Serologic Survey of the Sentinel Animals for Plague Surveillance and Screening for Complementary Diagnostic Markers to F1 Antigen by Protein Microarray

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Abstract. Plague is a deadly infectious disease caused by the gram-negative bacterium, Yersinia pestis. In 2005, five plague patients were confirmed in the Yulong County of the Yunnan Province, China. In this study, the serologic survey of > 2,900 serum samples from domestic dogs and cats in and around the county, where human plague occurred, confirmed that domestic dogs and cats could serve as sentinel animals for plague surveillance. Meanwhile, the antibody responses in the infected dogs and cats were profiled by microarray containing 218 proteins of Y. pestis. In addition to F1, LcrV, YPCD1.28c, and YPO2118 induced humoral responses in all or most of the individuals, providing complementary candidates to F1 antigen for diagnostic markers of plague.

INTRODUCTION

Plague, a category A infectious disease caused by Yersinia pestis, is endemic in China, Mongolia, Burma, Vietnam, Indonesia, India, the large parts of South Africa, the United States, and South America.1

In China, there remain 12 natural plague foci, covering > 291 counties in 19 provinces.2,3 The Yunnan Province includes two plague foci, Focus F and Focus E,4 the Rattus flavivectus plague focus and the Apodemus chevriieri and Eothemomyis milietus plague focus, respectively. Y. pestis isolated from these foci belongs to biovar Orientalis and Antiqua, respectively.2 In October 2005, five people in the Yulong county of Yunnan province, where no plague had been reported in the last 100 years, experienced fever, cough, and hard breath. Two of them died in 3 days after onset of the symptoms. Detection of a specific antibody against F1 antigen in the sera of the three survivors by the passive hemagglutination assay (PHA) showed a 4-fold rise between the convalescent and post-convalescent phase. A field investigation for finding the cause and the ranges of this epidemic was initiated.

The domestic dog, one of the animal hosts of Y. pestis, seems to be highly resistant to plague and has an inapparent or mild disease if the plague bacterium does infect it.1 The serologic study of dogs can give us a clue of infectious origin and areas. In contrast to the domestic dog, orally infected domestic cats become acutely ill and generally develop buboes and pneumatic lesions similar to those seen in humans with plague. Over one third die within 10 days, whereas 44% become ill but recover.5 In the United States, there are some plague cases that acquired Y. pestis through contact with infected domestic cats.6 In China, the roles of the domestic cat in the spread and maintenance of Y. pestis are still unknown.

In this study, > 2,900 serum samples of domestic dogs and cats in and around the epidemic county were collected, and a serologic survey on antibodies against the F1 antigen was conducted by PHA to determine the infected range of animals. Meanwhile, to overcome the false-negative results caused by Y. pestis mutants lacking the F1 antigen in the serologic survey to plague, the antibody responses of these two animals to 218 proteins of Y. pestis were profiled by microarray to understand the immunoresponses for finding complementary diagnostic markers to the F1 antigen.

MATERIALS AND METHODS

Collection of serum samples. Serum samples from domestic cats (Felis catus). One hundred fifty-one serum samples of domestic cats in and around the village (blue dot and red dots in Supplementary Figure S1; found online at www.ajtmh.org) with plague epidemics in 2005 were collected. Eleven serum samples were collected from nearby counties (yellow dots in Supplementary Figure S1) where there is no reported human plague cases in the past 10 years.

Serum samples from domestic dogs (Canis familiaris). Six hundred eighty-nine serum samples of domestic dogs were collected from the villages where the serum samples of cats were collected. Two thousand one hundred thirty-six serum samples were collected from nearby counties.

All of the above serum samples were collected in May 2006, ~7 months after human infection. The serum samples were first collected from the village with human plague epidemics and then extended to the surrounding villages until no positive serum of domestic dogs or cats was detected according to the Yunnan Animal Care and Use Protocol. To collect the sera, the captured animals were anesthetized by barbital, and 1.5 mL blood was collected from the vein on the back leg of animals. The sera were separated and stored at −20°C until use.

