Table 1:

Primer	Position	Nucleic acid sequence	PCR product
External (outer primers)			
TE1	91–110	(5)-AGGACGCAGAAATAGCAGTA (3)	810-bp
TE2	881–900	(5)-ATTTAATTG AGTGGCGTGAG (3)	
Internal (nested Primers)			
TE3	311–330	5)-CTTTTATACGAGGAG GGGA (3)	270-bp
TE4	561–580	5)-TATGGGCGTGCAGATTTC AC (3)	
Semi-nested 1			
TE1	91–110	5)-AGGACGCAGAAATAGCAGTA (3)	490-bp
TE4	561–580	5)-TATGGGCGTGCAGATTTC AC (3)	
Semi-nested 2			
TE3	311–330	5)-CTTTTATACGAGGAG GGGA (3)	590-bp
TE2	881–900	5)-ATTTAATTG AGTGGCGTGAG (3)	

Extraction of DNA from tissue samples

Five ml distilled water was added to 1 gm of each tissue sample collected from lungs, Liver and spleen of experimentally infected mice. The tissue sample was then mixed by homogenization. The homogenate was treated by freezing and thawing and finally centrifuge at 3000 RPM for 10 minutes. 200 μl of the supernatant was used for DNA extraction following the same procedure used for extraction of DNA from blood as described above.

Primers selection

Primers (TE1 and TE2) were selected from a highly conserved region of the published sequence of nuclear repetitive gene of Indonesian strain of *T. evansi* [24]. This pair of primers was used for the synthesis of the primary PCR amplification product. TE1 included bases 91–110 of the positive sense strand of *T. evansi* putative gene (5)-AGG ACG CAG AAA TAG CAG TA-(3). TE2 included bases 881–900 of the complementary strand: (5)-ATT TAA TTG AGT GGC GTG AG-(3). The PCR using primer TE1 and TE2 will result in a 810-bp PCR product. For the nested amplification step, oligonucleotide primers (TE3 and TE4) were selected from the same published sequence cited above. TE3 consisted of bases 311–330 of the positive strand (5)-CTT TTA TAC GAG GAG AGG GA-(3). TE4 was designed from the complementary strand between bases 561–580 (5)-TAT GGG CGT GCA GAT TTC AC-(3). PCR amplification using TE3 and TE4 will result in a 270 bp PCR product, internal to the annealing sites of TE1 and TE2. Using the semi-nested pair of primers (TE1 and TE4), the PCR assay will result in amplification of a 490-bp semi-nested PCR product. The pair of semi-nested primers (TE3 and TE2) will be expected to produce a 590-bp semi-nested PCR product. Details for primers design including position of nucleotides, nucleotide sequences, and expected PCR products are shown in (Table 1).

All primers were synthesized on a DNA synthesizer (Milliigen/Biosearch, a division of Millipore Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA.) as per manufacturer's instructions.

Polymerase chain reaction (PCR)

A stock buffered solution containing 250 μl 10× PCR buffer, 100 μl of magnesium chloride, 12.5 μl of each dATP, dTTP, dGTP and dCTP was prepared in 1.5 ml eppendorf tube. The primers were used at a concentration of 20 pg/μl, and double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml. Two μl of primers, 5.0 μl of the target DNA and 42 μl of the stock solution were added onto 0.5 ml PCR tubes and mixed by vortexing. One μl of Taq DNA polymerase (Perkin Elmer) was used at a concentration of 5.0 U/μl. All PCR amplification reactions were carried out in a final volume of 50 μl. The thermal cycling profiles were as follows: a 2 min initial incubation at 95°C, followed by 40 cycles of 95°C for 1 min, 55°C for 30 sec and 72°C for 45 sec, and a final incubation at 72° for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 15 μl from each PCR containing amplified product were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and *T. evansi* primary PCR products were easily identified following visualization under UV light.

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 (2 μl of nested primers and; 42 μl of stock PCR buffer and Taq DNA polymerase was used at a concentration of 5.0 U/μl. The nested pair of primers (TE3 and TE4) was expected to amplify a 270 bp PCR product, internal to the annealing sites of primers PSL1 and PSR2. All PCR amplifications were carried out in a final volume of 50 μl. The thermal cycling profiles were as follows: a 2 min incubation at