

Qualitative and quantitative assessment of *Theileria annulata* in cattle and buffaloes Polymerase Chain Reaction

Kundave, V.R.¹, Patel, A.K.², Patel, P.V.¹, Hasnani, J.J.¹ and Joshi, C.G.^{2*}

¹Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, AAU, Anand, Gujarat- 388001, India

²Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, AAU, Anand, Gujarat- 388001, India

*Corresponding author email: cgjoshi@aau.in

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Abstract. Bovine tropical theileriosis caused by *Theileria annulata* is a tick-borne disease associated with high morbidity and mortality in the livestock. The conventional method of diagnosis is by the demonstration of the parasite stages by microscopic examination. This method suffers from low sensitivity, making it even more difficult to detect piroplasm in the carriers. PCR based assays are known to be more sensitive. The present study was undertaken to detect and quantify *T. annulata* in the blood of clinically infected and carrier animals using a quantitative PCR protocol targeting the gene encoding the major merozoite piroplasm surface antigen Tams 1. A total of 116 samples were collected from infected as well as apparently healthy cattle and buffaloes. Of these, 74 samples (63.79%) were positive for *T. annulata* by real-time PCR, including the 15 samples that were positive by Giemsa staining. The parasite load ranged from 1.39×10^6 to 3.35×10^9 and 0.35×10^6 to 2.83×10^7 ml⁻¹ of blood in cattle and buffalo samples, respectively by qPCR. Our study suggests that real-time PCR assay can be used to detect and quantify the load of *T. annulata* in the blood of cattle and buffaloes. It also serves as a support to clinical diagnosis and assessment of carrier status in apparently healthy animals.

INTRODUCTION

Tropical and subtropical regions are prone to tick and tick-borne diseases, which cause a great economic loss due to its impact on the productivity and health of livestock (Uilenberg, 1995). The domestic and wild ruminants in Africa, Europe, Australia and Asia are usually affected by *Theileria* species (Allison & Meinkoth, 2010). Ticks, apart from being involved in disease transmission are also known to cause paralysis or toxicosis and physical damage to the livestock (Rajput *et al.*, 2005). Tropical bovine theileriosis, caused by *Theileria annulata*, poses a great threat to the livestock. Upgradation of the indigenous breeds was the main idea behind the

adaptation of large scale cross-breeding programmes in India, which eventually lead to increase in the milk production and decreased resistance to theileriosis (Anandan, 1992). The outcome of theileriosis may range from clinically inapparent to rapidly fatal disease (Taylor *et al.*, 2007). The common clinical signs noticed in *T. annulata* infection are pyrexia, anorexia, enlargement of superficial lymph nodes, salivation, nasal and ocular discharge (Osman & Al-Gaabary, 2007). Adverse effects of the disease are more prominent in the crossbreds than the indigenous population. The inefficient tick control practices provide little chances of rearing exotic and crossbred cattle free from infection (Mehta *et al.*, 1988). Tentative

diagnosis of theileriosis in the field is primarily based on clinical signs and history of tick infestation, and is confirmed by demonstration of parasite stages in Giemsa stained blood and lymph node smears (Aktas *et al.*, 2006). Post-recovery phase is characterized by the presence of low number of piroplasm infected erythrocytes and such animals can act as carriers. The widely used blood smear staining method could be unreliable in such cases, and so the need for special diagnostic techniques arises (d'Oliveira *et al.*, 1995).

The advent of real-time PCR has revolutionized the detection of pathogens in clinical specimens. The simultaneous amplification of the target sequence and analysis of the products within a single instrument is made possible by incorporating fluorescent probes or dyes into the PCR reaction mix which obviate the need for subsequent gel electrophoresis. In addition, it also allows continuous monitoring of emissions from the fluorescent reagent corresponding to amplification of target hence a *in situ* amplification of the target (Zarlenga and Higgins, 2001). The molecular techniques such as quantitative Polymerase Chain Reaction (qPCR) to estimate genome copy alleviate the need for microscopic calculations. The real-time assay is admired for its reduced requirement for specialist parasitological knowledge, speed, specificity, discriminatory power, objectivity, high throughput and automation potential (Dhinakar Raj *et al.*, 2013). Jeong *et al.* (2003) had carried out TaqMan based PCR assay to detect and quantify *T. sergenti* in which the 33KDa protein gene was amplified. He could detect a minimum level of 0.00005% parasitaemia and concluded this test to be sensitive. Criado-Fornelio (2007) developed a SYBR Green based PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina* and a minimum of 1000 copies of template DNA were detected. Santos *et al.* (2013) designed a novel primer set that targeted the conserved regions of Tams 1 gene of *T. annulata* to carry out the realtime assay, in which the specificity and the reliability of the assay have led to the conclusion that

Tams 1 gene can be used as a species specific target for the detection of *T. annulata*. In this study, a quantitative real-time PCR assay system was developed using a pair of primers specific to the Tams1 gene of *T. annulata* for accurate diagnosis and to detect the pathogen load in clinically affected as well as asymptomatic carrier animals.

