

Original Paper

A comparison between serological and molecular tests in diagnosis of *Toxoplasma gondii* infection among stray cats in Ahvaz, southwestern Iran

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ABSTRACT

Toxoplasma gondii infects all species of warm-blooded animals, including humans, and domestic cats and other felidae are its definitive hosts. The aim of the present survey was to compare serological and molecular tests in accurate diagnosis of *T. gondii* infection among stray cats in Ahvaz, Iran. A total of 100 cats were examined for the detection of serum antibodies against *T. gondii* (chronic phase) by enzyme-linked immunosorbent assay (ELISA) and modified agglutination test (MAT) and for the presence of antigen in the blood (active phase) by polymerase chain reaction (PCR). The studied cats were divided into seven age groups. According to the obtained results from ELISA, MAT, and PCR, the prevalence rates of *T. gondii* infection were 30%, 39%, and 8%, respectively. Kappa test showed that there was a good agreement between ELISA and MAT results (0.80), but it was obtained 0.24 for MAT and PCR and 0.34 for ELISA and PCR, indicating a fair agreement between them. The seroprevalence of this parasite was 53.33% and 48.71% in male cats and 46.66% and 51.28% in females based on ELISA and MAT, respectively. Gender did not significantly affect the prevalence of this infection ($P>0.05$), but there was a significant difference between age groups, with the highest rate belonging to cats aged less than three years ($P<0.05$). It can be concluded that the serologic tests such as ELISA and MAT have a good agreement in diagnosis of toxoplasmosis. Our findings showed that a considerable percentage of the cats were infected with chronic form of toxoplasmosis.

Keywords: *Toxoplasma gondii*, ELISA, MAT, PCR, Cat

Comparaison des tests sérologiques et moléculaires dans le diagnostic de l'infection causée par *Toxoplasma Gonda* chez les chats errants de la ville d'Ahvaz au sud-ouest de l'Iran

Résumé: *Toxoplasma Gonda* contamine toutes les espèces d'animaux à sang chaud y compris les humains. Les chats domestiques et les autres féliniformes sont les hôtes définitifs. Le but de la présente études est la comparaison des tests sérologiques et moléculaires dans le diagnostic de l'infection causée par *Toxoplasma Gonda* chez les chats errants dans la région d'Ahvaz. Un total de 100 chats ont été examinés par la méthode ELISA et test d'agglutination modifié (MAT) pour déterminer les anticorps sériques dirigés contre *Toxoplasma Gonda* (phase chronique), ils ont aussi été examinés par la méthode PCR pour déterminer les antigènes dans le sang (phase aiguë). Selon l'âge, les chats étudiés ont été divisés en 7 groupes. D'après les résultats obtenus des techniques ELISA, MAT et PCR, la prévalence de l'infection par *Toxoplasma Gonda* était respectivement 30, 39 et 8 pour cent. Le test de Kappa a montré que dans la comparaison entre les tests ELISA et MAT, l'accord était bon (0.80), mais ce chiffre était 0.24 pour les tests de MAT et PCR et 0.34 pour

les tests ELISA et PCR et l'accord entre eux était faible. Séroprévalence chez les chats mâles était 53.33 et 48.71 et chez les femelles était 46.55 et 51.28, selon les méthodes ELISA et MAT. Le genre des animaux n'a eu aucun effet significatif sur l'incidence de l'infection ($p>0.05$). Mais la différence entre les divers groupes d'âge était significative. Le taux d'infection le plus élevé chez les jeunes chats de moins de 3 ans était moins de 3 ($p<0.05$). On peut conclure que les tests sérologiques comme ELISA et MAT sont en bon accord l'un avec l'autre dans le diagnostic de la toxoplasmose. Nos résultats ont indiqué qu'un pourcentage important des chats étaient infectés par des formes chroniques de toxoplasmose.

