibility, bacteremia, and fleas (93, 104, 146); and the long-term carriage of *Y. pestis* by unblocked fleas during the hibernation or renewal of their hosts (depending on the flea stage [147], temperature [139–142], and sex [148] and the host population affecting the dynamics of the plague) (130). Interplay among all of these factors could occur in a particular space and season.

Transmission of *Y. pestis* to human populations. Plague is a zoonotic infection that can be contracted through direct contact with animals, including contact with animal carcasses, animal bites, and the consumption of animal meat and its derivatives, and through indirect contact, mainly mediated by animal ectoparasite contact (9, 40, 41, 99). Plague transmission has been reported after handling the carcasses of mountain lions (84, 149), wild coyotes (150), camels (151–153), rats (27), goats (153), marmots (154), Tibetan sheep (155), guinea pigs (156), rabbits (157), and dogs (158). In some cases, carcass manipulation during necropsy may lead to primary pneumonic infection through the inhalation of infectious aerosol droplets (149, 155), to primary bubonic plague following presumed passage through any skin breach caused by butchering carcass (47, 150–153, 155–157), or to intestinal plague (155). However, the precise mechanisms and exact type of skin breach leading to *Y. pestis* transmission have not been clearly explored, and whether all *Y. pestis* strains are transmissible through any type of skin breach remains unknown. Animal carcass handling results primarily in bubonic plague, characterized by enlarged lymph nodes around the area of the lymphatic drainage of the portal of entry and local skin lesions, which have been poorly described under these conditions (47, 150–153, 155–157). In the Chinese plague focus on the Qinghai-Tibet Plateau, plague cases were traced to the slaughtering or skinning of diseased Tibetan sheep and exhibited a high mortality rate of 60%. Genome sequence data indicated an epidemiological chain from the local marmot *Marmota himalayana* to sheep and, then, to humans. Interanimal transmission from marmots to sheep was hypothesized to take place through marmot ectoparasites (155). In Tibetan sheep, seropositivity for the F1 antigen was detected in 5/7 provinces on the Qinghai-Tibet Plateau, with a prevalence ranging from 0.33% to 5.2% (159). Dogs and cats are occasional sources of transmission of pneumonic, bubonic, and septicemic plague to their owners after the ingestion of *Y. pestis*-infected marmots (160), the sniffing of a dead prairie dog (161), contact with dead chipmunks, squirrels, wood rats, and their fleas (41, 162–164), or contact with unknown environmental sources (158, 165, 166). Animal bite-transmitted plague has been reported in Gunnison's prairie dog in New Mexico (167). The consumption of *Y. pestis*-contaminated animal meat and products is a reemerging form of zoonotic plague transmission. *Y. pestis*-contaminated products may include urine, which is recommended as a remedy in the Islamic world (168), dairy

products, meat, and liver from goats, dromedaries, sheep, and guinea pigs (151–153, 155, 156). Liver and meat cooked at temperatures of ≤68.3°C might represent a risk for *Y. pestis* transmission, as it has been experimentally documented that *Y. pestis* is inactive above this temperature in meat (169). The consumption of uncooked or insufficiently cooked *Y. pestis*-contaminated food may result in a rare form of pharyngeal and meningeal plague, as reported in Maghreb and Asian areas (Table 1) (151–153). The classic pattern of transmission of *Y. pestis* from rodent populations, such as rat populations, to humans through ectoparasites, such as rat fleas, was established as dogma after the work of Paul-Louis Simond in 1898 in India (125) during the third pandemic wave. The zoonotic transmission of plague from wild animals and fleas leads to sporadic cases and limited outbreaks of plague; it may not by itself explain large epidemics. Furthermore, this transmission route has not resumed during the current epidemiology of the plague observed in the United States and Maghreb (Table 1). The current epidemiological cycle of plague most often involves an animal reservoir (rodents, mainly *R. rattus*) and a person infected by the inoculation of the bacterium via the bite of a flea that previously fed on an infected animal. This model, which excludes all human-to-human transmission (Fig. 3 and 4) (and thus implies a relatively slow spread of the disease), appears to be incompatible with the high rate of the territorial expansion of the Black Death recovered from historical sources (on the order of 1.5 to 6 km/day) (170). The spread of this epidemic to northern Europe, where the black rat was absent in the Middle Ages, also undermines this model (171–176). Finally, while it is agreed that the "eastern" rat flea (*X. cheopis*) has been the main vector of plague epidemics since the end of the 19th century, its role in spreading the Black Death is currently disputed. Because this species is of tropical origin, it would be difficult for it to acclimatize to the European climate, in accord with the absence of fossil discoveries of *X. cheopis* in Europe, despite its highly resistant exoskeleton, whereas remains of *Pulex irritans* have been discovered at these latitudes (177).

(i) Aerosol transmission. Once *Y. pestis* has been introduced into a human population, it can be transmitted from one pneumonic plague patient to other individuals via droplet transmission. The Manchurian plague episode of 1910 to 1911 is the classic example cited to illustrate the interhuman droplet transmission of *Y. pestis* (178–180). The major limitation of the interpretation of more recent episodes attributed to pneumonic plague is the lack of the appropriate documentation of patients. For example, the 1924-1925 Los Angeles plague outbreak (8, 181) was thought to be pneumonic and highly contagious, but quarantine measures were ineffective because the majority of the patients diagnosed suffered from a secondary pulmonary form consecutive to a bubonic form caused by rodents and wild fleas (182). The recent investigation of a pneumonic plague outbreak in Madagascar led to the isolation of *Y. pestis* from two patients and the seroconversion of two additional patients, resulting in four microbiologically diagnosed cases from 14 suspected cases (28.7%) (183). Therefore, there has been an overestimation of the droplet transmissibility of pneumonic plague, which seems to be an effective route of transmission only in the final stage of the disease, when the patient can no longer move, and only through very close contact (≤1 m; based on experimental data) (182) for a prolonged period of time (182, 184). In Uganda, a precise investigation indicated that two index patients transmitted *Y. pestis* to only one caregiver each and not to 23 additional untreated close contacts (184). In China, an investigation indicated that three index patients exposed 214 contacts over a period of 3 to 13 days. All contacts were quarantined, and no secondary cases were reported (166). Furthermore, it was estimated from eight documented pneumonic outbreaks that a pneumonic plague patient can infect an average of 1.3 other persons (185). The careful analysis of documented pneumonic plague clusters indicates that the transmission of *Y. pestis* via respiratory droplets requires face-to-face exposure to a coughing patient, as can occur during funerals via close contact with coughing people who may have been exposed to the pathogen while visiting or attending plague victims before they died (58). The droplet transmission of plague by pneumonic patients remains very difficult to evaluate, but field observations and mathematical

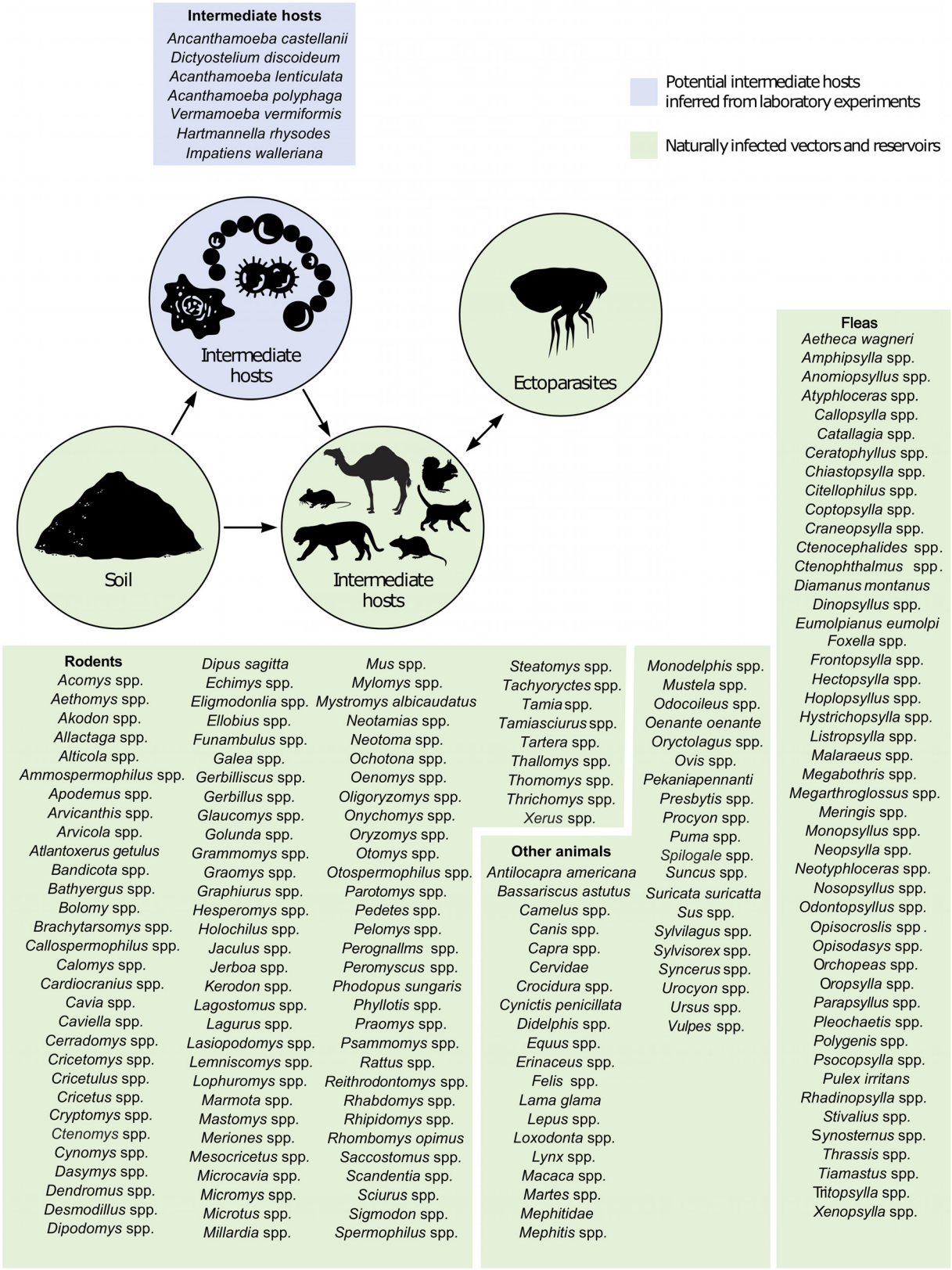

FIG 3 Scheme of the natural epidemiology of plague representing all documented natural and animal reservoirs, intermediate reservoirs, sources of infection, and vectors for humans described in the literature. Green represents field isolation and laboratory-confirmed sources. Blue represents potential sources/reservoirs inferred from laboratory experiments.

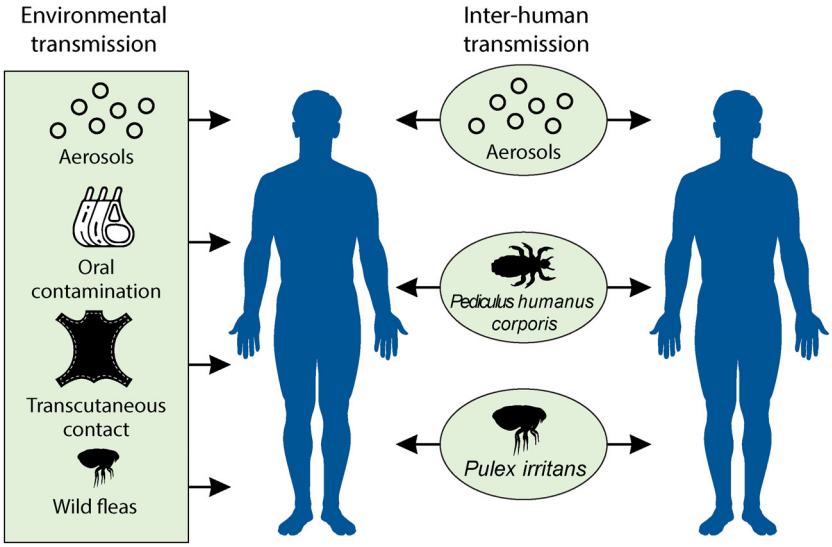

FIG 4 Different routes of interhuman transmission and human infection from plague sources (as described in Fig. 3). Green represents field observations of confirmed plague sources, such as aerosol transmission, the consumption of raw or poorly cooked meat, transcutaneous contamination by carcass skinning, and nonhuman flea bites. Once humans are infected, effective interhuman transmission can occur through aerosols (in the case of pulmonary plague) and human ectoparasites, such as body and head lice and human fleas (*P. irritans*).

models indicate that the rate of transmission is very low and cannot sustain a large epidemic among the human population (186).

