ORIGINAL ARTICLE

Molecular and Serological Evaluation of *Toxoplasma gondii* among Female University Students in Mamasani District, Fars Province, Southern Iran

Mohsen Kalantari[1], PhD; Qasem Asgari[2], PhD; Khadijeh Rostami[3], MD; Shahrbano Naderi[3], MSc; Iraj Mohammapour[3], PhD; Masoud Yousefi[4], PhD candidate; Mohammad Hassan Davami[5], PhD; Kourosh Azizi[1], PhD

[1]Research Center for Health Sciences, Institute of Health, Department of Medical Entomology and Vector Control, Shiraz University of Medical Sciences, Shiraz, Iran;
[2]Basic Sciences in Infectious Diseases Research Center, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran;
[3]Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran;
[4]Department of Environmental Health, Mamasani Higher Education Complex for Health, Shiraz University of Medical Sciences, Shiraz, Iran;
[5]Department of Parasitology and Mycology, School of Medicine, Jahrom University of Medical Sciences, Jahrom, Iran

Correspondence:
Kourosh Azizi, PhD;
Research Center for Health Sciences, Institute of Health, Department of Medical Entomology and Vector Control, Shiraz University of Medical Sciences, Shiraz, Iran
Tel:+98 9177168336
Email: Azizik@sums.ac.ir
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Abstract

Background: Anti-*Toxoplasma* antibodies were identified in female university students referred to Valie-Asr hospital of Mamasani from Azad and Payame-Noor Universities, using serological and molecular methods.

Methods: Based on the prevalence and characteristics method, 504 serum samples were collected from female university students, during 2015, and evaluated by Enzyme-Linked Immun-Sorbent Assay (ELISA), Modified Agglutination Test (MAT), and Polymerase Chain Reaction (PCR) based on B1 gene for detection of *Toxoplasma gondii*. The data were analyzed using SPSS 19 software.

Results: Out of 504 studied female students, 27 (5.36%) and 36 (7.14%) cases were found to be positive for anti-*Toxoplasma* IgG antibodies by MAT and ELISA, respectively. Moreover, 5 (0.99%) cases were found to be positive for *anti-Toxoplasma* IgM. PCR detected the *Toxoplasma* DNA in 58 out of 504 (11.51%) samples.

Conclusion: Findings of the current study revealed that *Toxoplasma* was a common infection among female university students in Mamasani district in Fars province. Seronegative individuals are at risk for the disease, as well as congenital toxoplasmosis in later stages of their life. Preventive measures should be taken to reduce the rate of infection.

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Keywords: *Toxoplasma gondii*, Polymerase Chain Reaction, Enzyme-Linked Immuno-Sorbent Assay, Modified Agglutination Test, Iran

Introduction

Toxoplasmosis is one of the most common parasitic infections between human and animals.[1, 2] This infection has a worldwide distribution and birds, cats, and domestic animals are the most important reservoirs of *Toxoplasma*.[3] Eating infected vegetables and fruits, blood transfusion and transplantation, transmission through the placenta, and infected undercooked meat are the main route of transmission to humans.[4] Most people infected with toxoplasmosis are asymptomatic and the infection can persist for many years in the body.[5] The main symptoms of this disease include severe brain and ocular complications of newborns as well as abortion.[3]

Various serological and molecular assays are used to detect *Toxoplasma*. In serological methods, anti-*Toxoplasma* IgG antibodies are detected in the serum, plasma and other bodily fluid samples of animals. Furthermore, formalin-treated *Toxoplasma* tachyzoites are used as the antigen. In the samples, the existence of anti-*Toxoplasma* IgG antibodies causes the antigens in suspension to form a mat of cells covering the bottom of a U-shaped well. Samples without/with pellets at the bottom of wells are reported as positive and negative, respectively.[5] The application of the polymerase chain reaction (PCR) diagnostic assay is

reported to be very sensitive for *Toxoplasma*. This method is a powerful tool for detection of *Toxoplasma* species in different hosts.[6]

The highest prevalence of toxoplasmosis has been reported among Paris residents (in France) up to 93%.[3] The lowest prevalence has been reported from Northern Mexico (7.4%).[7, 8] In Iran, the infection rate of toxoplasmosis is also remarkable (Tehran 82%, Shiraz 77%, and Isfahan 57%).[9-11] However, it seems that the infection is reduced in Shiraz (19.3%).[12] This cross-sectional study was conducted to identify the anti-*Toxoplasma* antibodies from female university students of Mamasani, during 2015, using MAT, ELISA, and PCR methods.

