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J Vet Res 67, 79-85, 2023
DOI: 10.2478/jvetres-2023-0016

Detection of *Babesia* spp., *Theileria* spp., and *Anaplasma ovis* in *Ornithodoros lahorensis* from southern Xinjiang, China

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Received: September 20, 2022 Accepted: March 6, 2023

Abstract

Introduction: Ticks are blood-sucking arthropods that have negative economic impacts and can spread a variety of diseases through their bites. There are few reports on soft ticks (Acari: Argasidae) and tick-borne pathogens in southern Xinjiang, China. This investigation supplements the available information for this region and is concerned with an argasid tick, apicomplexan parasites of the *Babesia* and *Theileria* genera and a bacterium of the *Anaplasma* genus. **Material and Methods:** In this study, 330 soft ticks were collected from nine sampling sites in southern Xinjiang between 2020 and 2021. The ticks were identified according to their morphological characteristics and confirmed as *Ornithodoros lahorensis* using mitochondrial 16S rDNA sequences. *Babesia* and *Theileria* were identified at the species level based on two fragments of the 18S rRNA gene, and one set of primers targeting the 16S rRNA gene was used to identify the *Anaplasma* genus. **Results:** Among the 330 samples, one *Babesia* species (*Babesia* sp.), two *Theileria* species (*T. ovis* and *T. annulata*), and one *Anaplasma* (*A. ovis*) species were detected. **Conclusion:** This study provides fundamental evidence for the occurrence of *Babesia*, *Theileria* and *Anaplasma* spp. in soft ticks. To the best of our knowledge, this is the first report of the detection of *Babesia* sp. and *T. annulata* in *O. lahorensis*. Therefore, the potential threat of soft ticks to livestock and humans should not be ignored.

Keywords: *Ornithodoros lahorensis*, southern Xinjiang, *Babesia*, *Theileria*, *Anaplasma*.

Introduction

Ticks are important vectors of diseases that affect the health of humans and livestock. Particular geographical conditions are beneficial to the survival of particular tick species and determine those species' global distribution (14). To date, approximately 900 tick species have been identified worldwide (10). Approximately 132 known tick species have been detected in China, including 113 species of Ixodidae in seven genera and 19 species of Argasidae in two genera (30). Forty-two species of ticks from nine genera, which represent more than one-third of the total tick species found in China, have been identified in Xinjiang (4), the most prevalent species being *Ornithodoros lahorensis* (Argasidae) and *Rhipicephalus turanicus* (Ixodidae) (28). As an important pathogenic vector, argasid ticks feed on a variety of hosts, especially migratory birds and mammals. With the movement of their hosts, these ticks have become distributed worldwide and are considered to be reservoirs for several diseases (6). However, because of the short feeding duration and nidicolous lifestyle, fewer studies have been conducted on argasid ticks and argasid tick-borne diseases than on ixodid ticks.

Babesiosis and theileriosis are significant zoonoses caused by obligate red blood cell parasites of the *Babesia* and *Theileria* genera, respectively (15, 26). *Babesia* and *Theileria* are piroplasms that include tick-borne pathogens infecting humans, wildlife and domestic animals. Infected animals show increased heart and respiratory rates, fever, loss of appetite,

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anaemia, jaundice, depression, and cessation of rumination, which lead to decreased productivity, increased stillbirths, and death (34). More than 100 *Babesia* species have been recorded, including nine (*B. microti*, *B. divergens*, *B. duncani*, *B. venatorum*, *B. bovis*, *B. bigemina*, *B. microti-like*, *B. divergens-like* and *B. crassa-like*) associated with human infections and several variants likewise harmful to humans (11, 26). Thirteen validated *Babesia* spp. have been reported in China (22). Eighteen *Theileria* spp. have been recognied worldwide (8). Recently, several of them have been reported in China, including *T. uilenbergi*, *T. annulata*, *T. orientalis*, *T. luwenshuni*, *T. lestoquardi*, *T. separata*, *T. ovis* and *T. sinensis* (16, 23).