(ii) Human ectoparasites. *(a) Human lice.* In 1909, during an outbreak of epidemic typhus in Tunis, Charles Nicolle and his team discovered a role of lice in the spread and transmission of the disease. Through his observations, he also demonstrated that the clothes of patients suffering from typhus could infect other people through aerosol transmissions (187, 188). This observation made 110 years ago remains crucial to this day for understanding the vector role of lice. The human transmission of *Y. pestis* by lice remains a controversial issue, even though several lines of evidence support this route of interhuman transmission of *Y. pestis* by the human louse *Pediculus humanus corporis* (186, 189). This hypothesis is supported by the paleomicrobiological observation in second-pandemic plague victims of coinfection with *Y. pestis* and *Bartonella quintana*, whose main vector is human body lice (190, 191), and the recent observation of *Y. pestis* in body lice collected from people living in areas of plague foci in the Congo (192); additionally, the codetection of *B. quintana* and *Y. pestis* in head and body lice (193) and experimental data that clearly demonstrate the capacity of body lice to transmit *Y. pestis* have been reported (194–198). Such transmission of *Y. pestis* was observed by G. Blanc and M. Baltazard in a cluster of bubonic plague in households in Morocco during World War II (195). These authors demonstrated that a body louse could be infected when feeding on a septicemic patient and then remain alive for 7 days while producing infectious feces and could, thus, transmit plague. Spontaneous infection was also demonstrated in *Pediculus humanus capitis* in 1903 by Herzog and in 1916 by de Raadt (194). When there is a plague outbreak involving an infested louse population, plague-infected lice have always been identified in epidemic situations when they are looked for. All reported evidence indicates that the louse is a unique vector that can be infected by deadly pathogens, including *Rickettsia prowazekii*, *B. quintana*, and *Borrelia recurrentis*. The only conditions required for lice to transmit any pathogen, including *Y. pestis*, are (i) that the pathogen ingested with blood remains viable in the digestive tract and the feces and (ii) that repeated louse bites provoke a local allergic response, inducing itching. The skin lesions that develop following self-scratching allow the penetration of pathogens present in the feces into the broken skin (Fig. 5A) (199).

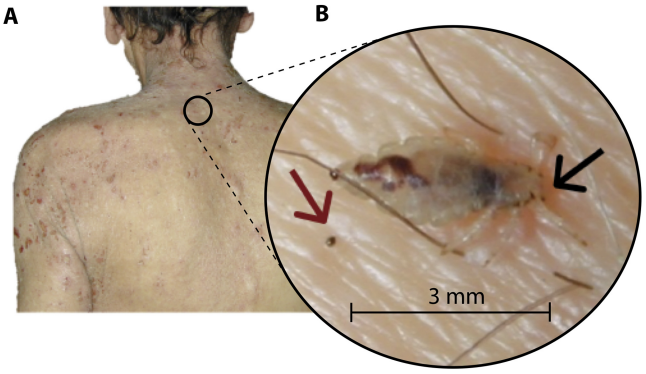

FIG 5 Example of pediculosis, showing a body louse defecating on skin while feeding. (A) Photograph of a severe pediculosis affecting the back, arms, and neck found on a homeless person in Marseille. (B) Photograph of a *Pediculus humanus corporis* collected from a homeless person's skin in Marseille. In this photograph, we see a body louse defecating (body louse feces are indicated by a red arrow) while taking a blood meal (the biting point is indicated by a black arrow). The body louse feces are deposited approximately 3 mm from the biting point. This proximity greatly increases the chance of the penetration of feces inside the broken skin (biting point) during scratching. (Both photos courtesy of Philippe Brouqui, reproduced with permission.)

Indeed, the louse often defecates while feeding on the skin, and the distance between the bite point and the infected feces that are deposited is $<$4 mm (Fig. 5B). As seen in Fig. 5A, it is clear that such skin lesions in heavily louse-infected patients make it possible for them to be infected by pathogens. In fact, similar to all other louse-transmitted diseases, *Y. pestis* is introduced by autoinoculation by scratching the skin where the lice defecate. In contrast to the situation in fleas, mosquitoes, or ticks, the vectorial capacity of lice does not depend on a complex cycle shaped by genetic markers leading to the inoculation of the bacteria through saliva during blood meals. In lice, viable bacteria swallowed with the blood will remain intact and infectious in louse feces. Based on these data, human lice could be an effective vector of plague because of their continuous presence on the human body or clothes (200). This hypothesis is supported by an animal model demonstrating the transmissibility of the bacillus by body lice on rabbits (196, 198), the observation of small family plague outbreaks related to body lice in Morocco in the 1940s (195), and the current detection of *Y. pestis* in head and body lice in the Congo (192, 193). However, the presence of *Y. pestis* in ancient human ectoparasites has never been tested. Nevertheless, a recent model of second-pandemic mortality in nine European localities indicated that the human ectoparasite model (including *P. humanus* and *P. irritans*) fit the historical data better than the rat ectoparasite model and the pneumonic plague model at seven of the nine localities (186). Indeed, massive plague outbreaks (25 to 50% of the human population affected) can occur in the event of massive infestation of humans by body lice causing widespread pediculosis, increasing the chance of *Y. pestis* entry into the blood (Fig. 5).

(b) Human fleas. *P. irritans*, commonly known as the "human flea," has been present in Eurasian human populations since the fourth millennium BC and has been recorded at more than 220 archeological sites dating from the Neolithic to the postmedieval period (177). Since the discovery of the rat flea (*R. rattus-X. cheopis*) model in 1898 by Paul-Louis Simond (27), scientists have hypothesized that plague is always transmitted from rodents to humans. Therefore, in the early 20th century, studies focused mainly on rodent ectoparasites, and research on the role of (nearly) strictly human fleas (9) in the spread of plague, have long been neglected (133). However, the Indian commission reported the collection of 85 *P. irritans* specimens in the houses of plague victims and the identification of only one infected flea (133). In 1904, Verjbitski succeeded in infecting *P. irritans* and showed that a batch of 10 fleas could transmit plague to a rat (194). During the Moroccan epidemic of Aït Imour in 1940, the French scientists Blanc and Baltazard (437) studied the role of *P. irritans* (after having demonstrated the

transmission ability of body lice) to explain part of the family plague cluster that they observed in this area (in 1932, a French scientist noted that the number of human fleas in Moroccan dwellings was extremely high, suggesting that fleas might have played a role in the interhuman transmission of plague [201]). Their results showed the presence of *P. irritans* in the houses of plague victims during the epidemic episode. This observation was later confirmed by M. Baltazard in Turkey, Iraq, Syria, Iran, and Kurdistan, where rats were not found, while *P. irritans* was found in the houses and tents of nomads, and *Pediculus humanus corporis* was found in clothes (197). Experiments conducted in 1940 with the fleas harvested in Moroccan houses showed the ability of *P. irritans* to conserve the plague bacillus in its body for at least 21 days (after feeding on last-stage septicemic plague victims) and the contamination of flea feces with virulent *Y. pestis* for at least 5 days under natural conditions. Concerning the transmission of plague by flea bites, three guinea pigs bitten twice daily by 600 *P. irritans* fleas developed typical plague lesions, such as carbuncles and buboes, and died from plague (133). Nevertheless, the *P. irritans* blocking capacity was incredibly low (119), and its ability to transmit plague via EPT is almost nonexistent. Indeed, only 3 of 38 EPT experiments involving 20 fleas led to a host infection (119); however, as demonstrated by Blanc and Baltazard, the fleas can transmit plague via EPT under extreme conditions (600 fleas fed twice per day on a single guinea pig). However, *P. irritans* was found to be spontaneously infected during plague outbreaks (93, 126, 133, 202). Although the individual flea transmission rate remained low throughout EPT (0 to 10%), the percentage of rodents infected (rats, guinea pigs, and squirrels) by a group of 10 to 100 fleas ranged between 10 and 100% (135). While some authors argue that early-phase/mass transmission might explain the rapid spread and great mortality during epizootic episodes in populations of species such as prairie dogs (93, 130, 203), other scholars emphasize that given the mass transmission ability of *P. irritans* and the alleged absence of *X. cheopis*, *P. irritans* might have been among the most effective interhuman vectors during the Black Death (130, 204, 205). Accordingly, a debated epidemiological study suggested that *P. irritans* and *P. humanus corporis* may mediate interhuman transmission, explaining the ancient outbreak dynamics to some extent (194, 206). Although the vectorial capacity of *P. irritans* associated with biting seems to be extremely low, human infection may follow the introduction of infected feces at skin breaches, as reported for lice (196). Indeed, it has been shown that *P. irritans* digests its blood meal rapidly and defecates large amounts of feces containing virulent *Y. pestis* shortly after feeding to clear itself of infection (119). Human ectoparasite transmission via infected feces is currently the most parsimonious hypothesis for explaining ancient and modern interhuman transmission of plague.

Clinical Aspects

The clinical characteristics of plague partially depend on the route of contamination. People of all ages and both sexes are susceptible to plague, although plague cases have been reported in children, with a low predominance of males, over the last several decades (108). Genetic susceptibility or resistance to plague remains a controversial issue. Observations have been reported regarding whether the protective role of the CCR5-delta32 mutation, which confers resistance to HIV infection, also confers resistance to plague (207–209). The most recent studies identified the FPR1R190W allele (109) or pyrin variants (210) as possible candidates implicated in human plague resistance. As the flea-borne transmission of *Y. pestis* is its most common route of transmission worldwide, bubonic plague is the most frequent clinical form of plague, developing 2 to 10 days after inoculation with *Y. pestis* (108). Intriguingly for a vector-borne infection, skin lesions at the portal of entry are not well described and are seldom mentioned in recent reports, whereas skin lesions described as carbuncles, which were the cardinal sign of the infection during the second historical pandemic and were sporadically reported at the beginning of the third pandemic, are no longer being reported in the 21st century (27, 205, 211). According to Simond, the carbuncle is a result of necrosis following the development of a phlycten. A phlycten occurs at the

inoculation point of *Y. pestis* via an ectoparasite bite, and the carbuncles reported to occur systematically during the second pandemic represent a bodily indicator of the point of entry of *Y. pestis* (27). Patients suffer from nonspecific signs and symptoms, including chills, fever, myalgias, arthralgias, and weakness (99). Much more evocative of the diagnosis in patients exposed to areas of plague endemicity are enlarged lymph nodes, which are painful, tender, and swollen and are referred to as "buboes," draining the site of inoculation. Femoral (~31%) and inguinal (~24%) nodes are the most frequent, followed in frequency by axillary (~22%) and cervical (~9%) nodes (80, 99). While the development of any lesion at the site of inoculation is rarely reported, a careful examination of the site may reveal a local skin inflammation papule, pustule, scab, or ulcer (9). The buboes resulting from plague are distinguishable from enlarged lymph nodes due to other causes because of their association with systemic signs of toxemia and rapid onset (212). Moreover, plague is a cause of clustered cases of febrile, enlarged lymph nodes that can be confused with tularemia but lead to an unambiguous diagnosis in a deadly epidemic situation. Bubonic plague rapidly responds usually to appropriate antibiotic therapy (reducing mortality from 60% to 5%) (9), while the lymph nodes remain enlarged and tender for 1 week. If not treated with an effective antibiotic, the patient can become increasingly toxemic and develop a septicemic form of the plague. Septicemic plague can be either primary, in the absence of buboes, or secondary to a bubonic form and is characterized by rapidly progressive, overwhelming toxemia. The patient may present gastrointestinal symptoms, including nausea, vomiting, diarrhea, and abdominal pain, confusing the diagnosis. Septicemic plague is diagnosed from a positive blood culture (213) and may evolve to a pneumonic form. In the absence of rapid supportive therapy combined with effective antibiotic treatment, septicemic plague is fulminant and fatal (mortality range of 30 to 100% according to the WHO). Pneumonic plague is the most rapidly fatal form of plague and is characterized by two clinical phenomena: primary pneumonic plague with an incubation period of 2 to 4 days after contact with a coughing patient, and secondary pneumonic plague occurring after the dissemination of *Y. pestis* bacteria to the lungs during an episode of primary bubonic or septicemic plague. In primary pneumonic plague, the onset is sudden, including chills, fever, chest pain, cough, dyspnea, and hemoptysis. Without treatment, the case fatality rate approaches 100% but is between 25 and 50% when appropriate treatment is administered within 24 h after the onset of symptoms (59, 214).

