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A nested polymerase chain reaction (nPCR) based assay for sensitive detection of latent *Trypanosoma evansi* infection in water buffaloes

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Surra, caused by *Trypanosoma evansi*, is a severe constraint for livestock development and productivity in many tropical and subtropical countries of South East Asia, Africa and Latin America (OIE 2004). The infection is prevalent in large ruminants in most parts of the Indian sub-continent with no apparent pathognomonic clinical signs (Singh and Chhabra 2008); the mortality rate may be as high as 20 to 90%, particularly in bovines (Gill 1991). Animals under stress, malnutrition, pregnancy and over work are more prone to infection (OIE 2004). The gold standard for diagnosis of trypanosomosis still remains the microscopical demonstration of parasites in blood smear, however, the method suffers from limitations of sensitivity (Tewari *et al.* 2012, Singh and Tewari 2012). The literature on the sensitive diagnosis of trypanosomosis especially in buffaloes is on finger tips (Holland *et al.* 2001a, 2001b).The DNA based molecular detection techniques are reliable yet their applicability suffers due to latent infections in buffaloes (Shyma *et al.* 2012). The polymerase chain reaction (PCR) is widely used as highly sensitive and specific assay for detection of trypanosomes. The concentration of *T. evansi* DNA in suspected samples may be below the detection limit of the hybridization assay and hence positive cases are likely to be missed (Viseshakul and Panyim 1990). In this study, we described a simple, rapid, highly sensitive and specific assay for detection of *T. evansi* in water buffaloes (*Bubalus bubalis*) using nested polymerase chain reaction (nPCR) amplification.

Collection of blood samples: Blood samples (1ml aliquot from each animal) were collected in clean sterile vacutainers, **c**ontaining ethylene diamine tetra acetic acid (EDTA), from the jugular veins of earlier *T. evansi* confirmed water buffaloes (microscopic observation of blood smears).

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DNA isolation from whole blood: DNA was isolated using standard phenol chloroform method with minor modifications (Pruvot *et al.* 2013). Briefly, 100 microliter blood was added into a 1.5 ml tube containing 500 µl of denaturing solution (guanidinium thiocyanate) and vortexed at high-speed for 5 min. 150 µl of chloroform and 150 µl of phenol were added and vortexed at high speed for 5–10 min. The microtube was centrifuged at 13,000 rpm (15,493× g) for 5 min, and the supernatant (upper phase) 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 13,000 rpm for 5 min. The 400 µl of supernatant was collected, 1 ml of absolute ethanol was added into a 1.5 ml tube, and left the sample to precipitate at –20 °C overnight. After 10 min of centrifugation at 13,000 rpm, the pellet was washed with 75% alcohol twice. The pellet was finally air dried before re-suspension into 50 µl of TE buffer. Thus, the final prepared sample was concentrated 2:1 compared to the initial blood sample.

Primers selection: Primers TE1: (5–AGG ACG CAG AAA TAG CAG TA-3) and TE2: (5-ATT TAA TTG AGT GGC GTG AG-(3) were custom synthesized using sequence from a highly conserved region of the published sequence of nuclear repetitive gene of *T. evansi* (Artama *et al.* 1992). This pair of primers were used for the synthesis of the primary PCR amplification product of 810-bp. For the nested amplification step, oligonucleotide primers, viz. TE3: (5-CTT TTA TAC GAG GAG AGG GA- 3) and TE4: (5-TAT GGG CGT GCA GAT TTC AC-3) were also selected and custom synthesized and resulted in 270 bp PCR product. Using the semi-nested pair of primers (TE1 and TE4) and (TE3 and TE2) as mentioned above, 2 PCR assays with a semi-nested PCR product size 490-bp and 590-bp were employed, respectively. Details for primers design including position of nucleotides, nucleotide sequences, and expected PCR products are shown in Table 1.

