**Occurrence of *Theileria* and *Babesia* species in water buffalo (*Bubalus bubalis*,
Linnaeus, 1758) in the Hubei province, South China**

Lan He^{a, b}, Hui-Hui Feng^{a, b}, Wen-Jie Zhang^{a, b}, Qing-Li Zhang^{a, b}, Rui Fang^{a, b}, Li-Xia Wang^{a, b}, Pan Tu^{a, b}, Yan-Qin Zhou^{b, c}, Jun-Long Zhao^{a, b*}, Marinda C. Oosthuizen^{d*}

^a*State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Hubei Wuhan 430070, China*

^b*College of Veterinary Medicine, Huazhong Agricultural University, Hubei Wuhan 430070, China*

^c*Key Laboratory Preventive Veterinary of Hubei province, Huazhong Agricultural University, Hubei Wuhan 430070, China*

^d*Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag x04, 0110 Onderstepoort, South Africa*

***Corresponding authors.** Marinda C. Oosthuizen (marinda.oosthuizen@up.ac.za), Jun-Long Zhao (zhaojunlong@mail.hzau.edu.cn)

Mailing address: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa. Phone: +27 12 529 8390. Fax: +27 12 529 8312. E-mail: marinda.oosthuizen@up.ac.za

Abstract

The presence and prevalence of tick-borne haemoparasites in water buffalo from the Hubei province, south China was investigated using the reverse line blot (RLB) hybridization assay and phylogenetic analysis of the parasite 18S rRNA gene. *Theileria buffeli* (19.1%) was the most frequently found species in all of the locations, followed by *Babesia orientalis* (8.9%), *B. bovis* (1.0%) and *B. bigemina* (0.7%). Only

12 (3.9%) of the samples had mixed infections. Eleven samples with single infections were selected for further characterization using 18S rRNA gene sequence analysis. Phylogenetic analysis showed that the eight *T. buffeli* 18S rRNA gene sequences obtained grouped into four clusters, of which three grouped with the known *T. buffeli* types B and D. The remaining five grouped separately from the previously describe *T. buffeli* types, constituting new *T. buffeli* types. The two *B. bigemina* 18S rRNA gene sequences obtained grouped closely with *B. bigemina* Kunming; this serves as the first report of *B. bigemina* in the Hubei province. The *B. orientalis* Daye 18S rRNA gene sequence obtained grouped closely with the previously reported *B. orientalis* Wuhan strain and with *Babesia* sp. Kashi1 and Kashi2.

Keywords: *Theileria*, *Babesia*, water buffalo, reverse line blot hybridization assay, phylogenetic tree

1. Introduction

Theileria and *Babesia* species, collectively known as piroplasms, are tick-transmitted apicomplexan parasites that infect wild and domestic animals worldwide (Mehlhorn and Schein, 1984). They are an important constraint to livestock production in developing countries, and are responsible for high morbidity and mortality resulting in decreased production of meat, milk and other livestock by-products (Uilenberg, 2001).

In China, *Theileria annulata*, *T. mutans* and the *T. buffeli*/*T. sergenti*/*T. orientalis* group have historically have been reported as the three most economically important bovine *Theileria* species (Yang et al., 1964), of which *T. annulata* is considered to be the most pathogenic and *T. sergenti* the most prevalent (Liu et al., 2010). More recently *T. sinensis*, a benign *Theileria* species of cattle and yaks, was discovered in the central part of Gansu Province (Bai et al., 2002). *Theileria annulata*, transmitted by ticks of the genus *Hyalomma*, causes Mediterranean or tropical theileriosis in cattle and domestic

water buffalo with a mortality rate of 10-90% (Levine, 1985). The benign *T. buffeli*/*T. sergenti*/*T. orientalis* group has a worldwide distribution. The taxonomy and nomenclature of this group is complex and is usually based on the geographic origin of the parasite (Chae et al., 1998). *Theileria sergenti* causes bovine theileriosis in cattle in Japan and Korea (Hagiwara et al., 2005) and is also known to cause disease, and even death, in water buffalo and cattle in China (Jin et al., 2007). *Theileria buffeli* is a less pathogenic *Theileria* species infecting cattle and water buffalo, is considered to be a common parasite of cattle in many parts of the world (Neitz, 1957). It causes benign bovine theileriosis with a mortality rate of less than 1% (Levine, 1985). In China, *T. mutans* has only been reported in cattle from the GuiZhou province and XinJiang Uygur Autonomous Region (Lv et al., 1995).