Pharyngitis, gastrointestinal, or tonsillar plague is a rare form of plague characterized by anterior cervical lymphadenitis that is diagnosed in patients who consume raw or poorly cooked contaminated meat, such as camel meat (112, 151–153). It can also occur in persons who catch human ectoparasites such as fleas and lice with their teeth (a common practice among indigenous people in Ecuador) (99, 194) or who acquire infection from patients with pneumonia (206). Meningeal plague is an unusual form of plague that follows insufficiently treated bubonic plague, while pleuritis, endophthalmitis, and myocarditis are exceptional forms of plague (99, 215).

Diagnosis of plague. The laboratory diagnosis of plague remains tedious. Early diagnosis is of major interest to start antibiotic treatment as quickly as possible and prevent severe complications leading to death. Its diagnosis relies on the isolation and culture of *Y. pestis* and the detection of *Y. pestis*-specific biomolecules from clinical samples. Many types of clinical samples can be used for *Y. pestis* diagnosis, including bubo aspirates, respiratory tract samples (i.e., sputum), blood, pharyngeal swabs, and urine. Sample quality is important, and the sampling procedure must be adapted to the suspected clinical form. For example, in the case of suspected pneumonic plague, deep respiratory secretions are required for diagnosis (33) due to tropism toward the lower respiratory tract, and viscous samples should be liquefied and homogenized (216). The gold standard is based on the isolation and detection of *Y. pestis* by culture from clinical samples. Clinical samples could be handled in a biosafety level 2 laboratory, but it is mandatory to perform the isolation, culture, and manipulation of *Y. pestis* in a biosafety level 3 laboratory, in line with national regulations. The easily culturable bacteria can

grow under routine culture in solid or liquid media (brain heart infusion broth, sheep blood agar, or MacConkey agar) after 24 to 72 h of incubation at 28 to 37°C (28°C is the optimum temperature) under aerobic conditions. Selective solid agar medium supplemented with cefsulodin-irgasan-novobiocin (CIN medium) is recommended by the WHO to limit the growth of contaminant bacteria from respiratory tract, pharyngeal, and sputum samples. CIN medium can be improved with the addition of irgasan, cholate salts, crystal violet, and nystatin (BIN medium) for the isolation of *Y. pestis* from complex samples, such as respiratory tract, pharyngeal, or environmental samples (217). Previously commonly used automated identification systems may fail to identify *Y. pestis* colonies (218), and the first-line identification of colonies can be achieved by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (219, 220), PCR, or phage lysis (221). To improve sensitivity, molecular assays have been developed as standard PCR assays, allowing *Y. pestis* DNA detection within 3 to 4 h or in less than 2 h for real-time PCR (33). Four main genes are targeted: the *pla*, *caf1*, *inv*, and *yopM* genes (108). The *pla* gene, encoding a plasminogen activator regarded as a major virulence factor of *Y. pestis* (222, 223), is present at 150 to 200 copies per bacterium (17), resulting in a high sensitivity of detection (100 CFU/ml in sputum) (224). However, the *pla* gene has also been detected in *Citrobacter koseri* (225), *Escherichia coli* (226), and rats (227), thus limiting the specificity of this detection method. The *yopM* gene, located on the *Yersinia* pYV/pCD1 virulence plasmid, which is present in approximately four copies per bacterium (33), shares 99.84% similarity with sequences from *Y. pseudotuberculosis* and 93.91% similarity with sequences from *Y. enterocolitica* strains. The chromosomal *inv* gene is also present in *Y. pseudotuberculosis*, but an insertion has increased the size of this gene in *Y. pestis* (228) (1,100 bp versus 400 bp for *Y. pseudotuberculosis*). The *caf1* gene is considered to be specific to *Y. pestis* and is present in approximately one or two copies per bacterium (17). PCR can be multiplexed; for example, in the case of suspected bubonic plague, a multiplex quantitative PCR (qPCR) assay targeting the *pla* and *caf1* genes has been validated only on bubo aspirates (229). In cases of positive results for *pla* and *caf1*, the presence of *Y. pestis* can be considered to be positively identified (33), but in the case of discordant or uncertain results, it is recommended to perform another multiplex qPCR assay, including the 1,100-bp *inv* gene (33, 230). Moreover, a portable real-time PCR instrument has been developed (231) for the triplex detection of *Y. pestis*, *Bacillus anthracis*, and *Francisella tularensis*. This instrument includes an embedded *Y. pestis* assay based solely on the detection of the *pla* gene but presents the limitations mentioned above. Furthermore, several commercially available assays incorporate *Y. pestis* as a target in a multiplex format in association with *B. anthracis* and *F. tularensis* (232) or target 17 pathogens, including *Y. pestis* (233). Both types of assays are reported to be usable at the point of care. During field trials, plague diagnosis can be achieved in approximately 90 min. Real-time PCR reagents with specific primers and probes targeting the nonspecific *3a* sequence (234) and *caf1* sequence that can be stored at room temperature have been developed to facilitate diagnosis in remote locations (235). Alternatively, the indirect detection of live *Y. pestis* cells can be achieved by the qPCR detection of *Y. pestis*-specific bacteriophages, such as ϕA1122 and L-413C (236). This method has been established with artificial clinical samples and presents a low sensitivity of 10^3 CFU/ml for ϕA1122 and 10^5 CFU/ml for L-413C and a high specificity for L-413C, while ϕA1122 can be detected in some *Y. pseudotuberculosis* strains. The detection of *Y. pestis* DNA can be achieved using the loop-mediated isothermal amplification method, but this method has been validated using only mock-infected animal samples and needs to be approved for sputum samples (237).

The development of rapid diagnostic tests (RDTs) allows F1 antigen detection in 15 min at concentrations as low as 0.5 ng/ml, which is of major interest in a deadly epidemic situation (238). This assay has been validated for the diagnosis of bubonic plague with both a specificity and a sensitivity of 100% in clinical samples and *Y. pestis* strains but needs to be evaluated for pneumonic plague because of false-positive and false-negative results observed in sticky sputum or saliva (33, 238). RDTs are easy to use,

provide rapid results at remote locations, and are 5 to 50 times less expensive than other molecular tests (238). RDTs are available at the Pasteur Institute of Madagascar but have not been commercialized. Hsu et al. developed another specific strip assay that has been favorably evaluated in clinical and mouse samples, with a minimum concentration of 4 ng/ml for the F1 protein and 10^3 CFU/ml for *Y. pestis*; to the best of our knowledge, this assay is not commercially available (239). Upconverting phosphor technology-based immunochromatographic assay (UPT-ICA) has been developed for the quantitative detection of *Y. pestis* in 15 min with a high specificity and an effective minimum concentration of 10^4 CFU/ml (100 CFU/test) (240, 241). This test can be applied in the field to various sample types, including blood, fresh or decomposed viscera (useful for zoonotic surveillance), and powdered material (useful for bioterrorism investigation), and can tolerate a wide pH range (pH 2 to 12) and high viscosity (unlike RDTs). Serological diagnoses are also commercially available, such as F1 capsular antigen capture ELISA, requiring a minimum concentration of 4 ng/ml and exhibiting a specificity of approximately 98% in sera and a sensitivity of 90.1% in serum and 100% in bubo aspirates (242). The results of these assays are similar for bubo aspirates but are more sensitive for serum than those reported by Chanteau el al. (sensitivity of 100% in bubo aspirates, 52% in serum, and 58% in urine specimens) for the F1 antigen test developed by the Naval Medical Research Institute (243). The gold standard diagnosis of plague remains difficult in countries of endemicity because infrastructure, resources, and logistics are often limited in remote areas. Furthermore, its diagnosis is time-consuming and is impossible outside biosafety level 3 laboratories. RDTs have become an effective (showing the same sensitivity and specificity as other molecular tools), time-saving, economical alternative to molecular diagnostic tests (PCR) adapted for field diagnosis at the point of care and can be performed by nontechnically trained personnel (213). Nevertheless, the diagnosis of plague is often uncertain, depending on the sample quality, invasion stage, and/or available technical support (244); for example, only 23% of patients with suspected pneumonic plague and 35% of patients with suspected bubonic plague received a laboratory-confirmed diagnosis (by rapid antigen detection, PCR, or culture) during the 2017 plague epidemic in Madagascar (59). This situation of underdocumentation is clearly detrimental to patients and to people in contact with them, as the epidemiological and clinical aspects of epidemics may not achieve a reasonable positive predictive value for the plague and may obscure other infectious diseases, such as leptospirosis (245).

Treatment of plague. At the beginning of the microbiological era, Alexandre Yersin built upon his discovery of *Y. pestis* and became the first to develop an antiserum for the treatment of plague patients. He developed the antiserum in collaboration with Calmette, Roux, and Borrel at the Pasteur Institute in Paris in 1895. The antiserum was first used to treat 23 Chinese plague patients in 1896, and only two of these patients died, lowering the mortality rate to 9% (246). These encouraging results led other physicians to use this treatment, but the lack of standardization among the methods they applied, such as the great diversity of the animals used for antiserum production and the variation in the doses given, led to unconvincing results. However, according to a report by Meyer et al. (247) based on the use of antiserum over a decade in Asia, Africa, and South America, this treatment is predicted to have reduced mortality from 82% without treatment to 32% following the injection of the serum. Antiserum can be coupled with sulfapyridine to achieve better efficiency; for example, in Egypt in 1940, only 12 of 69 plague patients treated in this manner died (248). Treatment with antisera, which confers only short-term protection and causes severe side effects, was progressively replaced by the development of new molecules, such as sulfonamides (preceded a year earlier by the intramuscular injection of sulfonamide prontosil with encouraging results [249] and used for the first time in East Africa and India in 1938 [250, 251]). In Madagascar in 1940, Girard reported the curing of 28 of 37 bubonic plague patients (mortality rate, 24%) by the injection of sulfapyridine, whereas all treated pneumonic plague patients died (252). In fact, this treatment proved effective only when administered within 3 days of the onset of symptoms, even in the case of

pneumonic plague, as demonstrated by the recovery of three such patients in Madagascar in 1947 (253). At the beginning of the 1940s, Wagle et al. demonstrated the superiority of sulfathiazole over classic antiserum in patients suffering from all forms of plague in India (254). In 1947, the first case of pulmonary plague was cured with streptomycin (255), which later cured five infected patients in Argentina (249). This new antibiotic was very effective and was able to cure patients in whom sulfonamide failed (256). Chloramphenicol and oxytetracycline were used in the early 1950s with the same efficiency as streptomycin (249). Cumulative evidence led to an updating of the recommendations regarding the antibiotic treatment of suspected and confirmed cases of plague. Streptomycin, which is the historical reference antibiotic and is still used in Madagascar, is no longer available in most countries, and it has been shown that gentamicin alone or in combination with tetracycline is an acceptable substitute (257). Gentamicin and doxycycline were shown to be equivalent in curing patients, except during the terminal stage (258). According to WHO and Centers for Disease Control and Prevention (CDC) recommendations, antibiotics that are commonly used against *Enterobacteriaceae*, such as streptomycin, gentamicin, levofloxacin, ciprofloxacin, doxycycline, moxifloxacin, and chloramphenicol, are proven to be effective against plague if given promptly (257–261). Antibiotic treatments should be continued for 10 to 14 days, and improvement is clinically evident 2 to 3 days after the initiation of antibiotic treatment, although fever may persist for several more days. Supportive therapy should be undertaken in the case of septic shock and septicemic plague. Very few antibiotic-resistant *Y. pestis* isolates have been described. In Madagascar, one isolate was reported to be resistant to eight antimicrobial agents, including those recommended for the treatment (streptomycin, chloramphenicol, and tetracycline) and prophylaxis (sulfonamide and tetracycline) of plague, as well as ampicillin, kanamycin, and spectinomycin (262). Streptomycin resistance may be plasmid transferable (263). A second isolate was reported to be resistant to streptomycin (264). In these strains, all resistance genes were carried by a conjugative plasmid consisting of approximately 150-kb and 40-kb sequences. Horizontal gene transfer in fleas may be the source of the antibiotic-resistant *Y. pestis* strains isolated from plague patients in Madagascar (263). Further, ampicillin- or tetracycline-resistant isolates have been generated in fleas and rats in Madagascar (264), whereas the 150-kb plasmid backbone has been shown to be broadly disseminated among multidrug-resistant zoonotic pathogens associated with agriculture (265). However, none of the 50 *Y. pestis* isolates generated during the 2017 epidemic in Madagascar were antibiotic resistant (59). Experimental data indicate that *Y. pestis* strains that are resistant to fluoroquinolones and rifampin can be easily selected in the laboratory, with no medical relevance thus far (266).

