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RESEARCH ARTICLE

Comparative sensitivity of different primers in detection of *Trypanosoma evansi* infection in experimentally infected mice by Polymerase Chain Reaction vis-a-vis conventional Parasitological techniques

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Abstract

Sensitivity of four different sets of *Trypanosoma evansi* specific primers viz. ITS1/ CF/BR, ESAG 6/7, TBR1/2 and 21/22mer was evaluated by targeted amplification by polymerase chain reaction using different DNA extraction methods. PCR products obtained by using the ITS1/ CF/BR, ESAG 6/7, TBR1/2 and 21/22mer primers yielded 480 bp, 237 bp, 164 bp multiples and 227 bp products, respectively. Overall results revealed TBR1/2 primers most sensitive followed by ESAG6/7 and 21/22-mer, where as ITS1 set of primers were least sensitive for detection of *T. evansi* DNA from infected mice blood and purified trypanosomes. Further PCR and parasitological diagnostic techniques viz. wet blood film (WBF), buffy coat (BC) and thin blood smear (TBS) were applied on blood samples collected from experimentally *T. evansi* infected mice at different intervals post infection for their comparative diagnostic evaluation. The results indicated PCR almost twice more sensitive as compared to CPT in terms of detection of *T. evansi*. PCR was able to detect the trypanosomes in pre patent phase.

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Introduction

Trypanosoma evansi the causative agent of 'Surra' was discovered by Griffith Evans, the then British Veterinary Officer at Dera Ismail Khan in erstwhile Punjab, India (now in Pakistan) in 1880 from the blood of equines and camels (Evans, 1880). The lack of maxi circle DNA appears to facilitate the utilization of a wide range of mechanical vectors by *T. evansi* (Masiga and Gibson, 1990) and thus making it widespread in Africa, Asia & South America in horses, camels, dogs, cattle, buffaloes and several wild animals (Hoare, 1972; Gill 1991; Singla et al., 2003; Sumbria et al., 2014).

Despite improvement in parasitological techniques for diagnosis of trypanosomes, a high proportion of infections remain undetected (Luckins, 1992) mainly due to constant antigenic variation (Jones and Mckinnell, 1984). Clinical signs of the disease are also non-specific and not sufficiently pathognomonic (Killick-Kendrick, 1968; Gill, 1991; Aulakh et al., 2005; Juyal et al., 2005). The direct detection of *T. evansi* in the blood of infected host is the only true "Gold Standard" diagnostic test available for 'Surra' in India. The conventional parasitological techniques (CPT) viz. wet blood film examination, Giemsa stained thin or thick smears and buffy coat smear related to direct detection of parasite are insensitive mainly due to scanty parasitaemia in peripheral blood during the chronic, intermission and prepatent phase (Gill, 1991; Juyal et al., 1994; Singla et al., 2013). Mouse inoculation

(MI) is generally regarded as the most sensitive method to detect parasites in blood circulation and is generally discouraged in the event of any other alternative because of use of live animals and it takes longer time (Reid et al., 2001). Various serological/antigen antibody based tests such as enzyme linked immunosorbent assay (ELISA), card agglutination test for trypanosomiasis (CATT), indirect fluorescent antibody technique (IFAT) for diagnosis of trypanosomiasis do not distinguish between current infection and residual antibodies from previous infection (Weiss, 1995).

The polymerase chain reaction (PCR) based assays permit identification of parasite at levels far below the detection limit of the commonly used parasitological techniques. To optimize the diagnosis by PCR it is necessary to consider several factors that affect the PCR sensitivity; such as the quantity and quality of the DNA present in the samples, type of primers used etc. (Gonzales et al., 2006; Sharma). PCR primers inherently exhibit high target-specificity but the sensitivities of the PCR when applied to field samples are often lower than expected considering the detection limit of the PCRs themselves (Masake et al., 2002). Present study was envisaged to evaluate PCR sensitivity to detect *T. evansi* DNA using four different primers and to compare efficacy of PCR with those of CPT in detecting *T. evansi* infection.

2. Materials and Methods

2.1. Parasite/Biological Material: *T. evansi* cattle strain isolated from the clinical case presented in Veterinary Clinics, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU). The parasite was identified as *T. evansi* on the basis of morphological features i.e. monomorphic thin trypomastigote parasite, long free flagellum and thin posterior extremity with subterminal small kinetoplast and length ranges from 17-34 microns (**Fig. 1-A**) as measured by software DPZ-BSW (OLYMPUS). Swiss albino mice were used for maintenance and isolation of strain after experimental infection with cattle blood and were observed daily for parasitaemia by wet blood film examination of tail blood. Mice showed paralysis in terminal stages of disease (**Fig. 1-B**) with teeming parasitaemia and presence of trypanosomes in leishman stained blood smear (**Fig. 1-C**).

The separation and purification of blood stream form of *T. evansi* from host blood cells was accomplished by combination of centrifugation and chromatography using the anion exchange, diethyl amino ethane (DEAE) column chromatography (**Fig. 1-D**) (Lanham and Godfrey, 1970). Host cell free trypanosomes were obtained (**Fig. 1-E**) for quantitative experimental infection and molecular diagnostic studies. Counting of trypanosome was done by the method of Janeen et al., (1972).

2.2. Parasitological Techniques (PT):

2.2.1. Wet blood film (WBF): One drop of fresh mice tail blood on a glass slide after putting a cover slip over it, putting a cover was examined under microscope high power (40x) to observe for motile trypanosomes directly without staining.

2.2.2. Buffy coat smear examination using microhaematocrit centrifugation technique (BC): Trypanosomes were concentrated using centrifugation of blood collected in heparinized capillary tubes. After centrifugation of the capillary tube, the buffy coat was used to prepare thin blood smears. The slides were stained with Leishman stain and examined under microscope.

2.2.3. Microscopic examination of stained thin blood smear (TBS): Thin blood smears were also prepared at the time of collection of blood. They were examined under an oil immersion objective of a microscope after staining with Leishman stain.

