A Dog-Associated Primary Pneumonic Plague in Qinghai Province, China

Hu Wang,1,a Yujun Cui,2,a Zuyun Wang,1,a Xiaoyi Wang,3,a Zhaobiao Guo,3 Yanfeng Yan,3 Chao Li,1 Baizhong Cui,1 Xiao Xiao,2 Yonghai Yang,1 Zhizhen Qi,1 Guojun Wang,1 Baiqing Wei,1 Shouhong Yu,1 Duolong He,1 Hongjian Chen,1 Gang Chen,2 Yajun Song,3 and Ruifu Yang3

1Qinghai Institute for Endemic Diseases Prevention and Control, Xining 811602; 2Hainan Center for Disease Control and Prevention, Gonghe 813000, Qinghai Province; and 3State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, People’s Republic of China

Background. Primary pneumonic plague (PPP) caused by Yersinia pestis is the most threatening clinical form of plague. An outbreak was reported in July 2009 in Qinghai Province, China.

Methods. This outbreak was investigated by clinical, epidemiological, bacteriological, and immunological methods. Multilocus variable number tandem repeat analysis (MLVA) was used to track the source of the outbreak.

Results. The index case, a patient with PPP, contaminated 11 close contacts. All the 12 cases, including the index patient, experienced sudden onset of fever, headache, and productive coughing with bloody sputum. Three of them died. Nevertheless, another 61 direct and 256 indirect contacts were not infected during the 2-week quarantine. Antibodies to F1 antigen were detected in 9 survival cases, with a 4-fold increase in titers in serum samples collected at different periods. Seven strains of Y. pestis were isolated from dogs and patients. Field investigation and MLVA of the isolated strains revealed that this outbreak was started by a deceased dog.

Conclusion. Dogs are believed to be an indicator animal for plague surveillance, but their association with PPP is rare. Our results provide evidence for this possibility, which suggests the public health significance of dogs as a source of plague.
METHODS

Clinical and Epidemiological Investigation
Clinical symptoms were observed by clinical doctors, and conventional epidemiological investigation on this outbreak was conducted by epidemiologists. The landscape of the outbreak site is alpine meadow steppe with bare rock in the Qinghai-Tibet plateau (a well-known plague focus). There are 324 families with 1472 people in the outbreak village. *Marmota himalayana* was the primary host of plague in this area. Other rodents (*Mus musculus*, *Cricetulus migratorius*, *Allactaga sibirica*, *Microtus oeconomus*, and *Ochotona dauurica*) and some wild or domestic animals (foxes, cats, goats, lynxes, badgers, and dogs) are sometimes found to be infected by *Y. pestis*. The mean temperature during plague outbreak is 11.5°C, with rainfall of 113.6 mm. To trace the source of this outbreak, domestic animals (3 dogs, 2 goats, 1 cat, 1 pig, and 1 cow), and wild animals (35 marmots and 1 field mouse) were captured in the vicinity of the epidemic area (~873 km² around the epidemic village). Serum samples were taken to detect antibody against F1 antigen by indirect hemagglutination assay (IHA) and to isolate *Y. pestis*. All the animals were handled according to the national criterion for animal plague investigation of China. All the experiments were performed according to our institute’s guidelines for animal operation. This study was approved by the bioethic subcommittee of the scientific committee in the Qinghai Institute for Endemic Diseases Prevention and Control.

Determination of F1 Antibody and Antigen
IHA [12] was used to measure the specific F1 antibody titer [13] by a 2-fold serial dilution of serum samples collected from the 9 survival patients and all their contacts. All the samples were taken specifically for this study, and all the live subjects provided signed informed consent; the samples taken from the dead patient was agreed by its direct relative’s signed informed consent; the samples taken specifically for this study, and all the live subjects provided signed informed consent. IHA was performed by including F1 antigen inhibition control and negative and positive controls [14]. Reverse IHA was used to detect the F1 antigen of *Y. pestis* from human and animal samples [15].