Methods

Study Area

Noorabad Mamasani is located in the west of Shiraz in Fars province. It has a rather cold climate in the north and warm in the south. In old history books, Mamasani was mentioned as "Shoulestan", derived from Shoul, which was one of Lur tribes. Noorabad is located about 58.7 Kilometers from Kazerun, 80. 5 Kilometers from Yasuj (Kohgiluyeh-e Boyerahmad Province), and 100. 2 Kilometers from Borazjan (Bushehr Province) (Figure 1).

Sampling

In the current study, according to the prevalence and characteristics formula, the sample size was calculated to be about 504 cases for the prevalence, reliability and error of 15%, 95%, and 0.04, respectively. During 2015, 5 cc whole blood samples were randomly prepared from native female students of Azad and Payame-Noor in Noorabad district. Serum samples were evaluated by MAT, ELISA, and PCR for detection of *Toxoplasma gondii* after signing the consent form containing questionnaire by volunteers. The questionnaire contained questions on: 1) age and occupation of students; 2) the level of exposure to cats (high, to some extent, or rarely); 3) washing the hands and time (with/without soap, before/after meal); 4) washing vegetables(with/without disinfectants); 5) the consumption of meat (using boiled broth/barbecued); 6) the amount of direct contact with the soil (high, rare, or none); 7) Living place (town/village); 8) the results of each serology test (MAT positive/negative, ELISA IgM and IgG positive/negative); and 9) the clinical signs noticed in the last three months (swollen lymph nodes, ocular discomfort, and/or troubled brain).[5]

Toxoplasma Modified Agglutination Test (MAT)

For MAT assay, essential reagents contained: (a). TgMAT antigens, 2x10^8/ml *Toxoplasma* whole-cell antigen, formalin-fixed tachyzoites. (b). Alkaline buffer (7.02 g NaCl, 3.09g boric acid (H3BO3), 24 ml of 1 N NaOH, 4g of bovine plasma albumin in 1 liter of distilled water, pH was adjusted to 8.7, 1g sodium azide was added (0.1% final) as a preservative), final pH 8.2-8.4. (c). Phosphate Buffered Saline (PBS), pH 7.2. (d). Positive control serum. (e). Negative control serum. (f). Evans blue dye (2 mg/ml in H2O). (g). 2-mercaptoethanol. (h). 96-well U-bottom microtiter plates. (i). Serum samples. All reagents were stored at 4 ºC, but the sera were stored at -20 ºC.

For sample test, 3 µl serum samples were diluted in 72 µl PBS buffer (1:25) in a total volume of 75 µl and mixed well. Then, 50 µl of diluted samples were transferred to the first and fifth rows from column 1 to column 10 of the plate. Afterward, 50 µl of diluted negative and positive controls was transferred to the first wells of columns 11 and 12, respectively (20 samples, 1 negative and 1 positive control). Afterwards, 25 µl of PBS was added to the rest of the wells. Using a multichannel pipette, 25 µl of diluted samples (10 samples) was taken from row

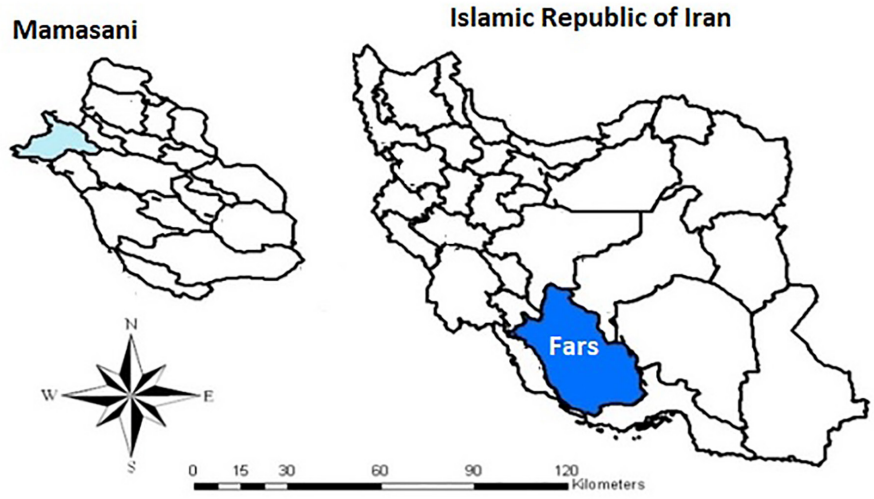

Figure 1: The geographical coordinates of Fars province, southern Iran including the studied area of Mamasani during 2015.

