RESEARCH ARTICLE

Molecular Investigation of Bartonella melophagi and the First Report of Trypanosoma melophagium in Melophagus ovinus from Southern Xinjiang, China

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ABSTRACT

Bartonella melophagi and Trypanosoma melophagium are intracellular bacteria and blood protozoan parasites, respectively, that can be transmitted by Melophagus ovinus and pose risks to human and animal health. Eight pupae and 214 M. ovinus were collected from three sampling sites in southern Xinjiang, China, between March 2019 and June 2023. The morphological characteristics and 18S rRNA sequences were used to identify M. ovinus. The gltA gene was used to detect B. melophagi and the 18S rRNA gene was used to detect T. melophagium in all samples. The DNA of B. melophagi was detected in 214 (100.00%, 214/214) samples and the DNA of T. melophagium was detected in 101 (47.20%, 101/214) samples using PCR and sequencing. Eight pupae produced two different outcomes: B. melophagi was positive and T. melophagium was negative. This study presents conclusive evidence for the presence of B. melophagi and T. melophagium in M. ovinus. To the best of our knowledge, this is the first report of T. melophagium in M. ovinus in China. These findings offer a significant reference for the advancement of the knowledge and prevention of M. ovinus, B. melophagi, and T. melophagium in China.


INTRODUCTION

Melophagus ovinus, also known as sheep ked, is a blood-sucking ectoparasite that primarily infests sheep. It is a wingless fly belonging to the family Hippoboscidae (Diptera, Hippoboscoidea) (Small, 2005). M. ovinus, which is 4–6 mm in size, has an adamanate exoskeleton, densely distributed bristles, three pairs of legs terminated with hook-like claws, and robust, sharp mouthparts (Li et al., 2023b). Their life cycle comprises three definite stages: larva, pupa, and wingless adult. They usually parasitize the animal’s head, neck, shoulder, abdomen, hind legs, perineal regions, and tail, and by direct contact, they can be transferred from one sheep to another (Zhang et al., 2023). Although M. ovinus mainly parasitizes the body surface of sheep, it can also parasitize humans, domestic animals (goats, dogs, and rabbits), and wild animals (Tibetan antelope, red foxes and European bisons) (Werszko et al., 2021).

M. ovinus blood-feeding behavior harms its host in two ways. On the one hand, the bite of M. ovinus cause itching, pain, and inflammation in the host. The host relieves the discomfort caused by gnawing, scratching, and rubbing; however, this process may lead to skin damage and wool shedding, creating conditions for secondary microbial infections and cutaneous myiasis. If M. ovinus parasitizes sheep, it will seriously damage their health and affect the economy of the livestock industry. (Duan et al., 2017a). On the other hand, according to reports, M. ovinus acts as a vector for the spread of zoonotic pathogens such Borrelia burgdorferi, Thelidia spp., Bartonella spp., Trypanosoma spp., Rickettsia spp., Anaplasma spp., Border disease virus, Bluetongue virus, et al. (Li et al., 2023b). The majority of sheep-rearing locations have been reported to have M. ovinus present in Asia, North America, Europe, Africa, and Oceania; it has been reported in Tibet, Gansu, Xinjiang, Qinghai, and Liaoning, China, and has been found in sheep and their products that are imported to China’s quarantine ports (Liu et al., 2018; Werszko et al., 2021).

Belonging to the family Bartonellaceae, Bartonella spp. are arthropod-borne intracellular Alphaproteobacteria that require haemotropic gram-negative bacteria. It infects the endotheliocytes and erythrocytes in mammalian hosts.
They are aerobic bacilli that are slow-growing, fastidious, and highly adapted to vertebrate hosts (Kumsa et al., 2014). Ever since Bartonella was reclassified in 1993, within the family Bartonellaceae, there is only one genus, Bartonella (Brenner et al., 1993). At present, the genus comprises 39 species and three subspecies that hold an effective taxonomic position, with at least 20 of them being linked to human ailments (Gonçalves-Oliveira et al., 2023). They have been associated with endocarditis and/or persistent bacteremia, retinitis, peliosis, hepatitis, myocarditis, uveitis, and bacillary angiomatosis. However, Bartonella spp. normally cause continuous asymptomatic bacteremia (Kumsa et al., 2014). Bartonella spp. use humans, cats, dogs, mice, cows, sheep, horses, and various wild animals as hosts worldwide. It can be transmitted via insect vectors such as ticks, M. ovinus, human body lice, sand flies, and cat fleas. Currently, five types of Bartonella are linked to ruminants: B. melophagi, B. bovis, B. capreoli, B. schoenbuchensis, and B. chomelii (Ni et al., 2021). Among these, Chinese scholars detected B. melophagi in M. ovinus from Gansu, Xinjiang, and Tibet (Duan et al., 2017a; Liu et al., 2018; Liu et al., 2022). Maggi et al. detected and cultured B. melophagi in the blood of two female patients with dry cough, fatigue, muscle pain, muscle weakness, shivering, and pericarditis. Both patients had been in contact with, or even scratched and bitten by, domestic or wild animals and had a history of being bitten by mosquitoes, flies, fleas, or ticks (Maggi et al., 2009). Although a causal relationship between the development of clinical symptoms and B. melophagi in these two female patients has not been established, it highlights the potential harm caused by B. melophagi as a zoonotic pathogen.

