

Research Article

**DETECTION OF *TOXOPLASMA GONDII* TARGETING THE REPETITIVE
MICROSATELLITE SEQUENCE BY PCR**

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ABSTRACT: *Toxoplasma gondii* RH strain tachyzoites were inoculated in mice intraperitoneally (1.5x10⁴) and serially propagated. Tachyzoites were cryopreserved using 10-20% glycerol and aliquoted in cryovials in liquid nitrogen. The viability of the preserved tachyzoites were further checked by passage in mice followed by PCR amplification of the highly repetitive sequence of *T. gondii*. The sequence analysis of the repeated sequence of *T. gondii* (GenBank Acc. No. KC607824) showed 99.2% homology with Strain SH and Strain PYS (GenBank Acc. no. DQ779192, DQ779189), 98.9% homology with Strain ZS1 (GenBank Acc. no. DQ779196), and 92.5% homology with Strain RH (GenBank Acc. no. DQ779191). The study showed that this repetitive microsatellite sequence could be a good target for detection of toxoplasmosis.

Key words: *Toxoplasma gondii*, Tachyzoites, Repetitive sequence, PCR.

INTRODUCTION

Toxoplasma gondii is an obligate, intracellular protozoan parasite with facultative heteroxenous life cycle. It remains a widely prevalent zoonotic disease affecting man and animals (Dubey and Beattie 1988). It constitutes a potential source of infection to all warm-blooded animals, viz., sheep, goat, pig, dog, cat, bear and chicken. The parasite can able to multiply virtually in all the nucleated cells of body and as a result encystment occurs.

This parasites was first described by Nicolle and Manceaux in the year 1909 in a rodent, but its widespread distribution in animal kingdom was realized after 20 years (Jones and Hunt 1983). Felines serve as definitive host, while non-felines (all warm blooded mammals including man and birds) act as intermediate host of the parasite with disseminated tissue infections (Frenkel *et al.* 1970). Intermediate hosts are infected by ingestion of sporulated oocysts, cyst-contaminated meat, milk contaminated by tachyzoites or trans-placentarily (Pepin *et al.* 1997). Meat from *T. gondii* infected pigs and sheep, and goat milk are shown to be primary sources of infection for men (Smith 1991, 1993). Presence of the parasite in semen, urine, saliva and secretion from the infected host may be also

responsible for transmission of this parasite from one animal to other (Spence *et al.* 1978; Chiari *et al.* 1984, Vickers *et al.* 1992).

The genus *Toxoplasma* has only one species, *T. gondii*, with different strains (Binas and Johnson 1998), which have been classified according to their biological differences (Literac *et al.* 1998) and virulence in mice. Some studies on genetic variability of these strains have been employed with different approaches such as antigen analysis (Bohne *et al.* 1993), isoenzyme assays (Darde *et al.* 1992, Asai *et al.* 1995), randomly amplified polymorphic DNA, PCR detection (Guo *et al.* 1997) and restriction fragment length polymorphism (Christina *et al.* 1991, Christina *et al.* 1995, Howe *et al.* 1997).

The diagnosis of toxoplasmosis classically relies on serology and demonstration of pathogen. Infected body fluid or tissue may also be inoculated intraperitoneally into mice or used to infect cell cultures *in vitro*. Mouse inoculation is still considered to be the reference method because of its high sensitivity and specificity. However, this is time consuming and need nearby specialized laboratory in order to inoculate the animals and the involvement of large number of laboratory animals. Therefore, polymerase chain reaction of *T. gondii* gene(s)

