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REGULAR ARTICLES

A duplex PCR-based assay for simultaneous detection of *Trypanosoma evansi* and *Theileria annulata* infections in water buffaloes

Vikrant Sudan[1] · **Amit Kumar Jaiswal**[1] · **Rahul Parashar**[1] · **Daya Shanker**[1]

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Abstract Trypanosomosis and bovine tropical theileriosis are important vector-borne protozoan diseases imposing some of the serious constraints on the health and productivity of domestic cattle in tropical and subtropical regions of the world. Following recovery from primary infection of both these conditions, animals become persistent carriers and act as reservoirs of infection thereby playing a critical role in disease epidemiology. The present study describes development and evaluation of duplex polymerase chain reaction (PCR) assays for simultaneous detection of *Trypanosoma evansi* and *Theileria annulata* in buffaloes. Following in silico screening for candidate target genes representing each of the pathogens, an optimized duplex PCR assay was established using TBR F/R and TAMS F/R as primer sets encoding for products of 164 and 721 bp for *T. evansi* and *T. annulata*, respectively. The results were compared and correlated with conventional Giemsa-stained thin blood smear examination and the single PCR assay. The duplex PCR detected each pathogen with the same level of sensitivity, irrespective of whether its DNA was amplified in isolation or together with DNA of another pathogen. Moreover, single and duplex PCRs were able to detect each species with equal sensitivity in serially diluted DNA representing mixtures of *T. evansi* and *T. annulata*, and no evidence of nonspecific amplification from nontarget species was observed. The developed assay may be seen as a good tool for epidemiological studies aiming at assessing the burden of dual infections and improving control of the associated diseases in endemic regions.

Keywords Buffaloes · Duplex PCR · *Theileria annulata* · *Trypanosoma evansi*

✉ Vikrant Sudan
viks.sudan@gmail.com

[1] Department of Parasitology, College of Veterinary Sciences and Animal Husbandry, U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura 281001, India

Introduction

Trypanosomosis (Surra) and theileriosis are among the most economically important diseases encountered in India. *Trypanosoma evansi* is mechanically transmitted by tabanid flies while *Theileria annulata* is transmitted mainly by the tick *Hyalomma anatolicum* (Soulsby 1982). Both of the diseases inflict significantly in terms of morbidity and mortality. Single-handedly, bovine tropical theileriosis (BTT) accounts for annual losses to the tune of US$ 800 million (Brown 1997) globally and US$ 384.3 million per annum in India alone (Minjauw and McLeod 2003); no reliable data is available on the economic impact of the *T. evansi* from India. To complicate the etiology of these diseases, both *T. annulata* and *T. evansi* infected chronic cases show almost similar types of symptoms and the arthropod vectors of these two diseases usually exist together making it prerequisite for the development of an economical, specific, and sensitive technique for simultaneous detection of these two diseases in susceptible animals. Routine diagnosis of both *T. evansi* and *T. annulata* is done by microscopic demonstration of the parasites on stained blood smears; however, the technique suffers drastically in cryptic cases of surra and latent carrier stages of BTT (Figueroa et al. 1993). The carrier state of infections with *T. annulata* is very important in the epidemiology of theileriosis and is characterized by very low levels of piroplasms in peripheral blood which are infective to ticks. So, polymerase chain reaction (PCR) remains one of the most significantly reliable tool for diagnosis

in such cases. Serological tests like indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) are capable of detecting antibodies in carrier animals and hence are routinely used for monitoring surveillance and export certification (Singh et al. 2014), but they too suffer on the grounds that antibodies can be detected even years after recovery of infection, though no active infection is prevalent, thereby obscuring the exact picture of prevalence of infection at that particular point. PCR is the most accurate tool for the diagnosis of subclinical and latent infections. Nevertheless, detection of this carrier stage is equally important as the carriers play a very critical role as reservoirs of infection and are a constant thread of introduction of a disease in a naïve demographical location. This stresses the relevance of molecular PCR-based techniques. Individual PCR assays are time consuming and expensive when applied to a large population especially when it is coinfected with a number of other species. The use of a single reaction in detection of both of these hemoprotozoans simultaneously by duplex PCR is both time and cost effective. In the present study, we describe a duplex PCR assay for simultaneous detection of clinical, subclinical, and latent carrier forms of *T. evansi* and *T. annulata* infections in water buffaloes (*Bubalis bubalis*). The test was laboratory standardized and validated on suspected animals.

