Comparison of agglutination test, microscopy and nPCR for diagnosis of *Toxoplasma gondii* isolated from sheep and goat of Central Ethiopia

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Abstract

Toxoplasmosis is one of the most globally widespread zoonoses with considerable health and economic impacts. Toxoplasmosis is conventionally diagnosed by serology. Bioassay is used for isolating strains of *T. gondii,* for assessing their pathogenicity in mice or for further molecular detection and genotyping. The aim of the present study was to compare the performance of microscopic cyst detection (MCD) and Direct Agglutination Test (DAT) with nested Polymerase Chain Reaction (nPCR) for the detection of *T. gondii* infection in mice (n=399) inoculated with heart tissue homogenates from seropositive sheep (n=47) and goats (n=44) in Central Ethiopia.Comparison of the diagnosis of *T. gondii* infection using DAT, MCD and nPCR revealed positive results on 30.58%, 28.82% and 53.13% of mice examined, respectively. There was a substantial agreement between DAT and MCD (Kappa = 0.69) for evidence of *T. gondii* infection in mice. Moderate agreement was observed between nPCR and MCD (Kappa = 0.43) and nPCR and DAT (Kappa = 0.47). Nested-PCR is more sensitive to diagnose *T. gondii* infection in mice compared to DAT (Sensitivity = 53.3%; specificity = 95.2%) and MCD (Sensitivity = 49.5%; specificity = 94.7%), however, the joint use of the three techniques increased the sensitivity of detection. This is the first report on nPCR based detec-

tion of *T. gondii* DNA in mice infected with tissue homogenates of sheep and goats of Ethiopia.

Keywords: Bioassay, DAT, Ethiopia, Microscopic cyst detection, nPCR, *Toxoplasma gondii*

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Introduction

Toxoplasmosis is one of the most globally widespread zoonoses with considerable health and economic impacts. Toxoplasmosis is caused by the protozoan parasite, *Toxoplasma gondii,* which infects nearly all warm-blooded vertebrates including mammals and birds. Humans get infections with *T. gondii* by ingesting raw or undercooked meat, sporulated oocysts via contaminated soil, food or water and congenitally by transplacental transmission of tachyzoites (Dubey, 2010).

Toxoplasmosis is conventionally diagnosed by serology. However, a positive serological result does not mean that viable parasites are present in all tissues. Mouse bioassay consists of inoculating mice with extracts of tissues and detecting infection by microscopic, serological or molecular assays. The bioassay can also be used for isolating strains of *T. gondii* for assessing their pathogenicity in mice or for further genotyping (Dubey, 2010; Su and Dubey, 2010). Although mousebioassay is used as a reference standard in *T. gondii* test evaluation previously, it often yields false-negative results. Use of such imperfect reference standard yields biased estimates of sensitivity and specificity of the test under evaluation (Staquet*et al.*, 1981).

Although serological studies have demonstrated that toxoplasmosis is highly prevalent in Ethiopia, the parasite has not been isolated from animal tissues yet. Furthermore, no PCR based detection of the parasite DNA has been performed. The aim of the present study was to compare the performance of microscopic cyst detection (MCD), direct agglutination test (DAT) and nested PCR (nPCR) for the detection of *T. gondii* infection in mice inoculated with heart tissue homogenates of DAT positive sheep and goats in Central Ethiopiato evaluate the results of the bioassay.

Material and Methods

Bioassay of sheep and goats hearts for *T. gondii*

The study was undertaken from September, 2011 to June, 2013. Hearts of 91 DAT seropositive sheep and goats from previous study (Endrias Zewdu *et al.*, 2013)were available for isolation of *T. gondii* cysts in mice and subsequent nPCR detection of the B1 and 529-bp repeat elements of *T. gondii*.

Heart samples from seropositive sheep and goats weighing approximately 50 g were processed for isolation of *T. gondii*as described previously (Dubey, 2010). Briefly, eachsample was cut in small pieces of approximately $1cm^3$, homogenized, digested by incubating in acidic pepsin, homogenate was filtered, centrifuged andthe sediment neutralized with sodium bicarbonate. After centrifugation the pellet was resuspended in saline mixed with penicillin (1000 U/ml) and streptomycin (100 µg/ml) and each homogenate sample was inoculated intraperitoneally into 5 Swiss white albino mice. The mice used for the experiment were *T. gondii* seronegative female Swiss Albino mice, obtained from the animal facility of the National Veterinary Institute, DebreZeit, Ethiopia. Non-infected mice (n=5) were kept separately as negative controls.

