academicJournals

Vol. 8(4), pp. 389-397, 22 January, 2014
DOI: 10.5897/AJMR2013.6531
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Investigation of *Anaplasma phagocytophilum* among agrarian residents and domestic animals in Anhui Province, China

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Accepted 27 December, 2013

A total of 596 blood samples from agrarian residents, and 132 from goats, 12 from dogs and six from cattle were collected from 3 rural Counties including Guangde, Mingguang and Huaiyuan in Anhui Province. The survey questions presumed exposure risk of *Anaplasma phagocytophilum* were recorded for each participant and statistically analyzed. The antibodies against *A. phagocytophilum* were determined using an immunofluorescence assay (IFA) and the *A. phagocytophilum* 16S rRNA gene was amplified and analyzed for the DNAs of the blood samples of febrile participants and domestic animals using nested polymerase chain reaction (PCR). The average percentage of *A. phagocytophilum* was 44.6% for human, 33.3% for dogs, 0.8% for goats and 0% for cattle, respectively. The positive rate of the 16S rRNA gene (389 bp) of *A. phagocytophilum* in dogs, goats and cattle was 25.0, 33.3 and 0%, respectively. Two genotypes of *A. phagocytophilum* were identified and group A was dominantly endemic in Huaiyuan County while group B was mainly located in Guangde County. Living Mountain regions, outdoor activities, contacting animals, fever history, more than 3 h of working per day and more than 2 years of servers might be increased at risk of exposure *A. phagocytophilum* in these areas of Anhui Province.

Key words: *A. phagocytophilum*, seroepedemiology, prevalence, Anhui Province of China.

INTRODUCTION

Human granulocytic anaplasmosis (HGA) is an emerging tick-borne zoonoses that is caused by the obligate intracellular bacteria *Anaplasma phagocytophilum* (Chen et al., 1994; Walker et al., 2008; Chapman et al., 2006; Dumler et al., 2001; Petrovec et al., 1997). In 2006, an unusual nosocomial human-to-human transmission of HGA occurred in a hospital in Anhui Province (Zhang et al., 2008a). In this event, five relatives of the index patient and four medical workers who participated in rescuing the index patients were secondary infected for direct contacting with blood or respiratory secretions. Despite clear laboratory evidence of an outbreak of anaplasmosis, most cases of HGA is likely misdiagnosed or underrecognized because of limited epidemiological,

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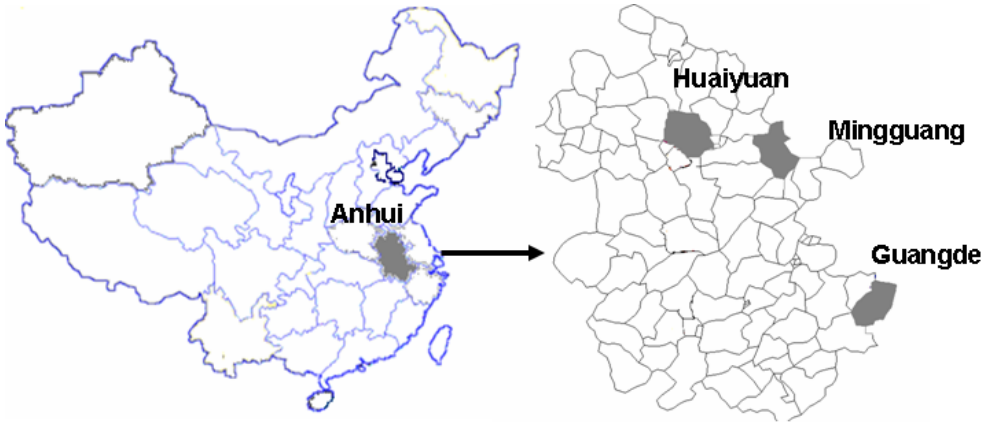

Figure 1. Map of Anhui Province and the 3 investigated sites.

clinical and microbiological data in these areas of Anhui Province (Wu et al., 2010; Cao et al., 2010). Therefore, an investigation to assess the epidemiologic status of emerging infectious diseases caused by *A. phagocytophilum* among farm residents and domestic animals in Guangde County (Figure 1), where the index patient from the nosocomial transmission of HGA lived, and in Huaiyuan County and Mingguang City in Anhui Province was undertaken by a collaboration project between the Department of Anaplasma and Rickettsiology, National Institute for Communicable Disease Control and Prevention, China CDC and the Department of Epidemiology, Centers for Disease Control and Prevention of Anhui Province from April to May in 2009.