Materials and methods

Collection of blood samples

Blood samples (1 ml aliquot from each animal) were collected in clean sterile vacutainers, with anticoagulant, from the jugular veins of earlier theileriosis- and trypanosomosis-confirmed water buffaloes (microscopic observation of blood smears) for isolation of positive controls for laboratory standardization of PCR assay. Later on, blood samples from 80 suspected buffaloes were also collected for validation of the PCR assay. Alongside, peripheral blood smears were also made and stained by routine Giemsa staining.

DNA isolation from whole blood and lymph node aspirate

DNA was isolated using standard phenol chloroform method with minor modifications (Pruvot et al. 2013). Briefly, 100 μl of blood/lymph node aspirate was added into a 1.5-ml tube containing 500 μl of denaturing solution (guanidinium thiocyanate) and vortexed at high speed for 5 min. Total volumes of 150 μl of chloroform and 150 μl of phenol were added and vortexed at high speed for 5–10 min. The microtube was then centrifuged at 15,493*g* for 5 min, and the supernatant was collected. Another 150 μl of chloroform and 150 μl of phenol were added to the supernatant, vortexed for 5–10 min, and centrifuged at 15,493*g* for 5 min. A volume of 400 μl of supernatant was collected, and 1 ml of absolute ethanol was added into a 1.5-ml tube, and the sample was left to precipitate at −20 °C overnight. After 10 min of centrifugation at 15, 493*g*, the pellet was washed with 75 % alcohol twice. The pellet was finally air dried before resuspension into 50 μl of TE buffer (Tris, EDTA). Thus, the final prepared sample was concentrated 2:1 compared to the initial blood sample.

Primer selection

Oligonucleotide primers used for duplex PCR targeted the mini-chromosome satellite DNA of *T. evansi* (TBR F/R) and merozoite surface antigen of *T. annulata* (TAMS F/R), respectively. The primers (TBR F/R) were custom synthesized and coded for a highly repeated sequence of mini-chromosome satellite DNA (Masiga et al. 1992) which is regarded as the best technique for PCR-based detection of *Trypanozoon* DNA. Alongside, primers TAMS F/R were also custom synthesized using a sequence published elsewhere (d'Oliveira et al. 1995). Details for primer design including position of nucleotides, nucleotide sequences, and expected PCR products are shown in Table 1.

Generation and visualization of duplex PCR amplification

The PCR reactions were set up into 25 μl volume containing 12.5 μl PCR Master Mix (0.05/μl Taq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 1.5 μl of each primer (TBR F/R and TAMS F/R, 20 pmol of each primer), and 2 μl of the extracted DNA template, and the total volume was made up to 25 μl using nuclease-free water. The details of thermal cycling profile are given in Table 2. Alongside, PCRs with the same single primers were also done so as to compare for the sensitivity of duplex PCR in comparison to single PCR as well as standard blood smear examination. The PCR amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel.

Sensitivity of single and duplex PCR

In order to determine the detection limit of the single and duplex PCRs, equal amounts (200 ng) of DNA from known positive *T. evansi* and *T. annulata* were used to create two individual series of 10-fold dilutions using distilled water. In addition, equal quantities of DNA representing these two species were mixed and 10-fold serially diluted in distilled water to evaluate the sensitivities of the single and duplex PCR assays to amplify from samples containing mixed DNA templates.

Table 1 Primer sequences along with expected amplicon size and the thermal cycling profile

Primer name	Primer sequence	Product size (bp)		Reference	
TBR F TBR R	(5′)-GAATATTAAACAATGCGCAG-(3′) (5′)-CCATTTATTAGCTTTGTTGC-(3′)	164		Masiga et al. 1992	
TAMS F TAMS R	(5′)-GTAACCTTTAAAAACG-(3′) (5′)-GTTACGAACATGGGTT-(3′)	721		d'Oliveira et al. 1995	
Thermal cycling profile					
	Initial denaturation	Denaturation	Hybridization	Extension	Termination
Duplex PCR	94 °C; 60 s	94 °C; 45 s X 30 cycles	55 °C; 60 s	72 °C; 60 s	72 °C; 300 s

Results

Specificity of PCR primers

PCR amplification employed on each individual DNA sample (*T. evansi* and *T. annulata*) using their specific primers led to the detection of expected fragments of size 164 and 721 bp, respectively. Each set of the primers was found to be specific for the respective parasite DNA, and amplification of nontarget DNA samples did not lead to the production of PCR products when other hemoprotozoan samples were used. Primer specific for one parasite species did not produce PCR products from any of the other parasite species (Fig. 1).

