Tropical Biomedicine 33(1): 27–34 (2016)

Development and application of Real-time PCR assay for detection of *Theilera annulata* infection in cattle in Xinjiang Uygur Autonomous Region, China

Guo, Q.Y.[1], Cao, W.L.[1], Wang, B.J.[1], Wang, Z.B.[2], Wang, S.[3] and Bayin Chahan[1*]
[1]College of Veterinary Medicine, Xinjiang Agricultural University, Urumqi, Xinjiang, 830052, PR China
[2]Yili Entry-exit Inspection and Quarantine Bureau, Yining, Xinjiang, 835000, PR China
[3]Department of Human Parasitology, School of Basic Medical Science, Xinxiang Medical College, Xinxiang, Henan, 453003, PR China
[*]Corresponding author email: bynch@hotmail.com.
Received 26 March 2015; received in revised form 30 December 2015; accepted 31 December 2015

Abstract. *Theileria annulata* (*T. annulata*), the causative agent of tropical theileriosis, is a protozoan parasite that also causes lymphoproliferative diseases in cattle. Development of reliable and fast methods are necessary in the epidemiological investigation of *T. annulata* in ticks and animals. Real-time PCR possesses merits of rapidity, accuracy, reliability, automation and ease of standardization, which is widely used for the detection of blood borne parasites. In this study, species-specific primers and *Taq*Man probe were designed on the basis of the 18s rRNA gene sequence of *T. annulata*, and the real-time PCR assay was developed by optimizing the reaction parameter. The performance of real-time PCR was assessed by testing 47 blood samples from cattle and comparing with the results from conventional PCR. The results show that this real-time PCR assay could specifically detect 10 copies DNA of *T. annulata*, which is 10-fold sensitivity more than conventional PCR. No cross-reactions were observed with *Babesia bovis*, *Babesia bigemina*, *Trypanosoma evansi* and *Theileria equi*. Of the 47 field samples collected from Xinjiang Uygur Autonomous Region, China, 36.17% were detected by real-time PCR, and 25.53% were found positive for *T. annulata* infection by conventional PCR. These results indicated that the real-time PCR assay is a useful approach for detecting *T. annulata* infections and has potential as an alternative tool for ecological and epidemiological surveillance of ovine theileriosis.

INTRODUCTION

Tropical theileriosis is caused by a tickborne parasite *Theileria annulata* (Mhadhbi *et al.*, 2010). *T. annulata* is one of the major protozoan parasites in cattle, and is common in North Africa, Southern Europe, India, the Middle East, and Central Asia where its *Hyalomma* tick vector is endemic (Taha *et al.*, 2013; Weir *et al.*, 2011). In China, *T. annulata* is the most virulent of the four reported bovine *Theileria* species and is mainly distributed in semi-dry and desert grasslands in Northern China (13 provinces), which include Xinjiang, Gansu, Ningxia, Inner Mongolia, Shaanxi, Shanxi, Heilongjiang, Jilin, Liaoning, Hebei, Shandong, Henan and northern areas of Hubei (Luo and Lu, 1997).

In Xinjiang Uygur Autonomous Region, the infection rate of *T. annulata* in cattle was up to 65%, and the mortality rate of acute theileriosis cases was 30%-50% (Meng *et al.*, 2014). It is therefore important to develop a new method for early diagnosis and disease surveillance, and eliminating persistently infected cattle, which is one of the effective measures to prevent and control the outbreaks of this disease.