Polymerase chain reaction (PCR): The PCR reaction was 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 (TE1 and TE2),

Table 1. Primer sequences along with expected amplicon size used in n PCR assay

Primer name	Position	Primer sequence	Product size (bp)
External (outer primer)			
TE 1	91–110	(5)-AGGACGCAGAAATAGCAGTA-(3)	810
TE 2	881–900	(5)-ATTTAATTGAGTGGCGTGAG-(3)	
Internal (inner primer)			
TE 3	311–330	(5)-CTTTTATACGAGGAGGGGA-(3)	270
TE 4	561–580	(5)-TATGGGCGTGCAGATTTCAC-(3)	
Semi nested 1			
TE 1	91–110	(5)-AGGACGCAGAAATAGCAGTA-(3)	490
TE 4	561–580	(5)-TATGGGCGTGCAGATTTCAC-(3)	
Semi nested 2			
TE 3	311–330	(5)-CTTTTATACGAGGAG GGGA-(3)	590
TE 2	881–900	(5)-ATTTAATTGAGTGGCGTGAG-(3)	

2 µl of the DNA template and total volume was made up to 25 µl using Nuclease free water. The details of thermal cycling profiles are given in Table 2. The PCR amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel.

Nested polymerase chain reaction (nPCR): For the nested PCR amplification, 2.0 µl of the primary amplified product produced by TE1 and TE2 were transferred to 0.5 ml PCR tube 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 (TE3 and TE4) and total volume was made up to 25 µl using nuclease free water. The details of thermal cycling profiles are given in Table 2. The PCR amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel. Likewise semi nested PCR were also performed using TE1 and TE4 as one primer set and TE3 and TE2 as other primer set under similar conditions as described above.

Sensitivity and specificity assay: The sensitivity of the present assay was determined firstly by spectrophotometry estimation of purified DNA concentration (1,240 µg/ml). Later, serial dilutions of the purified *T. evansi* DNA were performed to obtain regular scale of dilution from 1/100 to 1/200,000. Estimations of DNA quantity (pg) introduced in the reaction and equivalence in number of *T. evansi* genomes are compiled in Table 3 [calculated from initial concentration, dilution rate and a mean *T. evansi* genome weight of 0.075 pg (Borst *et al.* 1982)]. In order to check the specificity of these primers, PCR were performed with DNA extracted from the blood of buffaloes parasitologically positive (microscopic observation of blood smears, and confirmed by PCR) for *Babesia* sp. and *Theileria* sp. using standard primers (d'Oliveira *et al.* 1995, Singh *et al.* 2007).

The nPCR-based assay afforded sensitive and specific detection of *T. evansi* all naturally infected buffaloes. The outer pair of primers TE1 and TE2 produced a primary 810-bp PCR product from *T. evansi DNA*. The primary 810-bp PCR product was visualized on ethidium bromide- stained gel from ≥ 10 pg of *T. evansi* DNA (Fig. 1). Using the nested

Table 2. Thermal cycling profiles of PCR and nPCR used in the study

	Thermal cycling profile				
	Initial denaturation	Denaturation	Hybridization	Extension	Termination
PCR	95°C; 120 sec × 30 cycles	95°C; 60 sec	55°C; 30 sec	72°C; 45 sec	72°C; 600 sec
nPCR	95°C; 120 sec × 30 cycles	94°C; 60 sec	55°C; 30 sec	72°C; 45 sec	72°C; 600 sec

Table 3. Purified DNA dilutions: equivalence in DNA amount and in number of *T. evansi* genomes (calculated from Borst *et al.* 1982, Pruvot *et al.* 2010)

Reference DNA dilutions	1/100	1/500	1/1000	1/5000	1/10,000	1/25,000	1/50,000	1/75,000	1/100,000	1/125,000	1/150,000	1/200,000	1/400,000
Estimated *T. evansi* DNA amount (in pg/µl)	11.4	2.28	1.14	0.228	0.114	0.04560	0.0228	0.0152	0.0114	0.0091	0.0076	0.0057	0.00285
Equivalence in number of *T. evansi* genome (/µl)	152.0	30.40	15.20	3.040	1.520	0.6080	0.3040	0.2027	0.1520	0.1216	0.1013	0.0760	0.0380

