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ORIGINAL PAPER

Identification of piroplasm infection in questing ticks by RLB: a broad range extension of tick-borne piroplasm in China?

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Abstract Sensitive and specific diagnostic method for rapid and simultaneous detection and discrimination of the different species is needed for an effective control of piroplasmosis. Here, a reverse line blot (RLB) assay was developed for piroplasm detection. A general pair of primer based on 18S ribosomal RNA (rRNA) gene was used to amplify V4 region of 18S rRNA gene. General and specific probes for 13 piroplasm species were cited from previous publications or designed according to the alignment of 18S rRNA gene sequences. For sensitivity test of RLB assay, serially diluted plasmids of the different species were used to access the sensitivity of the RLB. Four hundred and fifty tick samples collected from grass from different provinces of China were then detected. The result indicated that the RLB assay is highly specific and sensitive, detecting up to 10^2 copies/μl of recombinant plasmid DNA. Multiple piroplasms were detected as single or mixed infection from tick species. Eight piroplasm species, most of which were *Theileria annulata* (33/450, 7.3 %) or *Babesia* sp. Xinjiang (30/450, 6.7 %), were found to infect with 89 tick samples in four tick species; no infections with *Babesia major*, *Babesia ovata*, *Babesia bigemina*, *Theileria sergenti*, or *Theileria equi* were detected. The piroplasms species-specific RLB assay may have potential clinical application in the simultaneous detection and differentiation of *Babesia* and *Theileria* species.

Keywords Piroplasm · Reverse line blot · Detection · Differentiation

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Introduction

Piroplasms are naturally tick-transmitted protozoan parasites of the genera *Babesia* and *Theileria* and are pathogenic to cattle, yak, sheep/goat, horses, pigs, dogs, and cats and even human. During infection, the vertebrate hosts suffer high fever, anemia, and hemoglobinuria and even death (Li et al. 2014). Piroplasms are responsible for vast economic losses: around 250 million domestic cattle in the world are threatened by tick-borne disease (TBD) (Slodki et al. 2011); the total annual loss of small ruminants in China is estimated to be around 70 million USD (Ahmed et al. 2002).

Ixodid ticks can transmit a variety of pathogens (bacteria, virus, and protozoa) of veterinary and economic importance. The geographic distribution of piroplasms is influenced by their tick vectors. A recent study reported that ticks and TBD are a growing threat worldwide due to expanding tick populations, climate changes, and increased contacts between human, animals, and ticks (de La Fuente and Estrada-Peña 2012). However, little is known about the presence of these pathogens in questing ticks in China. Moreover, several piroplasm species with known tick vectors display a broader host range than formerly thought (Aktas et al. 2015), which could imply that additional unknown vectors might be involved in their transmission. Monitoring the presence of piroplasms in unfed ticks collected from the vegetation is important to understand the roles of the different tick species as reservoir for the infection and assess the risk of piroplasm transmission in order to better control the disease.

At present, four species of ovine *Babesia* (*Babesia motasi*, *Babesia* sp. Xinjiang, *Babesia ovis*, and *Babesia crassa*) have been identified. *B. motasi* phylogenetic group forms two sister clades consisting European isolates and the *B. motasi*-like clade identified in the Chinese isolates (*B. motasi*-like Lintan, Ningxian, Hebei, Tianzhu, and Liaoning) (Liu et al. 2007; Guan et al. 2010). *Haemaphysalis punctata* transmits the *B. motasi* European isolates, while *Haemaphysalis qinghaiensis* and *Haemaphysalis longicornis* were recently described as the vectors of *B. motasi*-like Lintan isolate (Guan et al. 2010), and *H. longicornis* is the vector of Ningxian isolate (Bai et al. 2002). The vectors of other *B. motasi*-like Chinese isolates remain unknown. It was recently reported that *Hyalomma anatolicum anatolicum* transmits *Babesia* sp. Xinjiang in China (Guan et al. 2009). The known vectors of *B. ovis* are *Rhipicephalus bursa*, *Rhipicephalus turanicus*, and *Rhipicephalus sanguineus* (Uilenberg 2006). Seven species of bovine *Babesia* (*Babesia bovis*, *Babesia bigemina*, *Babesia major*, *Babesia divergens*, *Babesia ovata*, *Babesia orientalis*, and *Babesia* U sp. Kashi) have been reported to threat cattle, buffalo, and/or yak (Qin et al. 2015; Luo et al. 2005a). *B. bovis* and *B. bigemina* are the major causative agents of bovine babesiosis that are transmitted by *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus annulatus*, and *Rhipicephalus geigyi* and therefore often result in co-infection (Liu et al. 2014). *B. major* and *B. ovata* have low virulence and are transmitted by *H. longicornis*, *H. punctata*, while *B. orientalis* is highly pathogenic to only buffalo and can be transmitted by *Rhipicephalus* (He et al. 2012). Beside, *Babesia* U sp. Kashi is transmitted by *Hy. a. anatolicum*, which has been reported only in China (Luo et al. 2005b). *B. divergens* is transmitted by *Ixodes ricinus* and has been detected in anemic patients but not in cattle (Moreau et al. 2015).