Mots clés: *Toxoplasma Gonda*, ELISA, MAT, PCR, chat

INTRODUCTION

Toxoplasma gondii is a protozoan parasite that can infect almost all warm-blooded animals, including humans. The definitive host of this parasite is cat and other felidae, which excrete it in the highly infectious and environmental-resistant oocyst stage (Montoya and Liesenfeld, 2004). It is generally assumed that cats play a major role in *T. gondii* transmission through fecal contamination of soil, food, or water, because they can excrete millions of oocysts in a short period of time (1-2 week) (Dubey, 2008). Carnivorous animals become infected mostly by ingestion of meat containing bradyzoite in the tissue cyst (Dubey and Lappin, 2012). *T. gondii* infection has become a major public health concern in recent years due to the immunosuppressive effects of the ravaging HIV pandemic (Lindstrom et al., 2006). In Iran, large numbers of cats are found roaming in residential streets, which can endanger public health. Previous studies showed that the prevalence of *T. gondii* antibodies in the cat population is quite variable from 0% to 100% depending on the method, number of animals studied, and the geographic area (Dubey and Lappin, 2012). Some studies carried out in Iran demonstrated a high prevalence of *Toxoplasma* infection in cats. For example, the seroprevalence of *Toxoplasma* infection was obtained 40% in stray cats of Sari (Sharif et al., 2009), 36-90% in stray and household cats of Tehran (Haddadzadeh et al., 2006), 32.1% in cats in Kerman (Akhtardanesh et al., 2010), and 24.75-54% in companion and feral cats in Ahvaz

(Hamidinejad et al., 2011; Mosallanejad et al., 2011). Recent ecological and etiological investigations concerning risk factors and new sources of toxoplasmosis must be updated to solve the unexplained equation of high prevalence of this infection in various hosts (Dubey and Lappin, 2012). There are many different serological techniques for diagnosis of toxoplasmosis. For instance, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), modified agglutination test (MAT), latex agglutination assay (LAT), indirect hemagglutination assay (IHA), and indirect fluorescent antibody test (IFAT). Though these tests are sensitive and specific, they are expensive and need specialized laboratories (Zhang et al., 2009a). Specificity and sensitivity of the ELISA kits were found to be 97% and 100%, respectively, when the test is performed according to the manufacturer's instructions. Among the reliable methods, PCR has the highest accuracy, sensitivity, and specificity compared to the conventional diagnostic methods. PCR is frequently used to detect *T. gondii* DNA in clinical samples. It is performed by direct detection of parasite DNA, and its results do not depend on the immunological status of the animal. The presence of *Toxoplasma* DNA in maternal blood most probably indicates a recent infection (Dubey and Lappin, 2012; Hassanain et al., 2013). Combination of two or three tests may help improve diagnosis. Thus, this study aimed to compare serological (ELISA and MAT) and molecular (PCR)

tests in accurate diagnosis of *T. gondii* infection in stray cats in Ahvaz, southwestern Iran.

MATERIALS AND METHODS

Study area and sample population. This study was performed in Ahvaz, southwest of Iran, that is situated at an elevation of 12 meters above sea level and the climate is warm-humid. In the present study, a total of 100 stray cats of different ages were examined for the detection of serum antibodies against *Toxoplasma gondii* (chronic phase) by ELISA and MAT methods and for the presence of antigen in whole blood (active phase) by PCR from February to December 2013. The studied cats were captured in cages and brought alive to the Veterinary Hospital of Shahid Chamran University, Ahvaz, Iran. Most of the cats were apparently healthy and did not show any specific signs associated toxoplasmosis. In general, 93% of the studied cats were domestic short hair (DSH). Classification was made by age and gender. The cats were divided into seven groups based on age (less than 1 year, 1-2, 2-3, 3-4, 4-5, 5-6, and above 6 years). Ketamine (15 mg/kg) and acepromazine (0.15 mg/kg) were injected for sedative effects. Blood samples were drawn from jugular vein and poured in test tubes containing heparin as an anticoagulant, and then digested using the PCR kit. Thereafter, blood samples were allowed to clot and centrifuged for 5 min at 1800×g. Sera were removed and stored at -20 °C until assayed.

Laboratory methods: Serological test. All the serum samples were tested for anti-*T. gondii* antibodies with an indirect ELISA test using a commercial ELISA kit (Institute Pourquier, France) and MAT. In ELISA test, the tachyzoite antigen was prepared as the procedure described by Waltman et al. (1984). Briefly, tachyzoites were repeatedly frozen, thawed, sonicated, and centrifuged at 12000 rpm for 45 min at 4 °C. The supernatant was separately collected and its protein content was determined. The optimum antigen, serum, and conjugate concentrations were determined by checkerboard titration and test procedures were carried out according to the method. In the present survey, sera