Field diagnosis is normally achieved by observing clinical signs in infected animals. Theileriosis caused by *T. annulata* is indicated by a fever, enlarged lymph nodes,

and is associated with tick vector infestation. In addition, acute disease with a high mortality rate on farms without effective tick control may also indicate theileriosis. The disease can be confirmed by finding *Theileria* parasites in Giemsa-stained blood smears and lymph node fine needle aspirate smears (OIE, 2008). This method is useful in the detection of acute cases but has limited value in carrier cases, where low numbers of erythrocytes remain infected with piroplasms. There have been numerous serological diagnostic assays, such as the indirect fluorescence antibody test (Billiouw *et al.*, 2005), the indirect ELISA (Rajendran and Ray, 2014) and the cELISA (Renneker *et al.*, 2009; Renneker *et al.*, 2008). IFAT is knowingly restricted due to a subjective interpretation, low throughput and inherent cross-reactivity problems (Billiouw *et al.*, 2005). An ELISA that uses a defined recombinant *T. annulata* antigen appears to perform better than IFAT and shows good sensitivity and specificity (Rajendran and Ray, 2014; Renneker *et al.*, 2009; Renneker *et al.*, 2008). However, serological assays are unable to differentiate between current and past infections due to the persistence of antibodies.

PCR has been used for the sensitive and specific detection of *T. annulata* (Bilgic *et al.*, 2010; Bilgic *et al.*, 2013; Meng *et al.*, 2014; Orkun *et al.*, 2012; Ros-Garcia *et al.*, 2012). However, few of them allow reliable quantification of the parasite. Here, we described a real-time PCR assay that can be used for both the detection and the quantification of *T. annulata*.

MATERIALS AND METHODS

Ethics statement

The collection of animal blood samples was approved by the Ethical Committee of the College of Animal Medicine, Xinjiang Agricultural University, China (Approved No. 2011011).

Parasite isolates and DNA samples

Theilera annulata strain was isolated from blood of naturally infected cows and conserved in Laboratory of Veterinary Parasitology, Xinjiang Agricultural University College, China. DNA samples of *Babesia bovis*, *Babesia bigemina*, *Trypanosoma evansi*, *Theileria equi*, *Theileria sergenti* were supplied by Yili Entry-Exit Inspection and Quarantine Bureau.

Field blood specimens and DNA extraction

Forty seven blood samples were randomly collected from cattle in the affected areas in Xinjiang Uygur Autonomous Region. DNA was extracted from 300 ìL of cattle blood using a Gentra Puregene Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA of each sample was eluted in 100 µL DNA hydration solution and stored at -20°C.

Specific primers and probe

The 18S rRNA gene sequences of *T. annulata* and other *piroplasm* species available in GenBank were aligned and a pair of primers (TAF:5'- AATTTCTGCTGCATTGCTTG-3'; TAR:5'-GTAAACATTCAGACAAAGAAACT CC-3'), were designed to amplify a 83 bp fragment. A TaqMan probe specific for *T. annulata* was designed (TAP: 5'- CCTCT GGGGTCTGTGCATGTGG-3') and synthetized by Shanghai Yingjun biotechnological Co. Ltd (Invitrogen, Shanghai, China).

Conventional PCR for *T. annulata* 18S rRNA gene

Specific primers were TAF and TAR, which generated a product of 83 bp. PCR amplifications were performed in a final volume of 25 µL, containing 1 µL DNA template, 2.5 µL of 10xPCR Buffer (Mg^{2+} free), 4 µL of 25mM $MgCl_2$, 2 µL of 2.5 mM dNTPs, 0.5 µL of rTaq DNA polymerase (5U/ µL) (TaKaRa, Dalian, China), 1 µL of a 10 µM solution of each primer. PCR was carried out in a GeneAmp PCR system 9700 (Applied

Biosystems, California, USA). The cycling program was as follows: 95ºC for 5 min, 30 cycles (95ºC for 45 s, 57ºC for 45 s, 72ºC for 30 s), followed by a final extension step of 72ºC for 8 min. The PCR products were subjected to electrophoresis on 1% agarose gels containing 0.5 µg/ml ethidium bromide and visualized under UV light.