MATERIALS AND METHODS

Ethics statement

The study and the protocol of field investigation were approved by the China CDC Institutional Review Board (No.201103). A written consent form was obtained from each participant before sampling blood. Parents informed consent on behalf of their child. All procedures of sampling animal blood were conducted to conform to institutional guidelines for the care and use of laboratory animals as described by the China CDC, Beijing, China, and confirmed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Sampling animal blood was conducted after the owners consented.

The survey sites and sampling

According to the geographic location and previous epidemiological information on rickettsia infection (Zhang et al., 2008a), Guangde County, which lies in the south areas of Anhui, was selected because the first outbreak of HGA in China occurred there (Figure 1). The other two sites studied were Huaiyuan County and Mingguang City, which are located in the north and east areas of Anhui Provence, respectively (Figure 1). Guangde County and Mingguang City are typical hill and mountainous areas and most local people make a living by farming, raising domestic animals and growing fruit trees. Huaiyuan County is a typical plain area, and crop farming is the primary occupation there. As in many other mountainous areas, goats, dogs and cattle are the most common domestic animals raised in Guangde County and Mingguang City, and all of these animals are bred outside during the day in spring and summer. A high density of ticks was observed on the body of animals although pesticides were being sprayed twice (morning and evening) a day to kill them.

In each County, three to five villages were chosen based on their geographic locations (for example, the eastern, southern, western, northern and central areas of each). Participants were randomly selected based on the odd or even number of each registration permanent resident. The information collected included demographics; gender; age; living areas; past medical history; occupation (outdoor activities or not, if unemployment such as retried people, housewives, students and preschool children); contact with domestic animals including dog, cat, cattle, sheep or goat; and exposure to ticks or bitten by ticks; length of working time per day and length of service time; All participants were asked if they had a fever on the day of the survey and whether they had high fever (temperature of 38.0≥°C) during the preceding 12 month and if so, we asked their body temperature and the main clinical manifestation such as myalgias or headache, and whether they had received any antibiotic, and if so, what types. Two ml sample of non-anticoagulated blood was collected for each participant. At the same time, three or five of goats or sheep or cattle, and 1 or 2 dogs were selected for sampling blood. Five-ml of non-anticoagulated blood was collected from each animal if the participant owned domestic animals and consented the animal blood sampling. In the local County CDC, Sera were separated to test antibody and the remaining blood clot of the febrile person and the animals were used to extract DNA using a DNeasy® Blood and Tissue Kit (QIAGEN, Cat No. 69506) according to the manufacturers' instructions. All the samples were stored at -20 or -40°C at local CDC and then transferred to the Department of Anaplasma and Rickettsiology, National Institute for Communicable Disease Control and Prevention, China CDC by air within 48 or 72 h for laboratory test.

Antibody assay

Immunofluorescence assays (IFA) were performed as previously described (Bakken et al., 2002; Raoult et al., 1985). *A. phagocytophilum* (Webster strain) crude antigen and its positive human serum were provided by the J. S. Dumler at the Johns Hopkins University School of Medicine, USA. In order to evaluate the

the reactivity with other rickettsiae, another commen 6 rickettsiae crude antigen were simultaneously tested by IFA. These rickettsiae antigen were as follows: *E. chaffeensis* (Arkansas strain) antigen was provided by Dr. Robert Massung at the United Stated CDC. *Rickettsia typhi*, *Orientia tsutsugamushi* types Karp, *Rickettsia heilongjiangensis*, *Bartonella henselae* and *Coxiella burnetii* were kindly provided by Dr. Didier Raoult from WHO Rickettsial Diseases Collaborating Center (Marseille, France).