Prevention of plague. The measures for primary prevention prior to potential exposure to *Y. pestis* may include the avoidance of areas with known epizootic plague (42, 51, 63, 65, 66, 71, 96, 99, 267–290). In areas where plague is endemic, it is good practice to avoid contact with obviously sick or dead animals and to report such animals to the health department. Regarding potentially contaminated ectoparasites, people should avoid exposure to fleas from diseased rodents, dress in protective clothing, use repellents to avoid exposure to ectoparasites when outdoors, and apply insect repellent containing diethyltoluamide to the legs and ankles. Additionally, it is good practice to apply repellents and insecticides to clothes and outer bedding, to wear gloves and masks when handling dead animals and carcasses, and to cook meat on an open-flame grill or a clamshell-type electric grill (169). In 1946, killed whole-cell vaccines were developed and preventively administered to soldiers, but these vaccines conferred only short-term protection and did not protect against primary pulmonary forms of the disease (291). Furthermore, a live vaccine developed from an attenuated *Y. pestis* EV76 strain was demonstrated to protect against bubonic and pulmonary plague but potentially caused major side effects, such as general malaise, severe headaches, and pyrexia (292). Several vaccines, including a live attenuated *Y. pestis* EV76 strain, formalin-inactivated whole-cell plague, and heat-killed whole-cell plague (F1 fraction), can be administered via aerosol (EV76), subcutaneously (EV76, heat-killed

CSL vaccine; CSL Ltd., Victoria Australia) or intramuscularly (formalin-inactivated Greer vaccine; Greer Laboratories Inc., North Carolina). F1 fraction vaccines appear to show low efficacy (<60%) and cause side effects in 35% of vaccinees (293). There is not enough evidence to evaluate the effectiveness of any plague vaccine or the relative effectiveness of the vaccines and their tolerability. Circumstantial data from observational studies suggest that killed types of vaccines may be more effective against bubonic plague and have fewer adverse effects than attenuated types of vaccines. There is no evidence regarding the potential of vaccines to help control plague outbreaks (293). Furthermore, there is currently no vaccine recommended by the WHO or the CDC for preventing plague. Some vaccines are under development but will not be available in the immediate future (294). Secondary prevention in the case of potential exposure to *Y. pestis* relies on the administration of tetracycline or trimethoprim-sulfamethoxazole to people who are bitten by fleas during a local outbreak, are exposed to tissues or fluids from a plague-infected animal, live in households with a bubonic plague patient (since they may also be exposed to infected fleas), or are in close contact with a person or pet with suspected plague pneumonia (257). In a mass casualty situation, oral therapy with doxycycline, tetracycline, or ciprofloxacin is recommended (261, 295), and the use of the last option has been supported by an animal model, although a recent evaluation indicated that doxycycline should be considered a first-line antibiotic in the management of bioterrorism agents, including *Y. pestis* (296). The prevention of human-to-human transmission from patients with pneumonic plague must be achieved by maintaining confirmed and suspected cases under droplet precaution and negative-pressure isolation when available (297) for at least 48 days after the initiation of antibiotic treatment (298). Additionally, wearing gowns, gloves, surgical masks, and eye protection is strongly recommended to stop the spread of the disease (299).

Plague control is partly based on the active surveillance of sentinel animals such as wild carnivores, which generally produce antibodies against *Y. pestis* without suffering mortality (with the exception of some felids) (9). Wild carnivores become infected by eating infected prey (rodents) and are therefore a good indicator of infection among rodent populations; thus, testing one carnivore is equal to the testing of hundreds of rodents (300). In areas of epizootics, it is mandatory to eliminate food and shelter for rodents around homes, workplaces, and certain recreation areas, such as picnic sites or camping grounds, where people congregate. Bushes, rock piles, junk, and food sources, including pet food, should be removed. Pets (cats and dogs) should be treated for fleas regularly (9). Similarly, the control of human plague outbreaks relies mainly on the rapid confirmation of the diagnosis and treatment of confirmed and suspected cases. Killing rodent fleas and rodents using appropriate licensed insecticides and rodenticides or rodent traps is mandatory during plague outbreaks according to WHO recommendations. Nevertheless, during an epizootic situation involving endangered species, flea control must be prioritized (9). New control strategies are essentially reliant on monitoring (301, 302) or on the modeling of susceptible rodent populations combined with climate variation and environmental factors to predictively evaluate epizootic risk and human-related cases (9, 303, 304). The active long-term surveillance of plague foci coupled with the rapid response of health care professionals during epizootics helps to successfully reduce human cases.

ANCIENT PLAGUE

History of Plague

Historical sources. Several historical epidemics referred to as "plague" by ancient authors have been related in historical sources, based largely on the accounts of direct witnesses of epidemic episodes. The information from these accounts is of great value to scientists, who can extract valuable data from these documents to broaden the scope of the knowledge of plague, including its epidemiology. Sources of modern knowledge about plague include preserved historical archives, diaries of witnesses and contemporaries, preserved historical paintings, related artifacts, and biological archives,

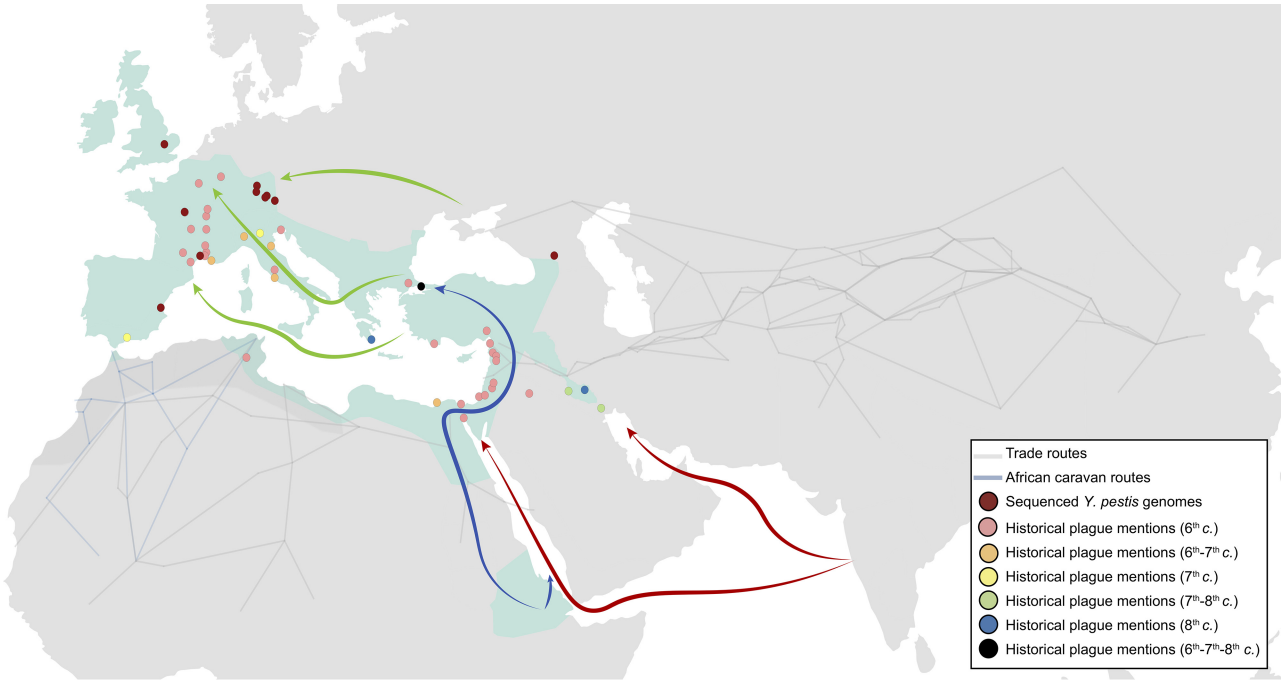

FIG 6 World map of the first plague pandemic (541 to 750/767). Regions historically affected by plague are represented in green, and regions potentially affected by plague (western part of North Africa) are in dark gray. The biological hypothesis of plague diffusion is represented by green (Eastern European origin) and red (Indian origin) arrows, while the debated historical hypothesis of plague diffusion is represented by a blue arrow (Ethiopian origin). The map was generated in QGIS 3.4. The mapped regions and roads are based on the Digital Atlas of Roman and Medieval Civilizations (DARMC; https://darmc.harvard.edu).

including human remains (most often the skeletons of the victims of these past epidemics). Furthermore, plague is the subject of a wealth of artistic output in Europe. Plague epidemics were the archetype disaster, threatening all of humankind and leading to a profusion of representations, attesting to their impact on people's mentality (305–311). This historical material constitutes a source of knowledge that can be interpreted in a scientific manner after distinguishing between representations of reality (e.g., regarding the localization of buboes and the management of plague-infected corpses) and symbolic and pictorial material (e.g., a living baby feeding from the breast of its dead mother). All of these representations have made deep impressions on the collective imagination of the ancient and contemporary European population.

(i) First pandemic: the Plague of Justinian (541 to 750/767). This pandemic, which was named after the emperor of the Roman Empire of the Orient, Justinian I (emperor from 527 to 565), started in 541 in the Egyptian port of Pelusium (312, 313) and was initially recorded around the Mediterranean Basin between 541 and 544 during the first wave called the "plague of Justinian," after which it returned from 558 to 750/767 in 14 to 21 additional waves called the "first pandemic" (1, 8, 314). According to Evagrius Scholasticus, the plague originated in Ethiopia (315), although this assumption remains controversial (316, 317), and an alternative hypothesis has been proposed (see below). The disease exhibited an endemic character, spreading through recurrent epidemic outbreaks over a long period (from the mid-6th to mid-8th centuries) across a wide geographical area (Europe and Mediterranean Basin). Indeed, the Justinian Plague primarily affected banks and ports and then penetrated deep inland via human movements and trade routes and along the Loire and Rhone valleys to Germany (2, 318, 319), England, and Ireland (2) (Fig. 6). Based on contemporary sources, the symptoms that were described (headache, fever, buboes, and rapid death) provide a clinical picture clearly indicating that plague was the etiological agent of this pandemic (314). The historian Procopius described these epidemics as follows: "It generally happened that those whose buboes grew large and suppurated, recovered from the disease,

which seemed to spend its violence upon these tumors; while in those whose buboes remained without suppuration, it had an unfavorable termination" (320). According to Gregory of Tours, "Death was sudden. A wound the shape of a serpent would appear on groin or armpit and the man would be so overcome by the poison as to die on the second or third day" (321). The origins of the plague and causes of its decline and temporary eradication over 3 centuries in affected European regions remain unknown (2). The human and social consequences of this pandemic remain controversial. Some authors put forward estimates ranging from 15 to 100 million victims, equivalent to 25% to 60% of the estimated human population (322, 323), and hypothesize severe social and economic consequences that initiated the collapse of classical antiquity (322, 324). However, in a recent study based on historical and archaeological data, it was suggested that the death toll has been greatly overestimated and was probably 0.1% of the estimated human population, suggesting that the Justinian Plague was not significantly influential of demographic, political, and social change (1).

