Parasitol Res (2015) 114:2715–2721
DOI 10.1007/s00436-015-4478-z

ORIGINAL PAPER

Development of a multiplex PCR assay for detection and discrimination of *Theileria annulata* and *Theileria sergenti* in cattle

Liu Junlong[1,2] · **Youquan Li**[1,2] · **Aihong Liu**[1,2] · **Guiquan Guan**[1,2] · **Junren Xie**[1,2] · **Hong Yin**[1,2] · **Jianxun Luo**[1,2]

Received: 10 March 2015 / Accepted: 8 April 2015 / Published online: 18 April 2015

Abstract Aim to construct a simple and efficient diagnostic assay for *Theileria annulata* and *Theileria sergenti*, a multiplex polymerase chain reaction (PCR) method was developed in this study. Following the alignment of the related sequences, two primer sets were designed specific targeting on *T. annulata* cytochrome b (COB) gene and *T. sergenti* internal transcribed spacer (ITS) sequences. It was found that the designed primers could react in one PCR system and generating amplifications of 818 and 393 base pair for *T. sergenti* and *T. annulata*, respectively. The standard genomic DNA of both species *Theileria* was serial tenfold diluted for testing the sensitivity, while specificity test confirmed both primer sets have no cross-reaction with other *Theileria* and *Babesia* species. In addition, 378 field samples were used for evaluation of the utility of the multiplex PCR assay for detection of the pathogens infection. The detection results were compared with the other two published PCR methods which targeting on *T. annulata* COB gene and *T. sergenti* major piroplasm surface protein (MPSP) gene, respectively. The developed multiplex PCR assay has similar efficient detection with COB and MPSP PCR, which indicates this multiplex PCR may be a valuable assay for the epidemiological studies for *T. annulata* and *T. sergenti*.

✉ Liu Junlong
jliu1211@gmail.com

[1] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science, Xujiaping 1, Lanzhou, Gansu 730046, People's of Republic China

[2] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, People's of Republic China

Keywords Multiplex PCR · *Theileria annulata* · *Theileria sergenti*

Introduction

Theileriosis is an important disease of bovine, caused with several *Theileria* species including *Theileria annulata*, *Theileria sergenti/orientalis*, and *Theileria parva*. Among of these pathogens, *T. annulata* is known as the pathogen of tropical theileriosis and widely distributed in North Africa, Southern Europe, India, the Middle East, and Asia (Bilgic et al. 2010). *T. sergenti* is the major vector-borne protozoan parasite of grazing cattle and buffalo in Japan and Korea and belongs in the group of *T. sergenti/Theileria buffeli/T. orientalis* (Wang et al. 2010; Liu et al. 2010). Both of the *Theileria* species were reported in China, *T. annulata* was considered to be the most pathogenic, and *T. sergenti* is prevalent (Luo and Lu 1997; Liu et al. 2010). In the case of blood samples collected from grazing animals, *T. annulata* and *T. sergenti* are often found co-infected in the host (Gubbels et al. 1999; Georges et al. 2001). Because they shared similar clinical signs, including fever, anorexia, icterus and anemia, it is very difficult to discriminate the pathogens.

Traditional diagnosis of bovine theileriosis is mainly based on the microscopic examination of blood smears for the presence of the merozoites stage of *Theileria* (Ahmed et al. 2002). However, the low sensitivity and the requiring well-trained technicians of this method do not permit its application in epidemiological studies (Liu et al. 2014). One of important character of theileriosis is that once the animal recovered from the primary infection, the animal will become a carrier for a long time. At this stage, the animal has very low parasitemia which is difficult to detect by a

microscope (Figueroa et al. 1993). Therefore, high sensitivity and specific assays for detection of these pathogens from the subclinical animals are needed.

Compare to the microscopic method, serological tests, which aim to detect the circulating antibodies in the later phase of the disease when the parasitemia drops, are much better. To date, many proteins such as Tasp, Tams-1, and PIM were indentified and used for the development of ELISA (Katende et al. 1998; Gubbels et al. 2000; Schnittger et al. 2002; Seitzer et al. 2010; Mohamed et al. 2012; Rajendran and Ray 2014). However, cross-reactivity of antibodies across different *Theileria* species is always following with these methods, particularly when the pathogenic and non-pathogenic pathogens are co-infection. Moreover, ELISA could not give the right proposal for drug treatment after detection, because even the pathogens disappeared a long time, the antibodies are still positive against it.