Anaplasmosis, caused by small, gram-negative, obligate intracellular bacteria of the *Anaplasma* genus (Rickettsiales: Anaplasmataceae), is also a common tick-borne disease. The *Anaplasma* genus includes *A. phagocytophilum*, *A. bovis*, *A. platys*, *A. marginale*, *A. centrale*, *A. ovis*, *A. capra* and *A. caudatum*. Three distinct species of *Anaplasma* (*A. phagocytophilum*, *A. capra* and *A. platys*) have been known to cause zoonoses (2). *Anaplasma ovis* is a tick-borne intra-erythrocytic pathogen in goats, sheep and wild small ruminants that can cause fever, weakness, progressive anaemia, mucous membrane pallor, weight loss, icterus, lethargy, abortion, a decrease in milk yield, and sometimes death (5).

The Xinjiang Uygur Autonomous Region, the largest administrative division in China with a territory of approximately 1,660,000 km^2, is approximately one-sixth of mainland China (21). Southern Xinjiang occupies an area of approximately 1,020,000 km^2 and is surrounded by multiple land forms, including the Gobi Desert, valleys, mountains, grassland and flatland. The economic drivers of southern Xinjiang are mainly agriculture and animal husbandry; therefore livestock diseases, especially babesiosis, theileriosis, and anaplasmosis, which have caused great harm and significant economic losses to animal husbandry in the region, have impact upon the entire Xinjiang economy (21). Despite being important tick-borne diseases, babesiosis, theileriosis and anaplasmosis are still understudied in Xinjiang. Previous studies have mainly focused on ixodid ticks and ixodid tick-borne pathogens (32), and the negative effects of argasid ticks and argasid tick-borne pathogens on animal husbandry and public health have been underestimated. The present study is a systematic investigation of argasid tick–borne pathogens within the piroplasmid *Babesia* and *Theileria* genera and the rickettsial *Anaplasma* genus in southern Xinjiang. This is the first report of *Babesia* sp. and *T. annulata* in *O. lahorensis* from southern Xinjiang, China.

Material and Methods

Sampling area. In the peak tick seasons from March to mid-September in 2020 and 2021, questing and parasitic ticks were collected from nine sampling sites in southern Xinjiang: ZePu, KuChe, MoYu, XinHe, AWaTi, BaChu, WuShi, YeCheng, and ATuShi (Table 1, Fig. 1). Questing ticks were collected from the surrounding environment using the flagging method, while parasitic ticks were collected directly from cattle, sheep and dogs. In addition, whole blood samples were drawn from tick-bearing sheep into individual tubes containing ethylenediaminetetraacetic acid. The animal handling protocol was revised and approved by the Ethics Committee of Tarim University, Xinjiang, China. Under these guidelines, adult ticks which had not fed were collected from the entire body of each animal, including ears, chest, neck, muzzle, axillae, abdomen, forehead, limbs, perianal area and tail. The location, host, and number of ticks were recorded. The ticks were immediately introduced into 75% ethanol, stored at 4°C, and transported to the laboratory for classification and identification based on morphological characteristics (13).

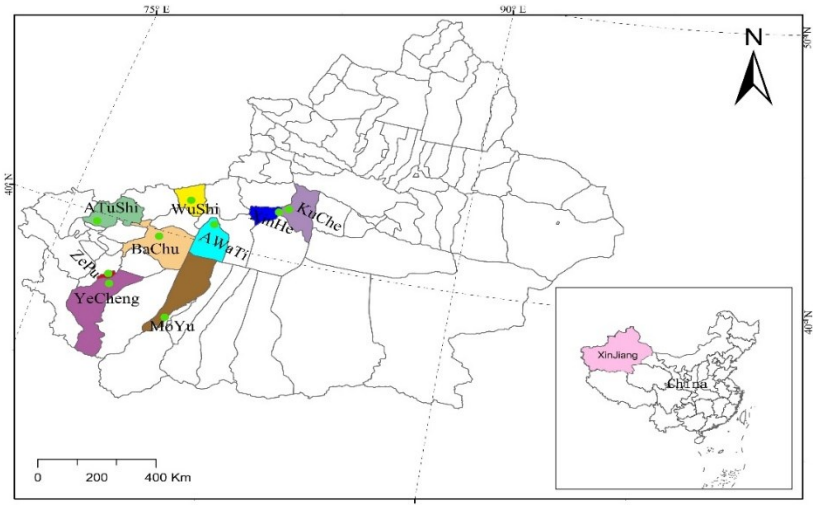


Fig. 1. Sites where ticks were collected in southern Xinjiang, China. The nine surveyed counties are marked with green dots

