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Molecular analysis of *Anaplasma ovis*, *Theileria ovis* and *Brucella abortus* in adult *Ornithodoros lahorensis* soft ticks (Acari: Ixodida: Argasidae) isolated from the Xinjiang Uygur Autonomous Region, China

Dandan Liu, Jinming Wang, Yutong Liu, Shuiyi Wang, Huiru Zhu, Bingbing Jiang, Yongchang Li, Yang Zhang, Bayin Chahan, Wei Zhang✉

College of Veterinary Medicine,
Xinjiang Agricultural University, Ürümqi 830052, China
zw2017xjau@163.com

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Abstract

Introduction: Ticks are obligate blood-feeding arthropods that cause significant economic losses in domestic animal husbandry and threaten public health. However, information about soft ticks (Acari: Argasidae) and tick-borne pathogens in the Xinjiang Uygur Autonomous Region (XUAR) of China is scarce. **Material and Methods:** In this study, PCR assays and gene sequencing were used to detect and analyse the epidemiological features of *Anaplasma ovis*, *Theileria ovis* and *Brucella abortus* parasitic infections in 366 *Ornithodoros lahorensis* soft ticks collected from five sampling sites in the XUAR from October 2019 to March 2022. The ticks were identified by morphological and molecular methods as *O. lahorensis*. The PCR was conducted using primers complementary to the major surface protein 4 (*Msp4*) gene of *A. ovis*, the 18S ribosomal RNA (18S rRNA) of *T. ovis* and the outer membrane protein 22 (*Omp22*) gene of *B. abortus*. **Results:** The overall infection rate was 91/366 (24.9%) for *A. ovis*, 127/366 (34.7%) for *T. ovis* and 94/366 (25.6%) for *B. abortus*. Sequencing analysis indicated that *A. ovis Msp4*, *T. ovis* 18S rRNA and *B. abortus Omp22* genes from XUAR isolates showed 99.58–100% identity with documented isolates from other countries. **Conclusion:** This study provides fundamental evidence for the occurrence of *A. ovis*, *T. ovis* and *B. abortus* in *O. lahorensis*. Therefore, the potential threat of soft ticks to livestock and humans should not be ignored. This study expands the understanding of the existence of tick-borne pathogens in *O. lahorensis* and is expected to improve the strategies for prevention and control of ticks and tick-borne diseases in China.

Keywords: *Ornithodoros lahorensis*, tick-borne pathogens, *Anaplasma ovis*, *Theileria ovis*, *Brucella abortus*.

Introduction

As obligate blood-feeding arthropods, ticks are currently considered to be second only to mosquitoes as vectors of human, domestic and wild animal infectious diseases in the world, and they have caused significant economic losses in livestock farming and threatened public health. Ticks are monophyletic and comprise the Ixodidae, Argasidae and Nuttalliellidae families, in which approximately 900 species have been identified worldwide. Up to the present time, approximately 10% of the tick species currently known have been reported to act as vectors of numerous pathogens, including viruses, bacteria, protozoa and helminths. Generally, each tick species has its preferred environmental conditions and biotopes, which distributes ticks and the risk areas for particular tick-borne diseases heterogeneously over the world.

Besides the Ixodidae (hard ticks), the Argasidae (soft ticks) also have high medical significance. The Argasidae differ from the Ixodidae by having no scutum on the dorsum. This family survives at high temperatures and under relatively dry conditions, resists starvation, lives nidicolously in the nests, burrows and caves of vertebrate animals or in human and livestock habitations, and takes short-duration bloodmeals. The preference of Argasidae for sheltered microhabitats and their characteristically short blood feeding duration

usually hides their presence, permitting their threat to human and animal health to generally be ignored. The Argasidae family is a complex and diverse assemblage of four genera and 183 species, namely *Argas*, *Carios*, *Ornithodoros* and *Otobius*, and at least 11 species in two genera (*Ornithodoros* and *Argas*) have been reported in China. Typically, the argasid ticks of recognised medical and veterinary importance belong to the *Argas*, *Ornithodoros* and *Otobius* genera. As haematophagous arthropods, in addition to harming the host while sucking blood through causing, for instance, toxicosis, paralysis, irritation, allergies and blood loss, argasid ticks can spread a variety of tick-borne disorders, including human tick-borne relapsing fever, viral encephalitis, African swine fever, fowl spirochaetosis or anaplasmosis-like diseases and epizootic bovine abortion syndrome.