Polymerase chain reaction (PCR) is a highly sensitive and specific method for detection of different pathogens. Since PCR method was introduced in the detection of *Theileria* (Kawazu et al. 1992; Bishop et al. 1993), many gene sequences such as 18S rRNA, internal transcribed spacer (ITS), major piroplasm surface protein (MPSP), Tams1, and cytochrome b (COB) were used for development of PCR method for detection of different *Theileria* species (Tanaka et al. 1993; d' Oliveira et al. 1995; Han et al. 2009; Bilgic et al. 2010; Kamau et al. 2011). Recently, some PCR-related assays including real-time PCR and loop-mediated isothermal amplification (LAMP) methods were developed for diagnosis of the pathogens of theileriosis (Wang et al. 2010; Ros-García et al. 2012; Chaisi et al. 2013; Liu et al. 2013). Those individual PCR methods are highly sensitive and effective for detection of the single pathogen at a time, but when they are applied to detect a large number of field samples which are co-infections with different *Theileria* species, it will be time-consuming and material-consuming. Aim to cover those problems, reverse line blot (RLB) which could detect multiple pathogens in one reaction was used for detection of piroplasms, but the time-consuming and requirement of special equipment restricted its application in epidemiological studies (Georges et al. 2001; Almeria et al. 2002; Iqbal et al. 2013). Multiplex PCR offers significant advantages over individual PCR for assessment of multiple species pathogen infection from large number of samples. These assays were already widely used to detect the co-infection of *Babesia*, *Theileria*, and *Anaplasma* (Figueroa et al. 1993; Alhassan et al. 2005; Bilgiç et al. 2013; Zhang et al. 2014). In the present study, a multiplex PCR was developed for simultaneous detection of *T. annulata* and *T. sergenti* from cattle blood samples, and compared its application with the single PCR methods targeting on the MPSP and COB gene for *T. sergenti* and *T. annulata*, respectively.

Materials and methods

Positive parasites DNA samples

The preserved laboratory standard stocks including *T. annulata* (Inner Mongolia) and *T. sergenti* (Liaoyang) were used as positive control in this study and preserved at −70 °C. The concentration of the DNA was evaluated by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Field blood samples

In order to evaluate the multiplex PCR assay for the diagnosis of field samples, a total of 378 blood samples of grazing cattle were collected from eight provinces, including Jilin, Inner Mongolia in the northern of China, Henan, Hebei and Shandong in central China, Guangdong, and Guangxi in southern China. Genomic DNA from the field samples was extracted by using genomic DNA extraction kit (Gentra, Germany). The extracted DNA was stored at −20 °C until use.

Primer design

The nucleotide sequence of cytochrome b gene of *T. annulata* (KP731977, XM949625) and the internal transcribed spacers sequence of *T. sergenti* (EF547929, EF547930) were used for designing the primers after aligned with relative sequences. Specific primer sets with similar annealing temperature were designed by using Primer 5. For detection of *T. annulata*, primer set AnCb (forward: 5′-CGGTTGGTTT GTTCGTCTTT-3′; reverse: 5′- GCCAATGGATTTGAAC TTCC-3′) was designed to amplify a 393-bp sequence. Primer set SerITS (forward: 5′-CAACCCAGCTGCTTTTGAGG-3′; reverse: 5′-CAACAGAATCGCAAAGCGGT-3′) was used to amplify a 818-bp sequence from the ITS gene of *T. sergenti*. The specificity of each primer set was evaluated by using the BLASTN algorithm from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Single and mutiplex PCR

The primer sets AnCb and SerITS were evaluated individually aim to determinate their specificity. All PCR reactions were performed in a final volume of 25 μl containing 12.5 μl Premix Taq DNA polymerase (TaKaRa, China), 0.8 μM of each primer, and 1 μl of DNA template. PCR reaction system comprised of one step initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation (94 °C for 1 min.), primer annealing (61.5 °C for 50 s), and extension (68 °C for 1 min.). The final extension was performed with one step at 68 °C for 5 min. The relative *Theileria* species (*Theileria sinensis*) and *Babesia* species

(*Babesia bigemina*, *Babesia bovis*, *Babesia ovata*, *Babesia major*, and *Babesia* sp. Kashi) were used for testing the specificity of the primer sets. PCR product was electrophoresed on a 1.0 % agarose gel containing 10 μl of Goldview (SolarBio, China) in Tris-acetate-EDTA (TAE) buffer at 120 V for 40 min and visualized under UV light.

