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Metagenomic sequencing of epidemic pathogens in *Ornithodoros lahorensis* from southern Xinjiang*

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Summary

Tick-borne diseases have continually remained a significant public health concern and represent a critical frontier in the prevention of zoonotic diseases. *Ornithodoros lahorensis* (*O. lahorensis*) belonging to the Argasidae family, primarily feeds by biting livestock directly and can transmit disease. However, the current understanding of pathogens carried and transmitted by soft ticks, particularly *O. lahorensis* remains limited. The samples in this study were collected from Qiemo County, Shache County, Shaya County, and Bachu County in Xinjiang, and then underwent DNA extraction and metagenomic sequencing. The study indicates that *O. lahorensis* from four regions in Xinjiang carries a total of 157 species of microorganisms belonging to 87 genera from 13 phyla. Notably, *Coxiella burnetii* exhibit higher prevalence and greater pathogenicity among ticks in these regions. Concurrently, Jaagsiekte sheep retrovirus (JSRV), a virus of significant concern to sheep, was detected. This study is the first to conduct metagenomic sequencing of *O. lahorensis*, providing direction for the prevention and control of tick-borne diseases in the Xinjiang region and offering scientific data for the academic community to study the pathogenicity of soft ticks.

Keywords: metagenomic sequencing, *Ornithodoros lahorensis*, soft tick, Xinjiang

At present, more than 960 species of ticks have been found in the world, including 740 species of hard ticks (Ixodidae), 218 species of soft ticks (Argasidae) and Nuttalliellidae (2). A total of 125 species, comprising 111 hard ticks and 14 soft ticks, have been identified in China (23). To date, the limited population of soft ticks and their unique geographical distribution have resulted in little attention from the academic community.

The *Ornithodoros lahorensis* (*O. lahorensis*) is a blood-sucking ectoparasite and the pathogen it carries can infect many different animals. It goes through four stages of development: egg, larva, nymph, and adult. The nymphs can parasitize multiple hosts, such as sheep, cattle, and camels, which belong to the multi-host mode (19). Adults can lay eggs only after engaging in blood-sucking mating. The *O. lahorensis* feeds on sheep blood after parasitizing sheep, causing pain and swelling at the site of the bite, which makes the sheep agitated. In severe cases, it can lead to anemia, wasting, dermatitis, and wool shedding in the sheep (20). It can indirectly carry and transmit a range of pathogenic microorganisms, such as *Anaplasma ovis*, *Theileria ovis*, and Crimean-Congo Hemorrhagic Fever Virus (CCHFV), making it a significant potential vector for their spread (24, 25). The infestation of *O. lahorensis* among animals leads to the transmission of several pathogenic microorganisms, resulting in the widespread prevalence of infectious diseases. Such infestations result in significant economic losses for animal husbandry industries.

Coxiella burnetii (*C. burnetii*), the causative agent of the zoonotic disease Q fever, is an obligate intracellular bacterium characterized by high infectivity and a unique requirement for acidic media for replication. It is primarily transmitted between animals and humans through vectors such as mammals, birds, and ticks. In 2017, Stephen R. Graves and John Stenos reported

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that rickettsial infections and Q fever (caused by *C. burnetii*) were the only systemic bacterial infections known to be transmitted by tick bites in Australia. Patients bitten by ticks exhibited symptoms including allergies, paralysis, and autoimmune disorders (8). By 2019, surveillance in the Lazio region of Italy detected *C. burnetii* in 16% of collected ticks (6). More recently, a 2024 survey in South Africa revealed a positivity rate of 9.2% for the bacterium in local tick populations (4). Given its wide distribution in nature, broad range of vectors, and significant impact on both human and animal health, *C. burnetii* poses a substantial threat to public health security.