Table 1. Molecular detection of *Babesia* spp., *Theileria* spp., and *Anaplasma* spp. in *Ornithodoros lahorensis* at specimen collection sites in nine counties and cities in southern Xinjiang, China

Location	Hosts	Number of ticks	Number of male/female ticks	Number of positive/Positive rate (%)		
				Babesia spp.	*Theileria* spp.	*Anaplasma* spp.
AWaTi	Environment	8	7/1	0/0	0/0	0/0
	Sheep	35	18/17	0/0	*T. ovis* 1/2.9 % 95% CI: −2.9–8.7	0/0
ATuShi	Cattle	17	5/12	0/0	0/0	0/0
BaChu	Sheep	31	11/20	0/0	*T. annulate* 1/3.2 % 95% CI: −3.4–9.8	0/0
KuChe	Sheep	26	14/12	0/0	*T. ovis* 1/3.8 % 95% CI: −4.1–11.8	0/0
MoYu	Sheep	22	14/8	0/0	*T. ovis* 1/4.5 % 95% CI: −4.9–14.0	0/0
WuShi	Sheep	43	18/25	0/0	*T. annulate* 1/2.3 % 95% CI: −2.4–7.0	0/0
XinHe	Dog	23	7/16	0/0	0/0	0/0
YeCheng	Cattle	16	5/11	0/0	0/0	0/0
ZePu	Environment	18	11/7	0/0	0/0	0/0
	Sheep	91	36/55	*Babesia* sp. 3/3.3% 95% CI: −0.4–7.0	*T. ovis* 2/2.2% 95% CI: −0.9–5.3	*A. ovis* 5/5.5% 95% CI: 0.7–10.3
Total		330	146/184	3/0.9% 95% CI: −0.1–1.9	7/2.1% 95% CI: 0.6–3.7	5/1.5% 95% CI: 0.2–2.8

Tick morphological identification and total DNA extraction. Morphological characteristics comprising basis capituli, legs, tarsi, dorsal surface, ventral surface, punctation, scutum, and anal groove were identified with a Leica stereomicroscope M165 C (Solms, Germany). The ticks were washed with 70%, 50%, 30% and 10% ethanol under a constant-temperature culture oscillator for 30 min, washed with sterile distilled water three times to eliminate contamination due to tick faeces and other substances, and then dried on filter paper. Total DNA was extracted from each individual tick and sheep blood sample using the TIANamp Genomic DNA Kit (Tiangen, Beijing, China) following the manufacturer's instructions. Each tick sample was minced with a disposable sterile scalpel in a microtube and digested with proteinase K (20 μg/mL), then the lysate was transferred to the columns of the TIANamp kit for DNA absorption. Finally, DNA was eluted in 200 μL of the buffer provided with the kit and stored at −20°C to avoid degradation. DNA was quantified using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), with a total DNA concentration threshold >50 ng/μL to ensure pathogen detection.

Molecular identification of tick species. Three ticks from each specimen collection site, making a total of 27 representative samples, were selected for molecular identification. These ticks were adults which had not consumed blood. They were subjected to polymerase chain reaction (PCR) amplification with primers targeting a 460-base-pair (bp) fragment of the tick mitochondrial 16S rDNA gene as previously described (18). Amplification by PCR was performed using 2× Taq PCR Master Mix (Tiangen) according to the manufacturer's instructions. The reaction mixture was initially denatured at 94°C for 5 min, then cycled 38 times through denaturation at 92°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, and finally extended in a step at 72°C for 8 min. The amplicons were visualised by electrophoresis on a 1.5% agarose gel containing GelStain (TransGen Biotech, Beijing, China). Bands of DNA of the correct size were purified and sequenced by GENEWIZ (Suzhou, China), and the 16S rDNA sequencing results were submitted to the GenBank database.

