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Research Article
Immunological, Histopathological, Molecular Identification and Genotyping of *Toxoplasma gondii* in Small Ruminants in Egypt

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Abstract

Background and Objective: Toxoplasmosis is an infective zoonotic disease caused by protozoan *Toxoplasma gondii* (*T. gondii*). Molecular identification of *T. gondii* followed by studying the hereditary variety range of *T. gondii* isolates in Egypt was investigated. **Materials and Methods:** Blood samples were acquired from 138 live ewes and 212 she-goats from 5 governorates of Egypt, also the blood and its related tissue samples (uterus, diaphragm, heart and thigh muscles from each animal) were collected from slaughtered 180 ewes and 206 she-goats from Cairo and Giza abattoirs. **Results:** Using ELISA, the total seropositivity of live ewes and she-goats was 26.8 and 21.2%, respectively, while it was 16.6 and 33% in slaughtered ewes and she-goats, respectively. *T. gondii* tissue cysts with the associated characteristic histopathological changes were detected in different organs. Twenty-eight *T. gondii* isolates were confirmed using PCR, while among 24 milk samples from seropositive live ewes and she-goats, only 12.5 and 6.25%, were positive using PCR, respectively. Genotyping using multiple nested PCR (n-PCR) combined with restriction enzyme analysis (RFLP) of the surface antigen 2 (SAG2) gene confirmed 26 isolates (92.8%) as type II and 2 (7.1%) as type III. **Conclusion:** Type II and III are the most common *T. gondii* genotypes in Egyptian small ruminants with additional importance for public health in Egypt. Further studies are needed on the role of milk in the transmission of toxoplasmosis.

Key words: *Toxoplasma gondii*, small ruminants, ELISA, histopathology, PCR, Genotyping, oocysts

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Toxoplasmosis is a disease caused by *Toxoplasma gondii* which is obligate intracellular protozoa that infect the uterus especially the placenta causing placentitis, abortion, fetal death or premature birth and miscarriage with the high probability of cysts formation in the meat of affected animals, especially small ruminants[1,2].

Half of the world's public is affected by *T. gondii*[3]. In Egypt, many reports recorded high seroprevalence of *T. gondii* in different governorates[4,5]. All warm-blooded mammals including, humans and birds, act as intermediate hosts of the parasite with dispersed tissue cyst while felines serve as definitive hosts[1].

Several methods are applied for the diagnosis of toxoplasmosis but the molecular methods are the main ones for *T. gondii* identification with superior sensitivity and accuracy than the ordinary screening approaches[6]. Strains of *T. gondii* have been categorized into 3 genetic varieties according to their virulence influence on mice which is reflected in the formation of meat tissue cysts and environmental oocysts, lethality to mice and zoonotic value[7].

Therefore, this study aimed to evaluate the seroprevalence and diagnosis of toxoplasmosis in small ruminants including isolation, histopathological examination, molecular confirmation and investigating the genotypes circulating in small ruminants in Egypt.

MATERIAL AND METHODS

Study area: The study was carried out at the Departments of Animal Reproduction and Zoonotic Diseases, National Research Center, Egypt from September, 2020-May, 2021.

Samples: Blood samples were obtained from 5 governorates of Egypt (Giza, EL-Beheira, Qalioubia, Bani Sweif and Kafr EL-Sheikh). The history of each examined case was documented which include the age of the examined animal, cases of abortion and other reproductive disorders. Blood samples were gathered from 138 ewes and 212 she-goats. Sera were get separated and preserved at -20°C until explored immunologically for brucellosis and toxoplasmosis (Table 1).

Milk samples were collected from 100 ewes and 120 she-goats and rapidly frozen at -20°C until used for PCR assay. Moreover, Aborted fetuses (liver, kidney and lung) were collected from a single case of late-stage aborted goat fetuses from Kafr EL-Sheikh governorates and fixed in neutral buffered formalin 10% for histopathological examination.

