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Molecular and serological prevalence of *Toxoplasma gondii* in pregnant women and sheep in Egypt

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ABSTRACT

Objective: To investigate molecular and serological prevalence of *Toxoplasma gondii* (*T. gondii*) in pregnant women and sheep in Egypt.

Methods: Blood samples collected from healthy 364 pregnant women and 170 sheep were investigated for *T. gondii* antibodies and parasitemia using highly specific and sensitive surface antigen (*Tg*SAG2) based enzyme linked immunosorbent assay (ELISA) and real time-polymerase chain reaction (RT-PCR).

Results: Overall prevalence of *T. gondii* was 51.76%, 17.65% in sheep, 33.79%, 11.81% in pregnant women, using ELISA and RT-PCR respectively. Significant differences in *T. gondii* prevalence were observed on the basis of contact with cats or soil in pregnant women using either RT-PCR or ELISA. In pregnant women, a significant increase was detected in aged and those eating under-cooked mutton using simultaneous ELISA/RT-PCR.

Conclusions: Consumption of under-cooked infected mutton is an important source of human infection and the combination of the two assays provide accurate and precise data during infection.

1. Introduction

Toxoplasmosis is a zoonotic disease spread worldwide and caused by intracellular protozoan parasite *Toxoplasma gondii* (*T. gondii*) that infects humans and wide range of animals [1,2]. In the host, as it caused abortion, it could result in significant reproductive failures, economic and public health problems, since consumption of infected meat could facilitate zoonotic transmission [3]. In sheep, *T. gondii* infection could result in stillbirth, miscarriage and neonatal mortality [4,5]. In human, this disease is asymptomatic in healthy individuals during primary infection. On the other hand, toxoplasmosis can be fatal for congenitally infected fetus and immunodeficient patients (transplant recipients or HIV-infected patients), as a result of either reactivation of the parasite or acute recent infection [6–8]. *T. gondii* become very important during pregnancy, especially when occurred with early gestational age, as it can spread to the developing fetus across the placenta and thereby cause hydrocephalus, intracranial calcifications, chorioretinitis, and even stillbirth [9–11].

In Egypt, antibodies against *T. gondii* have been detected in pregnant women using the IgG avidity assay, indirect hemagglutination test (IHAT), direct agglutination test, indirect immunofluorescence test [12,13], enzyme linked fluorescent assay [14,15], latex agglutination test and enzyme linked immunosorbent assay (ELISA) [16–18]. Moreover, *T. gondii* has been identified in sheep by microscopy [19], IHAT, Latex agglutination test, modified agglutination test, ELISA, indirect immunofluorescence test [20–23]. Proper diagnosis of the zoonotic diseases through sensitive and specific surveillance is required to monitor and improve the public health status. An epidemiological prevalence using *T. gondii* specific surface antigen (*Tg*SAG2) based ELISA/real time-polymerase chain reaction (RT-PCR) approach to detect toxoplasmosis in important hosts like sheep and pregnant women will therefore provide highly desirable data for adequate control and prevention of the disease in Egypt.

Previous reports considered the consumption of under-cooked infected meat products as the main risk and suspected mutton, to be a major source of human infection [1,24–26]. Mutton is traditionally consumed throughout Egypt [27].

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Considering the big distribution of sheep in the study regions and the zoonotic burden of *Toxoplasma* infection, the current study aimed to investigate *T. gondii* prevalence in sheep and pregnant women in Menoufia and Gharbiya Provinces of Egypt through cross-sectional prevalence depend on specific ELISA and RT-PCR.

2. Materials and methods

2.1. Ethical statement

The current study was conducted in accordance with the Declaration of Helsinki and the Guidelines for Good Clinical Practice and approved by the Review Board of the National Liver Institute, Menoufia University, Egypt (approval number 00109/2015). The procedures and purpose involved in the current study were explained and written informed consent was obtained from all participants in this study. Moreover, the sheep sampling was started after consent of the Institutional Animal Ethical Committee, Menoufia University, Egypt (approval ID: MUFS/F/IM/1/15).

