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Comparison of three diagnostic methods for the detection of *Toxoplasma gondii* in free range chickens

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Abstract. Detection of *Toxoplasma gondii* in free range chickens is an indicator of the prevalence and distribution pattern of *T. gondii* in the environment. For this purpose, serologic assays especially modified agglutination test (MAT) is the main approach in the literature. The main goal of this study was to compare the polymerase chain reaction (PCR) based on amplification of first internal transcribed spacer (ITS-1) of ribosomal DNA gene, ELISA, and MAT to demonstrate *T. gondii* infection in free range chicken. A total of 106 adult free - range chickens were killed. Blood, whole heart and brain samples were taken. Sera were examined for the presence of *T. gondii* antibodies by ELISA and MAT as well. Selected tissues were used for PCR and bioassay in mice. The results revealed that 48.11%, 51.89%, 46.23% and 27.36% of chickens were positive in ELISA, MAT, PCR and bioassay in mice respectively. Good correlation between the results of PCR, ELISA and MAT were detected, but not with bioassay in mice. Compared with PCR, the sensitivity and specificity of ELISA were 92.16% and 96.36% respectively and also for MAT, the sensitivity was 81.81% and the specificity was 92.15%. The specific diagnosis of *T. gondii* infection in chickens is central to a better understanding of the epidemiology and dynamics of transmission among the various host population and is particularly important for planning effective optimal prevention and control programs. Our data in the present study demonstrated that PCR, ELISA and the MAT are helpful and precise methods to detect *T. gondii* in naturally infected free-range chickens.

INTRODUCTION

Toxoplasmosis is a zoonosis of increasing concern in both human and warm-blooded animals. The disease has the highest human incidence among the parasitic zoonoses (Dubey, 2008, 2009a; Dubey & Beattie 2010). Toxoplasmosis is caused by an obligatory intracellular, protozoan parasite *Toxoplasma gondii* that is a member of the phylum Apicomplexa, order Coccidia (Rorman, 2006). Approximately one third of the world's human population is infected with the protozoan parasite *T. gondii* (Dubey & Beattie 2010). Felids are the most important definitive and reservoir hosts for this parasite (Dubey 2009b, 2009c, 2009d). Humans become infected with *T. gondii* in three principal ways: congenitally, through the ingestion of raw or undercooked meat containing tissue cysts and ingestion of water or poorly washed raw fruits and vegetables contaminated with sporulated oocysts from the feces of infected cats (Dubey & Beattie 2010).

Since free range domestic chickens (*Gallus gallus*) feed from the ground, these animals are the suitable indicators of soil contamination (Dubey *et al*, 2002; Dubey, 2010). Hence, many researchers consider this

criterion for characterization of *T. gondii* isolates (Sreekumar *et al.*, 2003; Dubey *et al.*, 2006; Basso *et al.*, 2009; Rajendran *et al.*, 2012). On the other hands, detection of infection in chicken is often performed by serological tests specially, with modified agglutination test (MAT) (Asgari *et al.*, 2008; Murao *et al.*, 2008) and little is known about the performance of other techniques including ELISA or molecular approaches such as polymerase chain reaction (PCR) to detect the *T. gondii* in free range domestic chicken. Therefore, the aim of the present study was to compare the performance of PCR, ELISA and MAT for diagnosis of *T. gondii* infection in naturally infected free range chicken.

MATERIALS AND METHODS

Animals and sampling

This study was carried out according to national ethical framework for research animal welfare and ethics in Iran (Mobasher *et al.*, 2008). The work was conducted on 106 purchased adult free - range chickens without fencing. Age and sex of examined chickens were not considered in this study. Chickens had not any clear symptom of diseases before sampling. All of the chickens were killed and blood, whole heart and brain samples were taken. The blood samples were centrifuged at 1000g and the supernatants were frozen at -20ºC until serologic examinations and the heart and brain samples were kept in 4ºC until transportation to the laboratory.

ELISA

The *T. gondii* tachyzoites were prepared from parasites harvested from cells grown in mice. After purification, tachyzoites were sonicated three times for 30 seconds on ice. To measure the appropriate concentration of antigen, conjugate and the sera, dilutions checkerboard procedure was performed. After setting up the measures, 2.5 µg of sonicated antigens were coated in 96 microtitre ELISA plates in 0.1 M carbonate buffer (pH=9.6) and kept overnight at 4ºC. The plates were blocked by 5% skim milk (Merck) containing 0.05% Tween 20 (Merck) for 2 h at 37ºC and subsequently diluted sera at 1:100 were added in duplicate and incubated for 60 min at 37ºC. Positive and negative control sera also were included in each assay. Donkey anti-chicken immunoglobulin, labeled with horseradish peroxidase (Sigma), was used as enzymatic conjugate at 1:5000 dilutions and incubated once more for 60 min at 37ºC. The reaction was revealed with a solution of enzymatic substrate and chromogen consisting of 0.03% H_2O_2 (Sigma) and TMB (Merck). The optical density (OD) was determined in a microplate reader (Dynatech, Netherlands) at 450 nm. The cut-off was calculated as the mean OD values from negative controls plus two standard deviations. The samples were considered seropositive when the OD value of sample was ≥OD of cut-off point.