1; serial dilutions were made to row 4, and finally 25 µl was removed from the last dilution and discarded. The procedure was repeated for the 10 samples in row 5. For negative and positive controls, serial dilutions were made to 1:3, and 25 µl was removed from the last dilutions.

For preparation of antigen mixture, 2.5 ml alkaline buffer, 35 µl 2-mercaptoethanol, 50 µl Evans blue dye (2 mg/ml in H2O), and 150 µl TgMAT antigen were needed for each 96-well plate. Antigens were mixed well by pipetting; immediately 25 µl antigen mixture was transferred to each well using multichannel pipette. To prevent carryover of the serum, the pipette tips should not touch the bottom of the wells. The plate was tapped lightly to bring the liquid to the bottom of the wells. Each well had 3x10^5. tachyzoites. The plate was covered with sealing tape and incubated at 37°C for 16-24 hours. Consequently, observing a pellet at the bottom of the well led to negative result and samples without pellets were positive. For positive samples with titers ≥1:200, further tests were performed to determine the titers. Serial dilutions included 1:25, 50, 100, 200, 400, 800, 1600, and 3200.[13]

Toxoplasma Enzyme-Linked Immunosorbent Assay (ELISA)

Prevalence of anti-*Toxoplasma* antibodies in female university was checked according to the immunoglobulin class, age, and different locations of Nourabad district. After centrifugation, the serum samples were separated and maintained at−70 °C before testing. Afterwards, IgM and IgG antibodies against *Toxoplasma* were checked by ELISA kit (Dia-Pro, Milan, Italy). ELISA was performed based on the manufacturer's procedural instructions; briefly, the samples were added to the 96-well plates and then incubated for 30 min. Then, the plates were washed three times to remove additional particles. After further incubation and washing, chromatogen was added to the well containing the substrate material. A colorless substrate was converted to a colored end product in the presence of peroxidase after 5 to10 min. The color changes were checked by spectrophotometer at a wavelength of 450 nm.[5]

PCR Assay for Toxoplasma
DNA Extraction

Totally, 504 serum samples were prepared from females. For extraction of DNA, approximately 200 µl of the sera was diluted with Proteinase K (10 µL) and lysis buffer (50 ml of Tris–HCl, pH=7.6; 1 mM of EDTA, pH=8.0; 1% Tween 20) was added to 500 µl of each sample and then incubated for 24 h at 37°C. The lysate was then extracted twice with phenol/chloroform/isoamyl (25:24:1) before the DNA was precipitated with pure absolute ethanol. The precipitated DNA was re-suspended in 100 µL of double-distilled water and stored at 4°C before use for PCR.

Nested PCR Assay

Bioneer primer sets (Bioneer, Korea) were used for amplifying fragments of the B1 gene for detection of toxoplasmosis. The external primers (5′-GGA ACT GCA TCC GTT CAT GAG-3′) and (5′-TCT TTA AAG CGT TCG TGG TC-3′) were amplified products of 193 bp. PCR reaction procedures were performed in a programmable thermocycler (Eppendorph, Mastercycler gradient). The first 25 µL of PCR reaction mixture contained outer primers at a final concentration of 50 pmol each, 2.5 mmol dNTPs, 1 µg of template, and 1.5 U recombinant *taq* DNA polymerase (GENET BIO, Korea, A-type Prime *Taq* TM DNA polymerase), in 1× PCR reaction buffer (50 mmol/L KCl and 10 mmol/L Tris–HCl, 1.5 mmol/L MgCl2, and 0.1% triton X-100; Sinagen Co., Iran). The first step of denaturation was amplified at 94 °C for 5 min, and then followed by 40 cycles 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 0ne min). The final cycle was followed by an extension step at 72 °C for 10 min.