The IFAs were simply performed as follows. The serum samples were diluted 1:40 (IgM) or 1:80 (IgG) in PBS containing 3% nonfat powdered milk, and 25 µl of the diluted serum was placed in a slide well and incubated for 60 min in a moist chamber at 37°C. After washing in PBS to remove unbound antibody, the slides were labeled with FITC-conjugated rabbit anti-human immunoglobulin (IgM or IgG; Sigma Co., NY, New York State, United States) as a secondary antibody, which was diluted 1:400 with Evans blue, for another 60 min in a moist chamber at 37°C. The slides were then washed in PBS to remove unbound secondary antibody. The slides were air dried at 37°C and examined using a fluorescent microscope (Nikon, Tokyo, Japan). Samples were interpreted as reactive if there was strong green fluorescence corresponding to bacterial morulae within the cells on the slide. PBS-milk and the mixed sera from healthy human (workers in our institute but not members of our laboratory) were used as negative control respectively. Samples that were reactive at the 1:80 screening dilution were deemed IgG positive based on the reference criteria (Bakken et al., 2002; Raoult et al., 1985) and IgM titer of 1:40 were considered positive. In order to reduce the perform errors, every 4 antigens mentioned above were spotted in different rows in the same slide. If a serum sample had reactivity with the other 6 reckettsial antigens mentioned above, further dilution and titration were conducted, and a two-fold or higher titer increase was read as positive.

Amplification and sequencing of the 16S rRNA gene

A previously developed nested PCR assay, based on the 16S rRNA gene (389bp) of *A. phagocytophilum,* was performed (Wen et al., 2002) with the DNAs extracted from febrile patients' blood and animals' blood as templates. Sterile deionised water and DNAs extracted from healthy person, goat, sheep, dog and cattle were used as a negative control, and the DNA of *A. phagocytophilum* strain Webster, which kindly provided by the J. S. Dumler at the Johns Hopkins University School of Medicine, USA, was used as a positive control. Positive results were confirmed by commercial sequencing (Shanghai Shengong Biotechnology Co.) using an ABI 3730 sequencing apparatus (Life Technologies, USA) and a Bigdye Terminator V3.1 sequencing kit. The sequences were compared with sequences in GenBank by Blast (http://www.ncbi.nlm.nih.gov/). All of the sequences obtained from the domestic animals or febrile patients were deposited in GenBank.

Statistical analysis

Statistical analysis was conducted using SAS software (version 9.1, SAS Institute, Inc., Cary, NC). A comparison of the prevalence in human and animals from different areas was performed using the $\chi 2$ and Fisher's exact tests. Age was converted into a categorical variable (2 -19, 20 - 29, 30 - 39, 40 - 49, 50 - 59 and >60 years of age). $\chi 2$ and Fisher tests were used to compare distributions of seropositivity or to examine association between pairs of categorical measures. Logistic regression analysis was used to calculate odds ratios for seropositivity among variables. The survey questions regarding variables "living plain areas", "living hilly regions", outdoor activity or works", "livestock breeding or contact domestic animals", "length of working hour per day" and "length of service time" were created to the associated with presumed risk among permanent residents of agrarian areas. The significance for these analyses was defined as a P value of 0.05. Phylogenetic analysis was conducted using MEGA 4.0 software and phylogenetic tree was constructed by using neighbor-Joining (NJ) methods.

RESULTS

Survey people and animals

From April to May in 2009(tick season), a total of 596 farm residents from the 3 sites investigated were enrolled in the study. Of those who participated, 244 were male (average age 50 years, range, 5 - 76 years), and 352 were female (average age 49 years, range, 4 - 66 years). Ninety five percent of people investigated were engaged in outdoor activity (92% of people for farming and 3% of person for feeding domestic animals and planting fruit trees) and their working time were all more than 3 h per day at least 4 days per week. Five percent of investigated individuals were preschool children, students, housewives and elderly people who were retired and could not work anymore. Ninety five percent of residents owned or contacted with domestic animals. Person reported that they had been bitten by ticks in the past two years accounted up 10.6% and all of the people recognized ticks but nobody could tell the species of the ticks. Nobody was bitten by lice or flea but all of the people recalled that they had been by mosquitoes during the last summer or autumn. About 7.7% residents recalled fever in the last 12 months and most people (85%) had headache and generalized myalgias but nobody could describe what drug had been used. Five residents had fever (≥38°C) on the day of their survey and all people had fatigue weakness and 3 of them had headache.

In this survey, a total of 132 blood samples were collected from 114 goats, 12 from dogs and 6 from cattle owned by the families of the participants during the tick season in 2009. A high density of ticks was noticed on the bodies of the animals although the pesticides were daily used. The detail geographic distribution of the animals was summarized in Table1.