Ornithodoros lahorensis is a species of argasid tick which infests large domestic animals and can usually be collected from sheep, camels, cattle, goats, horses and donkeys. In these animal hosts, *O. lahorensis* can induce anaemia, toxic reactions and paralysis. More importantly, it has strong vectorial capacity for several pathogens. As far as we know, *O. lahorensis* may carry or transmit the Crimean–Congo haemorrhagic fever virus, *Rickettsia sibirica*, *Rickettsia conorii*, *Brucella abortus*, *Francisella tularensis* and *Coxiella burnetii*. However, there have been few relevant research undertakings into *O. lahorensis* and associated tick-borne pathogens in China, and pathogens of only three genera (*i.e. Theileria* spp., *Anaplasma* spp. and *Babesia* spp.) have been reported in studies so far.

The Xinjiang Uygur Autonomous Region (XUAR) is the largest administrative division of China, occupying one-sixth of its mainland and having an area of approximately 1,660,000 km^2. It is halfway along the old Silk Road between eastern Asia and Europe and borders eight countries; therefore, international livestock trade is frequent in the XUAR. The complex geographical and diverse ecological environments, including the Gobi Desert, valleys, mountains, grassland and flatland, provide a natural habitat for ticks, making the XUAR one of the regions in China with the largest tick diversity.

Previous studies have mainly focused on ixodid ticks and ixodid tick–borne pathogens, and the potential threats of argasid ticks and argasid tick–borne pathogens to animal husbandry and public health have been underestimated. At least seven species of argasid tick have been identified in the XUAR alone, *Ornithodoros lahorensis* being the dominant one. In order to further investigate the vectorial capacity and pathogen burden of *O. lahorensis*, 366 adult *O. lahorensis* from five main habitats in the XUAR were collected and identified in the present study. In addition, molecular techniques were utilised to assess the presence of *O. lahorensis*-associated pathogens, namely *Anaplasma ovis*, *Theileria ovis* and *Brucella abortus*. To the best of our knowledge, this is the first experimental report of *Brucella abortus* in *O. lahorensis* from China.

Material and Methods

Tick collection. Adult *O. lahorensis* (n = 366) were collected in Shanshan County of Turpan City (n = 209), Awat County of Aksu City (n = 93), Hejing County of the Bayingolin Mongol Autonomous Prefecture (n = 24), and Yutian County (n = 20) and Qira County (n = 20) of Hetian City in the XUAR in China from October 2019 to March 2022 (Fig. 1 and Table 1). The samples were collected from the body surface (neck, back, axillary and thoracic regions) of sheep and were preserved in tubes containing moistened absorbent cotton. Subsequently, the samples were transported to the laboratory of the College of Veterinary Medicine at Xinjiang Agricultural University and identified by morphological and molecular methods. Briefly, preliminary identification was performed under a stereo microscope by the characteristics of the dorsal cuticle, anus, Haller's organ, hypostome, spiracle and gonopore. Then the *16S rDNA* genes of the ticks were amplified and the tick species were identified by sequence alignment.

DNA isolation. Fasting tick samples were ultrasonically washed in 70% ethanol for 30 min and washed with phosphate-buffered saline solution for 10 min. After drying, the tick samples were put into a porcelain mortar with liquid nitrogen and ground to a fine powder. A TIANamp Genomic DNA Kit (Tiangen Biotech Co., Ltd, Beijing, China) was used to isolate genomic DNA of each tick, following the manufacturer's instructions, and the resulting DNA samples were stored at −20°C until subsequent use.

