Acta Parasitologica (2024) 69:343–350
https://doi.org/10.1007/s11686-023-00752-4

ORIGINAL PAPER

Molecular Characterization of Tick-borne Pathogens in Bactrian Camels and Ticks from Gansu Province, China

Hong-xi Zhao[1] · Xiao-qing Zan[1] · Jin-zhong Tao[1] · Xin-gang Dan[1]

Received: 16 February 2023 / Accepted: 13 November 2023 / Published online: 13 December 2023

Abstract

Purpose Ticks are dangerous ectoparasites for humans and other animals, and tick-borne pathogens of Bactrian camels have been epidemiologically surveyed in Gansu Province, China. We aimed to determine the current distribution of tick-borne pathogens among Bactrian camels in Gansu during August 2013 using molecular tools.

Methods All ticks underwent morphological identification. We extracted DNA from the blood samples and ticks, screened them for *Theileria*, *Babesia*, *Anaplasma*, *and Ehrlichia* using standard or nested PCR with specific primers.

Results All ticks collected from the skin were identified as *Hyalomma asiaticum*. The blood and tick samples harbored similar pathogens, including the *Theileria* species, *T. annulata*, *T. luwenshuni*, *T. uilenbergi*, and *T. capreoli*, the *Anaplasma* species *A. bovis* and uncultured *Anaplasma*, the *Ehrlichia* species *E. canis* and uncultured *Ehrlichia*, and a new haplotype of *Babesia* species.

Conclusion Our findings of anaplasmataceae and piroplasmida in Bactrian camels in Gansu provide a theoretical basis for deeper investigation into the epidemiology of tick-borne pathogens in these camels.

Keywords Bactrian camel · *Theileria* · *Babesia* · *Anaplasma* · *Ehrlichia*

Introduction

China is home to the most Bactrian camels worldwide, and Zhangye City (Gansu Province, China) is one of their main production sites [1]. Bactrian camels have become an important natural way for local peasants and herdsmen to eliminate poverty, and they also provide local residents with milk, meat, and fur. To date, few studies have investigated the occurrence of tick-borne pathogens in these camels from Gansu [2]. Recently, 125 species of ticks have been identified in China, including 111 hard and 14 soft types [3]. *Hyalomma asiaticum* is a major tick species that is widespread in Zhangye City. Semi-desert and desert environments are conducive to the survival and reproduction of *H. asiaticum*, a 3-host tick that parasitizes cattle, goats, camels, sheep, pigs, horses, hedgehogs and humans [4]. It is widely distributed in northern China, Mongolia, and southern Russia [5].

Ticks are vectors of many zoonotic pathogens, that have recently increased the incidence of livestock diseases, and have impacted human health and the development of animal husbandry. *H. asiaticum* is an important disease vector that serves as a host for various microorganisms. Currently known pathogenic microorganisms carried by *H. asiaticum* in China include: Crimean-Congo hemorrhagic fever virus (CCHFV), Rift Valley fever virus (RVFV) [6], and Q fever and Tamdy virus [7]. Theileriosis, babesiosis, rickettsioses and anaplasmosis have been investigated in *H. asiaticum* [8]. Few studies have investigated hemoparasite infection in Bactrian camels in China, despite many species of anaplasmataceae and piroplasmida have been reported in domestic animals and wild animals [9].

Anaplasma and *Ehrlichia* belong to the anaplasmataceae. Anaplasmosis is caused by obligate intracellular bacteria of the genus *Anaplasma*. To date, eight *Anaplasma* species have been identified worldwide [10]. Known *Anaplasma* species that infect humans include *Anaplasma phagocytophilum*, *Anaplasma ovis* and *Anaplasma capra* [11, 12]. Infection often manifests as fever, thrombocytopenia, leukopenia, and organ dysfunction] [13]. *Ehrlichia* species

Hong-xi Zhao and Xiao-qing Zan have Contributed equally to the manuscript.

