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Short Communication

Molecular evidence of coinfection of *Anaplasma* species in small ruminants from Anhui Province, China

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ABSTRACT

The species of the genus *Anaplasma* are obligate intracellular pathogens that threaten the health of both humans and animals. In this study, we investigated the presence of *Anaplasma phagocytophilum*, *A. ovis* and *A. bovis* in 203 healthy small ruminants (117 goats and 86 sheep) in Anhui Province, China. The overall coinfection of *Anaplasma* species occurred in 33.0% (67/203) of all studied samples. The infection rates of *A. ovis*, *A. bovis*, and *A. phagocytophilum* were 14.5%, 12.0%, and 4.3% in goats and 26.7%, 17.4% and 3.5% in sheep, respectively. Coinfection of *A. ovis* + *A. bovis* was predominant in this study, with overall rates of 21.4% in goats and 20.9% in sheep, while the overall coinfection rates of *A. ovis* + *A. phagocytophilum* and *A. bovis* + *A. phagocytophilum* were 7.7% and 2.6% in goats and 7.0% and 4.7% in sheep, respectively. The occurrence of three-pathogen coinfection was also found in the studied ruminants, with a rate of 0.9% in goats and 1.2% among sheep. Phylogenetic analysis based on *msp4* sequences showed that there were differences in the *A. ovis* genotype between sheep and goats in this study.

Species of the genus *Anaplasma* are Gram-negative obligate intracellular pathogens causing tick-borne diseases of animals and humans [1], and the six major species of this genus—viz., *Anaplasma phagocytophilum*, *A. ovis*, *A. bovis*, *A. marginale*, *A. centrale* and *A. platys*—are animal and/or human pathogens of veterinary and public health significance [2].

Among *Anaplasma* species, *A. ovis* is the main intraerythrocytic pathogen of bovine and ovine animals. It causes bovine and ovine anaplasmosis in tropical and subtropical regions [3]. *A. ovis* is the main pathogen found in ruminants in northern China [4], while limited studies have been carried out in central and southern China [5]. *A. bovis* is a leukocyte pathogen of ruminants that is generally observed in monocytes and other professional phagocytes [6]. The presentation of *A. bovis* infection in domestic ruminants has mainly been reported in African countries, but in recent years, the agent has also been found in Asian countries, including China and Japan [5,7,8]. *A. phagocytophilum* infects neutrophil granulocytes of many wild and domestic animal hosts and humans [1]. In ruminants, it is the causative agent of tick-borne fever, and the frequent symptoms of this disease are characterized by high fever, malaise, anorexia, and lower milk yield [9]. *A. phagocytophilum* infections have been detected not only in ticks, rodents and some ruminants, including goats, cattle and sika deer, but also in human patients [10,11].

To our knowledge, molecular surveys have shown *A. phagocytophilum* infection in goats in Anhui Province, China. For instance, 22.1% (29/131) of goat samples were PCR-positive for *A. phagocytophilum* in Anhui [12]. However, there have been no reports on the infection of *A. ovis* and *A. bovis* in small ruminants in Anhui to date. To provide further information on the epidemiology of *Anaplasma* spp. in small ruminants, the aim of this study was to investigate the prevalence of these pathogen infections in goats and sheep sampled in Anhui Province.

Between May and September 2017, a total of 203 samples were collected from 117 healthy goats and 86 healthy sheep. These samples were taken from three localities (Yijiang, Fanchang and Wuwei) in Wuhu, Anhui Province, China (Fig. 1). Blood samples were collected in sterile EDTA tubes from the jugular vein of each animal and stored at −20 °C until use. DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's protocol.

This study was approved by the Animal Ethics Committee of Wannan Medical College (Permit No. 14.03.2017-15).