MAT

The sera were also tested for the presence of *T. gondii* antibodies using the MAT based on the direct agglutination of fixed parasites with sera pre-treated with 2-mercaptoethanol to prevent non-specific IgM agglutination, as described by Desmonts and Remington (1980) and Dubey and Desmonts (1987). Sera were started at 1:10 serum dilution. MAT was conducted in 96 well U-bottomed microtitre plates. Sera were diluted in a phosphate buffer (PBS) 0.01 M pH 7.2 The antigen suspension composed of 2.5 ml of borate buffer pH 8.9 containing 0.4% BSA, 35 µl of 2-mercaptoethanol, 50 µl of Evans blue at 2 mg/mL and 140 µl of the suspension of whole tachyzoites. In each well, 50 µl of antigen solution and 50 µl of the serum to be tested were mixed and the microtitre plate incubated at 37ºC for 12 hours.

Furthermore, the sera were tested by the *Neospora* agglutination test, NAT, as described by Romand *et al.* (1998). This test is identical to MAT for *T. gondii* that *N. caninum* tachyzoites are used instead of *T. gondii*. Sera were ran at 1:10 serum dilution.

For two above tests, a complete carpet of agglutinated parasites was considered as positive result. Clear and cut button-shaped

deposit of parasite suspension at the bottom of the well was interpreted as a negative reaction.

Polymerase chain reaction

Heart, brain and liver from each killed chicken were cut and homogenized, then, DNA were extracted using the genomic DNA extraction Kit (Cinnagen, Iran) according to manufacturer instructions. Next, the extracted DNA was stored at -20ºC until PCR was performed.

Determination of *T. gondii* DNA was carried out by PCR according to amplification of first internal transcribed spacer (ITS-1) of ribosomal DNA gene. PCR protocol and primer selection were adopted according to the previously described method by Xie *et al.* (2005) and Yana *et al.* (2010).

Briefly, amplification of the ITS-1 gene was carried out in 25 µl reaction volumes containing 3 µl of DNA template, 5 pmol of reverse and forward primers, 2.5 mM $MgCl_2$, 2.5 µl 10 X PCR Buffer, 2.5 mM of each dNTP and 0.625 U Taq DNA Polymerase. Forward and reverse genus-specific primer sequences used in this study were 5'-AGTTTAGGAAGCAATCTGAAAGCACATC-3' and 5'-GATTTGCATTCAAGAAGCGT GATAGTAT-3' respectively. The thermal program of PCR was as follows: 94ºC for 5 minutes, 35 cycles of 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 30 seconds, followed by a final extension step at 72ºC for 7 minutes. To verify the results, 10 µl of each PCR product was electrophoresed in a 1% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator. The PCR products were identified by size based on 100 base pair DNA ladder (Fermentas).

To determine the possibility of the cross reaction with related protozoan, *Neospora caninum*, the whole tachyzoites of this parasites was also analyzed by the same genus-specific primers. Positive amplicons were sequenced utilizing an automatic DNA Sequencers.

Bioassay in mice

Heart and brain of all samples were bioassayed individually in females BALB/c mice. The assay was identical to those described by Dubey and Beattie (2010). Briefly, the tissues were homogenized in 0.85% NaCl and digested in acidic pepsin and then incubated in a water bath for 1 h at 37ºC. Digested suspension was centrifuged for 15 minutes and supernatant was discarded afterwards. After that, penicillin and streptomycin were added to the sediment and finally 1 ml of the sediment injected separately via sub cutaneous (S.C.) and intraperitoneally (IP) to the mice. Also, 50 mice were considered uninfected as controls. Tissues of died mice were examined for both tachyzoites and tissue cysts and also PCR as described above. Survivors were euthanized and followed up for *T. gondii* infection using the MAT and PCR as mentioned.

Statistical analyses

The Kappa statistic test (ê) was used to test the level of agreement between the PCR, ELISA and the MAT for detection of *T. gondii* infection in free range chicken. Kappa and its 95% Confidence Interval (CI), was used further to measure the degree of agreement between the procedures after taking into account the probability of agreement by chance alone. Strength of agreement based on ê was judged according to the following guidelines: <0.2=slight; 0.2–0.4=fair; 0.4–0.6=moderate; 0.6–0.8=good; >0.8=very good. McNemar's test was also utilized for comparing the percentage of positive reactions (Dohoo *et al.*, 2003).

RESULTS

As presented in Table 1, ELISA detected anti-*T. gondii* antibodies in 51 of 106 chickens (48.11%), whereas 55 of 106 (51.89%) samples were positive by MAT using a positive cut-off of an 1/5 titer. MAT titers ranged from 5 to 5120 and *N. caninum* were detected in 8 out of the 106 examined chickens respectively. Only 2 sera were positive for both parasites.