Six *Theileria* species including *Theileria luwenshuni*, *Theileria uilenbergi*, *Theileria lestoquardi*, *Theileria ovis*, *Theileria recondita*, and *Theileria separata* are known to cause ovine theileriosis in sheep and goats in tropical and subtropical regions (Yin et al. 2007). Only *T. uilenbergi* and *T. luwenshuni* are highly pathogenic and are transmitted by *H. qinghaiensis* and *H. longicornis* in China (Yin et al. 2007; Li et al. 2009). In contrast, *T. ovis* and *T. separata* are non-pathogenic or mildly pathogenic to small ruminants and *Hy. a. anatolicum* could play an important role in *T. ovis* transmission (Yin et al. 2007; Li et al. 2010). Four bovine *Theileria* species (*Theileria annulata*, *Theileria mutans*, *Theileria sergenti*, and *Theileria sinensis*) have been reported; *T. annulata* caused tropical or Mediterranean theileriosis and transmitted by *Hy. a. anatolicum*, *Hyalomma asiaticum*, *Hyalomma detritum*, *Hyalomma excavatum*, *Hyalomma dromedarii*, and *Hyalomma marginatum* (Sayin et al. 2003). *T. sergenti* is the most prevalent benign *Theileria* and is transmitted by *H. longicornis*, while *T. sinensis* infects cattle and yaks and is transmitted by *H. qinghaiensis* (Liu et al. 2010).

Equine piroplasmosis is caused by either *Theileria equi* or *Babesia caballi*. Thirty-three ixodid species belong to the genera *Amblyomma*, *Ixodes*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus* that might implicate as competent vectors for *B. caballi*, *T. equi*, or both (Scoles and Ueti 2015; Hawkins et al. 2015; Rothschild 2013; Wise et al. 2013).

In the field, the risk of co-infection between *Babesia* and *Theileria* species is very high and the species are morphologically indistinguishable. There are several molecular detected methods including conventional PCR, nested PCR, multiplex PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP) which allow improvement in sensitivity, quantification, and speed of detection of the presence of piroplasm in hosts or ticks (Pienaar et al. 2011; Odongo et al. 2010; Liu et al. 2013, 2015; Ros-García et al. 2012), while methods such as reverse line blot (RLB), bead arrays, pan-FRET assays, and high-resolution melt analysis allow detection of multiple species or genotypes at the same time (Niu et al. 2012; Ros-García et al. 2013; Yang et al. 2014; Salim et al. 2013). Moreover, the RLB method is very convenient and useful for diagnosis, detection, quarantine, and epidemiological survey. More often, the marker gene for this application is the well-characterized 18S ribosomal RNA (rRNA) gene (Liu et al. 2013; Niu et al. 2009). The advantage of RLB is it uses

multiple highly specific probes to enable the simultaneous detection and direct identification of several piroplasms in hosts or ticks, either in single or mixed infection (Iqbal et al. 2013; Ros-García et al. 2011). This technique has been used in integrated epidemiological monitoring of tick-borne diseases (Niu et al. 2012). However, RLB was rarely used to detect piroplasm in unfed ticks in China.

In this study, we described the development of the RLB assay for the simultaneous detection and identification of piroplasms from questing ticks collected from vegetation of several provinces of China. The different tick-parasite associations and comparison with previous reports were discussed.

Materials and methods

Parasites

B. motasi-like, *Babesia* sp. Xinjiang *T. luwenshuni*, *T. uilenbergi*, *T. ovis*, *B. bigemina*, *B. bovis*, *B. major*, *B. ovata*, *T. annulata* China, *T. sergenti*, *T. sinensis*, and *T. equi* were cryopreserved in liquid nitrogen at the Vector and Vector-borne disease (VVBD) laboratory of Lanzhou Veterinary Research Institute (LVRI), CAAS Lanzhou, China. The parasites were isolated by inoculating infected blood cryopreserved in liquid nitrogen into hemoprotozoa-free splenectomized animal. When parasitemia was greater than 5 %, infected venous blood was collected into heparinized tubes, genomic DNA were extracted and then used as a positive control to develop the RLB assay. Genomic DNA from two *T. annulata* isolates (India and Turkey) were kindly provided by Professor Jabbar S. Ahmed (Forschungszentrum Borstel, Borstel, Germany). Two bacteria (*Anaplasma marginale* and *Borrelia burgdorferi* sensu stricto) were used as a negative control.

Collection and identification of tick samples

A total of 450 unfed ticks, *H. qinghaiensis* ($n=242$), *H. longicornis* ($n=57$), *H. punctata* ($n=6$), *R. sanguineus* ($n=19$), *Ixodes persulcatus* ($n=42$), and *Dermacentor silvarum* ($n=84$), were randomly collected from vegetation in five provinces of China, including Xinjiang Uygur Autonomous Region, Gansu, Heilongjiang, Henan, and Jilin provinces. The ticks were identified according to standard taxonomic keys (Teng and Jiang 1991). Each tick was placed in separate 1.5-ml Eppendorf tubes and washed in 70 % ethanol, rinsed in sterile phosphate-buffered saline, and dried. The ticks were then powdered with a sterile pestle after freezing in liquid nitrogen. Genomic DNA was extracted from the tick samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions and stored at −20 °C until use.