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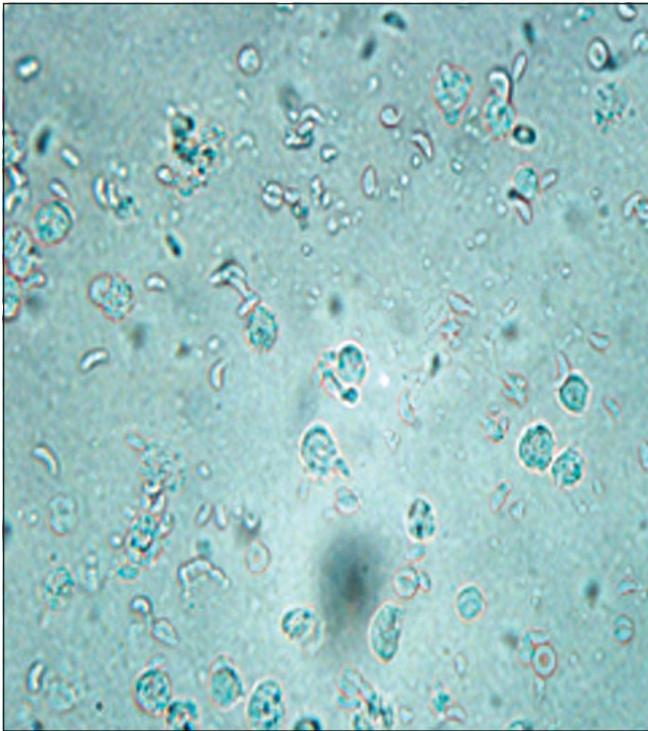


Fig. 1. Tachyzoites in peritoneal exudates of *Toxoplasma gondii* infected mice (X100).

is considered a valuable alternative. Expert laboratories have reported good correlations of PCR with animal inoculation; however, the use of a standardized *T. gondii* PCR in routine laboratories is still in its initial phase of development. Of the PCR targets described for *T. gondii*, the 35-copy number B1 gene (Burg 1989) is the most widely used. In order to lower the detection limit, the B1 gene is amplified by nested PCR. Since the sensitivity of the *T. gondii* PCR also depends on the copy number of the gene amplified, highly repeated sequences in the *T. gondii* genome can serve as new PCR target candidates (Homan *et al.* 2000). The present work was carried out with the objective of amplifying a repetitive sequence of *T. gondii* (RH Strain) for diagnosis of toxoplasmosis and sequence characterization of the said repeated sequence.

MATERIALS AND METHODS

Adult Swiss albino mice of either sex of 6-12 weeks of age, weighing 25-30g, were maintained in mice cage with congenial room temperature, standard diet and *ad libitum* water. A mouse adopted-RH strain of *T. gondii* was obtained from All India Institute of Medical Sciences, New Delhi for this study. Since all stages of *T. gondii* are infective, every safety precautions were taken during its handling.

Maintenance and Propagation of *T. gondii* in mice

Mice were inoculated with mouse adopted-RH strain of *T. gondii* tachyzoites (1.5×10^4) intraperitoneally. The

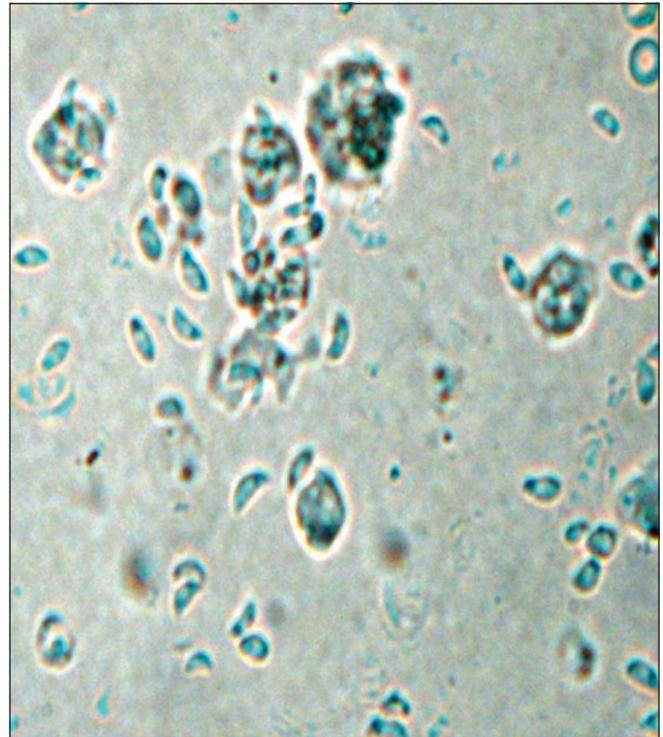


Fig. 2. Tachyzoites inside the macrophages (X400).