After modulation, the multiplex PCR was perform in a final volume of 25 μl containing 12.5 μl of Premix Taq DNA polymerase (TaKaRa, China), 0.3 μM of each AnCb primer, 0.8 μM of each SerITS primer, and 1 μl of each *Theileria* DNA template. The reaction was carried out as a single PCR. The PCR results were analyzed under UV light as described above.

Sensitivity of single and multiplex PCR

The *T. annulata* (Inner Mongolia) and *T. sergenti* (Liaoyang) DNA was initially diluted to10 ng/μl and then serially diluted tenfold in distilled water. The diluted DNA was used as a template in the PCR to evaluate the sensitivity of each pair of primers. Additionally, the sensitivity of the multiplex PCR for co-infection was also evaluated using a mixture of *T. annulata* and *T. sergenti* DNA.

Examination of field samples

In order to evaluate the application of the multiplex PCR, DNA from 378 field bovine blood samples was used as template to detect the presence of *T. annulata* and *T. sergenti*. Two published PCR methods for detection of *T. annulata* (Bilgic et al. 2010) and *T. sergenti* (Liu et al. 2010) were used for comparison the efficiency of the multiplex PCR detection.

Cloning and sequencing

Multiplex PCR products with the expected size of 393 bp for *T. annulata* and 818 bp for *T. sergenti* were electrophoresed on a 1.5 % agarose gel. After being visualized under UV, the PCR product was excised from the gel and purified using Axygen Gel Purification Kit. The DNA fragment was cloned into the pGEM-T Easy vectors (Promega, USA). The *Escherichia coli* JM 109 (TaKaRa, China) was transformed, and plasmid DNA from the selected clones was identified using PCR using the primers as mentioned above (program and reaction mixtures were used as same as PCR amplification described above) to verify the presence of correct inserts in selected clones before for the sequencing process by the Big Dye Terminator Mix of TaKaRa Company (China). For the field samples from each province, one positive PCR product for each parasite was selected for sequencing.

Results

Specificity of designed primers in single PCR

The specificity of the primer AnCb and SerITS was evaluated in single PCR reaction with a total of eight species of *Theileria* and *Babesia* which are present in China. And uninfected bovine DNA and water were used as negative and blank control, respectively. From the gel, the expected size of fragments were generated from *T. annulata* and *T. sergenti* DNA with the specific primers, while no products were found from either other *Theileria* and *Babesia* DNA or the controls (Fig. 1), which indicate the designed primer sets are specific targeting on the *T. annulata* COB gene and *T. sergenti* ITS sequence.

Sensitivity of the single and multiplex PCR

The sensitivities of the single PCR were evaluated using a tenfold serial diluted *T. annulata* and *T. sergenti* DNA which the concentration ranged from10 ng/μl to 10^{-8} ng/μl for each parasite. A mixed DNA representing both parasites was used to evaluate the sensitivity of the multiplex PCR assay. As shown in Fig. 2, single PCR could detect 10^{-8} ng/μl and 10^{-7} ng/μl DNA of *T. annulata* and *T. sergenti*, respectively. The multiplex PCR (mPCR) was able to detect 10^{-7} pg/μl DNA of *T. annulata* and *T. sergenti*, which was similar with the single PCR reactions.

Detection of *T. annulata* and *T. sergenti* infections in field samples

A total of 378 bovine blood samples from six provinces of China were examined for *T. annulata* and *T. sergenti* infection by using mPCR and single PCR targeting on *T. annulata* COB gene and *T. sergenti* MPSP gene, respectively. With multiplex PCR method, 11 samples are positive for *T. annulata* and 81 samples are positive for *T. sergenti*. Among the positive samples, three samples are mix infection from Jilin and Chongqing. Published PCR method target on *T. annulata* COB gene have detected six positive samples, and PCR target on *T. sergenti* MPSP gene have detected 80 positive samples. Only one mix infection sample was found with those two single PCR methods from Chongqing. The detail of the results could be found in Table 1.

