Occurrence of *Anaplasma phagocytophilum* in goats and sheep in Hebei Province, China¹⁾

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Summary

Anaplasma phagocytophilum is an emerging pathogen known to cause human granulocytic anaplasmosis (HGA). Here we determined the prevalence and genetic characterization of *A. phagocytophilum* in Hebei Province, China. A total of 253 samples were taken from goats and sheep in Hebei Province, and 52 (20.6%) were positive for *A. phagocytophilum*. There was a higher positive rate in sheep (23.8%, 20/84) than in goats (18.9%, 32/169). Analysis of the partial 16S RNA gene sequences of *A. phagocytophilum* revealed that the isolates in this study were members of the same clade and were 100% homologous with each other. This study provides information on the epidemiologic features of *A. phagocytophilum*.

Keywords: 16S RNA, *Anaplasma phagocytophilum*, genotype, Hebei Province, China

Anaplasma phagocytophilum is an obligate intracellular bacterium that infects a wide range of mammalian hosts. It is associated with human granulocytic anaplasmosis (HGA) and tick-borne fever in ruminants (5, 11). Ixodidae ticks are the principal hosts of *A. phagocytophilum*, with their geographic distribution and species influencing the epidemiology of *A. phagocytophilum* (6). Across China, *A. phagocytophilum* infection of rodents, small ruminants, and wild animals by ticks has been reported extensively, while the number of HGA cases reported from mainland China has been on the rise (5). This has caused *A. phagocytophilum* to be considered an emerging pathogen of global health importance.

A. phagocytophilum was originally considered to be of veterinary importance. The first confirmed human infection, known at the time as human granulocytic ehrlichiosis (HGE), was described in the United States as late as 1994 and in Europe in 1997. HGE was subsequently renamed HGA after reclassification in 2001 (5). Serologic and molecular evidence for *A. phagocytophi-*

lum infection in livestock, rodents and ticks has been reported in many countries, including Korea, Japan, and China. Its prevalence in these countries has ranged from 0 to 73% (2, 8, 9, 12, 16). In China, *A. phagocytophilum* infections have been confirmed in humans, ticks, rodents, and ruminants, leaving its presence and risk to human health beyond doubt (19). In Hebei Province specifically, previous research has confirmed the presence of *A. phagocytophilum* in *Haemaphysalis longicornis* (*H. longicornis*) and *Dermacentor nuttalli* (*D. nuttalli*), but there is still little information available on its presence in sheep and goats (21). In this study, a survey of the prevalence and genetic diversity of *A. phagocytophilum* in sheep and goats was conducted in Hebei Province.

Material and methods

To perform this study, blood samples were taken from randomly selected sheep (84) and goats (169) in rural areas of Qinghuangdao City (39°56'N, 119°36'E) and Cangzhou City (38°30'N, 116°83'E) located in eastern Hebei Province, Zhangjiakou City (40°46'N, 114°56'E) Xingtai City (37°07'N, 114°48'E) and Handan City (36°20'N, 114°03'E) located in northern Hebei Province between April 2013 and May 2015. No clinical symptoms were found in goats and sheep. All blood samples were stored at -20°C until DNA extraction. DNA was extracted using EasyPure Blood Genomic DNA Kit (TransGen Biotech, Beijing,

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China) according to the manufacturer's instructions. Nested PCR was performed using primers designed to amplify the partial 16S rRNA gene of *A. phagocytophilum*, as previously described (21). In brief, a pair of universal primers of the ehrlichial 16S rRNA gene (GenBank accession No. AF414399), Eh-out1 (5'-TTGAGAGTTTGATCCTGGCT-CAGAACG-3', located at positions 1 to 27) and Eh-out2 (5'-CACCTCTACACTAGGAATTCGCTATC-3', at positions 653 to 627), were used for the primary amplification of a 653-bp fragment. Primers HGA1 (5'-GTCGAACGGATTATT CTT TATAGCTTG-3', at positions 167 to 187) and HGA2 (5'-TAT AGG TACCGT CAT TAT CTT CCC TAC-3', at positions 448 to 428), which were designed based on the conserved positions in the sequence of the 16S rRNA genes of *A. phagocytophilum*, were used in the nested amplification of a 389-bp fragment. A plasmid containing the 16S rRNA gene of *A. phagocytophila* (kindly provided by Dr. Pengpeng Liu, National Research Center for Wildlife-Borne Diseases at the Institute of Zoology, Chinese Academy of Sciences) was used as a positive control. A negative control of distilled water was included in the assay to avoid a false positive (1, 3). A total of 52 positive PCR products were cloned and then sequenced by BGI-Beijing (Beijing, China). The partial nucleotide sequences of the *A. phagocytophilum* 16S rRNA (Hbe001, Hbe016, and Hbe103), randomly selected from three different regions, were submitted to GenBank (GenBank Accession No. KU171074, KU171075, and KU171076). The representative 16S rRNA genes of *A. phagocytophilum* strains Hbe001, Hbe016, and Hbe103 were aligned with previously published sequences deposited in GenBank (22). Phylogenetic trees were constructed using the neighbor-joining algorithm of MEGA 7.0.26 with the Kimura two-parameter model (10).

Statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL), and Chi-square test or Fisher's exact test was used to compare the prevalence rates (wherever necessary). The differences were considered statistically significant when the *p*-value was < 0.05 (18).

