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# IDENTIFICATION OF 12 PIROPLASMS INFECTING TEN TICK SPECIES IN CHINA USING REVERSE LINE BLOT HYBRIDIZATION

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ABSTRACT: Piroplasmosis, a disease of domestic and wild animals, is caused by tick-borne protozoa in the genera of *Theileria* and *Babesia*. There is limited information available about the prevalence of piroplasmosis in ticks in China, and to assess the potential threat of piroplasmosis in China, we investigated the infections of ovine and bovine *Babesia* and *Theileria* species in ticks collected from cattle, yaks, sheep, horses, and camels in several regions of China where tick-borne diseases have been reported. In total, 652 ticks were collected from the animals in 6 provinces of China. *Babesia* spp. and *Theileria* spp. were detected with a PCR-RLB method and identified by sequencing. Overall, 157 ticks (24.1%) were infected with 5 *Babesia* and 4 *Theileria* species. Among tested tick samples, 134 (20.6%) were single infections with 1 of 7 piroplasm species, with *Theileria annulata* (118/652, 18.1%) being dominant. Only 23 (3.5%) tick samples were double or triple infected, *Theileria luwenshuni* and *Theileria sinensis* (18/652, 2.8%) were frequently observed in co-infections. Some piroplasm species were carried by ticks that were not previously reported to be vectors.

Piroplasmosis is naturally caused by tick-transmitted, and generally host-specific, protozoan parasites in the genera *Babesia* and *Theileria*. It is a disease of domestic and wild animals as well as humans, and it is therefore responsible for economic losses. The impacts include lowered meat and milk production, abortions, fertility in bulls, control measure costs, trade or import restrictions, and a general impact on the global cattle industry (Bock et al., 2004).

Ticks are important vectors of a large variety of pathogens that infect animals and cause diseases of veterinary importance (Jonsson et al., 2008). Piroplasms are infective when both their definitive (ticks) or intermediate hosts (vertebrates) are encountered. Some tick species can carry more than 1 piroplasm species that affects the same host or different animal hosts. Furthermore, some piroplasm species are transmitted by different tick species, even those with close phylogenetic relationships (Yin et al., 2002).

*Babesia* cf. *motasi* and *Babesia* sp. Xinjiang were reported to cause ovine babesiosis in China (Liu et al., 2007). *Haemaphysalis qinghaiensis* and *Haemaphysalis longicornis* were described as the vectors of *B.* cf. *motasi* (Bai et al., 2002a; Guan et al., 2010). *Hyalomma anatolicum* was reported to transmit *Babesia* sp. Xinjiang (Guan et al., 2009). Four *Babesia* species, *Babesia bovis*, *Babesia bigemina*, *Babesia major*, and *Babesia ovata*, were described as responsible for cattle, buffalo, and yak babesiosis in China (Luo et al., 2005; Qin et al., 2015). *Babesia bigemina* and *B. bovis*, which are major causative agents of bovine babesiosis, share the same vectors *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus annulatus*, and *Rhipicephalus geigyi*, and often present in co-infection (Ravindran et al., 2006; Tavassoli et al., 2013). *Babesia major* is transmitted by *H. longicornis* and *Haemaphysalis punctata,* while *B. ovata* is transmitted by *H. longicornis*, which have low virulence in the bovine species (Yin et al., 1996).

Ovine theileriosis is mainly caused by *Theileria luwenshuni*, *Theileria uilenbergi*, and *Theileria ovis* in China (Yin et al., 2007). *Theileria uilenbergi* and *T. luwenshuni* are considered highly pathogenic to small ruminants, such as sheep and goats, and are transmitted by *H. qinghaiensis* and *H. longicornis* (Li et al., 2009), while *T. ovis* is non-pathogenic or mildly pathogenic for small ruminants and can be transmitted by *Hyalomma anatolicum* (Li et al., 2010). Three *Theileria* species, *Theileria annulata*, *Theileria sergenti*, and *Theileria sinensis*, have been reported in China as agents of bovine theileriosis (Liu et al., 2010, 2015). *Theileria annulata* is transmitted by *Hy. anatolicum*, *Hyalomma detritum*, *Hyalomma excavatum*, *Hyalomma dromedarii,* and *Hyalomma marginatum* (Sayin et al., 2003). *Theileria sergenti* is the most prevalent, which is transmitted by *H. longicornis* (Liu et al., 2010; Zhao et al., 2017). *Theileria sinensis* infects cattle and yaks and is transmitted by *H. qinghaiensis* (Bai et al., 2002b).