Sequencing analysis

Positive PCR products amplified from the standard DNA of *T. annulata* and *T. sergenti* were sequenced to confirm the specificities of the AnCb and SerITS. In addition, one positive sample for each parasite from each province was selected for sequencing and compared with the published sequences. From the sequencing results, fragments from standard samples were 99.5 and

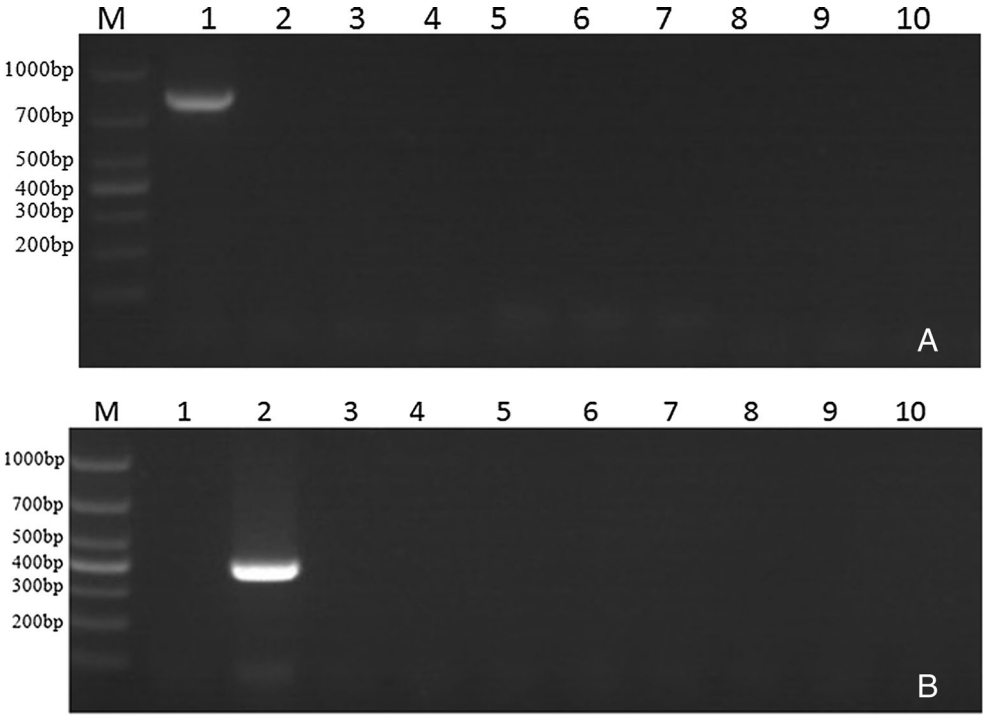

Fig. 1 Specific test of the primer sets SerITS (**a**) and AnCb (**b**). M, DL2000^{TM} Molecular marker (TaKaRa); *lane 1 T. sergenti*; *2 T. annulata*, *3 T. sinensis*, *4 B. bigemina*, *5 B. bovis*, *6 B. ovata*, *7 B. major*; *8*, *B* .sp. Kashi, *9* uninfected bovine DNA, *10* water. The bands from **a** and **b** shows the 818 and 393 base pair sequences generated from SerITS and AnCb primer sets, respectively

99.9 % identical with the *T. annulata* COB gene and *T. sergenti* ITS sequence. From the wild positive samples, four sequences for *T. annulata* were sequenced and compared with the *T. annulata* cytochrome b gene which shown the identities range between 98.7 and 99.7 %. For the samples positive for *T. sergenti*, eight single plasmid clones were selected for sequencing. The derived sequences showed the identities range from 97.1 to 99.5 % with the *T. sergenti* Liaoyang ITS sequence.