(ii) Second pandemic (1346 to 18th century). "Consult historians, they are silent; ask physicians, they are stupefied; seek the answer from philosophers, they shrug their shoulders, furrow their brows, and with fingers pressed against their lips, bid you be silent. Will posterity believe these things, when we who have seen it can scarcely believe it, thinking it a dream except that we are awake and see these things with our open eyes, and when we know that what we bemoan is absolutely true, as in a city fully lit by the torches of its funeral we head for home, finding our longed-for security in its emptiness? O happy people of the next generation, who will not know these miseries and most probably will reckon our testimony as a fable!" These few lines from the Florentine poet Petrarch clearly set the scene for the return of plague in the middle of the 14th century (325). The second pandemic likely began at the end of the 1330s in central Asia, probably in present-day Kazakhstan, Russia, or China (3, 326–329). After the alleged first episode of bacteriological warfare in 1346 (330, 331), plague spread via sea routes from the port of Caffa to Constantinople and then to the whole of North Africa and western Europe (Fig. 7). During the 14th century, people testified to this first epidemic assault, and Father Franciscan Michel Platensis of Piazza described it as follows: "In the month of October 1347, arrived from Genoa in the port of Messina, 12 ships having fled the plague that the Lord had sent them as punishment for their sins. They brought a disease so contagious that it was enough to talk to those who were afflicted to be mortally wounded without hope of healing" (332). During the first few months, it was easy to follow the route of the disease and the chronology of the infected cities. The route of dispersion then became more erratic because the plague followed travelers and commodities and stopped at their staging posts. The first attack of the plague, which occurred during the first 7 years of the second pandemic (1346 to 1353), killed between one-quarter and one-third of the human population and is historically referred to as the Black Death (333). The name Black Death does not refer to the color of the corpses of the plague victims. The expression refers to the figurative meaning of the adjective (e.g., lugubrious and appalling). Accordingly, the term Black Death does not date from the 14th century and is completely anachronistic (334). The people of the Middle Ages used the terms "great mortality," "bumps disease," and "epidemic" to describe the plague. Furthermore, some places were affected several times by recurrent epidemics, as reported by a chronicler of the city of Orvieto (335): "The first general plague occurred in 1348 and was the strongest." Then, this author adds, "Second plague, 1363; Third plague, 1374; Fourth plague, 1383; Fifth plague, 1389. . .Sixth Plague, 1410." Biraben summarized the endemicity of the disease perfectly as follows: "From that point onward, and until 1670, the plague raged every year in Europe, sometimes in vast territories, sometimes only in certain localities, but without skipping a single annual link in this long and painful chain" (336). From 1347 until at least the middle of the 17th century, the plague became a common fact of life in western European societies. After 1670, the plague became rarer, although some outbreaks still occurred during the 18th century, as recorded in Marseille and Provence between 1720 and 1722 (337), in Messina and its surrounding area in 1743 (338), and in Moscow and the surrounding area in 1771 (339, 340). Plague had considerable consequences for social life. It contributed to ending the Hundred Years' War between the French and English king-

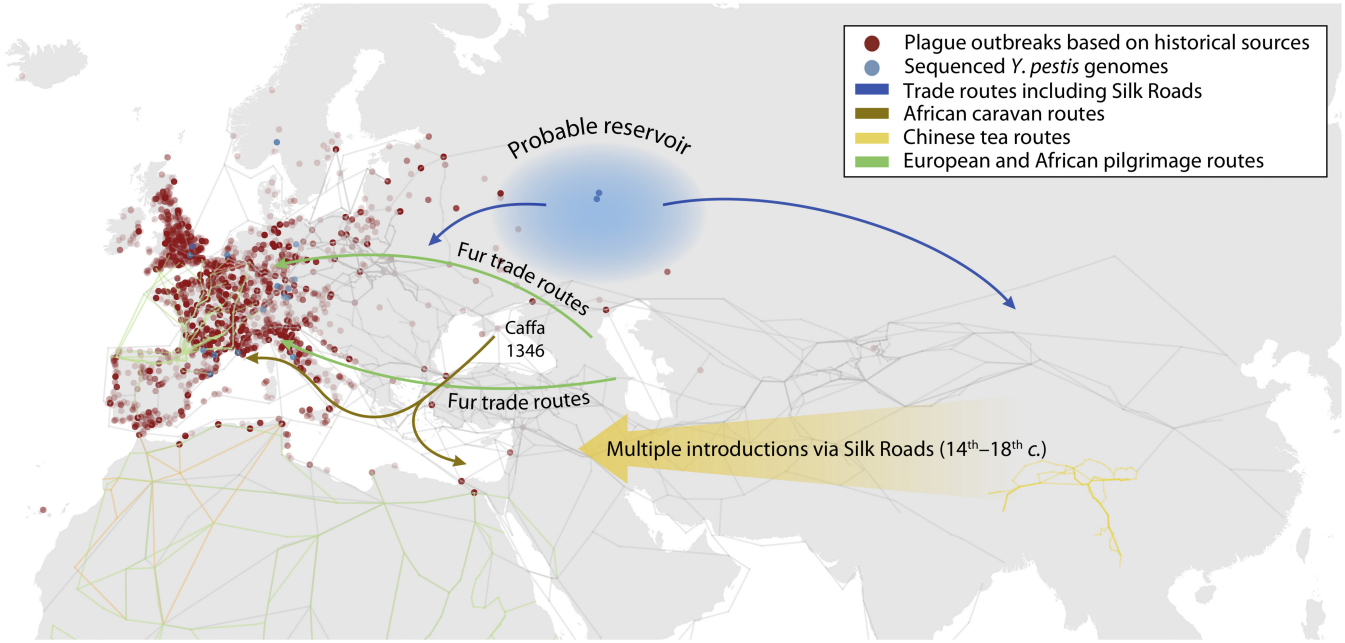

FIG 7 Map of the second pandemic (1346 to 18th century), including the so-called Black Death (1346 to 1353) and *pestis secunda* (1357 to 1366) episodes. Brown arrows indicate the well-known starting point of the Black Death in 1346 (city of Caffa) and the probable spread of the plague to Europe and Africa via land and maritime routes. Blue arrows indicate the first hypothesis regarding the dynamics of the second pandemic, in which plague was introduced from Eastern Europe to Western Europe before settling in one or several reservoirs and disappearing, followed by reintroduction in Asia giving rise to the third pandemic. The yellow arrow indicates the second hypothesis, indicating that plague was also introduced to Europe from Central Asia by successive waves from the 14th to the 18th centuries. This hypothesis excludes the existence of temporary plague reservoirs in Europe. The plague would have been introduced by successive waves over 4 centuries in Europe, mainly via silk roads and fur roads, by establishing several permanent foci (still existing) along these roads and spreading to Europe along maritime and terrestrial routes. The map was generated in QGIS 3.4. The mapped regions and roads are based on the Digital Atlas of Roman and Medieval Civilizations (DARMC; https://darmc.harvard.edu).

doms, which disrupted the economic expansion of the continent and affected the workforce (341). It is difficult to pinpoint the terms of the second pandemic. Indeed, Antoine-Jean Gros painted plague-affected soldiers of Bonaparte engaged in the Campaign of Egypt in Jaffa in 1799 (342). The plague was still present in Malta in 1813 (343), in Tunisia between 1818 and 1820 (344), and in Egypt between 1834 and 1835 (345). Finally, the epidemic that struck Constantinople in 1839 (346) is considered the last manifestation of the second pandemic in Europe (332). As reported above, although a period of several centuries separates the first from the second pandemic, the chronological break between the second and third pandemics is much less obvious. Indeed, the first manifestation of the third pandemic occurred in 1772 in Yunnan Province in southwestern China (3, 6, 347), while the plague that was still raging in Europe at that time was attributed to the second pandemic.

(iii) Third historical pandemic, 1772 to 1945. The third plague pandemic is likely to have originated from the province of Yunnan in southwestern China, where it was registered as early as 1772 in the city of Dali (348) before becoming endemic in southwestern China in the 1850s and 1860s (8). Thriving because of troop and refugee movements during the rebellion of the Mohammedans (99), the plague reached the city of Canton in March 1894, causing more than 60,000 deaths and rapidly spreading to Hong Kong in May of the same year (10). Between 1899 and 1900, the plague invaded all continents, including Asia (India in 1896 and Japan in 1899), the Middle East (Saudi Arabia and Turkey in 1897) (332, 349), Africa (Madagascar in 1898), Oceania (Brisbane in 1899 and Sydney in 1900), Europe (Lisbon in 1899 and Glasgow in 1900), and North and South America (Brazil, Paraguay, and Honolulu in 1899 and San Francisco in 1900) (8, 332, 350). Plague was thus recorded in more than 100 countries during the ongoing third pandemic (350). The rapid spread of plague along steamboat and rapid train routes worried health authorities in Europe. Because of the memory of previous, devastating epidemics, an emergency meeting of European health authorities took place in Venice in 1897 to attempt to stem and control the

possible return of the plague to Europe (332). Despite the reinforced quarantine system, bubonic plague returned to Lisbon in 1899 and Glasgow in 1900, claiming 37 lives (351, 352). Furthermore, the plague reached territories where it had never previously been documented, including South America, the United States, South Africa, and Australia. Following its introduction to these supposedly plague-free regions, *Y. pestis* established permanent plague foci in all of these regions, with the exception of Australia. It is interesting to compare the outcome of third plague pandemic in the United States and Australia. The plague entered both countries during approximately the same period (San Francisco, California, United States, in 1896 and 1900 [99, 353]; Brisbane and Sydney, Australia, in 1899 and 1900 [354, 355]) via the maritime route. In the United States, the plague remained a disease of harbor towns until the 1930s, and it emerged as an inland endemic infection in the 1950s, affecting populations of local rodents, including prairie dogs, squirrels, and chipmunks, and establishing endemicity in the semiarid southern states (41, 76, 353, 356). The plague entered Australia through Brisbane harbor in 1899 and Sydney 1 year later, where the affected districts were isolated, the inhabitants were evacuated, and more than 100,000 rats were killed to prevent the spread of the disease (354). The reasons why the plague did not become permanently established in Australia after its introduction, in contrast to the situation in the United States, remain unknown and are possibly linked to the inability of *Y. pestis* to establish permanent plague foci in the Australian soil (6, 76, 356). From 1910 to 1911, a massive presumed pneumonic plague outbreak occurred in Manchuria, causing approximately 60,000 deaths (180). Following this epidemic, the "ten thousand nations" international plague conference in Mukden (now Shenyang) was held from 3 to 28 April 1911 (357) and marked a turning point in the international fight against the plague. At this meeting, international plague scientists (including S. Kitasato) and government delegates discussed disease reservoirs, vectors, transmission, and etiology and preventive measures to be applied internationally in the event of new plague epidemics. The Mukden conference established a system of international cooperation to fight the spread of the plague during a time when steamboats and railroads were emerging (357). In France, the final cases were recorded in Marseille in 1919 to 1920 (358), and in Paris, the final cases were recorded in June 1920 ("Ragman's plague") (359). During this episode, a total of 90 cases of bubonic and pulmonary plague resulted in 30 deaths. The first cases were recorded in children after a boat transporting coal from London arrived in northern Paris. Notably, this pulmonary plague episode began at the same time as Spanish influenza, and confusion existed between the two diseases. Genomic analyses indicated that, in most cases, a single introduction was followed by regional dispersion, as recently illustrated in Brazil (360). Following World War II, the last cases of plague in Europe occurred in 1945 in Corsica and Italy and in 1947 in Kaliningrad (332, 351). The third pandemic had already accounted for more than 26 million cases and more than 12 million deaths in India and China alone (8, 99).