Epidemiologic studies concerning tick-borne diseases provide information about endemic instability of these diseases (Ekici et al., 2012). Varied diagnostic methods have been used for the detection of *Theileria* and *Babesia* species in ticks, but the application of molecular methods provides improved sensitivity and specificity and allows direct detection of parasites (Altay et al., 2008; Aydin et al., 2013). Recently, PCR-RLB was used for rapid, simultaneous detection and differentiation of numerous species of *Babesia* and *Theileria* even in mixed infection (Schnittger et al., 2004; Niu et al., 2009; Iqbal et al., 2013). Although there have been several studies of the frequency of ixodid tick species and the prevalence of tick-borne diseases in most areas in China (Chen et al., 2014a; Li et al., 2014a; Liu et al., 2015; Yu et al., 2015), little information is available about the prevalence of pathogens in ticks. Thus, the investigations of known parasites and new tick-piroplasms associations are important and significant for the animal health and livestock industry.

In this study, our aim is to apply a previously developed PCR-RLB assay to detect ovine and bovine piroplasms in ixodid ticks collected from animals in different provinces of China, to better

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quantify the flow of piroplasm infection in field ticks, and to provide new molecular parasitological data.

## MATERIALS AND METHODS

### Collection and identification of tick samples

In total, 652 adult ticks were collected from animals (cattle, yak, sheep, horses, and camels) from 13 different regions of 6 provinces: Xinjiang Uygur Autonomous Region, Gansu, Henan, Jilin, Guangdong, and Hunan provinces. Ticks were removed manually from the host body and placed in labeled bottles with water soaked cotton swabs. The ticks were identified to the species level as *H. punctata* (n = 29), *H. longicornis* (n = 107), *Hy. anatolicum* (n = 20), *Hyalomma asiaticum* (n = 14), *Hy. detritum* (n = 18), *Dermacentor marginatus* (n = 85), *Dermacentor silvarum* (n = 14), *Rhipicephalus sanguineus* (n = 168), *R.* (*Boophilus*) *microplus* (n = 168), and *Ixodes ovatus* (n = 29), according to taxonomic keys by Teng and Jiang (1991). Genomic DNA was extracted from ticks using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at –20 C.

### PCR amplification of 18S rRNA gene of *Theileria*/*Babesia*

To amplify the hyper-variable 4 (V4) region of the 18SrRNA gene of *Theileria* and *Babesia* species, primers RLB-F and RLB-R were used (Gubbels et al., 1999). The PCR and cycling conditions were as described in the previous study (Abdallah et al., 2016). The PCR products were purified using a MiniBEST DNA Fragment Purification Kit (TaKaRa®, Liaoning Province, China) and cloned into pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin), and then transformed into JM109 *Escherichia coli* cells according to the manufacturers' instructions. The plasmids with inserts were extracted by using MiniBEST Plasmid Purification Kit version 2.0 (TaKaRa®), and the inserts were sequenced. Positive colonies were selected and screened using vector primers (T7/SP6). Recombinant plasmids were extracted from overnight bacterial cultures and the V4 region of 18S rRNA was sequenced using vector primers.