Serological detection

The seroprevalence of *A. phagocytophilum* among people was shown in Table 1. There were 6 samples existing weak reactivity with *E. chaffeensis* at 1:80 cut off, and these samples were confirmed to be *A. phagocytophilum* by further titration using Fuller diagnose kit of *A. phagocytophilum* and *E. chaffeensis*, in which the 2 antigens of *A. phagocytophilum* and *E. chaffeensis* were spotted in the same well of the slice in order to avoid of laboratory errors. Overall, the average seropositive rate of *A. phagocytophilum* among farm residents was 44.6%. Of the 3 sites investigated, Guangde County

Table 1. Seroprevalence of *A. phagocytophilum* in humans and animals.

Area	Human %（No. of positive/No. of tested sera）				χ^2 test			Total			Animal% (No. of positive/No. of tested sera)		
	Male		Female										
	Seropositive rates%	95%CI	Seropositive rates%	95%CI	P value	OR	95%CI	Seropositive rates%	P value	OR(95%CI)	Goat	Cattle	Dog
Huaiyuan	9.3(10/108)	3.8-14.8	11.3(16/141)	6.1-16.5	1	0.8	0.35-1.83	10.4(26/249)	<0.0001[a]	0.08（0.05-0.14）	0(0/43)	0(0/1)	0(0/5)
Mingguang	40.3 (21/52)	27-53.6	69.5 (66/95)	60.2-78.8	0.37	0.3	0.15-0.6	59.2(87/147)	0.0006[b]	0.45（0.28-0.71）	0(0/38)	0(0/5)	57.1(4/7)
Guangde	83.3 (70/84)	75.3-91.3	71.5 (83/116)	63.3-79.7	0.59	2	0.99-4.0	76.5%(153/200)	<0.0001[c]	27.9（16.6-47.0）	2.0(1/51)	-	-
Total	41.4 (101/244)	35.2-47.6	46.9 (165/352)	23.7-33.1	0.82	1.8	1.3-2.5	44.6(266/596)	-	-	1.5(2/132)	0(0/6)	33.3(4/12)

a: Huaiyuan vs. Mingguang, b: Mingguang vs Guangde, c: Guangde vs Huaiyuan.

had the highest seroprevalence (76.5%), and Huaiyuan County had the lowest (10.4%). The seroprevalence of *A. phagocytophilum* in Guangde were significantly higher than in Huaiyuan (p<0.0001, OR 27.9, 95%CI 16.6, 47.0) and in Mingguang (p=0.0006, OR 0.45, 95%CI 0.28, 0.71), respectively. Moreover, the seroprevalence of *A. phagocytophilum* in Mingguang was higher than in Huaiyuan (p<0.0001, OR 0.08, 95%CI 0.05, 0.14). However, the differences between the male and the female residents in each County investigated was not statistically significant (Table 1). Statistical analysis of age distribution revealed that the seroprevalence differed across strata of age and the seroprevalence increased with age growth (Table 2). For the 5 people who were febrile on the day investigated in the study, 2 sera reacted with *A. phagocytophilum* in IFA testing. The IgG antibody titers of these 2 patients were 1:160 and 1:80 respectively while the IgM antibody titer were 1:80 and 1:80, respectively. However, no confirmed diagnoses were made because sera from the patients' covalent stage of illness were unavailable.

Considering the questionnaires in the study, Living Mountain or hill regions, outdoor activities, contacting animals, fever history in the last 12 month, more than 3 h of working time per day and more than 2 years of servers length were associated with the high seroprevalence of the participants (Table 3).

The total seropositive rate of the 3 species of animals in the study was calculated because of the limited number of samples (Table 1). The seroprevalence of *A. phagocyophilum* in dogs, goats and cattle was 33.3, 0.8 and 0%, respectively.

The seroprevalence between dogs and goats was statistically significant (p<0.001) as was the difference between dogs and cattle (P<0.001). However, the difference in seroprevalence between cattle and goats was not statistically significant (P=1.00). There was no reactivity with the six other common rickettsiae mentioned in the part of material and methods at 1:80 cut off, and only 1 dog sample showing weak reactivity with *E. chaffeensis* at 1: 80 dilution sera, but failed to be reactivity with *E. chaffeensis* at 1:160.