(iv) Undocumented historical epidemics. Regarding ancient periods, it is difficult to recognize the clinical symptoms of plague by reading the very rare and imprecise available sources. Thus, the descriptions of Greek and Chinese authors are very uncertain (361, 362). Other sources may report epidemics originating from an entirely different pathogen under the name "plague." Sometimes, we can only infer the possible occurrence of an epidemic on the basis of metaphorical texts, which are mostly theological in nature, making it impossible to recognize objective clinical symptoms in the episodes described in the Bible and Koran and in nonreligious texts, which may report plague episodes lacking any current microbiological confirmation. The most ancient record of a putative plague outbreak can be found in the Old Testament in the Book of Samuel, where the plague was interpreted as a divine punishment. God allegedly sent the plague to the Philistine city of Ashdod in retaliation for the theft of the Ark of Covenant (363). In antiquity, the first epidemic is reported by Homer's Aoidos at the beginning of *The Iliad*, where he describes an outbreak of plague driven by Apollo against the Greek army of Agamemnon (364). "The Plague of Thebes" described by Sophocles in the tragedy *Oedipus Rex* was recently reported as being attributable to *Brucella abortus* (365). The same applies to the "Plague of Athens" reported by Thucy-

dides in 430 to 426 BC (366), which was controversially documented as typhoid fever (367, 368). The final outbreak reported by a Greek historian was the "Plague of Syracuse" in 396 BC among the Carthaginian army during the siege of the city. In this chronicle, Diodorus Siculus, who was not a direct witness of the epidemic, reported that "now the plague first attacked the Libyans, and, as many of them perished, at first they buried the dead, but later, both because of the multitude of corpses and because those who tended the sick were seized by the plague, no one dared approach the suffering . . . For by reason of the stench of the unburied and the miasma from the marshes, the plague began with a catarrh; then came a swelling in the throat; gradually burning sensations ensued, pains in the sinews of the back, and a heavy feeling in the limbs; then dysentery supervened and pustules upon the whole surface of the body." According to Diodorus, these symptoms were similar to those endured by the Greeks 30 years earlier during the Plague of Athens (369). The occidental Roman Empire also experienced several large-scale epidemics that were allegedly attributable to plague, but this hypothesis was never formally tested. According to Saez, the tragic event of the "Antonine Plague" during the 2nd century killed approximately 10% of the Roman population and played a substantial role in initiating the decline of the Roman Empire (370). The Greek surgeon Galen witnessed this outbreak, and a critical reading of his writings suggests that smallpox might have been the etiological agent (371, 372). Subsequently, the plague of Cyprian, named after the bishop of Carthage, occurred on the periphery of the Mediterranean Basin from 249 to 270 (373). Although Cyprian left some testimonials about this epidemic, documentation related to this episode is extremely sparse. Nevertheless, coins have been discovered from this period that are inscribed with the words "Apoll Salutari," indicating the seeking of protection from Apollo, who governs illness, and archaeologists uncovered a Roman mass grave in the ancient city of Thebes dating from the same period (374). Considered together, these observations support the veracity of an epidemic episode, but questions remain as to whether the etiological agent was *Y. pestis*. In addition to historical documentation, archaeological documentation is currently essential to determine the history of plague and its epidemiology. Recently, several research teams have verified the presence of *Y. pestis* in the remains of individuals dating from the Neolithic era. These molecular biological studies, which were performed using skeletons discovered in Russia, Sweden, central Europe, and Asia, showed the presence of *Y. pestis* DNA in individuals aged approximately 4,000 to 5,000 years (375–379). Despite the extreme importance of these recent studies that have identified plague bacilli, there are no data other than genetic data that might help to understand the impacts of these epidemics.

Archaeology of Plague

The interest of archaeologists and biological anthropologists in plague burials is relatively recent. It was only in the mid-1980s to 1990s that the first excavations of the mass graves or funeral complexes of plague victims were carried out (380–384). Since that time, researchers have understood the interest of these sites and these human remains, which constitute true "biological archives." These "archives" provide information on both the process of death (practices implemented for the care of the deceased during a health crisis) and the dead themselves, including certain parameters that make it possible to characterize the victims (sex, age, health status, etc.). In the years that followed, archaeologists and anthropologists continued to explore burial sites with large numbers of multiple and simultaneous burials. The first sites that attracted attention were associated with the end of the second pandemic (17th and 18th centuries). These efforts notably enabled research projects to connect these mass graves with conserved historical archives and to place the archaeological human populations studied in their social and chrono-cultural context. It is therefore at this type of site (major complexes and epidemics of the modern era) that the first collaborations with paleomicrobiologists were established (385), allowing the formal identification of the pathogenic agent responsible for the Marseille and Provençal epidemic of 1720 to 1722. Since the mid-1990s, research on the burial sites of plague victims has

continued to increase in many European countries and in chronological contexts much broader than those initially considered. Today, nearly 100 sites with variable numbers of burials of plague victims have been the subject of cross-discipline studies, bringing together various complementary academic fields (archeology, anthropology, microbiology, and genetics). At the first sites, archeologists thought that the funeral areas related to plague epidemics were composed only of mass graves, within long trenches or large pits. This type of structure is found, for example, in the long parallel trenches of the Abbey of San Salvatore (3, 386), the long trenches and large pits, respectively, at the London sites of East Smithfield (387, 388) and the New Churchyard (326, 389), the great pit of Saints Màrtirs Just i Pastor in Barcelona (390, 391), the three large pits, including over 300 individuals, from the Rue des Trente Six Ponts site in Toulouse (326), and the Observance pit in Marseille (392, 393). All of these sites range from the 14th to the beginning of the 18th centuries and therefore cover the entire second plague pandemic. However, archaeoanthropologists have recently questioned the presence of burials presenting slight anomalies from conventional burial practices, such as the existence of small multiple burials (grouping from 2 to 10 individuals) in regular cemeteries. The observation of certain archaeological characteristics does not mark a profound change in funeral practices, as imposed by a major demographic crisis, but provides signs hinting at the simultaneous death of some members of the community. This type of anomaly might be related to the burial of the first or last victims of an epidemic or to the funeral management practices inherent to an epidemic that is successfully controlled by administrative and health measures. In all cases, these small multiple burials deserve special attention because some of them may have been associated with the burial of plague victims according to paleomicrobiological studies. This is particularly likely in several contexts from the 14th century, such as the cemetery of the churches of St. Nicolay and St. Clement of Oslo in Norway (3, 394), the Saint-Laurent-de-la-Cabrerisse cemetery in France (3, 395), or the Laishevo III cemetery in Russia (326). It is also the case for archeological contexts related to the Justinian Plague, such as the cemetery of Dittenheim in Germany (2, 396) or the cemetery of the Horts in Lunel-Viel in France (2, 397). Thus, relevant paleoepidemiological studies are no longer based solely on the large funeral complexes known as "disaster mass graves" but are also based on small multiple burials and even individual burials in "regular" cemeteries. Based on these discoveries, researchers have therefore reconsidered the data from ancient excavations. These advances have made it possible to associate other small multiple burials initially discovered in traditional funeral spaces with plague episodes. Thus, the presence of *Y. pestis* has been confirmed in skeletons excavated in 1984/85 in Germany at the site of St. Leonhardi in Manching-Pichl and in 1979 at the Russian site of Laishevo III (326). In some cases, this has allowed a reexamination of old excavation data, such as data from the Estonian site of Kunila II studied in 1948, which revealed that two adult men died from plague (375, 398). The recovery of these ancient data combined with renewed scientific attention toward these small original funeral structures has allowed us to "excavate" the plague bacillus from burials prior to our own era and in totally unexpected chronological contexts (thousands of years before the "first known historical pandemic" of antiquity), such as at the Kunila II site in Estonia, Beli Manastir in Croatia, Gyvakarai in Lithuania, Rasshevatskiy in Russia, and even Haunstetten graves in Germany (375, 398–401). These data have demonstrated the importance of close scientific collaboration between historians, archaeologists, anthropologists, microbiologists, and geneticists, enabling the identification of *Y. pestis* from a funerary context and within a well-established chronological framework.

Paleomicrobiology of Plague

Detection of ancient biomolecules. In 1998, one of the first ancient bacterial DNA studies was performed on ancient dental pulp, which is a complex tissue embedded in the teeth and a source of DNA and proteins, from which a 133-bp fragment of the *Y. pestis rpoB* gene was amplified (385). While the use of dental pulp was fortuitous, we now understand that ancient dental pulp still contains morphologically intact blood

cells (402), which may include blood-borne pathogens such as *Y. pestis* (7). Furthermore, dental pulp is sufficiently protected from the external environment by dentine and enamel to be characterized by low levels of contamination (403). Regarding *Y. pestis* DNA, initial studies aimed to detect specific sequences of *Y. pestis,* including the chromosomal sequences of the *rpoB* gene, encoding the beta-subunit of RNA polymerase (385, 404), the *glpD* gene (190, 191, 404, 405), the F1 antigen gene (*caf1M*), located on the pMT1 plasmid (3, 404, 406, 407), and the pPCP1 plasmid-borne *pla* gene sequence (2, 3, 318, 319, 326, 385, 387, 390, 392, 404, 408–410). The detection of ancient DNA sequences can be performed by sequencing PCR products resulting from multiplex real-time PCR (190) and suicide PCR (409). Suicide PCR avoids the need for positive controls and uses PCR primer pairs only once to avoid any risk of in-laboratory contamination (409). The detection of ancient *Y. pestis* can also be achieved by immuno-PCR or rapid diagnostic testing based on the detection of the F1 antigen specific to *Y. pestis* (404, 407, 411–413) or by paleoproteomics relying on the detection of host and blood-borne pathogen proteins (414).

(i) Ancient genome sequencing. Studies have aimed to recover large genomic fragments, starting with pPCP1 plasmid sequences from the London Black Death site of East Smithfield and dated from 1348 to 1350 (406) and entire genomes of ancient *Y. pestis* strains (7). These studies have indicated a higher yield of ancient DNA recovered from teeth (37%) than from bones (5.7%) (406). The first draft genome dating from 1348 to 1350 was reported in 2011 (East Smithfield strain) and was reconstructed using ancient DNA from teeth and bones from the plague-confirmed East Smithfield collection (387). The obtained reads were mapped to the CO92 *Y. pestis* bv. Orientalis reference genome, and the unmapped reads were assembled separately. The size of this first ancient *Y. pestis* genome reached approximately 4 million base pairs. The coverage of pPCP1 plasmid DNA reached 98.68%, C and T nucleotide damage was revealed, and an average length of 55 bp was observed for chromosomal and plasmid DNA reads, which is characteristic of ancient DNA (average length $<$ 100 bp) (387, 415). With regard to the applied experimental approach, the total DNA extracted from ancient specimens is enriched for *Y. pestis* DNA using microarrays (318, 319, 387, 390, 392, 406) or via in-solution capture (2, 326, 375, 378, 408), followed by high-throughput next-generation sequencing (NGS) (7, 416). Alternatively, specific sequences of *Y. pestis* can be extracted *in silico* from the ultradeep sequencing of human genomes from anthropological material to reconstruct complete ancient genomes (376, 377, 379, 417). By combining the technical approaches reported above, a total of 88 ancient *Y. pestis* genomes have been reported to date (available from the European Nucleotide Archive or NCBI GenBank databases) (Fig. 8). These ancient genomes were all recovered from individuals from Eurasia, covering a long period from 5000 BP (before the present) to 1722 (376, 392). The analysis of ancient *Y. pestis* genome sequences offers a unique opportunity to explore the genetic evolution of the pathogen through observation rather than deduction from phylogenetic reconstructions based on the whole-genome sequencing of modern isolates (17). Indeed, subsequent deductions have indicated that *Y. pestis* may have diverged from *Y. pseudotuberculosis* approximately 13,000 to 79,000 years ago (7), based on genes obtained from the acquisition of two associated virulence plasmids, pFra/pMT1 and pPLA/pPCP1 (418), and that a genome reduction of approximately 10% occurred, including the loss of genes associated with virulence and metabolism in *Y. pseudotuberculosis* (genome size, 4.72 Mb) (33, 419, 420).