2.3. Polymerase Chain Reaction (PCR):

2.3.1. Genomic DNA extraction/DNA template preparation:

i) Whole blood: DNA extraction from whole blood was carried out in two ways as following:

a) DNA template preparation was carried out by modifications in method used by Basagoudanavar et al., (1998) and Ravindran et al., (2008). Approximately 50-100 μ l of trypanosome positive (mice/cattle/horse) blood (freeze thawed) was dissolved in equal amount of TE buffer and boiled for 10 minutes in hot water bath/thermo cycler. After centrifugation (8000-10000 rpm) supernatant was kept and centrifuged again to get more purified supernatant as template DNA.

b) The protocol described by Sambrook and Russell (2001) was adopted. The whole blood in heparin (~500 μ l) was centrifuged (10,000 rpm) for 5 minutes, plasma was removed and remaining buffy coat and red blood cells were suspended in 500 μ l of Lysis buffer, incubated at 37°C for one hour and further processed for DNA extraction. The DNA pellet obtained was air dried and dissolved in an appropriate quantity of 1x TE buffer, pH 8.0 and stored at -20°C for further use.

ii) Purified trypanosomes: DNA template preparation was carried out as described above for whole blood (Basagoudanavar et al., 1998; Ravindran et al., 2008). Purified trypanosomes were boiled in TE buffer for 10 minutes. After centrifugation (8000-10000 rpm) supernatant was taken and used as template DNA.

2.3.2. Purity and concentration of DNA: The concentration of the isolated DNA was determined by UV spectroscopy using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). The DNA purity was estimated by determining the ratio of A260/A280 which for pure sample should fall between 1.65 and 1.85.

2.3.3. PCR amplification using genomic DNA: PCR was performed using DNA extracted from whole blood and purified trypanosomes. The reaction was carried out in Biometra (T-Gradient) thermal cycler. Four sets of primers viz. 21/22mer, ITS1, TBR1/2 and ESAG6/7 were used to check their sensitivity (**Table 1**). ESAG6/7, 21/22-mer and TBR1/2 primers are subgenus Trypanozoon specific, and ITS1 is of genus specific. PCR was performed using different temperature setups for annealing and concentrations of reagents/chemicals (**Table 1**). The PCR amplified products were analyzed on 1.5 % agarose gel stained with ethidium bromide (0.5 µg/ml) at 70 V (45- 55 minutes) after electrophoresis and visualized under UV light and were photographed using gel documentation system (Bio Rad). The molecular size of PCR product was estimated.

2.3.4. Sensitivity of different Primers: Different concentrations of the DNA (100 ng/µl, 50 ng/µl, 10 ng/µl, 1 ng/µl, 0.1 ng/µl, 0.01 ng/µl) were prepared to know the minimum concentration detectable by each primer (21/22- mer, ITS1, TBR1/2 and ESAG 6/7). For 50 µl PCR reaction, 1 µl of each of these dilutions was added as DNA template. Different dilutions of following samples were prepared: a) purified trypanosomes by boiling method (Ravindran et al. (2008)), b) trypanosome positive mice blood by Sambrook and Russell (2001) method, c) trypanosome positive mice blood by boiling method (Ravindran et al. (2008)).

2.3.5. Specificity of different Primers: PCR was performed by using each set of primers and DNA extracted from *Theileria* spp., *Babesia* spp., *Anaplasma* spp., *T. evansi*-cattle strain (positive control) and healthy cattle blood (host DNA) to check any cross amplification if any.

2.4. Comparison of PCR and parasitological techniques for detection of experimental *Trypanosoma evansi* infection in mice: Seventy two apparently healthy swiss albino mice of either sex (random bred) approximately 6-8 weeks of age weighing 34-43 gms (average 38.10 ± 0.30 gm) were divided into six groups of twelve each. Animals of five groups (Group I, II, III, IV and V) were inoculated intraperitoneally with 10^2 , 10^3 , 10^4 , 10^5 & 10^6 parasites/mice, respectively using *T. evansi* cattle strain maintained in mice.

Animals of one group (Group VI) were kept as uninfected healthy control. Approximately 1 ml blood was collected from two mice by cardiac puncture 6, 12, 24, 48, 72 and 96 hours post infection to know the patency by CPT and PCR signals. DNA extraction for PCR was performed

Results

Four different primers viz. ITS1/ CF/BR, ESAG 6/7, TBR1/2 and 21/22mer yielded 480 bp, 237 bp, 164 bp multiples and 227 bp PCR products, respectively (**Fig. 2**) with DNA from the *T. evansi* positive mice. The PCR products from TBR 1/2 set of primers were of 164 bp multiples bands due to the tandem repeat nature of the target.

3.1. Sensitivity of PCR:

Results indicated that in case of purified trypanosomes 21/22mer and ITS1/ CF/BR primers were able to detect 0.1 ng of DNA. However at 100 ng no product was visualized in case of ITS1 primers. The TBR1/2 and ESAG 6/7 primers were able to detect a minimum of 0.01 ng of DNA in case of purified trypanosomes. The minimum amount of DNA detectable by PCR from DNA from infected mice blood by standard PCI extraction was 1 ng for ITS1 and 0.1 ng for 21/22- mer, ESAG6/7 and TBR1/2 primers. In case of DNA extracted by boiling method from trypanosome positive mice blood, the minimum amount detectable by PCR was 10 ng for ITS1, 1 ng for 21/22- mer and ESAG6/7 and 0.1 ng for TBR1/2 primers (**Fig. 3**). Overall it was observed that ITS1 primers were found to be least sensitive and TBR 1/2 most sensitive in terms of detection of trypanosomal DNA from mice blood and purified trypanosomes.

3.2. Specificity of PCR/ primers:

It was observed that TBR1/2, ESAG6/7, ITS1 and 21/22-mer primers did not show any amplification with *B. bigemina*, *T. annulata* and *A. marginale* as well as host DNA. However some nonspecific bands observed in case of few cattle blood samples.

3.3. Comparison of PCR assay and parasitological techniques for detection of experimental *Trypanosoma evansi* infection in mice:

It was observed that both the methods viz. PCR and parasitological techniques were unable to detect parasites in group I (10^2 parasites/animal) even after 96 hrs post infection. However in case of group II (10^3 parasites/animal), PCR (using TBR1/2 primers) was able to detect trypanosomes after 96 hrs of infection. In case of groups III (10^4 parasites/animal) and IV (10^5 parasites/animal) infection was detected after 72 hrs by PCR as compare to 96 hrs post infection by CPT as evident from presence of trypanosomes in TBS as well as BC and WBF. In case of group V (10^6 parasites/animal), PCR was able to detect the infection at 48 hrs as compare to 72 hrs by CPT. However in animals of group VI (negative control) infection was not detected by CPT and PCR. As far as different

parasitological techniques viz; WBF, BC and TBS are concerned, BC was more sensitive as compared to TBS in terms of detection of *T. evansi* from mice blood (**Fig. 4**). Among different primers used, TBR1/2 set of primers were able to detect more number of samples (15) followed by 21/22-mer (13), ESAG6/7 (12) and ITS1 (9). Thus TBR1/2 were most sensitive primers for detection of trypanosomes in mice blood, followed by 21/22-mer, ESAG6/7 and ITS1 (**Fig. 5**), where as overall it was observed that PCR was twice more sensitive as compared to CPT in terms of detection of *T. evansi* from mice blood and can detect the trypanosomes in pre patent phase (**Table 2**).