The *O. lahorensis* is the dominant species of soft tick in Xinjiang, and is widely distributed throughout the region (18). Currently, it has been observed that the *O. lahorensis* is primarily found in Xinjiang, Inner Mongolia, Tibet, Gansu, Henan, and other provinces in China (14). Additionally, it is primarily distributed in Central Asian countries, such as Russia, Pakistan, Afghanistan, and Iran (1). Recent years have witnessed a steady increase in the global incidence of tick-borne diseases, alongside a constant stream of reports identifying new tick-borne threats, posing a growing public health concern (12). Therefore, studying the pathogens carried by *O. lahorensis* is essential for the development and implementation of prevention and control measures for ticks and tick-borne diseases.

The methods for detecting pathogens carried by animals have advanced due to the development of molecular biology techniques, evolving from Sanger sequencing technology to high-throughput sequencing (21). High-throughput sequencing methods are of great significance in examining the genes and gene composition of microbial communities (16). In comparison to Sanger sequencing, high-throughput sequencing technology has brought about revolutionary innovations, characterized by its high throughput, automation, and cost-effectiveness (9). As a result, it is being rapidly and extensively utilized in the fields of microbiology, medicine, and other related areas, serving as an effective means of detecting microbial species and diversity (3). Metagenomic sequencing is a method of applying high-throughput sequencing technology. Ticks play an important role in the transmission of infectious diseases (13). Therefore, in this study, metagenomic sequencing was employed to investigate and analyze the pathogens carried by soft ticks.

The microbial profile of *O. lahorensis* in Xinjiang remains poorly characterized. Therefore, this study sought to characterize the species and abundance of microorganisms carried by this tick in sheep across four distinct regions of Xinjiang, using metagenomic sequencing. This study provides foundational data for investigating pathogens carried by *O. lahorensis* and furthermore enhances our understanding of soft ticks.

Material and methods

Sample collection and preservation. A total of 85 soft ticks were collected from sheep in four distinct regions of Xinjiang. Soft ticks are mostly parasitic on the exposed parts of the host, and are primarily collected from the ears, abdomen and tail of sheep. After the soft tick is collected from the host, it is placed in 75% alcohol and then stored at 4°C in a refrigerator for sample preservation. For subsequent experiments, ticks from the four regions were pooled into four groups (RP1-RP4). Each group was composed of four soft ticks randomly selected from one of the regions. The sample information is in Table 1.

Morphological identification. The collected ticks were classified and morphologically identified using stereomicroscopy (Shanghai Qianxin Instruments, SMZ18), and it was determined that they belonged to the species *O. lahorensis*. The body of the adult tick is slightly oval in shape, tapering toward the anterior end and broadening into a wide, rounded posterior margin. The dorsal surface is uneven and textured, with a wrinkled appearance and numerous small, star-shaped pits scattered across it. The ventral side exhibits a similar epidermal structure to the dorsal side, though it bears a greater number of longer hairs, which are especially prominent near the anterior margin. The genital opening of the female tick is transversely slit-shaped, while that of the male is semicircular. The morphology of *O. lahorensis* is shown in Figure 1.

DNA extraction and fragmentation. Before DNA extraction, all samples were washed in a thermostatic culture shaker with 90%, 70%, 50%, 30%, and 10% ethanol at 37°C, 180 rpm, in each of which *O. lahorensis* was washed for 30 min, respectively, to facilitate morphologi-

Tab. 1. *O. lahorensis* sample information

County	Sample name	Sampling time	Host	Altitude	Longitude and latitude
Qiemo	RP1	2022.4.21	sheep	1295 m	38°08'46"N 85°31'48"E
Shache	RP2	2022.2.23	sheep	1232 m	38°23'27"N 77°13'24"E
Shaya	RP3	2021.4.7	sheep	978 m	41°13'48"N 82°48'21"E
Bachu	RP4	2021.3.1	sheep	1119 m	39°46'43"N 78°34'30"E