Pathogen detection. All the samples were screened with species-specific primers for *Anaplasma ovis*, *Theileria ovis* and *Brucella abortus* by PCR analysis (Table 2). The PCR reaction mixture (25 μL) was composed of 1 μL of the stored DNA templates, 1 μL of forward and reverse primers (10 μM), 12 μL of 2× EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China) and 10 μL of nuclease-free water. The PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and then visualised under UV light.

Sequencing and phylogenetic analysis. All the evaluated amplification products were sequenced with an ABI PRISM 3730 XL DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA), and the generated sequences were subsequently aligned using the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore/) and MEGA version X (20). In order to analyse the phylogenetic relationships, the gene sequences of *Anaplasma ovis*, *Theileria ovis* and *Brucella abortus* from different regions were downloaded from GenBank, and phylogenetic trees were constructed using the maximum likelihood algorithm in MEGA version X.

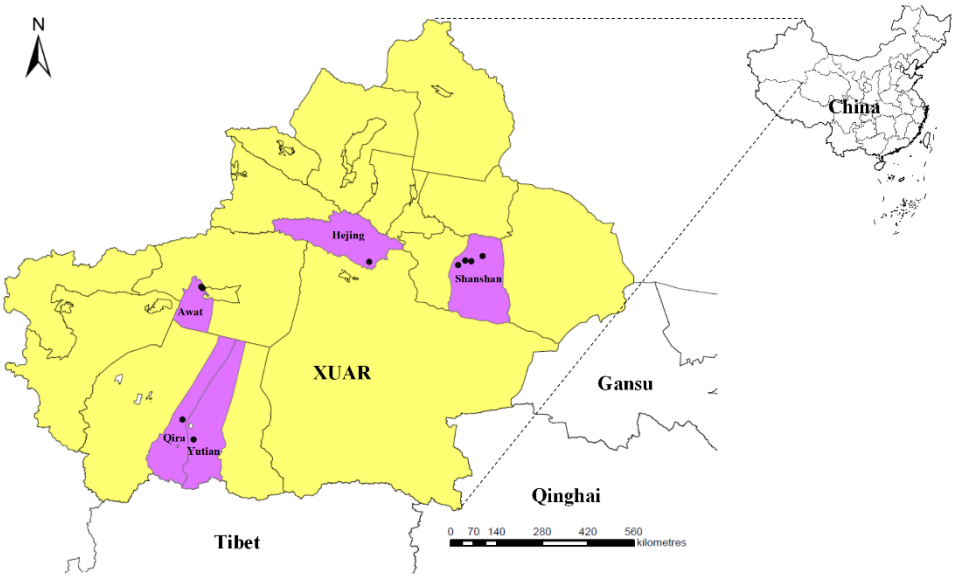

Fig. 1. Map of the Xinjiang Uygur Autonomous Region (XUAR), China. The black dots indicate localities where samples were collected

Table 1. Collection of *Ornithodoros lahorensis* soft tick samples in the Xinjiang Uygur Autonomous Region, China

Collection date	Collection season	Region	Number of samples collected	Altitude (m), latitude and longitude
Oct. 2020 and Mar. 2021	Autumn and spring	Shanshan	209	979; 42°82′ N, 90°25′ E
Mar.–Apr. 2021	Spring	Awat	93	1028; 40°76′ N, 80°25′ E
Oct. 2019	Autumn	Hejing	24	1100; 42°32′ N, 86°38′ E
Mar. 2022	Spring	Yutian	20	1628; 36°41′ N, 81°29′ E
Mar. 2022	Spring	Qira	20	1361; 37°3′ N, 80°46′ E

Table 2. PCR amplification of genetic material of pathogens isolated from *Ornithodoros lahorensis* soft ticks in the Xinjiang Uygur Autonomous Region, China

Pathogen	Target gene/region	Primer (5′–3′)	EPS (base pairs)	AT (°C)	Reference
Anaplasma ovis	*Msp4*	Forward: CGCCTGCTCCCTACTTGTT Reverse: TTCCACTCTGGCTCCTCCT	322	58	(13)
Theileria ovis	18S rRNA	Forward: TCGAGACCTTCGGGT Reverse: TCCGGACATTGTAAAACAAA	520	52	(1)
Brucella abortus	*Omp2*	Forward: TGATGGGAGGGACCGACTA Reverse: TGGTTCTTCAGGTTGTTACGC	494	55	(24)