Discussion

Theileria species are of the most importance pathogens of East Coast fever and tropical theileriosis in cattle. These parasites can be sorted as host cell transforming and non-transforming species. The sporozoites of *T. annulata* invade B cells, macrophage cells, and dendritic cells, while the *T. parva* infect mainly with T cells (Ahmed et al. 1984; Hostettler et al. 2014). The parasite-infected cells could produce cytokines which might impair the function of host immune cells and thus enhance the establishment of the infection (Ahmed and Mehlhorn 1999). In addition, the infected cells disseminate through lymphoid tissue and some organs including the heart, lungs, and brain which leading to hemorrhagic lesions (Jensen et al. 2009). The benign species of *Theileria* such as the group *Theileria buffeli /orientalis/sergenti* is one of the important pathogens in some area especially when infected with *T. annulata* or *T. parva*. Aim to eliminate the parasites, halofuginone lactate and buparvaquone were confirmed available against *T. annulata* and *T. parva*, but no drugs were found useful against *T. sergenti* infection for practical field application (Mehlhorn and Raether 1988; Shiono et al. 2003). Therefore, to define the infected parasite species will be helpful for control of the disease.

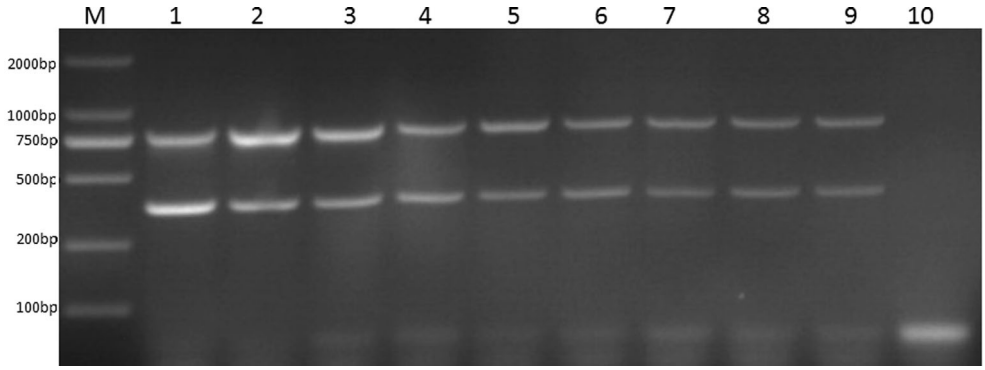

Fig. 2 Sensitivity tests of multiplex PCR with serial dilution of mixture of DNA from *T. annulata* and *T. sergenti*. M, DL2000^{TM} Molecular marker (TaKaRa); *lanes 1–10*, tenfold series dilution of mixed DNA sample ranging from 10 ng/μl to 10^{-8} ng/μl

Table 1 Multiplex PCR and the reference PCR methods tests on field bovine blood samples

Province	No. of samples	Positive *T. an* samples		Positive *T. ser* samples		Mix infection	
		mPCR	COB	mPCR	MPSP	mPCR	COB+MPSP
Guangdong	60	2	3	3	1	0	0
Guangxi	32	0	0	3	5	0	0
Henan	51	0	0	33	31	0	0
Hebei	25	0	0	9	8	0	0
Jilin	23	3	0	11	8	1	0
Inner Mongolia	93	1	0	4	5	0	0
Shandong	39	0	0	3	1	0	0
Chongqing	55	5	3	15	21	2	1

T. an Theileria annulata, *T.ser Theileria sergenti*

In China, bovine theileriosis mainly caused by *T. annulata*, *T. sergenti and Theileria mutans* (Luo and Lu 1997). Among of these pathogens, *T. annulata* and *T. sergenti* are the most prevalent. Both of the *Theileria* species were usually found co-infection from the field samples and cause important health and management problems which lead to reduce the productivity of the milk and meat. Its long lasting carrier stage with lower parasitemia which was difficult to detect by a microscope is a potential risk for the next outbreak of theileriosis. PCR method with higher sensitivity and specificity could directly detect the parasite genomic material to reveal the presence of live parasite in the animal. Therefore, an efficient PCR diagnosis assay is very helpful for making the control strategy for theileriosis. Multiplex PCR, which could simultaneously detect different pathogens in one reaction, was confirmed as a valuable tool for detection of pathogens in co-infection case (Figueroa et al. 1993; Alhassan et al. 2005; Chan et al. 2013; Bilgiç et al. 2013; Zhang et al. 2014).