Among the most significant and widespread parasites worldwide, trypanosomes (Euglenozoa, Kinetoplastea, and Trypanosomatida) are responsible for several serious diseases in humans and animals. These digenetic blood parasites are mostly spread by insects that feed on the blood. In the T. theileri group, T. melophagium is species-specific to sheep and is only transmitted by M. ovinus (Brotankova et al., 2022). Infection in T. theileri group is usually implicit and nonpathogenic but has been detected in ruminants with fever, anorexia, malnutrition, and anemia (Bittner et al., 2021; Hajihassani et al., 2020; Sood et al., 2011). Thus, the nonpathogenic T. theileri group may still cause minor damage to the host, especially in highly prevalent host populations that are easily overlooked (Oldrieve et al., 2022). Although T. melophagium may cause serious infections in humans and animals, it is harmless to M. ovinus and does not cause insect-to-insect transmission. Sheep become infected when they consume M. ovinus (Martinkovic et al., 2012). A sheep experimentally infected with T. melophagium had a maximum infection duration of three months, and there was no permanent immunity because the sheep could contract T. melophagium again after being isolated for several months (Gibson et al., 2010). T. melophagium uncertainty is a threat to human and animal public health.

With a total area of approximately 1,664,900 km², the Xinjiang Uyghur Autonomous Region is the largest autonomous region in China, comprising approximately one-sixth of the total area of mainland China. The length of the land border exceeds 5,700 km. It is a vital export channel for worldwide trade and adjoins many nations, including Pakistan, Mongolia, Russia, Kazakhstan, and India. Southern Xinjiang occupies an area of approximately 1,020,000 km² and is surrounded by various landforms, including the Gobi Desert, flatlands, grasslands, mountains, and valleys (Li et al., 2023c). The main economic industries in Southern Xinjiang are agriculture and livestock production. The main livestock species include cattle (e.g., Kazakh and Mongolian) and sheep (e.g., Hotan and Duolang). Therefore, insect vectors and insect-borne diseases have a significant impact on the livestock economy. At the same time, zoonotic diseases transmitted by M. ovinus must be considered.

The number of reports on M. ovinus and the different pathogens it carries has gradually increased in recent years, indicating that it poses a risk to human and animal health. (Hao et al., 2020; Li et al., 2023a; Liu et al., 2022; Zhang et al., 2021). In the current study, to better understand the genetic heterogeneity of B. melophagi and T. melophagium in southern Xinjiang, China, we conducted comprehensive molecular detection and analysis of B. melophagi and T. melophagium in M. ovinus samples obtained from this area and determined the prevalence and geographical distribution of M. ovinus in the region.

MATERIALS AND METHODS

Study area and sample collection: The selection of sites for the collection of M. ovinus were based on the presence of sheep ked, good flock health, lax implementation of deworming measures, and obtained consent from the head of the sheep farm. From March 2019 to June 2023, 214 M. ovinus and eight pupae were collected from 146 sheep captured at three sheep farms in three counties in southern Xinjiang: CeLe (CL), WuShi (WSW), and WenSu (WSE) (Table 1 and Fig. 1). The research locations were all situated in southern Xinjiang’s border regions, and they served as central points for animal husbandry in these areas. The head, neck, shoulder, abdomen, hind legs, perineal regions, and tail were carefully examined by separating the wool in the direction opposite to the fleece rest. All pupae and adult M. ovinus samples were collected using fine-tipped forceps, preserved in 75% alcohol and kept at 4°C. Finally, the samples were sent to the Tarim Animal Disease Diagnosis and Control Engineering Laboratory (Alar, China). Information on the samples, such as the number of samples, hosts and locations, was recorded.

