

Molecular identification of tick-borne pathogens in tick *Haemaphysalis longicornis* from sheep in Henan, China

Yongshuai PENG^{1,2,*}, Meng QI^{3,*}, Fuchun JIAN¹, Jinhong WANG¹, Yali LV¹,
Jiujian WEI¹, Rongjun WANG¹, Longxian ZHANG¹, Changshen NING^{1,**}

¹College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou, P.R. China

²Animal Medical Science, Henan University of Animal Husbandry and Economy, Zhengzhou, P.R. China

³College of Animal Science, Tarim University, Alar, P. R. China

Received: 29.07.2015 • Accepted/Published Online: 25.05.2016 • Final Version: 21.02.2017

Abstract: Tick-borne diseases are one of the most important classes of disease in animal husbandry and cause severe economic losses. In this study, 660 adult female ticks were collected from sheep in nine localities in Henan Province, China. All were identified as *Haemaphysalis longicornis*, confirmed with light microscopy and PCR amplification. The pathogens identified in these ticks included bacteria of the genus *Anaplasma* (*Anaplasma ovis*, *A. bovis*, *A. phagocytophilum*) and piroplasmal protozoans (*Theileria luwenshuni*, *Babesia motasi*). Our results show the high prevalence of *A. bovis* (20.4%, 135/660) and *T. luwenshuni* (14.4%, 95/660) in the ticks from sheep, whereas *A. ovis*, *A. phagocytophilum*, and *B. motasi* occurred in 2.3% (15/660), 1.5% (10/660), and 0.75% (5/660) of ticks, respectively. This is the first report of *B. motasi* in *H. longicornis* in China. In contrast, no *T. uilenbergi* infection was found in this study. These results confirm that *H. longicornis* is the most common tick species in sheep in Henan Province and can transmit *A. phagocytophilum*, a well-known zoonotic pathogen of public-health and veterinary significance. Importantly, these results suggest that *A. bovis*, transmitted by *H. longicornis*, is a predominant pathogen of sheep in this region.

Key words: *Haemaphysalis longicornis*, tick-borne pathogens, sheep, China

1. Introduction

Ticks are hematophagous arthropods and transmit more pathogen species like fungi, viruses, bacteria, and protozoa to humans, livestock, and companion animals than any other group of blood-feeding arthropods worldwide (1). Tick-borne diseases are one of the greatest obstacles to livestock production in developing countries, causing direct damage to animals and thus reducing the quality of hides, live weights, and milk production (2).

Haemaphysalis longicornis is the most common tick species in China and plays an important role as a vector of several pathogens that cause anaplasmosis, babesiosis, and rickettsiosis (3). These diseases are very important to the livestock industry. The study of tick-borne pathogens with molecular screening methods began in the early 1990s. The collection of ticks from hosts or vegetation and their analysis with molecular tools are efficient ways to assess the occurrence of tick-borne pathogens and the risk of tick-borne diseases in a specific geographic area (4–9).

The genus *Anaplasma* includes tick-borne pathogens that affect human and animal health. *Anaplasma phagocytophilum*, in particular, causes human and animal granulocytic anaplasmosis, an important immunopathological vector-borne disease in the United States, Europe, and Asia (10).

Tick-borne piroplasms can be divided into the protozoan genera *Theileria* and *Babesia*. Sheep and goat theileriosis is caused by *T. ovis*, *T. luwenshuni*, and *T. uilenbergi* in most regions of northwestern China (11). In susceptible sheep, it can be highly pathogenic. Babesiosis has been reported in several European countries, as well as Egypt, India, Japan, Korea, Taiwan, and South Africa (12). Although *Haemaphysalis longicornis* is a well-known tick vector of *Theileria* spp. and *Babesia* spp., as well as *Anaplasma* spp., little is known about the diversity of tick-borne diseases in China. This study aims to determine the occurrence of *Anaplasma* spp., *Theileria* spp., and *Babesia* spp. in *H. longicornis* from sheep in Henan Province, China.