Five *Babesia* species, namely *B. bigemina*, *B. bovis*, *B. major*, *B. orientalis* and *B. ovata*, have been identified in bovine in China. *Babesia bovis* and *B. bigemina* are regarded as the major causative agents of bovine babesiosis in China (Yin et al., 1997). They infect both cattle and water buffalo, and are transmitted by the one-host tick *Rhipicephalis microplus*. *Babesia major* and *Babesia ovata* are large *Babesia* species infective to cattle, and are transmitted by *Haemaphysalis punctata* and *Haemaphysalis longiconis*, respectively (Liu et al., 2008). It is difficult to discriminate between *B. major* and *B. ovata*; traditionally the discrimination was based on the tick vectors or morphology and pathogenicity (Bai et al., 1990; Higuchi et al., 1991). *Babesia major* was first reported in 1988 in China in the Henan province and is generally not regarded as such an important pathogen, although it frequently occurs in combination with other tick-borne haemoparasites and exerts a synergistic pathogenicity (Yin et al., 1997). *Babesia ovata* was not identified in China until 1990 (Bai et al., 1990). It causes anemic diseases among grazing cattle, and is regarded to be of relatively low virulence (Tsuji et al., 1999). *Babesia orientalis* causes water buffalo babesiosis, one of the most important diseases of buffalo in central and south China (Liu et al., 2005). It has recently been shown to have spread to the north of the Yangtse River, posing a serious threat to the water buffalo industry (He et al., 2009).

In this study, a survey was conducted in the Hubei province, south China to determine the occurrence of *Theileria* and *Babesia* species in water buffalo (*Bubalus bubalis*, Linnaeus, 1758) using the reverse line blot (RLB) hybridization assay. The objective was to investigate the presence and prevalence of haemoprotozoan parasites in field water buffalo. The full-length parasite 18S rRNA gene of selected samples were cloned, sequenced and subjected to phylogenetic analysis.

2. Materials and methods

2.1 Samples collection and DNA extraction

A total of 304 EDTA blood samples were collected from water buffalo (*Bubalus bubalis*, Linnaeus, 1758) from nine geographic locations in the Hubei province, south China. The genomic DNA was extracted from 200 µl of blood using the QIAamp blood and tissue extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was eluted in 100 µl elution buffer and stored at -20°C until further analysis. The standard positive control DNA of *B. orientalis*, *B. bigemina*, *B. bovis* and *T. buffeli* were preserved in our lab.

2.2 PCR amplification and reverse line blot hybridization assay

The V4 hypervariable region of the parasite 18S rRNA gene was amplified using the *Theileria* and *Babesia* genus-specific primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTGACA GT-3') (Gubbels et al., 1999). A touch-down PCR was followed and the PCR products were analyzed with the RLB hybridization assay as previously described (Gubbels et al., 1999; Nijhof et al., 2005). The genus and species-specific oligonucleotide probes included on the membrane are listed in Table 1. The 18S rRNA gene sequences of *Babesia occultans* (EU376017), *Babesia orientalis* (AY596279), *Babesia* sp. (Xinjiang) (DQ159073), *Theileria sinensis* (EU277003), *T. luwenshuni* (AF081136, AY262119, AY262118, AY262117, AY262115) and *T. uilenbergi* (AY262120,

AY262116, AY262121) were used to design RLB probes for the detection of these species. These were also included on the RLB membrane.

2.3 Cloning and sequencing of 18S rRNA gene

Eight *Theileria buffeli*/*T. sergenti*/*T. orientalis* positive samples, one *B. orientalis* positive sample and two *B. bigemina* positive samples were chosen for cloning and subsequent sequencing. The complete 18S rRNA gene was amplified using the universal primers forward-P1 (5'-AAC CTG GTT GAT CCT GCC AGT AGT CAT-3'), and reverse-P2 (5'-GAT CCT TCT GCA GGT TCA CCT AC-3') using the condition as described by Liu et al. (2005). The approximately 1 800 bp amplified products were purified and then ligated into the pMD18-T vector (TaKaRa Biotechnology), and the recombinant clones were sequenced using the ABI PRISM 377 DNA sequencer following the manufacturer's instructions. The primers forward-P1, reverse-P2 (Liu et al., 2005), RLB-F2 and RLB-R2 (Gubbels et al., 1999) were used to obtain the full-length 18S rRNA sequences.

