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Quantification of *Trypanosoma evansi* parasitic load in experimentally infected mice using real time PCR assay and its comparative evaluation with conventional parasitological techniques

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ABSTRACT

Trypanosoma evansi causes an economically important disease called *surra,* which is responsible for significant losses to livestock productivity in Asia, Africa, Central and South America. In the present study, a SYBR Green based quantitative PCR (qPCR) assay was optimized using primers targeting the internal transcribed spacer 1 (ITS–1) region of rRNA gene for quantitative estimation of *T. evansi* infection in experimentally infected mice. The sensitivity of qPCR assay was found approximate to the gold standard TBR1/2 primers based PCR assay and two times more than the conventional parasitological techniques, viz. wet blood film (WBF) stained thin blood smear (TBS) and microhaematocrit centrifugation test (MHCT). The detection limit of the assay was 1.5 parasite equivalence or 0.15 pg *T. evansi* DNA. On fourth day post infection, 100% (6/6) mice were detected positive by all parasitological techniques with parasitaemia ranging from 1.6×10^5 to 2.1×10^8 parasites. The study indicated that this assay can be applied for quantitative estimation of parasitaemia in animals suffering from *surra* and may be helpful for understanding disease status, risk analysis and efficacy of drug used in treatment of disease.

Key words: ITS-1, Molecular diagnosis, PCR, qPCR, Surra, SYBER, *Trypanosoma evansi*

Trypanosoma evansi, an extracellular protozoan parasite, causes an economically important wasting disease in animals commonly known as *surra*. It affects a wide range of domestic and wild animals (OIE 2012). The disease is prevalent in Africa, Central and South America, Middle East and Asia. In India, *surra* is endemic in equines in north and north-western regions of the country (Kumar *et al.* 2013, Sumbria *et al.* 2015). The economic losses to the livestock industry due to the disease are mainly through mortality, reduced draught capacity, low milk and meat production, poor carcass quality, infertility, abortion and high costs of treatment. The diagnosis of *surra* is often difficult because of chronic and fluctuating nature of parasitaemia and the cryptic nature of the parasite into less accessible tissues making the diagnosis often difficult (Nantulya 1990). Further inherent limitations of various parasitological and serological techniques raises the need for more sensitive and specific DNA based assays. In recent years, the development and application of the PCR techniques with various primers, viz. 21/22 mer TBR1/2 ESAG6/7 ITS-1 etc. have improved the diagnosis of *T. evansi* infection in animals (Masiga *et al.* 1992, Wuyts *et al.* 1994, Njiru *et al.* 2005, Fernandez *et al*. 2009, Pruvot *et al.* 2010). The OIE recommended use of TBR1/2 primers in PCR assay based on detection sensitivity for *surra* in animals (OIE 2012). Although PCR assays have been reported with higher detection sensitivity of *T. evansi* infection than conventional parasitological techniques but it is not possible to quantify the level of parasitaemia, which is essential for assessment of disease stage and risk of transmission (Konnai *et al.* 2009, Sumbria *et al.* 2014). In the present study, a real time PCR assay (qPCR) was applied for quantitative estimation of *T. evansi* infection in experimentally infected Swiss albino mice and its comparative sensitivity was evaluated with PCR and conventional parasitological techniques.

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MATERIALS AND METHODS

Propagation of T. evansi *in mice:* In this study, Swiss albino mice used for experimental infection were procured from Disease Free Small Animal House of the University after getting approval of Institute Animal Ethics Committee and as per guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals

(CPCSEA), Animal Welfare Division, Government of India. For experimental infection of mice, a *T. evansi* isolate of pony origin (*T.ev*-India-NRCE-Horse1/Hisar/Haryana) was used. The mice were inoculated with 1×10^5 trypanosomes per animal by intra-peritoneal route. The parasitaemia in mice was examined daily and at peak of parasitaemia (10^8 trypanosomes/ml) blood was collected by cardiac puncture for further study. The parasitaemia was estimated in infected blood using Neubauer cell counting chamber.

