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RESEARCH ARTICLE
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Molecular detection of *Toxoplasma gondii* DNA in milk and risk factors analysis of seroprevalence in pregnant women at Sharkia, Egypt

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Abstract

Aim: Toxoplasmosis is one of the most important zoonotic parasitic diseases worldwide. Infection is not only acquired by contact with the definitive host, but also by ingestion of raw milk and milk products. The aim of the study was to detect *T. gondii* DNA in milk samples, and to estimate anti-*T. gondii* antibodies in pregnant women for the analysis of the associated risk factors.

Materials and Methods: The study involved molecular examination of 150 milk samples collected from rural settings at Sharkia, Egypt. Seroprevalence of anti-*T. gondii* antibodies was determined in 100 pregnant women by indirect haemagglutination test. The analysis of risk factors associated with seropositive results was assessed by univariate and multivariate logistic regression.

Results: *T. gondii* DNA was detected in 8% and 2% of goat and sheep milk samples, respectively, while none of cow milk samples were positive. Seroprevalence of *T. gondii* antibodies in pregnant women was 82%, anti-*T. gondii*-IgG and anti-*T. gondii*-IgM were detected in 63% and 11% serum samples, respectively. The results showed a significant correlation between the seroprevalence and the contact with cats and consumption of raw milk and homemade cheese.

Conclusion: The role of raw milk in transmission of *T. gondii* cannot be excluded. Contact with cats remains the main risk factor for acquiring *T. gondii* infection; also consumption of raw milk and milk products is considered an important risk factor. Further large scale studies are recommended to clarify the association of *T. gondii* seroprevalence with different risk factors.

Keywords: *B1* gene, pregnant women, raw milk, risk factors, seroprevalence, *T. gondii*.

Introduction

Toxoplasma gondii is a ubiquitous obligate intracellular blood protozoan which is widely distributed among warm-blooded animals and humans that are considered intermediate hosts [1, 2]. All animals and birds consumed by humans can be considered a potential source of infection [1]. The public health significance of toxoplasmosis is not only due to direct contact with the definitive host (Family Felidae, such as domestic cats) but also due to consumption of infected meat and milk which can facilitate zoonotic transmission [3]. The medical and veterinary importance of toxoplasmosis is because the disease may cause abortion or congenital diseases in the intermediate hosts [4]. It has been estimated that up to one third of human population worldwide are infected with toxoplasmosis [5, 6]. Human infection is usually subclinical; however, primary infection during pregnancy may cause spontaneous abortion or stillbirth. Moreover, the exposure of a newborn to the parasite in utero may develop congenital toxoplasmosis with major ocular and neurological complications [5, 6].

Different studies have reported that consumption of raw milk and milk products may play a role in the horizontal transmission of toxoplasmosis [7]. Contamination of milk with *T. gondii* could be originated from the infected animal or from contamination of milk with cats' feces since stray cats are commonly found around and within farms [3]. Several PCR assays have been developed for the detection of *T. gondii* DNA [8]. Such PCR reactions amplify different targets such as B1 gene, P30 or 18S rDNA [9]. The B1 gene is a 35-fold repetitive gene sequence with unknown function; PCR amplifying this target has shown high specificity for *T. gondii* DNA detection [9].

Seroprevalence of anti-*Toxoplasma* antibodies is so far the most frequently used test for the routine screening of toxoplasmosis in humans [10]. However, the results of sero-diagnosis are influenced by different factors such as geographic areas, the examined populations, cultural, hygienic and nutritional habits [4, 10, 11].

Knowledge on the prevalence and risk factors of infection with *T. gondii* is essential for estimation of the burden of the disease and to design appropriate

strategies for prevention of the disease.The aim of the current study was to examine raw milk of caprine, ovine and bovine sources collected from rural areas at Sharkia Province, Egypt, by amplification of *T. gondii* B1 gene. Moreover, the study aimed to estimate the seroprevalence of anti-*T. gondii* antibodies in serum samples collected from pregnant women at the same localities, to evaluate the associated risk factors.