Nested PCR was performed to detect the presence of *A. phagocytophilum* and *A. bovis* in the blood samples with the primers EE1/EE2 [13] in primary PCR. The *A. phagocytophilum*-specific primers SSAP2f/SSAP2r and *A. bovis*-specific primers AB1f/AB1r were then used in secondary PCR [14]. *A. ovis* infection in the collected blood samples was detected with *A. ovis*-specific primers MSP45 and MSP43 [15],

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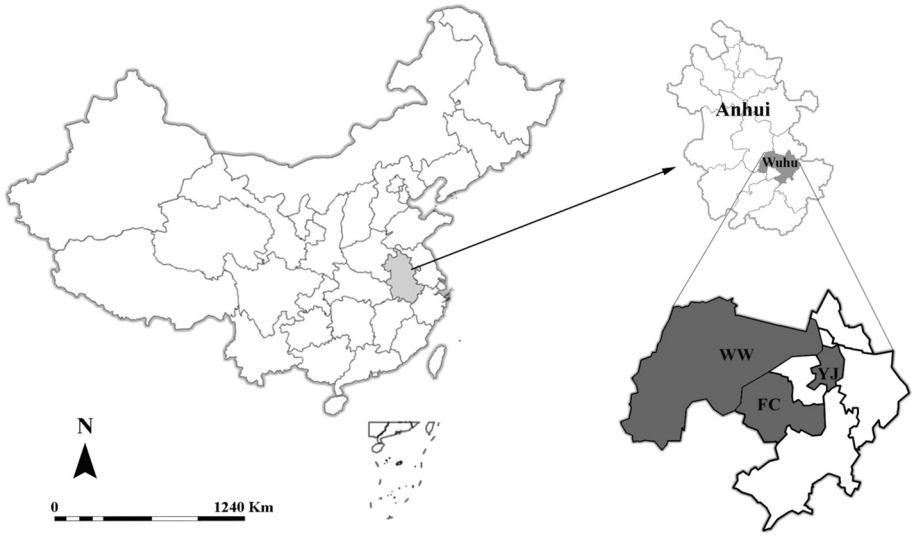

Fig. 1. Map of China and Anhui and the investigated localities. The sites Yijiang, Fanchang and Wuwei in this study are represented by YJ, FC and WW, respectively.

which amplify the major surface protein 4 (*msp4*) gene. All PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

Positive PCR products from primers SSAP2f/SSAP2r and AB1f/AB1r were selected for purification using a QIAquick Gel Extraction Kit (Qiagen, Germany). Purified DNA fragments were sequenced bidirectionally on an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). The obtained sequences were subjected to a BLASTn analysis in GenBank (http://blast.ncbi.nlm.nih.gov/) or to the ClustalX 2.0 software for aligning the level of their similarity to previously released sequences of the *Anaplasma* deposited in GenBank. Phylogenetic trees were constructed using the neighbor-joining (NJ) method implemented in MEGA7.0 software.

A chi-squared test was used to compare the prevalence of the *Anaplasma* species detected in goats and sheep with SPSS 20.0 (SPSS Inc.). A P value of < 0.05 was considered statistically significant.

The representative sequences identified in this study were submitted to the GenBank database (16S rRNA of *A. phagocytophilum* and *A. bovis*, MK129456-MK129461; *A. ovis msp4* gene, MK134854-MK134857).