MATERIALS AND METHODS

Collection of blood samples

A total of 116 (91 from crossbred cows and 25 from indigenous buffaloes) blood samples (3 ml blood in EDTA coated vacutainers) were collected from animals showing clinical signs arousing suspicion of theileriosis like anorexia, pyrexia, lymphnode enlargement, emaciation, depressed rumination, lacrimation, nasal discharge, terminal dyspnoea, frothy nasal discharge and also from apparently healthy ones. Blood smears were also prepared from all the animals and stained with Giemsa stain.

Isolation of DNA from blood samples

Genomic DNA was extracted from 200 μ l of blood samples arousing suspicion of theileriosis like anorexia, pyrexia, lymphnode enlargement, emaciation, depressed rumination, lacrimation, nasal discharge, terminal dyspnoea, frothy nasal discharge and also from apparently healthre stored at -20°C until further use.

Preparation of DNA standard for absolute quantification by qPCR

Plasmid DNA containing the respective target DNA sequence was used as DNA standard in qPCR. The target DNA was amplified using the species-specific primer sets 8 THLAN F 5'CCAGGACCACCCCTCAA GTTC3' and 437 THLAN R 5'GCATCTAGTT CCTTGGCGGA3' designed based on the coding sequence of the major merozoite piroplasm surface antigen of *T. annulata* (Tams 1) gene. After confirmation of the expected size amplification (430 bp) on an agarose gel, the PCR products were excised from the gel, purified using the QIAprep spin

miniprep kit (Qiagen, Valencia, CA, USA) and cloned in pTZR57T/A cloning vector using InsTAclone PCR cloning kit (Fermentas, UK). The recombinants were screened by colony PCR with respective primer sets and plasmids from positive clones were purified using a QIAprep spin miniprep kit (Qiagen, USA). The cloned insert was further verified by sequencing using vector specific M13 forward and reverse primers. The concentration of the plasmid was determined with the Nanodrop spectrophotometer.

Copy number of each standard plasmid was calculated using formula; Copy Number/ lony PCR with respective primer sets and plasmids ²³ / length of recombinant plasmid (bp) x 660, (660= Molecular weight of one basepair, 6.022×10^{23} = Avogadrof recombinant plasmid (bp) x 660, (660 = Molecular we⁹ to 10¹ copies were prepared for each target. Real-time PCR was performed with ABI 7500 FAST real time PCR system (Applied Biosystems, USA) using QuantiFast SYBR green PCR mastermix (Qiagen, USA). The 10 fold dilution series of the plasmid carrying cloned Tams 1 gene fragment was run along with the genomic DNA extracted from the samples in triplicate. The amplification reactions were performed in a total volume of 15 μ L, containing 1 μ L of template DNA,

7.5 μ L of 2X SYBR Green master mix, 0.5 μ L of each primer (10 pmol / μ L) and 5.5 μ L of sterile H₂O. The cycling conditions consisted of initial denaturation step at 95with ABI 75n, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The normalized fluorescence data were converted to a log scale and the threshold was determined to calculate the threshold cycle value (*Ct*; the cycle at which the threshold line crosses the amplification curve). Upon completion of real-time PCR run, data were automatically analyzed for melt curve and quantification by 7500 system Sequence Detection Software (SDS). The amplicons were subjected to sequencing for the confirmation of *T. annulata*.

RESULTS

Giemsa stained blood smears from 15 (12.93%) animals, suspected to be suffering from theileriosis, revealed the presence of *Theileria* piroplasms (Figure 1). Of the total of 15 samples that were positive by staining, an average of 1% parasitaemia (10 in 1000 RBC'S infected) was recorded.