Preparation of plasmid standards

For construction of a plasmid containing *T. annulata* 18S rRNA gene, the PCR product of 83 bp was cloned into pXT 19-T vector (Gen-View Scientific Inc, Wellington, FL, USA) and then propagated in competent Escherichia coli DH5a cells (TransGen Biotech, Beijing, China). Plasmid DNA was purified from transformed cells using EZNA TM plasmid miniprep kit (Omega, Doraville, GA, USA) and quantified by NanoDrop 2000 Spectrophotometer (ThermoScientific, Beijing, China). Copy numbers of the cloned 18S rRNA gene were derived from the molecular weights of the cloning vector and insert. To generate standard curves for quantitative determinations and to assess the amplification efficiency, 10-fold dilutions of the plasmid by Easy Dilution Solution (TaKaRa, Dalian, China) were made, representing $1.26x10^0$–$1.26x10^8$ copies/µL of DNA template. Aliquots of each dilution were frozen at -20°C until use.

Real-time PCR

The real-time PCR assay was carried out in a 25 µL reaction mix containing 1 µL of the forward and reverse primers (10 µM), 0.75 µL of the fluorescein-labelled probe (10 µM), 2 µL of dNTPs (2.5 mM each), 2.5 µL of 10xPCR Buffer (Mg^{2+} free), 4 µL of $MgCl_2$ (25 mM), 0.5 µL of Taq DNA polymerase (5 U/µL, TaKaRa, Dalian, China), 0.5 µL of ROX Reference Dye II and 1 µL of template DNA. Amplification was performed on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following program: initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 15 s, 57°C for 45 s. Amplification data were collected and analyzed by the ABI 7500 instrument's software.

Evaluation of specificity, sensitivity and reproducibility

Specificity of the real-time PCR assay was evaluated by testing positive genomic DNA from *Babesia bovis*, *Babesia bigemina*, *Trypanosoma evansi*, *Theileria equi*, *Theileria sergenti*, *Theileria annulata* isolate and the negative control with sterilized water instead of template DNA were incorporated.

To evaluate the detection limits of the real-time PCR and conventional PCR, 10-fold dilutions of the standard plasmid DNA were used to test the analytic sensitivity in the range of $1.26x10^0$–1.26×10^8 copies.

Reproducibility of the assay was evaluated by testing 100-fold dilutions of the standard plasmid DNA from $1.26x10^2$–$1.26x10^8$ copies, then evaluated reproducibility by coefficient of variation (CV) of cycle threshold values, as previously described (Xu *et al.*, 2011).

Comparison of real-time and conventional PCR by testing field samples

Genomic DNA of 47 blood samples from cattle was detected using real-time PCR and conventional PCR, respectively. Positive and negative controls were were incorporated in each run.

RESULTS

Specificity of the real-time PCR

The strains of *Theilera annulata* were identified correctly with the 18S rRNA specific probes and showed specifical fluorescence signals. As expected, no fluorescence was detected and no cross-reaction was observed with the negative DNA control, sterilized water and the above-mentioned pathogens (Fig. 1).

Sensitivity of the real-time PCR and the standard curve

The standard curve obtained ($y = -3.387 \text{Log}(X) + 41.30$, $R^2 = 0.999$) showed that there was a good linear relationship between the log of the plasmid DNA copy number and the