Blood collection and identification of seropositive mouse

Blood from experimentally infected mice, which survived the experimental period (n=399), was collected at scarification by neck piercing after anaesthetizing with diethyl ether. The blood was allowed to clot for about 3-4 h and then centrifuged at 2250 x g for 15 min. The serum was harvested using a disposable pipette. *Toxoplasma gondii*-specific IgG antibodies were detected by the Direct Agglutination Test (DAT) (Toxo screen DA, Biomerieux®, France) following the procedure described by the manufacturer of the kit. Sera were assayed at screening dilutions of 1/40 and 1/4000 in order to avoid false negative results that might occur at low dilutions when using sera with high antibody titers. Sedimentation of antigen at the bottom of the well and clear agglutination above half of the well at either dilution were recorded as negative and positive results, respectively. Positive and negative controls as well as an antigen control were included in each test for validation of the test results.

Detection of cysts from the mouse brain

Two months after intraperitoneal (i.p) inoculation, the brain from surviving mice (n=399) was removed by sagittal dissection after euthanasia with diethyl ether. Each brain was homogenized in 1 ml PBS(pH = 7.2)using a mortar and pestle. Presence of cysts was microscopically evaluated in three aliquots of each 10 µl under a light microscope with a 100X magnification(Goodwin*et al.*, 2008; Fritz*et al.*, 2012). Brain homogenates were stored in 1.5 ml Eppendorf tubes and frozen at -20°C until DNA extraction was done.

DNA extraction

DNA was extracted from mice brain as described previously by Su and Dubey (Su and Dubey, 2010) using the QIAamp Tissue kit (Qiagen). Briefly, 75-100 µl of homogenized brain (approximately 25 mg brain tissue), 180 µl of lysis buffer of the kit (ATL) and 20µl of proteinase K were added and incubated at 56°C until the tissue was completely lysed (60 to 90 min). The lysate was then mixed with 200 µl AL buffer and incubated for another 10 min at 70°C. DNA was precipitated by addition of 200 µl ethanol (96–100%). Then, the mixture was carefully applied to the QIAamp Mini spin column and centrifuged at 6000 x g for 1 min. The columns were then washed by centrifugation using buffers AW1 and AW2, according to the manufacturer's instruction. Finally, the DNA was eluted from the column using 50 µl of the elution buffer of the kit (AE) and stored at -20°C until tested by nested polymerase chain reaction (nPCR).

Detection of *T. gondii* DNA by nPCR

Nested-PCR was performed using primers directed at the B1 and 529-bp repeat element following the protocol of Su and Dubey(2010). Briefly,all PCR reagents (Qiagen) and template DNA samples were thawed at room temperature for 20 min, mixed and spun down. The first PCR amplification reaction (PCR1) was performed in a 50 µl total volume containing 10 µl template DNA, 5 µl of 10x PCR buffer (without Mg^{2+}), 4.0 µl of dNTP mix (2.5 mM each), 4 µl 25 mM $MgCl_2$, 0.3 µl external forward Primer (50 µM), 0.3 µl external reverse Primer (50 µM each), 0.2 µl FastStart Taq (5 U/µl) and 26.2 µl H_2O. To amplify the B1 gene the primers (Invitrogen) used were T8: ATGTGCCACCTCGCCTCTTGG, T5: GCAATGCTTCTGCACAAAGTG (forward) and T2: TGCATAGGTTG-CAGTCACTG, T7: TAAAGCGTTCGTGGTCAACT (reverse) (Nicoll*et al.*, 1996; Contini*et al.*, 2002). Similarly, to amplify the 529-bp repeat elements the primers (Invitrogen) used were Tox-8: GACGTCTGTGTCACGTAGACCTAAG,

Tox-5: CTGCAGACACAGTGCATCTGGATT (forward) and Tox-9: AGGAGAGATATCAGGACTGTAG, Tox-11: GCGTCGTCTCGTCTAGATCG (reverse) (Homan *et al.*, 2000; Reischl*et al.*, 2003). The cycling condition for PCR1 was 5 minutes of Taq activation and initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The finalization step consisted of a final extension at 72 °C for 10 min followed by cooling to 4 °C. PCR1 produced a 797bp and a 450bp product of the B1 gene and the 529-bp repeat element of the parasite, respectively on gel electrophoresis analysis of the PCR products.