Msp 4 – major surface protein 4; 18S rRNA – 18 Svedberg ribosomal RNA; EPS – expected product size; AT – annealing temperature

Results

Overall infection rate. The PCR assays revealed that out of the 366 samples, 256 (69.9%) carried one or two pathogens. Detailed tick-borne pathogen infection data are provided in Table 3. The highest rate was for infection with *T. ovis* at 34.7%, followed by the rate for infection with *B. abortus* at 25.6%), the *A. ovis* infection rate being 24.9%. The infection rates of the three pathogens showed obvious regional differences (Table 3).

Co-infection analysis. Rate data for co-infection with two of the three pathogens were also analysed. No infection with all three pathogens simultaneously was detected. In total, 56 (15.3%) ticks bore two pathogens. The most common co-infection was with *A. ovis* and *T. ovis*, accounting for 6.8% of the 366 tick samples, followed by *T. ovis* + *B. abortus* (5.2%) and *A. ovis* + *B. abortus* (3.3%) (Table 4).

Comparative gene sequence analyses and phylogenetic analysis. All the tested amplicons of *A. ovis*, *T. ovis* and *B. abortus* were of the expected sizes of 322 bp, 520 bp or 494 bp, respectively, and all the sequences of a particular pathogen were identical. The major surface protein 4 (*Msp4*) gene sequence of *A. ovis*, the 18 Svedberg ribosomal RNA (18S rRNA) gene sequence of *T. ovis* and the outer membrane protein 22 gene sequence (*Omp22*) of *B. abortus* were all deposited to GenBank with the accession numbers OL769317, OL774380 and OL769318, respectively. Phylogenetic analysis indicated that the *Msp4* gene sequence of *A. ovis* in this study and another three sequences of the same gene isolated from samples from different geographical regions grouped together in one clade, with identity of 99.65% (Fig. 2). Similarly, the 18S rRNA gene sequence of *T. ovis* in this study belonged to the same cluster as sequences from another eight isolates of *T. ovis*, with identity ranging from 99.58% to 100% (Fig. 3). Regarding the *Omp22* gene sequence of *B. abortus*, the identity between OL769318 which was tested in this study and the *Brucella abortus* strains XUAR1 and XUAR2 was 100% (Fig. 4).

Table 3. Prevalence of pathogens detected in *Ornithodoros lahorensis* soft ticks in the Xinjiang Uygur Autonomous Region, China

Pathogen	Prevalence (%)					
	Shanshan (n = 209)	Awat (n = 93)	Hejing (n = 24)	Yutian (n = 20)	Qira (n = 20)	Overall (n = 366)
Anaplasma ovis	28.2 (59/209)	23.6 (22/93)	0 (0/24)	40.0 (8/20)	10.0 (2/20)	24.9 (91/366)
Theileria ovis	45.0 (94/209)	35.5 (33/93)	0 (0/24)	0 (0/20)	0 (0/20)	34.7 (127/366)
Brucella abortus	24.4 (51/209)	39.8 (37/93)	25.0 (6/24)	0 (0/20)	0 (0/20)	25.6 (94/366)

Table 4. Prevalence of single and co-infection with one or more of *Anaplasma ovis*, *Theileria ovis* and *Brucella abortus* in *Ornithodoros lahorensis* soft ticks in the Xinjiang Uygur Autonomous Region, China

	Pathogen	Number of positive samples/percentage (%)
Single infection	*Anaplasma ovis*	54/366 (14.8)
	Theileria ovis	83/366 (22.7)
	Brucella abortus	63/366 (17.2)
Co-infection	*A. ovis* + *T. ovis*	25/366 (6.8)
	T. ovis + *B. abortus*	19/366 (5.2)
	A. ovis + *B. abortus*	12/366 (3.3)
	A. ovis + *T. ovis* + *B. abortus*	0/366 (0)