Table 1: List of primers used with sequences and product size.

Primers	Sequences	Product Size	Programme	Concentration in a final volume of 50 µl
21/22mer (genomic DNA) (Wuyts et al.,1995)	fwd5'TGCAGACGACCTGACGCTACT 3' rev5'CTCCTAGAAGCTTCGGTGTCTC 3'	227 bp	5' at 94 ⁰ C, 30 cycles: [30'' at 94 ⁰ C, 30'' at 60 ⁰ C, 30'' at 72 ⁰ C] and 7' at 72 ⁰ C	Taq Polymerase 2U, PCR Buffer (Tris/HCl) 1X, MgCl ₂ 1.5 mM, dNTPs 200 µM, 0.5 µM of each primer.
ITS1/ CF/BR (ribosomal DNA) (Njiru et al., 2005)	fwd5'CCGGAAGTTCACCGATATTG 3' rev5'TGCTGCGTTCTTCAACGAA 3'	480 bp	5' at 94 ⁰ C, 35 cycles: [40'' at 94 ⁰ C, 40'' at 58 ⁰ C, 90'' at 72 ⁰ C] and 5' at 72 ⁰ C	Taq Polymerase 2.5U, PCR Buffer (Tris/HCl) 1X, MgCl ₂ 1.5 mM, dNTPs 200 µM, 0.5 µM of each primer.
TBR1/2 (mini-chromosome satellite repeated monomers) (Masiga et al., 1992)	fwd5'GAATATTAACAATGCGCAG 3' rev5'CCATTTATTAGCTTTGTTGC 3'	164 bp multiples	3' at 94 ⁰ C, 30 cycles: [1' at 94 ⁰ C, 80'' at 60 ⁰ C, 1' at 72 ⁰ C] and 5' at 72 ⁰ C	Taq Polymerase 2.5U, PCR Buffer (Tris/HCl) 1X, MgCl ₂ 1.5 mM, dNTPs 200 µM, 0.5 µM of each primer.
ESAG 6/7 (heterodimeric complex of the transferrin receptor) Holland et al., 2001a)	fwd5'ACATTCCAGCAGGAGTTGGAG 3' rev 5'CACGTGAATCCTCAATTTTGT 3'	237 bp	4' at 94 ⁰ C, 35 cycles: [1' at 94 ⁰ C, 1' at 55 ⁰ C, 1' at 72 ⁰ C] and 5' at 72 ⁰ C	Taq Polymerase 2.5U, PCR Buffer (Tris/HCl) 1X, MgCl ₂ 1.5 mM, dNTPs 200 µM, 0.5 µM of each primer.

Table 2: Overall comparison of PCR and CPT for infected groups in terms of detection of *T. evansi*

Time post infection (Hrs)	CPT			PCR			
	Wet blood Film (WBF)	Buffy coat examination (BC)	Thin blood smear (TBS)	P-I (ITS-1)	P-II (21/22 mer)	P-III (ESAG6/7)	P-IV (TBR 1/2)
06	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0
48	0	1(G-V)	0	1(G-V)	1(G-V)	1(G-V)	2(G-V)
72	1 (G-V)	1 (G-V)	1 (G-V)	3 (G-IV=1,G-V=2)	6 (G-III=2,G-IV=2,G-	5 (G-III=1,G-IV=2,G-	6 (G-III=2,G-IV=2,G-V=2)

					V=2)	V=2)	
96	4 (G-III=1,G-IV=1,G-V=2)	5 (G-III=1,G-IV=2,G-V=2)	5 (G-III=1,G-IV=2,G-V=2)	5 (G-III=1,G-IV=2,G-V=2)	6 (G-III=2,G-IV=2,G-V=2)	6 (G-III=2,G-IV=2,G-V=2)	7 (G-II=1,G-III=2,G-IV=2,G-V=2)
Positive samples	5	7	6	9	13	12	15
Negative Samples	55	53	54	51	47	48	45
Detection % (<i>T. evansi</i>)	8.33	11.67	10	15	21.67	20	25.00

(G-I= 10^2 parasites per mice, G-II= 10^3 parasites per mice, G-III= 10^4 parasites per mice, G-IV= 10^5 parasites per mice, G-V= 10^6 parasites per mice)

* Mice in Group I remained negative for *T. evansi* by CPT and PCR up to 96 hrs. post infection

Percent detection (infected groups) = Total positive samples/Total tested samples x 100

