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PCR-based diagnosis of surra-targeting mini-chromosomal satellite DNA for unraveling the cryptic epizootiology of bubaline trypanosomosis

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Animal trypanosomosis, caused by unicellular eukaryotic haemoflagellate *Trypanosoma evansi,* often manifests in a chronic form in domestic ruminants as a classical disease entity-Surra. The gold standard for diagnosis of trypanosomosis still remains the microscopical demonstration of parasites in blood smear, however, it suffers from limitations of sensitivity (Singh and Singla 2012). PCR tools improved the parasitological techniques from the 1990s, when primers were described for amplification of satellite DNA of trypanosomes (Masiga *et al.* 1992). Pruvot *et al*. (2010) have documented highly repeated sequence of mini-chromosome satellite DNA (Masiga *et al.* 1992) as the gold standard for PCR based detection of *Trypanozoon* DNA. In this study, we have described a highly sensitive and specific assay for detection of *T. evansi* in water buffaloes (*Bubalis bubalis*) using PCR with TBR primer sets, its laboratory standardization and later field validation by comparing it with gold standard technique, viz. blood smear examination.

Collection of blood samples: Blood samples (1 ml 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) for isolation of positive controls for laboratory standardization of PCR assay. Later, blood samples from 80 suspected buffaloes were also collected for validation of the PCR assay. Along side, peripheral blood smears were also made using standard procedures.

DNA isolation from whole blood: DNA was isolated using standard phenol chloroform method with minor modifications (Pruvot *et al.* 2013). Briefly, 100 microliters of blood were 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; 400 µl of supernatant was collected; 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 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 (Tris, EDTA). Thus, the final prepared sample was concentrated 2: 1 compared to the initial blood sample.

Primers selection and polymerase chain reaction (PCR): Primers TBR1/2 were custom synthesized using sequence from a highly conserved region of the published sequence of nuclear repetitive gene of *Trypanozoan* genus (Masiga *et al*. 1992). The forward primer consisted of 5/-GAATATTAAACAATGCGCAG-3/ while the reverse primer consisted of 5/- CCATTTATTAGCTTTGTTGC-3/ sequence. This pair of primers was used for the synthesis of the primary PCR amplification product of 164-bp repeated sequence of mini-chromosome satellite DNA (Masiga *et al.* 1992). 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 (TBR1/2), 2 µl of the DNA template and total volume was made up to 25 µl using nuclease free water. The PCR consisted of initial denaturation at 95°C for 5 min followed by 32 cycles each consisting of denaturation at 95°C for 50 sec followed by primer annealing at 62°C for 75 sec and elongation at 72°C for 50 sec. This was followed by final elongation at 72°C for 12 min. The PCR amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel.

Sensitivity and specificity assay: The sensitivity of the present assay was determined firstly by spectrophotometry estimation of purified DNA concentration (1,020 µg/ml). Later, serial dilutions of the purified *T. evansi* DNA were

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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 were calculated from initial concentration, dilution rate and a mean *T. evansi* genome weight of 0.075 pg as per Borst *et al.* (1982). 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).

Statistical analysis: The results of the PCR were compared with that of blood smear examination. The sensitivity and specificity of the PCR was calculated and compared with that of blood smear examination, as a gold standard, using online software (http: //graphpad.com/quickcalcs/kappa1.cfm). The kappa value, hence calculated, was compared and results were formulated.

The PCR-based assay afforded sensitive and specific

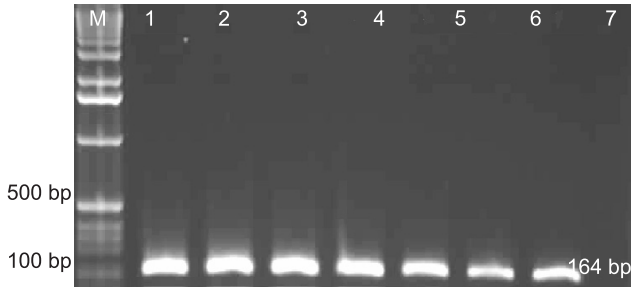

Fig. 1. Sensitivity of the specific-PCR product. Lane M: molecular weight marker (1 Kb ladder); lane 1–7: *T. evansi* DNA at a 10 fold dilutions.