* These authors contributed equally to this work.

** Correspondence: nnl1986@163.com

2. Materials and methods

2.1. Sample collection

From July 2011 to September 2012, 660 adult ticks were collected from 132 adult sheep at nine localities in Henan Province, China (Figure). Sample collection was equally distributed among these localities. All the ticks were first identified morphologically with stereomicroscopy and then verified by molecular analyses.

2.2. DNA extraction

Genomic DNA was extracted and purified from the complete bodies of the adult ticks using the Blood & Tissue Gene DNA Kit (Beijing Kangweishiji Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. All DNA samples were stored at -20°C until their molecular analysis.

2.3. PCR for detecting tick-borne *Anaplasma* pathogens

The purified DNA was used for the PCR-based detection of tick-borne *Anaplasma* species using a primer that amplified a 116-bp fragment of the 16S rDNA gene of bacteria belonging to the *Anaplasma* family, including species *A. ovis*, *A. bovis*, and *A. phagocytophilum* (Table). PCR was performed in a final volume of 25 μL of TaKaRa LA Taq (TaKaRa, Japan) containing 2.5 U of DNA polymerase, 2.5 mM LA Taq buffer, and 4 mM of

each dNTP. The conditions used for amplification were as follows: initial denaturing at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 10 min.

PCR-positive DNA samples were used to detect *A. ovis*, *A. bovis*, and *A. phagocytophilum* by conventional and nested PCR techniques using species-specific primers (Table). PCR amplification of genomic DNA from *A. ovis* was performed using conventional PCR with species-specific primers (Table). The primer sets used to detect *A. bovis* and *A. phagocytophilum* DNA in the nested PCR were derived from the 16S rDNA gene sequences, with the same pair of outer primers and a different set of inner primers.

Conventional and nested PCR was also performed using TaKaRa LA Taq (TaKaRa, Japan) in a total volume of 25 μL containing 50 to 500 ng of sample DNA for the first PCR and 1 μL of the first PCR product for the second PCR. The conventional PCR conditions were as follows: 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 10 min. The first conditions used for amplification were as follows: initial denaturing at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 10 min. The second conditions

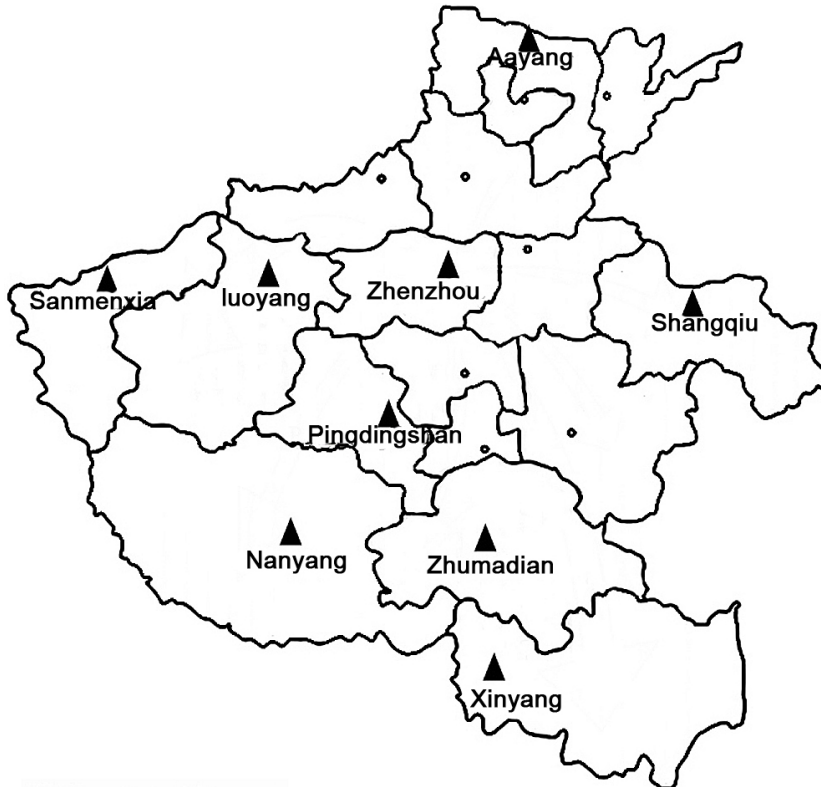


Figure. Map of Henan Province indicating the areas where samples were collected (marked with triangles).

