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Anaplasmosis in farmers and domestic animals in Anhui province, China

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ABSTRACT

Objective: To investigate the epidemiological status of anaplasmosis among farmers and domestic animals in Guangde County where the unusual nosocomial human to human transmission of human granulocytic anaplasmosis occurred in Anhui province and the index patient in this event lived, as well as Huaiyuan County and Mingguang City in Anhui province. **Methods:** From April to May in 2009, 596 farmers, 132 goats, 12 dogs and 6 cows was collected from Guangde, Mingguang and Huaiyuan counties and the IgG antibodies against *A. phagocytophilum* in farmers, goats, dogs and cattle was determined using an immunofluorescence assay (IFA). The *A. phagocytophilum* 16S rRNA gene was amplified from blood samples from domestic animals using nested PCR, and the genetic diversity of the 16S rRNA gene was analysed. **Results:** The percentage of farmers with IgG antibodies against *A. phagocytophilum* in the 3 survey counties was 33.7%, and the individual percentages for Guangde, Mingguang and Huaiyuan counties were 76.5%, 59.2% and 10.4%, respectively. The total seroprevalence in dogs, goats and cattle was 33.3%, 0.8% and 0%, respectively. The percentage of samples positive for amplification of the 16S rRNA gene of *A. phagocytophilum* in goats, dogs and cattle was 33.3%, 25.0% and 0%, respectively. Analysis of the genetic diversity of the 16S rRNA gene showed that there were two genotypes of *A. phagocytophilum*. Group A was endemic in Guangde County, which is located in the southeast of Anhui province, where the nosocomial human granulocytic outbreak of anaplasmosis in 2006 occurred. Group B was located north of Huaiyuan County. **Conclusions:** We concluded the prevalence of *A. phagocytophilum* among farmers and domestic animals in Anhui province was demonstrated, and diagnosis and differential diagnosis of zoonotic “rickettsial” infections should be emphasized in clinical practice. A systemic ecological survey should be conducted to reduce the public health threat to humans and animals.

1. Introduction

Anaplasmosis, or human granulocytic anaplasmosis (HGA) is an emerging tick-borne zoonoses that is caused by the obligate intracellular bacteria *Anaplasma phagocytophilum* (*A. phagocytophilum*) [1,2]. In 2006, an unusual nosocomial

human-to-human transmission of HGA occurred in Anhui province [3]. Despite clear evidence of an outbreak of anaplasmosis, few epidemiologic investigations into human and domestic animal infections have been performed in this area. Therefore, an investigation to assess the epidemiologic status of emerging infectious diseases caused by *A. phagocytophilum* among farmers and domestic animals in Guangde County, where the index patient from the nosocomial transmission of HGA lived, and in Huaiyuan County and Mingguang City in Anhui Province was performed from April to May in 2009.

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2. Materials and methods

2.1. The survey sites and sampling

According to the geographic location and previous epidemiological information on rickettsia infection, Guangde County, which lies in the south areas of Anhui, was selected because the first outbreak of anaplasmosis in China occurred there. The two other sites studied were Huaiyuan County and Mingguang City, which are located in the north and east areas of Anhui Province, respectively (Figure 1). Farmer families were randomly selected for survey from the registered residences. The information collected included demographics; gender; age; occupation; contact with pigs, cattle, sheep or poultry; and exposure to ticks, mites and fleas. Approval for the study was obtained from the China CDC Institutional Review Board.

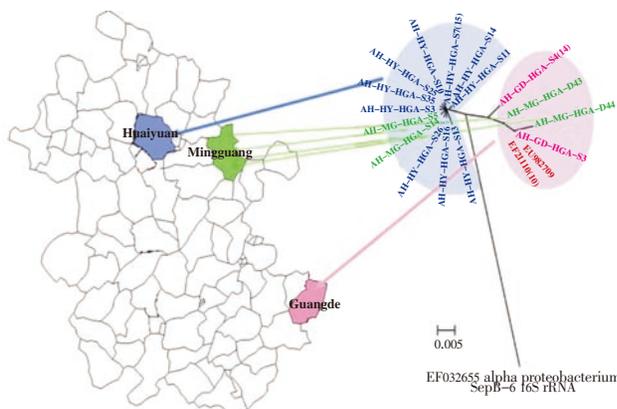


Figure 1. Genetic characteristics of *A. phagocytophilum* from goat, cattle and dog blood samples collected in Guangde, Mingguang and Huaiyuan Counties in Anhui province. The tree was based on the partial segment sequences by using neighbor-joining (NJ).