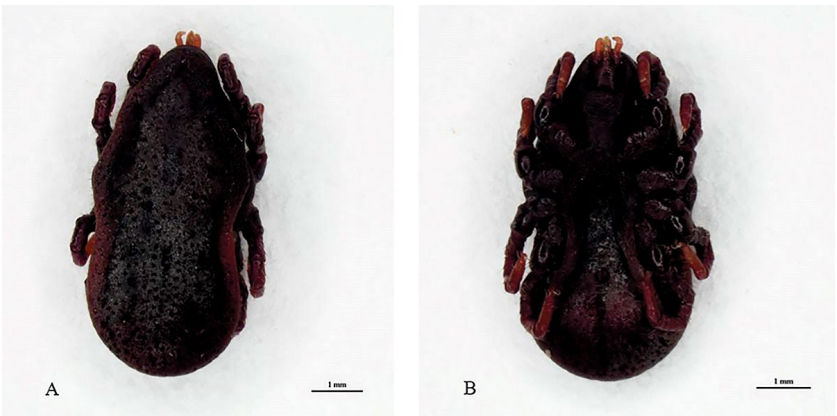

Fig. 1. The morphology of *O. lahorensis*. (A is the dorsal side, and B is the ventral side of *O. lahorensis*)

cal identification and molecular biology identification. The extraction of DNA was performed using a E.Z.N.A.Soil DNA Kit (Omega, M5635-02, USA), following the operating steps provided in the kit strictly. To ensure that the extracted DNA is not contaminated, the extraction operation should be performed on an ultraclean workbench, and the extracted DNA should be stored in a refrigerator at –20°C.

The Qubit ds DNA HS Assay Kit (ThermoFisher, Q32854) was used to accurately quantify the genome concentration, thereby determining the total amount of DNA added to the library construction. A library with a length of approximately 500 bp was prepared, and the initial total DNA amount was approximately 500 ng. The DNA was diluted to 130 µL using the elution buffer in a 0.5 mL Covaris DNA fragmentation tube. Fragmentation was performed with the Covaris instrument. The broken DNA fragments were concentrated and recovered using double HieffNGStm DNA Selection Beads (Yeasen, 12601ES56). After purification, Qubit 4.0 (Thermo, USA) was used for detection.

Library construction. End repair of DNA fragments using mixture A of end-preparative enzymes, including end completion and the addition of a tail to the 3' segment. Next, the joint is selected for connection, and magnetic beads are used to purify the connection product. Then, each sample was amplified using the polymerase chain reaction (PCR) for eight cycles with Primer Mix (P5, P7), the sequences of the P5 and P7 primers are listed in Table 2. Both primers contain sequences that can be annealed to the flow cell for bridging PCR; the P7 primer also includes a six-base index for multiplexing. Clean and validate PCR products using Agilent 2100 bioanalyser (Model G2939A). Qualified libraries were sequenced by double-ended PE 150 on Illumina Novaseq 6000 system.

Data analysis. The original image data files obtained by second-generation sequencing are converted into sequenced reads (raw data) by base calling analysis. The sequencing results were stored in FASTQ file format, which contained sequencing sequence information (reads) and corresponding sequencing quality information. The fastp software was used to analyze the quality of the raw data and other information. Raw data from sequencing, which contains low-quality sequences with junctions. To ensure the quality of information analysis, the original data must be filtered through the fastp software to obtain Clean Data. After quality control of the original data, host contamination should be removed to minimize its impact on the sample. Reads were compared to human or other host genomes using bowtie2, and reads from host genomes or contaminated with high similarity were removed.

After quality control and host decontamination, high-quality sequencing data were obtained. Megahit, a splicing software based on De Bruijn graphs, was used to splice multiple copies of clean reads. After that, the clean reads of each sample were compared to the assembled contigs using bowtie2 to obtain unmapped PE reads. The unmatched reads were continued to be mixed and spliced with SPAdes. For the contig generated by two splicing assemblies, sequences shorter than 500 bp were filtered for downstream analysis, including statistics and subsequent gene prediction. ORF prediction was performed on spliced contigs by Prodigal, and genes with a length greater than or equal to 100 bp were selected as candidate gene sets.