Detection of *Babesia* and *Theileria* spp. Two pairs of primers, PIRO-A/B (19) and BJ1/BN2 (3), targeting two fragments of the 18S rRNA gene were used to detect *Babesia* and *Theileria* spp., amplifying 408- and 487-bp fragments, respectively. A further PCR was performed using 2× Taq PCR Master Mix (Tiangen) according to the manufacturer's instructions. Sterile water was used as the negative control, and DNA from *B. motasi* and *T. ovis* as the positive controls. The amplicons were cloned into the pGM-T vector and sequenced using T7 primers (Promega, Madison, WI, USA). The sequencing results were compared to reference sequences downloaded from GenBank, and a phylogenetic tree based on two concatenated fragments of 18S rRNA partial gene sequences was constructed using MEGA 10 software.

Detection of *A. ovis*. One pair of primers, An-F: 5′-GAGAGTTTGATCCTGGCTCAGAAC-3′ and An-R: 5′-TATAGGTACCGTCATTATCTTCCCTAC-3′, targeting the 16S rRNA gene (24), was developed to identify the genus *Anaplasma*, amplifying a 452-bp fragment. The PCR mixture, which had a total volume of 25 μL, contained 13 μL of 2× Taq PCR Master Mix (Tiangen), 1 μL of the relevant primers (10 μM final concentration), 9 μL of nuclease-free deionised water, and 1 μL of template DNA. Amplification by PCR was performed on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s; and a final extension at 72°C for 10 min. The amplicons were electrophoresed on a 1.5% agarose gel containing GelStain (TransGen Biotech, Beijing, China).

Results

Tick collection and identification. After two years of intermittent collection, 330 soft ticks were captured from nine counties or cities in southern Xinjiang. Morphologically, 146 (44.2%, 146/330) were identified as male and 184 (55.8%, 184/330) as female (Table 1). Further morphological identification and molecular analysis confirmed that all ticks were *O. lahorensis*, and the identified sequences were deposited in the GenBank database (accession nos OM673115, OM673116, OM673118–OM673120 and OM673122–OM673125).

Analysis of *Babesia* and *Theileria* spp. Of the 330 soft ticks, 3 and 7 were positive for *Babesia* and *Theileria* DNA, respectively (Table 1). Sanger sequencing of these PCR products and BLAST analysis identified one *Babesia* (0.9%, 3/330; *Babesia* sp.) and two *Theileria* spp. (2.1%, 7/330; *T. ovis* and *T. annulata*). Phylogenetic analysis of the *Babesia* sp. based on two concatenated fragments of 18S rRNA revealed that our sequences shared 98.7–99.8% identity with those obtained from *Hyalomma anatolicum anatolicum* (Ixodidae) and sheep specimens from China (accession nos DQ159073.1 and HQ730762.1) (Fig. 2). The *T. ovis* sequence shared 99.8% identity with THOD2 (MN625903.1) reported in Egypt (Fig. 2). The concatenated sequences of *T. annulata* detected in our study were classified into the cluster including *T. annulata* Kashgar (MK415058.1) and shared 98.6–99.8% identity with the corresponding sequences (Fig. 2).



Fig. 2. Phylogenetic analysis of *Theileria annulata*, *T. ovis* and *Babesia* sp. based on two concatenated fragments of 18S rRNA partial gene sequences detected in *Ornithodoros lahorensis* from southern Xinjiang, China. The tree was constructed with maximum likelihood phylogenetic analysis with 500 bootstrap replicates using MEGA 10 software. Sequences of the *T. annulata*, *T. ovis* and *Babesia* sp. identified in this study are shown as (■), (▲) and (●), respectively