Morphological identification and total DNA extraction of M. ovinus: Using a Leica stereomicroscope M165 C (Solms, Germany), the morphological features of M. ovinus, including its basic capituli, ventral surface, dorsal surface, legs, tarsi, anal groove, and scutum, were identified. The main points of identification were as follows: the compound eyes were small and degenerated; the wings were completely degenerated; the head and thorax were densely covered with bristles; and there were no spots or stripes (Duan et al., 2017b). All M. ovinus were washed in a thermostatic culture shaker with 90%, 70%, 50%, 30%, and 10% ethanol at 37°C, 180 rpm,
Table 1: Molecular detection of *B. melophagi* and *T. melophagium* in *M. ovinus*.

<table>
<thead>
<tr>
<th>Cities</th>
<th>Year</th>
<th>Number of hosts</th>
<th>Number of <em>M. ovinus</em> pupa</th>
<th><em>B. melophagi</em> Positive Number(%)</th>
<th><em>T. melophagium</em> Positive Number(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CeLe (CL)</td>
<td>2023</td>
<td>27/68</td>
<td>143</td>
<td>4</td>
<td>143 (100%)</td>
</tr>
<tr>
<td>WuShi (WSW)</td>
<td>2021</td>
<td>9/31</td>
<td>28</td>
<td>0</td>
<td>28 (100%)</td>
</tr>
<tr>
<td>WenSu (WSE)</td>
<td>2019</td>
<td>16/47</td>
<td>43</td>
<td>4</td>
<td>43 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>52/146</td>
<td>214</td>
<td>8</td>
<td>214 (100%)</td>
</tr>
</tbody>
</table>

Note: Number of hosts: *M. ovinus* number of infected sheep/ total number of sheep; eight pupae produced two different outcomes: *B. melophagi* was positive and *T. melophagium* was negative.

in each of which *M. ovinus* was washed for 30 minutes respectively. They were then cleaned three times in sterile distilled water to remove any remaining debris and finally dried on filter paper. They were then placed in a 1.5 mL sterile centrifuge tube and cut into the smallest pieces using sterilized surgical scissors. Following the provided instructions, we extracted DNA from the processed *M. ovinus* samples utilizing the TIANamp Genomic DNA Kit (manufactured by TIANGEN Corporation, Beijing, China). Before PCR was carried out, the extracted DNA was kept at -20°C.

**Molecular identification of *M. ovinus*, *B. melophagi* and *T. melophagium***: To identify *M. ovinus*, polymerase chain reaction (PCR) targeting the Hippoboscidae 18S rRNA gene was performed. The detection of *B. melophagi* was performed using the *gltA* gene and *T. melophagium* using the 18S rRNA gene. Table 2-1 shows the primer sequences used for amplification of 520bp, 379bp and 650bp fragments. The PCR mixture, which had a total volume of 25 μL, contained 9 μL nuclease-free deionized water, 13 μL of 2× Taq PCR Master Mix (Tiangen), 1 μL of the relevant primers (10 μM final concentration) and 1 μL template DNA, and was performed under the following amplification conditions in Table 2-2.

To monitor for contamination, nuclease-free sterile distilled water was used as a negative control, and the DNA of *B. melophagi* and *T. melophagium* stored in our laboratory was used as a positive control. For each experiment, 5 μL of the PCR product was applied to a 1.5% agarose gel that contained GelStain (Beijing TransGen Biotech Co., Ltd., Beijing, China) for gel electrophoresis. Experimental data were evaluated using a gel documentation system (FluorChem, ProteinSimple, CA, USA). The PCR products were sent to GENEWIZ Inc. (Suzhou, China) for sequencing. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare and analyze the genomic sequences. Finally, the genotypes of *B. melophagi* and *T. melophagium* based on *gltA* and 18S rRNA sequences were determined using DNAStar and MegAlign software (DNASTAR, Inc., USA). The positivity rate was calculated using the following equation:

\[
\text{Positivity rate} (\%) = \frac{\text{number of positive pools}}{\text{number of examined pools}} \times 100
\]

The sequences of the *B. melophagi* and *T. melophagium* nucleotides were inferred from their respective genomic sequences using DNASTar and MEGA 7.0 software. Using maximum likelihood algorithms and 1000 bootstrap repetitions, a phylogenetic tree was constructed using the MEGA software (version 7.0) (Kumar et al., 2018; Saitou and Nei, 1987). Representative nucleotide sequences from this study were submitted to the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/) (18S rRNA of *M. ovinus*: OR921412 and OR921413; *gltA* of *B. melophagi*: OR936726, OR936727, OR936728, OR936729, OR936730, and OR936731; 18S rRNA of *T. melophagium*: OR921417, OR921418, OR921419, OR921420, and OR921421).
RESULTS