CPT detection % = $18/180 \times 100 = 10.00\%$

PCR detection (%) = $49/240 \times 100 = 20.42\%$

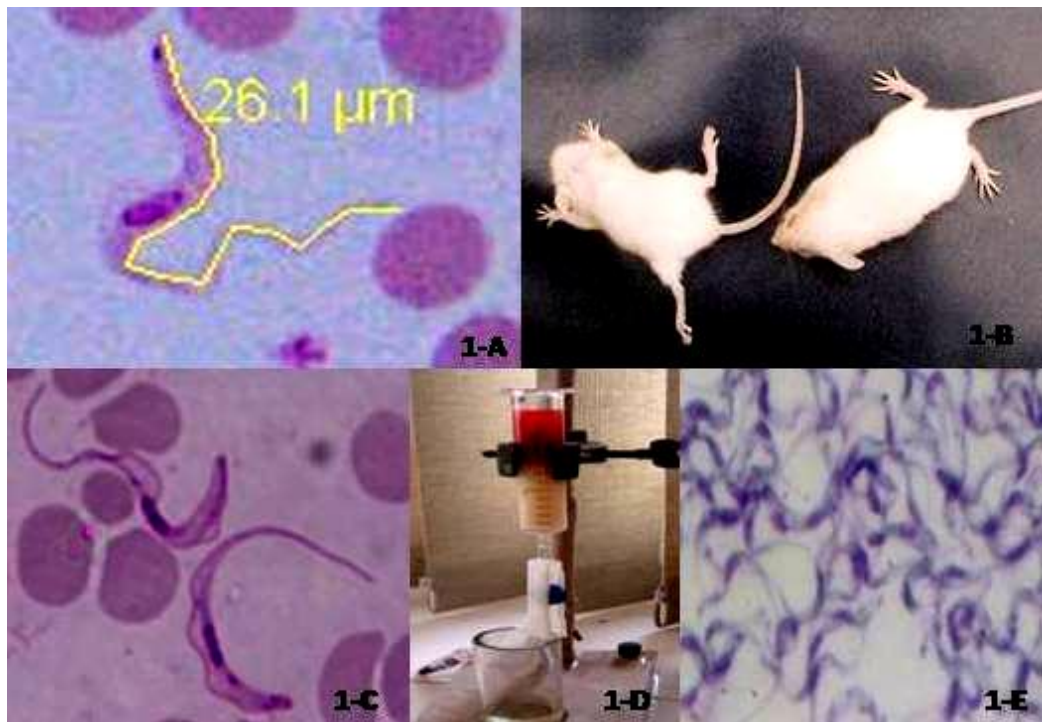


Figure 1. Maintenance and isolation of host cell free trypanosomes; 1-A: *Trypanosoma evansi* in leishman stained cattle blood smear identified on the basis of morphometric parameters, 1-B: Swiss albino mice (experimentally infected with *T. evansi* cattle strain) showing paralysis in terminal stages of disease, 1-C: Trypanosomes in stained mice blood, 1-D: Isolation of trypanosomes by using anion exchange, diethyl amino ethane (DEAE) column chromatography, 1-E: Host cell free (purified trypanosomes).

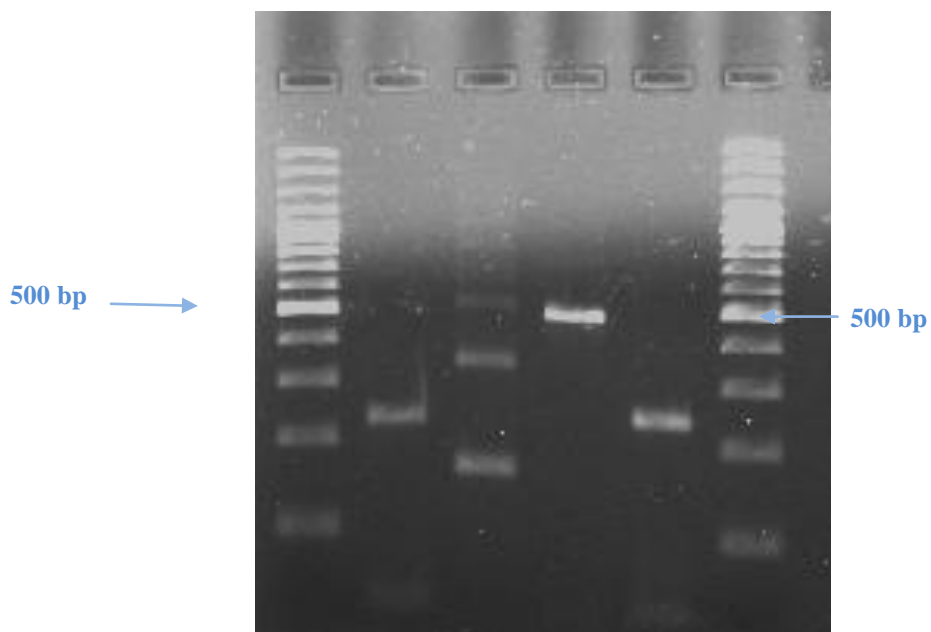
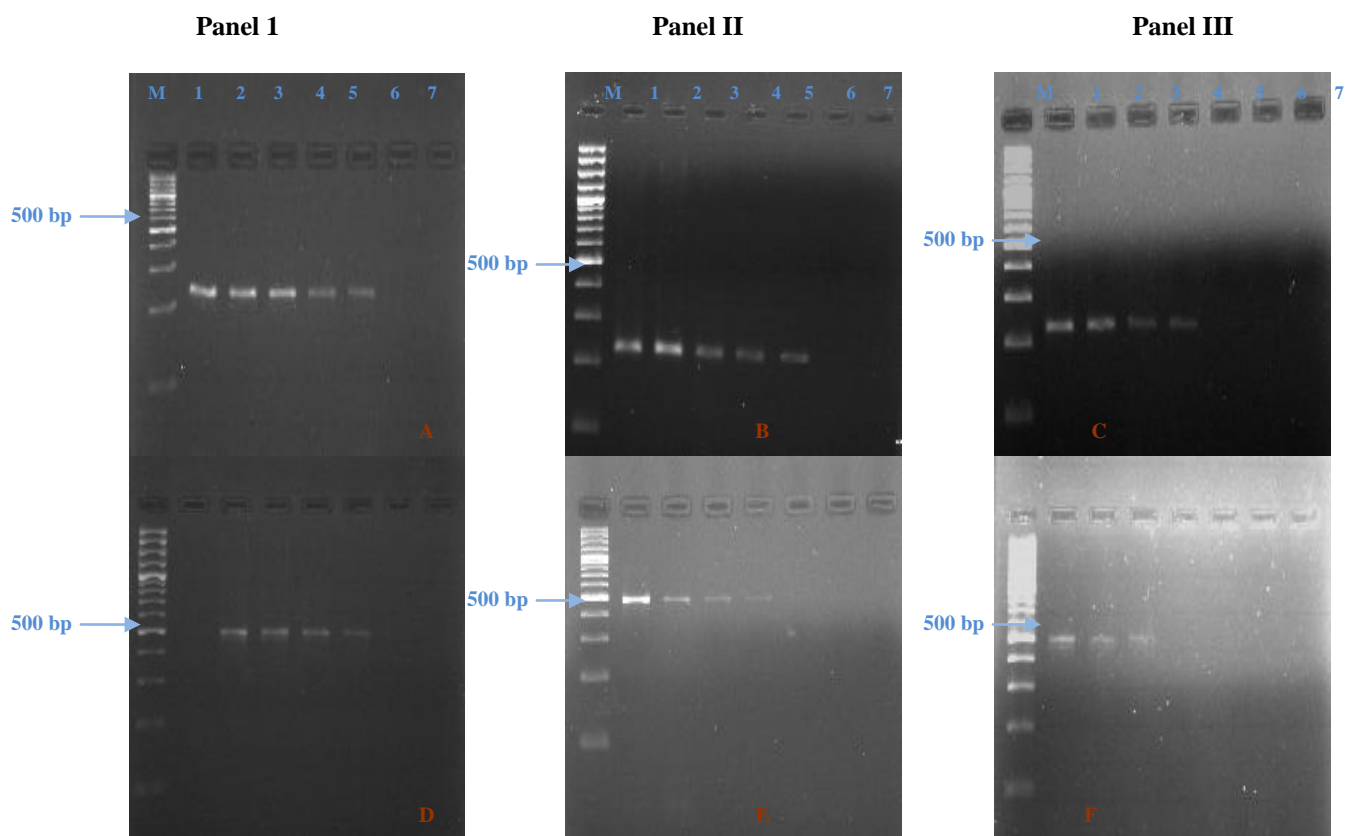