Detection of antibodies against F1 by PHA. The anti-F1 antibody titers in the sera were detected by the PHA.9 The serum samples whose antibody titers against F1 antigen were ≥ 1:40 were considered positive. All the PHA measurements were validated by an accompanying inhibition assay.10 Meanwhile, all of the positive samples by PHA were confirmed by the F1 antigen-based up-converting phosphor technology.11

Diagnostic markers screening by protein microarray. The protein microarray included 218 proteins of Y. pestis and was constructed according to our previous reports12,13 (Supplementary Table S1; found online at www.ajtmh.org). The purified Y. pestis proteins were printed on silylated glass slides.
from CEL (Gene Company, Hong Kong) by allowing covalent binding by amino groups on the molecules that couple to aldehyde groups on the glass slide surface. The printed slides were stored at 4°C for further use. According to the results of PHA against F1 antigen, 14 serum samples of cats and dogs at different antibody titers (from 1:80 to 1:20,480 and from 1:80 to 1:1,280, respectively) were selected to profile the antibody amounts and response magnitude against the antigens on the microarray. Eight serum samples of cats and four serum samples of dogs that were negative for the F1 antibody were used as negative controls.

To determine the results in domestic cats and dogs, the cut-off value for each protein was calculated as the average value plus 2 SD of the fluorescence intensity (FLI) from the negative control sera. The proteins whose antibody increased in at least three individuals were considered to be immunogens in the animals.

RESULTS AND DISCUSSION

**Prevalence of serologic antibodies against F1 in domestic cats and dogs.** All the collected serum samples were initially screened by PHA to detect the IgG antibody titer against the F1 antigen. Using the result of PHA ≥ 1:40 as positive criterion, 162 of 689 (23.5%) sera from domestic dogs and 40 of 151 (26.5%) sera from domestic cats were positive (Table 1). None of the serum samples of dogs and cats from the control counties were positive for F1 antibody by PHA. The seropositive rates of 23.5% and 26.5% in domestic dogs and cats in and around the village where the human plague occurred were significantly higher than 0% in the dogs and cats from the regions in which plague was not identified in the past 10 years (Table 1; Supplementary Figure S1). It was concluded that the animals could serve as sentinel animals for plague surveillance, and seropositivity may be a clue for recent active prevalence of plague in the area.

The domestic dog plays an important role as a plague carrier, and serologic survey of them is an index for plague surveillance in China. Domestic cats can also be infected by *Y. pestis* through ingestion of plague-infected rodents or by the bite of infected fleas, and exposure to infected cats has recently been recognized in human plague in the United States.7,14 Our study showed that the seropositive rates of domestic cats in the villages around a human-infected village were much higher than that in villages further away (Supplementary Figure S1), and high antibody titers against the F1 antigen were detected in domestic cats (from 1:80 to 1:20,480), suggesting that domestic cats might play an important role in the spread of plague in the Yunnan Province and that serologic survey of cats would help determine the infected origin and range.

According to the results of the PHA, the positive serum samples of domestic dogs and cats were detected in 35 villages around the village with human plague patients, covering ~210 km² where no *Y. pestis* infection was reported in the past 100 years. This area has been classified as a novel natural plague focus by extended epidemiologic study and bacteriologic studies (unpublished data).

**Antibody profiling showed different immunologic responses of dogs and cats against *Y. pestis*.** To better understand the different susceptibility to *Y. pestis* between domestic dogs and cats, a protein microarray containing 218 known or putative virulence-associated proteins of *Y. pestis* was used in this study to profile humoral immune responses of domestic dogs and cats whose antibodies against the F1 capsule antigen were > 1:40. Figure 1 gives an overall picture of these antibody profiles for different hosts: 1) for domestic cats, antibody responses to 45 proteins were found in at least three individuals (Figure 1, @); 2) for domestic dogs, 26 proteins were identified as immunogens (Figure 1, &). In total, 59 proteins were detected to induce humoral immune responses by using the above-mentioned protein microarray. Thirty-four of them are hypothetical or putative proteins. The detection of antibodies suggests that the corresponding proteins can be expressed by the bacterium in vitro and/or during the course of infection. Overall, the numbers and response magnitude of antibodies in domestic cats were above those in domestic dogs (Supplementary Figure S2 and Table S2; found online at www.ajtmh.org). The immunogenicity of 33 proteins was found in domestic cats but not in dogs, indicating that these proteins might be expressed specifically when the bacterium invaded cats or is processed specifically by cat immune cells.