In the present study, a multiplex PCR method was established for simultaneously detection of *T. annulata* and *T. sergenti*. The internal transcribed spacers with higher evolutionary rates were already used in phylogenetic analysis and as a target gene for detection of *Theileria s*pecies (Aktas et al. 2007; Kamau et al. 2011). Previously, a study has shown that *T. annulata* cytochrome b gene is a very sensitive and specific gene target, and a PCR method was already established based on this gene for detection of *T. annulata* (Bilgic et al. 2010; Bilgiç et al. 2013). Therefore, two primer sets were designed based on the *T. annulata* COB gene and *T. sergenti* ITS sequence, which the expected products are 393 and 818 bp, respectively. After optimizing the reaction system, two primer sets could specifically amplify its target gene successfully in one reaction. No cross-reaction was found with other bovine piroplasms DNA. And the sensitivity of the established PCR method could detect the 10^{-7} ng/μl DNA of *T. annulata* and *T. sergenti*, which is higher than the method for detection of *T. annulata* based on COB and *T. sergenti* based on MPSP gene (Liu et al. 2010; Bilgiç et al. 2013).

Aim to evaluate the application of multiplex PCR, 378 field blood samples were used for detection of *T. annulata* and *T. sergenti*. Two PCR methods for detection of *T. annulata* and *T. sergenti* based on the published papers were used as reference methods (Liu et al. 2010; Bilgiç et al. 2013). From the results, 2.91 % (11/378) samples from Guangdong, Jilin, Inner Mongolia, and Chongqing were positive for *T. annulata* by using multiplex PCR. However, samples only from Guangdong and Chongqing were positive (1.59 %) for *T. annulata* by using published COB PCR method. The infection of *T. sergenti* were found present in every investigated province by both multiplex PCR and MPSP PCR method, and the positive rate was 21.43 and 21.16 % of total sample number, respectively. For the co-infection detection, one sample from Jilin and two samples from Chongqing were positive for both parasites by using multiplex PCR, and one sample from Chongqing was found infected with both parasites by using the two published PCR methods. From the result examination of field samples, the multiplex PCR has a higher sensitivity than the single COB PCR for *T. annulata* but with the similar sensitivity of MPSP PCR for *T. sergenti*. Of the two *Theileria* species detected in the present study, *T. sergenti* was found to be the most prevalent in all investigated provinces which confirmed again this parasite exists in most part of China, while the infection of *T. annulata* mainly found in the north or northwest of China. However, both methods found *T. annulata* infection from Guangdong province belongs to a sub-tropical area. Because there has been no report for the presence of *T. annulata* and its vector in this area before, it is needed further works to analyze the distribution of ticks in Guangdong province.

In conclusion, the developed multiplex PCR method in the present study provides an efficient and higher sensitivity assay for detecting of *T. annulata* and *T. sergenti* in

the carrier cattle. The method should be useful for addressing the prevalence of both parasites in more areas in China and will be helpful to make the control strategies for the co-infection by these pathogens.

Acknowledgments This study was financially supported by the NSFC (No. 31402189, No. 31372432, No. 31201899, No. 31272556, No. 31471967), ASTIP, FRIP (2014ZL010), CAAS; Creative Research Groups of Gansu Province (No. 1210RJIA006); "948" (2014-S05), NBCIS CARS-38, Special Fund for Agro-scientific Research in the Public Research (No. 201303035, No. 201303037), MOA; 973 Program (2015CB150300,2010CB530206), Supporting Program (2013BAD12B03, 2013BAD12B05), MOST, China; Jiangsu Co-innovation Center program for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, State Key Laboratory of Veterinary Etiological Biology Project. The research was also facilitated by CRP No. 16198/R0 IAEA.