Isolation, purification and DNA extraction: Trypanosomes were isolated and purified from the infected whole blood by DEAE-cellulose anion exchange column chromatography as per Lanham and Godfrey (1970). Live parasites were eluted in PBS-G pH 8.0 (57 mmol/l Na_2HPO_4, 3 mmol/l NaH_2PO_4, 43.8 mmol/l NaCl with 1% Glucose). The eluted trypanosomes were washed twice with PBS (pH 7.2) and centrifuged at 3000×g for 5 min. Finally, the genomic DNA was extracted by phenol-chloroform extraction method (Sambrook and Russel 2001) and concentration of extracted DNA was determined by nanodrop.

*PCR optimization with TBR1/2 primers:*The PCR was performed with TBR1/2 primers which amplified repetitive sequence of mini-chromosome satellite DNA (Masiga *et al.*1992) considered as gold standard by OIE for detection of *Trypanosoma evansi* DNA. The PCR mix consisted of 12.5 µl of Dream Taq PCR Master Mix, 1.0 µl of each primers (10 µM), 1.0 µl Template DNA (15 ng) and nuclease free water up to 25 µl. The PCR was performed in 96 universal gradient thermocycler with cycling conditions 1 cycle of 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 30 sec at 72°C and finally 1 cycle of 2 min at 72°C. The PCR products were electrophoresed in 2.0% agarose in 1× TAE buffer and visualized under UV light. The optimum conditions of amplification were determined for primer TBR1/2.

Primer selection, optimization and development of qPCR assay: The ITS-1 region of six *T. evansi* isolates collected from different agro-ecological zones of India maintained in National Fellow laboratory were sequenced and the region exhibiting a high degree of sequence conservation by alignment of these sequences was used for designing of primers for qPCR assay. The details of primer sets (ITS1–RT-F and ITS1-RT-R) are given in Table 1. The experiments were performed to determine the optimum concentration of the primers by using different combinations. The PCR reaction mix consisted of 10 µl of Power SYBR® Green PCR Master Mix, 1.0 µl of each primer (forward and reverse) concentration ranging from 5 µM to 0.625 µM, 1.0 µl Template DNA (15 ng) and 7 µl nuclease free water. The reactions were performed in step one real-time PCR thermocycler having software version 2.2.2. with thermal cycling conditions of 10 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at 54°C and 30 sec at 60°C and finally a melting stage of 15 sec at 95°C, 1min at 60°C and 15 sec at 95°C. All the reactions were performed in duplicate. The optimized primer concentrations giving the highest ΔRn with minimum Ct value were selected for qPCR amplification. The product identity was confirmed by melting curve analysis and agarose gel electrophoresis.

Table 1. Detail of primers used in qPCR assay

Primer name	Sequence 5'-3'	Length	T_m (°C)	GC (%)	Product size
ITS1-RT-F	5'-CAGTAGTAATA ACACAGAGAAT- 3'	22	49.4	31.8	151 bp
ITS1-RT-R	5'-AATAGGAAG CCAAGTCAT- 3'	18	53.5	38.8	

Enumeration of parasite and concentration of genomic DNA: The number of parasites in per ml infected mice blood sample and purified parasite elute were counted as 2×10^8 parasites/ml and 4×10^7 parasites/ml respectively. The concentration of DNA isolated with known quantity of trypanosomes was measured by nanodrop spectrophotometer which was 15 ng/µl.