(ii) Paleogenomic study of prehistoric plague, 5000 BP to 800 BC. Sixteen genomes predating historical pandemics have been sequenced, proving the endemicity of plague infection among human populations in the Late Neolithic-Bronze Age period (LNBA) ($\sim$5000 BP to $\sim$3500 BP). The most ancient strain of Gok2 (which diverged from all other *Y. pestis* strains 5,700 years BP) is basal to all known modern and ancient *Y. pestis* genomes, while the other Bronze Age genomes were found to belong to an independent lineage emerging between 6000 and 5000 BP in Eurasia and probably spreading in the context of Yamnaya-related steppe expansion during the fifth millennium BP (376, 379). This first divergence yielded the basal lineages 0.PE7 and 0.PE2,

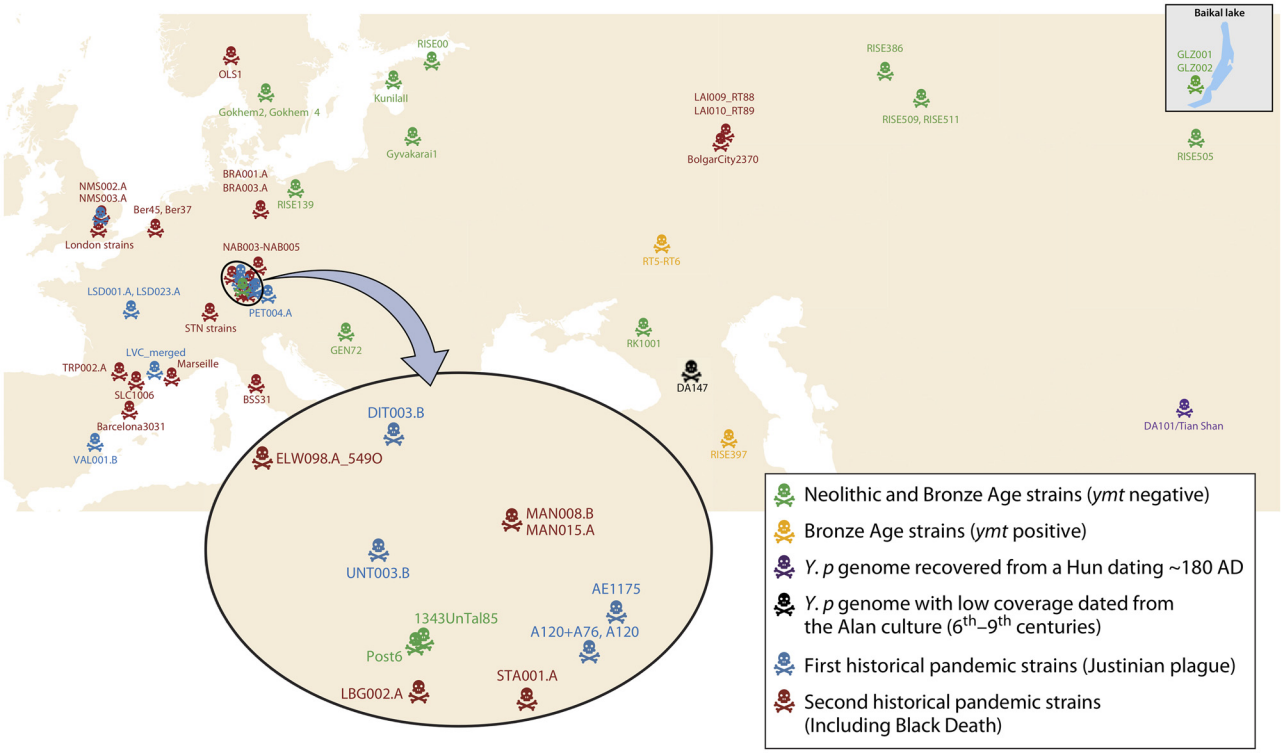

FIG 8 Map of ancient *Yersinia pestis* (*Y. p*) genomes.

which still exist today, and two extinct lineages from the Neolithic (Gok2) and Bronze Ages (376). The second divergence occurred 4,000 years ago on the Eurasian steppes and yielded the extinct RT5 lineage, the extant 0.PE4 lineage (Microtus), and other lineages that may be ancestral to those responsible for the historical pandemics (378). These genomes specifically lack a 20-kb plasmid region comprising the virulence-associated *Yersinia* murine toxin gene (*ymt*) (375–377, 379). The *ymt* gene encodes a phospholipase D implicated in the survival and replication of *Y. pestis* inside the flea gut. The presence of several virulence-associated genes, including *pla*, *caf1*, *ureD*, *rcsA*, *flhD*, *pde2*, and DFR4, indicates that these genomes belong to ancestral strains of *Y. pestis* associated with reduced invasiveness and, possibly, host adaptation (375, 378). The two easternmost Bronze Age genomes (GLZ001 and GLZ002) exhibit a lack of *ympt1.66c*, which is involved in the initial within-macrophage survival of the bacterium and, thus, bubonic plague progression (379). Furthermore, the lack of the *ymt* gene, which plays a key role in the flea-borne transmission of plague through late-stage biofilm-dependent transmission (18), also suggests that alternative transmission scenarios might have occurred during the Late Neolithic-Early Bronze Age (LN-EBA) period. However, fleas do not require the *ymt* gene for the early-phase transmission (EPT) of plague (203, 421), but this mechanism is considered to be poorly efficient, and it has been extensively debated whether it has the capacity to drive massive epidemics (119, 130, 186, 204, 422). All of these elements may explain the absence of mass graves during this period. The appearance of the *ymt* gene combined with altered sequence-suggested inactivation or loss of the *ureD*, *rcsA*, *flhD*, *pde2*, and *pde3* genes contributed to effective flea-borne transmission and an increase in bubonic plague forms. This event was dated to approximately 3,800 years ago based on two individuals found in Russia but possibly began as early as 5,000 years ago (33, 378). These data highlight the coexistence of *ymt*-negative and *ymt*-positive strains in Eurasia between 5,000 and 3,000 years ago, suggesting different transmission cycles and different disease symptoms, although the exact mechanisms of transmission and the vectors and sources involved remain unclear (7). Concerning the spread of plague, researchers have hy-

pothesized the occurrence of dispersion through the early trade networks, rather than through human migration (376), and have suggested that plague might have contributed to the decline of Neolithic societies by causing large deadly outbreaks, although there is currently no genomic (absence of the *ymt* gene) or archaeological (absence of mass graves) evidence that plague could have been epidemic in nature. Nevertheless, Neolithic plague may have migrated from the Eurasian steppes toward western Europe, which potentially continued during the Bronze Ages through lineages that eventually became extinct (376).

(iii) Paleogenomic study of the first historical pandemic, 541 to 750/767. The pandemic of 541 to 750/767 was confirmed as being caused by *Y. pestis* by three paleomicrobiological studies that recovered genomes from 10 individuals from Germany, France, England, and Spain (2, 318, 319). Genomic analyses suggested the existence of great diversity among the *Y. pestis* strains that were circulating during the sixth to eighth centuries in Europe (2), classifying all of these strains into the same lineage, with no contemporary representatives, diverging between the clades 0.ANT1, 0.ANT2, and 0.ANT5. The ED1001 strain recovered from Edix-Hill (Great Britain) is ancestral to all the other Justinian strains. Interestingly, four strains, including the Lunel-Viel and Saint-Doulchard strains, diverged from the Altenerding cluster through a polytomy; these strains belonged to a subsequent wave that was different from the original wave of 541 to 544. In Saint-Doulchard, one of the two studied genomes was ancestral to the second one, illustrating for the first time the coexistence of two independent *Y. pestis* genomes at the same site (2). These data led to a reinterpretation of the historical sources that had once suggested that the Justinian Plague was restricted to the shores of the Mediterranean Basin (8). Paleomicrobiological data indicated that the Justinian Plague was a continental plague that reached what is currently Germany and the United Kingdom (2, 318, 319). Three unique deletions were observed in *pheA*, the YPO2283 region, and the *celB* gene. Notably, a 45-kb and a 49-kb deletion including the *mgtB* and *mgtC* virulence-associated genes have been observed in some tardive strains of both the first and second pandemics (2, 326). It has been hypothesized that this genomic decay found only within end-of-epidemic strains might have been significant for the disappearance of these two pandemics (2). Concerning the origin of the Justinian Plague, some researchers have hypothesized a central Asian localization (417). Indeed, the framework for these genomes comes from the 0.ANT1, 0.ANT2, and 0.ANT5 lineages, which were isolated in China or Kyrgyzstan (66, 327). The basal strain of all the first-pandemic genomes was isolated from the Hun people in the Tian Shan Mountains, China (417). The Tian Shan mountain lineage may be the origin of the Justinian Plague, although 3 centuries separate this genome and the beginning of the pandemic in the Mediterranean Basin. Alternatively, the Justinian Plague may have spread along the Red Sea and Indian Ocean maritime routes connecting the Byzantine Empire and India (322) (Fig. 6). However, these claims based on genomic and phylogenetic analyses contradict certain historical sources. Procopius, who experienced this pandemic, described the plague as originating from Egypt and then spreading to Palestine and, finally, Constantinople (423). Evagrius described an "Ethiopian" origin of this outbreak. The possible African origin of the first pandemic is among the most scientifically debated issues (316, 317). In fact, in the absence of an ancient African *Y. pestis* genome, the current data do not allow the determination of the origin and spread of this pandemic (2).

(iv) Paleogenomic study of the second historical pandemic, 1346 to 18th century. In total, 56 *Y. pestis* genomes dating from the second pandemic in Eurasia have been sequenced (3, 326, 387, 390, 392, 408) (Table 2). These genomes have been identified through phylogenetic analyses as belonging to the second-pandemic branch, indicating that this pandemic was caused by a single introduction of one *Y. pestis* lineage (326, 390). In this lineage, four genomes have been shown to be identical to that of the Black Death strains (Barcelona3031, NAB003, NMS003, and OSL1), supporting the hypothesis of a single wave entering Europe and further spreading via maritime and terrestrial routes (326, 390). The Laishevo strain (LAI009), dating from 1300 to 1400 in Russia, was

TABLE 2 Summary of all sequenced genomes of *Y. pestis* belonging to the second pandemic[a]

Archeological site	City	Country	No. of genome(s)	Dating (yrs)	Strain name(s)	Sample accession no.	Study accession no.	Reference(s)
East Smithfield burial ground	London	England	5	1348–1350	Individual 8124	SRX096049	SRP008060	387
					Individual 11972	SRX096048		
					Individual 8291	SRX096047		
					Individual 6330	SRX096046		
					East Smithfield	SRX096021		
Observance	Marseille	France	5	1722	OBS107	ERS1020860	PRJEB12163	392
					OBS110	ERS1020861		
					OBS116	ERS1020862		
					OBS124	ERS1020863		
					OBS137	ERS1020864		
Saints Màrtirs Just i Pastor	Barcelona	Spain	1	1300–1420	Barcelona3031	ERS1124787	PRJEB13664, PRJEB29990	326, 390
Ust'-Jerusalem necropolis and Bolgar City mausoleum	Bolgar	Russia	1	1298–1388	BolgarCity2370	ERS1124788		
Marktplatz	Ellwangen	Germany	1	1485–1627	Ellwangen549_O	ERS1124789		
						ERS1124790		
						ERS3607398		
Saint-Laurent-de-la-Cabrerisse	Saint-Laurent-de-la-Cabrerisse	France	1	1348	SLC1006	ERS2865271	PRJEB24499	3
Bergen op Zoom	Bergen op Zoom	The Netherlands	2	1358–1360	Ber37	ERS2865267		
					Ber45	ERS2865268		
Churches of St. Nicolay and St. Clement	Oslo	Norway	1	1349–1350	OSL1	ERS2865270		
Abbadia San Salvatore	Abbadia San Salvatore	Italy	1	1348	BSS31	ERS2865269		
Laishevo III cemetery	Laishevo	Russia	2	1300–1400	LAI009	ERS3607399	PRJEB29990	326
					LAI010	ERS3607400		
16 Rue des Trente Six Ponts	Toulouse	France	1	1347–1350	TRP002	ERS3607426		
The New Churchyard	London	England	5	1560–1635	BED024	ERS3607391		
					BED028	ERS3607392		
					BED030	ERS3607393		
					BED034	ERS3607394		
					BED038	ERS3607395		
Augustinian Friary	Cambridge	England	2	1475–1536	NMS002	ERS3607408		
					NMS003	ERS3607409		
Sankt Johans Freidhof	Nabburg	Germany	3	1292–1392	NAB003	ERS3607404		
					NAB004	ERS3607405		
					NAB005	ERS3607406		
						ERS3607407		
St. Leonhardi	Manching-Pichl	Germany	2	1283–1390	MAN008	ERS3607402		
					MAN015	ERS3607403		
Possenhofener Str. 3	Starnberg	Germany	1	1433–1523	STA001	ERS3607410		
Kirchhof St. Johannis	Landsberg am Lech	Germany	1	1455–1632	LBG002	ERS3607401		
Nägeligasse	Stans	Switzerland	15	1485–1635	STN002	ERS3607411		
					STN004	ERS3607412		
					STN005	ERS3607413		
					STN007	ERS3607414		
					STN008	ERS3607415		
					STN011	ERS3607416		
					STN012	ERS3607417		
					STN013	ERS3607418		
					STN014	ERS3607419		
					STN018	ERS3607420		
					STN019	ERS3607421		
					STN020	ERS3607422		
					STN021	ERS3607423		
					STN031	ERS3607424		
					STN032	ERS3607425		
Domlinden 12	Brandenburg an der Havel	Germany	2	1618–1648	BRA001	ERS3607396		
					BRA003	ERS3607397		
Aguonu g. 10	Vilnius	Lithuania	4	1440–1621	AGU007	ERS4398260	PRJEB37508	408
					AGU010	ERS4410876		
					AGU020	ERS4398261		
					AGU025	ERS4398262		
						ERS4398263		

[a]Based on the phylogenetic tree or dating and including project numbers for read accession.