Molecular analysis

The 16S rRNA gene of *A. phagocytophilum* was PCR amplified from 33.3, 25.0 and 0% of the goat, dog and cattle blood samples, respectively. The 44 goat sequences deposited in GenBank included 24 sequences from Huaiyuan (GQ499896,GQ499897,GQ499898,GQ499899,GQ499900,GQ499901,GQ499902,GQ499903,Q499904,GQ499905,GQ499906,GQ499907,GQ499914,Q499909,GQ499895,GQ499910,GQ499911,GQ499912,GQ499913,GQ499915,GQ499916,GQ499917,GQ499918,GQ499930), 9 from Mingguang(GQ499922,GQ499923, GQ499921,GQ499924, GQ499927, GQ499925,GQ499926,GQ499932, GQ499928) and 11 from Guangde(GQ499885,GQ499886,GQ499887,GQ499888 GQ499889, GQ499890,GQ499891, GQ499892, GQ499893,GQ499894, GQ49993). The 3 dog sequences included 2 from Mingguang (GQ499919, GQ499920) and 1 from Huaiyuan (GQ499929).

Based on the analysis of Blast and alignments, 15 represented sequences were selected from the sequences obtained in the study for constructing the phylogenetic tree. In addition, some sequences of *A. phagocytophilum* identified in different ticks, animals, patients from different areas of China and counties around of China as

Table 2. Seroprevalence of *A. phagocytophilum* in humans by age.

Age group	Seropositivity rate% (No. of positive/No. of tested)	Age group					
		2 - 19	20 - 29	30 - 39	40 - 49	50 - 59	>60
2-19	28.8(15/52)	-	0.4[a] 0.7[b] (0.4, 1.5)[c]	0.06 0.5 (0.3, 1.0)	0.01 0.4 (0.2, 0.8)	0.009 0.4 (0.2, 0.8)	0.006 0.3 (0.1, 0.7)
20-29	35.7(40/112)		-	0.2 0.7 (0.4, 1.2)	0.03 0.6 (0.3, 0.9)	0.03 0.5 (0.3, 0.9)	0.02 0.4 (0.2, 0.9)
30-39	43.8(64/146)			-	0.3 0.8 (0.5, 1.3)	0.3 0.8 (0.5, 1.2)	0.2 0.6 (0.3, 1.2)
40-49	50.0(66/132)				-	0.9 1 (0.6, 1.6)	0.5 0.8 (0.4, 1.5)
50-59	51.0(53/104)					-	0.6 0.8 (0.4, 1.6)
>60	56.0(28/50)						

a: P value, b: odds ratio, c: 95%CI.

Table 3. Analysis of survey questions which presumed risks for *A. phagocytophilum* among agrarian residents.

Variable	No. (%) of resident					
	Total cohort (n=596)	IFA positive (n=266)	IFA negative (n=330)	OR	95%CI	P Value
Living plain areas	249(41.8)	26(9.8)	223(67.6)	0.05	(0.03,0.08)	<0.0001
Living hill or mountain areas	347(58.2)	240(90.2)	107(32.4)			
Outdoor activity	566(95.0)	261(98.1)	305(92.4)	4.3	(1.6,11.3)	0.002
Contact farm animals	566(95.0)	259(97.4)	307(93.0)	2.7	(1.2,6.6)	0.02
Tick bite in last 24 month	60(10.6)	25(9.4)	35(10.6)	0.9	(0.5,1.5)	0.6
Fever in last 12 month	46(7.7)	5(1.9)	41(12.4)	0.1	(0.05,0.4)	<0.0001
Working time >3 h per day	566(95.0)	261(98.1)	305(92.4)	4.3	(1.6,11.3)	0.002
Service time>2 years	536(90.0)	256(96.2)	280(84.8)	4.6	(2.3,9.2)	<0.0001

well as other areas of the world were included (Figure 2). There were two dominant genetic groups of *A. phagocytophilum* in the surveyed areas based on the Phylogenetic analysis and they were distributed in two distinct geographic areas. Group A was mainly found in Huaiyuan, which is in the north plain areas of Anhui Province, and Group B was found in Guangde, which is in the southeast. Both groups of *A. phagocytophilum* were found in Mingguang City, which is in east Anhui Province. Notably, a total of 10 sequences obtained from the animals in the study were 100% homologues with the sequences (EF211110) from patients involved in the nosocomial outbreak of anaplasmosis in Guangde County, Anhui Province, in 2006 (Zhang et al., 2008a) and the other sequences from patients in Yiyuan County (EU982709), Shandong Province (Zhang et al., 2011), and these sequences were grouped in the same group (group B), which was predominantly distributed in the Guangde area. None of the positive PCR results was found on the 5 DNA samples from the febrile person