2.2. Parasite genomic DNA and TgSAG2t antigen

Parasite genomic DNA, *T. gondii*, RH strain, the glutathione *S*-transferase (GST) and GST-*Tg*SAG2t were received as gift from Prof. Dr. Xuenan Xuan and Dr. Yoshifumi Nishikawa, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan, and were used in RT-PCR and ELISA, respectively.

2.3. Sample collection and questionnaire

The sampling was carried out during January–December, 2015. Blood samples were collected from the brachial vein of 364 pregnant women, (20–35 years of age, 6–18 wk of gestation). A structured questionnaire was used to assess risk factors, which included such as age, residential area, pregnancy status, stage of pregnancy, previous abortion, contact with cats, contact with other animals, and consumption of undercooked mutton, and exposure to soil. Based on locations human and sheep samples were obtained from Menoufia and Gharbiya provinces in the Delta of Egypt. Blood samples were collected from the jugular vein by local veterinary practitioners from 170 sheep obtained from public markets (with consideration of sex). Sheep from 1 to 4 years old were divided into two groups based on their age, young sheep (2 years or less) and aged sheep (more than 2 years). Each blood sample was divided into two tubes, one was mixed with EDTA and the other was permitted to clot. Blood was incubated at room temperature for 1 h, and then centrifuged at 1 000 × *g* for 10 min, and the serum was collected and stored at −20 °C for further use.

2.4. ELISA

ELISA was done according to the modified procedure described previously [25,28,29]. The recombinant antigens (GST-*Tg*SAG2t, or GST, 5 μg/mL) in a coating buffer (50 mM carbonate/bicarbonate) were coated in the 96 well plates and incubated overnight at 4 °C. The plates were washed one time with washing buffer (phosphate buffer saline plus 0.05% Tween 20), blocked with blocking solution (phosphate buffer saline plus 3% skim milk) at 37 °C for 2 h. After washing the plates one time with washing solution, 50 μL of serum diluted one hundred times in blocking solution was added to duplicate wells for each sample and kept at 37 °C for 1 h. Then the plates were washed six times and incubated with 50 μL of horseradish peroxidase-conjugated rabbit anti-sheep IgG – H&L (Abcam, Cambridge, MA, USA), and horseradish peroxidase-conjugated goat anti-human IgG (Sigma, St. Louis, MO, USA) diluted in blocking solution four thousand times per well at 37 °C for 1 h. After washing six times, the 96 well plates were incubated with one hundred microliter substrate 2,2′-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid (ABTS)] in an ABTS buffer solution (0.1 M citric acid, 0.2 M sodium phosphate) per each well at room temperature for 1 h. The absorbance was detected at 405 nm using a microplate reader (Seac, Radim Company, Italy). The ELISAs data were calculated on the base of the mean optical densities at the value of 405 nm (OD_{405}) for the recombinant antigen (GST-*Tg*SAG2t) subtracted from those of the GST protein. The cutoff values were estimated as the OD_{405} value for *T. gondii* negative sera plus three standard deviations; which were 0.096 and 0.039 in sheep and pregnant women sera, respectively, (n = 20). The negative sera from sera stock were tested and confirmed negative by western blot and RT-PCR of its corresponding whole blood samples.

2.5. DNA isolation and real time-PCR

DNA was extracted from the whole blood samples and chemically purified by phenol-chloroform extraction and ethanol precipitation [30]. Amplification of *Toxoplasma* DNA was done using designed primers specific for the *T. gondii B1* gene (5′-AAC GGG CGA GTA GCA CCT GAG GAG A-3′ and 5′-TGG GTC TAC GTC GAT GGC ATG ACA AC-3′) that widely occurred in all strains the parasite [31]. The reaction mixture (20 μL) contained 1 × SensiFAST SYBR Lo-ROX Mix (SensiFAST™ SYBR Lo-ROX Kit, Bioline, France), 3 mM $MgCl_2$, 0.5 μ moles of each primer and 50 ng of genomic DNA was prepared. Amplification was performed by following a standard protocol recommended by the manufacturer (2 min at 50 °C, 2 min at 95 °C, then 40 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 10 s). Amplification, data acquisition, and data analysis were performed using an ABI7500 Fast Real-Time PCR System (AB Applied Biosystems), and cycle threshold values were exported to Microsoft Excel for further analysis. Melting curve analyses were utilized to confirm positive sample specificity of each amplified PCR product. Positive and negative controls were used with each PCR run.