The real-time assay could detect *T. annulata* in 74 out of 116 samples, which included 66 and 8 blood samples from cattle and buffaloes, respectively. The parasite load of *T. annulata* in the positive samples

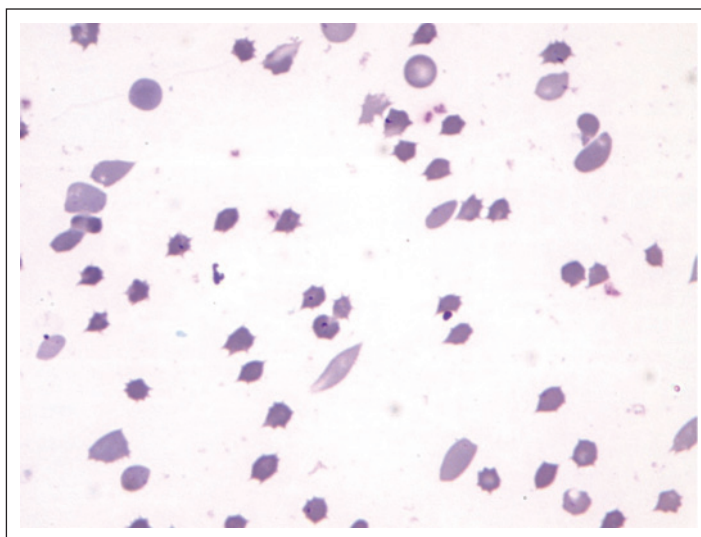


Figure 1. Piroplasmic forms of *Theileria annulata* in a microscopic field by Giemsa staining method.

of cattle ranged from 1.39×10^6 to 3.35×10^9 ml^{-1} and that of the buffalo samples ranged from 0.35×10^6 to 2.83×10^7 ml^{-1} , indicating the sensitivity of the diagnostic assay and determination of degree of infection in the infected as well as carrier animals. Figure 2 represents the histogram depicting the parasite load, frequency and degree of infection, in which 11 cattle were asymptomatic but had shown the presence of *Theileria* parasites in the blood by realtime PCR. The number of cattle with degree of infection +, ++, +++, ++++ were 12, 22, 13 and 8, respectively. The grouping of animals based on clinical signs and parasite load is shown in Table 1, which shows that the carrier cattle had low level of parasitaemia and were clinically uninfected, while the clinically positive animals had a higher quantity of parasite. Additionally, the degree of clinical signs was significantly

correlated with the load of parasites in the blood as revealed by the Kruskal-Wallis test.

The detection limit of the assay was assessed to be 10^1 DNA copies of plasmid template. Ten-fold dilutions of standard plasmid DNA were tested and used to construct the standard curve by plotting the plasmid copy number logarithm against the measured Ct values. The threshold value for positive sample was 357 organisms μl^{-1} of blood. The generated standard curve covered a linear range of nine orders of magnitude (from 10^1 to 10^9 copies of standard DNA) and showed linearity over the entire quantification range (slope = -3.456), providing an accurate measurement over a very large variety of starting target amounts. Melt curve analysis of the standard plasmid DNA showing the specificity of amplification is shown in Figure 3.

