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Detection of *Anaplasma* in *Haemaphysalis longicornis* from Hilly Area in Central China

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KZ and XDJ designed and supervised the experiment and improved the manuscript. HJW, RXS, YJC and YD performed the experiment. YZW reviewed the manuscript and made improvement.

Key words
***Anaplasma, Haemaphysalis longicornis*, Hilly area, Central China**

ABSTRACT

Anaplasma are zoonotic intracellular bacteria transmitted mainly by ticks. Ticks and tick-borne *Anaplasma* in the hilly area in Central China are not well studied. During June to July in 2018, ticks were collected from six counties in the hilly area of Funiu Mountainsinin in Central China. All ticks were identified by morphology and confirmed by *16S rDNA* gene sequencing. *Anaplasma* were detected by nested PCR amplification of *16S rDNA* gene. In total, 686 ticks including two species *Haemaphysalis longicornis* (n=683) and *Rhipicephalus microplus* (n=3) were obtained. All of *H. longicornis* tick were clustered into 166 pools (n=1-10), and the minimum infection rate (MIR) of *Anaplasma* was 4.10% (28/683), *Anaplasma phagocytophilum* carriage rate was 3.66% (25/683) *and Anaplasma centrale* was 0.44% (3/683). The *Anaplasma 16S rDNA* gene phylogenetic analysis showed that all *Anaplasma*-positive samples were divided into four clades (Clade 1, 98.23-100% identity to *A. phagocytophilum* from Shandong and Zhejiang Province in China; Clade 2, 98.23% identity to *A. phagocytophilum* from North Korea; Clade 4, 98.48% identity to *A. phagocytophilum* from South Korea; Clade 3, 100% identity to *Anaplasma centrale* from South Korea). In the hilly area of Funiu Mountains in Central China, *H. longicornis* was the dominant tick species with high MIR 3.66% of *A. phagocytophilum* and low MIR 0.44% of *A. centrale*. Ticks and tick-borne *Anaplasma* had high identity to them from other region of Asia. These results indicated that *H. longicornis* tick carried high prevalence *A. phagocytophilum*, which may be a challenge for public health in the study area.

INTRODUCTION

Anaplasma are zoonotic obligate intracellular bacteria mainly transmitted by *Ixodes* spp. ticks, which causes considerable economic losses in the livestock industry and serious public health concernss (Ismail *et al*., 2010). *Anaplasma* genus includes seven species: *A. phagocytophilum*, *A. ovis*, *A. bovis*, *A. centrale*, *A. marginale* *A. platys* and *A. capra* (Dumler *et al*., 2001; Li *et al*., 2015). *A. phagocytophilum* can cause human granulocytic anaplasmosis with several features ranging from mild illness such as fever, headache, myalgia, malaise, thrombocytopenia, and leukopenia to severe disease such as gastrointestinal, respiratory distress, myocarditis, neurological complications, septic shock-like disease, and even death (Dumler *et al*., 2007; Bakken and Dumler, 2000). *A. marginale* can cause bovine anaplasmosis with severe anaemia and death in infected cattle and is responsible for economic losses due to high morbidity and mortality, reduced weight gains and milk production, abortions, and treatment costs among cattle worldwide (Battilani *et al*., 2017). *A. centrale* called *Anaplasma marginale* variety *centrale* before generally causes a milder, less virulent form of the disease. Due to infection with *A. centrale* conferring some cross-protection against *A. marginale*, it has been employed as a live vaccine (Potgieter and Stoltsz,

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2004; Theiler, 1912). *A. ovis* can causes anaplasmosis of sheep, goats and wild ruminants, rarely cattle with mild clinical disease (Friedhoff, 1997; Kuttler, 1981; Ryff and Weible, 1964). Although *A. ovis* infects the erythrocyte where it is phenotypically similar to, but does not provide protection against *A. marginale* infection (Splitter, 1956).

Funiu Mountains located in central China is the climate zoning line of the north subtropical and warm temperate zone in China. The mountains in the southeast section usually called hilly area are gradually low and scattered with an altitude of 400-1000 meters, even 200-400 meters, and the slope of the hillside is reduced from 20-40° to 20-35°, even lower than 20°. In the hilly area, traditional sheep, goat and occasionally cattle grazing, and planting of corn, soybean and wheat are both popular. Although ticks and tick-borne *Anaplasma* were reported in North western China (Guo *et al*., 2016; Yan *et al*., 2020; Yang *et al*., 2013), in eastern China (Qin *et al*., 2018), in southeastern China (Liu *et al*., 2017), even in Xi'an (Guo *et al*., 2018), in Hebei province (Zou *et al*., 2011), in Xinyang in Dabieshan Mountains (Zhuang *et al*., 2018), in the Funiu Mountains, especially in the hilly area, ticks and tick-borne pathogens are not well studied. The aim of this study was to investigate ticks and tick-borne *Anaplasma* in the hilly area of Funiu Mountains in Central China.