✉ Hong-xi Zhao
zhaohongxi2006@163.com

[1] College of Animal Science and Technology, Ningxia University, Yinchuan 750021, People's Republic of China

are obligate intracellular Gram-negative bacteria [14]. Ten *Ehrlichia* species have been identified since the first strain was isolated from a canid in 1935 [15]. Piroplasmida that mainly includes *Babesia* and *Theileria* are considered parasites with high host specificity that can infect host red blood cells, lymphocytes, or macrophages, causing a series of clinical symptoms such as fever, anemia, and jaundice [16]. Currently, over 100 species of *Babesia* have been identified, and the prevalence of infection is increasing worldwide [1]. Approximately, 13 species of *Theileria* have been reported [18, 19]. *Rickettsia*, *Theileria* and *Babesia* are common tick-borne pathogens that infect domestic animals worldwide, but little is known about hemoparasitic infections in Bactrian camels in China [20]. There is limited information on tick-borne diseases in the Gansu and genetic characterization of tick-borne pathogens (TBPs) in that area. Therefore, we aimed to investigate the occurrence and genetic diversity of tick-borne pathogens in Bactrian camels and ticks from Gansu. Our findings provide a scientific reference for the epidemiology of tick-borne diseases in this region of China.

Materials and Methods

Sample Collection

Zhangye city is located in the middle of the Hexi Corridor at the northern base of the Qilian Mountains in Gansu, a long narrow stretch of terrain in northwestern China with a complex landform and a variable climate. A unique desert oasis provides a natural pasture suitable for the survival of Bactrian camels [21]. The study was conducted in dry August 2013 at sites in Zhangye City (Fig. 1). The climate is also conducive to the prevalence of ticks and tick-borne diseases. We collected 45 blood samples and 33 unfed or partially engorged ticks from 45 Bactrian camels. All ticks