Identification of *M. ovinus*: In the present study, 52 (35.6%) sheep were infested with *M. ovinus* between March 2019 and June 2023 at three sampling points in southern Xinjiang. A total of 214 *M. ovinus* and eight pupae were collected from the infested sheep (Table 1 and Fig. 2). Each sheep was infected with *M. ovinus* at an average of 1.5 *M. ovinus*. Through the identification of their morphological features and molecular biological studies using 18S rRNA, all these ectoparasites were verified to be *M. ovinus*. The 18S rRNA sequences found in *M. ovinus* were identical according to molecular analysis. BLAST analysis of the 18S rRNA gene sequence of *M. ovinus* showed 100% nucleotide identity with GenBank reference sequences (*M. ovinus*: ON211915 and FN666411).

Prevalence of *B. melophagi*: Overall positivity rates for *B. melophagi* in sheep and pupae were 100% (214/214) and 100% (8/8), respectively (Table 1). In this study, the gltA gene sequence of *B. melophagi* was amplified from the total DNA extracted from *M. ovinus*. After BLAST comparison, these sequences showed 98.60%~100.00% similarity to the gltA gene sequences of *B. melophagi* reported elsewhere (e.g., MT154631, OQ924085, AY724768, OQ924089 and OQ924091). Based on the gltA of the characteristic *Bartonella* spp., a phylogenetic tree revealed that all sequences acquired for our examination belonged to the same clade, encompassing strain K-2C (*B. melophagi*: AY724768) previously discovered in the USA (Fig. 3).

Prevalence of *T. melophagium*: The prevalence of *T. melophagium* was 51.05% (73/143), 53.57% (15/28), and 30.23% (13/43) in *M. ovinus* at the CeLe, WuShi and WenSu, respectively (Table 1). The total positivity rate was 47.20% and the positive results did not include pupae. The PCR products were subjected to Sanger sequencing, and BLAST analysis of the 18S rRNA gene sequence revealed the presence of 101 *T. melophagium* isolates (47.20%, 101/214). Through phylogenetic analysis of the *Trypanosomes* spp., it was discovered that our sequences exhibited 99.11~100.00% similarity to the accession numbers of *T. melophagium* ON637625, HQ664912, OM256700, and FN666409 (Fig. 4).

DISCUSSION

The presence of *B. melophagi* and *T. melophagium* in our samples was verified by molecular identification analysis of *Bartonella* spp. and *Trypanosoma* spp. in *M. ovinus*, utilizing gltA and 18S rRNA genes. As China's largest province, Xinjiang is home to most of the country's arid regions, and *M. ovinus* has been reported multiple times (Li et al., 2023b; Liu et al., 2019; Zhao et al., 2020). Studies have indicated that *M. ovinus* is present in Xizang, Gansu, the Eastern Tibetan Plateau, and other regions of China (Duan et al., 2020; Liu et al., 2022; Zhang et al., 2021). Approximately 75% of emerging infectious diseases are zoonotic and 28% are vector-borne (Regier et al., 2016). Therefore, a one-health strategy is required to reduce the risk of zoonotic illnesses in both humans and animals.

Based on the results of standard PCR, sequencing, and sequence analyses conducted at three sampling locations in southern Xinjiang, China, we determined that 214 *M. ovinus* and eight pupae contained *B. melophagi*. The prevalence of *B. melophagi* DNA in *M. ovinus*

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Table 2-1: Details of primers, target gene, nucleotide sequence and product size of PCR assays.

<table>
<thead>
<tr>
<th>Species</th>
<th>Target gene</th>
<th>Primer(s)</th>
<th>Nucleotide sequences (5'→3')</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Melophagus ovinus</em></td>
<td>18S rRNA</td>
<td>45F</td>
<td>AAC TTG TGC TTC ATA CGG G</td>
<td>520</td>
<td>(Duan et al., 2017b)</td>
</tr>
<tr>
<td>Bartonella</td>
<td>gltA</td>
<td>S64R</td>
<td>GCG ACT GAG AGA GCC ATA A</td>
<td>379</td>
<td>(Li et al., 2015)</td>
</tr>
<tr>
<td>Trypanosomes</td>
<td>18S rRNA</td>
<td>TrypF 150</td>
<td>GAA ACA CGG GAG CGG TTC G</td>
<td>650</td>
<td>(Werszko et al., 2020)</td>
</tr>
</tbody>
</table>

Table 2-2: Amplification conditions (temperature and time) corresponded to each set of primers.