Standard curve: With the consideration that a single parasite genomic weight is 0.1pg (Borst *et al.* 1982), the number of parasitic equivalence were calculated from extracted DNA. Stock DNA of *T. evansi* having concentration 15 ng/µl was considered equal to 1.5×10^5 parasite equivalence/µl of DNA. A 10 fold serial dilutions of extracted stock DNA were prepared in nuclease free water to obtain a regular series of standards which contains known

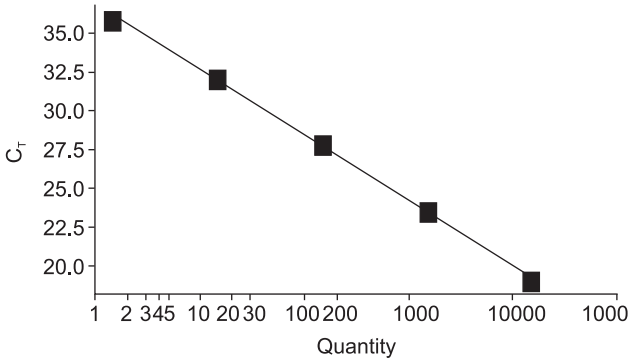

Fig. 1. Standard curve from 5 dilutions of *T. evansi* DNA (1.5×10^4, 1.5×10^3, 1.5×10^2, 15, 1.5 parasitic equivalence) for qPCR assay.

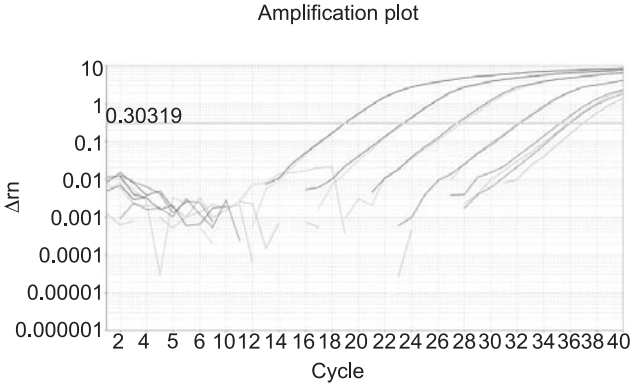

Fig. 2. Real time amplification plot with different dilution of DNA comprising 1.5×10^4 to 1.5 parasitic equivalence of *Tryapanosoma evansi.*

number of parasitic equivalence ranging from 1.5×10^5 to 1.5×10^{-4} and standard curve was generated using these serial dilutions of DNA (Figs 1, 2).

Sensitivity and specificity of qPCR assay: The evaluation of sensitivity was performed by using standards prepared by 10 fold serially diluted extracted DNA and parasitic equivalence was calculated on basis of concentration of DNA. 1µl of each dilution was used for each qPCR reaction and sensitivity was obtained in the form of parasitic equivalence. Primer specificity of assay was confirmed by using DNA samples extracted from *Theileria equi*, a commonly observed haemo-protozoan parasite in Indian equids.

Calculation of parasitaemia

*In mice:*Sensitivity in terms of parasites/µl of blood was derived by using extracted DNA with a known level of parasitaemia. The extracted DNA having a parasitaemia 2×10^8 parasites/ml and finally eluted in 100 µl of elusion buffer (stock DNA). 1 µl of 1:10 diluted stock DNA was used in five replicates and parasite equivalence was calculated. A difference factor was derived based on difference in parasite equivalence count (counted by real-time PCR thermocycler) and actual number of parasites in whole blood (counted by Neubaur chamber) which was equal to1:1.0955 (Table 2).

The parasitaemia in experimentally infected mice was

Table 2. Calculation of difference factor for estimation of parasitaemia

Replicate no	Ct value of replicates	Parasite equivalence calculated by real time thermocycler in 1 µl DNA extracted from infected blood	Parasite no. in infected blood calculated by Neubaur chamber in corresponding 1 µl DNA extracted from infected blood
1	13.56133	35,676.66	40,000
2	13.67609	31,937.59	
3	13.39992	36,798.87	
4	13.32028	39,737.77	
5	13.34668	38,405.79	
Mean		36,511.33	
	Difference factor	40,000/36,511.33= 1.0955	

calculated as:

Parasitaemia/ml of blood of infected mice = Parasite equivalence × 1.0955 (calculated difference factor) × 5000 (dilution factor).