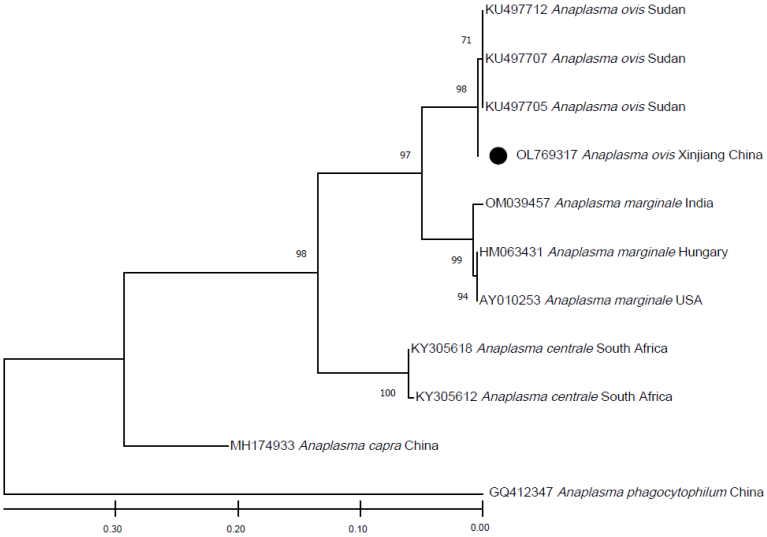

Fig. 2. The phylogenetic analysis of *Anaplasma ovis* identified in this study based on the *Msp4* gene sequences. The tree was constructed using the maximum likelihood method. The numbers at nodes represent the percentage occurrence of the clade in 1,000 bootstrap replications. The sequence from this study is indicated by a black dot

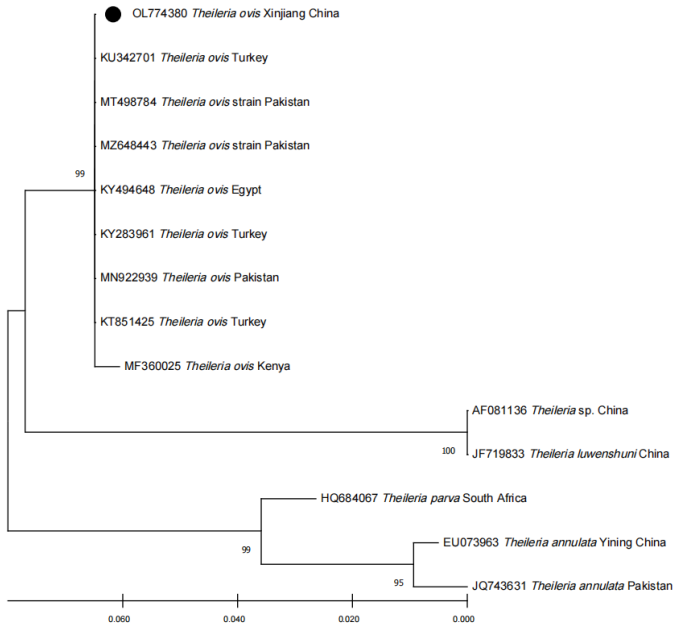

Fig. 3. The phylogenetic analysis of *Theileria ovis* identified in this study based on the 18S rRNA gene sequences. The tree was constructed using the maximum likelihood method. The numbers at nodes represent the percentage occurrence of the clade in 1,000 bootstrap replications. The sequence from this study is indicated by a black dot