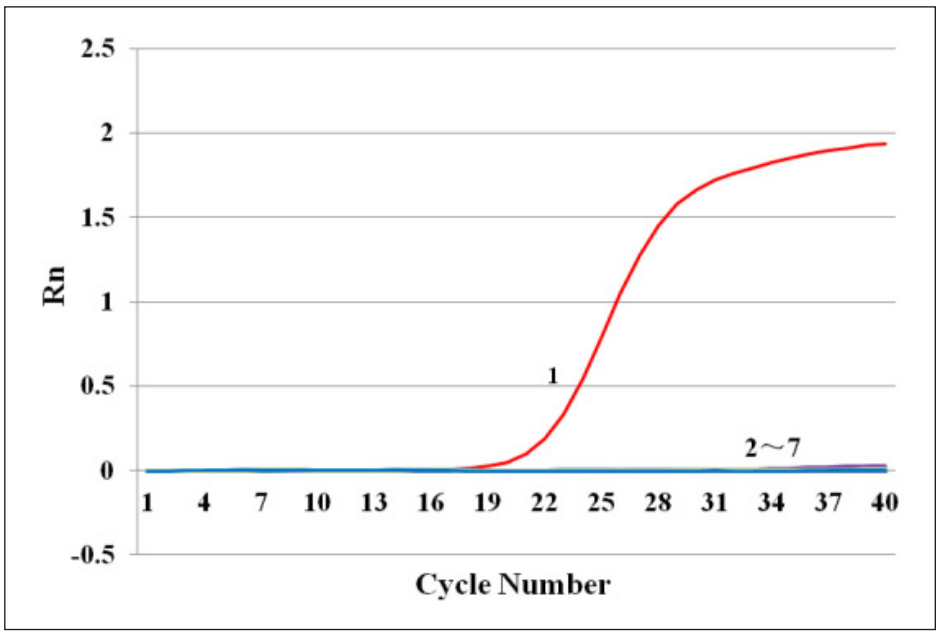

Figure 1. The specificity of the real-time PCR. The template DNA was genomic DNA of *Theilera annulata* (1), *Babesia bovis* (2), *Babesia bigemina* (3), *Trypanosoma evansi* (4), *Theileria equi* (5), *Theileria sergenti* (6), and sterilized water (7) as blank control.

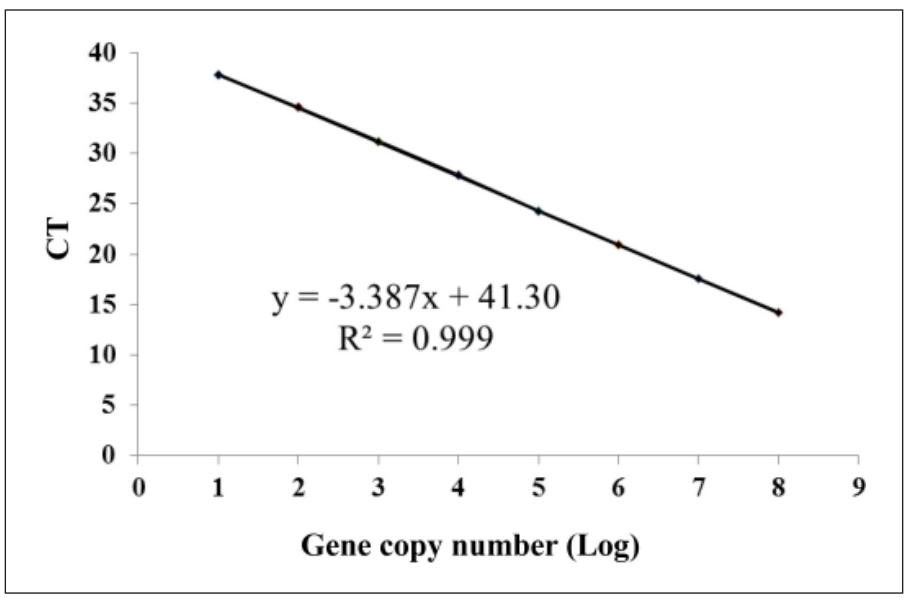

Figure 2. Standard curve of real-time PCR. It showed DNA amplification plots with cycle threshold (CT) values plotted against the logarithm of the input copy number.

calculated cycle threshold (CT) value across the specified concentration range (Fig. 2).

The detection limits of the real-time PCR assay in serial dilutions of the standard plasmids were as low as $1.26x10^1$ copies for *Theilera annulata* (Fig. 3). In contrast, the conventional PCR could only detect minimal $1.26x10^2$ copies for *Theilera annulata* (Fig. 4). The real-time PCR was more sensitive than the conventional PCR.