Isolation and Identification of *Y. pestis*
Clinical specimens from patients, dogs, marmots, and field rats, including bloody sputum, throat swabs, or necropsy organs (liver, lungs, spleen and heart from the index case and the 2 dead dogs, marmots, and rats), were first injected intraperitoneally into mice (1 sample for 5 mice) for observing disease symptoms (eg, fever, ruffled fur, and dysthymic) for 7 days. If the animal was dead, the liver, spleen, and lungs of the animal were obtained immediately for inoculating onto 2 selective agar plates (termed BIN agar, which is based on brain heart infusion agar with adding the selective agents of irgasan and cholate salts) for bacterial isolation at 28°C [16]. If the animal showed no obvious symptoms, the animals were autopsied on day 5–7, with 1 animal killed each day; the liver, spleen, and lungs were emulsified for inoculating more mice as mentioned above. If the sample was negative for the third round of aforementioned inoculation for bacterial isolation, it was considered to be negative for *Y. pestis*. Biochemical tests, such as arabinose, glycerine, rhamnose, and melibioseuse; Gram staining; bacteriophage lysis test; and specific polymerase chain reaction tests targeting F1 and pla genes, were used to identify the suspected isolates. The in vitro antibiotic susceptibility of them to streptomycin, ceftriaxone, kanamycin, and ciprofloxacin was evaluated by disc diffusion test [17].

Multilocus Variable Number Tandem Repeat Analysis (MLVA) for Source-Tracing
MLVA with 46 loci was performed on the 7 *Y. pestis* isolates from both dogs and patients in this outbreak and 5 other isolates from the same natural focus around Qinghai Lake, as previously described [18, 19]. Fluorescently labeled amplicons were visualized by polyacrylamide gel electrophoresis on an Applied Biosystems 3100 DNA sequencer using GeneScan fragment analysis. Data were imported in the BioNumerics software package, version 5.10 (Applied-Maths) as character data sets. Clustering analysis was done using the categorical coefficient and the unweighted pair-group method with arithmetic means. Minimal spanning tree was constructed from the similarity matrix.

RESULTS

Overview of the Outbreak
The index case (patient A) was a 34-year-old male herdsman who moved from his winter grassland to his summer one in Zhihaigou, Xinghai County shortly before the outbreak. When his family arrived at Zhihaigou on 20 July 2009, the herdsman found one of his dogs lost and he searched for it. He found it 6 km away beside a river, lying there short of breath. He took it home and buried it. Another dog in his family died with similar symptoms the next day.

In the afternoon of 24 July, the herdsman felt uneasy, and he developed a fever and cough on 25 July. On 26 July, his younger brother took him by a motorcycle to the village clinics, where 2 doctors received him. One of the doctors examined the herdsman’s throat by tongue depressor without any personal protection for himself. On 29 July, this doctor became ill (patient L). The other doctor, who was only in contact with the...
patient’s arm to give transfusion of physiological saline with 4.0 g ceftriaxone sodium, did not get infected. The doctors described the herdsman’s symptoms as fever, coughing with blood-tinged sputum, a pale face, and chest pain. During the treatment in this clinic, the patient had a face-to-face contact with one of his friends in the same village (patient K). After initial treatments, the doctor advised that the patient should be sent to the XCT hospital for further treatments. On the way to the hospital, accompanied by his 2 elder brothers (patients G and J) and 1 younger brother (patient F), the patient presented with abrupt dysphoria and vomiting. He died at ~5 PM on 26 July. In the evening of the same day, the herdsman’s corpse was driven home, where his 2 elder brothers (patients G and J), 2 younger brothers (patient F and I), his father-in-law (patient E), and his nephew (not infected) helped to prepare him in a fetus shape according to the Tibetan tradition. Early next morning (~5 AM), the victim was buried. During the development of his disease, his wife (patient B), 2 sons (patients C and D), and younger brother (patients H) also had close contact with him.

During epidemiological investigation, we defined a close contact as a person who lived with or had face-to-face contact (within 1 m) with the patient, a direct contact as a person who once was together with a patient in the same room or in the same car with closed windows, and an indirect contact as a person who met a patient in the hospital, in the open air, or in a bus without face-to-face contact. All 11 close contacts were infected (Figure 1), and the 61 direct or 256 indirect contacts were not infected. This observation is similar to previous reports that the transmissibility of PPP was not as high as was thought to be [4, 5, 20–23].