The overall infection rate of *Anaplasma* spp. was 63.2% (74/117) in goat samples, while the corresponding value was 81.4% (70/86) in sheep samples (Table 1). The one-pathogen infection rates of *A. ovis*, *A. bovis* and *A. phagocytophilum* in goats were 14.5%, 12.0% and 4.3%, respectively. In sheep, the rates were 26.7%, 17.4% and 3.5%, respectively (Table 1). *A. ovis* was the most dominant of the three pathogens found in the examined animals, with an infection rate of 44.4% in goats and 55.8% among sheep. It was correspondingly followed by *A. bovis* with a rate of 36.8% and *A. phagocytophilum* with a rate of 15.4% in goats, as well as *A. bovis* with 44.2% and *A. phagocytophilum* with 16.3% among sheep. Mixed infection was found in the examined samples. *A. ovis* + *A. bovis* coinfection was the most dominant type of infection. In goats, the overall rates of *A. ovis* + *A. bovis* infection were 21.4%, which were higher than the rates (7.7%) of *A. ovis* + *A. phagocytophilum* infection and the rates (2.6%) of *A. bovis* + *A. phagocytophilum* infection (Table 1). Likewise, among sheep, the overall rates (20.9%) of *A. ovis* + *A. bovis* were also higher than the rates (7.0%) of *A. ovis* + *A. phagocytophilum*, as well as the rates (4.7%) of *A. bovis* + *A. phagocytophilum* (Table 1). Furthermore, three-pathogen coinfections were detected in only 0.9% of the goat samples and 1.2% of the sheep samples (Table 1). In brief, the overall coinfection rate for the above-mentioned *Anaplasma* species was 33.0% (67/203) in all samples of this study (Table S1). The high coinfection rates found in this study suggest the potential difficulty in precise etiological diagnosis of anaplasmosis in small ruminants. Additionally, the prevalence of *Anaplasma* in goats was higher in females (34.7%) than in males (28.9%), while in sheep, the prevalence of *Anaplasma* was higher in ewes (35.8%) than in rams (30.3%) (Table 2). Moreover, the *Anaplasma* prevalence in adult goats (≥ 2 years) (38.2%) was higher than that in young goats (< 2 years) (22.4%) (Table 2). This difference could be because adult goats were exposed to several tick seasons and had greater chances of exposure to tick infestation.

Although several investigations have indicated the presence of anaplasmosis in small ruminants from northwestern, central and southern China [4,5,8,16], few surveys have focused on *Anaplasma* coinfection in small ruminants from Anhui Province, in which studies are only limited to the report of one of the *Anaplasma* species in goats and cattle [12]. In the present study, the molecular epidemiology of *Anaplasma* species was investigated in small ruminants collected from the Wuhu area, Anhui. Our results show evidence of *A. phagocytophilum*, *A. bovis* and *A. ovis* infections in both goats and sheep collected from the sampling sites.

A. phagocytophilum can cause public health concerns due to its zoonotic potential. Molecular or serological evidence has suggested that *A. phagocytophilum* has been detected in many hosts in China, including ticks, rodents, goats, sheep, and human patients [5,10,11,17]. Our findings showed that the infection rates of *A. phagocytophilum* were 3.8–13.3% in goats and 9.5–19.5% among sheep. This was higher than that (1.1–6.0%) reported in a previous study of ticks along the Sino-Russia border [17]. Phylogenetic trees based on 16S rRNA showed that the *A. phagocytophilum* isolates (GenBank acc. no. MK129456-MK129457) obtained in the present study formed an independent cluster, which displayed a close relationship with isolates from Chinese goats, sheep, cattle, ticks and Japanese deer (JN558815, KM285232, KX115432, KF569915 and AB196721) (Fig. S1-A). In addition, the *A. phagocytophilum* isolate (MK129456) from sheep was 99.4% identical to the human isolate from the USA (AF093789). Although there is no direct evidence for the transmission of *A. phagocytophilum* between ruminants and humans that live in the same environment through tick vectors, there is a potential risk for those people who contact animals that are infected with the causative agent.

The *A. bovis* infection rates were 36.9% and 44.2% in the goat and sheep samples, respectively. The rates were obviously higher than those in wild deer (9.0%) and ticks (12.0%) in Japan [14] but comparable to those in cattle from Japan (53.3%) [7]. Although the occurrence of *A. bovis* was detected by PCR in the study sites, the investigated animals showed no clinical signs of infection. This finding agrees with previous reports [5,8] that predicted that *A. bovis* in sheep and goats could have limited pathogenicity. In addition, phylogenetic analyses indicated that

Table 1
Results of nested PCR for detection of *Anaplasma* pathogens in goats and sheep in this study.