used for amplification were as follows: initial denaturing at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 90 s, annealing at 55 °C for 90 s, and extension at 72 °C for 10 min.

Positive DNA samples with *A. ovis*, *A. bovis*, and *A. phagocytophilum* from the International Joint Research Laboratory for Zoonotic Diseases of Henan, China, were used as a positive control for detecting *A. ovis*, *A. bovis*, and *A. phagocytophilum* species. Distilled water was used as a negative control. The positive control and negative control were included in each PCR experiment.

2.4. PCR for detecting tick-borne Babesia and Theileria species

The PCR for detecting *Theileria* and *Babesia* spp. was performed using primers that amplified a 403-bp fragment of the V4 region of the 18S rDNA gene. PCR-positive DNA samples were then used for the detection of *T. luwenshuni*, *T. uilenbergi*, and *B. motasi* by nested PCR. The positive DNA sample with *T. luwenshuni*, *T. uilenbergi*, and *B. motasi* from the International Joint Research Laboratory for Zoonotic Diseases of Henan, China, was used as a positive control for detecting tick-borne protozoan species. Positive and negative (distilled water) controls were included in each PCR experiment. The specific primer pairs and annealing temperatures are shown in the Table.

Conventional and nested PCR was performed in a final volume of 25 µL of TOYOBO KOD FX Taq (TOYOBO, Japan) containing 2.5 U of DNA polymerase, 12.5 mM KOD FX Taq buffer, and 5 mM of each dNTP. The conditions used for amplification were as follows: initial denaturing at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 10 min.

2.5. PCR products testing

After purification, the PCR products were sequenced directly with secondary PCR primers on an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, USA). Sequence accuracy was confirmed with two-directional sequencing, and a new PCR product was sequenced if necessary. The sequences were identified by their alignments with reference sequences downloaded from GenBank (<http://www.ncbi.nlm.nih.gov>), using MEGA 4 software. The sequences were as follows: *H. leporispalustris* (L34309), *H. juxtakochi* (AY762323), *A. hebraeum* (L34316.1), *A. aureolatum* (AF541254), *I. scapularis* (L34293), *I. persulcatus* (L34295), *I. acutitarsus* (U95877), *D. reticulatus* (JF928522), *H. doenitzi* (JF979402), *H. longicornis* (FJ712721), and *H. anatolicum anatolicum* (JX392003).

Table. PCR primers used to detect tick-borne pathogens from sheep.

Target organism	Gene	Primer sequence (5'-3')		Fragment size, bp
		First	Second	
Tick DNA	12S rRNA	T1B: CTGCTCAATGAATATTTAAATTGC	T2A: CGGTCTAAACTCAGATCATGTAGG	454
<i>Anaplasma</i>	16S rDNA	F: AGTCCACGCTGTAAACGATGAG R: TTCCTTTGAGTTTTAGTCTTGCGAC		116
<i>A. ovis</i>	16S rDNA	F: CCGGATCCTTAGCTGAACAGGAATCTTGC R: GGGAGCTCCTATGAATTACAGAGAATTGTTTAC		867
<i>A. bovis</i>	16S rDNA	F1: TCCTGGCTCAGAACGAACGCTGGCGGC R1: GTCACTGACCCAACCTTAAATGGCTG	F2: CTCGTAGCTTGCTATGAGAAC R2: TCTCCCGGACTCCAGTCTG	641
<i>A. phagocytophilum</i>	16S rDNA		F2: GCTGAATGTGGGGATAATTTAT R2: ATGGCTGCTTCCTTTCGGTTA	551
Piroplasma	18S rDNA	F: GACACAGGGAGGTAGTGACAAG R: CTAAGAATTCACCTCTGACAGT		403
<i>T. luwenshuni</i>	18S rDNA	F1: CATGGATAACCGTGCTAATT R1: ATCGTCTTCGATCCCCTAACT	F2: GGTAGGGTATTGGCCTA CTGA R2: TCATCCGGATAATACAAGT	388
<i>T. uilenbergi</i>	18S rDNA		F2: GGTAGGGTATTGGCCTACCGG R2: AACTGGAAAATGCAAGCT	389
<i>B. motasi</i>	18S rDNA		F2: TAAACCAA TTTGTTGGT R2: TCTGCCAGGGTTTAAGTCGG	294