The reaction products of PCR1 were then submitted to a second PCR run (PCR2) containing per reaction: 5µl PCR1 product, 5.0 µl of 10x PCR buffer (Mg-), 4.0 µl dNTPs (2.5 mM each), 4.0 µl 25 mM $MgCl_2$, 0.30 µl nested forward Primer (50 µM each), 0.30 µl nested reverse Primer (50 µM each), 0.20 µl Fast-Start Taq (5 U/µl) and 31.2 µl H_2O to obtain a final volume of 50.0 µl.

The PCR2 reactions were heated at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min followed by cooling at 4 °C. The PCR2 products were analyzed by gel electrophoresis as follows: 5 µl of each amplification product was mixed with 2 µl of 5x loading dye and run on a 2% agarose gel (containing 0.3 µg/ml ethidium bromide) in 1x tris–borate–EDTA (TBE) buffer at 120 V for ~ 1 h. The Gelplot DNA marker (100 bp; Qiagen) was used as DNA ladder to estimate the fragment lengths. The DNA bands were visualized under UV illumination. The amplified fragments of B1 and 529-bp repeat element were approximately 126 bp and 162 bp, respectively.

During each amplification DNA of *T. gondii* from tachyzoites of the RH strain, collected from experimentally infected mice (Scientific Institute Public Health, Brussels, Belgium) and double distilled water were used as positive and negative controls, respectively.

Data analysis

Data generated were recorded and coded using Microsoft Excel and analyzed using STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA). Descriptive statistics were used to summarize the data. The results of nPCR, serology (DAT) and microscopic cyst detection were compared using the Chi-square test and their concordances were determined by calculating the

Kappa coefficient (k). Using nPCR as reference test, the diagnostic sensitivity (Se), specificity (Sp), negative predictive value (NPV) and positive predictive value (PPV) of DAT and microscopic cyst detection along 95% CI were calculated and interpreted following the recommendations of Dohoo*et al.*(2003), which states Kappa values as: <0.2: slight agreement, 0.2–0.4: fair agreement, 0.4–0.6: moderate agreement, 0.6–0.8: substantial agreement and >0.8: almost perfect agreement. The 95% confidence interval and a significance level of α = 0.05 were used.

Ethical issues

This research project was approved by the College of Veterinary Medicine and Agriculture, Addis Ababa University. All efforts were made to minimize animal suffering during the course of the study.

Results

Prevalence of *T. gondii* using nPCR, DAT and Microscopic examination

Prevalence of *T. gondii* in mice by nPCR, DAT and microscopic cyst detection (MCD) were 53.13% (95% CI: 48.22-58.05), 30.58% (95% CI: 26.04-35.12) and 28.82% (95% CI: 24.36-33.29), respectively. A total of 23 sheep samples (48.94%) gave seropositive results on mice (out of 198 surviving mice, 73 became seropositive, 36.87%). Similarly, a total of 18 goat samples (40.91%) gave seropositive results on mice (out of 201 surviving mice, 49 became seropositve, 24.38%). A significantly higher percentage of mice inoculated with sheep samples reacted positively (36.87%) in DAT as compared to mice inoculated with goat samples (24.38%) [χ^2 (1) = 7.3309, P = 0.007]. In contrast, no significant difference was observed with respect to microscopic cyst detection, nPCR and overall result between mice inoculated with sheep and goat samples (P> 0.05) (Table 1).

Out of the total of 459 mice inoculated with tissue homogenates of seropositive sheep (n=236) and goats (n=223), 63 mice (38 from sheep and 25 from goat samples) died before two months. Among these dead mice 54 died between 1 – 3 days after inoculation while 9 mice died after 3 days post inoculation.

Table 1: Proportion of mice classified as positive based on microscopic cyst detection, DAT and nPCR.

Hosts		Cyst/microscopy	DAT*	nPCR	Overall
Species	No. of mice	Positive (%)	Positive (%)	Positive (%)	Positive (%)
Sheep (n=47)	198	63 (31.82)	73 (36.87)	100 (50.51)	109 (55.05)
Goat (n=44)	201	52 (25.87)	49 (24.38)	112 (55.72)	119 (59.20)
Total (N=91)	399	115 (28.82)	122 (30.58)	212 (53.13)	228 (57.14)

*Statistically significant ($P< 0.05$)

Detection of *T. gondii* in mice using nPCR

Nested-PCR gave positive results on mice for 81.8% (36/44) goat and 78.9% (35/47) sheep samples. On the other hand, nPCR gave positive results for 212 (53.13%; 95% CI: 48.22, 58.05) of the 399 examined mice samples. Ten cyst positive samples, of which 3 were also DAT positive, returned negative results using n-PCR. In addition, 9 DAT positive samples gave negative results by n-PCR. Both DAT and MCD gave negative results for mice samples #. 230-1, 230-3, 230-5, 231-2, 231-3, 231-4 and 259-1; however these samples were positive by nPCR. An example of a typical nPCR result obtained is depicted in Figure 1.