MATERIALS AND METHODS

Ticks collection and identification

In 2018, between June and July, at the tick peak time, the ticks were collected from eleven sites of six counties including Wugang, Baofeng, Lushan, Ruzhou, Jiaxian, and Xincheng in the hilly area of Funiu Mountains (Fig. 1). In summer, average daily temperature of the study area ranges from 23°C to 34°C and -2°C to 10°C in winter. The average rainfall is about 790 mm/year. In the study area, the altitude of rolling hills ranges mostly from 200 m to 600 m and the altitude of flatlands is less than 70m.

The parasitic ticks were collected over the livestock's entire body including ears, neck, thorax, armpits, abdomen, interfeminium, crissum and so on (Zhang *et al*., 2016). The free-living ticks were collected by the flagging method and artificial trapping method (Zhang *et al*., 2016). All the collected ticks were subjected to starvation for 2-3 days, and a stereomicroscope was used to examine their morphological features including back, abdomen, shield plate, gas door plate, false head base, lateral furrow, and genital orifice. One to ten (mean= 4.06, 686/169) ticks of the same species collected from one site were mixed into one pool with no separation of males and females. Then, the pooled ticks were analyzed individually. Partial *16S rDNA* sequences of the 60 representative ticks, with 1–8 pool of each tick species from each sampling site were sequenced to validate the findings in the morphology of the ticks (Black and Piesman, 1994). A brief summary of this experiment is illustrated in Table I. Then, ticks were stored at -80 °C until DNA extraction.

DNA extraction

DNA extraction of ticks was carried out following a previously reported procedure (Zhang *et al*., 2021). In brief, sterile deionized water and 75% ethanol were used for washing and disinfecting of ticks before extraction of DNA. The TIANamp Genomic DNA Kit (TIANGEN Biotech Co., Ltd., Beijing) was used for the extraction of DNA according to the instructions specified by the manufacture.

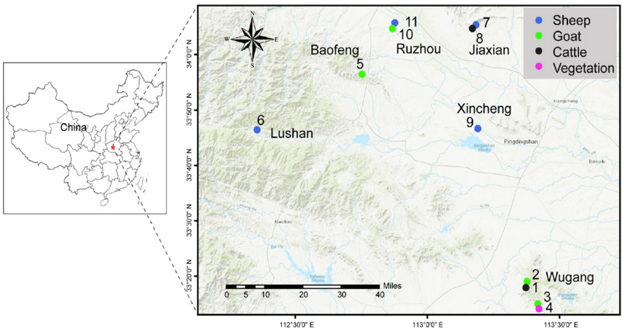

Fig. 1. Map of eleven ticks' collection sites in six counties in the hilly area of Funiu mountains in central China. The purple circle, black circle, green circle, and red circle represent the vegetation, cattle, goat, and sheep, respectively.

Table I. Molecular identification of ticks and tick-borne *Anaplasma* spp.

Ticks and tick-borne agent	Target gene	Primer	Sequences (5'-3')	Tm (℃)	Length (bp)	Ref
Ticks	16S rDNA	*16s F*	CTGCTCAATGATTTTTTAAATTGCTGTGG	54	460	Black and Piesman (1994)
		16s R	CCGGTCTGAACTCAGATCAAGT			
Anaplasma spp.	16S rDNA	*out1*	TTGAGAGTTTGATCCTGGCTCAGAACG	55	650	Wen *et al*. (2002)
		out2	CACCTCTACACTAGGAATTCCGCTATC			
		HGA1	GTCGAACGGATTATTCTTTATAGCTTG	55	390	
		HGA2	TATAGGTACCGTCATTATCTTCCCTAC			

Table II. Number of tick species recovered from the 11 sampling sites.