Nested reactions contained 1 µl first-round product, 10 mM Tris–HCl, pH 8.3 (at 25 °C), 50 pmol each, 2.5 mmol dNTPs, 1 µg of template, and 1.5 U recombinant taq DNA polymerase. Internal primers (5′-TGCATAGGTTGCAGTCACTG-3′ and 5′GGCGACCAATCTGCGAATACACC-3′) were amplified products of 96 bp. The second round thermocycler program was similar to the first, but only the secondary annealing temperature was 62.5 °C. *T. gondii* strain and doubled distilled water were used as positive and negative control, respectively. The amplification products were detected by gel electrophoresis using 2% agarose gel in 1× Tris–borate–EDTA buffer. DNA bands were visualized in the presence of ultraviolet light, following the staining with 0.5% ethidium bromide.[6]

Analysis

Statistical analyses of the tables and graphs were done using SPSS software. Descriptive statistics were displayed in either frequency tables or as mean standard deviation note. The relationships between the categorical variables were evaluated by Chi-square test. *P*-values less than 0.05 were considered as statistically significant.

Results

Serological Findings

In serological assays, out of 504 studied female students, 36 (7.14%) and 27 (5.36%) of cases were found to be positive for anti-*Toxoplasma* IgG antibodies by ELISA and MAT, respectively. Besides, 5 (0.99) cases

were found to be positive for *anti-Toxoplasma* IgM.

Statistical results of *Toxoplasma* risk factors and descriptive relationships between the categorical variables are displayed in Table 1.

PCR findings

In general, using nested PCR method, we found that 11.5% of the sera were positive to *Toxoplasma*. The amplicons produced in the Nested PCR based on the B1 gene primers from positive samples are shown in Figure 2.

Level of agreement between MAT and PCR techniques in the diagnosis of female toxoplasmosis was analyzed. Consequently, the correlation between MAT and PCR was obtained 59.2%, whilst this figure between ELISA and PCR was 66.7%. P values of P=0.02 and P=0.0001, respectively, were reported for dual correlations between the two above mentioned tests.

Discussion

Toxoplasmosis is a global parasitic infection and its prevalence is as high as 50 percent in some parts of the world including some parts of Iran.[12] The disease infects humans and a wide range of mammalians and birds.[14-17] In the current study, the seroprevalence of *T. gondii* was obtained 5.36% and 7.14% in the female sera by MAT and ELISA, respectively. Serological assays such as ELISA, with a specificity of 97% and sensitivity of 100%, is considered as one of the best laboratory methods used to detect the serum antibodies levels against toxoplasmosis.[5] In similar studies, the serological infection rate of toxoplasmosis was obtained 10% in Fasa districts, which was nearly close to the result of the present research.[18] However, the seroprevalence rate was less than other study results obtained from Jahrom, Tehran, Shahre Rey, Isfahan, and Bushehr districts,[5, 19-21] which might be due to dissimilar conditions in the same geographical districts of Mamasani and other studied regions.

In the present study, 5 (0.99%) cases were found to be positive for *anti-Toxoplasma* IgM. In another study, 5.47% and 12.3% of the blood donors were seropositive for IgM and IgG in Shiraz, respectively. Furthermore, 1.6% were positive for both IgG and IgM. IgG immunoglobulin is usually chronic form of the disease and is related to the past infection separated from the patient (mostly observed in the older adult), especially in cases which are contaminated for the first time and cyst formation in different tissues. In two different studies conducted in Meshkinshar and Isfahan, the infection rates of toxoplasmosis increased with age, and the highest infection rates were reported in those aged over 40 (25.4% and 50%, respectively).[20, 22]

Nested-PCR was a valuable technique for molecular detection of *T. gondii*. Indeed, the prevalence of *T. gondii* infection was considerable (11.51%) by

Table 1: Statistical results of Toxoplasma risk factors among female students in Mamasani County, Southern Iran, during 2015

Risk factors	Students (n=504)	Statistics
Age (years)	24.30±4.24	P<0.001
Having contact with cat(s)	102 (20.23%)	$\chi2$=2.02, P=0.224
Wash hand and time (with/without soap)	345/159	$\chi2$=14.47, P=0.091
Washing vegetable (with/without disinfectants)	383/121	$\chi2$=7.86, P=0.083
Consumption of raw or uncooked meat	158 (31.35%)	$\chi2$=8.34, P=0.211
Contact with soil	332 (65.87%)	$\chi2$=19.02, P=0.395
Living place (town/village)	323/181 (64.09%/35.91%)	$\chi2$=0.545, P=0.031
Clinical signs*	2 (0.40%)	$\chi2$=11.41, P=0.074

*Swollen lymph nodes, ocular discomfort, and/or troubled brain.