Comparative evaluation of diagnostic sensitivity of qPCR with conventional parasitological techniques and PCR in experimentally infected mice

Six Swiss albino mice were experimentally infected with *T. evansi* isolate of pony origin (*T.ev*-India-NRCE-Horse1/Hisar/Haryana) at 1×10^5 parasites/animal by intra-peritoneal route. Three mice were kept as negative control. The blood samples were collected at 24 h interval for evaluation of comparative diagnostic sensitivity with conventional parasitological techniques (WBF, MHCT, stained smear examination) PCR and qPCR.

For wet blood film examination, a drop of freshly collected blood was placed on a clean glass slide covered with a cover slip and was scanned microscopically. Thin blood smear was examined under microscope after staining with Giemsa's stain (1:10 dilution in PBS, pH 7.2). MHCT was performed for the presence of *T. evansi* as per method of Woo and Rogers (1974). The PCR and qPCR were applied as per procedure described above.

RESULTS AND DISCUSSION

Primer optimization for qPCR assay: Different combinations of ITS1 primers were used ranging from 5 µM to 0.625 µM and primer combination giving highest ΔRn with minimum Ct value (5 µM) was selected for qPCR amplification. Melting temperature of PCR product was confirmed by dissociation curve analysis and identity of PCR product was confirmed by agarose gel electrophoresis which revealed a 151 bp product by qPCR amplification.

Sensitivity and melting curve analysis for specificity of qPCR assay: To evaluate the specificity and sensitivity of the qPCR assay for detection and quantification of level of parasitaemia, first the limit of detection was determined using 10–fold serial dilutions. Lowest level of parasitaemia detected by the assay was 1.5 parasites equivalence or 0.15 pg *T. evansi* DNA. Product melting temperature and its identity were confirmed with melting curve analysis. All replicates from 1.5 ng (1.5×10^4 parasite equivalence) to 0.00015 ng (1.5 parasite equivalence) were showing linear relation on standard curve plot with a R^2 value of 0.998. The specificity of qPCR was determined by qPCR melting curve programme and melting temperature of amplicon was established as approximately 79°C. In qPCR assay using ITS-1 NRCE primers 2 melting peaks with melting temperature of approximately 79°C and 73°C were obtained. Similar patterns of 2 melting peaks were observed with DNA from other five different isolates of *T. evansi* of donkey, camel and cattle origin indicating specific amplification at 2 melting temperatures (Fig. 3).

Application of qPCR in experimentally T. evansi *infected mice and its comparative evaluation with conventional parasitological techniques and PCR*

The diagnostic sensitivity of various techniques were compared and it was observed that conventional parasitological techniques were less sensitive as compared to PCR and qPCR. The qPCR assay sensitivity for detection of *T. evansi* was found near to TBR-PCR. Both PCR and qPCR assays were able to detect the infection 24 h post infection (hpi) in 33% (2/6) of experimentally infected mice, whereas, other conventional parasitological techniques detected infection on second day post infection (dpi). The parasitaemia estimated using qPCR ranged from 1.5×10^4