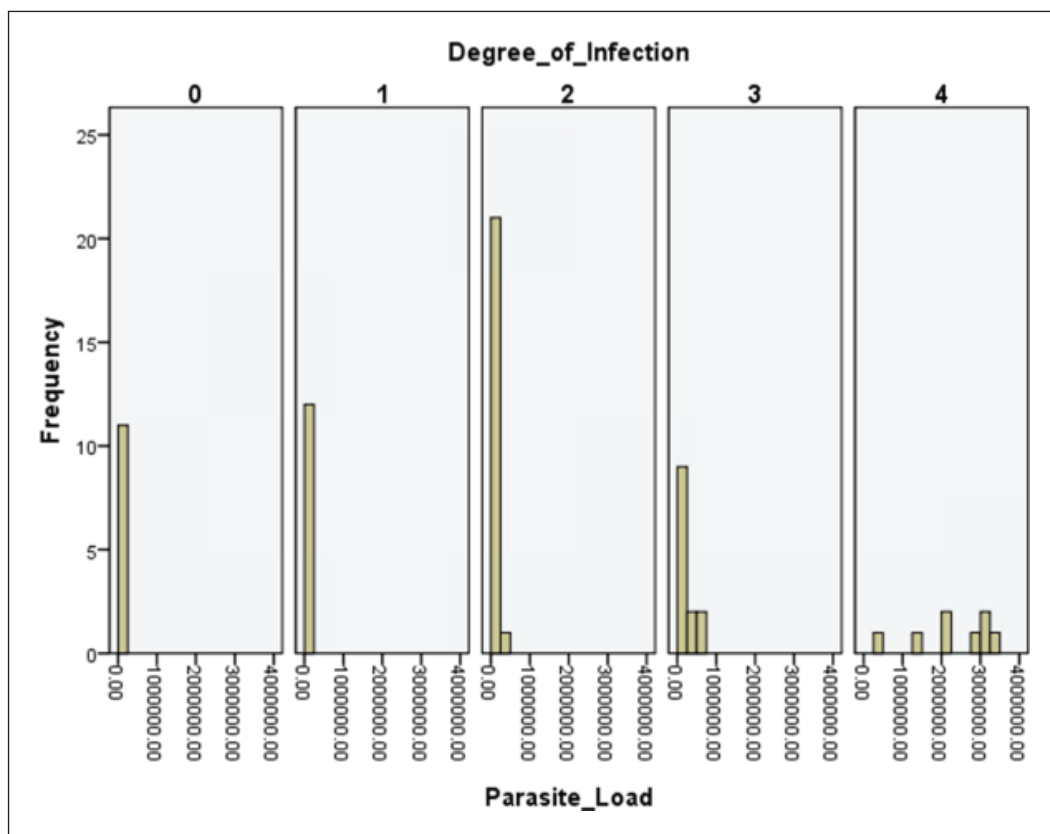


Figure 2. Histogram showing parasite load (X-axis), frequency (Y-axis) and the degree of infection. The degree of infection 0, 1, 2, 3, 4 corresponds to asymptomatic, +, ++, +++, ++++ with respect to the clinical signs exhibited.

Table1. Grouping of animals based on the clinical signs with the range of parasite load

Degree of infection	Clinical signs	Parasite load (Nx10 ³) organisms /ad (Nx10 ani)
0	Asymptomatic	1.39 – 3.61
1 (+)	Inappetence, slight lymphnode enlargement, reduction in milk yield	5.32 – 28.28
2 (++)	1 + Pyrexia, pale mucous membrane, salivation, depressed rumination, Anorexia	27.28 – 401.02
3 (+++)	2 + severe enlargement of cervical lymphnodes, bruxism, bilateral nasal discharge, lacrimation, emaciation, cessation of rumination, icteric and anaemic appearance of mucous membrane	104.73 – 609.59
4 (++++)	3+ Frothy nasal discharge, severe dyspnoea, open mouth breathing, hypothermia	334.66 – 3359.57

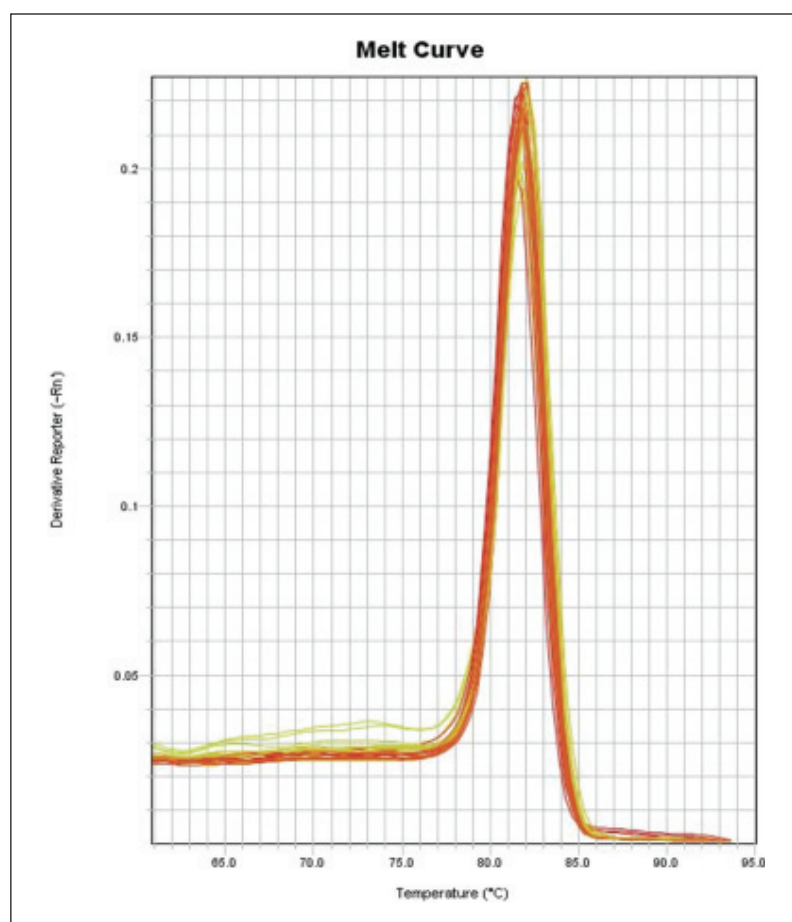


Figure 3. Melt curve analysis of the standard plasmid DNA showing the specificity of amplification.