Location	Site No. (Date)	Altitude (m)	Origin	*H. longicornis*/ (♂/♀)	*R. microplus*/ (♂/♀)
Wugang	1#(2018/6/5)	269	Cattle	6(3/3)	2(1/1)
	2#(2018/6/5)	234	Goat	65(29/36)	0(0/0)
	3#(2018/6/5)	221	Goat	92(56/36)	0(0/0)
	4#(2018/6/5)	215	Vegetation	6(2/4)	0(0/0)
Baofeng	5#(2018/7/3)	578	Goat	137(39/98)	0(0/0)
Lushan	6#(2018/7/13)	476	Sheep	110(19/91)	0(0/0)
Jiaxian	7#(2018/7/13)	377	Sheep	153(31/122)	0(0/0)
	8#(2018/7/13)	377	Cattle	25(3/22)	0(0/1)
Xinhua	9#(2018/7/30)	114	Sheep	4(0/4)	0(0/0)
Ruzhou	10#(2018/7/30)	209	Goat	51(17/34)	0(0/0)
	11#(2018/7/30)	202	Sheep	12(0/12)	0(0/0)
Total number				683	3
Percentage (%)				99.56%	0.44%

Detection of Anaplasma *spp. using PCR*

The amplification of the *16S rDNA* gene (390 bp) was carried out via employing nested PCR and sequencing for the molecular identification of *Anaplasma* spp. (Wen *et al*., 2002). The PCR products of the *16S rDNA* gene for *Anaplasma* was purified using the TIAN gel Midi Purification Kit (TIANGEN, Beijing, China) and then cloned into the pGEM-T Easy vector and sequenced by ABI 3730 with the Sanger sequencing technique on both strands in the Beijing Genomics Institute. A brief summary of these experiments is illustrated in Table I.

Sequence analysis

The analysis of gene sequences was carried out with the basic local alignment search tool of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed using Neighbor-Joining approach in the Mega 5.0 software (Tamura *et al*., 2011; Saitou and Nei, 1987). Bootstrap analysis was tested with 1000 replicates (Felsenstein, 1985).

RESULTS

Collection and identification of ticks

A total of 686 ticks were collected from eleven sampling sites in the six counties in the hilly area of Funiu Mountains in Central China (Table II). Of them, 683 (pool=166) ticks were identified as *H. longicornis* and only 3 ticks (pool=3) were identified as *R. microplus* by morphological methods and confirmed by DNA sequencing of the tick *16S rDNA* gene. Compared to the date from GenBank, the *16S rDNA* genes of *H. longicornis* showed 99.51%-100% identity to *H. longicornis* tick from Henan (KJ652225.1), Hubei (KJ710084.1), Beijing (KC203355.1), Hebei (JF979374.1), Gansu (FJ712721.1), Sichuan (JF979373.1), Shanghai (KP324925.1) from China and Aomori (AB819205.1) from Japan (Fig. 2A). The *16S rDNA* genes of *R. microplus* showed 98.67%-99.76% identity to Henan (KX450285.1) from China and Itanagar (MK621328.1) from Southeast Asia (Fig. 2B).

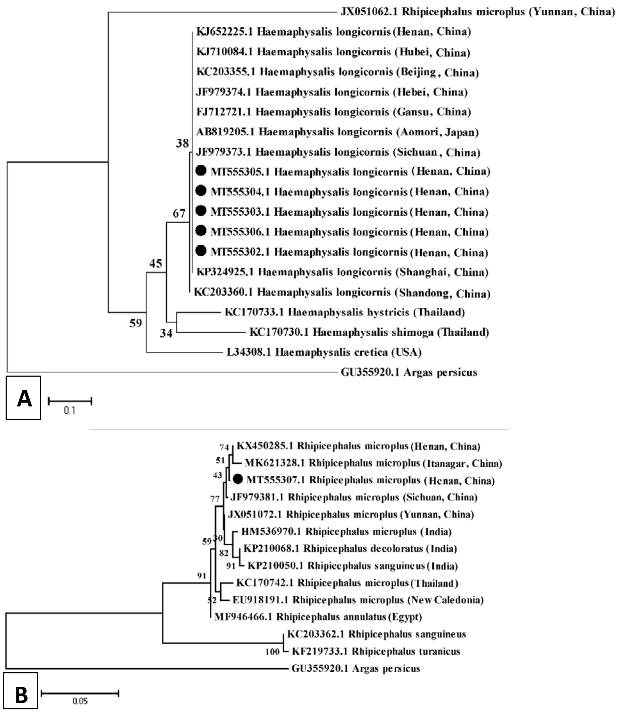

Fig. 2. Phylogenetic analysis of ticks from hilly area in the Funiu Mountains in Central China on the basis of on tick *16S rDNA* (460 bp). The phylogenetic tree for *H. longicornis* (A) and *R. microplus* (B). The bootstrap was 1000 replicates, while at each node, the numbers present bootstrap values. The outgroup is *Argas persicus* (GU355920).