3. Results

In this study, morphological examination of 660 ticks collected from sheep identified them all as *H. longicornis*. All the 16S rDNA gene sequences isolated with PCR were 454 bp in length and showed 98.6%–100% similarity to the *H. longicornis* sequence obtained from the GenBank database (accession number FJ712721). These results suggest that *H. longicornis* is the most common tick species in sheep in Henan Province.

Of the ticks tested, 150 specimens (23.5%) were infected with *Anaplasma* spp., including *A. bovis*, *A. ovis*, and *A. phagocytophilum*. The prevalence of *A. bovis* was higher (20.4%, 135/660) than that of *A. ovis* (2.3%, 15/660) or *A. phagocytophilum* (1.5%, 10/660). Coinfection with *A. bovis* and *A. phagocytophilum* was observed in two tick specimens (an infection rate of 1.5%). Piroplasmas were detected in 100 (15.2%) tick specimens. A BLAST analysis of their 18S rDNA sequences assigned them to *T. luwenshuni* (95/660; 14.4%) and *B. motasi* (5/660; 0.75%), with 100% sequence similarity.

4. Discussion

A previous study showed that *H. longicornis* is the tick species most commonly detected in grass and other vegetation in China (13). Another epidemiological study revealed that *H. longicornis* is the tick species most frequently recovered from sheep and goats in central China, including Henan Province (14).

A. ovis causing anaplasmosis is the most frequent pathogen in sheep and goats, with high serological and biomolecular prevalence (15,16). In Italy, *A. ovis* is reported to occur with a prevalence of 82.9% in sheep and 74.9% in goats (17,18). A PCR-based molecular analysis demonstrated that *Anaplasma* spp. are highly prevalent in goats in central and southern China, and the average prevalence of single infections with *A. ovis*, *A. bovis*, or *A. phagocytophilum* was 46.6%, 49.6%, or 14.5%, respectively. *Anaplasma ovis* is transmitted by ticks of the species *Rhipicephalus bursa*, *R. turanicus*, *Dermacentor silvarum*, *D. marginatus*, *D. andersoni*, and *H. sulcata* (16). The lower prevalence of *A. ovis* in *H. longicornis* in this study suggests that *H. longicornis* might not be the main host tick for this pathogen.

Anaplasma bovis is another major pathogen of ruminants. In a previous study, the prevalence of *A. bovis* and *A. phagocytophilum* in cattle was 80.0% and 40.0%, respectively (19), whereas the prevalence of *A. phagocytophilum* was 6.7% in both sheep and goats (19). Domestic ruminants infected with *A. bovis* have been

reported predominantly in Turkey and African countries (19, 20). However, *A. bovis* DNA was recently detected in *H. longicornis* ticks collected in Korea (21) and Honshu Island, Japan (1). These results indicate that *A. bovis* is more common than other *Anaplasma* species in *H. longicornis*. *Anaplasma bovis* was also observed in the monocytes of experimentally infected sheep in a previous study, suggesting that *A. bovis* is a predominant pathogen of sheep, transmitted by *H. longicornis* ticks.