### RLB hybridization

Oligonucleotide probes (catch-all *Theileria* and *Babesia*, *B.* cf. *motasi* isolates, *Babesia* sp. Xinjiang, *B. major*, *B. ovata*, *B. bigemina*, *B. bovis*, *T. uilenbergi*, *T. luwenshuni*, *T. ovis*, *T. annulata*, *T. sinensis*, and *T. sergenti*) containing a *N*-(trifluoroacetamidohexyl-cyanoethyl, *N*, *N*-diisopropyl phosphoramidite [TFA])-C6 amino linker were synthetized (Gubbels et al., 1999; Schnittger et al., 2004; Niu et al., 2009, 2012; Abdallah et al., 2016). Positive plasmids from *Babesia* or *Theileria* species genomic DNA, 2 species of bacteria (*Anaplasma marginale* and *Borrelia burgdorferi* sensu stricto), and water were used as positive, negative, and blank control, respectively, to perform the specificity and sensitivity of the RLB assay (Abdallah et al., 2016). RLB procedure was performed according to Gubbels et al. (1999).

### Statistical analysis

The 95% confidence intervals (CIs) for the overall prevalence values of each parasite were calculated using IBM SPSS Statistics version 19.0.

## RESULTS

### Detection and identification of *Theileria* and *Babesia* species in tick samples by PCR-RLB

The most frequently detected parasite was *T. annulata*, mostly found in *R. sanguineus* from cattle in Huidong, reaching 33.9% (57/168). A high infected rate of *T. annulata* was also found in *Hy. asiaticum* (30%, 3/10), *H. longicornis* (26.3%, 21/80), and *R.* (*B.*) *microplus* (19.6%, 33/168) from the camels and cattle in Gansu, Xinjiang, and Hunan province, respectively. In addition, high *T. luwenshuni* (88.9%, 24/27) and *T. sinensis* (70.4%, 19/27) infections were observed in *H. longicornis* from sheep in Henan province. Species-specific positive signals were only obtained with 9 piroplasm species, and the average positive rates were 0.2% (95% CI = 0–0.7) for *B.* cf. *motasi*, *B. major*, and *B. bovis*; 0.6% (95% CI = 0–2.9) for *Babesia* sp. Xinjiang and *B. bigemina*; 3.9% (95% CI = 0.3–17.5) for *T. luwenshuni*; 18.7% (95% CI = 4.5–25.3) for *T. annulata*; 0.5% (95% CI = 0–1.9) for *T. sergenti*, and 2.9% (95% CI = 0–13.2) for *T. sinensis* (Table I).

Among total of 652 adult *ixodid* ticks, 157 (24.1%) ticks were infested with at least 1 piroplasm species, most of which corresponded to *Theileria* infection. No *I. ovatus* sample was found infected with any parasite. Single infections (20.6%, 134/652) were found in most of the positive ticks, most of which were infected by *T. annulata* (118/652, 18.1%). Single infections were also observed for other pathogens including *Babesia* sp. Xinjiang, *B. major*, *B. bigemina*, *B. bovis*, *T. luwenshuni*, and *T. sergenti*. Mixed infections (23/652, 3.5%) involved 7 pathogens, some of which could affect the same host (cattle or sheep), or 2 different animal hosts (cattle and sheep). The highest co-infection (18/652, 2.8%) was with *T. luwenshuni* + *T. sinensis* infecting *H. longicornis*. Other double infections were detected in *Hy. anatolicum*, *R. sanguineus*, and *R.* (*B.*) *microplus* with low infection rate. Triple infection was observed in 1 *H. longicornis* infected by *B.* cf. *motasi* + *T. luwenshuni* + *T. sinensis* (Table II).

The highest parasite infection occurred in *H. longicornis* (43%) and *R. sanguineus* (33.9%), while the positive rate of *Babesia* or *Theileria* in *Hy. anatolicum*, *R.* (*B.*) *microplus*, and *Hy. asiaticum* reached 20% (Table III).