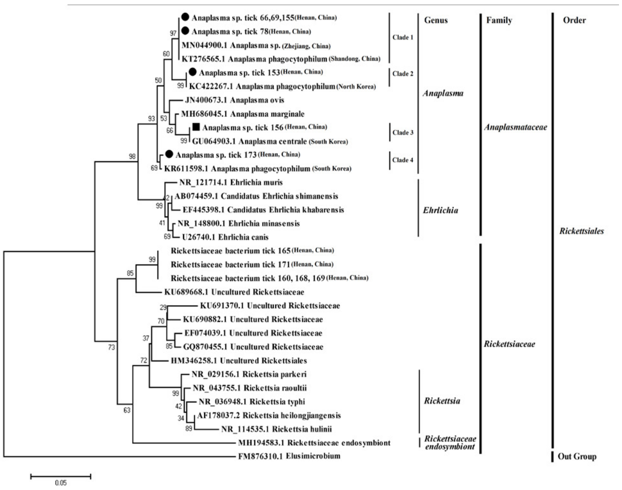

Fig. 3. Phylogenetic analysis of *Anaplasma* spp. based on *16S rDNA* (390 bp) sequences. The bootstrap was replicates, while at each node, the numbers present bootstrap values. *Elusimicrobium* (FM876310.1) is the outgroup.

Detection of Anaplasma

Anaplasma were detected by nested PCR based on the *16S rDNA* gene amplification and sequencing. Two species including *A. phagocytophilum* and *A. centrale* were detected. Among the 166 pools of *H. longicornis*, 28 were positive for *Anaplasma* with MIR (MIR, Minimum Infection Rate, number of positive pools/total specimens tested) 4.10% (28/683) in *H. longicornis*. The MIR is high for *A. phagocytophilum* (25/683, 3.67%) and low for *A. centrale* (3/683, 0.44%) in *H. longicornis*. In the three pools of *R. microplus*, *Anaplasma* was not detected.

Based on the phylogenetic analysis, *A. phagocytophilum* we detected were classified into three clades including clade 1, 2 and 4 (Fig. 3). Clade 1 (detected at Xinzhuang Village in Yaoshan town in Lushan County, site 6; and at Zhushadong Village in Xincheng County, site 9) revealed 99.74-100% identity to *A. phagocytophilum* from *H. longicornis* in Shandong (KT276565.1) and Zhejiang Province (MN044900.1). Clade 2 (detected at Zhushadong Village in Xincheng County, site 9) revealed 98.23% identity to *A. phagocytophilum* detected in *H. longicornis* from goats in North Korea (KC422267.1). Clade 4 (detected at Zhushadong Village in Xincheng County, site 9) revealed 98.48% identity to *A. phagocytophilum* detected in blood of cervids (Chinese water deer) in South Korea (KR611598.1). The *A. centrale* (Clade 3) we detected at Shizhuang Village in Xiaotun Town in Ruzhou County (site 10) showed 100% identity to *A. centrale* in *H. longicornis* from Jeju Island in South Korea (GU064903.1).

DISCUSSION

H. longicornis is widely distributed in Northeast China, Russian Far East, Japan, Republic of Korea, New Zealand, Australia, and certain Pacific Islands (Hoogstraal *et al*., 1968). In this study, we performed that in the hilly area of Funiu Mountains in central China, *H. longicornis* is the most predominant tick species. These results indicated that the local ecological environment is suitable for the survival and reproduction of *H. longicornis*. In addition, the marker genes of ticks and tick-borne *Anaplasma* have high identity with them from Shandong Province and Zhejiang Province in China, North Korea, South Korea and Japan. Considering that all of these regions are located in the East Asian-Australasian Flyway of migratory birds (Kasahara *et al*., 2020; Somveille *et al*., 2013), these results prompted us that the blood meal and free air tickets provided by migratory birds may promote the marker gene high homology of ticks and tick-borne pathogens in the region of East Asian-Australasian Flyway. In addition to the above factors, the formation of the global distribution pattern of *H. longicornis* and the pathogens its transmission should also include the ecological environment factors such as low altitude mountainous areas, hot and rainy summer, and sufficient hosts to provide blood meals. However, the recent prevalence of *H. longicornis* in18 states across eastern USA is maybe different from the above situation, because the initial report found that they may come from imported domestic animals (Egizi *et al*., 2020; Keirans and Durden, 2001).