Study sites	Goat							Sheep							Total
	No. infected (%)							No. infected (%)							
	No. tested	Single pathogen			Two pathogens			Three pathogens	No. tested	Single pathogen			Two pathogens			Three pathogens	
		Ao	Ab	Ap	Ao + Ab	Ao + Ap	Ab + Ap	Ao + Ab + Ap		Ao	Ab	Ap	Ao + Ab	Ao + Ap	Ab + Ap	Ao + Ab + Ap	
Yijiang	60	8 (13.3)	7 (11.7)	3 (5.0)	13 (21.7)	5 (8.3)	2 (3.3)	1 (1.7)	36	9 (25.0)	6 (16.7)	2 (5.6)	7 (19.4)	4 (11.1)	2 (5.6)	1 (2.8)	96
Fangchang	31	5 (16.1)	4 (12.9)	2 (6.5)	6 (19.4)	3 (9.7)	1 (3.2)	0 (0)	29	8 (27.6)	5 (17.2)	1 (3.4)	6 (20.7)	2 (6.9)	0 (0)	0 (0)	60
Wuwei	26	4 (15.4)	3 (11.5)	0 (0)	6 (23.1)	1 (3.8)	0 (0)	0 (0)	21	6 (28.6)	4 (19.0)	0 (0)	5 (23.8)	0 (0)	2 (9.5)	0 (0)	47
Total	117	17 (14.5)	14 (12.0)	5 (4.3)	25 (21.4)	9 (7.7)	3 (2.6)	1 (0.9)	86	23 (26.7)	15 (17.4)	3 (3.5)	18 (20.9)	6 (7.0)	4 (4.7)	1 (1.2)	203

Note: Ao, Ab and Ap represent *Anaplasma ovis*, *Anaplasma bovis* and *Anaplasma phagocytophilum*, respectively.

Table 2
The prevalence of *Anaplasma* infection based on gender and age of goats and sheep in this study.

Variables	Goats		Sheep	
	No. tested	Prevalence (positive/tested)	No. tested	Prevalence (positive/tested)
Gender				
Male	45	28.9 (13/45)	33	30.3 (10/33)
Female	72	34.7 (25/72)	53	35.8 (19/53)
Age				
< 2 year	49	22.4 (10/49)	25	32.0 (8/25)
≥ 2 year	68	38.2 (28/68)	61	34.4 (21/61)
Total	117	32.5 (38/117)	86	33.7 (29/86)

the *A. bovis* sequences obtained in this study fell within a clade together with the sequences from Chinese cattle and goat (HQ913644, FJ169957, and KJ782393) as well as deer from Japan and South Korean (AB211163 and EU682764) (Fig. S1-B).

A. ovis was the most frequently detected species in this study, and the prevalence was as high as 49.3% (100/203) among all samples. This prevalence was similar to that reported by previous studies (40.5% [4]; 46.6% [5]). The high prevalence of *A. ovis* in the studied ruminants may be the result of multiple vectors contributing to the transmission of this agent. As a result, *A. ovis* is the predominant species in the most common coinfections observed in this study. In addition, sheep had a slightly higher *A. ovis* prevalence than goats (see Table 1), indicating a high susceptibility of sheep to anaplasmosis compared with goats. However, this finding was not consistent with those reported in previous studies in South Africa and Turkey [18,19], suggesting possible differences in the susceptibility of goats and sheep to anaplasmosis in different bioclimatic regions. Phylogenetic analyses indicated that the *A. ovis msp4* genotypes obtained in this study formed two main clusters (Fig. S1-C). This result suggested that *A. ovis msp4* genotypes may vary between goats and sheep. Similar studies have been reported among sheep and deer and in the hosts of other *Anaplasma* species [15,20].

In conclusion, this study first reported the presence of *A. ovis*, *A. bovis*, and *A. phagocytophilum* in domestic small ruminants from the investigated area. Coinfection of these three species was found in this study. Phylogenetic analysis showed that there are differences in the *A. ovis* genotype between sheep and goats in this study. These results may provide essential information for the control and prevention of *Anaplasma* spp. in Anhui.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2019.04.004.

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