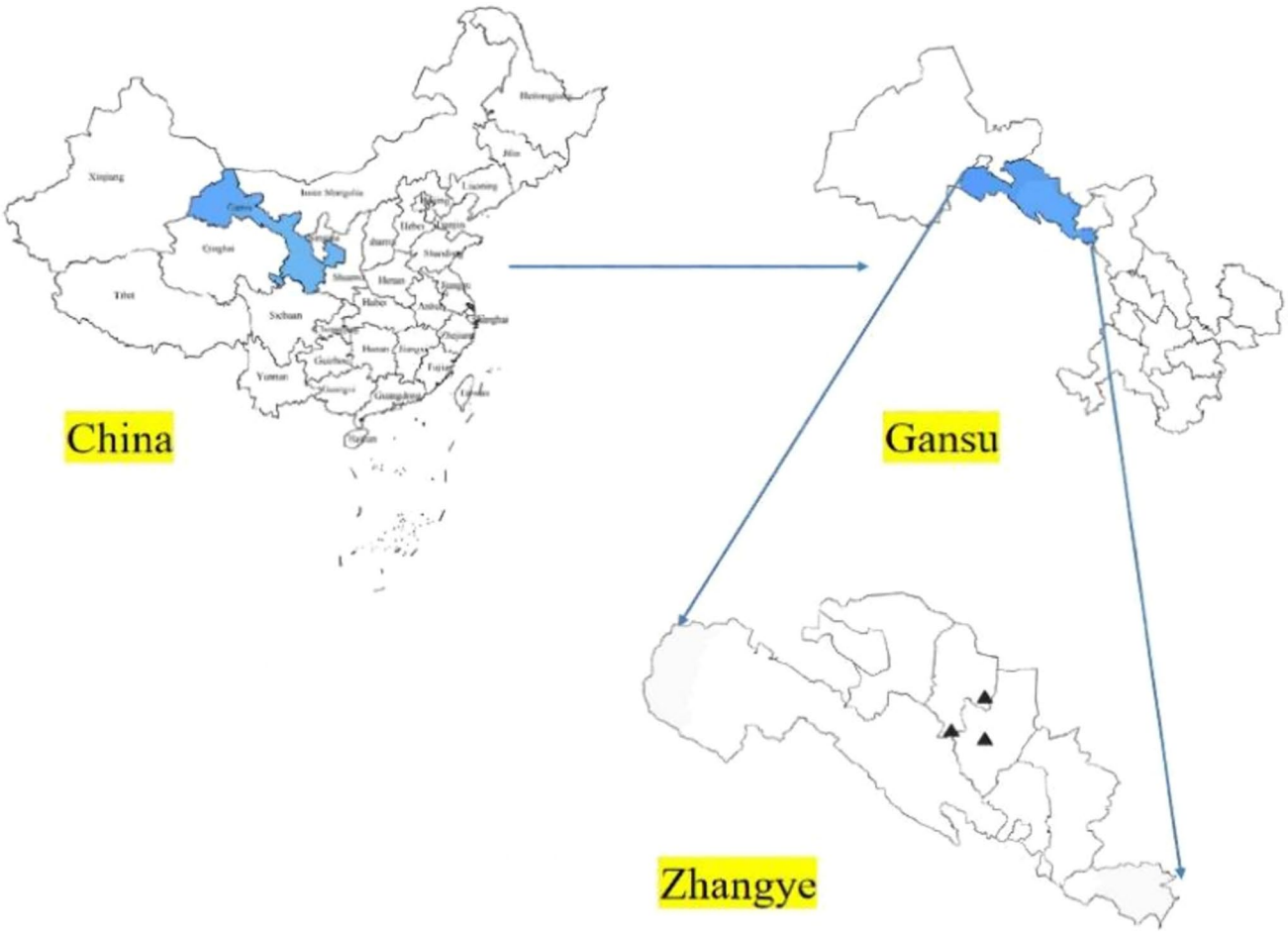

Fig. 1 Sampling sites in Zhangye, Gansu Province, China. The map is produced using NB Map (https://www.nbcharts.com/map/map) and WPS

were morphologically identified according to previous report [22] and stored at − 20℃.

DNA Extraction

Before DNA extraction, each tick was washed three times with 75% alcohol and followed by one wash of normal saline to remove surface impurities. Then, the ticks were homogenized by grinding rod. Seventy-eight genomic DNA was extracted from 45 camel blood samples and 33 ticks using DNeasy Blood & Tissue Kits (Qiagen, Germany) as described by the manufacturer, and stored at − 80 °C.

PCR Amplification

All DNA samples were initially screened by standard PCR-specific primers to detect and amplify piroplasmida, *Anaplasma*, and *Ehrlichia* shown in Table 1, and the DNA samples of piroplasmida were amplified by nested PCR. *Anaplasma* and *Ehrlichia* and piroplasmida were used for the preliminary genera-specific identification of the samples. *A. bovis* and *A. phagocytophilum* were used for the species-specific identification of the positive samples about *Anaplasma* and *Ehrlichia*. Fragments were amplified in a total volume of 25 µL in PCR Amplifier (Bio-Rad, Hercules, CA, USA) containing 2.5 µL of PCR buffer (10×), 2.0 µL of dNTPs (2.5 mM), 1.25 U of Taq DNA polymerase (Takara Bio Inc., Kusatsu, Japan), 2.0 µL of template DNA, 1.0 µL of each primer (10 pmol) and 16.25 µL of distilled water. Amplicons were separated by 0.5 µg/mL ethidium bromide-stained 1% agarose gel electrophoresis (Proteinsimple Alphalmager HP, USA) and visualized under ultraviolet light.

Sequencing and Phylogenetic Analyses

All positive PCR products derived from the 18S rRNA of piroplasmida and the 16S rRNA of *Anaplasma* and *Ehrlichia* were purified using a TaKaRa agarose gel DNA purification kit version 2.0, ligated into a pGEM-T Easy vector (Promega Corp., Madison, WI, USA), and transformed into *Escherichia coli* JM109 competent cells. Two recombinant clones of each sample were selected for sequencing using BigDye Terminator Mix (GenScript, Nanjing, China). Sequences from recombinant positive clones were compared with those in GenBank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were then phylogenetically analyzed using the neighbor-joining algorithm in Molecular Evolutionary Genetics Analysis (MEGA) 7 software [27] and analyzed with the Clustal W method in the MegAlign software (DNAStar, Madison, WI, USA). We then constructed a phylogenetic evolutionary tree of 16S rRNA sequences in *Anaplasma* and *Ehrlichia*, and 18S rRNA sequences in *Theileria* and *Babesia* using MEGA7.