Figure1. Nested PCR based detection of *T. gondii* DNA from mice brain tissue. Electrophoresis on 2% agarose gel. Lane 1: molecular weight marker (100–1500 bp); lane 2-7: positive samples; lane 9-11: negative samples; lane 8 faint

positive sample; lane 12: positive control; lane 13: negative control (double distilled sterile water).

Mice samples inoculated with tissues from positive goats showed a higher percentage of nested PCR positivity (55.72%) than sheep samples (50.51%).

Test agreement

T. gondii specific IgG antibodies from sera using DAT, bradyzoite cysts from brain using MCD and specific gene bands of *T. gondii* using nPCR were detected each from 399 mice.

In 97 out of 228 positive cases, *T. gondii* infection was detected in 7 mice only by microscopy, in 6 mice by DAT and in 84 mice using nPCR. All tests returned positive results on 90 samples (22.6%). Out of the 115 cyst positive mice 22 (19.13%) were DAT negative and out of 122 DAT positive mice 29 (23.77%) were cyst negative. Out of 212 nPCR positive mice 99 (46.7%) and 107 (50.47%) were DAT and cyst negative, respectively.

Results of the calculations of prevalence, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) of DAT and MCD using nPCR as a reference test revealed that DAT and MCD have comparable results (Table 2).Kappa index and extent of agreement between the diagnostic tests are shown in Table 3.

Table 2: Comparison of sensitivity, specificity, PPV and NPV of DAT and MCD on artificially infected mice using nPCR as a reference test.

Diagnostic method	[a]Sensitivity (%) (95% CI)	[b]Specificity (%) (95% CI)	[c]PPV (%) (95% CI)	[d]NPP (%) (95% CI)
DAT	53.3 46.3-60.2	95.2 91.1-97.7	92.6 86.5-96.6	64.3 58.3-69.9
Cyst detection	49.5 42.6-56.5	94.7 90.4-97.4	91.3 84.6-95.8	62.3 56.4-68

Sensitivity: the percentage of mice with *T. gondii* infection (determined by reference test) that gave positive result in DAT or microscopic cyst detection (MCD)

[b]Specificity: the percentage of mice uninfected by *T. gondii* (determined by reference test) that gave a negative result in DAT or microscopic cyst detection (MCD)

[c]PPV: percentage of *T. gondii* positive mice that test positive for both DAT and reference test or MCD and reference test divided by total mice positive in DAT or MCD

[d]NPP: percentage of *T. gondii* uninfected mice that test negative for both DAT and reference test or MCD and reference test divided by total mice negative in DAT or MCD

Table 3: Concordance results among MCD, DAT and nPCR.

Diagnostic tests	Kappa (95% CI)	*P*-value	Interpretation
DAT vs MCD	0.69 (0.62, 0.77)	<0.001	Substantial agreement
DAT vsnPCR	0.47 (0.40, 0.55)	<0.001	Moderate agreement
MCD vsnPCR	0.43 (0.35, 0.51)	<0.001	Moderate agreement

DAT= direct agglutination test, MCD = microscopic cyst detection, nPCR = nested polymerase chain reaction, CI = confidence interval

Discussion

The present study indicates that. 53.13% (212/399), 30.58% (122/399) and 28.82% (115/399) of examined mice were positive for *T. gondii* DNA, IgG antibodies and brain tissue cysts, respectively. In some bioassayed sheep and goat hearts overall positive for cysts, some inoculated mice became negative for cysts suggesting low numbers as well as uneven distribution of *T. gondii* in the inocula. On the other hand, 99 nPCR and 22 cyst positive samples gave negative DAT result. These mice that didn't seroconvert might be those challenged with an insufficient quantity of bradyzoites to adequately stimulate a detectable humoral immune response. On the other hand, such a small quantity of parasite was detected from the brain of mice by nPCR due to its higher sensitivity.