2.4 Phylogenetic analysis

The sequences were assembled and edited using GAP4 of the Staden package (version 1.6.0 for Windows) (Staden et al., 2000). A search for homologous sequences in GenBank was performed using BLASTn (www.ncbi.nlm.nih.gov/BLAST/). The sequences were aligned using MAFFT 6 employing the FFT-NS-i algorithm (Kato et al., 2002). The alignment was manually edited using BioEdit 7.0.9.0. (Hall, 1999). The best-fit model of nucleotide substitution was determined by JmodelTest 0.1.1 (Posada, 2008) selected by AIC calculations. A general time reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (TIM1+I+G) substitution mode was used in PAUP* v4b10 (Swofford, 2002) to explore neighbour-joining, parsimony and maximum likelihood methods. MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) was used to explore Bayesian phylogeny. Consensus trees were edited in MEGA v4.0.2 (Tamura et al., 2007).

2.5 Nucleotide sequence accession numbers:

The 18S rRNA gene sequences obtained in this study was submitted to GenBank under the following accession numbers: *Babesia bigemina* 493 (Hong'an): HQ840959; *B. bigemina* 563 (Hong'an): HQ840960; *B. orientalis* Daye: HQ840969; *Theileria buffeli* Daye4: HQ840961; *T. buffeli* Daye56: HQ840962; *T. buffeli* Hong'an1: HQ840963; *T. buffeli* Hong'an2: HQ840964; *T. buffeli* Suizhou117: HQ840965; *T. buffeli* Xiaogan17: HQ840966; *T. buffeli* Xiaogan19: HQ840967; *T. buffeli* Xiaogan20: HQ840968.

3. Results and Discussion

Blood samples collected from 304 water buffalo from nine distinct geographical locations of the Hubei province were screened for the presence of *Theileria* and *Babesia* species. Results are summarized in Table 2. *Theileria buffeli*, *B. orientalis*, *B. bigemina* and *B. bovis* were detected either as single or mixed infections. The most prevalent species detected was *T. buffeli* (19.1%). This was in concordance with the finding of Zhao (2008) who found *T. buffeli* as the most prevalent *Theileria* spp present in water buffalo in the Hubei province. Of the *Babesia* species detected, *B. orientalis* was the most prevalent (8.9%), followed by *B. bovis* (1%) and *B. bigemina* (0.7%). This serves to be the first report of *B. bigemina* in the Hubei province. Only 3.9% (n = 12) of the samples had mixed infections. Ten samples tested positive for the presence of *T. buffeli* and *B. orientalis*, one tested positive for *T. buffeli* and *B. bovis* and one sample tested positive for the presence of *T. buffeli*, *B. bovis* and *B. orientalis*.

The full length parasite 18S rRNA genes from 11 single infected samples were cloned and sequenced. BLASTn homology searches indicated that five of these sequences were closely related to published *T. buffeli* sequences, two to *T. orientalis*, one sequence was closely related to *T. sinensis*, one to *B. orientalis* and two sequences were closely related to published *B. bigemina* sequences. The two sequences, *T. buffeli* Xiaogan19 and *T. buffeli* Xiaogan20, were closely related (99% identity) to published *T. buffeli* 18S rRNA gene sequences previously identified from cattle and

buffalo in China (accession numbers HM538212, HM538211, HM538205 and DQ104611). *T. buffeli* Suizhou117 was closely related (99% identity) to *T. buffeli* HM538208 and HM538206 both identified in buffalo. *T. buffeli* Daye4 and *T. buffeli* Daye56 obtained from buffalo were closely related (99% identity) to *T. orientalis* HM538218 (identified from a bovine), *T. orientalis* AB520958 (identified from cattle) and *T. sergenti* Ikeda AY661515 (identified in cattle). *T. buffeli* Xiaogan17 was closely related to a *T. sinensis* (HM538213) identified from a buffalo in China. For the cloned *Babesia* isolates, *B. bigemina* Hongan493 and Hongan563 were closely related (99% identity) to the *B. bigemina* Kunming (AY603402) which was previously isolated from *Rhipicephalis microplus* ticks collected from Kunming, Yunnan province, China (Luo et al., 2005). The *B. orientalis* Daye were closely related to *B. orientalis* (AY596279) identified from a water buffalo, *Babesia* sp. Kashi 2 (AY726557) identified in bovines from China and an unnamed *Babesia* species (U09834) that was detected in a bovine in 1994 in South Africa.