DIAMOND was used to compare the gene aggregation protein sequences with the Nr database for blastp homology, and functional annotation and homologous species information were obtained. The screening criteria were: E-value < 1e-5 and Score > 60. At the same time, according to the NCBI microbial taxonomic information database, gene annotation information was obtained for species classification. The relative abundance of Species was measured at the taxonomic levels of Kingdom, Phylum, Class, Order, Family, Genus, and species. Finally, the data were visualized using ggplot2.

Tab. 2. Sequences of the P5 and P7 primers

Primer name	Sequence
P5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
P7	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Results and discussion

DNA quality. A total of 298,054,250 high-quality sequences were obtained after quality control and dehostcontamination, of which 70,085,686 were obtained for RP1, 88,593,648 for RP2, 63,526,488 for RP3 and 75,848,428 for RP4. Q20 accounted for more than 97% of species (98.34%, 97.91%, 98.24%, 97.74%), and Q30 accounted for more than 93% of data (95.13%, 95.44%, 93.68%, 94.18%) (Tab. 3).The raw sequencing reads generated in this study have been deposited in the NCBI Sequence Read Archive under the BioProject accession number SRR30596778, SRR30591852, SRR30588612, SRR30570823.

General statistics. After concatenating and assembling 298,054,250 high-quality sequences, 67,214 contigs were obtained. The ORF of the obtained contig was predicted using the Prodigal software, and 1,551,550 genes with a length greater than 100 bp were identified. The results show that the content and types of microorganisms contained in the samples are relatively large.

Tab. 3. Quality parameters of the sequencing data for the DNA of each sample

Sample	Raw date (Mbp)	Clean date (Mbp)	Clean Q20 (%)	Clean Q30 (%)	Clean GC (%)	Effective (%)
RP1	10596	10406	98.34	95.44	46.75	99.59
RP2	13357	13108	97.91	94.18	47.96	99.77
RP3	9586	9437	98.24	95.13	47.20	99.53
RP4	11421	11274	97.74	93.68	48.31	99.76

Explanations: "Clean Q20" represents the percentage of base error recognition rate at 1%, while "Clean Q30" represents the percentage of base error recognition rate at 0.1%. The larger the value, the more accurate the sequencing. "Clean GC" represents the proportion of GC bases.

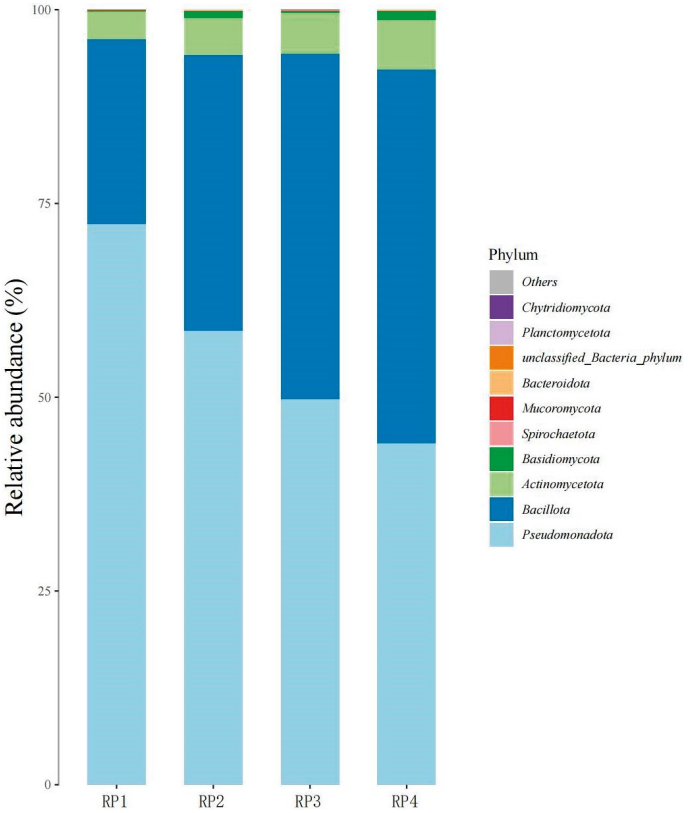

Fig. 2. Microbiome bar maps of four *O. lahorensis* samples (Phylum)

Explanations: The horizontal axis represents the sample names, while the vertical axis represents the relative abundance content. Each color represents a phylum.