Materials and Methods

Sampling: A cross-sectional study was conducted from December, 2013 to March, 2014, in rural areas at Sharkia Province, Egypt. A total of 150 milk samples from different milking animals (goats, sheep and cows, 50 each) were collected from small local outlets that buy the produced milk from in-house reared animals at the villages under investigation. Moreover, 100 venous blood samples without EDTA were collected from pregnant women presenting to antenatal clinic of the Obstetrics and Gynecology Department, Zagazig University Hospital, Sharkia, Egypt. The choice of the hospital for sample collection was based on its location in close proximity to the rural areas from which milk samples were collected. During sampling, a questionnaire was prepared to estimate the predisposing risk factors associated with seroprevalence.

Molecular amplification of *B1* gene:

DNA extraction: From each examined animal, 50 ml milk sample was collected and centrifuged at 2200 g for 5 minutes for concentration [12]. One ml of the sediment was re-suspended re-suspended in 200µl TE (1mM EDTA, 10mM Tris-HCl (pH = 7.6)) and 300 µl 0.5MEDTA (pH = 8), and centrifuged at 3000 g for 10 minutes, to avoid interference by casein [13]. Milk pellet was then diluted in 200 µl of PBS and DNA was extracted using the Thermo Scientific GeneJET Genomic DNA purification kit (Thermo Fisher Scientific, NYSE: TMO) according to the manufacturer guide.

PCR: Extracted DNA from milk was subjected to PCR for the amplification of the highly conserved 35-fold-repetitive B1gene [14]. The reaction was performed using the oligonucleotide primers TOXOB22 (Forward) 5'-AACGGGCGAGTAGC ACC TG AG GAGA-3' and TOXOB23 (Reverse) 5'-TGGGTC-TACGTCGATGG CATGACAAC-3' (synthesized by Biosearch Technologies Inc., USA) that amplify 115 bp of the target. The reaction was performed in 20 µl reaction volume containing 10 µl of readymade 2x power Dream*Taq*Green PCR mastermix (Thermo Fisher Scientific, NYSE: TMO), 20 pmol of B1 primers, each, and 2 µl of the purified DNA. The reaction conditions consisted of one cycle of 95°C for 5 min followed by 40 cycles of 94˚C for 30 sec, 60 ˚C for 30 sec and 72˚C for 60 sec and a final extension at 72˚C for 10 min. The reaction was carried out in Primus Thermal Cycler (MWG-Biotech Thermal Cycler, Ebersberg, Germany). Amplification products were resolved in 1.2% (w/v) agarose gels along with 100 bp molecular weight ladder (BioTeke Corporation, Shanghai, China). The agarose gel was prepared in 1 x TBE (89 mMTris- Borate, 2 mM EDTA, and pH 8.3) stained with 5 µM ethidium bromide. The gels were run in 1xTBE, 5 µM ethidium bromide for at least 45 minutes at 100 volts and then visualized under ultraviolet light of a transilluminator (Spectroline, Westbury, NY, USA).A positive control *T. gondii* strain that was kindly obtained from Biotechnology Department, Animal health Research Institute, Dokki, Giza, and a reaction mixture with no added DNA were run in the PCR reaction as positive and negative controls, respectively.

Serology: Anti-*T. gondii* IgG and IgM antibodies were detected by indirect haemagglutination test using TOXO-HAI FUMOUZE® kits (FUMOUZE DIAGNOSTICS, France) with a cutoff point of 1:80. Blood samples without anticoagulant were allowed to clot at room temperature for 15 min, and then serum was collected for performing serology testing according to the manufacturer guidelines.

Risk factors analysis: A questionnaire included questions about the possible risk factors associated with *T. gondii* seroprevalence in pregnant women was filled individually for each participant during the study. The questionnaire included general information about the age, previous abortions and village of residence of the participants. Questions on the presence of cats, contact with soil (gardening or working in the field), rearing livestock and consumption of raw milk and homemade cheese were also included.

Statistical analysis: Bivariate and multivariate logistic regression models were fitted to determine factors associated with *T. gondii* infection using the computer program SPSS, Inc. version 22. Odds ratios (OR), COR: Crude Odd Ratio, AOR: Adjusted Odd Ratio and their 95% confidence interval [95% CI] were noted. P-values less than 0.05 were considered statistically significant.