In the hilly area in Funiu Mountains in Central China, where hemorrhagic fever with renal syndrome (HFRS) was prevalent in the 1970s, have disappeared. In recent ten years, brucellosis in domestic animals, especially grazing sheep, cattle carrying rate is very high. In a county, there are twenty to thirty new cases of human brucellosis each year, most of which come from cattle and sheep farmers. In the previous study, *Brucella* and *Ochrobactrum* were detected in ticks collected from the surface of livestock and vegetation (Zhang *et al*., 2021). In this study, we reported two species of *Anaplama* detected from ticks in the hilly area. The local prevalence of these pathogens may be attributed to economic activities, such as increased beef and mutton consumption and increased cattle and sheep breeding, and the local ecological environment.

In the world, *A. phagocytophilum* was transmitted mainly by several tick species of genus *Haemaphysalis* and *Ixodes* (Cao *et al*., 2003; Jiang *et al*., 2011; Yang *et al*., 2013). In addition, the DNA of *A. phagocytophilum* was detected in *Dermacentor albipictus*. Meanwhile, *A. centrale* was transmitted by ticks species mainly belonging to *Rhipicephalus* genus (Ngnindji-Youdje *et al*., 2022; Rehman *et al*., 2019) and the DNA of *A. centrale* was detected in *D. reticulatus* (Dunaj *et al*., 2021) and *Ixodes persulcatus* (Wu, 2013). In this study, we detected *A. phagocytophilum* and *A. centrale* in *H. longicornis*.

Of the genus of *Anaplasma*, *A. phagocytophilum* is the agent of human granulocytic anaplasmosis (HGA) (Parola and Raoult, 2001). Domestic animals and wildlife can also be infected by *A. phagocytophilum* (Hartwig *et al*., 2014; Yang *et al*., 2013). In China, since the first suspected human case described in 2006 (Zhang *et al*., 2008), an increasing number of HGA cases have been recorded (Fang *et al*., 2015). In the present study, high MIR of *A. phagocytophilum* were detected in *H. longicornis* and it is higher than the infection rate of 0.1% from Jiaonan County in Eastern China (Qin *et al*., 2018) and 2.4% from South Korea (Kim *et al*., 2003). The diversity could be attributable to different ecological environment of infected ticks or the sensitivity of various primers. Based on our study, high infection rate of *A. phagocytophilum* were in hilly area in Funiu mountains were detected, which was a challenge for public health and the prevalence of *A. phagocytophilum* in herders, livestock and wild life need to be further studied.

A. centrale mostly found in wild deer, swine (Portillo *et al*., 2011; Kawahara *et al*., 2006), sheep (Zhang *et al*., 2013) and vectored by several ticks. We detected *A. centrale* in *H. longicornis* from grazing livestock in Funiu Mountains in Central China and marker gene 100% identity to *A. centrale* in *H. longicornis* from South Korea. These results remind us that the people living here have risk to infection with *A. centrale*. Even if *A.centrale* causes a minor infectious disease (Shkap *et al*., 2002), it still threatens public health especially herdsmen and *A.centrale* cannot be completely ignored.

CONCLUSION

In the present study, we investigated the ticks and tick-borne *Anaplasma* in the hilly area of Funiu Mountains in central China. *H. longicornis* was the dominant tick species, and the marker genes had a high identity to the tick and tick-borne *Anaplasma* in East Asian-Australasian Flyway of migratory birds. These finding remind us that tick ecology related migration of birds maybe is an important strategy to understand ticks and tick-borne disease and regional cooperatives is needed for prevention and control of ticks and tick-borne disease. In addition, high minimum infection rate of *Anaplasma* especially *A. phagocytophilum* was detected. Our studies suggested ticks and tick borne *Anaplasma* may be a challenge to public health in study area. In future, the prevalence of *A. phagocytophilum* in human and animals need to be further studied.

DECLARATIONS

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IRB approval

This research was conducted with the approval of the Institutional Review Board (IRB). The IRB ensured that all procedures and protocols adhered to ethical guidelines and protected the rights and welfare of research participants. Their oversight was crucial in maintaining the integrity and validity of the study.

Ethical statement

The study was proved by the Animal Ethics Committee of Zhengzhou Railway Vocational and Technical College (numbered TEMAEC-2018-001).

Data availability

The nucleotide sequences were submitted to Genbank: *H. longicornis16S rDNA* gene MT555302-MT555306, *R. microplus16S rDNA* gene MT555307, *Anaplasma16S rDNA* gene OQ326846-OQ326850 and *Rickettsiaceae* bacterium *16S rDNA* gene OQ383347-OQ383349.

Statement of conflict of interest

The authors have declared no conflict of interest.

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