2.6. Assays agreement percentage calculation

Agreement between recombinant protein *Tg*SAG2 based ELISA and RT-PCR was calculated according to Ibrahim *et al.* [28,29].

$$\text{The percentage of agreement between the RT} - \text{PCR assay and ELISA} = \text{O}/\text{T} \times 100.$$

where the output (O) that represents the agreement between the two tests (ELISA & RT-PCR) in both the positivity and the

negativity = the total No. of tested samples (T) subtracted from the summation of disagreement between the two assays (S).

S = Samples that positive RT-PCR but negative ELISA + Samples that positive ELISA but negative RT-PCR.

2.7. Statistical analysis

Binary logistic regression was utilized to assess significant differences of infection rate in pregnant women and sheep samples of different conditions using SPSS version 16 (SPSS Inc., Chicago, IL, USA). A *P*-value less than 0.05 was considered as statistically significant.

3. Results

Overall prevalence of *T. gondii* was 51.76%, 17.65% in sheep, using ELISA and RT-PCR, respectively. In Menoufia and Gharbiya Provinces, *T. gondii* prevalence was 51.04%, 52.70% using ELISA and 17.71%, 17.57% using RT-PCR, respectively. The prevalence was higher in aged sheep 55.13%, 17.95% compared to younger animals 48.91%, 17.39% using ELISA and RT-PCR, respectively. On the basis of gender, the prevalence was higher in males 53.85%, 21.15% compared to female animals 48.48%, 12.12% using ELISA and RT-PCR, respectively. Finally, during autumn the prevalence was higher 54.81%, 19.23% than winter season 46.97%, 15.15% using ELISA and RT-PCR, respectively. The difference recorded on the basis of region, age, gender or period of the year for the detected parasite was not statistically significant.

In pregnant women, the overall prevalence of *T. gondii* was 33.79% and 11.81% using ELISA and RT-PCR, respectively. Although no significant difference was detected among human according to region, higher percentages were recorded in Menoufia Province than in Gharbiya Province, using ELISA and RT-PCR, significant ($P < 0.05$) high prevalence of *T. gondii* in pregnant women was observed in the age group of more than 25 years, 37.74%, 14.62%, respectively, when compared to the younger cases. While there are no significant differences were detected at the level of the other socio-demographic characteristics (Table 1).

Regarding the major risk factors that associated with *T. gondii* positivity, the molecular prevalence showed significant ($P < 0.05$) elevation in the pregnant women had contact with cats 16.36% than those had not any contact with cats 11.00%. Serological prevalence demonstrated significant ($P < 0.05$) increase in the pregnant women had contact with soil 38.89% compared with those who had not any contact with soil 33.53%. Both molecular and serological prevalence detected no significant ($P > 0.05$) difference between pregnant women had contact with other animals and those had no contact with other animals. Interestingly, both RT-PCR and ELISA, showed significant ($P < 0.05$) elevation in the pregnant women consumed undercooked mutton 20.00%, 57.37% compared to those that consumed well cooked mutton 2.87%, 8.05%, respectively (Table 2).

During the determination of *T. gondii* infection in pregnant women and sheep, the results of the ELISA were cross-tabulated with these of RT-PCR and summarized in Table 3. In the presented results, the agreement percentage between the ELISA and RT-PCR was detected. The agreement percentages between ELISA and RT-PCR were 78.02%, 65.88% in pregnant women and sheep, respectively.

Table 1
Socio-demographic characteristics and prevalence for toxoplasmosis among pregnant women in Egypt.