Primer and probe design

A pair of previously described primers based on the 18S rRNA gene conserved region was used for RLB (Gubbels et al. 1999). General (catch-all) and species-specific RLB oligonucleotide probes for each *Babesia* and *Theileria* species were deduced from the V4 region of the 18S rRNA gene sequences with a range of 2.5–800 μM and containing *N*-(trifluoroacetamidohexyl-cyanoethyl, *N*, *N*-diisopropyl phosphoramidite [TFA])-C6 amino linker. The primers and probes were synthetized by Sangon Biotech Company, China (Table 1).

Table 1 Sequence and concentration of oligonucleotide probes

Oligonucleotides	Sequences 5′–3′	Optimal concentrations (μM)	References
Catch-all	(NH2)-CTGTCAGAGGTGAAATTCT	2.5	(Niu et al. 2009)
B. motasi-like HB/NX	(NH2)-TTCAGCCCTTCGGCTTGCG	600	This study
B. motasi-like LT/TZ	(NH2)-GAGCTCGCGTGTGCGCCT	800	This study
Babesia sp. Xinjiang	(NH2)-CGGGTTTCGTCTACTTCGC	800	(Niu et al. 2009)
B. major	(NH2)-CGTTGCGCCGCGCGACTTTGG	600	This study
B. ovata	(NH2)-GCCCTTCGGCTTTTCCCTA	400	This study
B. bigemina	(NH2)-CGTTTTTTCCCTCTTTTCGG	200	This study
B. bovis	(NH2)-CAGGTTTCGCCTGTATAATTGAG	10	(Gubbels et al. 1999)
T. uilenbergi	(NH2)-TGCATTTTCCGAGTGTTACT	200	(Schnittger et al. 2004)
T. luwenshuni	(NH2)-ATCTTCTTTTTGATGAGTTG	200	(Niu et al. 2009)
T. ovis	(NH2)-TGCCTTTGCTCCTTTACGAG	50	This study
T. annulata	(NH2)-TGCTTGTGTCCCTCTGGGGTCTG	200	This study
T. sergenti	(NH2)-CATTTCTCTTTCTGAGTTTGT	600	This study
T. sinensis	(NH2)-TCGCATCTCTTGCTGAGTG	600	(Niu et al. 2012)
T. equi	(NH2)-CGTGGTTCTTCGCTATGTCGAG	200	This study

PCR amplification, cloning, and sequencing

PCR was performed to amplify the V4 region of the 18S rRNA gene from standard positive samples and all collected tick samples. The PCR reaction consisted of 2 μl genomic DNA, 25 μM of each primer (RLB-F and RLB-R), 5 μl 10 × reaction buffer, 2.5 μM of each dNTP, and 5 U/μl Taq polymerase in a total volume of 50 μl. The amplification conditions were as follows: an initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 45 s. Final extension was at 72 °C for 10 min. The amplified products were analyzed on 1 % agarose gels with ethidium bromide staining. An expected length of about 500 bp was obtained and ligated into the pGEM-T Easy Vector and cloning. Positive colonies were selected after screening by colony PCR 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. These plasmids with the inserts were used as positive controls to assess the specificity and sensitivity of RLB.

RLB hybridization

The RLB protocol was performed as described previously (Gubbels et al. 1999). Briefly, a Biodyne C blotting membrane (BNBCH5R, Pall BioSupport) was activated at room temperature by incubating in 16 % EDAC (E7750, Sigma) for 10 min, then washed in distilled water, and placed in a MN45 miniblotter (FZB, Germany). Specific oligonucleotide probes were diluted in 10–1000 μM/150 μl in 500 mM $NaHCO_3$ (pH 8.4), added to the miniblotter slots, and incubated for 2 min. Then, the membrane was incubated in 100 mM NaOH for 10 min and rinsed with demineralized water at 60 °C for 5 min in 2 × SSPE/0.1 % SDS. The membrane was then placed perpendicular to the probe orientation in the miniblotter. Twenty microliters of each PCR product was diluted in 2 × SSPE/0.1 % SDS to a final volume of 150 μl, heated to 99 °C for 10 min, and then cooled immediately on ice. The denatured PCR products were then added to the slots in the miniblotter and incubated for 60 min at 60 °C, and the membrane was washed twice at 60 °C for 10 min in 2 × SSPE/0.5 % SDS. Additionally, the membrane was treated at 42 °C for 60 min with peroxidase-labeled streptavidin diluted 1:4000 in 2 × SSPE/0.5 % SDS and washed twice at 42 °C for 10 min in 2 × SSPE/0.5 % SDS and twice at room temperature for 5 min in 2 × SSPE. Finally, chemiluminescence detection was performed according to standard procedures (Amersham).

Specificity and sensitivity of RLB

Specific probes of 13 piroplasms were designed on the basis of the hypervariable V4 region in the 18S rRNA gene. The V4 region of piroplasm species, two bacteria (*A. marginale* and *B. burgdorferi* sensu stricto), was amplified using a pair of universal primers, and water was used as the blank control.

To assess RLB sensitivity, the plasmid length was first calculated by adding the vector length (pGEM-T Easy—3018 bp) and the length of the partial 18S rRNA gene sequence from each pathogen. The plasmid concentrations were measured by spectrophotometry. Plasmid copy number was then calculated using the following formula:

$$\text{Copies}/\mu\text{l} = \frac{\text{Plasmid concentration (ng / μl)} \times 10^{-9} \times 6.02 \times 10^{23}}{\text{Plasmid length} \times 660}$$

Based on the copy number of each parasite species analyzed, plasmids with the inserts were serially diluted from an initial copy number of 10^{10} to 10^{0} copies/μl and then used as template for the RLB sensitivity analysis.