2.2. Antibody assay

Immunofluorescence assays were performed as previously described[4]. *A. phagocytophilum* (Webster strain) antigen was prepared by the Johns Hopkins University School of Medicine, USA. An additional *A. phagocytophilum* (RA2682, lot 03–0403N) antigen was provided by the United States CDC. The positive control serum was prepared from rabbits immunized with *A. phagocytophilum* in our lab. Samples that were reactive at 1:80 screening dilution were deemed positive. *Ehrlichia chaffeensis* (*E. chaffeensis*) (provided by the United States CDC) and *Rickettsia typhi* (*R. typhi*), *Orientia tsutsugamushi* (*O. tsutsugamushi*) types Karp, *Rickettsia heilongjiangensis* (*R. heilongjiangensis*), *Bartonella henselae* (*B. henselae*), *Coxiella burnetii* (*C. burnetii*) [provided by the WHO Collaborating Centre for Rickettsial Reference and Research (Marseille, France)] were also used

to evaluate the cross-reaction between each of them and *A. phagocytophilum*.

2.3. Amplification and sequencing of the 16S rRNA gene

DNA was extracted using the QIAamp blood and tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Sterile deionised water was used as a negative control, and a culture of *A. phagocytophilum* was used as a positive control. A previously developed nested polymerase chain reaction (PCR) assay, based on the 16S rRNA gene of *A. phagocytophilum*, was performed[5]. Positive results were confirmed by commercial sequencing (Shanghai Shengong Biotechnology Co.) and compared with sequences in GenBank.

2.4. Statistical analysis

Statistical analysis was conducted using SAS software (version 9.1). A comparison of the prevalence in animals from different areas was performed using the χ^2 and Fisher's exact tests. The significance for these analyses was defined as a *P* value of less than 0.05. Phylogenetic analysis was conducted using MEGA 4.0 software and phylogenetic tree was constructed by using neighbor-joining (NJ) methods.

3. Results

3.1. Survey area and lifestyle

Guangde County and Mingguang City are typical mountainous areas, and most local farmers make a living by raising domestic animals and growing fruit trees. Huaiyuan County is a plain, and crop farming is the primary occupation there. As in many other mountainous areas, goats, sheep 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 used daily to kill them. In total, we enrolled 596 farmers from the 3 areas investigated in the study. Of those who participated, 244 were male (average age 50 years), and 352 were female (average age 49 years). In addition, serum from 132 goats, 12 dogs and 6 cows was collected for the study.

3.2. Serological detection

The seroprevalence of *A. phagocytophilum* among farmers and animals is shown in Table 1. Overall, the average seropositive rate of *A. phagocytophilum* among farmers was

Table 1

Seroprevalence of *A. phagocytophilum* in humans and domestic animals.

Areas	Human seropositive rates (%)		Animal Seropositive rates (%)		
	(No. of positive/No. of tested sera)		(No. of positive/No. of tested sera)		
	Male	Female	Goat	Cattle	Dog
Huaiyuan	9.3 (10/108)	11.3 (16/141)	0 (0/43)	0 (0/1)	0 (0/5)
Mingguang	40.3 (21/52)	69.5 (66/95)	0 (0/38)	0 (0/5)	57.1 (4/7)
Guangde	83.3 (70/84)	71.5 (83/116)	2.0 (1/51)	–	–
Total	41.4 (101/244)	28.4 (100/352)	0.8 (1/132)	0 (0/6)	33.3 (4/12)

Table 2The GenBank accession numbers of *A. phagocytophilum* obtained in animals' blood samples in the study.

Areas	Goat		Cattle		Dog		
	PCR positive rates (%)	No. of GenBank	PCR positive rates (%)	No. of GenBank	PCR positive rates (%)	No. of GenBank	
Huaiyuan	55.8 (24/43)	GQ499896	GQ499897	0 (0/1)	–	20.0 (1/5)	GQ499929
		GQ499898	GQ499899				
		GQ499900	GQ499901				
		GQ499902	GQ499903				
		GQ499904	GQ499905				
		GQ499906	GQ499907				
		GQ499914	GQ499909				
		GQ499895	GQ499910				
		GQ499911	GQ499912				
		GQ499913	GQ499915				
		GQ499916	GQ499917				
		GQ499918	GQ499930				
		Mingguang	23.7 (9/38)				
GQ499921	GQ499924			GQ499920			
GQ499927	GQ499925						
GQ499926	GQ499932						
GQ499928							
Guangde	21.6 (11/51)	GQ499885	GQ499886	–	–	–	–
		GQ499887	GQ499888				
		GQ499889	GQ499890				
		GQ499891	GQ499892				
		GQ499893	GQ499894				
		GQ499931					
Total	33.3 (44/132)			0 (0/6)		25.0 (3/12)	

“–”No samples were available.

33.7%. Of the 3 sites that were investigated, Guangde County had the highest seroprevalence (76.5%), and Huaiyuan County had the lowest (10.4%). The statistical analysis indicates that the seroprevalence of *A. phagocytophilum* in Guangde and Mingguang were significantly higher than in Huaiyuan ($P < 0.001$; $P = 0.002$). However, there was no statistical significance between Guangde and Mingguang. The total seropositive rate was calculated for each animal species because of the limited number of samples. The seroprevalence of *A. phagocytophilum* 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 cross-reaction between *A. phagocytophilum* and *R. typhi*, *O. tsutsugamushi* types Karp, *B. henselae* and *C. burnetii*. Among 106 *A. phagocytophilum*-reactive sera (101 human sera, 4 dog and 1 goat sera) tested for *E. chaffeensis*, *R. typhi*, *O. tsutsugamushi* types Karp, *R. heilongjiangensis*, *B. henselae*, *C. burnetii*, no cross-reaction with *A. phagocytophilum* was noted for *R. typhi*, *O. tsutsugamushi* types Karp, *R. heilongjiangensis*, *B. henselae*, *C. burnetii* at a titer of 80 and only 10 samples from human and one sample from dog contained *E. chaffeensis* antibodies at a titer of 80, but no reactivity beyond that titer as a possible cross-reaction with *A. phagocytophilum* was noted.