Variables	Total	ELISA	Real-time PCR
Region			
Menoufia	171	63 (36.84%)	24 (14.04%)
Gharbiya	193	60 (31.09%)	19 (9.84%)
Age group (year)			
25 or less	152	43 (28.29%)	12 (7.89%)
>25	212	80 (37.74%)*	31 (14.62%)*
Parity			
One time	212	72 (33.96%)	23 (10.85%)
Two times or more	152	51 (33.55%)	20 (13.16%)
Maternity trimester			
First trimester	286	95 (33.22%)	31 (10.84%)
More than first trimester	78	28 (35.90%)	12 (15.38%)
Occupation			
Yes	18	6 (33.33%)	3 (16.67%)
No	346	117 (33.82%)	40 (11.56%)
Abortion history			
No	304	103 (33.88%)	35 (11.51%)
Yes	60	20 (33.33%)	8 (13.33%)
Abortion trimester			
1st	52	17 (32.69%)	6 (11.54%)
2nd	8	3 (37.5%)	2 (25.00%)
Total	364	123 (33.79%)	43 (11.81%)

*Prevalence of *T. gondii* is significantly different ($P < 0.05$) in aged women, more than 25 years old, compared to those 25 years old or less.

Table 2
Risk factors associated with *Toxoplasma* positivity in pregnant women from Egypt.

Variables	Total	ELISA	Real-time PCR
Contact with cat			
Yes	55	21 (38.18%)	9 (16.36%)*
No	309	102 (33.01%)	34 (11.00%)
Contact with other animals			
Yes	28	14 (50.00%)	7 (25.00%)
No	336	109 (32.44%)	36 (10.71%)
Exposure to soil			
Yes	18	7 (38.89%)*	2 (11.11%)
No	346	116 (33.53%)	41 (11.85%)
Consumption of undercooked mutton			
Yes	190	109 (57.37%)*	38 (20.00%)*
No	174	14 (8.05%)	5 (2.87%)

*Prevalence of *T. gondii* is significantly different ($P < 0.05$) between the current variables: contact with cats, exposure to soil and consumption of undercooked mutton.

Table 3
Summary on the detection of *T. gondii* infections in ELISA and real-time PCR.

T. gondii	ELISA[a]		Real-time PCR[b]	
			(+)	(−)
Human	(+)	123	43	80
	(−)	241	0	241
	Total	364	43	321
Sheep	(+)	88	30	58
	(−)	82	0	82
	Total	170	30	140

[a] The frequencies of positive and negative samples as results of ELISA.
[b] The frequencies of positive and negative samples as results of real-time PCR cross-tabulated with ELISA results.

4. Discussion

Using ELISA and RT-PCR, *T. gondii* overall prevalence was 51.76%, 17.65% in sheep and 33.79%, 11.81% in pregnant women, respectively. No significant difference was recorded between the two examined Provinces. Prevalence was higher in Menoufia Province than Gharbiya Province in pregnant women samples. Interestingly, the high prevalence of the parasite in sheep was accompanied by high human positivity. Shaapan *et al*. recorded high pattern of *T. gondii*, ranged from 37.0% to 43.7% using different serological assays in sheep samples from Cairo, Egypt [22]. At Tanta (Gharbiya) abattoir, prevalence of *T. gondii* antibodies were 52.4% among workers and 44.1% in slaughtered animals using IHAT [20]. The parasite cyst was monitored microscopically in the brains of a flock of sheep in private farm in Suez Province, Egypt [19]. Moreover, high positivity of *T. gondii* antibodies were detected in sheep samples from different regions in the Delta of Egypt [21,23]. Altogether, the recorded high prevalence of the parasite in sheep indicates the wide distribution of *T. gondii* in Egypt, and suggests that ovine meat might be a critical source of human infection, especially pregnant women as one of the major targeted categories. In Egypt, similar high patterns of *T. gondii* antibodies have been reported in pregnant women [12–18].