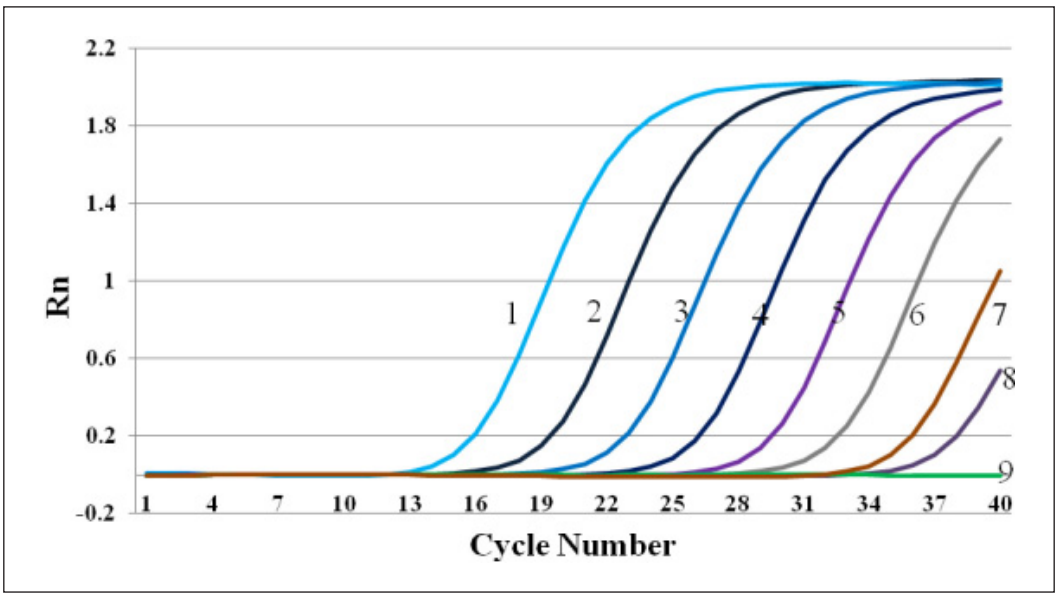

Figure 3. Amplification plots of real-time PCR for detecting standard plasmids containing the 18S rRNA gene. The copy number of standard plasmid ranged from $1.26x10^8$ to $1.26x10^0$ (plot 1 to 9).

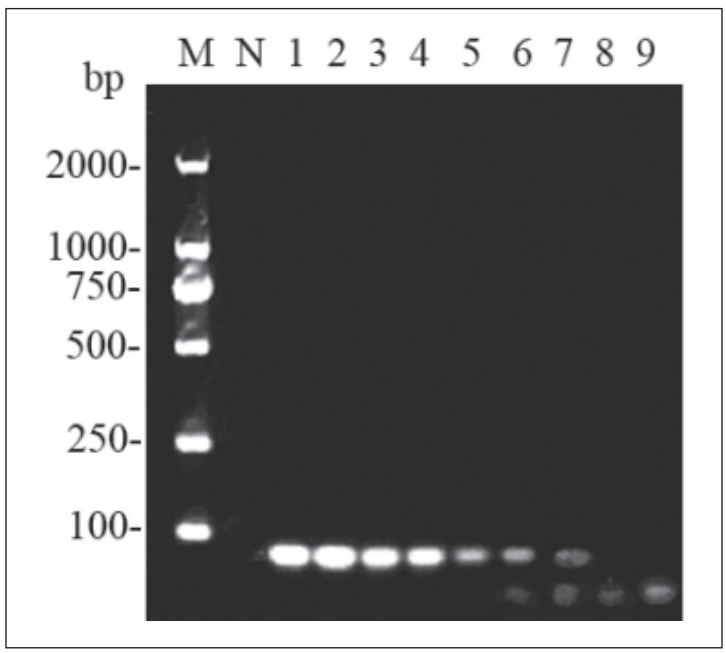

Figure 4. Sensitivity of conventional PCR. Lanes 1-9 represented standard plasmid dilutions ($1.26x10^8$ to $1.26x10^0$ copies) and N represented no-DNA control. M represented DNA size marker.