### Identification of pathogens from ticks by sequencing analysis

Sixty infected tick samples were randomly screened and sequenced, based on the 18S rRNA of *Babesia* or *Theileria* species, or rhoptry-associated-protein-1 (*rap-1*) gene of sheep *Babesia* species, or cytochrome b (COB), as well as internal transcribed spacer (ITS) genes of *Theileria* species of cattle to further confirm our findings. Sequences were then blasted with the published sequences from GenBank. Based on the 18S rRNA and *rap-1* genes to detect *Babesia* sp. Xinjiang, 2 *Hy. anatolicum*, and 1 *H. longicornis* samples were sequenced and showed 99% identity with these gene sequences (GenBank accession number: DQ159073 and KF811199). One *R. sanguineus* infected by *B. bigemina* was sequenced based on the 18S rRNA gene and showed 100% identity with this gene sequence (GenBank accession number: AY603402). In order to detect *T. annulata* infection, a total of 50 tick samples, including *Hy. anatolicum*, *Hy. asiaticum*, *R.* (*B.*) *microplus*, *R. sanguineus*, *H. longicornis*, *H. punctata*, *D. marginatus*, and *D. silvarum* samples, were sequenced based on the

TABLE I. Prevalence of piroplasms in field ticks collected from hosts in different regions of China during March–August 2016.*

| Province | Location | Tick species | Hosts | No | Infection of piroplasm (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *B.* cf. *motasi* | *B.* sp. XJ | *B. ma* | *B. bi* | *B. bo* | *T. lu* | *T. an* | *T. se* | *T. si* |
| Xinjiang | Chaxian | *H. longicornis* | Cattle | 80 | | 1 (1.3) | | | | | 21 (26.3) | 1 (1.3) | |
| | | *H. punctata* | Sheep | 29 | | | 1 (3.4) | | | | 2 (6.9) | | |
| | | *Hy. asiaticum* | Sheep | 4 | | | | | | | | | |
| | Kuerdening | *D. marginatus* | Cattle/ Sheep | 54 | | | | | | 1 (1.9) | | | |
| | Zhaosu | *D. marginatus* | Sheep | 12 | | | | | | | | | |
| | Yining | *D. marginatus* | Cattle | 16 | | | | | | 1 (6.3) | 1 (6.3) | | |
| | Shache | *Hy. anatolicum* | Cattle | 20 | | 3 (15) | | | | | 3 (15) | | |
| | Xinhe | *Hy. detritum* | Cattle | 11 | | | | | | | | 1 (9.1) | |
| | Xingyuan | *Hy. detritum* | Horse | 7 | | | | | | | | | |
| Gansu | Jinchang | *Hy. asiaticum* | Camel | 10 | | | | | | | 3 (30) | | |
| | Tianzhu | *D. marginatus* | Yak | 3 | | | | | | | | | |
| | | *I. ovatus* | Yak | 29 | | | | | | | | | |
| Henan | Linzhou | *H. longicornis* | Sheep | 27 | 1 (3.7) | | | | | 24 (88.9) | | | 19 (70.4) |
| Jilin | Qingshi | *D. silvarum* | Sheep | 14 | | | | | | | 2 (14.3) | | |
| Guangdong | Huidong | *R. sanguineus* | Cattle | 168 | | | | 1 (0.6) | | | 57 (33.9) | | |
| Hunan | Xinhuang | *R.* (*B.*) *microplus* | Cattle | 168 | | | | 3 (1.8) | 1 (0.6) | | 33 (19.6) | 1 (0.6) | |
| Total (%) | | | | 652 | 1 (0.2) | 4 (0.6) | 1 (0.2) | 4 (0.6) | 1 (0.2) | 26 (3.9) | 122 (18.7) | 3 (0.5) | 19 (2.9) |

* Abbreviations: *B.* cf. *motasi* = *Babesia* cf. *motasi*; *B.* sp. XJ = *Babesia* sp. Xinjiang; *B. ma* = *Babesia major; B. bi* = *Babesia bigemina; B. bo* = *Babesia bovis; D. marginatus* = *Dermacentor marginatus; D. silvarum* = *Dermacentor silvarum; H. longicornis* = *Haemaphysalis longicornis; H. punctata* = *Haemaphysalis punctata; Hy. asiaticum* = *Hyalomma asiaticum; Hy. anatolicum* = *Hyalomma anatolicum; Hy. detritum* = *Hyalomma detritum; I. ovatus* = *Ixodes ovatus; R. sanguineus* = *Rhipicephalus sanguineus; R.* (*B.*) *microplus* = *Rhipicephalus* (*Boophilus*) *microplus*; *T. an* = *Theileria annulata; T. lu* = *Theileria luwenshuni; T. se* = *Theileria sergenti; T. si* = *Theileria sinensis.*