Clinical Symptoms and Treatment
This outbreak involved 12 persons: 9 male adults, 2 male children, and 1 female adult. Three of them died. Clinical manifestations are summarized in supplementary Table S1. Unfortunately, because of the sudden outbreak, rapid handling requirement of public health emergency, and limited medical conditions in such a remote area, radiographs and conventional blood cell counting were not performed for these patients. All the cases were diagnosed by contact history, bacterial isolation and/or specific antibody production, and antibiotic treatment outcome. All cases presented with sudden onset of fever, coughing with bloody sputum, and chest pain. The incubation period was 2–4 days. Streptomycin, ceftriaxone sodium, and ciprofloxacin in combination were selected for treating the patients [24, 25]. Streptomycin was administered intramuscularly (6 g per day for adults and 2 g for children). Ceftriaxone sodium was given intravenously (6g per day for adults and 4g–5g for children). Ciprofloxacin was only given to adults (0.2g–0.3 g twice daily). The dose of the antibiotics was reduced 1 week later according to the symptoms of the patients. All patients were treated with conventional antishock and other supporting therapies (supplementary Table S1).

Bacterial Isolation and Immunoassays
Y. pestis strains were isolated in the lung samples of the index case, sputum samples from patients L and K, sputum and throat swab samples from patient J, and lung samples from the 2 dead dogs. Therefore, 7 strains of Y. pestis were isolated: 5 from patients and 2 from dogs. The bacteria were identified by Gram staining, biochemical assays, phage lysis test, and polymerase chain reaction targeting caf1 and pla genes. All isolates were confirmed to be biovar antiqua. The isolates were susceptible to all the antibiotics tested. No bacteria could be isolated from other patients because antibiotics were given immediately at admission. Although the index patient also received 4g ceftriaxone, he was not treated specifically with streptomycin and we isolated Y. pestis from his lung samples, suggesting the inhibition of streptomycin to Y. pestis during the early stage.
IHA was used to measure the specific antibody titer to F1 antigen by a 2-fold serial dilution of serum samples collected from the 9 survival patients and all their contacts. As shown in Figure 2, the IgG antibody could not be detected at day 6 after onset of symptoms, but dramatic increases are seen for all survivors at day 12. Of note, 1 patient (I) had much higher F1-antibody titer than did the others, which might reflect the biological variations of immunological responses among individuals. All the serum samples from the direct and indirect contacts were shown to be negative for antibody against F1 by IHA, indicating that no asymptomatic infections among them occurred during this outbreak.

F1 antigen of *Y. pestis* was detected in sputum samples from some of the patients by reverse IHA until day 10 after onset of symptoms. The necropsy samples from the index case and the sputum sample from patient J at day 3 after onset of symptoms were shown to be F1 positive. In patient L, who experienced an extended course of the plague, with complicating shock, F1 antigen could be detected in the sputum samples until day 20 after onset of symptoms. F1 antigen was not detected in sputum samples from the other patients.

**Molecular Investigation by MLVA**

*Y. pestis* was isolated from the 2 dead dogs, indicating the possible source of the pathogen. This is corroborated by the fact that the strains from both dogs and humans belong to the same genotype by MLVA [18, 19]. Twelve strains were investigated by MLVA, including 2 from dogs and 5 from patients in this outbreak and 5 representative strains collected in our previous work from different counties near Xinghai County. As shown in Figure 3, there is no difference in the MLVA profiles of all 7 isolates in the 2009 outbreak, and they are different from other isolates from nearby regions, indicating that the isolates from patients were transmitted from the infected dog.

**Epidemiological Investigation on Wild and Domestic Animals**

Among the animal serum samples detected by IHA, serum samples from 4 marmots (from the vicinity of the epidemic area where the diseased dog was found) had anti-F1 antibody, with titers of 1:20 for 1, 1:80 for 1, and 1:1280 for 2, which were all significant, compared with the zero background in marmots. Although no *Y. pestis* strain was isolated from animals captured, this serological evidence indicates that sylvatic plague is still ongoing, or was once prevalent in this region. Because shepherd dogs have a habitat of eating dead marmot in the field, it also implies that the dog might have been infected by hunting a diseased marmot. We had previously reported that in the neighboring county (Huangyuan) (Figure 3B), hunting marmots is a high-risk factor in this plague focus, and that hunters and their family members have a significantly increased plague seropositivity rate of 21.7% (26 of 120) because of asymptomatic infections [26].

**DISCUSSION**

On the basis of results of bacterial, serological, epidemiological, and genetic investigations, it can be concluded that the PPP reported here was caused by *Y. pestis* and was initialized by an infected dog.