Results

Tick Identification and PCR Detection of 18S RNA and 16S RNA Using Specific Primer Sets

All ticks used in this study were identified as *H. asiaticum* as described. The PCR result shows that among anticoagulant blood samples from Bactrian camels, 43 (95.56%) of 45 were positive for piroplasmida, including *T. luwenshuni*, *T. uilenbergi*, *T. capreoli*, *T. annulata*, uncultured *Babesia* sp., 11 (24.45%), and 24 (53.33%) of 45 were positive for *A. bovis* and *A. phagocytophilum*, respectively. All 33 (100%) tick samples were positive for piroplasmida, including *T. luwenshuni*, *T. uilenbergi*, *T. capreoli*, *T. annulata*, whereas 7 (21.21%) and 6 (18.18%) were also positive for *A. bovis* and *A. phagocytophilum*.

Sequence Alignment

Eleven of 45 and 6 of 33 sequences derived from Bactrian camel blood and from ticks in camel skin differed. We obtained 17 representative sequences and deposited them in GenBank (Table 2).

Table 1 Sequences of oligonucleotide primers

Pathogen	Target gene	Primers	Oligonucleotide sequences (5′→3′)	Amplicon size (bp)	References
Anaplasma and *Ehrlichia*	16S rRNA	EC9	TACCTTGTTACGACTT	1462	Kawahara et al. [23]
		EC12A	TGATCCTGGCTCAGAACGAACG		
A. bovis	16S rRNA	AB1f	CTCGTAGCTTGCTATGAGAAC	551	Kawahara et al. [23]
		AB1r	TCTCCCGGACTCCAGTCTG		
A. phagocytophilum	16S rRNA	SSAP2f	GCTGAATGTGGGGATAATTTAT	641	Kawahara et al. [23]
		SSAP2r	ATGGCTGCTTCCTTTCGGTTA		
Piroplasmida	18S rRNA	PIRO-A	AATACCCAATCCTGACACAGGG	1407–1409	Persing et al. [24] Olmeda et al. [25]
		PIRO-B	TTAAATACGAATGCCCCCAAC		
	18S rRNA	Piro1-S	CTTGACGGTAGGGTATTGGC	396–429	Yang et al. [26]
		Piro3-AS	CCTTCCTTTAAGTGATAAGGTTCAC		

Table 2 Comparison of 17 sequences determined herein with those in GenBank using BLAST

		Sequences				Closest BLASTN match
Pathogen	Blood or tick samples (n)	Accession no	Length (bp)	Identity (%)	Isolated pathogen	Accession no. (China)
T. luwenshuni	Blood (2)	KU554725	1017	98.52	*T. luwenshuni*	KC769996
	Tick (4)	KU554726	1305	99.85	*T. luwenshuni*	KC769996
	Blood (10)	KU554729	1379	99.85	*T. luwenshuni*	KC769996
	Blood (26)	KU554730	1295	100	*T. luwenshuni*	KC769996
T. uilenbergi	Tick (14)	KU554724	851	93.85	*T. uilenbergi*	MW404469
	Blood (8)	KU554728	1373	99.78	*T. uilenbergi*	JF719835
T. capreoli	Tick (11)	KU554727	1179	97.09	*T. capreoli*	MH179334
T. annulata	Blood (27)	KU554731	1373	99.93	*T. annulata*	KF429795
Babesia	Tick (9)	KU554723	358	97.21	*Babesia* sp.	HQ730762
A. platys	Blood (23)	KU554735	529	100	*A. platys*	KJ659044
	Blood (24)	KU554736	511	99.61	Uncultured *Anaplasma* sp.	KP939254
A. bovis	Blood (21)	KU554737	529	100	*A. bovis*	KJ639885
	Blood	KU554738	545	99.63	*A. bovis*	KJ639883
Uncultured *Ehrlichia* sp.	Tick (16)	KU554732	641	99.06	Uncultured *Ehrlichia* sp.	OL884435
	Blood (6)	KU554733	622	99.84	Uncultured *Ehrlichia* sp.	OL884435
	Tick (28)	KU554734	599	99.67	Uncultured *Ehrlichia* sp.	OP047988
	Blood (1)	KU554739	641	99.69	Uncultured *Ehrlichia* sp.	OL884435