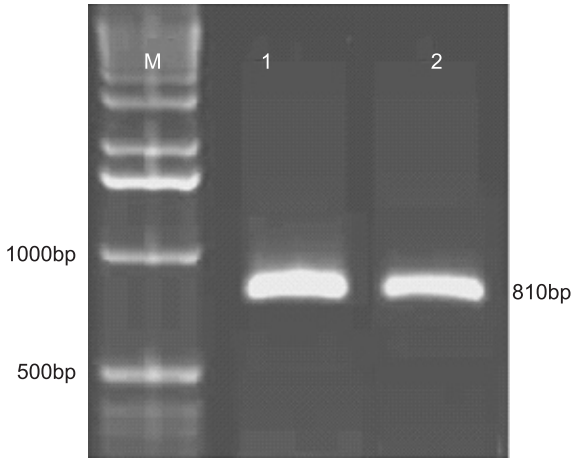

Fig. 1. Visualization of the primary 810-bp PCR product. Lane M: molecular weight marker (1 Kb ladder); Lane 1,2: 810 bp amplicon.

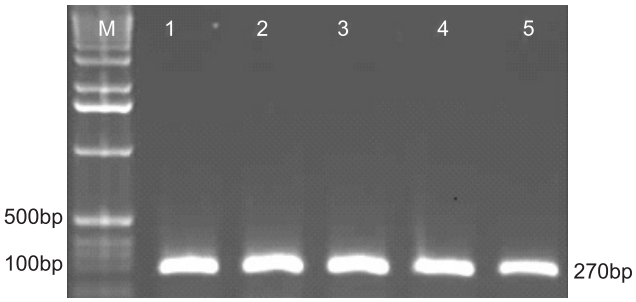

Fig.2. Amplifcation of the nested 270-bp specific-PCR product. Lane M: molecular weight marker (1 Kb ladder); Lane 1–5: *T. evansi* DNA at a 10 fold dilutions.

primers (T3 and T4), the PCR assay resulted in amplification of a 270 bp PCR product from the primary PCR product. The nested 270 bp PCR product (Fig. 2) was detected from as little as 10 fg of *T. evansi* DNA (equivalent to a single copy of nuclear repetitive gene of *T. evansi*). Using the pair of primers semi-nested 1 (TE1 and TE4), the PCR assay resulted in amplification of a 490-bp PCR product. Likewise, the use of pair of primers semi-nested 2 (TE3 and TE2) produced a 590-bp PCR product (Fig. 3).The nested amplification clearly increased the sensitivity of the PCR assay. The DNA from other blood parasites failed to produce the primary or the nested amplification products (Fig. 4) accounting for its high specificity.

No doubt, the conventional parasitological techniques will always remain important for understanding the biology, ecology and molecular epidemiology of different strains of the parasites yet there is need of a highly sensitive test that can detect the lowest levels of parasitemia (Singh and Singla 2012). The described nPCR assay provides simple, rapid, sensitive and specific method for detection of *T. evansi* in naturally infected latent carrier stages in buffaloes as well as in other species also and can be recommended for inclusion in survey and control programmes. Selection of the universal primers in the instant study was based on the observation that the nuclear repetitive gene has highly conserved nucleotide

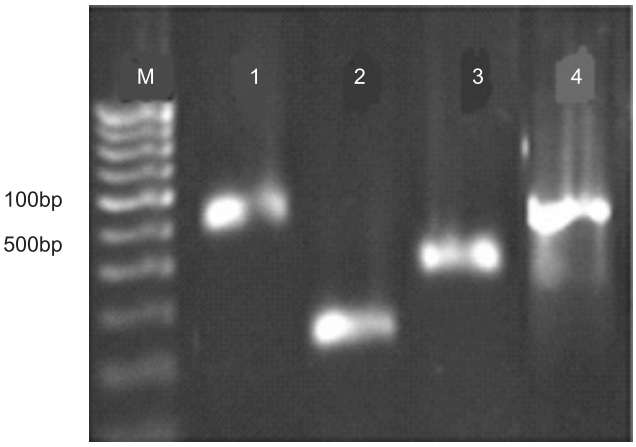

Fig.3. Detection of *T. evansi* DNA by PCR using 4 different primers (nPCR). Lane M: molecular weight marker (1 Kb ladder); Lane 1: amplification of the primary 810 bp-PCR product using the outer pair of primers (TE1 and TE2); Lane 2: amplification of the nested 270-bp PCR product using the internal pair of primers (TE3 and TE4); Lane 3: amplification of the semi-nested 490-bp PCR product using the pair of primers semi-nested 1 (TE1 and TE4);Lane 4: amplification of the semi-nested 590-bp PCR product using the pair of primers semi-nested 2 (TE3 and TE2).