Results

Molecular characterization of *T. gondii* DNA in milk samples by PCR: The presence of *T. gondii* DNA in milk was determined by amplification of *B1* gene using PCR reaction. The results in Table-1 show that 8% of the examined goat milk samples were positive for *T. gondii* DNA, while 2% of sheep milk samples were positive (Figure-1). None of the examined cattle (cow) milk samples were positive for *T. gondii* DNA.

Serology: *T. gondii* antibodies were detected in 82% [82/100 (95% CI: 73.1-89)] of the examined pregnant women serum samples by HAI test. Anti-*T. gondii*-IgG were detected in 63% [63/100 (95% CI: 52.8-72.4)], while anti-*T. gondii*-IgM were found in 11% [11/100 (95% CI: 5.6-18.8)]of the examined serum samples. Both IgG and IgM were detected in 8% [8/100 (95% CI: 3.5-15.2)] of the samples.

Risk factors associated with *T. gondii* seroprevalence:

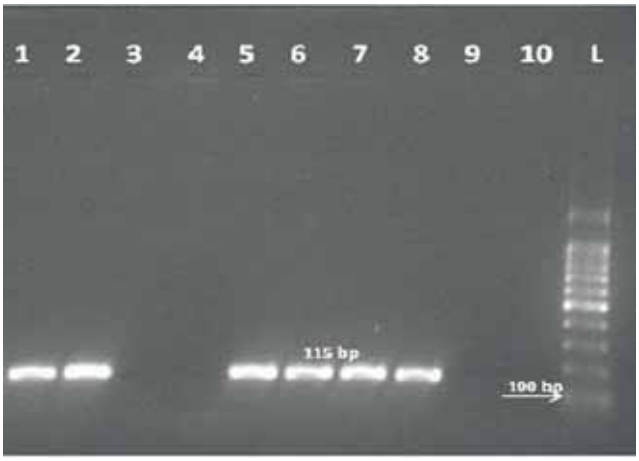

Figure-1: Agarose gel showing PCR results of *B1* gene amplification in the examined milk samples (L: 100 bp ladder, 1: Positive control, 2: positive sheep milk sample, 3, 4, 9: negative samples, 5-8: positive goat milk samples, 10: Negative control).

Table-1: Percent positivity of *T. gondii* DNA in animal milk samples as for detected by PCR.

Source	Number examined	Number positive [%(CI)]
Goat milk	50	4 [8% (95% CI: 2.2-19.2)]
Sheep milk	50	1 [2% (95% CI: 0.1-10.6)]
Cattle milk	50	0 [0 (95% CI: 0-16.8)]

Table-2: Factors associated with *T. gondii* seroprevalence in pregnant women

Characteristics	Seroprevalence		Bivariate		Multivariate	
	Positive n (%)	Negative n (%)	COR (95% CI)	P value	AOR (95% CI)	P value
Age						
31-35	40 (85.1)	7 (14.9)	1		1	
26-30	22 (81.5)	5 (18.5)	0.95 (0.31-2.99)	0.935	0.73(0.13-4.19)	0.722
21-25	20 (76.9)	6 (23.1)	0.65(.21-1.94)	0.436	0.15 (0.02-1.09)	0.061
Previous abortion						
Yes	10 (100)	0	4.04E+08 (0.0- .)	0.999	6.91E+08 (0.0-.)	0.998
No	72 (80)	18 (20)	1		1	
Presence of cats						
Yes	69 (84.1)	13 (15.6)	18.57 (5.27-65.44)*	< 0.000	39.33(6.49-238.22)	< 0.000
No	4 (22.2)	14 (77.8)	1		1	
Contact with soil						
Yes	60 (87)	9 (13)	2.73 (0.96-7.76)	0.060	2.46(0.62-9.83)	0.202
No	22 (71)	9 (29)	1		1	
Rearing goats						
Yes	62 (79.5)	16 (20.5)	.388 (0.08-1.83)	0.232	0.77(0.09-7.03)	0.818
No	20 (90.9)	2 (9.1)	1		1	
Rearing sheep						
Yes	23 (82.1)	5 (17.9)	1.01 (0.33-3.16)	0.982	0.55(0.10-3.02)	0.495
No	59 (81.9)	13 (18.1)	1		1	
Rearing cattle						
Yes	47 (83.9)	9 (16.1)	1.34 (0.48-3.73)	0.572	0.57(0.12-2.70)	0.476
No	35 (79.5)	9 (20.5)	1		1	
Consumption of raw milk and homemade cheese						
Yes	68 (87.2)	10 (12.8)	3.89(1.30-11.60)*	0.015	2.09(0.46-9.51)	0.339
No	14 (63.6)	8 (36.4)	1		1	