Confirmation and sequence analysis of positive samples

Based on the target genes of 18S rRNA (to detect all piroplasm species), as well as *rap-1a* or *rap-1b* gene (for *Babesia* sp. Xinjiang or *B. motasi*-like, respectively), COB gene (for *T. annulata*), or ITS genes (for *T. sinensis*), the PCR was performed to amplify positive samples, which were randomly screened and used to further confirm RLB-tested result. The positive PCR samples were cloned into pGEM-T easy vector and sequenced (GENEWIZ Biotech Company, Suzhou, China). The obtained sequences were subjected to blast search on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTn algorithm. Multiple sequence alignment was performed using ClustalW 2.0.12.

Statistical analysis

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

Results

Specificity of RLB

All probes from 13 piroplasms bound only to their respective target sequence, resulting in the recognition of individual isolate, species, or group. The nucleotide probes did not show any cross-reaction with *A. marginale* and *B. burgdorferi* sensu stricto DNA or water used as a blank control. The catch-all probe specifically detected any *Theileria* and *Babesia* species present. Each *Theileria* or *Babesia* was identified by two oligonucleotide probes: the catch-all probe and species-specific probes for either *Theileria* or *Babesia* species (Fig. 1).

Sensitivity of RLB

The RLB assay is capable of detecting about 10^2 copies (*B. major*), 10^3 copies (*Babesia* sp. Xinjiang, *T. luwenshuni* and *T. sergenti*), 10^4 copies (*B. ovata* and *T. uilenbergi*), 10^5 copies (*B. motasi*-like Hebei/Ningxian, *B. bigemina*, *B. bovis*, *T. ovis*, *T. sinensis*, *T. annulata*, and *T. equi*), and 10^6 copies (*B. motasi*-like Lintan/Tianzhu) (Fig. 2).

Detection of parasite DNA in field tick samples

In summary, 450 tick samples from eight regions of five provinces of China were detected for piroplasm infection by using RLB. The detail of the results was summarized in Tables 2 and 3. In general, four of six tick species were infected with eight piroplasm species with single or mix infections. *H. qinghaiensis* is the most prevalent to infect with two *Babesia* and four *Theileria* species with single or co-infection way, and *D. silvarum* less, with two *Babesia* and two *Theileria* species infection, mainly infected with *T. annulata*. Among these positive tick samples, 76 single infected ticks, most of which were infected by *T. annulata* (33/450, 7.3 %) or *Babesia* sp. Xinjiang (30/450, 6.7 %), were from Gansu and Jilin provinces. No infections with *B. major*, *B. ovata*, *B. bigemina*, *T. sergenti*, or *T. equi* were detected. Of all these positive samples, 13 tick samples involving two tick