18S rRNA and COB gene, and the similarity ranged from 87% to 100% with these gene sequences (GenBank accession number: KM288519; KP731977; KF732030). One *R.* (*B.*) *microplus* and 1 *Hy. detritum* DNA infected by *T. sergenti* were sequenced based on the ITS gene and showed 89% similarity (GenBank accession number: HM538261). One *H. longicornis* infected by *T. sinensis* was sequenced and showed 99% similarity with *T. sinensis* ITS gene from China (GenBank accession number: EF547931). The

TABLE II. Prevalence and nature of *Theileria* and *Babesia* infection in ticks collected from hosts in China during March–August 2016.*

| Nature of infection | Infected ticks by piroplasm (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *H. punctata* | *H. longicornis* | *Hy. anatolicum* | *Hy. asiaticum* | *Hy. detritum* | *D. marginatus* | *D. silvarum* | *R. sanguineus* | *R.* (*B.*) *microplus* | *I. ovatus* | Total (%) |
| Single infections | | | | | | | | | | | |
| *Babesia* sp. Xinjiang | | | 2 (10) | | | | | | | | 2 (0.3) |
| *Babesia major* | 1 (3.4) | | | | | | | | | | 1 (0.2) |
| *Babesia bigemina* | | | | | | | | | 3 (17.9) | | 3 (0.5) |
| *Babesia bovis* | | | | | | | | | 1 (0.6) | | 1 (0.2) |
| *Theileria luwenshuni* | | 5 (4.7) | | | | 2 (2.4) | | | | | 7 (1.1) |
| *Theileria sergenti* | | 1 (0.9) | | | 1 (5.6) | | | | | | 2 (0.3) |
| *Theileria annulata* | 2 (6.9) | 20 (18.7) | 2 (10) | 3 (21.4) | | 1 (1.2) | 2 (14.3) | 56 (33.3) | 32 (19) | | 118 (18.1) |
| Total single infections | | | | | | | | | | | 134 (20.6) |
| Two co-infections | | | | | | | | | | | |
| *B.* sp. XJ + *T. an* | | 1 (0.9) | 1 (5) | | | | | | | | 2 (0.3) |
| *B. bi* + *T. an* | | | | | | | | 1 (0.6) | | | 1 (0.2) |
| *T. lu* + *T. si* | | 18 (16.8) | | | | | | | | | 18 (2.8) |
| *T. an* + *T. se* | | | | | | | | | 1 (0.6) | | 1 (0.2) |
| Three co-infections | | | | | | | | | | | |
| *B.* cf. *motasi* + *T. lu* + *T. si* | | 1 (0.9) | | | | | | | | | 1 (0.1) |
| Total co-infections | | | | | | | | | | | 23 (3.5) |
| Total infections | 3 (10.3) | 46 (43) | 5 (25) | 3 (21.4) | 1 (5.6) | 3 (3.5) | 2 (14.3) | 57 (33.9) | 37 (22) | 0 | 157 (24.1) |

* Abbreviations as in Table I.