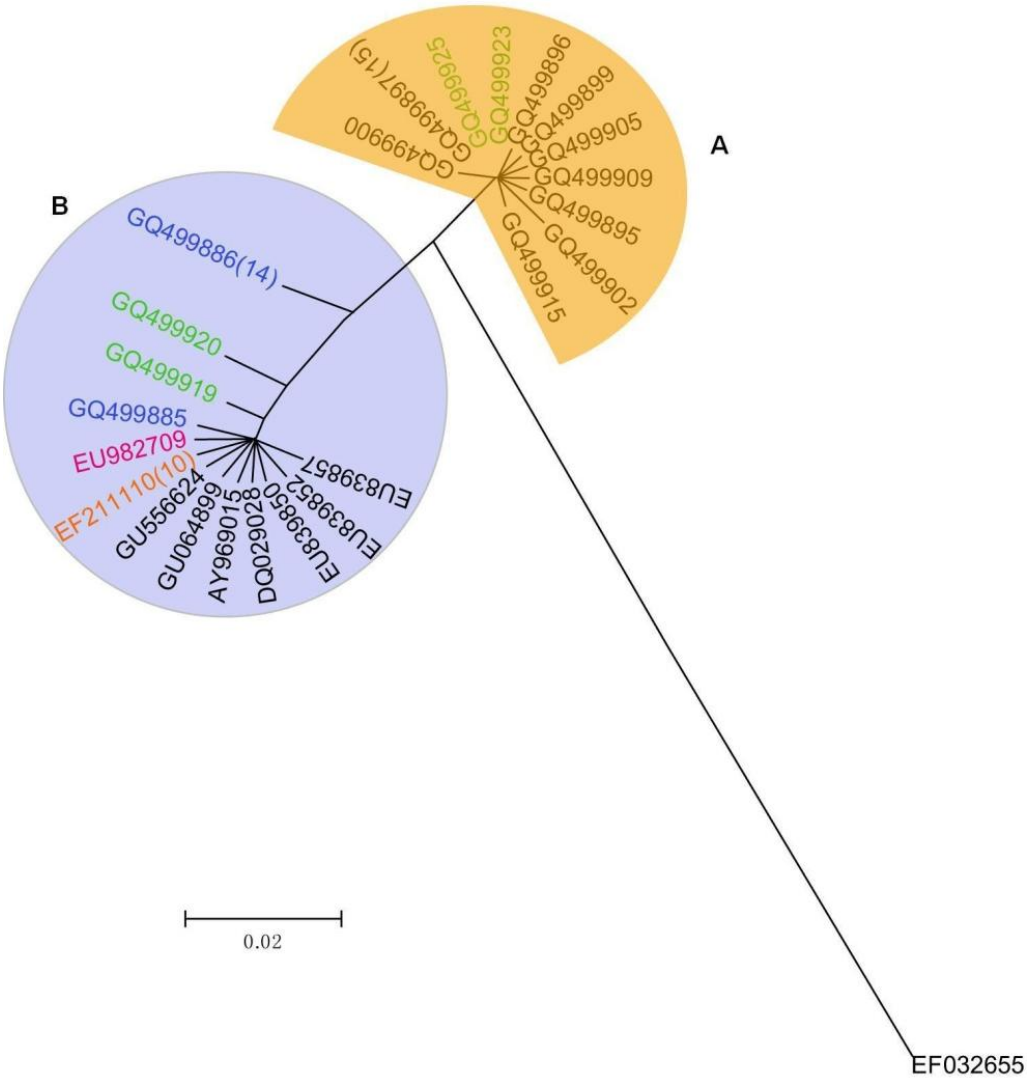

Figure 2. Phylogenetic analysis of *A. phagocytophilum* from goat, cattle and dog blood samples collected in Guangde, Mingguang and Huaiyuan Counties in Anhui Province. The tree was based on the partial segment sequences (389 bp) of *A. phagocytophilum* 16SrRNA by using neighbor-joining (NJ). In group A: green sequences were from goats in Mingguang County in this study, and the black sequences were from goats in Huaiyuan County in this study. In group B: orange sequences were from patients in unusual nosocomial outbreak of HGA in Anhui Province in 2006; purple sequences were from patients in Yiyuan County, Shandong Province; green sequences were from dogs in Mingguang County in this study; blue sequences were from goats in Guangde County in this study, and the black sequences were from different hosts, vectors and patients identified in other countries. The numbers in parentheses indicate the number of isolates in each genotype. EF032655: reference sequence (alpha proteobacterium SepB-6 16SrRNA).