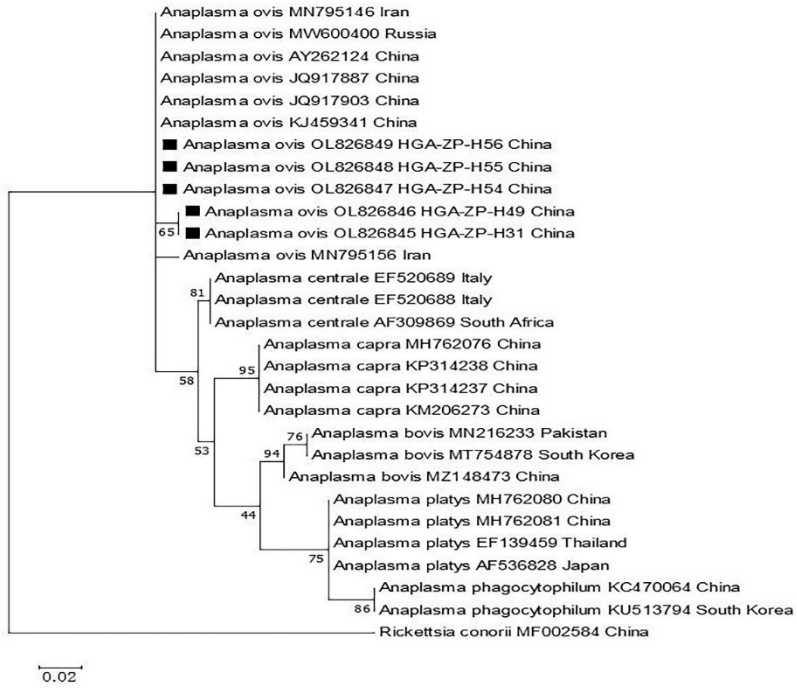


Fig. 3. Phylogenetic tree of *Anaplasma ovis* based on the 16S rRNA partial gene sequences detected in *Ornithodoros lahorensis* from southern Xinjiang, China. The tree was constructed with maximum likelihood phylogenetic analysis with 500 bootstrap replicates using MEGA 10 software. Sequences of the *A. ovis* identified in this study are shown as (■)

Analysis of *A. ovis*. The molecular survey showed an overall positivity rate of 1.5 % (5/330) for *A. ovis* in *O. lahorensis*. Sequence analysis of the five amplicons derived from the specimens collected from the ZePu sampling site showed that they shared 98.4–99.1% sequence identity. A phylogenetic tree based on the 16S rDNA of the representative *Anaplasma* spp. showed that all sequences acquired in our study clustered within the clade including the ovis5 strain (KJ459341.1) previously identified in China (Fig. 3). The identified sequences were deposited in the GenBank database (accession nos OL826845-OL826849).

Discussion

China has more than 120 diverse species of ticks (accounting for approximately 13% of species worldwide), among which the ticks identified in Xinjiang are the most abundant. As the ecological environment, climate, and human activity have changed, so have species' relative prevalences and the quantity of ticks. Birds and mammals are important hosts for most ticks and facilitate their migration, which increases the risk of tick-borne diseases (29). In recent years, several tick-borne pathogens from Pakistan, Kyrgyzstan, Afghanistan and Tajikistan, have been reported, as have tick-borne zoonotic diseases such as tularaemia, Crimean-Congo haemorrhagic fever and Q fever (12, 25, 27). Southern Xinjiang, which borders Pakistan, Afghanistan, Tajikistan, Kyrgyzstan and India, is a potential area for tick migration and transmission of tick-borne diseases. Several pathogens have been found in ixodid ticks in Xinjiang, such as Candidatus *Rickettsia barbariae*, *R. massiliae*, and *R. conorii* (32). In addition, a high prevalence of pathogens in soft ticks from this area has previously been reported (31). Southern Xinjiang has a multiplex ecological environment: not only is there the Gobi Desert but there are also partial oases that provide a suitable habitat for ticks that may carry various pathogens. Moreover, corridor much international livestock trade with neighbouring countries passes through southern Xinjiang, and given that tick-borne diseases including babesiosis and theileriosis can be disseminated by the long-distance movement of hosts, such diseases are a challenge in the region not only in veterinary medicine but also in human healthcare. Therefore, it is necessary to continuously investigate tick-borne diseases in this region.