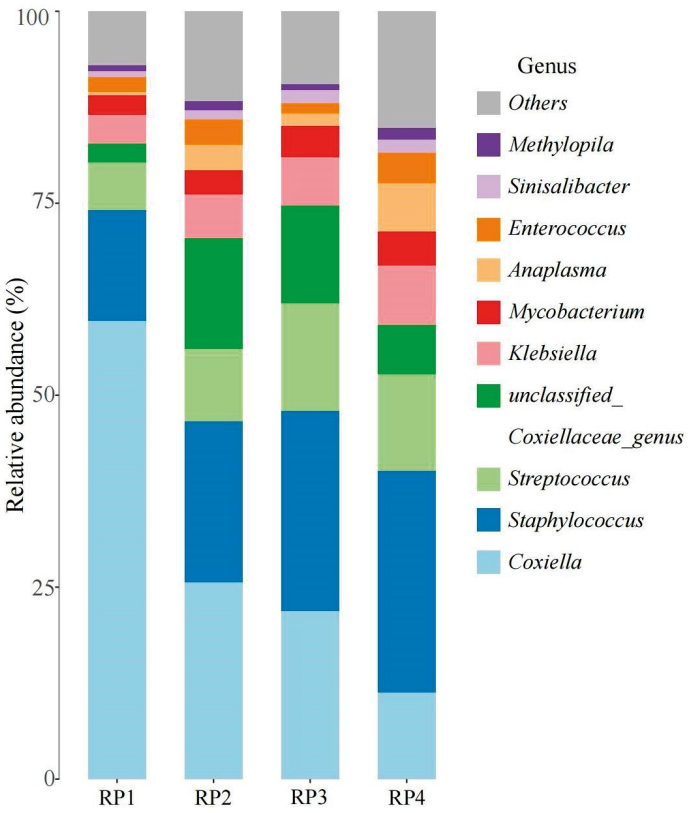

Fig. 3. Microbiome bar maps of four *O. lahorensis* samples (Genus)

Explanations: The horizontal axis represents the sample names, while the vertical axis represents the relative abundance content. Each color represents a genus

Relative abundance of microorganisms. A total of 13 bacterial phyla were detected in the four samples, 12 microbial phyla were identified, while 1 remained unclassified. They are Actinomycetota, Artverviricota, Bacillota, Bacteroidota, Basidiomycota, Chlamydiota, Chytridiomycota, Mucoromycota, Phixviricota, Planctomycetota, Pseudomonadota, Spirochaetota, and unclassified Bacteria phylum (The arrangement order has nothing to do with the relative abundance), respectively. Pseudomonadota and Bacillota were the dominant phyla in the four samples. The relative abundances of Pseudomonadota in each sample are 72.3%, 58.5%, 49.7%, and 44.0%, and the relative abundances of Bacillota in each sample were 23.9%, 35.6%, 44.6%, and 48.2%. Actinomycetota followed with relative abundances of 3.6%, 4.8%, 5.3%, and 6.4%, respectively. The relative abundances of the remaining phyla did not exceed 1%. Additionally, Chlamydiota was only present in the RP2 sample, with a relative abundance of 0.4% (Fig. 2).

A total of 87 microbial genera were identified in the four samples, 78 microbial genera were identified, while 9 remained unclassified. *Coxiella* and *Staphylococcus* were found to be the most abundant in each sample, with *Coxiella* accounting for 59.6%, 25.6%, 21.8%, and 11.2%, and *Staphylococcus* accounting for 14.4%, 21.0%, 26.0%, and 28.9% in the respective samples. Additionally, the abundance of *Adlercreutzia*, *Anaplasma*, *Enterococcus*, *Klebsiella*, *Methylopila*, *Mycobacterium*, *Nitratisporus*, *Pleurotus*, *Salmonella*, *Sinisalibacter*, *Streptococcus*, and the unclassified Coxiellaceae genus was relatively higher in the samples (Tab. 4). On the other hand, the remaining 73 genera had relative abundances of less than 1% (Fig. 3). It is notable that RP1 exhibited a markedly elevated relative abundance of *Coxiella* (59.6%), far greater than that detected in other groups. This may indicate a substantially higher prevalence of this bacterium in its corresponding region.