References

Ahmed JS, Mehlhorn H (1999) Review: the cellular basis of the immunity to and immunopathogenesis of tropical theileriosis. Parasitol Res 85:539–549

Ahmed JS, Rehbein G, Schein E (1984) Characterization of *Theileria annulata* infected lymphoblastoid cells. Z Für Parasitenkd Berl Ger 70:819–821

Ahmed J, Yin H, Schnittger L, Jongejan F (2002) Ticks and tick-borne diseases in Asia with special emphasis on China. Parasitol Res 88: S51–S55

Aktas M, Bendele KG, Altay K et al (2007) Sequence polymorphism in the ribosomal DNA internal transcribed spacers differs among *Theileria* species. Vet Parasitol 147:221–230. doi:10.1016/j.vetpar.2007.04.007

Alhassan A, Pumidonming W, Okamura M et al (2005) Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Vet Parasitol 129:43–49. doi:10.1016/j.vetpar.2004.12.018

Almeria S, Castella J, Ferrer D et al (2002) Reverse line blot hybridization used to identify hemoprotozoa in Minorcan cattle. Ann N Y Acad Sci 969:78–82

Bilgic HB, Karagenç T, Shiels B et al (2010) Evaluation of cytochrome b as a sensitive target for PCR based detection of *T. annulata* carrier animals. Vet Parasitol 174:341–347. doi:10.1016/j.vetpar.2010.08.025

Bilgiç HB, Karagenç T, Simuunza M et al (2013) Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. Exp Parasitol 133:222–229. doi:10.1016/j.exppara.2012.11.005

Bishop R, Sohanpal B, Morzaria S (1993) *Theileria parva*: detection of genomic polymorphisms by polymerase chain reaction amplification of DNA using arbitrary primers. Exp Parasitol 77:53–61. doi:10.1006/expr.1993.1060

Chaisi ME, Janssens ME, Vermeiren L et al (2013) Evaluation of a real-time PCR test for the detection and discrimination of *Theileria* species in the African buffalo (*Syncerus caffer*). PLoS One 8:e75827. doi:10.1371/journal.pone.0075827

Chan K, Marras SAE, Parveen N (2013) Sensitive multiplex PCR assay to differentiate Lyme spirochetes and emerging pathogens *Anaplasma phagocytophilum* and *Babesia microti*. BMC Microbiol 13:295. doi:10.1186/1471-2180-13-295

D' Oliveira C, van der Weide M, Habela MA et al (1995) Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. J Clin Microbiol 33:2665–2669

Figueroa JV, Chieves LP, Johnson GS, Buening GM (1993) Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Vet Parasitol 50:69–81

Georges K, Loria GR, Riili S et al (2001) Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. Vet Parasitol 99:273–286

Gubbels JM, de Vos AP, van der Weide M et al (1999) Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J Clin Microbiol 37:1782–1789

Gubbels MJ, d' Oliveira C, Jongejan F (2000) Development of an indirect Tams1 enzyme-linked immunosorbent assay for diagnosis of *Theileria annulata* infection in cattle. Clin Diagn Lab Immunol 7:404–411

Han J-I, Jang H-J, Na K-J (2009) Molecular detection of *Theileria* sp. in wild Chinese water deer (*Hydropotes inermis argyropus*). J Wildl Dis 45:1213–1216. doi:10.7589/0090-3558-45.4.1213

Hostettler I, Müller J, Stephens CE et al (2014) A quantitative reverse-transcriptase PCR assay for the assessment of drug activities against intracellular *Theileria annulata* schizonts. Int J Parasitol Drugs Drug Resist 4:201–209. doi:10.1016/j.ijpddr.2014.09.003

Iqbal F, Khattak R, Ozubek S et al (2013) Application of the reverse line blot assay for the molecular detection of *Theileria* and *Babesia* sp. in sheep and goat blood samples from Pakistan. Iran J Parasitol 8:289–295

Jensen K, Makins GD, Kaliszewska A et al (2009) The protozoan parasite *Theileria annulata* alters the differentiation state of the infected macrophage and suppresses musculoaponeurotic fibrosarcoma oncogene (MAF) transcription factors. Int J Parasitol 39:1099–1108. doi:10.1016/j.ijpara.2009.02.020

Kamau J, Salim B, Yokoyama N et al (2011) Rapid discrimination and quantification of *Theileria orientalis* types using ribosomal DNA internal transcribed spacers. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis 11:407–414. doi:10.1016/j.meegid.2010.11.016

Katende J, Morzaria S, Toye P, et al (1998) An enzyme-linked immunosorbent assay for detection of *Theileria parva* antibodies in cattle using a recombinant polymorphic immunodominant molecule. Parasitol Res 84:408–416.