Phylogenetic Analyses

Three phylogenetic trees were constructed based on the 18S rRNA sequences of *Babesia* and *Theileria*, and the 16S rRNA sequences of *Anaplasma* and *Ehrlichia* determined herein and GenBank (Figs. 2, 3, and 4).

The evolutionary history was inferred using the Neighbor-Joining method (bootstrap replications: 1000). The evolutionary distances were computed using the *p*-distance method. The scale bar indicated the estimation of evolutionary distance. The optimal tree with the sum of branch length = 0.50053544 is shown. The taxa marked by black triangle and bold font depict the sequences obtained in the

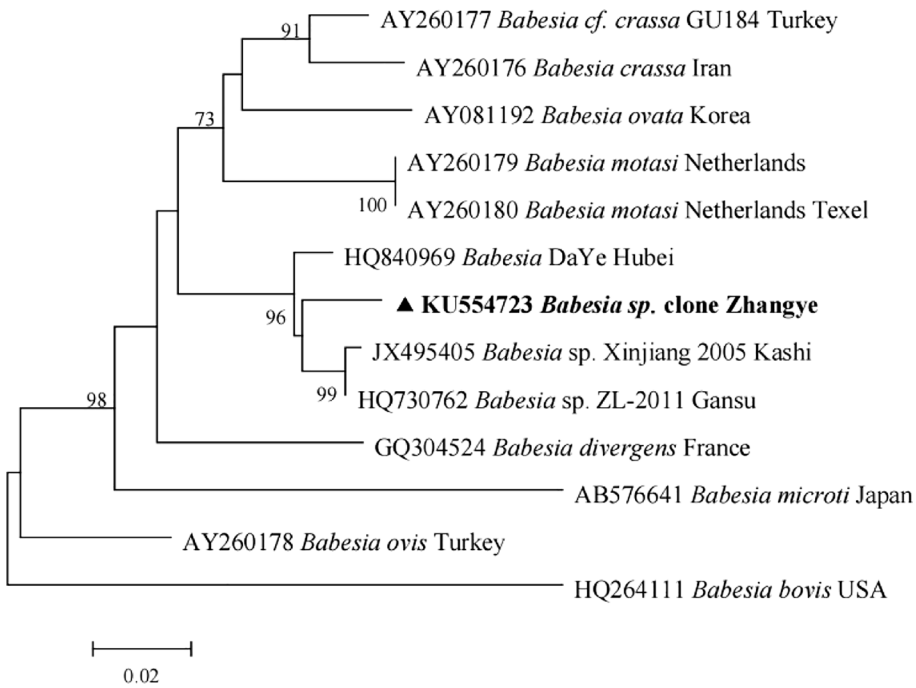

Fig. 2 Phylogenetic tree of *Babesia* based on 18S rRNA sequences

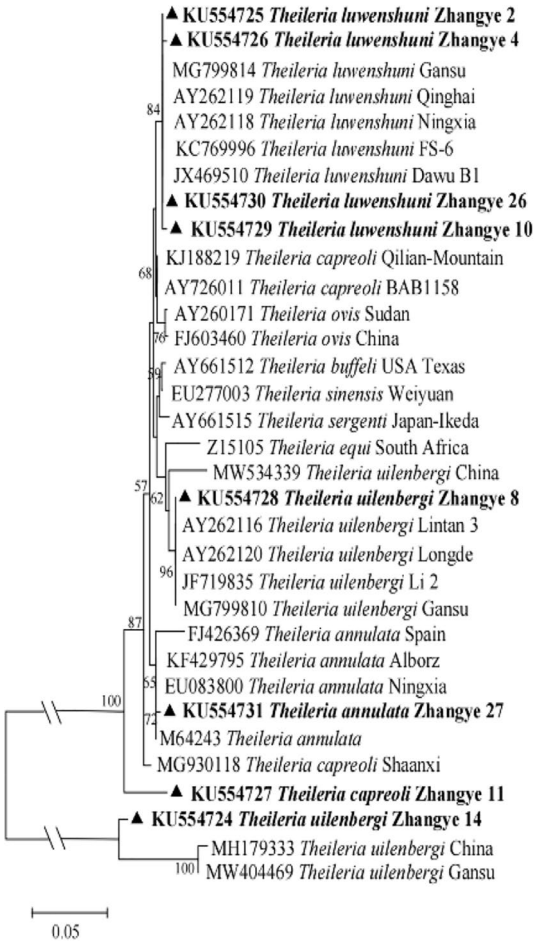

Fig. 3 Phylogenetic tree of *Theileria* based on 18S rRNA sequences

study, and GenBank accession number is provided in front of each strain name. Note: All trees were mid-point rooted for clarity only. Bootstrap values (> 50%) were shown for appropriate nodes. Scale bar represents number of nucleotide substitutions per site. The analysis involved 13 nucleotide sequences.

The evolutionary history was inferred using the Neighbor-Joining method (bootstrap replications: 1000). The evolutionary distances were computed using the p-distance method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 33 nucleotide sequences. Target size of the primer used to generate the phylogenies is 851–1379.

The evolutionary history was inferred using the Neighbor-Joining method (bootstrap replications: 1000). The evolutionary distances were computed using the p-distance method. The scale bar indicated the estimation of evolutionary distance. The taxa marked by black triangle and bold font depict the sequences obtained in the study, and GenBank accession number is provided in front of each strain name. Note: All trees were mid-point rooted for clarity only. Bootstrap values (> 50%) were shown for appropriate nodes. Scale bar represents number of nucleotide substitutions per site.

Discussion