were considered equal or greater than 50% to be from animals that had been in contact with *T. gondii*. The manufacturer's test report showed that the sensitivity and specificity of the ELISA kit were 97% and 100%, respectively. In MAT method, the *T. gondii* antigen was prepared from Razi institute of Shiraz. Sera were tested for the presence of *T. gondii* antibodies using the agglutination tests based on the direct agglutination of fixed parasites with serum pre-treated with 2-mercaptoethanol to prevent non-specific IgM agglutination. Sera were started at 1:25 serum dilution. A titer of 1:25 and higher was considered as *T. gondii* infection in cats. Sera with borderline result were re-examined. A complete agglutination was considered as a positive result. Clear-cut, button-shaped deposit of parasite suspension at the bottom of the well was interpreted as a negative reaction. MAT was carried out according to the method described by Dubey (2008).

Molecular test: DNA extraction. This method consists of extracting DNA by using the conventional phenol/chloroform. The presence of DNA was evaluated by 1.5% agarose gel electrophoresis, and nanodrop spectrophotometry method used for determining DNA purity. Genomic DNA was extracted from Toxo ITS-1 (Jauregui et al., 2001) with the following modifications to the manufactures protocols. Primers (Bioneer, South Korea) used in the reaction were the forward primer Np 21 with the sequence 5'-GATTTGCATTCAAGAAGCGTGATAGTA-t-3' and the reverse primer Np 6 with the sequence 5'-AGTTTAGGAAGCAATCTGAAAGCACATC-3', yielding a 330 bp product. PCR reactions included a negative control, consisting of the reaction mix and 2 µl of DNase/RNase-free water instead of DNA and a positive control consisting of DNA sample from the tachyzoites of *T. gondii* (NC- 1 isolate). Primers used for PCR were targeting the repetitive 35-fold B1 gene (Burg et al., 1989). The outer primers were only used in this study. Finally, the PCR products were stained with ethidium bromide solution, visualized under UV transilluminator, and photographed.

Statistical analysis. The cats were grouped according to age and gender to determine whether these factors were associated with *T. gondii* infection using Kappa test, McNemar's test (for comparison and agreement between ELISA, MAT and PCR methods), and Chi-square test in SPSS, version 16.0. P-value less than 0.05 was considered statistically significant.



Figure 1. Polymerase chain reaction after staining with ethidium bromide on 1.5% agarose gel electrophoresis. Lane 1-5: 330 bp band of *T. gondii* DNA. N: negative control, P: positive control.

RESULTS

In the present study, 100 cats were tested for the detection of antibodies against *T. gondii* using ELISA and MAT and for the presence of antigen in the blood by PCR. According to the data from ELISA, MAT, and PCR, the prevalence rates of *T. gondii* infection were 30%, 39%, and 8% in the studied cat's population, respectively. Kappa test showed that there was a good agreement between ELISA and MAT tests (0.80), but it was obtained 0.24 for MAT and PCR and 0.34 for ELISA and PCR, respectively, indicating a fair agreement between them. The seroprevalence rates of this parasite were 53.33% and 48.71% in males and 46.66% and 51.28% in females based on ELISA and MAT methods, respectively. In MAT and PCR methods, the prevalence of this infection was higher in females than males (Table 1). Nevertheless, gender did not significantly affect the prevalence of this infection ($P>0.05$), but there was a significant difference between age groups, with the highest rate belonging to cats less than three years old (up to 33.33% in ELISA, 30.76% in MAT, and 50% in PCR) compared to over

three years ($P<0.05$; Table 2). Results are summarized in tables 1 and 2.

DISCUSSION

ELISA and MAT methods showed that the seroprevalence of *T. gondii* infection was relatively high, especially in the chronic form (30-39%), in stray cat's population in Ahvaz, Iran. Results of PCR revealed that 8% of the examined animals were infected with *T. gondii*. As the results showed, ELISA and MAT data were higher than PCR. This indicates that the presence of *Toxoplasma*-specific antibodies is an insufficient criterion for identifying *Toxoplasma* infection. Accordingly, some animals are falsely identified as being infected and undergo unnecessary diagnostic amniocentesis and anti-parasitic treatments. In other words, our survey suggested that ELISA and MAT can be used as highly sensitive screening tests followed by PCR as a specific confirmatory test for diagnosis of toxoplasmosis in cats. Kappa test demonstrated that the serologic tests such as ELISA and MAT have a good agreement in diagnosis of toxoplasmosis. Statistical analysis was performed in the studied cats for one year and from different districts of Ahvaz, thus, it is a suitable sample of cat's population in this area. In our study, the prevalence of this infection was significantly higher in cats aged less than three years compared to those over three; these results were different than those described by Hill et al. (2000), Gauss et al. (2003), Miro et al. (2004), Haddadzadeh et al. (2006), Alvarado-Esquivel et al. (2007), De Craeye et al. (2008), and Sharif et al. (2009). Data from the present study indicates that cats become infected very early in life. Lower incidence in older cats (above 3 years) may be due to increased resistance of the immune system to this type of infection (Dubey and Lappin, 2012). Haddadzadeh et al. (2006) tested 100 serum samples from 50 stray and 50 household cats in Tehran, Iran, and found no significant differences in the *T. gondii* antibody titers between males and females. Similar findings were reported by Smielewska-Los and Dorny et al. (2002),