A total of 157 bacteria species were detected in the four samples, 151 bacterial species were identi-

Tab. 4. The Top 10 Genera by Relative Abundance

Genus	RP1 (%)	RP2 (%)	RP3 (%)	RP4 (%)
Anaplasma	0.4	3.3	1.6	6.3
Coxiella	59.6	25.6	21.8	11.2
Enterococcus	1.9	3.2	1.3	4.0
Klebsiella	3.7	5.7	6.2	7.7
Methylopila	0.8	1.1	0.7	1.5
Mycobacterium	2.6	3.2	4.1	4.4
Sinisalibacter	0.8	1.3	1.8	1.7
Staphylococcus	14.4	21.0	26.1	29.0
Streptococcus	6.2	9.4	14.1	12.6
Unclassified Coxiellaceae	2.5	14.4	12.7	6.5

Tab. 5. The Top 10 Species by Relative Abundance

Species	RP1 (%)	RP2 (%)	RP3 (%)	RP4 (%)
Anaplasma phagocytophilum	0.4	3.3	1.6	6.3
Coxiella-like endosymbiont	2.5	14.2	12.5	6.4
Coxiella burnetii	56.4	9.5	8.2	4.2
Coxiella endosymbiont of *Amblyomma nuttalli*	2.4	14.7	12.4	6.4
Enterococcus faecium	1.9	3.2	1.3	4.0
Klebsiella pneumoniae	3.7	5.7	6.2	7.7
Mycobacterium tuberculosis	2.6	3.2	4.1	4.4
Staphylococcus aureus	14.4	20.9	26.0	28.9
Streptococcus anginosus	1.7	2.5	2.3	3.5
Streptococcus pneumoniae	4.4	6.7	7.3	8.8

fied, while 6 remained unclassified. The relative abundances of these bacteria species are presented in Supplementary Material S1. Among them, 10 species have high relative abundance: *Anaplasma phagocytophilum*, *Coxiella*-like endosymbiont, *Coxiella burnetii*, *Coxiella* endosymbiont of *Amblyomma nuttalli*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Streptococcus anginosus*, *Enterococcus faecium*, *Staphylococcus aureus*, and *Streptococcus pneumonia* (Tab. 5). The species with the highest relative abundance in RP1 is *C. burnetii*, accounting for 56.4%, which is substantially higher than the relative abundance of *C. burnetii* recorded in the other three groups (9.5%, 8.2%, 4.2%). Among RP2, RP3, and RP4, the species with the highest relative abundance is *Staphylococcus aureus*, accounting for 20.9%, 26.0% and 28.9% respectively (Fig. 4).