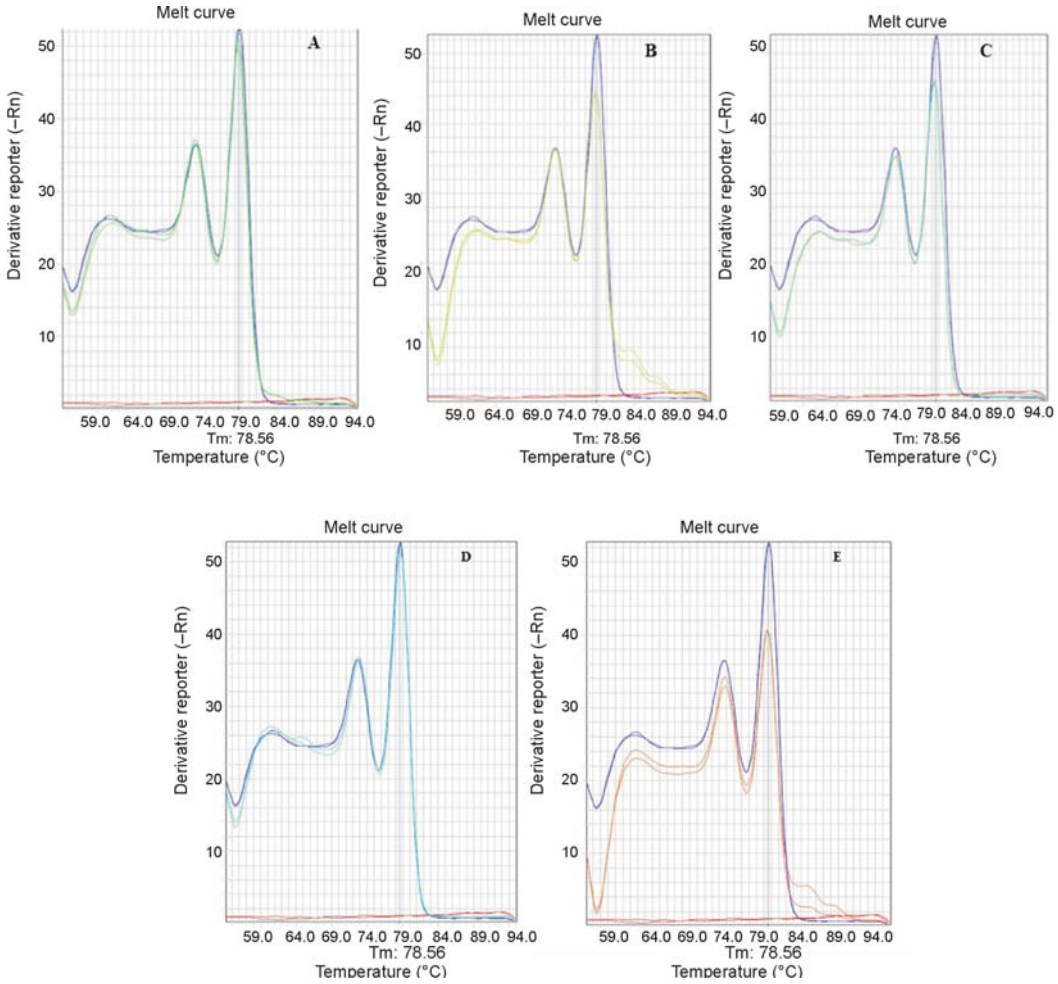

Fig. 3. Melting curve showing 2 melting peaks in real time PCR with genomic DNA isolated from 5 different isolates of *T. evansi.* A, *T. evansi* donkey isolate (Gujarat); B, *T. evansi* donkey isolate (Uttar Pradesh); C, *T. evansi* camel isolate (Rajasthan); D, *T. evansi* cattle isolate (Haryana) E, *T. evansi* horse isolate (Haryana).

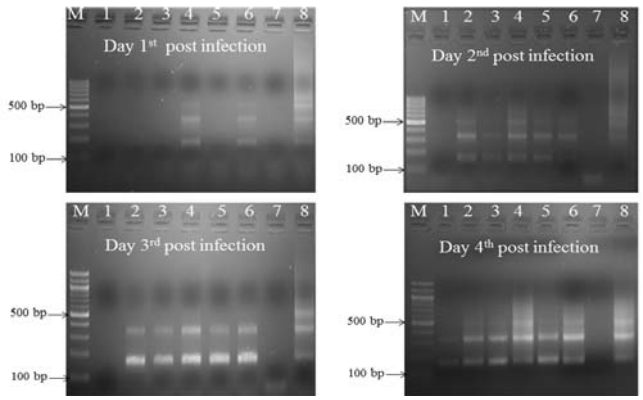

Fig. 4. PCR reaction with TBR1/2 primers showing 164 bp amplicons in multiple repeats with DNA from six experimentally *Trypanosoma evansi* infected mice. Lane M, 100 bp DNA ladder; lane 1–6, PCR products from 1–6 mice; lane 7, negative control; lane 8, positive control.