tachyzoite stage was maintained by serial passage in the peritoneal cavity of outbred Swiss albino mice, and examined daily for development of peritonitis. The mice were then euthanized by ether and chloroform anaesthesia and peritoneal lavage was done using 7-10 ml of PBS (pH 7.4) after cutting the abdominal cavity aseptically. The process was repeated thrice or until the lavage becomes clear. The contents were washed thrice in PBS (pH 7.2) and numbers of live tachyzoites were counted.

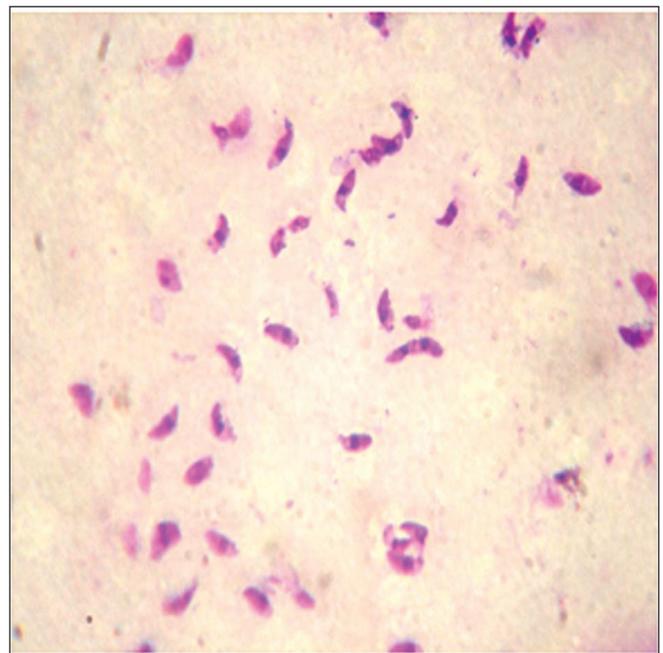


Fig. 3. Host cell free tachyzoites Giemsa stain, X1000).

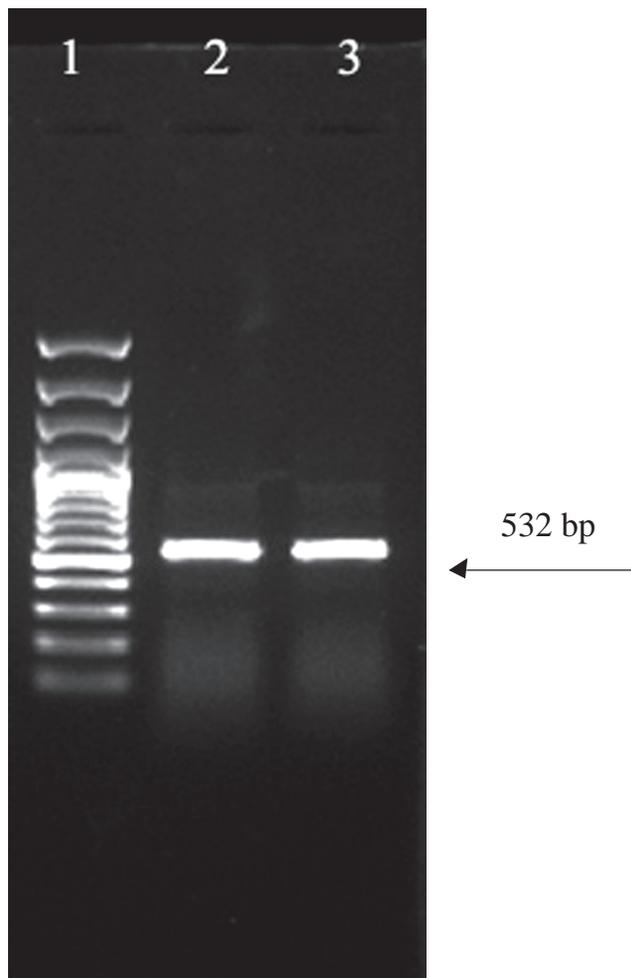


Fig. 4. Amplification of repeated sequence of *Toxoplasma gondii*
 Lane 1: 100bp DNA ladder plus.
 Lane 2&3: 532bp product.