**Antibody responses to some known virulence proteins were different in domestic dogs and cats.** F1 capsular antigen (YPMT1.84), PsaA (YPO1303), and the proteins encoded by type III secretion system (T3SS) are important virulence proteins, which are involved in inhibiting phagocytosis of *Y. pestis* by macrophages and neutrophils.15–21 The antibody response intensities to these proteins were obviously different between dogs and cats. The antibodies to YPCD1.39c (YopN), YPCD1.19 (YopK), and YPCD1.67c (YopH) were only detected in cats and those to YPCD1.26c (YopM), YPCD1.28c (YopD), and YPO1303 (PsaA) were detected in more cats than dogs. Although the antibodies against YPMT1.84 (F1) and YPCD1.31c (LcrV) were detected in all tested sera of both hosts, their increasing magnitude in dogs was obviously lower than that in cats (Figure 1; Supplementary Table S2). The above results implied that the fewer bacteria proliferate in dogs for inducing the immune responses, which might partially explain the dog’s high resistance to *Y. pestis*. In contrast to the domestic dog, the bacteria should proliferate rapidly in cats, and the expression of virulence-associated proteins helps *Y. pestis* counteract the defense mechanisms of cats, resulting in the apparent symptoms. The detection of antibodies against these known virulence proteins and 37 other proteins in the sera of cats showed that they might be synthesized during disease development and played roles in plague pathogenesis.

**Proteins whose antibodies were detected in both hosts.** A total of 12 proteins induced humoral responses in both hosts (Figure 1, *). Because the infection in domestic dogs is transitory, the antibodies detected in them indicated the dominant immunogenicity of these proteins. Seven of them were found to be immunogenic in a previous study12,22,23, the other five were found to be immunogenic for the first time (Figure 1, $). YPO2125, along with YPO2118 and YPO2108, locate in

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<td>Analysis of sera from different domestic hosts by PHA</td>
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a 33-kb chromosomal fragment YPO2095-2135 that was absent from the biovar Microtus strains, which are supposed to be avirulent to humans, although they are highly lethal to mice; therefore, this fragment likely contributes to the ability to infect humans with fully virulent strains. The detection of antibodies against the proteins encoded by this fragment showed that this fragment could play a role in plague pathogenesis. Additionally, the obvious increase of antibody to YPO2118 in plague patients and EV76-vaccinated rabbits makes it a potential target for vaccine research.

**Potential complementary diagnostic markers to F1 antigen.** The detection of F1 antibody by PHA is a standard method for plague serologic surveillance in mammalians and serologic diagnosis of human plague. It should also be noted that serodiagnosis of plague by detecting the F1 antibody only could miss the detection of strains lacking F1, which have been isolated from a number of different host species and from human infection. Although many alternative methods such as PCR analysis and DNA hybridization for diagnosing plague have been developed, these methods are not widely available for on-site applications. The handheld diagnostic assay should be developed for field applications in the daily surveillance of plague.

The serologic methods such as PHA, enzyme-linked immunosorbent assay (ELISA), and biosensor-based enzyme immunosassay (EIA) are simple and can be performed in small hospital. The LcrV captured enzyme-linked immunosorbent assay has been developed to help confirm the diagnosis of plague. Our results also showed that LcrV could be used with F1 for surveillance purposes and epidemiologic study. In all of the animals tested, the antibody against LcrV increased dramatically. Given the variability of the LcrV antigen and the fact that no antibodies were detected in five Yunnan patients who were infected with *Y. pestis* in 2003, other antigens are needed to help serologic surveillance and diagnosis. Except F1 and LcrV, the antibodies against YPCD1.28c (YopD) and YPO2118 were detected in most of the animals, human patients, and experimentally infected rabbits. YPCD1.28c locates in the plasmid pCD1, which is conserved among all three pathogenic *Yersinia* spp. and is essential for...
the virulence of Y. pestis. The serodiagnosis with multiple proteins located both in the pCD1 plasmid (LcrV and YPCD1.28c) and chromosome (YPO2118) will complement the plague diagnosis based on the F1 antigen to avoid missing infections caused by the F1-negative strains.

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Note: Supplementary Table S1 (Y. pestis proteins represented on the microarray), Supplementary Table S2 (The original data of antibody fluorescent value against the protein on the Microarray), Supplementary Figure S1 (The geographic distribution of the soropositive rate in the satellite map of Yulong and around County) and Supplementary Figure S2 (Antibody profile by the protein microarray) appear online at www.ajtmh.org.

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