Reproducibility of the real-time PCR

The assay was tested for both intra- and inter-assay reproducibility. For intra-assay reproducibility, 100-fold dilutions of the standard plasmid DNA from $1.26x10^2$–$1.26x10^8$ copies were tested four times in one run. Coefficient of variation values (CV) were found to range from 0.26% to 0.85%

Table 1. Intra-assay and inter-assay reproducibility of real-time PCR for detecting *T. annulata* standard plasmid

Copies/µL	Intra-assay				CV (%)	Inter-assay				CV (%)
	CT 1	CT 2	CT 3	CT 4		CT 1	CT 2	CT 3	CT 4	
$1.26x10^2$	34.94	34.28	34.59	34.36	0.85	34.78	34.54	34.61	34.64	0.36
$1.26x10^4$	27.82	28.03	27.95	27.70	0.52	28.14	27.88	27.86	27.96	0.56
$1.26x10^6$	21.08	20.84	21.11	21.10	0.62	20.96	21.03	20.95	20.98	0.21
$1.26x10^8$	14.30	14.22	14.23	14.25	0.26	14.30	14.25	14.22	14.26	0.30

CT, cycle threshold; CV, coefficient of variation.

Table 2. Comparative evolution of the real-time and conventional PCR methods for detection of field samples

qPCR	Conventional PCR		
	Positive	Negative	Total
Positive	12	5	17 (36.17%)
Negative	0	30	30
Total	12 (25.53%)	35	47

(Table 1). For inter-assay reproducibility, 100-fold dilutions of the standard plasmid DNA ($1.26x10^2$-$1.26x10^8$) were tested separately on 3 times, CV values ranged from 0.21% to 0.56% (Table 2).

Comparison of the real-time and conventional PCR by testing field samples

Real-time PCR showed that 17 of 47 (36.17%) blood samples were positive for *T. annulata*. In comparison, the positive rate using conventional PCR was 25.53% (12/47). The 12 positive samples were also positive for real-time PCR. The prevalence with the real-time PCR was significantly higher than with the conventional PCR ($X^2 = 24.25$, $P < 0.05$). In the 17 real-time PCR positive samples that were 5 blood samples negative by conventional PCR (Table 3). These results indicated the high sensitivity of real-time PCR compared with conventional PCR.

DISCUSSION

Development of reliable and fast methods is necessary in the epidemiological investigation of *T. annulata* in ticks and animals. Real-time PCR possesses merits of rapidity, accuracy, reliability, automation and ease of standardization, which is widely used for the detection of blood borne parasites. Therefore, in the present study, we developed a real-time PCR assay for the detection of *T. annulata*.

The selection of a suitable target gene for the accurate determination of animal infection status is crucial for the development of real-time PCR for pathogens. In the present study, the real-time assay primers were designed on the basis of the 18S rRNA gene of *T. annulata*.

Ribosomal RNA is the most abundant constituent of nucleic acids in any non-viral organism with the eukaryotic RNA transcription unit consisting of the large and small subunit (18S rRNA) and the 5.8S rRNA gene (Gubbels *et al.*, 2002). The 18S rRNA gene is increasingly accepted as a widely used marker for characterization, taxonomic classification, and phylogenetic analysis and this gene has been sequenced from a variety of different organisms, resulting in a large database for sequence comparisons (Chae *et al.*, 1999; Chaisi *et al.*, 2013; Khan *et al.*, 2013; Liu *et al.*, 2012a; Liu *et al.*, 2012b; Peckle *et al.*, 2013; Ros-Garcia *et al.*, 2012).

The conserved function and structure of the 18S rRNA molecule allow sequences to be aligned, even among divergent species. After alignment of the 18S rRNA gene sequences from 9 *T. annulata* isolates from different countries, the sequence identity was confirmed very high, from 99.2% to 100% (Habibi, 2012). Using such a conserved gene as a target for real-time PCR is critical. This ensured the specificity of the diagnostic method.

The sensitivity and linear detection range of the newly developed qPCR assay were found to be high and broad enough to reliably detect and quantify as little as 12.6 target gene copies per reaction tube, while the conventional PCR could only detect minimal 126 copies. Meanwhile, real-time PCR detected 17 positive samples in 47 field samples, five of them were negative with conventional PCR, also indicating the high sensitivity of the real-time PCR compared with the conventional PCR.