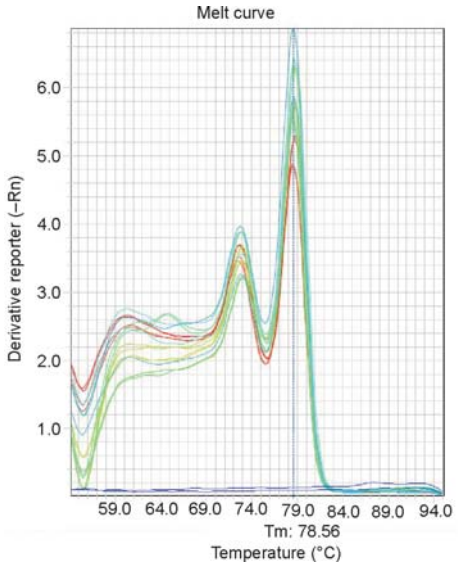

Fig. 5. Post amplification acquisition of melting curves with DNA of six experimentally *Trypanosoma evansi* infected mice on day third post infection showing specific amplification. Red line, known positive standard; bottom blue line, negative control showing no amplification.

to 5.2×10^4 parasites/ml of blood. On second dpi WBF Giemsa stained TBS, MHCT, PCR and qPCR detected 33% (2/6), 50% (3/6), 67% (4/6), 83% (5/6) and 67% (4/6), respectively, with parasitaemia ranging from 5.3×10^4 to

2×10^6. After third dpi, all the conventional parasitological techniques detected infection in 83% (5/6) of mice. PCR also detected 83% (5/6 mice) while 100% mice. (6/6) were positive by qPCR with parasitaemia ranging from 4.7×10^4 to 2.5×10^6/ml of blood. After day fourth dpi, 100% (6/6) mice were positive by all the parasitological techniques, PCR and qPCR with parasitaemia ranging from 1.6×10^5 to 2.1×10^8 (Tables 3, 4; Figs 4–6).

The qPCR primers were designed targeting conserved common sequence present in ITS-I (between 18s and 5.8s subunits of ribosomal RNA gene) of six *T. evansi* Indian isolates collected from different agro-ecological regions and hosts for wide application of the assay in Indian context. In our qPCR study melting curve analysis revealed two specific melting peaks one near 79°C and another near 73°C. Similar pattern of two melting peaks was observed with DNA of other *T. evansi* isolates of donkey, camel and cattle origin. Other workers had also reported such type of observation in qPCR studies which could be attributed to the presence of polymorphic nucleotide sites randomly distributed in the conserved regions of a gene giving rise to highly conserved sequence block alternated with slight variable sequence polymorphisms (Degrave *et al.* 1988, Moreira *et al.* 2013).

In the present study, limit of sensitivity of qPCR was determined to be 1.5 parasitic equivalence or 0.15 pg genomic DNA of *T. evansi* and equivalent to 0.82 parasite/

Table 3. Comparative efficacy of different methods (WBF, MHCT, Thin blood film, TBR-PCR and qPCR) for diagnosis of *Trypanosoma evansi* infection in experimentally infected mice

Day post infection	WBF	TBS	MHCT	PCR (TBR primer)	qPCR (ITS-1 NRCE primer)
Day 1	0/6 (0%)	0/6 (0%)	0/6 (0%)	2/6 (33%)	2/6 (33%)
Day 2	2/6 (33%)	3/6 (50%)	4/6 (67%)	5/6 (83%)	4/6 (67%)
Day 3	5/6 (83%)	5/6 (83%)	5/6 (83%)	5/6 (83%)	6/6 (100%)
Day 4	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)

specific primers (Konnai *et al.* 2009); 0.01ng of purified *T. evansi* DNA (0.33 genomic equivalent) and 0.1 ng of whole blood extracted DNA (6.12 genomic equivalent) in mice model using ITS-1 specific primers (Sharma *et al.* 2012); variation in sensitivity of real time PCR using ITS1-TeRT and TeRoTat primers were 1.6×10^{-2} parasites/µl, 5.1×10^1 parasites/µl of capybara blood, respectively (Eberhardt *et al.* 2014). The variation in the sensitivity as reported by previous workers may be due to difference in primer efficiency method and volume of blood used for DNA extraction reagents variation and targeted gene sequence.