The number of pregnant women with IgM against *T. gondii* was too low to estimate the risk factors associated with acute infection separately, therefore, IgM and IgG positive results were compiled for the risk factors analysis. The age of pregnant women under investigation ranged from 21-35 years with a mean of 26.95 years (Table 2). There was insignificant increase of *T. gondii* seroprevalence pattern in association with age (p=0.7). A total of 10 (10%) of the examined women had a history of previous abortion which had no significant effect on the seroprevalence of *T. gondii* antibodies (p=0.998).

The presence of domestic cats in 82 (82%) of the studied households was recorded, of which, 69 (84.1%) were seropositive (Table-2). The association of *T. gondii* seroprevalence with the presence of domestic cats in households was significant (OR=18.57, 95% CI: 5.27-65.44, p< 0.05). After multivariate analysis, there was also significant correlation between the presence of cats and *T. gondii* seroprevalence (OR=39.33, 95% CI: 6.49-238.22, p< 0.05). About 69% of the participants reported contact with soil during gardening or farming activities, of which, 87% were *T. gondii* seropositive (Table-2), however, there was no significant association with contact with soil and *T. gondii* seroprevalence (p=0.2).

Rearing goats, sheep and cattle were reported in 78%, 28% and 56% of the examined households, respectively, of which, the respective seroprevalence rates of *T. gondii* antibodies were 79.5%, 82.1%, 83.9%. The association of rearing animals with *T. gondii* seroprevalence was insignificant (Table-2). Consumption of raw milk and homemade cheese was reported in 78% of the pregnant women under investigation, of which, 87.2% were seropositive (Table-2). The association of raw milk and homemade cheese consumption with *T. gondii* seroprevalence was significant (OR=3.89, 95% CI: 1.3-11.6, p< 0.05), however, after multivariate analysis, there was no significant association (p=0.3).

Discussion

Tachyzoites of *T. gondii* have been detected in

milk of several intermediate hosts such as sheep, goats, cows and camels [4]. An experimental study verified the transmission of *T. gondii* to milk and offspring of female rat orally infected with the parasite [15]. In addition, breast milk of a mother with a history of acquiring toxoplasmosis due to consumption of raw sheep meat was reported to infect her infant after breast feeding [16]. Moreover, a study in Sudan has suggested that a high seropositivity for *T. gondii* (67%) of pastoral nomads was correlated to the consumption of cameline raw milk [17].

Tachyzoites are easily destroyed by gastric enzymes; however, the role of ingestion in getting infection should not be ruled out [18]. Previous studies have reported that ingestion of tachyzoites in milk especially in infants resulted in *T. gondii* infection due to the low concentration of gastric enzymes [1]. Moreover, the survival of tachyzoites in acid pepsin solution for up to 2 hours may support the hypothesis of acquiring infection through consumption of milk and other food [18]. Another explanation to the possibility of acquiring infection by ingestion is that, in rare occasions, tachyzoites may penetrate the mucosal tissues, thus, they gain access to circulation before reaching the stomach [1, 19, 20].

The results of the present study revealed that *T. gondii* DNA was detected in 8% of the examined goat milk samples (Table-1). Although the scarce data on prevalence of *T. gondii* in milk; the obtained results were nearly similar to a reported 10% prevalence of the parasite DNA in goat milk samples in Iran [21]. Moreover, 13% and 6.05% of *T. gondii* DNA detection in goat milk samples were also documented in Italy [22] and Brazil [23], respectively.