Relative efficacy of conventional parasitological method and duplex PCR Assay

Trypanosomes were found in 38 cases using blood smear examination while the duplex PCR was able to detect 49 cases as positive. Likewise, when compared with blood smear examination, PCR on blood detected 26 positive cases of theileriosis in comparison to blood smear examination, which detected 18 positive cases. Blood smear examination could detect only 3 cases of coinfection whereas duplex PCR was able to diagnose 11 cases of mixed infection.

Table 2 Comparison of sensitivities of species-specific primer sets in single and duplex PCR

DNA dilution series	Single PCR		Duplex PCR
	TBR	TAMS	
T.evansi alone	10^{-9}	–	10^{-7}
T.annulata alone	–	10^{-7}	10^{-6}
T.evansi in mixture	10^{-7}	–	10^{-7}
T.annulata in mixture	–	10^{-6}	10^{-6}

Relative efficacy of single PCR and duplex PCR assays

The sensitivities of the single and duplex PCR assays were evaluated using serially diluted DNA preparations. Starting with equal amounts of DNA from confirmed *T. evansi* and *T. annulata* samples, 10-fold serial dilutions were generated for DNA of each species individually and for a mixture representing both of them (Fig. 2). As summarized in Table 2, the duplex PCR was able to detect *T. evansi* and *T. annulata* at dilutions of 10^{-7} and 10^{-6}, with identical sensitivity for both single DNA and mixed DNA template dilutions. Since almost identical detection limits were observed when a single PCR was used to assay mixed DNA serial dilutions (Table 2), there was no significant loss of sensitivity using the duplex PCR protocol compared to the single PCR protocol. When the single PCR was used to amplify DNA dilutions representing each parasite species individually, the sensitivity of the assay increased to 10^{-9} and 10^{-7}, respectively (Tables 2).

Discussion

Duplex PCR-based diagnosis was able to detect 53.73 and 26.25 % prevalence rates of *T. evansi* and *T. annulata*, respectively. However, Giemsa-stained blood smears revealed much lower prevalence rates (*T. evansi* 47.5 % and *T. annulata* 22.5 %). Duplex PCR was able to detect 11.75 % of mixed infections. However, these prevalence rates are considered to be very much higher than expected. This could be very well explained by two facts firstly, that these samples are taken from clinically suspected ill animals and secondly, that the area under study is said to be endemic for both trypanosomosis and theileriosis (Sudan et al. 2014a, b) (Table 3). The high sensitivity of duplex PCR can very well be acknowledged as it was able to expose the subclinical and carrier cases of both *T. evansi* and *T. annulata* which would have led to further attention for treatment and control in lieu being obscured. This point further stresses the importance that due to

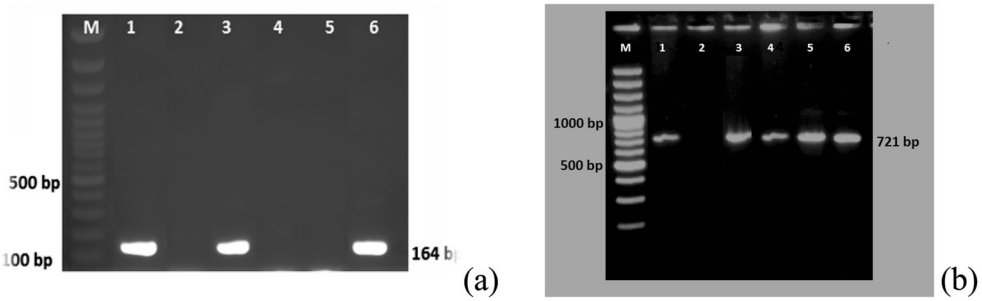

Fig. 1 Agarose gel electrophoresis of amplified DNA for *Trypanosona evansi* and *Theileria annulata* in single PCR using **a** TBR and **b** TAMS primer sets, respectively. *M*, 100 bp molecular size marker. Lane *1*, known positive control. Lane *2*, known negative control. Lanes *3–6*, suspected samples