Each healthy mouse of next successive batch was injected intraperitoneally with 1.5×10^4 tachyzoites.

Host cell-free tachyzoites were obtained by washing the peritoneal lavage thrice with PBS (pH 7.4) at 5000 rpm for 10 min. The intracellular tachyzoites were rendered by rupturing the intact parasitized macrophages mechanically and passing the contents through a 27 G needle fitted in a 10 ml syringe. The resultant tachyzoite suspension was washed again by resuspending in 20 ml of PBS (pH 7.4) and the debris was allowed to settle down in a centrifuge tube for 10 min. The supernatant was collected carefully and passed slowly at the rate of 1ml per 2-3 min through a pre-wetted (with PBS) polycarbonate membrane filter of 3.0 μ m pore size (Millipore) (Gross *et al.* 1991). The filtered suspension was centrifuged at 4000 rpm for 10 min to sediment the tachyzoites. The sedimented tachyzoites were resuspended in 1-2 ml of PBS (pH 7.2). The purified tachyzoites were used for various purposes. After filtration through polycarbonate membrane followed by

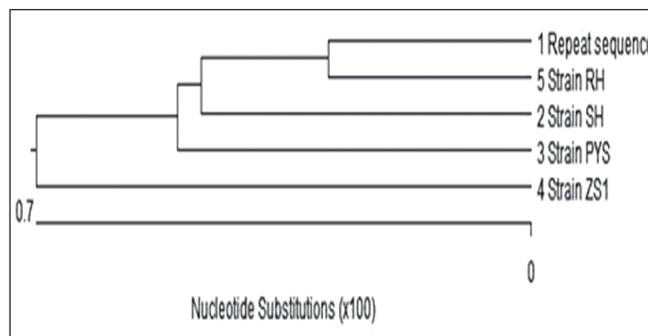


Fig. 5. Phylogenetic tree.

live and dead counts by Trypan blue dye exclusion test, tachyzoites were used for infecting experimental animals.

Host cell free tachyzoites were resuspended in PBS (pH 7.2) containing 10-20% glycerol and aliquoted in cryovials and preserved in liquid nitrogen.

Genomic DNA Extraction from host cell free Tachyzoites

Two hundred microlitre volume of cell free tachyzoites were used for extraction of DNA using DNEasy[®] Blood and Tissue Kit (Qiagen, USA) following manufacturer's protocol. The extracted DNA was stored at -20°C for further study.

PCR Amplification of repeated sequence of *Toxoplasma gondii*

Amplification of repeated sequence of *T. gondii* was done using primers of repeated sequence of *T. gondii*, viz. Forward primer: 5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3' and Reverse primer: 5'-CGCTGCAGACACAGTGCATCTGGATT-3'. The reaction was conducted in 50 μ l volume containing 25 μ l of 2X master mix, 5.0 μ l of genomic DNA, 1.0 μ l (10 p mole/ μ l) each of forward and reverse primer and nuclease free water. The amplification conditions were initial denaturation at 95°C for 10 min., followed by 30 cycles of 95°C for 1 min, 65°C for 30s, 72°C for 30s and final elongation by 72°C for 10 min. The amplification of specific PCR product was checked by gel electrophoresis of the PCR product in 1% agarose and viewed in UV transilluminator (BIO-RAD) system. The PCR product was purified using GeneJET[™] Gel extraction Kit (Fermentas) following manufacturer's protocol.

Sequence analysis of repeated sequence of *T. gondii*

20 μ l of the purified DNA was sequenced using automated sequencer (ABI prism) using Sanger's dideoxy chain termination method with 20 μ l primers at a commercial sequencing firm Xceltris, Ahmedabad, India.