Climate change and global warming have facilitated disease vector spreading into new geographic locations, 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, which is of utmost importance in devising appropriate vector and pathogen control interventions. This is the first study to report the presence of *T. luwenshuni*, *T. uilenbergi*, *T. capreoli*, *T. annulata*, uncultured *Babesia* sp., *A. platys*, *A. bovis*, *E. canis* and uncultured *Ehrlichia* sp. in Bactrian camels blood and in *H. asiaticum* in Gansu. Our results complement the vacancy of Bactrian camels carrying pathogens in Zhangye, Gansu. *H. asiaticum* has recently parasitized Bactrian camels in inner Mongolia from China [28].

Anaplasma and *Ehrlichia* were identified commonly of the tick-borne organisms, also occasionally infect humans. *Anaplasma* was initially detected in Jordanian dromedary camels [12]. *Anaplasma phagocytophilum* was originally identified detected in cattle on Yonaguni Island, Okinawa, Japan [29]. In recent years, *A. bovis* was initially thought to be only the agent of animal ehrlichiosis until it was the etiologic agent of human infection, when the first patient case was reported in 2019 [30]. In China, the report shows two cases of *A. bovis* infection in humans in Anhui Province [31]. This study shows that Bactrian camels contain *A. bovis* pathogens and indicates the spread of pathogens among livestock. Therefore, mixing several types of grazing livestock should be avoided. *Anaplasma platys* has been found mainly infecting dogs, cats, goats, cattle, deer and Bactrian camels [32, 33]. *Anaplasma platys* is a parasite with tropism for platelets having a wide host range, primarily being the causative agent of canine cyclic thrombocytopenia [34]. Although *A. platys* and *E. canis* is best known as a very common dog pathogen around the world, infections have also been described in people and other hosts include cattle, sheep, goats, rodents, and deer [15, 34]. *Ehrlichia* is a genus closely related to human diseases. *Ehrlichia canis* has also been detected in Bactrian camels, indicating the importance of epidemiologically detecting tick pathogens in these hosts. These ecological features give a hint about the possibility of transmission of these pathogens among domestic animals. *Ehrlichia canis* is considered a potential agent of human disease, although human infection cases have never been reported in China[35].