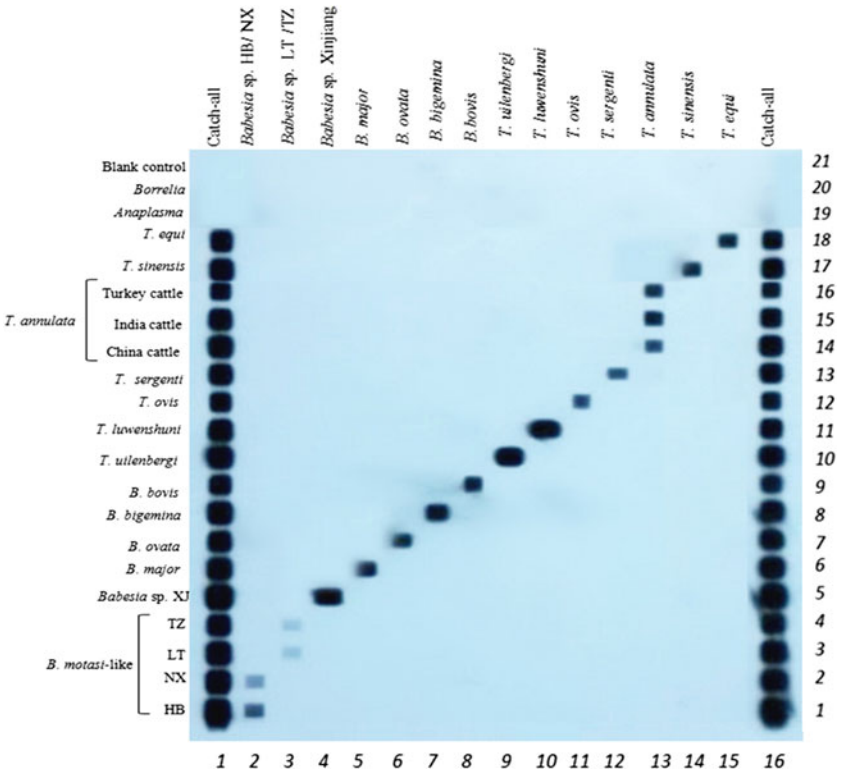

Fig. 1 RLB assay for specificity test of 13 piroplasms. Oligonucleotides probes are applied in *vertical lanes* and PCR products are applied in *horizontal rows*. Lanes *1* and *16* indicate catch-all, *2* and *3* indicate *B. motasi*-like (Hebei/Ningxian and Lintan/Tianzhu groups, respectively), *4* indicates *Babesia* sp. Xinjiang, *5* indicates *B. major*, *6* indicates *B. ovata*, *7* indicates *B. bigemina*, *8* indicates *B. bovis*, *9* indicates *T. uilenbergi*, *10* indicates *T. luwenshuni*, *11* indicates *T. ovis*, *12* indicates *T. sergenti*, *13* indicates *T. annulata*, *14* indicates *T. sinensis*, and *15* indicates *T. equi*. *Rows*: PCR product hybridization with probe. *1* to *4* indicate *B. motasi*-like (Hebei, Ningxiang, Lintan, and Tianzhu strains respectively); *5* indicates *Babesia* sp. Xinjiang; *6* indicates *B. major*; *7* indicates *B. ovata*; *8* indicates *B. bigemina*; *9* indicates *B. bovis*; *10* indicates *T. uilenbergi*; *11* indicates *T. luwenshuni*; *12* indicates *T. ovis*; *13* indicates *T. sergenti*, *14* to *16* indicate *T. annulata* isolates from Chinese cattle (*row 14*), Indian cattle (*row 15*), and Turkish cattle (*row 16*); *17* indicates *T. sinensis*; *18* indicates *T. equi*; *19* indicates *A. marginale* DNA; *20* indicates *B. burgdorferi* sensu stricto (B31) DNA; and *21* indicates blank control

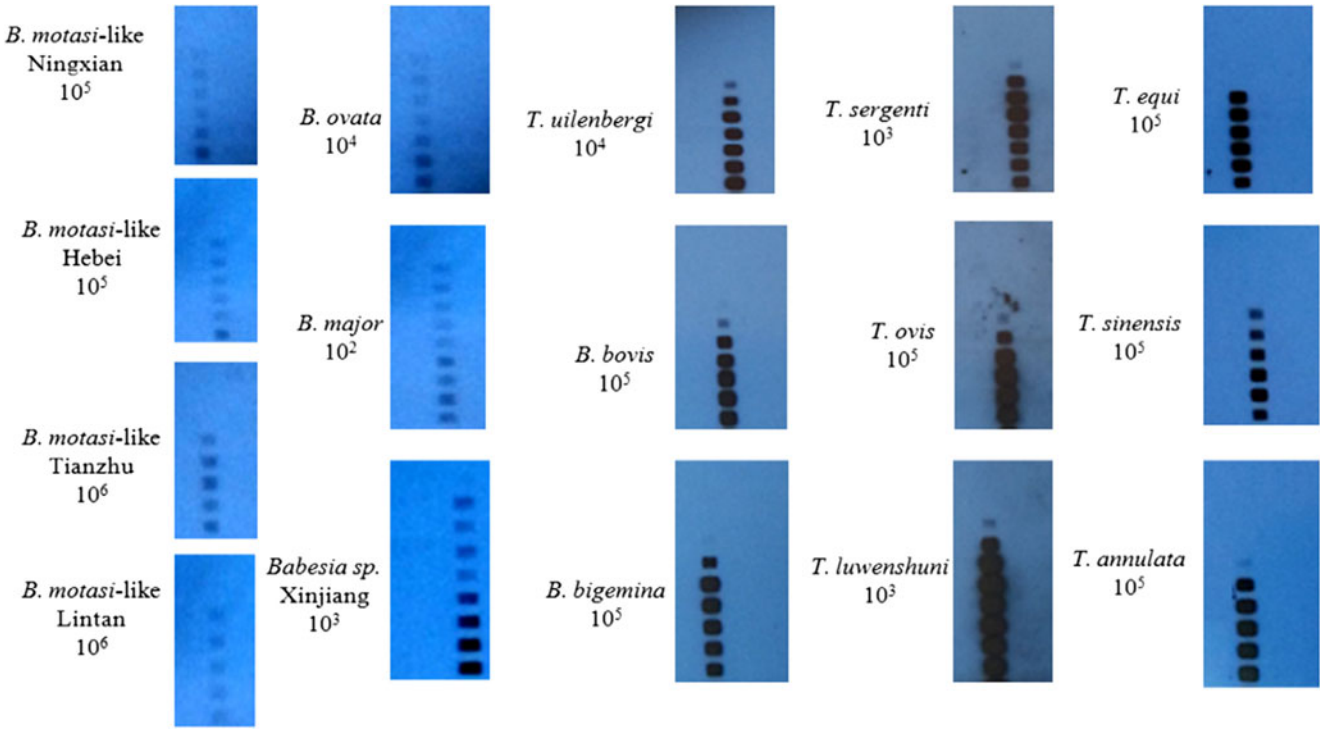

Fig. 2 RLB assay for sensitivity test for each of the 13 piroplasm species tested. Oligonucleotides probes are applied in *vertical lanes*, and diluted plasmids are applied in *horizontal rows*. The assay detected copy number of about 10^2 (*B. major*), 10^3 (*Babesia* sp. Xinjiang, *T. luwenshuni*, and *T. sergenti*), 10^4 (*B. ovata* and *T. uilenbergi*), 10^5 (*B. motasi*-like Hebei/Ningxian, *B. bigemina*, *B. bovis*, *T. ovis*, *T. equi*, *T. sinensis*, and *T. annulata*), and 10^6 copies (*B. motasi*-like Lintan/Tianzhu)

species (*H. qinghaiensis* and *D. silvarum*) were presented mix infection with two or three different piroplasm species.