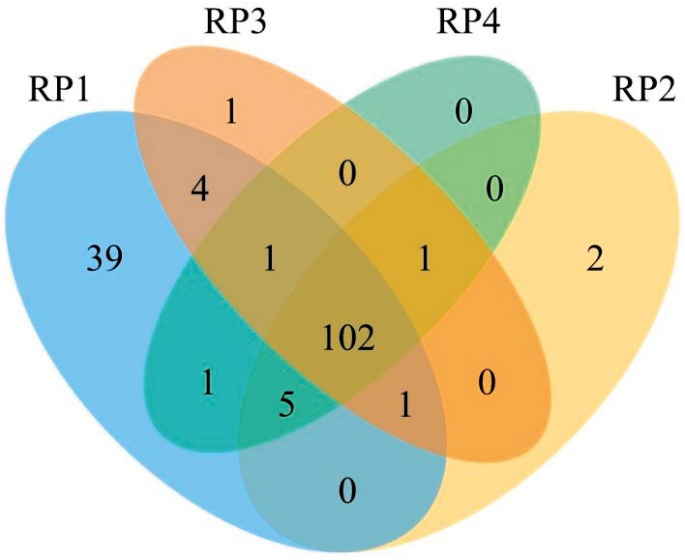

Fig. 5. Venn diagram of four *O. lahorensis* based on microbial species

Explanations: Each color represents a sample, and the numbers in the figure indicate the types of microorganisms present in the samples. The numbers in the overlapping areas represent the same types of microorganisms present in different samples simultaneously

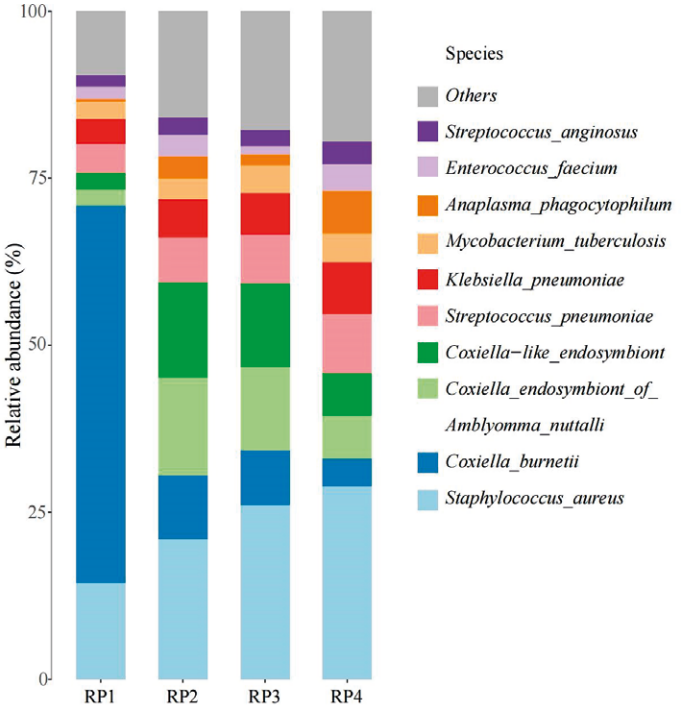

Fig. 4. Microbiome bar maps of four *O. lahorensis* samples (species)

Explanations: The horizontal axis represents the sample names, while the vertical axis represents the relative abundance content. Each color represents a species

Additionally, a retrovirus known as Jaagsiekte sheep retrovirus (JSRV) was detected in RP1, RP2, and RP4, which was the only virus detected in the four samples.

Analysis of the differences in microorganisms carried by four samples. As depicted in Figure 5, the Venn Diagram illustrates the number of microbial species detected in RP1, RP2, RP3, and RP4, which are 153, 111, 110, and 110, respectively. Among these, 108 types of microorganisms exhibit high similarity between RP1 and RP2, 108 types between RP1 and RP3, 103 types between RP1 and RP4, 104 types between RP2 and RP3, and 108 types between RP2 and RP4. RP3 and RP4 share 104 highly similar microorganisms, while 102 microorganisms are highly similar in the samples of RP1, RP2, RP3, and RP4 simultaneously.

Metagenomic analysis of tick samples from four Xinjiang regions revealed a diverse microbiome comprising 13 phyla, 87 genera, and 157 species, including taxa with known zoonotic potential. Consistent with previous studies (7), we detected *C. burnetii*, confirming its presence in the local tick population. A notable finding was the identification of previously unclassified bacterial taxa, suggesting that the microbial diversity within these ticks is not fully documented. The bacterial community was dominated by Pseudomonadota and Bacillota at the phylum level, which is a common profile in arthropod microbiomes, potentially reflecting their adaptability to the tick internal environment. At the genus level, seven dominant genera were identified – *Coxiella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Mycobacterium*, *Anaplasma*, and *Enterococcus* – all of

which have been previously isolated from ticks (5, 15). The concurrence of these pathogenic or opportunistic genera underscores a significant reservoir of bacteria capable of threatening both animal and human health, thereby highlighting an ongoing regional public health risk.