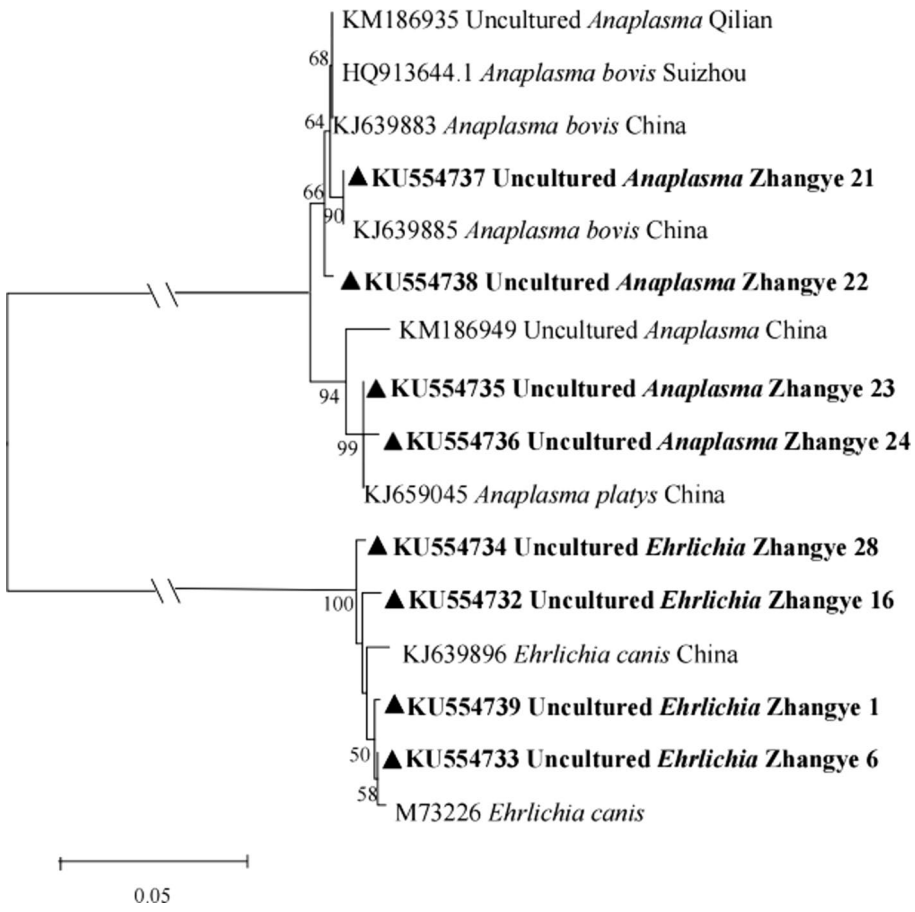

Fig. 4 Phylogenetic tree of *Anaplasma* and *Ehrlichia* based on nucleotide sequences of 16S rRNA sequences