The sequence information obtained was analyzed using Laser gene DNASTar software by ClustalW method and Megablast of Basic Local Alignment Search Tool (BLAST, NCBI). Repeated sequence of the *T. gondii* was compared with the gene of Strain SH (GenBank Accession no. DQ779192), Strain PYS (GenBank Acc. no. DQ779189), ZS1 (GenBank Acc. no. DQ779196), RH Strain (GenBank Acc. no. DQ779191).

RESULTS AND DISCUSSION

Mice with acute toxoplasmosis show clinical manifestations like being less active, mild responsiveness to novel stimuli, and lethargy (Hutchison *et al.* 1980). Other characteristic signs such as incoordination in movement, circling movement, body hair standing on one end, deep yellow urination, mucopurulent discharge from pubic opening, peritonitis, ascites with pendulous abdomen, tachypnoea marked by resting of fore legs either on walls of the cages or on the nozzle of water bottle or on other resting mice, described by Waree *et al.* (2007) were also observed in this study. The pendulous abdomen due to ascites caused by tachyzoites of *T. gondii* given intraperitoneally was also observed. From each mouse, approximately 5 ml and 10 ml peritoneal fluid was aspirated. Peritoneal fluid filled with *T. gondii* tachyzoites was evident under microscope (Fig. 1). The tachyzoites inside the macrophages were also observed (Fig. 2). The peritoneal lavage containing free tachyzoites as well as infected macrophages and infiltrated cells were removed by filtration of the tachyzoites through polycarbonate membrane of 3.0 µm pore size and host cell free tachyzoites were harvested (Fig. 3).

Depending on the zoonotic importance of toxoplasmosis the present study has been under taken which aimed at applying the glycerol as cryoprotectant for liquid nitrogen (LN₂) preservation of *T. gondii* tachyzoites to determine the easiest, safest and most economic method for parasite propagation and preservation. The preserved tachyzoites in liquid nitrogen (LN₂) showed its infectivity as expected, in experimentally infected group of mice and in agreement with earlier reports by Eyles *et al.* 1956, Bollinger *et al.* 1974, Gerges *et al.* 2005.

Mouse inoculation is still considered the reference method because of its high sensitivity and specificity (Homan *et al.* 2000). However, the process is time taking which is a concern in prenatal diagnosis. Therefore, PCR diagnosis of *T. gondii* is considered as a valuable alternative. PCR has been shown to be more sensitive for detection as compared to other methods (James *et al.* 1996). Since the sensitivity of PCR depends on the copy number of gene amplified, highly repetitive sequences in *T. gondii* can serve as a new and potential PCR target

candidates (Homan *et al.* 2000).

In present study, the repetitive DNA fragment was amplified using DNA extracted from the tachyzoites cultivated in mouse peritoneum (Fig. 4). Homan and co-worker (2000) also described the PCR amplification of 200-300 fold repetitive fragment and compared its performance with B1 gene. The combination of several factors such as sensitivity, specificity and detection in presence of host DNA made the 532bp repetitive fragment a worthwhile candidate for further investigation in diagnostic PCR. The repetitive sequence was also considered for copro-diagnostic method used for identification of cats infected with *T. gondii* (Salant *et al.* 2010).

20 µl of the PCR purified DNA was sent for sequencing (Xcelris, Ahmedabad). Badawy *et al.*, 2015 sequenced the ITS2 of *Haemonchus contortus* purified PCR product for studying evolutionary divergence.

The sequence data of repetitive sequence of *T. gondii* was analyzed and submitted to NCBI Genbank (GenBank Accession no. KC607824). The genetic relatedness of the identified strain was determined through phylogenetic analysis (Fig. 5). The present repetitive sequence resulted in 99.2% homology with SH strain and PY strain, 98.9% homology with ZS1 strain but only 92.5% homology with RH strain. Phylogenetic study had placed the repetitive sequences of RH strain in same clade although the sequence homologies amongst the RH strain from different places were 92.8% (Christina *et al.* 1991, Parmely *et al.* 1994, Costa and Bretagne 2012).

CONCLUSION

This 532 bp long 200-300 fold repetitive microsatellite fragments could be a sensitive and specific target for PCR diagnosis of *T. gondii*.

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