Anaplasma phagocytophilum was transmitted by ticks of the family Ixodidae, by species *I. persulcatus*, *D. silvarum*, *H. longicornis*, and *H. concinna*, at 14 sites near the China–Russia border, and the prevalence of *A. phagocytophilum* was 2.5% in *H. longicornis* (22). Our results show that the prevalence of *A. phagocytophilum* in *H. longicornis* in southern China (1.5%) is lower than that in *H. longicornis* ticks in the north. However, the importance of this vector in public health and agriculture is yet to be investigated in these areas.

Li et al. reported that *T. luwenshuni* is the most prevalent *Theileria* species in small ruminants in central China, whereas no *T. uilenbergi* or *T. ovis* infections were detected (13). In the present study, we also detected no *T. uilenbergi* in the ticks collected from sheep. *Theileria luwenshuni* and *T. uilenbergi* can be transmitted by both *H. qinghaiensis* and *H. longicornis*, and they are mainly distributed in the northwestern regions of China (23). However, *H. qinghaiensis* is a species specific to China and is distributed throughout the western plateau of the country. In this study, *H. longicornis* was the only tick species isolated from sheep in Henan Province, and therefore it plays an important role as a natural vector of *T. luwenshuni*.

The tick vectors of *B. motasi* have not been systematically studied, and its route of transmission is unknown. *Haemaphysalis qinghaiensis* has been shown to transmit *B. motasi* in Gansu Province (24,25). In this study, we found that *H. longicornis* is also a carrier of *B. motasi* and that the occurrence of *B. motasi* was 0.75% (5/660). To our knowledge, this is the first report of the detection of *B. motasi* in *H. longicornis* ticks in China.

Acknowledgments

We warmly acknowledge Haiyan Wang at the Henan Agricultural Vocational College for assistance with this study. Funding was supported by the earmarked fund for the China Modern Agro-industry Technology Research System (nycytx-39).