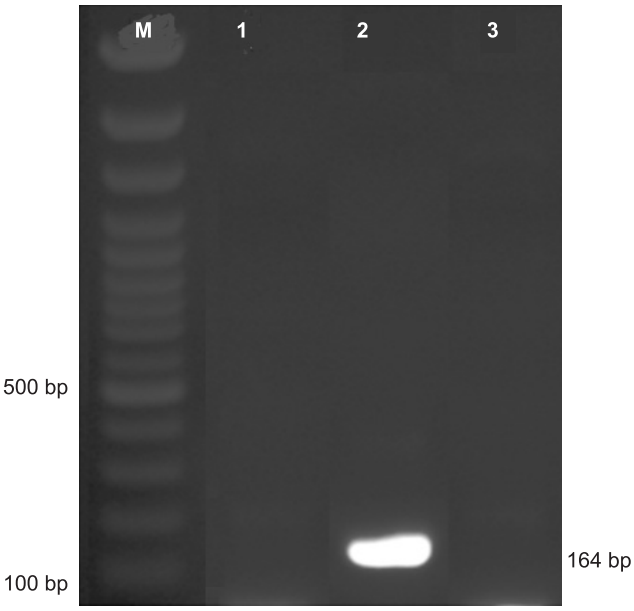

Fig. 2. Specificity of the polymerase chain reaction for *T. evansi*. Lane M: molecular weight marker (1 Kb ladder); lane 1: no amplification with *Babesia bigemina* DNA; lane 2, amplification with *T. evansi* DNA (positive control); lane 3: no amplification with *Theileria annulata* DNA.

detection of *T. evansi* in all naturally infected buffaloes. The PCR produced a 164-bp PCR product from *Trypanozoan DNA*. The serial dilution results revealed a very good response and the PCR was able to detect 0.10 pg of *Trypanozoan* DNA promising a higher levels of sensitivity (Fig. 1).The DNA from other blood parasites failed to produce the primary amplification products (Fig. 2) accounting for its high specificity.

When compared with blood smear examination, PCR detected 49 cases positive in comparison to blood smear examination, which detected 38 positive cases. Keeping blood smear examination as a gold standard for detecting actual number of confirmed positive cases, PCR was found to be 77.55 % sensitive and 100 % specific based on their kappa values estimation (Table 1).

Table 1. Kappa value prediction of PCR with blood smear examination

TEST	Blood smear			Sensitivity	Specificity	Kappa
PCR	Positive	Negative	Total	(95%ci)	(95%ci)	value
Positive	38	0	38	77.55%	100%	0.728
Negative	11	31	42			
Total	49	31	80			

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). Blood smear examination proved to be of limited value in diagnosis of subacute or chronic cases. Herbert and Lumsden (1976) reported non feasibility of microscopic detection when less than 2,500,000 parasites/ mL are present in blood samples. In serological tests, antibodies to *T. evansi* infection persist after drug treatment, complicating the differentiation of patent infection from non-patent infections. Hence, polymerase chain reaction, free of these hurdles, specifically amplifies genetically defined regions of the genome of the infectious agent. Although the detection of a single DNA molecule is possible, detection levels to a minimum of 5 trypanosomes by PCR assay are well documented (Artama *et al.* 1992). Amplification of repetitive *T. evansi* specific DNA sequence was possible even with DNA of a single trypanosome (Viseshakul and Panyim 1990). Mini-chromosomes of the nuclear DNA contain satellite DNA which has been the most favoured target in the development of species-specific primers able to detect very small amounts of parasite DNA. The selected primer in the instant study, is known to be the most potent proven primer set for the detection of trypanosomosis amongst all the tested primer sets (Pruvot *et al.* 2010). Though the primer is tested on other species of hosts like horses (Muieed *et al.* 2010), cattle (Pruvot *et al.* 2010), rodents (Pruvot *et al.* 2010), yet there is no record of using this primer for detection of trypanosomosis in bubaline

hosts. *T. evansi* genome size is considered to be between 0.05 and 0.1 pg of DNA (Panyim *et al.* 1993). TBR1/2 was able to detect 0.1 pg of DNA in the instant study, however it is known to detect 0.01 pg of purified DNA which is approximately equivalent to about 0.1 parasite (Pruvot *et al.* 2010). Again, Fernandez *et al.* (2009) were able to detect up to 0.001 pg of purified DNA using TBR1/2 primers. The variations are attributed to the differences in the methods of isolation of DNA which greatly interferes with the quality and purity of DNA isolated. This higher detection capacity is related to the highly repeated sequence targeted by these primers (10,000–20,000). From infected tsetse flies, Masiga *et al.* (1992) also reported PCR with TBR1/2 to be able to detect 1 parasite, and the equivalent of 0.01 parasite using Sau AI restriction enzyme in order to increase the concentration of the target sequence.

SUMMARY

The described PCR assay, using well characterized *Trypanozoan* primers, provided a simple, rapid, sensitive and specific detection of trypanosomosis in naturally infected buffaloes and can be used as a valuable tool during epidemiological surveys and control programmes. The higher sensitivity and repeatability confirmed that TBR1/2 should be used in any primer development and evaluation, as a gold standard for the detection of trypanozoon DNA by PCR. It is essential to carry out proper performance evaluation during primer development, and to use systematically TBR1/2 as a reference.

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