antigenic variations, cryptic infections are seen in *T. evansi* leading to immunosuppression and nondetectable parasitemias (Gill 1991; Singla et al. 2010; Nair et al. 2011), and there is every possibility that due to immunosuppression, the infection of theileriosis flares up, as most of the animals in the studied area are actually carriers of theileriosis owing to endemic status of the disease in the studied area. History of the presence of tabanid fly in the vicinity was frequently recorded in the vicinity of sugarcane fields which are very much conducive for the breeding of tabanid flies alongside the presence of ample numbers of *H. anatolicum* ticks in the studied area (Patel et al. 2013) further justifies the existence of much higher rates of coinfection. The higher prevalence of *T. evansi* can probably be explained by the fact that the tabanid flies have an interrupted feeding habit and can travel longer distances as compared to the ticks.

A peculiar similarity between both trypanosomosis and theileriosis is the subsequent carrier stage of the infected animal upon recovery from the primary infection (Callow 1984). This carrier stage is characterized by a serious drop in the parasitemia, making detection of parasites very difficult by blood smear examination (Figueroa et al. 1993). Duplex polymerase chain reaction (dPCR) is a variant of PCR in which two target loci from one or more organisms are amplified using a mixture of locus-specific primer pairs in a single reaction (Markoulatos et al. 2002). Thus, duplex PCR offers a significant advantage over single-species detection systems for assessment of coinfection in a large number of samples at almost half the financial input. The optimized duplex PCR in the present study was able to specifically detect *T. evansi* and *T. annulata* from both single and mixed parasite DNA preparations. The detection limit using either a single species or a mixture of DNA from both the species was seen to be almost at par in the present study. Both single and duplex PCRs were able to detect DNA of each species with equal sensitivity in serially diluted DNA mixtures of *T. evansi* and *T. annulata*. However, the higher sensitivity of a single PCR while using the DNA template of each species in isolation could be very much attributed to the fact that the amount of template DNA present in the reaction mixture coupled with the competition for a finite amount of reagents between the primers affects the amplicon production quantitatively (Henegariu et al. 1997). Nevertheless, the duplex PCR assay was able to amplify target amplicons in mixed DNA templates with the same sensitivity as the single PCR. Therefore, for

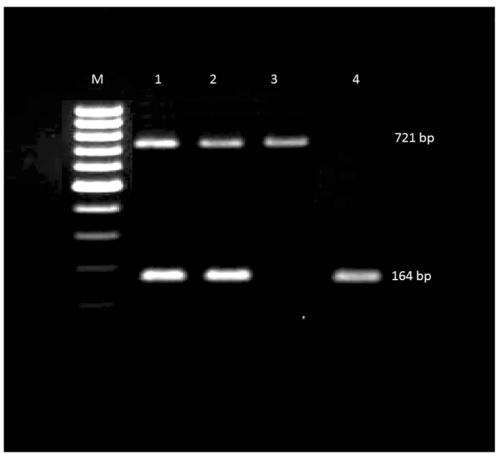

Fig. 2 Agarose gel electrophoresis of amplified DNA for *Trypanosona evansi* and *Theileria annulata* in duplex PCR. *M*, 100 bp molecular size marker. Lane *1*,*2*; sample positive for both *T. evansi* and *T. annulata.* Lane *3*, sample positive for *T. annulata.* Lane *4*, sample positive for *T. evansi*

Table 3 Single PCR, duplex PCR, and blood smear tests on suspected bubaline blood samples

Test		Number of positive animals			Number of negative animals	Total number of animals
		T.e	T.a	T.e+T.a		
Single PCR	TBR	49	0	–	31	8 0
	TAMS	0	26	–	54	80
Duplex PCR		43	21	11	16	80
Blood smear		38	18	3	24	80

T.e T. evansi, *T.a T. annulata*

epidemiological studies, the duplex PCR should be considered as an important alternative. Definitely the testing of a single species in isolation may be underestimating the overall risk, and development of suitable assays like this would be beneficial for a more complete assessment of vector borne diseases within a particular region. The duplex PCR assay developed in this study provides a simple test for detecting of two significant vector borne diseases in carrier animal and is useful for studies assessing the impact of productivity losses associated with coinfection of these infections.