Table III. Infection rate of field ticks collected from hosts in different regions of China during March–August 2016.

| Tick genera | No | Positive rate (%) | Tick species | No. | Positive rate (%) |
|---|---|---|---|---|---|
| *Haemaphysalis* | 136 | 49 (36.0) | *H. punctata* | 29 | 3 (10.3) |
| | | | *H. longicornis* | 107 | 46 (43) |
| *Hyalomma* | 52 | 9 (17.3) | *Hy. anatolicum* | 20 | 5 (25.0) |
| | | | *Hy. asiaticum* | 14 | 3 (21.4) |
| | | | *Hy. detritum* | 18 | 1 (5.6) |
| *Dermacentor* | 99 | 5 (5.1) | *D. marginatus* | 85 | 3 (3.5) |
| | | | *D. silvarum* | 14 | 2 (14.3) |
| *Rhipicephalus* | 336 | 94 (27.9) | *R. sanguineus* | 168 | 57 (33.9) |
| | | | *R. (B.) microplus* | 168 | 37 (22.0) |
| *Ixodes* | 29 | 0 (0) | *I. ovatus* | 29 | 0 (0) |
| Total | | | | 652 | 157 (24.1) |

sequences of 18S rRNA gene from 2 *D. marginatus* DNA samples that are infected by *T. luwenshuni* showed 100% similarity with published sequence (GenBank accession number: JF719833). Few samples produced weak positive hybridization signals with the catch-all probe only. One *Hy. detritum* sample was sequenced based on the 18S rRNA gene and sequence blasted with 18S rRNA sequence of *Babesia caballi*.

### Nucleotide accession numbers

All representative sequences obtained in this study have been deposited in GenBank with the following accession numbers: KY425610 (*Theileria* sp. ITS gene), KY464052–KY464056 (*Theileria* sp. COB gene), KY464057 (*Theileria* sp. 18S rRNA gene), KY464058–KY464062 (*Theileria* sp. COB gene), KY464045–KY464046 (*Babesia* sp. 18S rRNA gene), KY464047 (*Babesia* sp. *rap-1b* gene), and KY464048–KY464051 (*Babesia* sp. *rap-1a* gene).

## DISCUSSION

In our study, the prevalence of 12 piroplasms was investigated in a broad geographic range in the field. The findings revealed that *T. annulata* was the most widespread species in the tested ticks. In China, *T. annulata* and *T. sergenti* are the most virulent bovine *Theileria* species and mainly distributed in northern China. At present, *T. annulata* infection was also found from Guangdong province of South China (Liu et al., 2015). In this study, the highest positive rate of *T. annulata* infection was observed in Guangdong, as well as Hunan provinces (south of China). *Theileria annulata* and *T. sergenti* are usually found as co-infections in the field samples (Gubbels et al., 1999). Here, only 1 *R.* (*B.*) *microplus* displayed mixed infections with these 2 species. The prevalence of *T. sergenti* was very low (0.3%), in contrast to a previous study in China (Liu et al., 2015). This variability might have been caused by the distribution of the pathogen in different samples (tick or host) and sampling sites. Ticks of the genera *Hyalomma* could transmit *T. annulata* (Sayin et al., 2003). In our case, except *Hy. anatolicum* and *Hy. asiaticum*, *T. annulata* infection was for the first time detected in *H. longicornis*, *H. punctata*, *R.* (*B.*) *microplus*, *R. sanguineus*, *D. silvarum*, and *D. marginatus* (Table IV). Except for a study in France that reported *T. annulata* infection in *D. marginatus* (Bonnet et al., 2013), no related studies have reported *T. annulata* infection in the other 6 tick species. This can be explained by the fact that most of these ticks parasitized cattle and they probably carried *T. annulata* from an animal infected with a *T. annulata* infection. We found *H. punctata* and *D. silvarum* from sheep; these ticks may have carried *T. annulata* from a previous blood meal or from the sheep they were collected from, as *T. annulata* can naturally infect sheep (Zaeemi et al., 2011). In our study, *T. annulata* infections were found in 3 *Hy. asiaticum* ticks collected from camel, consistent with studies that reported *T. annulata* infection in *Hyalomma* tick parasitizing camels and *T. annulata* being the most abundant piroplasm in camels (El Kammah et al., 2001; Youssef et al., 2015). Our finding further confirms the evidence of low host specificity of *T. annulata*. The *T. sergenti* infection was detected as single infection in vector *H. longicornis*, consistent with the literature (Liu et al., 2010). In addition, *T. sergenti* was also detected for the first time in *Hy. detritum* and *R.* (*B.*) *microplus*. This is not surprising since these ticks were all collected from cattle.