Although PCR is the widely used molecular technique for *T. gondii* detection, it is typically limited to reactivate or acute infections as its significance reduces in chronic infections because Toxoplasma DNA will not be present in collected samples (especially, blood samples) [32]. In the present study, although the results of RT-PCR were coming in partial agreement with those of ELISA, serological assays detected higher prevalence compared with molecular determination of the *T. gondii* in the host blood. Zainodini *et al*. detected that RT-PCR is essential to detect acute *T. gondii* infections as compared to IgM detection using ELISA, but it recorded lower prevalence when compared to IgG detection by ELISA among Iranian blood donors [33]. Furthermore, the antibody response, especially IgG may be independent of *T. gondii* tachyzoites burden. Previous study reported that a serum-based ELISA is the more sensitive for detection of *T. gondii* than real time PCR, semi-nested PCR and direct PCR during naturally and experimentally infection in pigs [34].

Evaluation of relationship between ages, gender, periods of the year and infection level was performed by comparing the prevalence of *T. gondii* in sheep population. Although the prevalence was higher in aged animals than younger populations, no significant changes were demonstrated in the present study. This observation result may be caused by the increasing exposure of the old sheep population to the parasite oocysts for long periods [35]. The current results were in agreement with previous reports in Libya [36], Tunisia [37,38] and Egypt [23]. The current study showed high prevalence among male animals during autumn season when compared to female sheep during the winter season using ELISA and RT-PCR with no significant differences. Regarding gender, previous studies in horses and sheep come in agreement with the current study [39–41]. Moreover, Frequently *Toxoplasma* oocysts accumulation may be occurred during spring, summer and early autumn, when temperatures are convenient for the process of sporulation [42]. In contrary, prevalence of *T. gondii* in cold weather becomes low in arctic areas [11].

The present study was extended to assess the relationship between socio-demographic characteristics, risk factors and *Toxoplasma* positivity in Egyptian pregnant women. The current data showed that the positivity was observed more in those more than 25 years old than in the younger pregnant women. Previous studies stated that the positivity of *Toxoplasma* infection increases with age [43,44]. No significant association was found between the presence of *T. gondii* and the other socio-demographic characteristics. Gelaye *et al*. detected similar patterns on the different socio-demographic characters during toxoplasmosis in pregnant women in Ethiopia using LAT [45]. In the present study, regarding the risk factors and *T. gondii* positivity in pregnant women, a significant increase was recorded in the pregnant women had contact with cats compared to those had not contact with cats using RT-PCR. Many previous studies reported that ownership of cat or contact with cats was associated with positivity of *T. gondii* [44,46–49]. Cats, as the definitive host, are considered the major source of toxoplasmosis to animals and humans through excreting the oocysts with its feces [2]. Excreted oocysts may remain viable for years under some environmental condition [50]. Hence, exposure to soil could be considered as a potential source of infection for human's especially pregnant woman. The present results showed a significant elevation in the pregnant women had contact with soil compared to those had not any contact with soil using ELISA. This finding was in accordance with several studies performed in China and France which showed that exposure to soil is an important risk factor for pregnant women [51–54]. Finally, the current data revealed that significant increase in the pregnant women consumed undercooked mutton compared to those consumed well-cooked mutton using RT-PCR and ELISA. Mutton and Lamb are the preferable meats for the majority of the Egyptian people elevating risk of *T. gondii* infection in Egypt for the meat consumers. Consumptions of mutton kebab maximize the incidence of human toxoplasmosis [55,56]. Moreover, a previous study has identified the consumption of mutton/lamb meat as a highly significant risk factor for contracting *T. gondii* infection in pregnant women [24].

In the current study, various significant differences detected by either specific based ELISA or RT-PCR or both methods emphasize the importance of the simultaneous testing approach using the combination of the two assays in order to acquire accurate and precise data on the parasite prevalence during the acute and chronic infection. In conclusion, the current results indicated that *T. gondii* infection is frequent in sheep and pregnant women in Menoufia and Gharbiya Provinces of Egypt. Consumption of under-cooked infected mutton is a critical source of human infection. Finally, detection of *T. gondii* using ELISA and RT-PCR are accurate and perfectly suitable for control and prevention of the infection.

Conflict of interest statement

The Authors declare no conflict of interest related to this work.

Authors contributions

HMI conceived the study, HMI, HEE designed the experiments, HMI and HEE performed the experiments, HMI, AHM

and HEE analyzed results, HMI, AHM, AAA and HEE wrote the manuscript.

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