Sequencing analysis

The PCR products from selected field-positive samples (7 *D. silvarum* samples, 30 *H. qinghaiensis* samples, 1 *R. sanguineus* sample, and 1 *H. longicornis* sample) were sequenced and compared with the published sequences. One *D. silvarum* DNA was amplified and sequenced based on *rap-1b* gene to verify the presence of *B. motasi*-like; the sequence presented 99 % similar to the *rap-1b* gene of *Babesia* sp. Hebei from China (GenBank accession number: KJ205336). Nineteen *H. qinghaiensis* and one *D. silvarum* DNA were sequenced

Table 2 Piroplasm distribution in questing ticks collected from the vegetation in different regions of China

Province	County	Tick species	No.	Infection of piroplasm											
				B. m-like	*B.* sp. XJ	*B. bo*	*B. ma*	*B. ov*	*B. bi*	*T. ui*	*T. lu*	*T. o*	*T. an*	*T. si*	*T. se*	*T. eq*
Xinjiang	Zhaosu	*H. punctata*	6													
	Shaya	*R. sanguineus*	19									1 (5.3)				
Gansu	Lintan	*H. qinghaiensis*	242		32 (13.2)	1 (0.4)				13 (5.4)	7 (2.9)		25 (10.3)	1 (0.4)		
Heilongjiang	Luobei	*I. persulcatus*	22													
	Tonghe	*I. persulcatus*	15													
	Mudanjiang	*I. persulcatus*	5													
Henan	Linzhou	*H. longicornis*	57							1 (1.8)	1 (1.8)					
Jilin	Jiaohe	*D. silvarum*	84	3 (3.6)	1 (1.2)						2 (2.4)		15 (17.9)			
Total (%)			450	3 (0.7)	33 (7.3)	1 (0.2)				14 (3.1)	10 (2.2)	1 (0.2)	40 (8.9)	1 (0.2)		

H. punctata = *Haemaphysalis punctata*, *R. sanguineus* = *Rhipicephalus sanguineus*, *H. qinghaiensis* = *Haemaphysalis qinghaiensis*, *I. persulcatus* = *Ixodes persulcatus*, *H. longicornis* = *Haemaphysalis longicornis*, *D. silvarum* = *Dermacentor silvarum*, *B. m*-like = *Babesia motasi*-like, *B.* sp. XJ = *Babesia* sp. Xinjiang, *B. ma* = *B. major*, *B. ov* = *B. ovata*, *B. bi* = *B. bigemina*, *B. bo* = *B. bovis*, *T. ui* = *T. uilenbergi*, *T. lu* = *T. luwenshuni*, *T. o* = *T. ovis*, *T. an* = *T. annulata*, *T. se* = *T. sergenti*, *T. si* = *T. sinensis*, *T. eq* = *T. equi*

Table 3 *Theileria* and *Babesia* infection in ticks collected from grass

Piroplasm infection	Infected ticks						
	H. qin	*H. lon*	*H. pun*	*D. sil*	*R. sang*	*I. per*	Total (%)
Single infections							
B. m-like				1 (1.2)			1 (0.2)
B. sp XJ	30 (12.4)						30 (6.7)
B. bo	1 (0.4)						1 (0.2)
T. ui	6 (2.5)	1 (1.8)					7 (1.6)
T. lu	2 (0.8)	1 (1.8)					3 (0.7)
T. o					1 (5.3)		1 (0.2)
T. an	23 (9.5)			10 (11.9)			33 (7.3)
Total single infections							76 (16.9)
Two co-infections							
B. m-like + *T. an*				2 (2.4)			2 (0.4)
B. sp XJ + *T. an*	1 (0.4)			1 (1.2)			2 (0.4)
B. sp XJ + *T. ui*	1 (0.4)						1 (0.2)
T. ui + *T. lu*	4 (1.7)						4 (0.9)
T. ui + *T. an*	1 (0.4)						1 (0.2)
T. lu + *T. an*				2 (2.4)			2 (0.4)
Three co-infections							
T. ui + *T. lu* + *T. si*	1 (0.4)						1 (0.2)
Total co-infections							13 (2.9)
Total infections	70 (28.9)	2 (3.5)		16 (19)	1 (5.3)		89 (19.8)