To date, DNA of *Theileria equi*, *T. annulata*, *T. mutans*, *T. ovis* and *B. caballi* has been detected in blood of dromedaries [36]. In our study, we found five piroplasmid infections in Bactrian camel bloods and ticks, including *T. luwenshuni*, *T. uilenbergi*, *T. capreoli*, *T. annulata*, uncultured *Babesia* sp. *Babesia* and *Theileria* have been found not only in Bactrian camels, but also in horses in Gansu Province [37]. *Babesia* and *Theileria* have also been found in wild Reeves' muntjacs (*Muntiacus reevesi*) in China [26], as well as in horses and Bactrian camels in northeastern Mongolia [38]. *Theileria luwenshuni* and *T. uilenbergi* have previously infected small domestic ruminants, but we detected these pathogens in Bactrian camel blood. *Theileria luwenshuni* and *T. uilenbergi*, which have been previously reported to petits ruminants, were detected for the first time in blood samples from cattle and yaks on the Tibetan Plateau, and lack of relevant information infection in other animals [39]. Detecting *T. luwenshuni and T. uilenbergi* in Bactrian camels fortifies their roles as vectors of these economically threatening tick-borne pathogens [40]. *Theileria capreoli* was recorded in roe deer, red deer, fallow deer, roe deer and Chinese water deer [41]. This was first described in Bactrian camel in China.

Therefore, these bacteria are important for both veterinary and human public health. However, mortality associated with tick-borne diseases in livestock keepers has not been reported in Gansu. We investigated the molecular prevalence and genetic diversity of TBPs in Bactrian camels in Gansu to increase the amount of available epidemiological data on these pathogens in this area of China. Our result may contribute to the current knowledge of the biodiversity of part of tick-borne circulating in this area. The detection of *A. platys*, *A. bovis* and *E. canis* in Bactrian camel blood and ticks indicates that this issue requires more investigation and further measures should be taken to control and prevent of anaplasmataceae transmission between humans and other animals.

Bactrian camel blood and tick collection were not conducted at different times throughout the year, and as a result, the study did not investigate the seasonality of tick-borne diseases in Zhangye. The study is also limited in its selection of sampling sites and, therefore, could not cover all the Gansu ecological zones to provide a good representation of all Bactrian camel blood, tick and tick-borne pathogens in the entire country. Another limitation of this study is that the majority of the infections detected were in Bactrian camel blood samples or tick samples, and therefore, it could not be differentiated whether these represent actual coinfections or whether individual samples

among the Bactrian camel blood samples or tick samples each carried multiple pathogens.

Conclusions

The present findings confirm previous results and provide more details about the molecular detection of tick-borne pathogens in blood and ticks from camels in Gansu Province, China, including part of *Theileria*, *Babesia*, *Anaplasma*, and *Ehrlichia*, while highlighting the importance and relevance of molecular methods. In recent years, with rising global temperature, the range of tick activity has expanded, and our findings reflected the prevalence of tick-borne diseases in Gansu. We showed that Bactrian camel blood and *H. asiaticum* are carriers of *T. luwenshuni*, *T. uilenbergi*, *T. capreoli*, *T. annulata*, *Babesia*, *A. platys*, *A. bovis*, *E. canis* and *uncultured Ehrlichia* sp. These potential zoonotics suggests that preventive and control measures are needed to avoid transmission between humans and other animals and among other animals. Clarification of the roles of the Bactrian camels and ticks as reservoir hosts for some *Theileria*, *Babesia*, *Anaplasma*, and *Ehrlichia* species is critical to determine whether ticks contribute to the spread of ruminant piroplasmida and anaplasmataceae in China. Our results provide sufficient epidemiological data to enhance the understanding of the measures required to effectively control theileriosis transmission to Bactrian camels and other ruminants in Gansu.

Author Contributions HZ and XD conceived and designed the study. HZ, XZ and JZ Tao carried out the field work, and HZ and XZ drafted the manuscript. All the authors reviewed the original manuscript and agreed to the final version. All the authors read and approved the final manuscript.

Funding This work was funded by the Ningxia Natural Science Foundation (No.2023AAC02017, 2022AAC03017).

Availability of Data and Materials All data generated during this study are included in this published article.

Declarations

Conflict of Interest The authors declare that they have no competing interests.

Ethical Approval This study was approved by the Animal Ethics Committee of Ningxia University, NXU (No. NXU 2015–012). The use of these field samples was approved by the Animal Ethics Procedures and Guideline of China.

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