Babesiosis is a tick-borne disease caused by several species of *Babesia*, which can infect many domestic and wild animals, and is of public health importance. In China, *Babesia* sp. Xinjiang (accession no. DQ159073) was originally detected in Kashi, southern Xinjiang, and the same study found that *Babesia* spp. were transmitted to sheep by *H. anatolicum anatolicum* (9). *Babesia* sp. has also been reported in *H. asiaticum* from the border area of Xinjiang (21). To the best of our knowledge, the present study is the first to detect *Babesia* sp. in *O. lahorensis*. The detected species was closely related to *Babesia* sp. Xinjiang. Our findings extend the

potential vector spectrum of *Babesia* sp. and raise veterinary and public health concerns about soft ticks; however, further studies should be carried out to confirm whether *O. lahorensis* can transmit this pathogen to livestock and humans.

Theileriosis, a tick-borne disease caused by haemoprotozoan parasites, significantly impacts the development of animal husbandry and agriculture. *Theileria annulata* and *T. ovis* are the most common tick-borne pathogens that affect domestic ruminants (*e.g.* sheep, goats, and cattle) in parts of Africa, Asia and Europe (7, 27). *Theileria annulata* is transmitted by ticks, and the most common vectors are *H. marginatum*, *H. lusitanicum*, *H. excavatum*, *Rhipicephalus annulatus* (Ixodidae) and *R. appendiculatus* (1). In this study, we found *T. annulata* DNA in *O. lahorensis* from free-grazing sheep in the BaChu and WuShi counties. In the phylogenetic tree constructed based on 18S rRNA sequences, *T. annulata* isolated from *O. lahorensis* formed part of the clade which included *T. annulata* isolated from China. Interestingly, *O. lahorensis* collected from the same sheep were positive for *T. annulata*, but blood specimens from those sheep were negative for *T. annulata*. This finding suggests that *O. lahorensis* may act as a vector for *T. annulata*; however, it remains unclear whether *T. annulata* can be transmitted to other livestock and humans by *O. lahorensis*. *Theileria ovis* is a relatively widespread parasite: several hard ticks, including *R. bursa*, *R. turanicus*, *R. sanguineus*, *Haemaphysalis punctata* (Ixodidae) and *Hyalomma turanicum*, are major vectors of this species (20). Zhao *et al.* (33) first found *T. ovis* in *O. lahorensis* from southern Xinjiang, and the present study also confirmed that *T. ovis* is distributed in southern Xinjiang. However, further studies are needed to determine whether and how this pathogen is transmitted when carried by *O. lahorensis*.

Anaplasma ovis, an intraerythrocytic rickettsial pathogen, primarily infects sheep, goats, and wild ruminants, and its primary tick vectors include *H. asiaticum*, *Dermacentor nuttalli* (Ixodidae) and *R. pumilio* (17). In China, Zhao *et al.* (31) first detected *A. ovis* in *O. lahorensis* collected from Uqturpan County in southern Xinjiang, and our systemic investigation confirmed that adult *O. lahorensis* collected from ZePu before a blood meal can also carry *A. ovis*. Our study further expands the *A. ovis* area spectrum and enriches our understanding of the pathogens carried by ticks.

To the best of our knowledge, this is the first report on the detection of *Babesia* sp. and *T. annulata* in *O. lahorensis*. This study broadens the potential vector spectrum for these pathogens, and its findings imply that the role of soft ticks in disease transmission should not be ignored. Further studies should be carried out to explore the precise transmission routes of these pathogens. Taking in account southern Xinjiang's significance as a corridor for international domestic animal trade, pathogens in local ticks collected from transported livestock, wildlife, and humans should be investigated further to reduce the risk of tick-borne diseases.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research was supported in part by grants from the National Natural Science Foundation of China (Grants Nos 31960705 and 32160841)

Animal Rights Statement: This study was approved by the Ethics Committee of Tarim University, Xinjiang, China.

Acknowledgements: We would like to thank Professor Yuanzhi Wang (Department of Basic Medicine, School of Medicine, Shihezi University, Shihezi, Xinjiang Uygur Autonomous Region, China) for his critical suggestions on the manuscript.

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