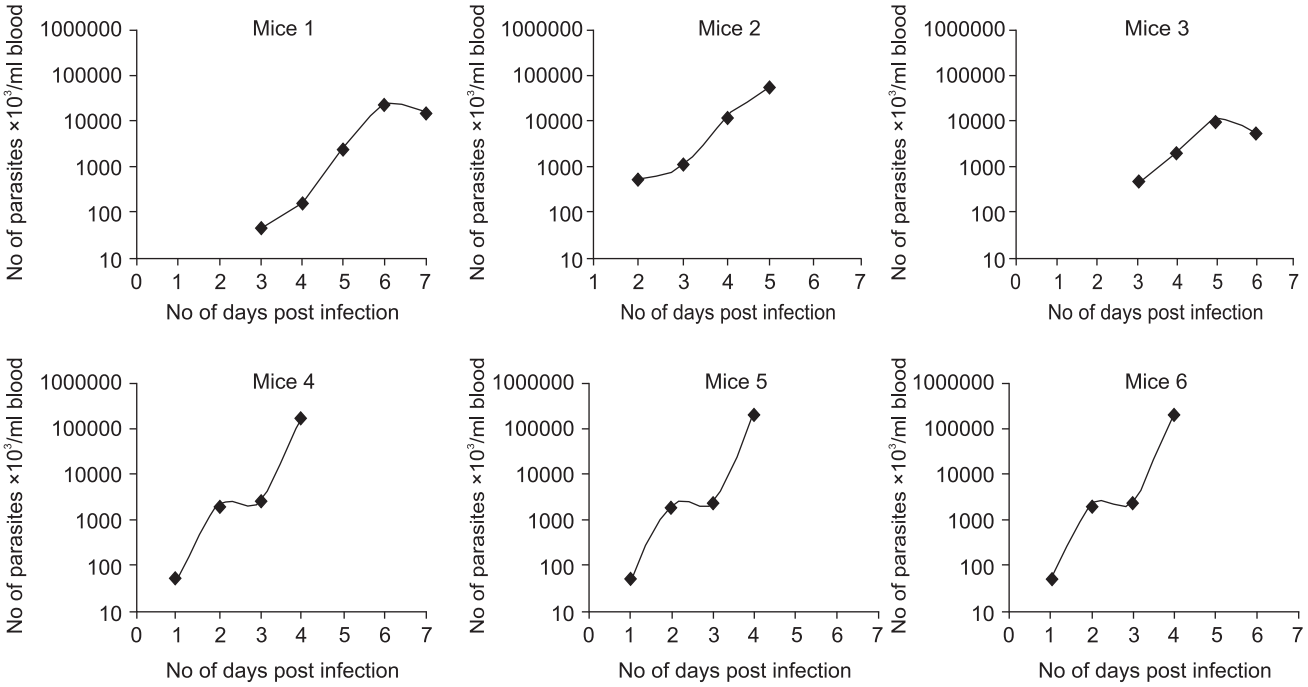

Fig. 6. Quantification of parasitaemia in six experimentally infected mice with *Trypanosoma evansi* from day 1–7 post infection.

µl of blood. The different levels of sensitivity have been achieved by different workers 0.3 genomic equivalent/ml of blood (Taylor *et al.* 2008) and qPCR was more sensitive than MHCT but less as compared to mouse inoculation assay; 100 parasite/ml of infected buffalo blood using VSG These factors play a very important role in determining the sensitivity of qPCR assay.