Although used as a reference standard in *T. gondii* test evaluation, mouse bioassay often yields false-negative results. Use of such imperfect reference standard yields biased estimates of sensitivity and specificity of the test under evaluation (Staquet *et al.*, 1981). In this study nPCR was used as a reference test (assuming that nPCR detected 100% of positive samples) for the purpose of comparative ranking of the relative sensitivity, specificity, PPV and NPV of DAT and MCD. Therefore, all measures of sensitivity, specificity, PPV and NPP are dependent upon the accuracy of this method. Comparison of results of DAT and MCD revealed substantial agreement (Kappa = 0.69). On the other hand, moderate agreement was observed between DAT and nPCR (Kappa = 0.47) as well as between MCD and nPCR (Kappa = 0.43). The high rate (53.13%) of detection of *T. gondii* DNA in the mice brain samples as compared to MCD (28.82%) and DAT (30.58%) indicates a higher sensitivity of nPCR in comparison to bioassay. The small volume of mouse brain homogenate ex-

amined by microscopy (30 µl homogenate in total) compared to the volume of brain homogenate used for DNA extraction (70-100 µl homogenate in total, equivalent to 25 mg brain tissue) subsequently used for nPCR might have played an important role in getting lower positive bioassay results compared to nPCR (Montoya *et al.,* 2009). Thus, sensitivity of MCD could be increased by increasing the volume of tissue examined beyond 30 µl, or by using concentration methods and sub-passaging of negative brains. However, this would be time consuming, laborious and expensive. The higher positivity rate observed in the nPCR might also be explained by the fact that this technique amplifies two conserved genes with higher copy numbers (Homan *et al.*, 2000) and makes use of 2 successive amplifications of the first amplification, increasing its sensitivity (Martins*et al.*, 2000). Therefore, nPCR is more preferred as a diagnostic method than DAT or MCD, not only because of its high sensitivity but also due to the fact that it does not rely upon the host immune response to the parasite. However, the difficulty in the practical elimination of risks of false positives in nPCR due to cross-contamination, even in an extremely controlled environment (Ajzenberg, 2010), and non-specific primer binding are shortcomings of these molecular methods. Such potential nPCR contamination may lead to the possibility of false positive cases, hence contributing to a lack of correspondence of the results between the different tests. Although nPCR performed better than the other two tests it would still be difficult to call it a gold standard or perfect test for *T. gondii* infected mice since it failed to detect some cyst (n = 10) and DAT (n = 9) positive mice samples. Also nPCR does not accurately indicate the risk to humans as it detects both viable and non-viable parasite DNA (Garcia*et al.*, 2006).

The inability of the mouse bioassay to detect non-viable parasites (Wastling *et al.*, 1993) and subjectivity in the interpretation of results (particularly DAT and nPCR) by the researcher might also account, to some degree, for the observed differences among detection methods. In agreement with the present findings, higher sensitivity of nPCR compared to bioassay has been reported in experimentally infected pigs (Yai*et al.*, 2003). On the other hand, unlike the present findings, absence of a significant difference ($P > 0.05$) between mouse bioassay and PCR was reported for detection of *T. gondii* from experimentally infected sheep (Wastling *et al.*, 1993), orally infected sheep (Esteban-Redondo *et al.,* 1999), congenital toxoplasmosis in neonates (Filisetti*et al.,* 2003) and natural infection in cats (Montoya*et al.,* 2009). Garcia *et al.*(2006) reported that isolation by mouse bioassay (64.6% in muscles and 46.0% in brains) was significantly more sensitive than 529 bp PCR (14.1% in muscles and 26.6% in

brains) ($P< 0.01$) in detecting *T. gondii* in tissues from experimentally infected pigs with the VEG strain (done on day 60 post infection). Moreover, Garcia *et al.* (2006) also reported DNA from pig tissue to interfere with the sensitivity of the 529 bp PCR.

Eleven mice identified as positive by DAT and/or MCD gave a negative result by nPCR (false negative). The reason might be due to the fact that the blood occasionally seen in the brain in relation to the killing of the mice contains components inhibiting the PCR reaction. Wamekulasuriya *et al.*(1998) and Homan *et al.*(2000) previously suggested that a negative PCR result in the presence of the parasite could be due to the presence of host DNA and defective templates, mainly linked to the quality of the DNA extraction.

Conclusion

Nested-PCR is more sensitive to diagnose *T. gondii* infection in mice compared to DAT and MCD, however, the joint use of the three techniques increased the sensitivity of detection. This is the first report on nPCR based detection of *T. gondii* DNA in mice infected with tissue homogenates of sheep and goats of Ethiopia.

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