Figure 2. Showing all the four PCR products lanes M, E: Gene Ruler/molecular weight marker (100 bp). Lane A-D: PCR product using 21/22 mer (227 bp), TBR 1/2 (164 bp multiples), ITS1 (480 bp) and ESAG 6/7 (237 bp) primers.



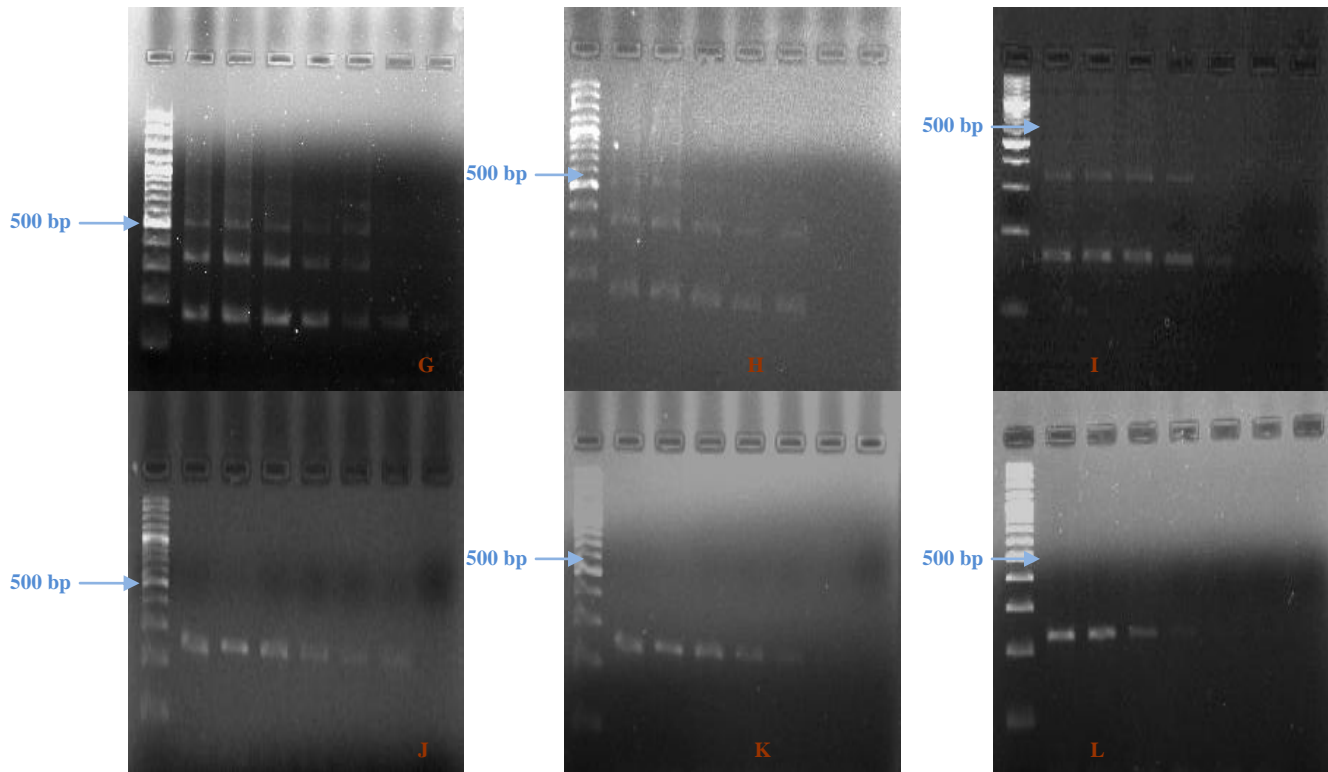


Figure 3. Evaluation of sensitivity of different primers viz. 21/22 mer (A,B,C), ITS1 (D,E,F), TBR 1/2 (G,H,I) and ESAG 6/7 (J,K,L) using DNA from purified *T. evansi* (Panel I), DNA extracted from *T. evansi* positive blood (PCI extraction) (Panel II), DNA template from *T. evansi* positive heperanized whole blood (by boiling method) (Panel III). Lane 1: DNA 100 ng, Lane 2: 50 ng, Lane 3: 10 ng, Lane 4: 1 ng, Lane 5: 0.1 ng, Lane 6: 0.01 ng, Lane 7: Blank sample (without DNA), Lane M: Gene Ruler/molecular weight marker (100 bp).