Gauss et al. (2003), Salant and Spira (2004), Pena et al. (2006), and Hooshyar et al. (2007). In this study, a higher seroprevalence was noted in female cats than males in methods of PCR and MAT, but the difference was not significant. Our results showed agreement with the above-mentioned results. In cats, the seroprevalence of *T. gondii* is variable depending on living type (stray or domestic), age, the diagnostic method used, and geographic area (Dubey and Lappin, 2012). The prevalence of *T. gondii* was reported to be different in cats in recent years in Iran. The prevalence of *T. gondii* was studied in some cities of Iran, showing that the prevalence rate of this infection was 36-90% in Tehran (Haddadzadeh et al., 2006), 86% in Kashan ((Hooshyar et al., 2007), 40% in Sari (Sharif et al., 2009), and 32.1% in Kerman (Akhtardanesh et al., 2010). Another study reported that the seroprevalence of *T. gondii* infection was relatively high (up to 54%) in serum of the feral cats in Ahvaz (Hamidinejat et al., 2011). It shows that stray cats are more important in the infection spread compared with companion cats. Other surveys of *T. gondii* prevalence have recorded 41% prevalence rate in America (Ladiges et al., 1982), 70.2% in Belgium (Dorny et al., 2002), 5.4% in Japan (Maruyama et al., 2003), 25.5-51.9% in Spain (Gauss et al., 2003; Miro et al., 2004), 44.1% in Czech Republic (Sedlak and Bartova, 2006), 87.3% in Brazil (Cavalcante et al., 2006), 21-91.8% in Mexico (Alvarado-Esquivel et al., 2007), 40.3% in Turkey (Ozkan et al., 2008), 8.1-38.9% in Korea (Kim et al., 2008; Lee et al., 2010), and 11.7- 17.98% in China (Zhang et al., 2009b; Wang et al., 2012). The prevalence data for the determination of *T. gondii* infection was extremely variable in cats in different areas of the world mainly based on different serological tests. Having an antibody titer to *Toxoplasma* usually means that the main shedding period of oocysts in cats has finished. A high number of chronic cases should be expected while studying cat population. Traditionally, seropositive cats are considered as chronically infected (Dubey and Lappin, 2012). The percentage of acute cases of toxoplasmosis found in this study (8%) was

very similar to the results of Galván Ramírez et al. (1999) in Mexico. Presence of acute cases indicates a constant dynamic of the disease and the high risk of contact of cats with the protozoan. The prevalence of acute cases may depend on the chance of cats to be in contact with infected cyst through hunting infected prey or food provided to them, indicating the importance of appropriate food for cats. Early acute cases are those that are positive by PCR before the production of any immune response (Dubey and Lappin, 2012). A high number of acute cases should be expected while studying cat population. Suh and Joo (1999) reported that 5.3% of cats were PCR positive for *T. gondii* infection. However, Lee et al. (2010) reported 47.2% positive cats by using nested PCR in South Korea. Reactivation in chronically infected stages involves reactivation of cysts and conversion of bradyzoites to tachyzoites and might involve a new enteroepithelial phase and excretion of oocysts. In chronically infected hosts with tissue cysts, they may rupture and release bradyzoites to the circulation, which are eventually destroyed by the immunocompetent host or might reactivate the infection in the animal (Dubey and Lappin, 2012). In chronic reactivated cases, a significant protective association was found with more than one cat per household. This result indicates that when living with other cats, cats are less likely to be infected and reinfected with *T. gondii*, similarly as reported in cats from Mexico, which indicates a change in environmental conditions and the immune status of cats living together (Galván Ramírez et al., 1999). A number of reports suggested that PCR is a sensitive method for the detection of *T. gondii* DNA in clinical settings and for epidemiological surveys (Dubey and Lappin, 2012). Burg et al. (1989) stated that the combination of sensitivity and specificity should make detection of the B1 gene based on PCR amplification a very useful method for diagnosis of toxoplasmosis both in immunocompromised hosts and congenitally infected fetuses. By combination of both ELISA and MAT, our study showed that 30-39% of stray cats in Ahvaz were in the chronic stage of infection, but with