Conflict of interest The authors declare that they have no conflict of interest.

References

Brown, C.G.D., 1997. Dynamics and impact of tick borne diseases of cattle. Tropical Animal Health and Production, 29,15–35.

Callow, L.L., 1984. Protozoan and rickettsial diseases. In: Australian Bureau of Animal Health, Animal Health in Australia, Vol. 5. Australian Government Publishing Service, Canberra. pp. 121–216.

d'Oliveira, C., Van-der Weide, M., Habela, A., Jacquiet, P. and Jongejan, F., 1995. Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. Journal of Clinical Microbiolology, 33(10), 2665–2669.

Figueroa, J. V., Chieves, L. P., Johnson, G. S. and Buening, G. M., 1993. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Veterinary Parasitology, 50(1–2), 69–81.

Gill B. S., 1991. *Trypanosomes and Trypanosomiases of Indian Livestock*, Indian Council for Agricultural Research, New Delhi, India.

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H., 1997. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques, 23, 504–511.

Markoulatos, P., Siafakas, N. and Moncany, M., 2002. Multiplex polymerase chain reaction: a practical approach. Journal of Clinical Laboratory Analysis 16, 47–51.

Masiga, D.K., Smyth, A.J., Hayes, P., Bromidge, T.J. and Gibson, W.C., 1992. Sensitive detection of trypanosomes in tsetse flies by DNA amplification. International Journal of Parasitology, 22, 909–18.

Minjauw, B. and McLeod, A., 2003. *Tick- borne diseases and poverty.* The impact of ticks and tick-borne diseases on the livelihood and marginal livestock owners in India and Eastern and Southern Africa. Research report, DFID animal health programme, centre of tropical veterinary medicine, University of Edinburgh

Nair, A. S., Ravindran, R. and Lakshmanan, B., 2011. Haemoprotozoa of cattle in Northern Kerala, India. Tropical Biomedicine,. 28(1), 68–75.

Patel, G., Shanker, D., Jaiswal, A.K., Sudan, V. and Verma S.K., 2013. Prevalence and seasonal variation in ixodid ticks on cattle of Mathura district, Uttar Pradesh. Journal Of Parasitic Diseases, 37(2), 173–176.

Pruvot, M., Kamyingkird, K., Desquesnes, M., Sarathapan, N. and Jittapalapong, S., 2013. The effect of the DNA preparation method on the sensitivity of PCR for the detection of *Trypanosoma evansi* in rodents and implications for epidemiological surveillance efforts. Veterinary Parasitology, 191, 203–208.

Singh, H., Tewari, A.K., Mishra, A.K., Maharana, B.R., Sudan, V., Raina, O.K. and Rao, J.R., 2014. Detection of antibodies to Toxoplasma gondii in domesticated ruminants by recombinant truncated SAG2 enzyme-linked immunosorbent assay. Tropical Animal Health and Production. DOI 10.1007/s11250-014-0703-5

Singla, L. D., Juyal, P. D. and Sharma, N. S., 2010. Immune responses to haemorrhagic septicaemia (HS) vaccination in *Trypanosoma evansi* infected buffalo-calves. Tropical Animal Health and Production. 42(4), 589–595.

Sudan, V., Jaiswal, A.K., Parashar, R. and Shanker, D., 2014. A nested polymerase chain reaction based assay for sensitive diagnosis of Trypanosoma evansi infection in water buffaloes. Indian Journal of Animal Sciences, 84 (12), 26–30.

Sudan, V., Sharma, R. L., Yadav, R. and Borah, M.K., 2014b. Turning sickness in a riverine buffalo naturally infected with *Theileria annulata* and its successful therapeutic management. Comparative Clinical Pathology, 23(1), 39–42.

Soulsby, E.J.L., 1982. Helminths, arthropods and protozoa of domesticated animals, ELBS-7. Bailliere Tindal, London, 728–737.