References

1. de la Fuente J, Estrada-Pena A, Venzal JM, Kocan KM, Sonenshine DE. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Front Biosci* 2008; 13: 6938-6946.
2. Simuunza M, Weir W, Courcier E, Tait A, Shiels B. Epidemiological analysis of tick-borne diseases in Zambia. *Vet Parasitol* 2011; 175: 331-334.
3. Yin H, Luo J. Ticks of small ruminants in China. *Parasitol Res* 2007; 101: S187-S189.
4. Zhang Y, Si BY, Liu BH, Chang GH, Yang YH, Huo QB, Zheng YC, Zhu QY. Complete genomic characterization of two tick-borne encephalitis viruses isolated from China. *Virus Res* 2012; 167: 310-313.
5. Movila A, Toderas I, Uspenskaia I, Conovalov J. Molecular detection of tick-borne pathogens in *Ixodes ricinus* from Moldova collected in 1960. *Ticks Tick Borne Dis* 2013; 4: 359-361.
6. Aktas M, Altay K, Ozubek S, Dumanli N. A survey of ixodid ticks feeding on cattle and prevalence of tick-borne pathogens in the Black Sea region of Turkey. *Vet Parasitol* 2012; 187: 567-571.
7. Aydin MF, Aktas M, Dumanli N. Molecular identification of *Theileria* and *Babesia* in ticks collected from sheep and goats in the Black Sea region of Turkey. *Parasitol Res* 2015; 114: 65-69.
8. Aktas M. A survey of ixodid tick species and molecular identification of tick-borne pathogens. *Vet Parasitol* 2014; 200: 276-283.
9. Ica A, Vatansever Z, Yildirim A, Duzlu O, Inci A. Detection of *Theileria* and *Babesia* species in ticks collected from cattle. *Vet Parasitol* 2007; 148: 156-160.
10. Severo MS, Stephens KD, Kotsyfakis M, Pedra JH. *Anaplasma phagocytophilum*: deceptively simple or simply deceptive. *Future Microbiol* 2012; 7: 719-731.
11. Yin H, Schnittger L, Luo J, Seitzer U, Ahmed JS. Ovine theileriosis in China: a new look at an old story. *Parasitol Res* 2007; 101 (Suppl. 2): S191-S195.
12. Gray J, Zintl A, Hildebrandt A, Hunfeld KP, Weiss L. Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity. *Ticks Tick Borne Dis* 2010; 1: 3-10.
13. Chen Z, Yang X, Bu F, Yang X, Yang X, Liu J. Ticks (Acari: Ixodoidea: Argasidae, Ixodidae) of China. *Exp Appl Acarol* 2010; 51: 393-404.
14. Li YQ, Zhang X, Liu ZJ, Chen Z, Yang JF, He HN, Guan GQ, Liu AH, Ren QY, Niu QL et al. An epidemiological survey of *Theileria* infections in small ruminants in central China. *Vet Parasitol* 2014; 200: 198-202.
15. Torina A, Galindo RC, Vicente J, Di Marco V, Russo M, Aronica V, Fiasconaro M, Scimeca S, Alongi A, Caracappa S et al. Characterization of *Anaplasma phagocytophilum* and *A. ovis* infection in a naturally infected sheep flock with poor health condition. *Trop Anim Health Prod* 2010; 42: 1327-1331.
16. Liu Z, Ma M, Wang Z, Wang J, Peng Y, Li Y, Guan G, Luo J, Yin H. Molecular survey and genetic identification of *Anaplasma* species in goats from central and southern China. *Appl Environ Microbiol* 2012; 78: 464-470.
17. Stoltz WH. Ovine and caprine anaplasmosis. In: Coetzer J, Tustin RC, editors. *Infectious Diseases of Livestock*. Oxford, UK: Oxford University Press; 2004. pp. 617-624.
18. Altay K, Dumanli N, Aktaş M, Özübek S. Survey of *Anaplasma* infections in small ruminants from east part of Turkey. *Kafkas Univ Vet Fak Derg* 2014; 20: 1-4.
19. Zhan L, Cao WC, Jiang JF, Zhang XA, Wu XM, Zhang WY, Liu W, Zuo SQ, Cao ZW, Yang H et al. *Anaplasma phagocytophilum* in livestock and small rodents. *Vet Microbiol* 2010; 144: 405-408.
20. Aktas M, Özübek S. Bovine anaplasmosis in Turkey: first laboratory confirmed clinical cases caused by *Anaplasma phagocytophilum*. *Vet Microbiol* 2015; 178: 246-251.
21. Kim CM, Yi YH, Yu DH, Lee MJ, Cho MR, Desai AR, Shringi S, Klein TA, Kim HC, Song JW et al. Tick-borne rickettsial pathogens in ticks from small mammals in Korea. *Appl Environ Microbiol* 2006; 72: 5766-5776.
22. Jiang JF, Jiang BG, Yu JH, Zhang WY, Gao HW, Zhan L, Sun Y, Zhang XA, Zhang PH, Liu W et al. *Anaplasma phagocytophilum* infection in ticks, China-Russia border. *Emerg Infect Dis* 2011; 17: 932-934.
23. Oh JY, Moon BC, Bae BK, Shin E, Ko YH, Kim YJ, Park YH, Chae JS. Genetic identification and phylogenetic analysis of *Anaplasma* and *Ehrlichia* species in *Haemaphysalis longicornis* collected from Jeju Island, Korea. *J Bacteriol Virol* 2009; 39: 257-267.
24. Li Y, Luo J, Guan G, Ma M, Liu A, Liu J, Ren Q, Niu Q, Lu B, Gao J et al. Experimental transmission of *Theileria uilenbergi* infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. *Parasitol Res* 2009; 104: 1227-1231.
25. Guan GQ, Yin H, Luo JX, Lu WS, Zhang QC, Gao YL, Lu BY. Transmission of *Babesia* sp to sheep with field-collected *Haemaphysalis qinghaiensis*. *Parasitol Res* 2002; 88: S22-S24.