H. qin = *Haemaphysalis qinghaiensis*, *H. pun* = *Haemaphysalis punctata*, *H. lon* = *Haemaphysalis longicornis*, *D. sil* = *Dermacentor silvarum*, *R. sang* = *Rhipicephalus sanguineus*, *I. per* = *Ixodes persulcatus*, *B. m*-like = *Babesia motasi*-like, *B.* sp. XJ = *Babesia* sp. Xinjiang, *B. bo* = *Babesia bovis*, *T. ui* = *Theileria uilenbergi*, *T. lu* = *Theileria luwenshuni*, *T. o* = *Theileria ovis*, *T. an* = *Theileria annulata*, *T. si* = *Theileria sinensis*

based on the *rap-1a* gene of *Babesia* sp. Xinjiang, and the derived sequences showed 99 % identity with *Babesia* sp. Xinjiang *rap-1aβ2* gene (GenBank accession number: KF811199). The 18S rRNA gene sequence from *H. qinghaiensis* infected by *B. bovis* was also clarified. Five *D. silvarum* and eight *H. qinghaiensis* infected by *T. annulata* were sequenced based on COB genes and shared 99 to 100 % homology with the *T. annulata* COB gene (GenBank accession number: KP731977 and KF732030). For *T. sinensis* infecting *H. qinghaiensis*, one sample based on ITS gene was sequenced and shared 99 % similarity with *T. sinensis* ITS gene from China (GenBank accession number: EF547931). Similarly, *R. sanguineus* infected by *T. ovis* as well as *H. qinghaiensis* and *H. longicornis* infected by *T. uilenbergi* or by *T. luwenshuni* were sequenced and their 18S rRNA gene sequences corresponded with this gene sequence on GenBank (GenBank accession number: FJ603460, JF719835, and JX469524, respectively).

Discussion

Diagnostic of piroplasms is considered with regard to sensitivity and specificity of the diagnostic method assays and their use in epidemiology. Molecular tests allow direct confirmation of the presence of parasite genomic material and are specific and highly sensitive for parasite detection. Specifically, PCR-based molecular diagnostic tool, RLB technique has been established for the detection and identification of different *Babesia* or *Theileria* species or genotypes at the same time. Recently, PCR-RLB was used for the rapid and simultaneous detection of co-infecting *Babesia* and *Theileria* species in animals or ticks (Iqbal et al. 2013; García-Sanmartín et al. 2008; Ros-García et al. 2011; Niu et al. 2012). In this study, specificity and sensitivity of RLB method were developed and used for the species-specific detection and differentiation of piroplasms.

After optimizing the reaction system of the RLB assay, no signal was observed with any of the bacteria or water used as a negative or blank control. This indicates that the species-specific probes used in this study were specific for *Babesia* spp. and *Theileria* spp. and that PCR-RLB method can be used to effectively amplify the target DNA and easily discriminate between piroplasm infections, which is in agreement with previous reports of RLB methods (Niu et al. 2012; Iqbal et al. 2013; Aktas et al. 2015).

The detection limit of RLB assays was lowest in 10^2 copies in this study. Several studies investigated the sensitivity of

RLB and demonstrated parasite detection with lower parasitemia than other diagnostic methods, like PCR, nest-PCR, or LAMP (Gubbels et al. 1999; Schnittger et al. 2004; Altay et al. 2008; Niu et al. 2009, 2012; Notomi et al. 2000). Our results confirmed that RLB assay is sensitive and could be used to detect piroplasm species in very low parasitemia.

The overall infection rates of *B. motasi*-like, *Babesia* sp. Xinjiang, *B. bovis*, *T. uilenbergi*, *T. luwenshuni*, *T. annulata*, *T. ovis*, and *T. sinensis* were 0.7 % (95 % CI = 0–1.13), 7.3 % (95 % CI = 0–12.13), 0.2 % (95 % CI = 0–0.38), 3.1 % (95 % CI = 0–5), 2.2 % (95 % CI = 0.13–3), 0.2 % (95 % CI = 0–0.38), 8.9 % (95 % CI = 0–11.88), and 0.2 % (95 % CI = 0–0.38), respectively, in tick samples using the RLB assay (Table 2), 19.8 % (89/450) tick samples were positive for piroplasms with *T. annulata* (40/450) and *Babesia* sp. Xinjiang (33/450), the most widespread hemoprotozoan species infecting ticks from Gansu and Jilin provinces. This is in agreement with the study which reported that *T. annulata* was mainly found in the north or northwest of China (Liu et al. 2015). It has been reported that the ticks of the genera *Hyalomma* spp. transmit *T. annulata* and *Hy. a. anatolicum* transmit *Babesia* sp. Xinjiang (Sayin et al. 2003; Guan et al. 2009). In our case, *T. annulata* and *Babesia* sp. Xinjiang were first detected in *H. qinghaiensis* and *D. silvarum* in China. *T. annulata* was previously detected in *Dermacentor marginatus* in France (Bonnet et al. 2013), but no related studies reported either *T. annulata* or *Babesia* sp. Xinjiang infecting the two *H. qinghaiensis* and *D. silvarum* ticks so far. Similar situation was observed with *B. motasi*-like and *T. luwenshuni* infection in *D. silvarum* or *B. bovis* detected in *H. qinghaiensis*. The role of these ticks in *T. annulata*, *Babesia* sp. Xinjiang, *B. motasi*-like, *T. luwenshuni*, and *B. bovis* transmission remains uncertain. It should be noted that *H. qinghaiensis* and *D. silvarum* could play a role as reservoir and risk for these piroplasm transmissions, since the highest positive rates for *T. annulata* and *Babesia* sp. Xinjiang were found in these tick species. Taking into account that *H. qinghaiensis* and *Dermacentor* spp. are three-host ticks, it is likely that these infections were acquired by the ticks during blood meals of previous tick stages, resulting from transstadial or transovarial transmission.