Table 1. Absolute and relative frequency distribution of *T. gondii* infection in stray cats based on different gender in Ahvaz district, Southwestern Iran, 2013.

Test	Gender	Positive		Negative		Total	
		Number	Percent	Number	Percent	Number	Percent
ELISA	Male	16	53.33	30	42.85	46	30
	Female	14	46.66	40	57.14	54	
MAT	Male	19	48.71	27	44.26	46	39
	Female	20	51.28	34	55.73	54	
PCR	Male	3	37.5	43	47.91	46	8
	Female	5	62.5	49	51.08	54	

Table 2. Absolute and relative frequency distribution of *T. gondii* infection in stray cats based on different age groups in Ahvaz district, Southwestern Iran, 2013.

Test/situation	Age (years)	< 1	1-2	2-3	3-4	4-5	5-6	> 6	Percent
ELISA	Positive	n=8 (%26.66)	n=10 (%33.33)	n=4 (%13.33)	n=5 (%16.66)	n=1 (%3.33)	n=2 (%6.66)	n=0 (%0)	30
	Negative	n=9 (%12.85)	n=22 (%31.42)	n=19 (%27.14)	n=9 (%12.85)	n=1 (%1.42)	n=6 (%8.57)	n=4 (%5.71)	70
MAT	Positive	n=10 (%25.64)	n=12 (%30.76)	n=6 (%15.38)	n=7 (%17.94)	n=1 (%2.56)	n=3 (%7.69)	n=0 (%0)	39
	Negative	n=7 (%11.47)	n=20 (%32.78)	n=17 (%27.86)	n=7 (%11.47)	n=1 (%1.63)	n=5 (%8.19)	n=4 (%6.55)	61
PCR	Positive	n=0 (%0)	n=3 (%37.5)	n=4 (%50)	n=1 (%12.5)	n=0 (%0)	n=0 (%0)	n=0 (%0)	8
	Negative	n=17 (%18.47)	n=29 (%31.52)	n=19 (%20.65)	n=13 (%14.13)	n=2 (%2.17)	n=8 (%8.69)	n=4 (%4.34)	92
Number		17	32	23	14	2	8	4	100

different molecular statuses (8% in active stage by PCR). Surprisingly, the *T. gondii* seroprevalence in cats observed in this study was not significantly different from those reported in the year 2011, which was 24.75% for companion cats in Ahvaz (Mosallanejad et al., 2011). In the present study, all the cats were outdoor and 93% of them were DSH. Therefore, the exact relationship between indoor and outdoor breeding types could not be differentiated. Due to close contact of cats with humans and the fact that children play outdoors in the soil, cats can be an important potential source of transmission of zoonotic parasites such as *T. gondii* (Dabritz and Conrad, 2010). Preventive efforts should focus on educating cat owners on the importance of collecting cat feces in litter boxes, spaying owned cats, reducing the number of feral cats,

and promoting rigorous hand hygiene (Meireles et al., 2004).

It can be concluded that the serologic tests such as ELISA and MAT have a good agreement in diagnosis of chronic form of toxoplasmosis. We determined that a considerable percentage of cats in Ahvaz, southwest of Iran, were infected with *T. gondii*. These infected cats may play an important role in the transmission of toxoplasmosis to humans. It seems that climatic conditions in this area (warm and humid) are relatively suitable for the spread and survival of the oocysts. Our results can be the basis of further studies that will deepen our knowledge of the epidemiology of *T. gondii*. Future studies in various areas are necessary to survey the overall epidemiological status of toxoplasmosis in cat population.

Ethics

I hereby declare that all the ethical standards were respected in preparation of the submitted article. Most animals were clinically healthy. All the procedures that might be associated with discomfort, including venipuncture, were performed by an experienced veterinarian.

Conflict of Interest

The authors declare that they have no conflict of interest.

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