<table>
<thead>
<tr>
<th>Primer(s)</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>45F</td>
<td>95°C/5 min</td>
<td>95°C/30 s</td>
<td>55°C/30 s</td>
<td>72°C/30 s</td>
<td>38</td>
<td>72°C/8 min</td>
</tr>
<tr>
<td>S64R</td>
<td>94°C/2 min</td>
<td>94°C/30 s</td>
<td>53°C/30 s</td>
<td>72°C/1 min</td>
<td>30</td>
<td>72°C/5 min</td>
</tr>
<tr>
<td>BhCS.781p</td>
<td>94°C/3 min</td>
<td>94°C/30 s</td>
<td>62°C/30 s</td>
<td>72°C/1 min</td>
<td>35</td>
<td>72°C/4 min</td>
</tr>
<tr>
<td>BhCS.1137n</td>
<td>94°C/3 min</td>
<td>94°C/30 s</td>
<td>62°C/30 s</td>
<td>72°C/1 min</td>
<td>35</td>
<td>72°C/4 min</td>
</tr>
</tbody>
</table>

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Fig. 2: The *M. ovinus* and pupae collected from sheep, in southern Xinjiang, China. Features of identification: The compound eyes on the head are small and degenerated (red arrows); Wings are fully degraded (yellow arrows); Males have convex ends and elongated genitalia, females have concave ends (blue arrows)
obtained from sheep in this study was 100.0%, which is similar to the prevalence of *B. melophagi* in *M. ovinus* reported previously in Ethiopia (Kumsa et al., 2014), the Czech Republic (Rudolf et al., 2016), the USA (Kosoy et al., 2016), Poland (Werszko et al., 2021) and China (Liu et al., 2019). However, their prevalence in Algeria is low (Boucheikhchoukh et al., 2019). This difference in prevalence can be attributed to differences in animal hosts, living conditions, vectors, and geographical and environmental factors. The identification of *B. melophagi* in non-feeding pupae of *M. ovinus* suggests the potential vertical transmission of *B. melophagi* in *M. ovinus*. In the USA, two female patients with pericarditis and skin lesions with a history of exposure to wild or domesticated animals were found to be infected with *B. melophagi* in 2009 (Maggi et al., 2009). Kosoy et al. detected not only the DNA of *B. melophagi* in sheep blood in the USA but also demonstrated that *M. ovinus* served as the primary vector, and its natural hosts were sheep, which was also shown to be zoonotic but requires further investigation (Kosoy et al., 2016).

A total of 214 *M. ovinus* and eight pupae were examined by PCR, and the average positivity rate was 47.2%. All the pupae tested negative. To date, there are no reports of *T. melophagium* being transported by *M. ovinus* in Asia. To our knowledge, this is the first study to report the presence of *T. melophagium* in *M. ovinus* and to provide molecular evidence that *M. ovinus* carried *T. melophagium*. This is the first report of *T. melophagium* being found in *M. ovinus* around the Taklimakan Desert. But its positivity rate was lower than 67.0% at Hořice of the Czech Republic, 86.0% in the Republic of Croatia and
Experimental infections with *M. ovinus* isolates were unsuccessful, in agreement with *M. ovinus* as a specific vector for *T. melophagium* (Brotankova et al., 2022). In an investigation of organic sheep farms, *T. melophagium* was present in 86% of *M. ovinus* but was not detected in non-feeding pupae of *M. ovinus*, suggesting that vertical transmission of *T. melophagium* in *M. ovinus* may not occur. This is also the first report.

Conclusions: To our knowledge, this is the first report of *T. melophagium* infection in China. Additionally, it has been proven that *B. melophagi* and *T. melophagium* can coexist in *M. ovinus* located in southern Xinjiang, China. These diseases have not been previously reported in this region, indicating potential negligence among local medical professionals and public health workers. To maintain healthy and sustainable growth of the sheep farming business and reduce threats to human health, our findings are crucial for Xinjiang herders in managing insect-borne diseases.

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Author contributions: WJ provided the research idea. ZXQ and ZX performed the collection and assembly of data. ZK and SL performed the experiments. HK and XN performed the data analysis and interpretation. HK wrote the manuscript. WJ handled the critical revision of the article. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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