Results and discussion

Our results showed that 52 samples were positive for *A. phagocytophilum*, with an overall prevalence of 20.6% (52/253) in Hebei Province, China. The positive rates for *A. phagocytophilum* in different regions varied from 0 to 69.2% (Tab. 1). Interestingly, no positive samples were found in Cangzhou City. This may be due to the geography of Cangzhou City, which consists of plains, unlike the other sampling sites, which are mountainous regions. The prevalence of *A. phagocytophilum* in Qinhuangdao City was significantly higher than in Zhangjiakou, Xingtai or Handan City ($p < 0.05$), the prevalence of *A. phagocytophilum* in Zhangjiakou City and Xingtai City was significantly higher than in Handan City (Tab. 1). Cangzhou in Hebei Province is located in North China Plain, while other regions, such as Qinhuangdao, Zhangjiakou, Xingtai, Handan, and other places, are all mountainous areas.

Ticks are more likely to occur in mountainous areas, so such areas may have a higher *A. Phagocytophilum* infection rate. The prevalence of *A. phagocytophilum* in goats and sheep amounted to 18.9% (32/169) and 23.8% (20/84), respectively. The positive rate was higher in sheep than in goats ($p < 0.05$). Moreover, significant differences were also found in the prevalence of *A. phagocytophilum* in sheep and goats from different regions ($p < 0.05$) (Tab. 1). Similar studies on *A. phagocytophilum* infection in sheep and goats have previously been conducted in many provinces of central, Southeastern, Northeastern and Northwestern China. In these studies, the infection rates at different sampling sites varied from 0 to 78.1%, which implies an extensive distribution of this pathogen in China (22). The prevalence of *A. phagocytophilum* infection in sheep and goats in the regions surveyed in the current study was lower than in sheep and goats from Gansu Province (40.0%, 56/140) and central and Southeastern China (25.2%, 106/421), but higher than in goats from Henan (13.0%, 6/46), Jilin (5.7%, 8/35), and Hubei (14.5%, 10/69) (23). This may have been due to the sampling sites chosen, temporal effects on samples, the sampling method used, the distribution of the vectors, the different diagnostic methods used, or any other factor related to the ecological environment.

Moreover, the fact that *A. Phagocytophilum* infection rates in goats and sheep are different in different regions may be attributed to vast territory, diverse geographical environment, and large regional differences in China, with higher incidence in mountainous areas with higher grazing rates. In addition, various samples may be collected at different times and other factors may cause the diversity of *A. phagocytophilum* infection rates.

Ixodidae ticks are the main vector of *A. phagocytophilum*, and the species and geographical distribution of this vector influence the epidemiology and genetic diversity of this pathogen. Sequence analysis of the partial 16S rRNA gene of *A. phagocytophilum* from goats and sheep revealed that the isolates from this study were 100% genetically homologous to each other. These sequences were also similar to a sequence of the *A. phagocytophilum* variant previously detected in *H. longicornis* and *D. nuttalli* in

Tab. 1. Prevalence of *Anaplasma phagocytophilum* in different regions of Hebei Province, China

Regions	No. of animals tested		No. of animals positive (%)	
	goat	sheep	goat	sheep
Qinghuangdao	32	20	23/32 (71.9)	13/20 (65.0)
Zhangjiakou	15	12	2/15 (13.3)	2/12 (16.7)
Xingtai	10	14	1/10 (10.0)	3/14 (21.4)
Cangzhou	31	19	0/31 (0.0)	0/19 (0.0)
Handan	81	19	6/81 (7.4)	2/19 (10.5)
Total	169	84	32/169 (18.9)	20/84 (23.8)

Hebei Province (GenBank Accession No. HQ651826, HQ651827, and HQ651828). Genetically, these variants have 96.9%-98.4% similarity to each other, but are all distinct from the other known *A. phagocytophilum* sequences deposited in GenBank (Fig. 1). These findings, together with the evidence accumulated from previous studies, suggest that *H. longicornis* and *D. nuttalli* are the main vectors of *A. phagocytophilum* in Hebei Province and that they directly influence its epidemiology and genetic diversity, resulting in this genetic distinction (21). Further phylogenetic analyses performed on these isolates revealed that the *A. phagocytophilum* variant identified in sheep and goats and the *A. phagocytophilum* variant identified in *H. longicornis* and *D. nuttalli* (GenBank Accession No. HQ651826, HQ651827, and HQ651828) are all from a clade distinct from *A. phagocytophilum* strains previously reported in ticks, humans, goats, sheep, rodents, and dogs from other provinces of China, as well as from Japan, Europe, and the United States. This indicates that the genetic differences found in the variants may be due to geographical segregation (Fig. 1) (14, 17). Environmental factors, such as climate, vegetation type, and abundance of appropriate hosts, also influence the geographical distribution of pathogens, and therefore may have also caused this genetic distinction. In this study, we analyzed only the partial 16S rRNA gene and inferred from it a phylogenetic tree. However, because of the limitations of partial 16S rRNA gene analysis, further phylogenetic studies involving different gene markers are needed to provide a better genetic characterization of *A. phagocytophilum* observed in this study (14, 17).

Our study adds to the knowledge of the epidemiologic features of *A. phagocytophilum* infections in Hebei Province and indicates that *A. phagocytophilum* may be endemic in sheep and goats in this region. These findings provide valuable information for management and control programs for anaplasmosis in small ruminants in Hebei Province.

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Fig. 1. The phylogenetic tree for the partial 16S rRNA gene of *Anaplasma phagocytophilum*. It was constructed using the neighbor-joining algorithm of MEGA 6.06. The neighbor-joining consensus tree used 1000 bootstrap replicates. The number represents bootstrap values. The species, GenBank accession number, and the country or region are indicated

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