T. sinensis is widespread among field cattle or yaks throughout China, including Lintao, Dingxi, and Weiyuan cities from the Gansu Province, which are endemic to *T. sinensis* (Yin et al. 2002; Liu et al. 2010). Here, one *H. qinghaiensis* infected by *T. sinensis* from Lintan of Gansu Province was found. Lintan is likely to be a new endemic region for *T. sinensis*. Among ovine *Theileria* species, *T. uilenbergi* was frequently found in tested ticks, especially in *H. qinghaiensis* ticks, than *T. luwenshuni*, which is in agreement with previous study that reported more *T. uilenbergi* detected in *H. qinghaiensis* ticks by RLB (Niu et al. 2012). *T. uilenbergi* has been reported in the southeastern parts of China (Zhang et al. 2014). In our study, *T. luwenshuni* was also found in Henan, consistent with previous studies (Chen et al. 2014), and Gansu, infecting *H. longicornis* and *H. qinghaiensis* ticks, respectively. In addition, *T. luwenshuni* was detected in one *D. silvarum* tick from Jilin. *T. luwenshuni* infection in *Dermacentor niveus* from Mongolian gazelle in northern China was reported (Li et al. 2014), but the occurrence of *T. luwenshuni* infection in *D. silvarum* ticks has not been reported yet. The potential role of *T. luwenshuni* transmission by this tick has to be further studied. *T. ovis* is a new isolate of ovine *Theileria* from Xinjiang Uygur Autonomous Region, and *Hy. a. anatolicum* could play an important role in transmission (Li et al. 2010). Interestingly, we found *T. ovis* in one *R. sanguineus* tick collected in Shaya, Xinjiang Uygur Autonomous Region, consistent with studies that reported the presence of *T. ovis* in *R. sanguineus* in Iran and Turkey (Zakkyeh et al. 2012; Aydin et al. 2015). In Turkey, *T. ovis* infection was also noticed in *R. bursa* and *R. turanicus* (Aydin et al. 2015). Taken together, these results suggest that *R. sanguineus* could act as an additional vector for *T. ovis* in China. It was reported that *B. bovis* could be transmitted by several *Rhipicephalus* species (Liu et al. 2014). In the present study, *B. bovis* infection in *H. qinghaiensis* was first reported, which is likely due to the feeding behavior of this tick on hosts; this tick species was likely co-infected during blood meal on different single infected hosts at different life stages. The ability of *H. qinghaiensis* to transmit *B. bovis* depends on host-pathogen compatibility and needs further investigation. *B. motasi*-like was also for the first time detected in *D. silvarum*, either in single or mixed infection. *Dermacentor* spp. lack host specificity and are able to infect sheep, goats, and cattle in the field, which could explain the presence of *B. motasi*-like in this tick species. Further studies on this tick species should be performed to better understand its role in the *B. motasi*-like circulation.

Among the six tick species examined in terms of *Theileria* and *Babesia* species, four were infected involving eight different piroplasm species. Among the infected ticks, the three-host ticks *H. qinghaiensis* and *D. silvarum* carried different piroplasm species that included double or triple infections and combinations of piroplasm species which were either from the same genus or both *Theileria* and *Babesia* (Table 3). Tick life cycles with two and three hosts increase the chance of co-infections. Moreover, the highest infection rate was also observed in *H. qinghaiensis* and *D. silvarum*. Our result indicates that *H. qinghaiensis* and *D. silvarum* play a role in the maintenance of these parasites in China. These two tick species showed a mixed profile of vector-pathogen combinations that were never described before which might explain the increase in infection prevalence of these piroplasms species. As these tick-pathogen associations came from ticks collected from vegetation after moulting, the presence of piroplasm infection in these ticks would suggest that this kind of infection

occurred at an earlier stage, implying that the piroplasm was able to multiply within the tick. All ticks used in this study are of epidemiological importance in China (Chen et al. 2014; Yu et al. 2015). The majority of ticks found in China belong to the genus *Haemaphysalis*, and *H. qinghaiensis* is an endemic species and is distributed in the western plateau while *H. longicornis* is widely distributed in China (19 provinces) and could support diverse pathogenic microorganisms (Yu et al. 2015; Chen et al. 2010, 2012). In the genus *Dermacentor*, *D. silvarum* could carry a large numbers of varying pathogenic microorganisms (Yu et al. 2015). *R. sanguineus* is distributed in 20 provinces in China and is a vector of *Coxiella*, *Rickettsia*, *Ehrlichia*, *Babesia*, and *Hepatozoon* (Chen et al. 2014). Our finding showed that even *Theileria* species could be carried by this tick species.

In conclusion, the RLB assay was able to simultaneously detect low numbers of pathogens in tick and sensitive and specific for the detection and identification of piroplasms, even when two or three species were present as multiple infections in field tick samples. The use of RLB technique guarantees that all new piroplasm species and genotypes can be detected at least at the group level by applying a catch-all probe. Moreover, this method can substantially improve the detection of piroplasm carriers and better protect livestock trade and facilitate preventive control programs. These findings that are of biological significance will be clarified in future experimental transmission studies. Meanwhile, the detection of piroplasm in field ticks provides a new perspective for the circulation of these pathogens in nature.

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