investigated in the study.

DISCUSSION

In 2006, we first reported the identification of HGA in China and first demonstrated nosocomial human-to-human transmission of HGA (Zhang et al., 2008a). In 2009, Zhou et al further reported the epidemic characterizes of HGA in this event and indicated that the infection rate of people contacting with the index patient after onset of illness was 14.3% while the incidence of people contacting the index patient with critical ill was 23.1% (Zhou et al., 2009). However, the seroepidemiological, clinical and microbiological information about HGA is very limited in these areas and the disease is likely underreported (Wu et al., 2010; Cao et al., 2010). Unlike HGA cases reported from US or European coun-

tries, Chinese HGA patients represented severe clinical manifestations and 52.1% of patients had systemic inflammation response syndrome (SIRS) and 34.2% of patients rapidly developed multiple organ dysfunction syndrome (MODS), and the fatality rate of Chinese HGA patients has been reported to be as high as 26.5% (Li et al.,2011). A more recent nationwide etiological investigation of HGA was conducted and a total of 46 confirmed and 16 probable HGA cases were obtained from 2009-2010. Among these cases, 41.2% of patients were diagnosed with multiple organ dysfunction syndrome (MODS), and the fatality rate was as high as 8.1% (Zhang et al., 2013). Four human HGA isolates and one tick isolate was obtained in this project mentioned above and the 16S rRNA gene (750bp) of the 5 Chinese HGA isolates were 100% identical to the sequences (EF211110) from patients involved in the nosocomial outbreak of anaplasmosis in Guangde County, Anhui province, in 2006 (Zhang et al., 2008a) and the other sequences from patients in Yiyuan County (EU982709), Shandong Province. This dominant sequence of HGA 16S rRNA gene accounted for up to 60% of the sequences identified in *H. longicornis* from Laizhou, Shandong Province (Zhang et al., 2013).

It is reported that HGA is a nonspecific febrile tick-borne illness and the infected patients generated specific antibody at 2 week and peaked at 4 week after the onset of clinical sympotoms (Bakken et al., 1996). Nearly 50% of patients maintain serum IFA antibody titers at 1:80 or higher for 18 months or longer and some people could remain seropositive for as long as 42 month (Bakken et al., 2002).

In this study, we conducted a broad seroepidemiological investigation of *A. phagocytophilum* among agrarian residents and domestic animals in Anhui Province; that was focused on Guangde County, where the index case of the nosocomial human-to-human transmission of HGA lived (Zhang et al., 2008a). The average seroprevalence (44.6%) of *A. phagocytophilum* among people in 3 surveyed sites is significantly higher than the 16.3% (Zhang et al., 2011) and the 2.8% (Cao et al., 2010) that was observed in a previously studies in Anhui. The reasons caused this big differences might be related with the different strains of *A. phagocytophilum* as antigens for IFA. In another words, the endemic strain of *A. phagocytophilum* in these areas is more genetically related with the *A. phagocytophilum* Webster strain used in this study.

Similarly, the seroprevalence (44.6%) obtained in the study is higher than the 8.8% of Tianjin areas (Zhang et al., 2008b) and it is also higher than the average seroprevalence 7.1% (from 2.4% in Henan Province to 17.6% in Shanxi Province) reported from a recent serological investigation in China (Hao et al., 2013) and higher than the average seroprevalance 14.1% of Beijing rural areas (Zhang et al. 2012). However, it is similar to the rates that have been reported in endemic areas in US (Dumler et al., 2005; IJdo et al., 2000). Notably, this study found that the seropositive rate of *A. phagocytophilum* among people in Guangde County is as high as 76.5% (with a cut off value of 1:80). We also found that the seroprevalence in humans from the mountainous areas (Guangde and Mingguang) was significantly higher than that from humans in the plain areas (Huaiyuan). In addition, contacting animals, outdoor working, fever history in the last 12 month, more than 3 h working per day and more than 2 year service were all independent risks of exposure to *A. phagocytophilum*. However, the seropositive rates between the males and the females in the investigated sites were not statistically significant.