Regarding the specificity of the assay, the real-time PCR in our study did not show any cross-reactions with the DNA from closely related organisms, including *Babesia bovis*, *Babesia bigemina*, *Trypanosoma evansi*, *Theileria equi*, *Theileria* isolate, which indicated the high specificity of the real-time PCR.

The intra-assay and inter-assay CVs were satisfactorily low (0.26–0.85% and 0.21–0.56%, respectively), indicating high reproducibility of the established real-time PCR.

In conclusion, we developed a real-time PCR method that was sensitive and specific for the detection of *T. annulata*. It could be used for the molecular epidemiology investigation of theileriosis in bovine, which will help to prevent and control theileriosis.

Acknowledgements. This work was supported by the Joint Funds of the National Natural Science Foundation of China and Xinjiang Uygur Autonomous Region of China (No. U1503188 & U1170301), the International Science & Technology Cooperation Program of Xinjiang Uygur Autonomous Region, China (No. 20126008), the Scientific Research Program of the Higher Education Institution of XinJiang (No. XJEDU2013I18) and the Doctoral Scientific Research Activation Foundation of Xinxiang Medical University (No. XYBSKYZZ201504).

REFERENCES

Bilgic, H.B., Karagenc, T., Shiels, B., Tait, A., Eren, H. & Weir, W. (2010). Evaluation of cytochrome b as a sensitive target for PCR based detection of *T. annulata* carrier animals. *Veterinary Parasitology* **174**: 341-347.

Bilgic, H.B., Karagenc, T., Simuunza, M., Shiels, B., Tait, A., Eren, H. & Weir, W. (2013). Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. *Experimental Parasitology* **133**: 222-229.

Billiouw, M., Brandt, J., Vercruysse, J., Speybroeck, N., Marcotty, T., Mulumba, M. & Berkvens, D. (2005). Evaluation of the indirect fluorescent antibody test as a diagnostic tool for East Coast fever in eastern Zambia. *Veterinary Parasitology* **127**: 189-198.

Chae, J.S., Allsopp, B.A., Waghela, S.D., Park, J.H., Kakuda, T., Sugimoto, C., Allsopp, M.T., Wagner, G.G. & Holman, P.J. (1999). A study of the systematics of *Theileria* spp. based upon small-subunit ribosomal RNA gene sequences. *Parasitology Research* **85**: 877-883.

Chaisi, M.E., Janssens, M.E., Vermeiren, L., Oosthuizen, M.C., Collins, N.E. & Geysen, D. (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.

Gubbels, M.J., Yin, H., Bai, Q., Liu, G., Nijman, I.J. & Jongejan, F. (2002). The phylogenetic position of the *Theileria buffeli* group in relation to other *Theileria* species. *Parasitology Research* **88**: S28-32.

Habibi, G. (2012). Phylogenetic Analysis of *Theileria annulata* Infected Cell Line S15 Iran Vaccine Strain. *Iranian Journal of Parasitology* **7**: 73-81.

Khan, M.K., He, L., Hussain, A., Azam, S., Zhang, W.J., Wang, L.X., Zhang, Q.L., Hu, M., Zhou, Y.Q. & Zhao, J. (2013). Molecular epidemiology of *Theileria annulata* and identification of 18S rRNA gene and ITS regions sequences variants in apparently healthy buffaloes and cattle in Pakistan. *Infection, Genetics and Evolution* **13**: 124-132.

Liu, A., Guan, G., Du, P., Gou, H., Liu, Z., Liu, J., Ma, M., Yang, J., Li, Y., Niu, Q., Ren, Q., Bai, Q., Yin, H. & Luo, J. (2012a). Loop-mediated isothermal amplification (LAMP) method based on two species-specific primer sets for the rapid identification of Chinese *Babesia bovis* and *B. bigemina*. *Parasitology International* **61**: 658-663.