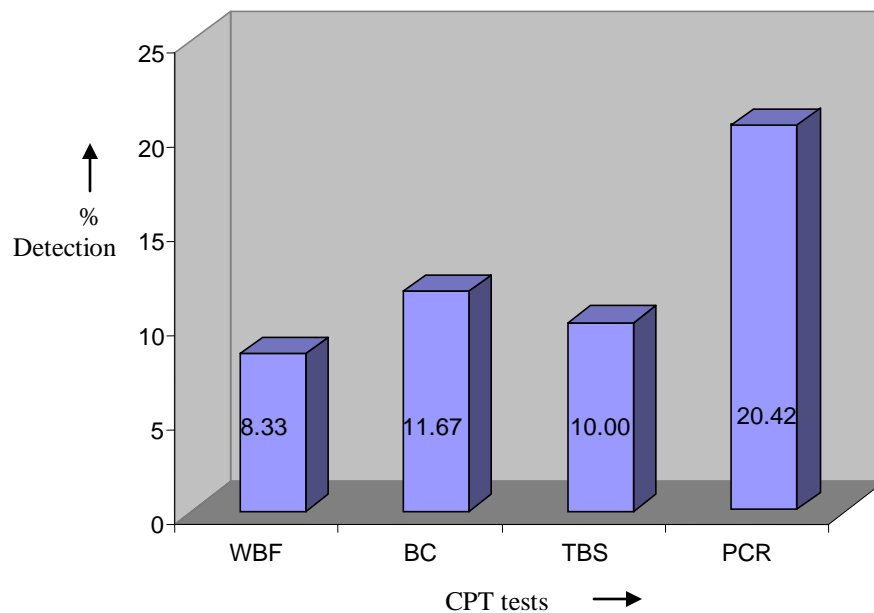
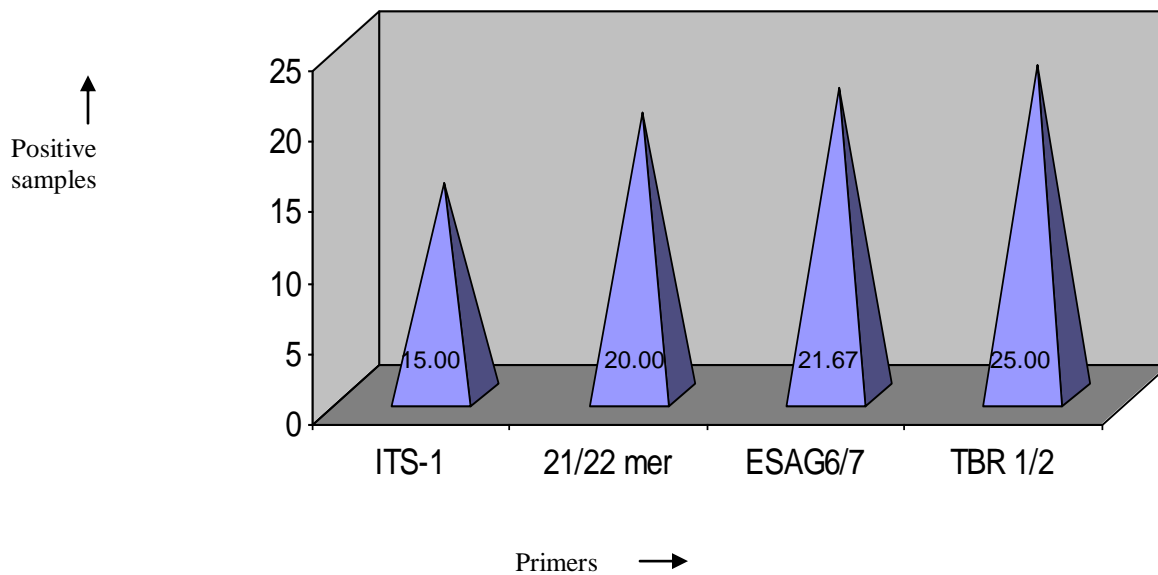


Figure 4: Comparative efficacy of CPT (WBF, BC, TBS) and PCR for detection of *T. evansi* from mice blood.



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Figure 5: Showing comparative efficacy of different primers in detection of *T. evansi* DNA from mice blood in increasing order.

Discussion

Sensitivity of PCR:

In case of purified trypanosomes at 100 ng no product was visualized in case of ITS1 primers. This inhibitory effect may be due to an excess amount of DNA in the reaction (Fernandez et al., 2009). It was observed that ITS1 primer set was least sensitive and TBR 1/2 most sensitive by DNA extracted from purified trypanosomes and trypanosome positive heparanized blood after boiling. While in case of DNA extracted from heparanized blood by standard PCI extraction, sensitivity of all the three primers except ITS1 (least sensitive) was same in terms of detection of trypanosomal DNA from mice blood.

Sensitivity was higher when DNA template from purified parasites was used as compared to DNA extracted from infected mice blood, which may be due to the presence of host DNA in the blood samples. Kabiri et al., (1999), while using the ESAG 6/7 primers, reported a minimum detectable quantity of 0.01pg of DNA for *T. brucei*. Njiru et al., (2005) reported minimum quantity of DNA detected as 10 pg of DNA for *T. evansi*, *T. congolense* and *T. vivax* from infected mice blood by using the ITS1 primers. However, Herrera et al., (2005), using the TBR1/2 primer detected 30 fg of *T. evansi* DNA from blood of infected mice. Garcia et al., (2005) and Wuyts et al., (1994) reported the detection of 10 pg and 0.5 pg of *T. evansi* DNA, using the 21/22-mer primers respectively. There are variations in the results of various workers as observed in the present study too. These differences may be due to several factors such as parasitaemia levels, relative amounts of specific parasite DNA in blood samples, DNA extraction methods, diversity of *Trypanosoma* strains, and/or differences in PCR programs and primers concentrations that diverge from one work to another.

Other factor which contribute to the sensitivity of primers is the number of copies of the target sequence in the genome of the organism, e.g. the PCR performed using TBR1/2 primers, which targets sequence having a multicopy satellite regions (~10,000–20,000), presented the highest sensitivity. High number of copies presents in the genome increases the probabilities to obtain an amplification product by PCR (MacLeod et al. 1997; Desquesnes and Davila 2002) and the sensitivity of the test. On the other hand, although the genome of *T. evansi* contains more copies (~100–200) of the ITS regions than the ESAG 6/7 genes (20 copies), the sensitivity of the PCR when using the ITS1 primers was lower, which might be due to a higher similarity between the ESAG6/7 primers and the target sequence in the genomic DNA of *T. evansi* (TEVA1 strain) (Fernandez et al. 2009).

Pruvot et al. (2010) observed that TBR1/2 primer set was able to detect 0.01 pg of purified DNA, although ESAG6/7 showed equivalent results with purified DNA and rat samples but presented non-specific products with Thai dairy cattle samples, leading to non interpretable results.

Overall our results indicate that TBR1/2 primers were most sensitive followed by ESAG6/7 and 21/22-mer, where as ITS1 set of primers were least sensitive for detection of *T. evansi* DNA from infected mice blood and purified trypanosomes.

Specificity of PCR/ primers:

Pruvot et al. (2010) also observed that using TBR1/2 and 21/22-mer primers there was no cross reaction with *Babesia* spp., *T. theileri* and *A. marginale*. None of the primers that were evaluated amplified the host DNA, corroborating its ability to detect specifically *T. evansi* in infected mice blood.