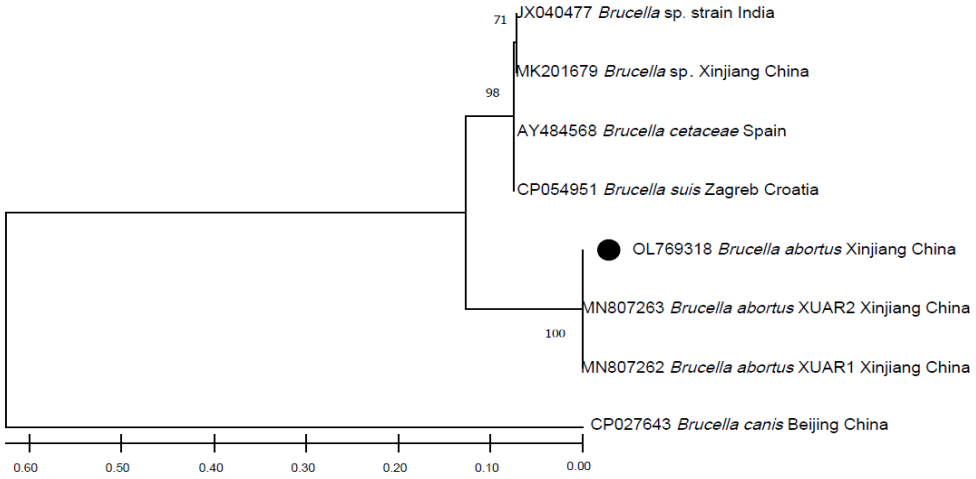

Fig. 4. The phylogenetic analysis of *Brucella abortus* identified in this study based on the *Omp22* gene sequences. The tree was constructed using the maximum likelihood method. The numbers at nodes represent the percentage occurrence of the clade in 1,000 bootstrap replications. The sequence from this study is indicated by a black dot

Discussion

As the dominant argasid tick species of the *Ornithodoros* genus in China, *Ornithodoros lahorensis* is widely distributed in the XUAR, especially in the regions around the Tarim Basin. As indicated by epidemiological investigation, *O. lahorensis* is mainly found in the Palaearctic region, including the five countries of central Asia, Russia, Georgia, Ukraine, Pakistan, Afghanistan, Iran, India and China (26, 34, 37). Regarding the ecology, the larvae of these ticks were reported to usually attach to hosts during the autumn or winter and the final nymph instars to moult to adults in the spring (15). In conformance with this seasonal pattern, according to Zhao *et al*. (41), *O. lahorensis* were collected from late February to early April in the XUAR – they only occurred in spring. However, in our research, besides their being found in spring, adult and nymph argasid ticks were also sampled in the autumn from the regions of Hejing and Shanshan (Table 1). Comparing annual temperature data for these two sampling sites, we found that the average temperatures in spring (4–18.5°C in March) and autumn (4–21°C in October) were almost the same, indicating that there might be two suitable seasons for *O. lahorensis* to moult to adults in the XUAR.

Anaplasma ovis is a tick-borne obligatory intraerythrocytic bacterium which can lead to ovine anaplasmosis, and usually infects sheep, goats and wild ruminants in tropical and subtropical regions. In general, the infection is subclinical; however, the acute phase can produce the symptoms of fever, progressive anaemia, icterus, weight loss and milk yield decrease, and can sometimes cause death (5). According to Chochlakis *et al.* (6), in 2007, a 27-year-old woman became infected with *A. ovis* after a tick bite in Cyprus, demonstrating that *A. ovis* is a zoonotic parasite. In 2018, *O. lahorensis* was first reported as a potential vector of *A. ovis* in the XUAR (41); however, the evidence was only derived from one sampling site of Uqturpan County of Aksu City, and the infection rate reports were lacking in detail. In this study, *A. ovis* was detected from *O. lahorensis* ticks collected from four sites around the Tarim Basin omitting Uqturpan, with a total carrier rate of 24.9%, which further indicated the important role for *O. lahorensis* as a vector of *A. ovis*. Among the five different sampling sites of Shanshan, Awat, Hejing, Yutian and Qira, *O. lahorensis* was only negative for *A. ovis* in Hejing. Further analysis found that only in the Hejing region was the sampling season the autumn, indicating that the *A. ovis* which was carried by *O. lahorensis* might be more prevalent in spring than in autumn.