PCR based upon the amplification of ITS-1 fragment revealed that 49 samples (46.23%) represented *T. gondii* positive reaction. Sequencing analysis of the amplicon

Table 1. Infection rate of *T. gondii* in examined chickens using PCR, ELISA and MAT

	PCR	ELISA	MAT
Positive	49 (46.23%)	51 (48.11%)	55 (51.89%)
Negative	57 (53.77%)	55 (51.89%)	51 (48.11%)
Total	106	106	106

Table 2. Relative sensitivity and specificity of Dot- ELISA in comparison with standard ELISA and NAT

	ELISA		PCR	MAT	
PCR	Positive	Negative		Positive	Negative
Positive	47 (a)	2 (b)	Positive	45 (a)	4 (b)
Negative	4 (c)	53(d)	Negative	10 (c)	47 (d)
Total	51 (a + c)	55 (b + d)	Total	55 (a + c)	51 (b + d)
Sensitivity %	92.16			81.81	
Specificity %	96.36			92.15	

Sensitivity = a/ (a + c); Specificity = d/ (b + d)

confirmed *T. gondii*. Compared with PCR, bioassay in mice detected *T. gondii* infections only in 29 of 106 (27.36%) samples.

Based upon the Kappa statistical test, a good correlation between the results of ELISA and MAT (k= 0.736), ELISA and PCR (k= 0.736), and MAT and PCR (k= 0.736) were detected. Furthermore, McNemar's test revealed that ELISA as well as the MAT has acceptable ability to discriminate the positive results.

Compared with PCR, relative sensitivities and specificities for the ELISA were 92.16% and 96.36%, and for the MAT, 81.81% and 92.15% respectively (Table 2).

There was poor agreement between the results of bioassay and other rendered tests (k=0.736).

DISCUSSION

Clinical toxoplasmosis in chickens has been rarely reported. In a study by Dubey *et al.* (2007), they reported clinical toxoplasmosis in three chickens that presented neurological signs on a farm in Illinois (Dubey *et al.*, 2007). On the other point of view, since free-range chickens usually get oocysts during their feeding from the soil, the presence of *T. gondii* in these animals is a good indicator of the prevalence and distribution patterns of *T. gondii* oocysts in the soil and therefore the estimation of the potential risk of transmission to human from this source of infection.

The specific diagnosis of *T. gondii* infections in chickens is central to a better understanding of epidemiology and dynamics of transmission among the various host population, especially in human, and is particularly important for planning an effective optimal prevention and control programs. On the other hands, characterization and genotyping of *T. gondii* isolates are dependent on serologic investigations especially the MAT.

During recent years, there have been significant advances in the development of molecular diagnostic tools. Several PCR based assays targeting different regions of the protozoan genome have been described. Since the ITS regions are less conserved than the rRNA genes, designing primers for detecting variations in this region of DNA sequence is straightforward and there is lower risk of cross reactions among different

species (Homan *et al.*, 1997; Nowzari *et al.*, 2004; Aigner *et al.*, 2010).

We employed ITS-1 rDNA as a species-specific marker for *T. gondii* for amplification with PCR, which can detect 10 or less parasites per reaction (Homan *et al.*, 1997; Hurtado *et al.*, 2001; Jauregui *et al.*, 2001; Xie *et al.*, 2005).

MAT examination of free-range chickens detected a much higher prevalence of *T. gondii* as compared with ELISA and PCR, but results of the present study revealed that in comparison with PCR, ELISA was more sensitive and specific than the MAT. However, it is widely accepted that the MAT is an easy to perform, cost-effective and accurate method without requiring expensive laboratory instrument, and therefore several studies have used the MAT for the detection the antibodies against *T. gondii* in chickens (Dubey *et al.*, 2002; Dubey *et al.*, 2006; Basso *et al.*, 2009; Dubey 2010; Rajendran *et al.*, 2012). It should be considered that by utilizing all performed methods, the occurrence of *T. gondii* infection in the area of the study (49-55%) was relatively high and transmission via oocysts originated from contaminated soil with cat feces is more likely important especially near the households.

Yan *et al.* (2010) showed that *T. gondii* can be detected by PCR in the tissues of naturally infected chicken from 21 days post infection onwards, but we expect that serologic procedures to detect antibodies during the acute phase of infection before division or localization in tissues. However, PCR with specific primers is very accurate and cost-effective for detecting *T. gondii* DNA (Su *et al.*, 2010).

Bioassay in mice only detected *T. gondii* infections in 29 of 106 chickens. As mentioned in other studies (Dubey and Beattie, 2010), our results also revealed that bioassay of tissues in mice may lead to false negative result. Data in the present study demonstrated that PCR, ELISA and the MAT are the sensitive and specific methods to detect *T. gondii* in naturally infected free-range chickens.

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