identified as the most ancestral form of the *Y. pestis* strain that entered Europe and caused the second pandemic, confirming the hypothesis of plague foci present approximately 2,000 km away in northeastern Crimea before the first plague was introduced to southern Europe in 1347 (326). Furthermore, the analysis of these 56 *Y. pestis* genomes (Table 2) provided considerable evidence of microevolution into the same

lineage at the end of the second pandemic (post-Black Death lineage), as follows: the Ellwangen strain gave rise to (at least) two distinct post-Black Death clades, where the first clade includes the German and Swiss strains from the 15th to 17th centuries and the second clade includes the Observance strains and the London strains from the 17th to 18th centuries. All the lineages were derived from the Black Death isolates and are likely to have evolved separately, leading to their divergence from the Black Death strains from approximately the 16th and 18th centuries. The post-Black Death lineage no longer exists, which could partially explain the disappearance of plague in the 18th century (326, 390). A study analyzing 34 ancient second-pandemic genomes identified a deleted region associated with virulence-related genes (*mgtB*, *mgtC*, and *inv* genes) that are vital for macrophage colonization (associated with decreased virulence in mice) (424) in genomes dated to the end of the second pandemic. The same deletion has been observed in late first-pandemic genomes. The functions of these genes in mammalian or arthropod plague conservation remain to be established (2, 326). The second-pandemic genomes are closely related to the genome sequence of current isolates, suggesting very few differences in virulence factors between current and ancient strains. Nevertheless, the Black Death strains and released Justinian Plague genomes (318, 319, 387) exhibit a 15-kb genomic island corresponding to DFR4 (difference region 4), which is deleted in all contemporary *Y. pestis* bv. Orientalis strain isolates but is present in some *Y. pestis* bv. Antiqua isolates, such as the Antiqua and Nairobi strains, some *Y. pestis* bv. Medievalis isolates, Pestoides A to D isolates, and *Y. pseudotuberculosis* (425). This region potentially includes virulence factor genes such as *ccm2A*, whose role in human infection is poorly understood. Analyses of genomes from the second historical pandemic suggest a single introduction of plague (based on the absence of genetic diversity among the sequenced strains) from Asia/eastern Europe into Europe in 1347 via terrestrial and maritime routes, followed by the persistence of plague in several temporary but undetermined foci (now extinct) to give rise to the western European phenomenon of endemic plague until the 18th century. Shortly after its introduction to Europe, the Black Death strain returned to Asia to give rise to the third pandemic (318, 326, 387, 390, 392). However, this hypothetical scenario seems very unlikely according to historical and epidemiological studies (3, 426, 427). An alternative second hypothesis argues that the plague originated in eastern Europe/central Asia and spread along trade routes (particularly the Silk Road), thus creating relatively permanent plague foci along these routes. The plague may have spread from such secondary foci through successive waves in western Europe along terrestrial and maritime trade routes (such as fur roads) (427) or through human migration (428) from the 14th to the 18th centuries (3, 429). Climate data showed that events such as episodes of aridity or significant fluctuations were decisive in the second-pandemic plague introduction and reintroductions (428, 429). Furthermore, the Asian origin of the second pandemic is debatable (3, 326–328, 430). A recent study indicates the lack of historical and molecular evidence of plague in 14th century eastern China. The whole-genome sequencing of *Y. pestis* animal isolates from modern plague foci in China showed genomic diversity greater than that observed in medieval European strains. Based on this observation, the most recent study led to the inference that the northeastern European plague reservoir acted as a second-pandemic source (326).

AREAS OF UNCERTAINTY: PROSPECTIVE STUDIES

This review highlights areas of uncertainty regarding plague dynamics. Clearly, the lack of any ancient genomes from outside Eurasia biases our understanding of historical plague origins and spreading, which may be partially related to cultural bias, as most ancient plague studies have been performed by European researchers, including the discovery of the plague bacillus by Alexandre Yersin. The recovery of ancient *Y. pestis* genomes in Africa might be of value for resolving the ongoing controversies regarding the sources of plague and their persistence over several centuries (2, 326). Concerning plague vectors, the role of bedbugs (*Cimex lectularius*), which have long been suspected to be an effective interhuman vector (27, 194), should be investigated on the basis of

current concepts and techniques and proper methodology. In 1897, Imagiva reported a human case of plague possibly caused by bedbugs (431). In 1910, Walker found *Y. pestis*-infected bedbugs in a camp where plague was raging and successfully transmitted the plague to rats via these bedbugs. Interestingly, Walker found no fleas during this outbreak (432). Subsequently, bedbugs were commonly found to be infected (sometimes in plague patients' beds) during plague outbreaks, as reported by Pollitzer (99). Unfortunately, the previous experiments were all carried out at the beginning of the 20th century using methods that did not allow researchers to indisputably prove the infection of bedbugs by *Y. pestis*. Nevertheless, these experiments indicated that virulent *Y. pestis* colonizes bedbug stomachs and feces for >100 days, thereby a host infection; however, the underlying mechanisms and efficiency remain to be investigated (99, 194, 433, 434). Outbreaks of the alleged pneumonic form have remained a debated issue since the confusing observation of *Streptococcus pneumoniae* in blood from plague patients by Kitasato in 1894. Kitasato's patients were clearly coinfected with *Y. pestis* and *S. pneumoniae*, and the culture conditions used by Kitasato favored the growth of *S. pneumoniae* (435). The propensity for these coinfections during plague outbreaks remains unquantified, as a syndromic approach is not routinely used in cases of suspected pneumonic plague. For example, during the last major outbreak in Madagascar in 2017, only 32 of 1,846 probable or suspected pneumonic plague cases were laboratory confirmed, leaving the possibility that other pathogens were responsible for pneumonia (59). This possibility is not only speculative, as a leptospirosis outbreak was retrospectively found to be nested within a suspected pneumonic plague outbreak (245). The relative efficiency of numerous plague vectors, such as fleas or body lice, remains extremely difficult to evaluate because of the lack of uniformity of the methods used in the laboratory (depending on the susceptibility of the hosts, the infectious doses used, the strains involved, and the choice of the temporal dynamics of infection and postinfection); sometimes, these methods are contrived and do not approach the actual reality that can be observed in the field.

CONCLUSION

Y. pestis is among the oldest reported and deadliest opportunistic human pathogens. During the Neolithic period at least, plague raged across Eurasia, causing outbreaks and possibly leading to a temporary decline in centenarian cultures. Approximately 3,800 years ago, *Y. pestis* acquired the *ymt* gene, enabling it to become a more efficient flea-borne bacterium. The Justinian Plague (541 to 750/767) remains controversial as to its demographic, political, and social consequences, unlike the second pandemic (1346 to 18th century), which killed at least 30% of the European population, made a profound impression on the collective imagination, and led to a deep restructuring of medieval society. The mortality rates resulting from the current third pandemic (1772 to present) have been incredibly low (except at its beginning) due to the first treatments and the use of antibiotics, which made it possible to reduce the mortality rate from 50 to 100% in the absence of treatment to approximately 10 to 25%. The most recent study indicates that *Y. pestis* is a telluric bacterium that exists in a free state, an L-form, or inside amoeba, and soil could serve as a reservoir. More than 200 mammals (mainly rodents) have been found to be infected by plague, probably orally, resulting from digging in soil. Human contamination from wild sources may occur from flea and tick bites, transcutaneous contact, aerosol inhalation, or oral ingestion (raw meat consumption). The most recent evidence indicates that interhuman transmission may be due mainly to human ectoparasites, such as body lice and fleas (aerosol transmission appears to be very low). The current data indicate that the epidemiology of plague is extremely complex because it is intrinsically linked to a given environment and time. For example, rats and fleas were found to be effective vectors in 1898 in India, but this scheme cannot be applied to effective reservoirs and vectors in ancient and modern plague outbreaks. The plague in the United States seems to be linked mainly to squirrels or chipmunks. In North Africa and the Middle East, outbreaks are linked to the consumption of raw or poorly cooked meat from camels or goats, and Tibetan cases

are linked to sheep and marmot skinning. In regions where plague is endemic, efforts have been made to prevent human cases by performing "sentinel" animal surveys and vaccination. In addition, to support the appropriate management of patients and provide a rapid and accurate microbiological diagnosis, we recommend evaluation in point-of-care laboratories, some of which are currently operating in a few remote regions in Africa. In addition to the direct diagnosis of disease in humans, the direct detection of *Y. pestis* at the point of care among potential sources and vectors could facilitate our understanding of how plague epidemics are sustained.

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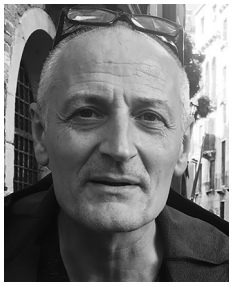
R. Barbieri has been a Ph.D. student in microbiology and biological anthropology at the IHU Méditerranée Infection, developing paleomicrobiology, with a particular interest in past plague, source, and dynamics.

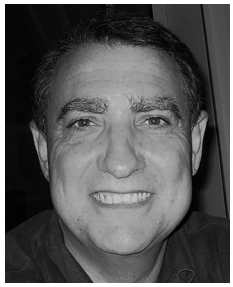


M. Signoli is Senior Researcher at the CNRS and Director of the ADES research unit (UMR 7268) at the Marseille Faculty of Medicine. An anthropologist specializing in the study of past epidemics, he works on these contexts using two types of sources: biological archives, i.e., the victims of these epidemics exhumed during archaeological operations, and historical archives, i.e., documents written by contemporaries of these epidemic crises. He has thus conducted and participated in numerous interdisciplinary studies on the plague, but also on other pathogens (tuberculosis, typhus, pox virus, etc.).

D. Chevé is an anthropologist, a Ph.D. in biocultural anthropology, and an associate researcher at UMR ADES (AMU-CNRS-EFS, Marseille, France), where she is coresponsible for a theme on the anthropology of life. Her work focuses on bodies (states, practices, and representations) at the real and symbolic levels and their relationships to health, the environment, and society, in particular to epidemics. She is codirector of the journal "Corps" (CNRS-Editions, Paris, France).

C. Costedoat is an Associate Professor in population genetics and HDR of Aix-Marseille University at the UMR 7268 Bio-Cultural Anthropology, Ethics and Health Law (ADES-AMU, CNRS, French Blood Establishment PACA Corse) research unit. Her research activities on genome-environment relations focus on the human model and in particular on the impact of the pathogenic environment on the genome and of the cultural footprint on the diversity of populations. Her dual affiliation in environmental science and anthropology allows her to address public health issues through the prism of interdisciplinarity, from field studies to data analysis.

S. Tzortzis is a Ph.D. in anthropology, an engineer of the French Ministry of Culture (Direction Régionale des Affaires Culturelles, Service Régional de l'Archéologie de Provence Alpes-Côte d'Azur, Aix-en-Provence), and a member of the Unité Mixte de Recherche 6578 ADES (Anthropologie Bio-Culturelle, Droit, Ethique, Santé, Marseille). As an archeoanthropologist, he has led excavations of multiple burial sites linked to plague epidemics of the Modern Times (17th to 18th centuries).



G. Aboudharam, a dentist by training, is a cofounder of the field of paleomicrobiology, inventing all the techniques for the manipulation of ancient dental pulp, leading to the discovery of *Yersinia pestis* in ancient human specimens, with further interest in ancient blood-borne infections at large.

D. Raoult M.D., Ph.D., who specializes in infectious diseases, is a professor of microbiology at the Faculty of Medicine of Marseille, Aix-Marseille University. In 1984, he created ex nihilo his research laboratory, the Rickettsia Unit, in the frame of which he serendipitously created the field of paleomicrobiology. He accordingly started working on plague more than 20 years ago, and his unit has now become the Research Unit in Infectious and Tropical Emergent Diseases (URMITE), collaborating with the CNRS (National Center for Scientific Research), the IRD (Institute of Research for Development), and INSERM (National Institute of Health and Medical Research). In 2011, he became the Director of the University Hospital Institute Mediterranée Infection, which is a 600-person medical institute focused on infectious diseases. This facility includes the largest diagnostic and research microbiology laboratory in France. As of 2020, Prof. Raoult has published over 2,800 indexed publications.

M. Drancourt M.D., Ph.D., is a professor of medical microbiology in IHU Méditerranée Infection, Marseille Medical School, and head of the Microbiology Laboratory at Marseille's public hospital. His research topics include paleomicrobiology and past plague epidemics.