Liu, A., Guan, G., Du, P., Liu, Z., Gou, H., Liu, J., Yang, J., Li, Y., Ma, M., Niu, Q., Ren, Q., Bai, Q., Yin, H. & Luo, J. (2012b). Loop-mediated isothermal amplification (LAMP) assays for the detection of *Theileria annulata* infection in China targeting the 18S rRNA and ITS sequences. *Experimental Parasitology* **131**: 125-129.

Luo, J. & Lu, W. (1997). Cattle theileriosis in China. *Tropical Animal Health and Production* **29**: 4S-7S.

Meng, K., Li, Z., Wang, Y., Jing, Z., Zhao, X., Liu, J., Cai, D., Zhang, L., Yang, D. & Wang, S. (2014). PCR-based detection of *Theileria annulata* in Hyalomma asiaticum ticks in northwestern China. *Ticks and Tick-borne Diseases* **5**: 105-106.

Mhadhbi, M., Naouach, A., Boumiza, A., Chaabani, M.F., BenAbderazzak, S. & Darghouth, M.A. (2010). *In vivo* evidence for the resistance of *Theileria annulata* to buparvaquone. *Veterinary Parasitology* **169**: 241-247.

OIE, A. (2008). Manual of diagnostic tests and vaccines for terrestrial animals. *Office International des Epizooties, Paris, France*: 1092-1106.

Orkun, O., Deniz, A. & Guven, E. (2012). Survey of *Theileria annulata* and *Theileria buffeli/orientalis* complex in cattle in the Kirsehir region using multiplex-PCR. *Acta Parasitologica Turcica* **36**: 9-11.

Peckle, M., Pires, M.S., Dos Santos, T.M., Roier, E.C., da Silva, C.B., Vilela, J.A., Santos, H.A. & Massard, C.L. (2013). Molecular epidemiology of *Theileria equi* in horses and their association with possible tick vectors in the state of Rio de Janeiro, Brazil. *Parasitology Research* **112**: 2017-2025.

Rajendran, C. & Ray, D.D. (2014). Diagnosis of tropical bovine theileriosis by ELISA with recombinant merozoite surface protein of *Theileria annulata* (Tams1). *Journal of Parasitic Diseases* **38**: 41-45.

Renneker, S., Abdo, J., Ahmed, J.S. & Seitzer, U. (2009). Field validation of a competitive ELISA for detection of *Theileria annulata* infection. *Parasitology Research* **106**: 47-53.

Renneker, S., Kullmann, B., Gerber, S., Dobschanski, J., Bakheit, M.A., Geysen, D., Shiels, B., Tait, A., Ahmed, J.S. & Seitzer, U. (2008). Development of a competitive ELISA for detection of *Theileria annulata* infection. *Transboundary and Emerging Diseases* **55**: 249-256.

Ros-Garcia, A., Nicolas, A., Garcia-Perez, A.L., Juste, R.A. & Hurtado, A. (2012). Development and evaluation of a real-time PCR assay for the quantitative detection of *Theileria annulata* in cattle. *Parasites & Vectors* **5**: 171.

Taha, K.M., Salih, D.A., Ali, A.M., Omer, R.A. & El Hussein, A.M. (2013). Naturally occurring infections of cattle with *Theileria lestoquardi* and sheep with *Theileria annulata* in the Sudan. *Veterinary Parasitology* **191**: 143-145.

Weir, W., Karagenc, T., Gharbi, M., Simuunza, M., Aypak, S., Aysul, N., Darghouth, M.A., Shiels, B. & Tait, A. (2011). Population diversity and multiplicity of infection in *Theileria annulata*. *International Journal for Parasitology* **41**: 193-203.

Xu, Z.Q., Cheng, W.X., Li, B.W., Li, J., Lan, B. & Duan, Z.J. (2011). Development of a real-time PCR assay for detecting and quantifying human bocavirus 2. *Journal of Clinical Microbiology* **49**: 1537-1541.