In this investigation, we found that simple countermeasures could effectively prevent the spread of PPP among contacts. Patient L, a doctor, has some knowledge of plague, and when he heard of the herdsman’s sudden death, he was worried that he had contracted contagious PPP. When he developed a sudden fever, chest pain, and cough with bloody sputum on 29 July 2009, he asked his wife not to contact him face-to-face and to sleep with him in a head-to-foot manner. When he took a bus to the Xinghai prefecture hospital for further treatment, he put a jacket over his head all the way. He did not contaminate any direct or indirect contacts. Patient K was sent by his elder brother-in-law to see another village doctor. The doctor had heard about a possible PPP outbreak and asked them to wear a mask before entering the clinics. The doctor also wore a mask when receiving them. Then, they were met by the quarantine personnel and assisted to the XCT hospital, and the patient was treated timely in the open air on the grassland for 6 h with transfusion of antibiotics before he was transferred to the hospital. No direct or indirect contacts were infected from this patient. Another example is the herdsman’s nephew who was not infected. He only contacted the herdsman’s corpse, indicating the relatively higher communicability of PPP during the bloody coughing period. Similarly, a Utah girl with pneumonic
plague contacted >200 people, and no one was contaminated, according to antibody analyses [8]. However, there is another report that some attendees of the funeral ceremonies for the patient with PPP were contaminated [6].

Most of the patients (6 of 11, excluding the index case) in this outbreak developed septicaemic plague complicated with shock, 2 of 11 developed multiple organ failure, and 1 of 11 developed meningitis. Effective antibiotics should be administered as early as possible because a delayed specific treatment will result in harmful outcomes [24]. Pneumonic plague reported recently were often traced to domestic or wild animal contacts, including cats [9], dogs [27–29], guinea pigs [11], and lions [3] or other wild animals [5]. This emphasized the important role of domestic animals in plague surveillance. Although the dogs were thought to be resistant to <i>Y. pestis</i> infection and a mediator to facilitate transfer of fleas as a source of bubonic or septicemic plague [27] and a surveillance sentinel by measuring the antibody to F1 in dog’s serum to predict the plague outbreak risk [29, 30], dogs indeed could be infected by <i>Y. pestis</i>, showing lethargy, pyrexia, and a purulent skin lesion [28]. To our knowledge, this is the first report to demonstrate pneumonic plague caused by dogs, which might contract plague from hunting a diseased or dead marmot, the main host of <i>Y. pestis</i> in the epidemic area. This result is significant for public health, because if a nonspecific fever and lethargy were found in a dog in a natural plague focus, we should keep in mind the possibility of <i>Y. pestis</i> infection. Although this outbreak was well-documented, it was difficult for us to determine a basic reproduction number for it because we could not identify whether there was a possible secondary transmission of plague among patients who lived in a poorly ventilated yurt.

The natural plague focus in Xinghai County, Qinghai Province, was first confirmed in August 1956 by isolating <i>Y. pestis</i> from a dead marmot. Since then, <i>Y. pestis</i> has been isolated from different hosts, including marmots and Mongolian 5-toed jerboas, and vectors, including fleas, ixodid ticks, and lice. The first outbreak of human plague occurred in September 1960, caused by flaying marmots. Twenty people were infected, and 7 of them died. In August 1962, 1 person died of septicemic plague caused by flaying marmots. There was no human or animal plague reported in this area from then until this outbreak. As revealed by MLVA analysis, the 2009 outbreak isolates were different from the 1962 isolates in Xinghai County (Figure 3), indicating that MLVA is a promising technology for source-tracing of suspected bacterial pathogen in a specific outbreak investigation, which has previously used for identifying the most likely source of 2 New York bubonic cases [31].

**Supplementary Material**

Supplementary materials are available at Clinical Infectious Diseases online (http://www.oxfordjournals.org/our_journals/cid/).

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**Potential conflicts of interest.** All authors: no conflicts.

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**Figure 3.** Relationship between 2009 outbreak isolates (larger red node) and other isolates from the same natural focus around Qinghai Lake. The origins of the isolates in panel A are shown in the map (B) by different colors. A. Minimal spanning tree with 46 VNTR loci [18, 19]. The isolating year and source of the strains are shown near the nodes, such as “1962 patient” for the upper-left smaller red node, indicating that this strain was isolated from a patient in 1962. Digits near the branches correspond to the number of VNTR loci differences between two nodes. The number in each node indicates the strain ID. B. Geographical distributions of the isolates analyzed in this study.
References