Comparison of CPT and PCR assay for detection of experimental *Trypanosoma evansi* infection in mice:

To detect *T. evansi* infections, PCR has been reported to be more sensitive than CPT in experimentally and clinically infected cattle (Wuyts et al., 1995, Sharma et al., 2013), water buffaloes (Holland et al., 2001a) and mice (Ijaz et al., 1998) and other mammals species (Herrera et al., 2005). Present results also showed major differences between the methods used to detect the presence of parasites (Tables 2), in both time and sensitivity. In experimentally infected mice, the parasitological tests were not totally effective since they yielded negative results in the periods when low parasitaemia levels occur. In the present study, PCR was capable of detecting parasite DNA much earlier than any of the CPT employed. These results were consistent with those reported by other authors (Wuyts et al., 1994; Ijaz et al., 1998; Holland et al., 2001a) and confirmed the superiority of PCR over parasitological tests. Ravindran et al., (2008) while comparing sensitivity of PCR and light microscopy for detection of *T. evansi* in camels, donkeys and dogs in India reported PCR as a more sensitive test for detection of *T. evansi* as compared to examination of thin blood smears by light microscopy. Similarly, Singh et al., (2004) compared parasitological (wet blood film, stained thin blood smear), immunodiagnostic (double antibody sandwich enzyme linked immunosorbent assay for antigen detection, Ag-ELISA) and DNA amplification by polymerase chain reaction (PCR) on samples from 217 camels for *T. evansi* infection. They also found PCR to be more sensitive as compare to other methods.

The period between the initial infection and the detection of the first parasites is related to the technique being employed, and also depends on the virulence of the isolate or strain and number of parasites that are inoculated (Holland et al., 2001a). Additionally, the percentage of detection and the sensitivity of the PCR vary depending of the primers employed, which are determined by the number of copies and the homology of the primers with the target sequence. Fernandez et al., (2009) observed percentage of detection for PCR varies depending on primer employed with 60% and 66% for ITS1 and 21/22-mer and 80% for ESAG6/7 and TBR1/2. Consequently, TBR1/2 and ESAG6/7 were the best primers to monitor *T. evansi* infections in mice.

In the present experiment, *T. evansi* parasites were detected almost one day (24 hrs.) earlier by PCR than microscopy. On the other hand, in earlier studies during the acute phase of experimental *T. evansi* infection in mice, the parasites were detected by PCR three days earlier than microscopy (Ijaz et al., 1998). But on the contrary, Holland et al., (2001a) observed that owing to the inherent detection limits of PCR and fluctuating levels of parasitaemia, the number of ongoing infections were underestimated.

Despite the low sensitivity of parasitological technique particularly WBF, they are widely used for rapid diagnosis in the field, because they have high specificity and are less expensive. However, samples must be examined within 3 hrs after sampling because of the limited viability of the *Trypanosoma* in a blood sample (Holland et al., 2001b). Unlike the parasitological tests, one advantage of PCR is that DNA samples can be processed afterwards, with no real time limit after collection (Singla et al., 2010).

Conclusion

As indicated by overall results TBR1/2 primers were most sensitive followed by ESAG6/7 and 21/22-mer, where as ITS1 set of primers were least sensitive for detection of trypanosomal DNA. Further PCR is almost twice more sensitive as compared to parasitological techniques in terms of detection of *T. evansi* from mice blood which is able to detect the trypanosomes in pre patent phase. Hence suitable application of this sensitive, rapid and precise diagnostic technique for early detection of *Trypanosoma evansi* can play a key role in implementation of trypanosomosis control programme.

Conflict of Interests

The authors declare that there is no professional or financial conflict of interests related to this paper.

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References

- Aulakh, G.S., Singla L.D. and Singh, J., 2005. Bovine trypanosomosis due to *Trypanosoma evansi*: clinical, haematobiochemical and therapeutic studies. In: New Horizons in Animal Sciences, Sobti RC and Sharma VL (eds). Vishal Publishing and Co., Jalandhar, pp 137-144.
- Basagoudanavar, S. H., Rao, J. R., Omanwar, S., Singh, R. K. and Butchaiah, G. (1998). Sensitive Polymerase Chain Reaction for detection of *Trypanosoma evansi* in Camels (*Camelus dromedarius*). J. Parasit. Dis. 22: 40-43.
- Desquesnes, M. and Davila, A. M. R. (2002). Applications of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. Vet. Parasitol. 109: 213–231.
- Evans, G. (1880). Report on Surra disease in Dera Ismail Khan. Punjab Government Military Department No. 493, p 446.
- Fernandez, D., Baradat, B. G., Eleizalde, M., Marcano, E. G., Perrone, T. and Mendoza, M. (2009). *Trypanosoma evansi*: A comparison of PCR and parasitological diagnostic tests in experimentally infected mice. Exp. Parasitol. 121: 1-7.
- Garcia, H., Garcia, M. E., Perez, H. and Mendoza-Leon, A. (2005) The detection and PCR based characterization of the parasites causing trypanosomiasis in waterbuffalo herds in Venezuela. Ann. Trop. Med. Parasitol. 99: 1–12.
- Gill, B. S. (1991). Trypanosomes and trypanosomiasis of Indian Livestock. Indian Council for Agricultural Research, New Delhi.
- Gonzales, J. L., Loza, A. and Chacon, E. (2006). Sensitivity of different *Trypanosoma vivax* specific primers for the diagnosis of livestock trypanosomosis using different DNA extraction methods. Vet. Parasitol. 136: 119–126.
- Hoare, C. A. (1972). The trypanosomes of mammals. A zoological Monograph, Blackwell Scientific Publications.
- Herrera, H. M., Norek, A., Freitas, T.P., Rademaker, V., Fernandes, O. and Jansen, A.M., (2005). Domestic and wild mammals infection by *Trypanosoma evansi* in a pristine area of the Brazilian Pantanal region. Parasitol. Res. 96: 121– 126.
- Holland, W. G., Claes, F., My, L. N., Thanh, N. G., Tam, P. T., Verloo, D., Buscher, P., Goddeeris, B. and Vercruyse, J. (2001a). A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. Vet. Parasitol. 97: 23–33.
- Holland, W. G., My, L. N., Dung, T. V., Thanh, N. G., Tam, P. T., Vercruyse, J. and Goddeeris, B. M. (2001b). The influence of *T. evansi* infection on the immuno-responsiveness of experimentally infected water buffaloes. Vet. Parasitol. 102: 225–234.
- Ijaz, M. K., Nur-K-Kamal, M. S., Mohamed, A. I. and Dar, F. K. (1998). Comparative studies on the sensitivity of Polymerase chain reaction and microscopic examination for the detection of *Trypanosoma evansi* in experimentally infected mice. Comparative Immunology, Microbiology and Infectious Diseases 21: 215-223.
- Janeen, D., Alan, S. R. and Sandra, A. T. (1972). *Trypanosoma lewisi*: In vitro growth in mammalian cell culture media. Exp. Parasitol. 31 : 225-231.
- Jones, T. W. and Mckinnell, C. D. (1984). Antigenic variation in *Trypanosoma evansi*: Isolation and characterization of variable antigen type population from rabbit infected with a stock of *T. evansi*. Tropenmedizin und Parasitologie 35: 237-241.