There have been academic reports indicating that *C. burnetii* is carried by ticks, with most cases involving hard ticks and a few cases involving soft ticks, such as *O. lahorensis* (17, 22). In this study, *C. burnetii* was detected in RP1, RP2, RP3, and RP4. The relative abundance of *Coxiella* in RP1 is as high as 56.4%, and the relative abundance in RP2, RP3, and RP4 is also high, being 9.5%, 8.2%, and 4.1%, respectively. This finding suggests that the presence of *C. burnetii* in ticks is a common phenomenon. The relative abundance of *C. burnetii* in ticks has a particular relationship with this region, perhaps due to the tick's host. Given the rivers and plains in Qiemo County, sheep grazing is a common practice. It is important to note that *O. lahorensis* utilizes mobile hosts, and the transmission of pathogens is a direct mechanism that occurs during tick feeding. These two factors, when combined, may lead to this situation and suggest that Q fever infectious zoonosis is more likely to occur in Qiemao County.

In this study, among the genera with high abundance, aside from the most predominant *Coxiella*, the relative abundances of *Staphylococcus*, *Streptococcus*, and *Enterococcus* were also notably high. This constitutes an interesting finding, as these bacteria are not typically regarded as utilizing ticks as vectors for transmission. However, the fact that ticks are not transmission vectors for these bacteria does not mean they do not carry them. Bacteria such as *Staphylococcus*, *Streptococcus*, and *Enterococcus* are common opportunistic pathogens. They often exist as commensals on the skin, in the oral cavity, respiratory tract, and intestines. Their primary transmission route is direct contact, which is far more efficient than a vector-borne mechanism involving ticks. The observed high relative abundance of these bacteria in ticks may be attributed to their ubiquitous distribution and host associations. These bacteria are pervasive in the environment, making it easy for ticks to acquire them on their body surfaces during natural activities. Furthermore, these bacteria proliferate on the skin of hosts with minor infections or lesions. When ticks feed on such hosts, the bacteria can be passively ingested along with the blood meal into the tick's digestive tract, leading to their detection at notably high concentrations.

In addition to the bacteria mentioned above, this study also identified more than 150 uncommon pathogens, including *Aeromonas allosaccharophila*, *Chlamydia trachomatis*, and *Psychrobacter immobilis*, among others. Although these bacteria are uncommon, they are pathogenic and present a risk to both humans and livestock. It was observed that some of these bacteria were only detected in one or two samples, suggesting that the epidemic pathogens carried by *O. lahorensis* varied across different regions. This finding offers valuable insights for controlling tick-borne diseases in southern Xinjiang.

In this study, the retrovirus Jaagsiekte sheep retrovirus (JSRV) was identified. JSRV primarily infects sheep, causing lung adenomatosis, with minimal impact on goats and other animals (11). The occurrence of sheep lung adenomatosis caused by JSRV was first documented in South Africa in 1825 and has since been reported in approximately 10 countries (10). Currently, sheep lung adenomatosis has been found in almost all countries and regions worldwide, indicating a global distribution. The initial detection of JSRV in *O. lahorensis* in Xinjiang suggests that these ticks may serve as a potential transmission vector for JSRV, leading to the development of sheep lung adenomatosis in sheep and goats in Xinjiang. However, further confirmation of whether JSRV can infect sheep through *O. lahorensis* requires extensive research experiments.

In conclusion, this is the first study to utilize metagenomic sequencing to analyze *O. lahorensis* in Xinjiang. The current study not only provides a reference for studying the pathogens carried by *O. lahorensis*, but also contributes to our knowledge of *O. lahorensis*. Furthermore, this study provides a theoretical foundation for investigating transmissible diseases caused by Xinjiang soft ticks, offering valuable insights for the academic community to explore the harm caused by soft ticks and potential control measures.

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