Different studies have documented the correlation between human infection with *T. gondii* and the consumption of unpasteurized goat milk [19, 20, 24, 25, 26]. In Brazil, human infection with *T. gondii* due to consumption of goat milk was reported and tachyzoites excretion was detected in milk of naturally infected goats [25].

The survival of *T. gondii* tachyzoites in milk was experimentally studied by Walsh *et al.* [27] who reported that the tachyzoites can survive in goat milk stored at 4 °C for 3-7 days. Furthermore, the infectivity of *T.gondii* cysts was maintained in bovine milk even after storage for 20 days at refrigerator temperatures [28]. The aforementioned studies support the hypothesis of the possible human infection due to consumption of raw milk and also the probability of tachyzoite shedding in milk.

Sheep milk samples were also examined for *T. gondii* DNA during the present study, 2% of the examined samples were positive by PCR (Table-1). Likewise, prevalence rates of 3.4% and 5% of *T. gondii* in sheep milk were also obtained in Italy [29] and Brazil [7], respectively.

Although none of the examined cow milk samples were positive for *T. gondii* DNA in the present study, the risk of acquiring infection due to consumption of bovine milk cannot be excluded. In accordance, Dubey [30] reported that the risk of acquiring toxoplasmosis by drinking cow's milk is minimal. A possible explanation of such condition could be that cattle seem to be able to reduce the number or even eliminate the parasite cysts from their tissues [31].

The obtained results put emphasis on the probability that any type of milk is a potential source of infection if consumed raw [1]. Therefore, pasteurization or boiling of milk is essential to prevent the public health hazard caused by *T. gondii* tachyzoites. Although the detection of *T. gondii* DNA in milk does not ensure the viability of the parasite in the milk sample [7], the detection of *T. gondii* DNA has an implication of the possible shedding of the parasite in milk. Therefore, the potential transmission of the parasite through consumption of raw milk cannot be underestimated.

Tests utilized for the detection of IgG and IgM antibodies in human blood are the most common for diagnosis of toxoplasmosis, however, these tests can not differentiate precisely between recent and previous infection [4]. The detection of *Toxoplasma*-specific IgM antibodies suggests an acute possible maternal infection during pregnancy and indicates the need for therapeutic intervention, whereas, IgG seroprevalence of *T. gondii* indicates past infection or acquired immunity [32, 33].

The present study showed an overall 82% seroprevalence of anti-*T. gondii* antibodies in the examined pregnant women serum samples. Similarly, in Nigeria, an overall 83.6% seroprevalence of anti-*T. gondii* antibodies among pregnant women was reported [33]. A study that was carried out in the same geographical area (Sharkia, Egypt) revealed that anti-*T. gndii* IgG and IgM seroprevalence in pregnant women were 30% and 10%, respectively [34]. In Menoufia, Egypt, seroprevalence of *Toxoplasma*-specific IgG and IgM antibodies was determined by enzyme-linked fluorescent assay [32]. The results showed that 70.3% of the samples were seropositive, of which, seropositivity rates of 67.5% and 2.8% for IgG and IgM, respectively, were found [32]. Another study in Menoufia, Egypt, reported the seroprevalence of 27.3% of sera from pregnant women [35]. In Alexandria and Dakahlia, Egypt, studies have shown that IgG antibodies specific for *T. gondii* were reported in 46.2% [36] and 51.5% [37] of the examined pregnant women by ELISA, respectively. In Qualyobia, Egypt, ELISA results showed that 57.9% of pregnant women were seropositive for *T. gondii* antibodies, of which, 10.5% were positive for IgM [38]. Also, El-Gozamy *et al.* [39] reported 57.6% seroprevalence among pregnant women in Qualyobia, Egypt. A possible explanation of the high seroprevalence in the present study (82%) compared to the aforementioned studies could be the residence of all the participants in rural areas. Previous studies corroborate with this finding highlighting that the risk of acquiring *T. gondii* infection is significantly greater

among pregnant women living in rural settings [32, 40, 41]. The variation of *T. gondii* seroprevalence could be related to the use of different serological tests, geographic location, socioeconomic conditions, eating and hygienic habits [32].The seroprevalence of IgG in the examined samples during the present study (63%) was nearly similar to 62.2% in Lebanon [42], 58.5% in Brazil [43] and 69.5% in Turkey [44].