Neighbor-joining, maximum likelihood, maximum parsimony and Bayesian phylogenetic analyses were used to reveal the relationship among the cloned 18S rRNA sequences and piroplasms. No significant changes in the topology of the trees or in the bootstrap values were found. As shown in Figure 1 (neighbor joining tree), the *Theileria* species could broadly be divided into two major groups; the virulent taxa (including *T. parva*, *T. annulata* and *T. taurotragi*) and the benign *Theileria* parasites (*T. buffeli*/*T. sergenti*/*T. orientalis* group). The benign group clustered into seven distinct groups according to the type distinction (A to G) derived from Chae et al. (1998). Of the eight cloned 18S rRNA sequences in this study, only *T. buffeli* Daye4 and Daye56 grouped with *T. buffeli* type B, and *T. buffeli* Xiaogan17 grouped with *T. buffeli* type D as well as the published *T. sinensis* sequences. The RLB hybridization assay results indicated, however, the presence of *T. buffeli* in this particular sample. The PCR amplicons did not bind to the newly developed *T. sinensis* RLB probe (which was based in the published *T. sinensis* sequence, accession nr EU277003). The sequence alignment revealed that there were 5 bp differences under the probe region,

explaining failure to bind. The other five *T. buffeli* sequences clustered separately from any of the *T. buffeli* types. Two new groups were identified which clustered separately from any of the *T. buffeli* types. The *T. buffeli* Suizhou117 grouped with *T. buffeli* previously identified in buffalo in China. Four sequences (Hongan1, Hongan2, Xiaogan19 and Xiaogan20) grouped with *T. buffeli* 18S rRNA gene sequences previously identified from cattle and buffalo in China as well as a published *T. buffeli* sequence from India (EF126184). We propose that these two groups constitute new *T. buffeli* types.

The *Babesia* spp 18S rRNA gene sequence obtained lied within the *Babesia* sensu stricto clade. The obtained *B. bigemina* sequences (493 and 563) grouped with to the *B. bigemina* Kunming (AY603402). The *B. orientalis* Daye formed a monophyletic group with *B. orientalis* Wuhan, *Babesia* sp. Kashi 1, *Babesia* sp. Kashi 2, *B. occultans*, and the unnamed *Babesia* species (U09834) detected in a bovine.

In conclusion, the presence and prevalence of tick-borne haemoparasites in water buffalo in the Hubei province were determined using the RLB hybridization assay. Four species were identified; *T. buffeli* was the most abundant haemoparasite, followed by *B. orientalis*, *B. bovis* and *B. bigemina*. Phylogenetic analysis indicated the existence of two new *T. buffeli* types in the surveyed areas. Our results also showed the existence of *B. bigemina* which is the first report of this parasite in the Hubei province. Further epidemiological surveys (both cattle and water buffalo) are required in the Hubei province to get a better understanding of the epidemiology of these important tick-borne haemoparasites.

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Figure 1: Neighbour-joining phylogenetic tree showing (a) relationship of the *T. buffeli* genotypes identified in this study with other *Theileria* species and (b) relationships of *B. bigemina* and *B. orientalis* genotypes with other related *Babesia* spp. based on the full-length 18S rRNA gene sequences. Values at the nodes are bootstrap values indicating the degree of support for each cluster. Vertical lengths in each tree are not significant and are merely set for clarity. GenBank accession numbers are indicated in parentheses. The tree was outgroup rooted using *Prorocentrum micans*, *Sarcocystis muris* and *Toxoplasma gondii*.

Table 1: *Theileria* and *Babesia* specific oligonucleotide probe sequences used in this study. The degenerate position R denotes either A or G, W denotes either A or T.