Infection by *T. sinensis* is widespread among cattle and yaks throughout China, and Lintao, Dingxi, and Weiyuan city from Gansu Province were described as endemic regions of *T. sinensis* (Yin et al., 2002; Liu et al., 2010). Here, *T. sinensis* infections were present in Linzhou of Henan province with prevalence (2.9%) comparatively lower than previous reports (Liu et al., 2010). Linzhou is likely a new endemic region for *T. sinensis*. In this study, *T. sinensis* was detected in 19 *H. longicornis* samples collected from sheep. This can be explained if *H. longicornis* could carry *T. sinensis*. Since *H. longicornis* can support diverse pathogenic microorganisms (Chen et al., 2012), this tick species may play an important role as the reservoir for *T. sinensis* in China.

Among ovine *Theileria*, only *T. luwenshuni* (3.9%, 26/652) was detected. A previous study reported a high infection rate of *T. luwenshuni* in ticks by RLB, or in small ruminants via PCR and FRET-qPCR (Niu et al., 2012; Li et al., 2014a; Yang et al., 2014). In our study, *T. luwenshuni* was mostly prevalent in Linzhou, Henan province, corresponding with a previous study, which reported *T. luwenshuni* infecting sheep in the same province (Chen et al., 2014b). We noticed that *T. luwenshuni* was mostly found parasitizing *H. longicornis*. *Theileria luwenshuni* was also detected in *D. marginatus* collected from cattle, but with a low infection rate. A study documented *T. luwenshuni* infection in *Dermacentor niveus* collected from Mongolian gazelle in northern China (Li et

TABLE IV. Ticks tested by RLB and reported vector ticks.

| *Babesia*/*Theileria* | Carrier or infected ticks in this study | Reported vectors of different piroplasm | References |
|---|---|---|---|
| *Babesia* cf. *motasi* | *Haemaphysalis longicornis* | *Haemaphysalis qinghaiensis* (Lintan), *H. longicornis* (Lintan, Ningxian), *H. punctata* (*Babesia motasi* Europe isolates) | Guan et al. (2010) |
| *Babesia* sp. Xinjiang | *Hyalomma anatolicum*, *H. longicornis*, | *Hy. anatolicum* | Guan et al. (2009) |
| *Babesia major* | *Haemaphysalis punctata* | *H. longicornis*, *H. punctata* | Yin et al. (1996) |
| *Babesia bigemina* | *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus sanguineus* | *R.* (*B.*) *microplus*, *Rhipicephalus* (*Boophilus*) *decoloratus*, *Rhipicephalus evertsi evertsi*, *Rhipicephalus bursa*, *Rhipicephalus annulatus*, *Rhipicephalus geigyi* | Tavassoli et al. (2013); Ravindran et al. (2006) |
| *Babesia bovis* | *R.* (*B.*) *microplus* | *R.* (*B.*) *microplus*, *R.* (*B.*) *decoloratus*, *R. evertsi evertsi, R. bursa*, *R. annulatus*, *R. geigyi* | Tavassoli et al. (2013); Ravindran et al. (2006) |
| *Theileria luwenshuni* | *H. longicornis*, *Dermacentor marginatus* | *H. qinghaiensis*, *H. longicornis* | Li et al. (2009) |
| *Theileria annulata* | *Hy. anatolicum*, *Hyalomma asiaticum*, *H. longicornis*, *H. punctata*, *D. marginatus*, *Dermacentor silvarum*, *R. sanguineus*, *R.* (*B.*) *microplus* | *Hy. anatolicum*, *Hy. asiaticum*, *Hy. detritum*, *Hy. excavatum* | Sayin et al. (2003) |
| *Theileria sergenti* | *H. longicornis*, *Hyalomma detritum*, *R.* (*B.*) *microplus* | *H. longicornis* | Liu et al. (2010) |
| *Theileria sinensis* | *H. longicornis* | *H. qinghaiensis* | Bai et al. (2002b) |

al., 2014b), but no finding concerning *T. luwenshuni* infecting *D. marginatus*. Ticks were collected from animals, and thus piroplasms infection could either come from previous meals or from the animal they were feeding on when collected. Thus, the potential transmitted role of this tick for *T. luwenshuni* has to be further studied, and blood from these animals should be tested for piroplasm infection.