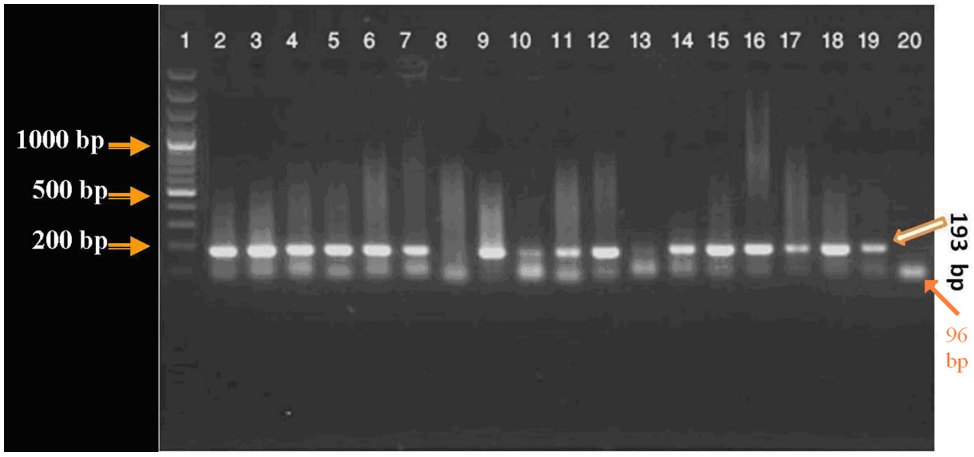

Figure 2: The amplicons produced, in the Nested PCR based on the B1 gene primers; ladder (Lane 1), Positive Samples (lane 3-20), and Reference positive sample of *Toxoplasma gondii* (prepared from Tehran University of Medical Sciences) (lane 2). The amplified product of 193 bp belonged to the first stage of nested PCR whereas the amplified product of 96 bp was the next stage of the test.

PCR method among female university students in Mamasani district. Because of high sensitivity and specificity, PCR method has been used many times for detection of toxoplasmosis. Indeed, PCR was able to detect up to 69% of *Toxoplasma* DNA in some mammalian reservoirs in Fars province.[6, 12]

Different factors are known to be effective in increasing the prevalence of toxoplasmosis. Some factors consist of age, gender, occupation, habits and behaviors, climatic conditions, soil or cat exposure, and education level.[23-25] These factors may directly enter into the human living or facilitate it indirectly and increase the incidence of toxoplasmosis.[24] Contact with cat feces (oocyst), consuming unwashed crops, and undercooked animal meat are the most important factors that increase the incidence of the disease.[26, 27] Gender, age, level of education, and occupation may indirectly have effect on the prevalence of the disease.[24]

No significant relationship was found between the frequency of the disease and different factors considered in the current study. In another similar study, no relationship was seen as well,[22] but in Fasa, the relationship between the increasing percentage of *Toxoplasma* infection and contact with the cats was significant.[18] In the present study, the prevalence of *T. gondii* in females who consumed grilled meat was higher than those who poached meat. However, the differences were not statistically significant. In a similar study, no correlation was seen between the type of cooking and *Toxoplasma* infection in Fasa.[18] In Iran, the prevalence of toxoplasmosis was reported higher in the rural areas of Islamshahr, Kermanshah, and Chaharmahal and Bakhtyari.[22, 28] *Toxoplasma* infection rate has been reported in rural areas more than in the urban parts of Isfahan and was statistically significant.[20] In the present study, the infection rates in rural areas were more than in urban areas, but the difference was not statistically significant.

Conclusion

Serological and molecular infection rates of toxoplasmosis obtained from this study indicated that the agreement between MAT/ ELISA and PCR (59.2% and 66.7%, respectively) was statistically significant. Results of the recent study confirmed that *Toxoplasma* is a common parasitic infection among female university students in Mamasani district in Fars province. Seronegative individuals are at risk for toxoplasmosis, as well as congenital form of the disease in later stage of their life. Preventive measures should be taken to decrease the rate of infection.

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Conflict of Interest: None declared.

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