Kawazu, Sugimoto C, Kambio T, Fusjiaki K (1992) Analysis of the genes encoding immunodominant piroplasm surface proteins of *Theileria sergenti* and *Theileria buffeli* by nucleotide sequencing and polymerase chain reaction. Mol Biochem Parasitol 56:169–175

Liu A, Guan G, Liu Z et al (2010) Detecting and differentiating *Theileria sergenti* and *Theileria sinensis* in cattle and yaks by PCR based on major piroplasm surface protein (MPSP). Exp Parasitol 126:476–481. doi:10.1016/j.exppara.2010.05.024

Liu A, Guan G, Du P et al (2013) Rapid identification and differentiation of *Theileria sergenti* and *Theileria sinensis* using a loop-mediated isothermal amplification (LAMP) assay. Vet Parasitol 191:15–22. doi:10.1016/j.vetpar.2012.08.006

Liu J, Guan G, Liu A et al (2014) A PCR method targeting internal transcribed spacers: the simultaneous detection of *Babesia bigemina* and *Babesia bovis* in cattle. Acta Parasitol Witold Stefański Inst Parasitol Warszawa Pol 59:132–138. doi:10.2478/s11686-014-0222-6

Luo J, Lu W (1997) Cattle theileriosis in China. Trop Anim Health Prod 29:4S–7S

Mehlhorn H, Raether W (1988) Effects of halofuginone lactate on lymphocytes and MDBK cells infected with *Theileria annulata* and/or vesicular stomatitis virus (VSV): an in vitro study. Parasitol Res 74: 441–447

Mohamed AM, Abdel-Rady A, Ahmed LS, El-Hosary A (2012) Evaluation of indirect TaSP enzyme-linked immunosorbent assay for diagnosis of tropical theileriosis in cattle (*Bos indicus*) and water buffaloes (*Bubalus bubalis*) in Egypt. Vet Parasitol 186:486–489. doi:10.1016/j.vetpar.2011.11.028

Rajendran C, Ray DD (2014) Diagnosis of tropical bovine theileriosis by ELISA with recombinant merozoite surface protein of *Theileria annulata* (Tams1). J Parasit Dis Off Organ Indian Soc Parasitol 38:41–45. doi:10.1007/s12639-012-0183-3

Ros-García A, Nicolás A, García-Pérez AL et al (2012) Development and evaluation of a real-time PCR assay for the quantitative detection of *Theileria annulata* in cattle. Parasit Vectors 5:171. doi:10.1186/1756-3305-5-171

Schnittger L, Katzer F, Biermann R et al (2002) Characterization of a polymorphic *Theileria annulata* surface protein (TaSP) closely related to PIM of *Theileria parva*: implications for use in diagnostic tests and subunit vaccines. Mol Biochem Parasitol 120:247–256

Seitzer U, Gerber S, Beyer D et al (2010) Schizonts of *Theileria annulata* interact with the microtubuli network of their host cell via the membrane protein TaSP. Parasitol Res 106:1085–1102. doi:10.1007/s00436-010-1747-8

Shiono H, Yagi Y, Chikayama Y et al (2003) Oxidative damage and phosphatidylserine expression of red blood cells in cattle experimentally infected with *Theileria sergenti*. Parasitol Res 89:228–234. doi:10.1007/s00436-002-0742-0

Tanaka M, Onoe S, Matsuba T et al (1993) Detection of *Theileria sergenti* infection in cattle by polymerase chain reaction amplification of parasite-specific DNA. J Clin Microbiol 31:2565–2569

Wang LX, He L, Fang R et al (2010) Loop-mediated isothermal amplification (LAMP) assay for detection of *Theileria sergenti* infection targeting the p33 gene. Vet Parasitol 171:159–162. doi:10.1016/j.vetpar.2010.02.046

Zhang X, Liu Z, Yang J et al (2014) Multiplex PCR for diagnosis of *Theileria uilenbergi*, *Theileria luwenshuni*, and *Theileria ovis* in small ruminants. Parasitol Res 113:527–531. doi:10.1007/s00436-013-3684-9