DISCUSSION

Theileriosis infected cattle exhibit clinical signs like anorexia, emaciation, depressed rumination, lacrimation, corneal opacity, nasal discharge, diarrhea, terminal dyspnoea and frothy nasal discharge (Fukasawa *et al.*, 2003). Col & Uslu (2006) recorded pyrexia, prescapular lymphnode enlargement, inappetence, lacrimation, nasal discharge, cessation of rumination, cachexia, dyspnoea, conjunctivitis and eyeball protrusion. Similar findings of the above mentioned signs were also noticed in our study, which gives sufficient grounds to suspect for theileriosis. Absolute quantification by qPCR reveals the number of target fragments in a given sample and helps to ascertain the parasitic load in the infected hosts. Ros-Garcia *et al.* (2012), had studied the evaluation of a real-time PCR assay for the quantitative detection of *T. annulata* in carrier cattle, and had estimated parasitaemia ranging between 2.75×10^3 and 8.14×10^6 *T. annulata* ml⁻¹, with the observed mean value of 1.1×10^6 *T. annulata* ml⁻¹ blood. In our study, the carrier status detection was found to be 40% in cattle (10 out of 25 cattle) that were apparently healthy and 26.08% in buffaloes (6 out of 23 buffaloes).

A SYBR green-based real-time polymerase chain reaction assay for quantitative detection of *Babesia gibsoni* (Asian genotype) DNA, targeting the p18 gene was developed and the detection limit of the assay was found to be 9 parasites ml⁻¹ of blood (Matsuu *et al.*, 2005). Realtime qPCR assays were also developed to detect and quantify the load of Potomac horse fever agent *Neorickettsia risticii* in horses and snails. The rickettsial load was observed in the range of 1×10^4 - 9×10^6 and 3.5×10^4 - 6.8×10^8 *N. risticii* equivalents per mg leukocyte DNA and snail DNA, respectively (Pusterla *et al.*, 2000). Carelli *et al.* (2007) in a study on the detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR, highlighted that real-time assay could be used for detection and quantification of rickettsemia in carrier, pre-symptomatic and

symptomatic cattle, and for assessment of the precise correlation between levels of rickettsemia and occurrence of clinical signs as well as for evaluation of the efficacy of vaccines and also follow-up after treatment with antirickettsial drugs.

Bhoora *et al.* (2010) developed real-time PCR assay for detection of *B. caballi* and *T. equi* in horses in which it was found that the real-time assay was more sensitive than the Reverse Line Blot hybridization assay. Sibeko *et al.* (2008) developed real-time PCR for the detection of *T. parva* infection in Cape buffalo and cattle. The primers and probes were designed targeting the 18S ribosomal RNA (rRNA) gene. The assay could detect parasitaemia as low as 8.79×10^{-4} % in the carrier animals and concluded that the real-time assay required less time and was more sensitive. TaqMan probe based real-time PCR was developed for the detection of *T. parva* in the blood samples and could detect parasitaemia level as low as 2×10^{-6} % (Papli *et al.*, 2011).

The clinical signs suggestive of anaplasmosis tend to be more prominent in the acute phase of infection, which is also characterized by high levels of parasitaemia while their low level occurrence in the blood indicates the carrier status of the animal (Kieser *et al.*, 1990). The carrier cattle and buffalo had low level of the parasite in the blood and were clinically uninfected, while the clinically positive animals had a higher level of parasitaemia (Table 1).

The real-time PCR assay facilitates increased laboratory throughput, simultaneous processing of several samples and is a potential tool for laboratory diagnosis (Kieser *et al.*, 1990). Our study suggests that real-time PCR is a sensitive technique, useful for the detection and quantification of parasite load in carrier cattle. On the other hand, microscopic examination although an easy and fast technique may not be suitable for detection of carrier or chronic phases of theileriosis. The native carrier cattle are the major source for spreading the infection to healthy population, thus accurate diagnosis is the key to prevention of the disease.

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