In the present study, to know the level of parasitemia we calculated difference factor between parasite equivalence (calculated by Real time PCR machine based on copy

Table 4. Quantification of *T. evansi* in experimentally infected mice by qPCR assay

Time ↓ interval	Animal no. →	M1	M2	M3	M4	M5	M6
Day 1	Parasite equivalence	No specific amplification	No specific amplification	No specific amplification	9.59	No specific amplification	2.82
	No. of parasite/ml blood				5.2×10^4		1.5×10^4
Day 2	Parasite equivalence	No specific amplification	96.77	No specific amplification	3.81×10^2	24.05	83.08
	No. of parasite/ml blood		5.3×10^4		2×10^6	1.3×10^5	4.5×10^5
Day 3	Parasite equivalence	8.63	2.14×10^2	77.81	4.7×10^2	1.32×10^2	2.82×10^2
	No. of parasite/ml blood	4.7×10^4	1.1×10^6	4.2×10^5	2.5×10^6	7.2×10^5	1.5×10^6
Day 4	Parasite equivalence	30.4	2.4×10^3	3.7×10^2	3.9×10^4	1.5×10^3	9.8×10^3
	No. of parasite/ml blood	1.6×10^5	1.3×10^7	2×10^6	2.1×10^8	8.5×10^6	5.4×10^7
Day 5	Parasite equivalence	4.3×10^2	1.15×10^4	1.9×10^3	Mice died on 4 dpi	1.1×10^4	1.1×10^4
	No. of parasite/ml blood	2.4×10^6	6.3×10^7	1×10^7		5.9×10^7	6.2×10^7
Day 6	Parasite equivalence	4.1×10^3	Mice died on 5 dpi	1×10^3	Mice died on 4 dpi	Mice died on 5 dpi	Mice died on 5 dpi
	No. of parasite/ml blood	2.2×10^7		5.6×10^6			
Day 7	Parasite equivalence	2.3×10^3	Mice died on 5 dpi	Mice died on 6 dpi	Mice died on 4 dpi	Mice died on 5 dpi	Mice died on 5 dpi
	No. of parasite/ml blood	1.6×10^7					

numbers used in standard curve based on genomic DNA equivalence) and actual number of parasites present in blood counted by Neubauer chamber. This factor may overcome the differences occurring due to methods used for extraction of DNA and differences due to different copy number of targeted gene sequences in genomic DNA of *T. evansi* used for designing of primers. The calculation factor to determine the parasitaemia may vary depending upon volume of blood used for extraction of DNA and dilution of DNA used in qPCR assay.

The sensitivity of qPCR assay was compared with conventional parasitological techniques (wet blood film examination, thin blood film examination and MHCT) and PCR. The qPCR was found to be having a sensitivity approximately gold standard TBR-PCR. Both gave positive signals after 24 hpi in 2 out of 6 mice while conventional parasitological techniques were positive after 48 hpi indicating that molecular techniques detected infection two times earlier as compared to conventional techniques. Our results slightly vary from the findings of Sharma *et al*. (2012) who detected parasitaemia after 36 hpi by real-time PCR earlier than MHCT and blood film examination that detected after 60 and 72 hpi respectively. Ijaz *et al.* (1998) detected parasitaemia 72–150 hpi in WBF examination in experimentally infected mice after inoculation of 104 *T. evansi* of camel origin. The variation in detection time may be attributed to variation in number of parasites inoculation for experimental infection of mice. The results indicated that PCR/qPCR were found twice more sensitive as compared to conventional parasitological techniques and this was in concordance with the findings of earlier workers (Muieed *et al.* 2010, Radwan and Madawy 2010, Elhaig *et al*. 2013). The additional advantage with qPCR was in determining the level of parasitaemia in experimentally infected mice.

The assays, PCR and qPCR revealed same level of sensitivity in experimentally *T. evansi* infected mice. The qPCR yielded additional information in terms of quantification of parasitaemia during different stages of infection. The qPCR assay demonstrated higher sensitivity than conventional parasitological techniques with the ability to detect low level of parasitaemia in quantitative form. In field, qPCR can be applied for quantitative estimation of parasitaemia and efficacy of anti-trypanosomal drug in treated animals suffering from surra. Data analysis is a crucial part due to biological variation, process variation and system variation. Therefore appropriate statistical tools and calculation factor should be used for analysis of results.

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