As a zoonotic pathogen, a wide variety of *A. phagocytophilum* strains circulate in different animal systems and some may cause zoonoses and some may not infect human. In this study, 3 species of animals were included and the seropositive rate of *A. phagocytophilum* were 33.3% for dogs, 0.8% for goats and 0% for cattle and the PCR positive rates of 16SrRNA gene were 33.3% for dogs, 25.0% for goats and 0% for cattle. There were not any positive evidences of *A. phagocytophilum* identified in cattle, which we proposed that one of the reasons is the limited samples (6 blood samples). In addition, the *A. marginale* that was considered as one of the most common pathogen for cattle anaplasomosis was unavailable in our laboratory. A big difference was noted between the serological positive rates and PCR positive rates for goats and this might be related with the different immunogenicity between the domestic strains of Anaplasma in China and the abroad strain of *A. phagocytophilum* Webster used in the study. However, the seropositive rate and the PCR positive rate for dogs were as high as 33.3 and 33.3%, respectively. Furthermore, phylogenetic analysis demonstrated that these sequences from dogs were grouped as the same clad (Group B) with the sequences from patients involved in the nosocomial transmission of HGA in Guangde County in 2006 (Figure 2).

There are two varieties of *A. phagocytophilum* from animals based on the phylogenetic tree constructed in this study and a striking geographic distribution was noticed between these two genetic groups. Group B is represented by the sequences from patients in the nosocomial outbreak of HGA in 2006; a patient (EU 9827709) in Yiyuan County; and 60% of *Haemaphysalis longicornis* collected from Laizhou Bay (Zhang et al., 2013), Shandong province; and water deer (GU556624) from Korea and *H. longicornis* (GU064899) collected from Jeju Island in Korea. This clad was also detected in *Ixodes ovatus* (AY969015) in Japan (Ohashi et al., 2005). In addition, a sequence that is 100% homologous to group B was identified in an Italian patient (DQ029028) with HGA from Sicily (de la Fuente et al., 2005), in wild ruminant animals (EU839850) and in 2 horses (EU839857 and EU839852) that were infected with *A. phagocytophilum* in the Czech Republic (Zeman and Jahn, 2009).

Group A was mainly endemic in Huaiyuan County, but sequences that were 100% homologous were also identified in goats (HM439432) from Zhejiang Province and in goats (FJ389576) from southeast China (Zhou et al., 2010), in *H. longicornis* (GU064899) collected from Jeju Island in Korea, and in deer (AB454075) from the Nara park in Japan. However, no sequences from patients grouped into this clad.

As an emerging tick-borne infectious disease, this study is the first and largest serological survey of *A. phagocytophilum* in Anhui Province based on the special and unusual nosocomial outbreak of HGA in 2006. The local documents from Anhui Provincial CDC (Zhou et al., 2009; Wu et al., 2010; Cao et al., 2010) and the sero-logical and molecular evidences in the study indicated that the rural residents especially residents in mountain or hill are at increased risk of *A. phagocytophilum* exposure.

Further systematic surveillance, including the role of the vector and host and monitoring of the trans-mission of HGA in these areas should be performed in the future. Etiological investigation based on the isolation of agents is urgently needed and differential diagnosis of HGA in clinical practice should be emphasized.

ACKNOWLEDGMENTS

We thank all of the epidemiologists, technicians and administrative personnel from the 9 villages in the 3 Counties in Anhui Province that were surveyed in this study. We would like to thank Dr Didier Raoult for providing the *Rickettsia* strains for this study. We also thank Robert Massung from the U.S. CDC for providing the *E. chaffeensis*. We appreciate Dr. J. S. Dumler at The Johns Hopkins University School of Medicine for providing the *A. phagocytophilum* Webster antigens for IFA. This study was supported by the National Basic Research Program of China (973 Program-2010CB530206) and the National Key Science and Technology Projects of China (Project no.2008ZX10004-008 and 2012ZX10004215).

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