Different possible risk factors that could be associated with seroprevalence of anti-*T. gondii* antibodies were evaluated in pregnant women during the current study. The increase in the age of pregnant women has been reported to be associated with *T. gondii* infection [11, 33, 43]. Although insignificant, the increase in seropositivity to anti- *T. gondii* antibodies with the increase of age was observed in the present study (Table 2). Similar results were previously reported in Brazil [11] and Ethiopia [33]explaining such correlation to the increased frequency of exposure to infection sources with age. In relation to the history of abortion, there was insignificant association between previous abortions and seropositivity for toxoplasmosis (Table-2). Likewise, a study in Brazil reported the same finding [43].

The obtained results revealed a significant association between *T. gondii* seroprevalence and the presence of domestic cats in houses (Table-2). This finding is in accordance with other results reported in Ethiopia [33, 45] and Brazil [43]. However, in contrast to the obtained results, El-Deeb *et al.*[32] found no significant correlation between seropositivity to anti-*T. gondii* antibodies and contact with domestic cats in Menoufia, Egypt. This discrepancy could be attributed to the abundance of domestic stray cats in the study area rather than pet cats. Domestic stray cats are reported to be more exposed to the parasite than pet cats [33, 46]. In Egypt, *T. gondii* infection of 97.4% feral cats was reported indicating the role of domestic cats in the dissemination of oocysts in the environment [32, 47].

Contact with soil (gardening or working in fields) has an insignificant effect on the seroprevalence of *T. gondii* in the participants of the current study (Table-2). This finding corroborates with other studies in USA [48], Ethiopia [33] and Brazil [11, 43]. However, the obtained findingwas inconsistent with El-Deeb *et al.*[32] who reported a correlation between contact with soil and *T. gondii* seroprevalence.

Significant association between the seroprevalence of *T. gondii* and contact with farm animals was reported previously in Egypt [32]. In contrast, the present study showed no significant correlation (Table-2).

Consumption of raw milk has been reported as an important source of acquiring *T. gondii* infection[7]. Similarly, El-Deeb *et al.*[32] in Menoufia, Egypt, reported significant correlation between raw milk consumption and *T. gondii* seroprevalence. In contrary, the current finding was inconsistent with other studies in USA [48], Ethiopia [45], Brazil [43] and Kyrgyz Republic [49] that reported no significant correlation between raw milk consumption and *T. gondii* seroprevalence. This could be attributed to the type of consumed milk and the habit of using raw milk for the manufacture of homemade cheese in the current study.

Egyptian rural areas are known to have the habit of eating homemade cheese (Kareish) which is made from raw milk. Such habit has been shown to be pertinent acquisition of *T. gondii* and other microbial infection [32]. In addition, previous studies have shown that local homemade cheese from raw milk is considered a risk factor for public health [29]. This was supported by an experimental study conducted by Hiramoto *et al.* [28] who found that *T. gondii* cysts were able to survive the production process of homemade fresh cheese and storage for a period of 10 days at refrigeration temperature.

Conclusion

The present study reported the detection of *T. gondii* DNA in milk of goat and sheep in rural settings of Sharkia, Egypt. The study revealed also a high seroprevalence of *T. gondii* antibodies in pregnant women of the same rural residence. The asso-ciated risk factors have been presented, highlighting the significant correlation between the seroprevalence and the contact with cats and consumption of raw milk and homemade cheese. Data presented herein recommend the need for a further large scale study to clarify the association of *T. gondii* seroprevalence with different risk factors for more reliable conclusions. Also, screening for *T. gondii* infection during pregnancy should be considered to prevent and minimize congenital toxoplasmosis.

Authors' contributions

HAA, SMS, MEMA and STE: Designed the study and shared in the practical part on milk samples. HAA and AAA collected and examined human serum samples. HAA drafted and revised the manuscript. All authors read and approved the final manuscript

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Competing interests

The authors declare that they have no competing interests.

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