The prevalence of *B. bigemina* and *B. bovis* reported was 0.6% and 0.2%, respectively, but high prevalence of *B. bigemina* and *B. bovis* in cattle was recently reported using PCR (Liu et al., 2014; Niu et al., 2015). Several studies showed that *B. bigemina* and *B. bovis* could be transmitted by the genus *R.* (*B.*) *microplus*, *Rhipicephalus* (*Boophilus*) *decoloratus*, *Rhipicephalus evertsi evertsi, R. annulatus*, *R. geigyi,* and *Rhipicephalus bursa* (Ravindran et al., 2006; Tavassoli et al., 2013). Our study showed that 3 and 1 *R.* (*B.*) *microplus* were positive in terms of *B. bigemina* and *B. bovis* infections, respectively (Table IV). An earlier study in China reported a higher prevalence of *B. bigemina* and *B. bovis* (7.3% and 5.8%) by multiplex PCR assay, and 2.3% and 1.5% by light microscopy, respectively (Liu et al., 2014). Moreover, 1 *R. sanguineus* displayed a co-infection of *T. annulata* and *B. bigemina*, which is a new finding and suggests that the role of *R. sanguineus* in *B. bigemina* transmission needs to be experimentally validated. In the past, *B. major* was isolated by infesting calves with adult *H. punctata* collected from pasture in Xinjiang Uygur Autonomous Region of China (Liu et al., 2014). In the present study, *B. major* with low prevalence (0.1%) was detected as a single infection in 1 *H. punctata*, which is also collected from Xinjiang. Although this *H. punctata* tick was removed from sheep, *B. major* was documented to be a bovine *Babesia* species (Yin et al., 1996).

The prevalence of the *Babesia* cf. *motasi* in our study was low (0.4%), consistent with previous data that detected *Babesia* cf. *motasi* in field blood samples by RLB assay (Niu et al., 2009). In the present study, only 1 *H. longicornis* collected from sheep was infected by *Babesia* cf. *motasi* (in a co-infection with *T. luwenshuni* and *T. sinensis*). It is possible that most of the *H. longicornis* tested in this study carried an undetectable amount of *Babesia* cf. *motasi*. *Babesia* sp. Xinjiang was originally isolated from a splenectomized sheep infested with *R. sanguineus* and *Hy. anatolicum*, but only *Hy. anatolicum* was experimentally shown to be a vector; *R. sanguineus* was incapable of serving as a vector for this parasite (Guan et al., 2009). In addition, a *Babesia* sp. Xinjiang infection was first detected in *H. longicornis*, removed from cattle. This tick might carry but not transmit *Babesia* sp. Xinjiang.

Additionally, 1 *Hy. detritum* DNA extract was found with *B. caballi*–like infection, for which species-specific probes were not included when developing the RLB method. This result reflects a great advantage of RLB. Moreover, *Hy. detritum* has never been reported to transmit *B. caballi*, especially when collected from cattle. This tick could have been collected in an area where cattle and horses shared the same habitat.

The present study shows that the prevalence of piroplasm species was in general lower than previously reported. Veterinary practitioners and stakeholders should be aware of the existence and prevalence of *T. annulata* in China, given the potential threat this parasite represents to the Chinese livestock industry. Many tick species examined during our investigation were associated with a broader pathogen range than previously reported, and the potential risk of these ticks to transmit those piroplasm species could exist. However, their vector competence needs to be validated by experimental transmission tests using tick and host blood sampled across the country.

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