- Juyal, P.D., Kalra, I.S. and Singla, L.D., 1994. Prevalence of haemoprotozoans in domestic animals in Punjab. In: Proceedings of the 6th national congress of veterinary parasitology, Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur, 22–24 Oct 1994.
- Juyal, P. D., Singla, L. D. and Kaur, P. (2005). Management of Surra due to *Trypanosoma evansi* in India: an overview. In: V. Tandon and B.N. Dhawan (ed) Infectious diseases of domestic animals and zoonosis in India. P. Natl. A. Sci. India B. 75:109-120.
- Kabiri, M., Franco, J. R., Simarro, P. P., Ruiz, J. A., Mario, S. M. and Steverding, D. (1999). Detection of *Trypanosoma brucei gambiense* in sleeping sickness suspects by PCR amplification of expression-site-associated genes 6 and 7. Trop. Med. Int. Health. 4: 658–661.
- Killick-Kendrick. (1968). The diagnosis of trypanosomiasis in livestock- A review of current techniques. Vet. Bull. 38: 191-197.
- Lanham, S. M. and Godfrey, D. G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-Cellulose. Exp. Parasitol. 28: 521-534.
- Luckins, A. G. (1992). Protozoal diseases of camels. In Allen, W. R., Higgins, A.J., Mayhew, I.G., Snow, D.H. and Wade J.F. (eds.) Proceedings of the 1st International conference. R. and W. Publication New Market Limited., Suffolk, U.K. pp: 23-27.
- MacLeod, A., Turner, C. M., Tait, A. (1997). Detection of single copy gene sequences from single trypanosomes. Mol. Biochem. Parasitol. 84: 267– 270.
- Masake, R. A., Njuguna, J. T., Brown, C. C. and Majiwa, P. A. O. (2002). The application of PCR-ELISA to the detection of *Trypanosoma brucei* and *T. vivax* infections in livestock. Vet. Parasitol. 105: 179-189.
- Masiga, D. K. and Gibson, W. C. (1990). Specific probes for *Trypanosoma (Trypanozoon) evansi* based on kinetoplast DNA minicircles. Mol. Biochem. Parasitol. 40: 279-283.
- 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. Int. J. Parasitol. 22: 909–918
- Njiru, Z. K., Constantine, C. C., Guya, S., Crowther, J., Kiragu, J. M., Thompson, R. C. and Davila, A. M. (2005). The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol. Res. 95: 186–192.
- Pruvot, M., Kamyngkird, K., Desquesnes, M., Sarataphan, N. and Jittapalpong, S. (2010). A comparison of six primer sets for detection of *Trypanosoma evansi* by polymerase chain reaction in rodents and Thai livestock. Vet. Parasitol. 171 :185–193.
- Ravindran, R., Rao, J. R., Mishra, A. K., Pathak, K. M. L., Babu, N., Satheesh, C. and Rahul, S. (2008). *Trypanosoma evansi* in camels, donkeys and dogs in India: comparison of PCR and light microscopy for detection- short communication. Veterinarski. Arhiv. 78: 89-94.
- Reid, S. A., Husein, A. and Copeman, D. B. (2001). Evaluation and improvement of parasitological tests for *Trypanosoma evansi* infection. Vet. Parasitol. 102: 291–297.
- Sambrook, J. and Russell, D. W. (2001). Molecular cloning: A laboratory manual. 3rd edition. Cold Spring Harbor Laboratory Press, New York, U.S.A.
- Sharma, A., Singla, L. D., Tuli, A., Kaur, P., Batth, B. K., Javed, M. and Juyal, P.D. (2013). Molecular prevalence of *Babesia bigemina* and *Trypanosoma evansi* in dairy animals from Punjab, India by duplex PCR: A step forward to detection and management of concurrent latent infections. Biomed Res. Int. Article ID 893862

- Singh, N., Pathak, K. M.L. and Kumar, R. (2004). A comparative evaluation of parasitological, serological and DNA amplification methods for diagnosis of natural *Trypanosoma evansi* infection in camels. *Vet. Parasitol.* 126: 365–373.
- Singla, L. D., Juyal, P. D. and Sharma, N. S. (2010) Immune responses to haemorrhagic septicaemia (HS) vaccination in *Trypanosoma evansi* infected buffalo-calves. *Trop. Anim. Health. Pro.* 42: 589–595.
- Singla, L.D., Sharma, A., Kaur, P., Tuli, A., Bhat, S.A. and Bal, M.S. (2013). Bovine Trypanosomosis in Punjab: Assessment of seroprevalence by CATT/ *T. evansi*. *Int. J. Advanced Res.* 1:364-371.
- Singla, N., Parshad, V. R. and Singla, L.D. Potential of *Trypanosoma evansi* as a biocide of rodent pests. (2003). In: *Mice, rats and people: Rodent biology and management*, Singleton GR, Hinds LA, Krebs CJ and Spatt D (eds). ACIAR monograph, Canberra, Australia. pp. 43-46.
- Sumbria D, Singla L D, Sharma A, Moudgil A D and Bal M S. 2014. Equine trypanosomosis in central and western Punjab: Prevalence, haemato-biochemical response and associated risk factors. *Acta Trop.* 138: 44–50.
- Weiss, J. B. (1995), DNA Probes and PCR for diagnosis of Parasitic infections. *Clin. Microbiol. Rev.* 8:113-130.
- Wuyts, N., Chokesajjawatee, N. and Panyim, S. (1994). A simplified and highly sensitive detection of *Trypanosoma evansi* by DNA amplification. *Southeast Asian J. Trop. Med. Public. Health.* 25: 266–271.
- Wuyts, N., Chokesajjawatee, N., Sarataphan, N. and Panyim, S. (1995) PCR amplification of crude blood on microscopic slides in diagnosis of *Trypanosoma evansi* infection in dairy cattle. *Annales de la Societe Belge. De. Medecine. Tropicale.* 75: 229-237.