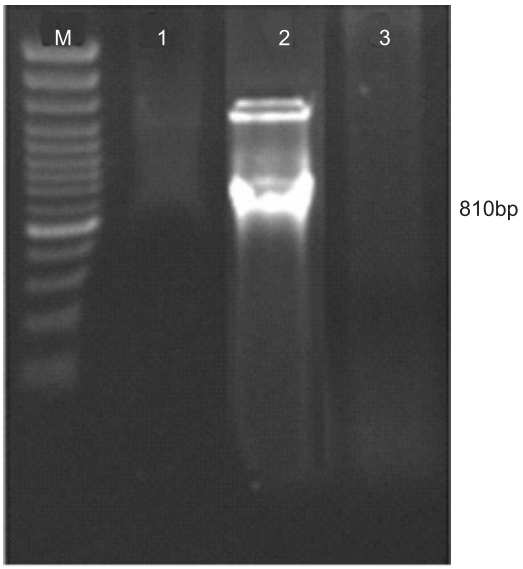

Fig. 4. Specificity of the polymerase chain reaction for *T. evansi*. Lane M: molecular weight marker (1 Kb ladder); Lane 1: no amplication with *Babesia bigemina* DNA;Lane 2: amplification with *T. evansi* DNA (positive control);Lane 3: no amplication with *Theileria anulata* DNA.

sequences (Artama *et al.* 1992). The nPCR assay was a simple procedure that efficiently detected all *T. evansi* strains under the stringency conditions. The laboratory detection limit indicated that the nPCR protocol was capable of detecting the amount of 10 fg of total *T. evansi* genomic DNA. This could be very detrimental in diagnosis of cryptic and latent carriers where parasite concentration is very much lower to be amplified by simple PCR technique or by conventional serological and microscopic techniques.

The specificity studies indicated that the primary specific

821–bp and the nested 270-bp PCR products were not amplified from DNA samples of other haemoprotozoans under identical conditions described in this study. Based on the sensitivity and specificity results of this study, the described nPCR should be considered as a highly sensitive and specific alternative compared to the some of the previously described PCR-based detection assays (Tewari *et al.* 2012). The second amplification step using the nested primers TE3 and TE4 is necessary to confirm the specificity of the primary amplified product and to increase the sensitivity of the PCR-based assay by at least 1,000 times particularly, when the concentration of the *T. evansi* DNA in the sample is less than 10 pg. The use of nested amplification for confirmatory diagnosis of *T. evansi* infection renders this PCR assay a rapid and a highly accurate assay. Although the literature on use of nPCR for diagnosis of trypanosomosis is limited in general but when it comes to buffaloes the literature is scanty. The authors did not find any suitable reference regarding use of nPCR in buffaloes for the sake of discussion and/ or criticism.

In conclusion, the described nPCR assay, using well characterized *T. evansi*-specific primers, provides a simple, rapid, sensitive and specific detection of *T. evansi* in naturally infected buffaloes and can be used as a valuable tool during epidemiological surveys and control programmes.

SUMMARY

A nested polymerase chain reaction (nPCR)-based assay was developed and evaluated for rapid detection of latent and cryptic cases of *Trypanosoma evansi* in buffaloes. Four oligonucleotide primers (TE1, TE2, TE3 and TE4), selected from nuclear repetitive gene of *T. evansi*, were designed and used for PCR amplifications. The first amplification, using a pair of outer primers TE1 and TE2, produced a 821–bp primary PCR product from *T. evansi* DNA. The second amplification, using nested (internal) pair of primers TE3 and TE4, produced a 270–bp PCR product. The nested primers TE3 and TE4 increased the sensitivity of the PCR assay and as little as 10 fg of *T. evansi* DNA (equivalent to a single copy of the putative gene of the parasite) was amplified and visualized onto ethidium bromide-stained agarose gels. Amplification products were not detected when the PCR-based assay was applied to DNA from other blood parasites including *Thieleria annulata* and *Babesia bigemina*. The described nPCR-based assay provides a valuable tool to study the epidemiology of *T. evansi* infection in buffaloes and other susceptible animal populations.

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