Theileria ovis, a tick-borne haemoprotozoan parasite, is a global livestock pathogen that can cause ovine theileriosis, which is a significant infectious disease affecting small ruminants (2). In China, *T. ovis* was first reported in 2011 in the XUAR, when its transmission vector was demonstrated to be *Hyalomma anatolicum anatolicum* (22). Zhao *et al.* (43) first found *T. ovis* in *O. lahorensis* from the southern part of the XUAR in 2020, and revealed that the infection rates were 42.86% in Kashgar and 26% in Aksu. Subsequently, Li *et al.* (21) confirmed that *T. ovis* was distributed in the southern part of the XUAR in 2023, but the infection rate of *O. lahorensis* with the parasite was only 1.5% (5/330). However, in the present study, the overall rate for this soft tick to bear *T. ovis* was 22.7% (83/366), which was in agreement with the data of Zhao *et al.* (43). Interestingly, in this study, *O. lahorensis* which were collected from Shanshan (45.0%) and Awat (35.5%) were positive for *T. ovis*, but specimens from the other three sampling areas were negative. The positive regions are at the lowest altitude among the five sampling areas; therefore, the reason for the different prevalence of *T. ovis* in different areas might be the altitude of the sampling sites. This potential correlation still needs further research.

Brucellosis is one of the most important zoonotic diseases caused by Gram-negative bacteria belonging to the *Brucella* genus (8). Among all *Brucella* species, *B. melitensis* and *B. abortus* are the most pathogenic and

virulent (10, 11, 18). As the main intracellular causative pathogen of brucellosis in cattle, *Brucella abortus* generally causes abortion and infertility in adult animals and is also an important cause of chronic infection in humans (7). According to Balashov (4) and Rodríguez *et al.* (31) *Brucella* could be cultivated from multiple tick species. Additionally, *B. abortus* which was cultivated from *Boophilus annulatus* hard ticks could be re-cultivated from the blood and organs of guinea pigs infected by tick bite (36). Similarly, Wang *et al*. (38) demonstrated that there was transovarial transmission of *Brucella* in *Dermacentor marginatus*. However, the current data are too scarce to clarify the relationship between *B. abortus* and Argasidae. According to Philip and Burgdorfer (29), in 1955 fasting *O. lahorensis* were found infected with *Brucella abortus* in areas where infected sheep had been kept one year before. Also, in 1962 the preservation of vaccinal strains of *B. abortus* 19-BA in *O. lahorensis* was reported (32). Since then, there has been no relevant experiment evidence found to demonstrate the relationship between *O. lahorensis* and *Brucella*. In 2020, Li *et al*. (24) tested 12 Ixodidae species collected from six counties in the XUAR and found that the infection rate with *Brucella* spp. was 26.2%. The present study gave a similar finding in Argasidae ticks: we found that *O. lahorensis* were positive for *B. abortus* in Shanshan, Awat and Hejing in the XUAR with an overall infection rate of 25.6%. Interestingly, *B. abortus* was not detected from the tick samples collected in Yutian and Qira, which had relatively high altitude and low latitude compared with the other regions.

Mixed infections of *O. lahorensis* involving more than one pathogen were observed in the present study. Most co-infections were caused by *A. ovis* + *T. ovis* or *T. ovis* + *B. abortus*. Although clinical cases were not observed in any of the sheep from which the *O. lahorensis* were collected, it is possible that animals infected with multiple pathogens may have more pronounced clinical signs or haematological abnormalities than those infected with single pathogens. It is suggested that *A. ovis*, *B. abortus* and *T. ovis* are potential pathogens that cause mixed infections in the sheep population of the XUAR.

Conclusion

This study revealed the existence of *A. ovis*, *B. abortus* and *T. ovis* in *O. lahorensis* collected from Shanshan, Awat, Hejing, Yutian and Qira, in the XUAR, China from October 2019 to March 2022. The current data determined the prevalence rates of the detected pathogens in *O. lahorensis* in that region and suggest the possible emergence of tick-borne diseases in local animals. This study also broadens the potential vector spectrum for these pathogens, and its findings imply that the role of Argasidae in disease transmission should not be ignored.

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

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Animal Rights Statement: All of the required procedures for the sample collection were carried out applying the ethical guidelines for the use of animal samples and were permitted by the Ethics Committee of Xinjiang Agricultural University, Xinjiang, China (Animal Experiment Permit No. 2018006).

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