3.3. 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 and the sequences were deposited in GenBank (Table 2). A phylogenetic tree was constructed based on these sequences, those reported in another study in China and some from other areas of the world (Figure 1). Phylogenetic analysis demonstrated that there were two dominant genetic groups of *A. phagocytophilum* in the surveyed areas and additionally, the analysis found that these two groups were distributed in two distinct geographic areas. Group A was mainly found in Huaiyuan, which is in the north 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, the sequences (EF211110) from patients involved in the nosocomial outbreak of anaplasmosis in Guangde County, Anhui province, in 2006 and other sequences from patients in Yiyuan County, Shandong Province, also grouped into group B, which was predominantly distributed in the Guangde area.

4. Discussion

In 2006, we first reported the identification of HGA in China and first demonstrated nosocomial human-to-human transmission of HGA. However, some rickettsioses experts were wary of these results because of the lack of supporting etiological evidence and the limited titer (the highest titer was 1:256 in the covalent sera from the 4 patients) of IgG antibody to *A. phagocytophilum*; even though a 4-fold increase of antibody was found in all patients. To evaluate the epidemiology of *A. phagocytophilum* in Anhui province, we conducted a broad epidemiological investigation of *A. phagocytophilum* in Anhui Province, which was focused on

Guangde County, where the index case of the nosocomial human-to-human transmission of HGA lived. The serological evidence in our study revealed that the average seroprevalence of *A. phagocytophilum* among farmers in 3 surveyed sites was 33.7%, which is significantly higher than the 8.8% that was observed in a previously study in Tianjin^[6], but similar to the rates that have been reported in endemic areas in US^[7,8]. Notably, this study found that the seropositive rate of *A. phagocytophilum* among farmers in Guangde County is as high as 76.5% (with a cut off value of 1:80). These results strongly support our previous findings in the outbreak of HGA in Anhui Providence in 2006. Furthermore, phylogenetic analysis not only demonstrated that the sequences from patients involved in the nosocomial transmission of HGA in 2006 were predominantly identified in local domestic animals and that they grouped into the clade that is endemic in Guangde County.

Additionally, we observed a different seroprevalence in humans when using the *A. phagocytophilum* (RA2682, lot 03–0403N) antigen as opposed to *A. phagocytophilum* Webster (0.8% vs. 35%, $P < 0.0001$). Similarly, 2.0% (1/51) of the goats were seropositive by IFA when using *A. phagocytophilum* (RA2682, lot 03–0403N) as the antigen, which was significantly lower than the 21.6% of samples that were positive by PCR. Therefore, we propose that the above phenomenon resulted from the different phenotypes of Chinese and US isolates of *A. phagocytophilum*. Another reason may be related to the different quality of the antigens due to inappropriate transportation, conservation, and so on.

Our study found that the seroprevalence in humans from the mountainous areas (Guangde and Mingguang) was significantly higher than that in humans from the plain areas (Huaiyuan). The observed difference between the seropositive rates of males and females in the investigated sites was not statistically significant.

There are two varieties of *A. phagocytophilum* that were isolated from animals in this study and a striking geographic distribution 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, Shandong province; and water deer (GU556624) from Korea and *Haemaphysalis longicornis* (GU064899) collected from Jeju Island in Korea. This clade was also detected in *Ixodes ovatus* (AY969015) in Japan^[9]. In addition, a sequence that is 100% homologous to group B was identified in an Italian patient (DQ029028) with HGA from Sicily^[10], in wild ruminant animals (EU839850) and in 2 horses (EU839857 and EU839852) that were infected with *A. phagocytophilum* in the Czech Republic^[11].

Group A was mainly endemic in Huaiyuan County, but sequences that were 100% homologous were also identified in goats (HM439432) from Zhejiang Province and (FJ389576) southeast China^[12], in *Haemaphysalis longicornis* (GU064899) collected from Jeju Island in Korea, and in deer (AB454075) from the Nara park in Japan. However, no sequences from patients were grouped into this clade.

As an emerging tick-borne infectious disease, this study is the first and largest serological survey of *A. phagocytophilum* in Anhui Province. The serological and molecular evidence indicates that anaplasmosis is widely distributed in Anhui province. Systematic surveillance, including the role of the vector and host and monitoring of the transmission of HGA in local areas should be performed in the future. Etiological

investigation based on the isolation of agents is urgently needed.

Conflict of interest statement

We declare that we have no conflict of interest.

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