Oligonucleotide probe	Sequence (5' – 3')	Reference
<i>Theileria/Babesia</i> genus-specific	ATT AGA GTG TTT CAA GCA GAC	Nijhof ^a , unpublished
<i>Theileria</i> genus-specific	ATT AGA GTG TTT CAA GCA GAC	Nijhof ^a , unpublished
<i>Theileria annulata</i>	CCT CTG GGG TCT GTG CA	Gubbels et al., 1999
<i>Theileria bicornis</i>	GCG TTG TGG CTT TTT TCT G	Nijhof et al., 2003
<i>Theileria buffeli</i>	GGC TTATTT CGG WTT GAT TTT	Gubbels et al., 2000
<i>Theileria equi</i>	TTC GTT GAC TGC GYT TGG	Butler et al., 2008
<i>Theileria lestoquardi</i>	CTT GTG TCC CTC CGG G	Schnittger et al., 2004
<i>Theileria luwenshuni</i>	GGC TTA TTT CGG ATG ATA C	This study
<i>Theileria mutans</i>	CTT GCG TCT CCG AAT GTT	Gubbels et al., 1999
<i>Theileria orientalis</i> 1	GGC TTA TTT CGG ATG ATA CTT GT	Nijhof (unpublished)
<i>Theileria orientalis</i> 2	GGC TTA TTT CGG ATG ATA CTT GT	Nijhof (unpublished)
<i>Theileria parva</i>	GGA CGG AGT TCG CTT TG	Gubbels et al., 1999
<i>Theileria separata</i>	GGT CGT GGT TTT CCT CGT	Schnittger et al., 2004
<i>Theileria sinensis</i>	TCG CAT CTC TTG CTG AGT GC	This study
<i>Theileria</i> sp. (buffalo)	CAG ACG GAG TTT ACT TTG T	Oura et al., 2004
<i>Theileria</i> sp. (kudu)	CTC CAT TGT TTCTTT CCT TTG	Nijhof et al., 2005
<i>Theileria</i> sp. (sable)	GCT GCA TTG CCT TTT CTC C	Nijhof et al., 2005
<i>Theileria taurotragi</i>	TCT TGG CAC GTG GCT TTT	Gubbels et al., 1999
<i>Theileria uilenbergi</i>	GCT TGC ATT TTC CGA GTG T	This study
<i>Theileria velifera</i>	CCT ATT CTC CTT TACGAG T	Gubbels et al., 1999
<i>Babesia</i> genus-specific 1	ATT AGA GTG CTC AAA GCA GGC	Nijhof (unpublished)
<i>Babesia</i> genus-specific 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
<i>Babesia bicornis</i>	TTG GTA AAT CGC CTT GGT	Nijhof et al., 2003
<i>Babesia bigemina</i>	CGT TTT TTC CCT TTT GTT GG	Gubbels et al. 1999
<i>Babesia bovis</i>	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al. 1999

<i>Babesia canis</i>	TGC GTT GAC CGG TTT GAC	Matjila et al., 2004
<i>Babesia crassa</i>	GTT GGC TTA TCT TTT TAC TTT	Schnittger et al., 2004
<i>Babesia major</i>	TCC GAC TTT GGT TGG TGT	Georges et al. 2001
<i>Babesia occultans</i>	CCT CTT TTG GCC CAT CTC GTC	This study
<i>Babesia orientalis</i>	CCT CTT TTG GCC GTC TCA CT	This study
<i>Babesia ovis</i>	TGC GCG CGG CCT TTG CGT T	Schnittger et al., 2004
<i>Babesia</i> sp. (sable)	GCG TTG ACT TTG TGT CTT TAG C	Oosthuizen et al. 2008
<i>Babesia</i> sp. (Xinjiang)	GCG GGT TTC GTC TAC TTC GCT TTG T	This study
<i>Babesia vogeli</i>	AGC GTG TTC GAG TTT GCC	Matjila et al., 2004

^a Dr. Ard M. Nijhof, (Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands)

Table 2: The occurrence of *Theileria* and *Babesia* species infections in water buffalo in the Hubei province, China as determined by the RLB

Occurrence of haemoparasites per collection site							
Single infections					Mixed infections		
<i>T. buffeli</i>	<i>B. orientalis</i>	<i>B. bovis</i>	<i>B. bigemina</i>		<i>T. buffeli</i> & <i>B. orientalis</i>	<i>T. buffeli</i> & <i>B. bovis</i>	<i>T. buffeli</i> , <i>B.</i> <i>bovis</i> , & <i>B.</i> <i>orientalis</i>
Total (n = 304)	58 (19.1%)	27 (8.9%)	3 (1%)	2 (0.7%)	10 (3.3%)	1 (0.3%)	1 (0.3%)