Table 1: Blood samples collected from live and slaughtered ewes and she-goats

Animals	Ewes	She-goats	Total
Live			
Clinically affected	14	32	46
Healthy	124	180	304
Total	138	212	350
Slaughtered	180	206	386
Overall total	318	418	736

The blood and its corresponding tissue samples (heart, diaphragm, uterus and thigh muscles) were collected from 180 ewes and 206 she-goats from selected abattoirs at Cairo and Giza governorates of Egypt (Table 1). Tissue samples were divided into 2 parts: First was preserved in ice for parasite isolation and the second one was fixed in neutral buffered formalin 10% for histopathological examination.

Enzyme-linked immunosorbent assay (ELISA): Sera were screened using sheep and goat *T. gondii* antibody ELISA Kit (Cat. No: CK-bio-20329 and 20330, Shanghai Biotech Co., China). Positive/Negative Controls or samples were tested as recommended by the manufacturer instructions. Readings were measured at 450 nm. Animals that demonstrated positive ELISA, their tissue samples were additionally tested (microscopic examination and mice inoculation).

Viability test: Twenty grams of the equal sheep and goat tissue samples from the diaphragm, heart, uterus and thigh muscles were collected from selected abattoirs in Egypt. Joint samples were presented for pepsin digestion. Digested samples were microscopically examined then the residue samples containing bradyzoites like protozoa were inoculated intraperitoneally (1 mL/mouse) into two seronegative Swiss Webster Albino mice. The injected mice were tracked daily for clinical evidence or death. If as cites have taken place, peritoneal exudates were collected within 72-84 hrs post-infection and microscopical inspection for tachyzoites was finished[8].

Histopathological examination: The fixed specimens were washed, dehydrated, embedded in paraffin wax, sectioned at 4-5 µm thickness and stained with hematoxylin and eosin (H and E) to observe histopathological changes in liver and lungs of aborted fetuses and uterus of slaughtered ewes. The entire slides were also investigated for *T. gondii* tissue cysts, including bradyzoites and tachyzoites[9].

Polymerase chain reaction (PCR): *T. gondii* isolates DNA was extracted using GF-1 Tissue DNA extraction kit (GF-TD-050, Vivantis, Malaysia) according to the company instructions.

Only 24 milk samples from serologically positive 16 lactating she-goats and 8 ewes were tested with PCR assay. Frozen milk samples (50 mL each) were thawed at room temperature then centrifuged at 2200 rpm for 5 min. Further 1 mL of the sediment was distributed in 200 µL TE (pH 7.6) and 300 µL of 0.5 M EDTA (pH 8.0) and centrifuged at 3000 rpm for 10 min to avoid interference with casein. Thus, the milk pellet was diluted in 200 µL of PBS and the extraction of DNA was done using the same kit. Then DNA was stored at -20°C until use. The extracted DNA was amplified by PCR targeting B_1 gene[10] with an expected PCR product of 96 bp.

The reaction was applied in 25 µL reaction volume containing 12.5 µL of 2x COSMO PCR RED Master Mix (Cat. W1020300X, Willofort Co., UK) 1 µL (0.1 mM) of each primer, 9.5 µL of DDW and 1 µL of the purified DNA. The reaction conditions consisted of one cycle of 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 55°C for 30 sec, 72°C for 45 sec and the final extension at 72°C for 10 min (GS-96 gradient thermocycler, hercuvan, Malaysia). The amplification products were analyzed by 1.5% agarose gel electrophoresis stained with ViSafe Red Gel Stain (Cat. No. SD0103, Vivantis Co., Malaysia). PCR products with positive control (*T. gondii* DNA), negative control (DDW) and GeneRuler 100 bp DNA ladder were electrophoresed at 100 volts and examined using InGenius[3] gel documentation system (Syngene, UK).

Genotype analysis: Equivalent capacities (2 µL) of 28 DNA specimens were examined at the SAG2 site by multiple nested-PCR tactics that exaggerated the 5′ and 3′ ends of the site[11]. Primers individually intensify the 5′ and 3′ ends of the *T. gondii* SAG2 gene. The amplified parts were cleaned using GeneJET™ Gel Extraction Kit (K0691, Thermo Fisher, USA) then digestion of the 3′ end amplicons with HhaI enzyme (RE-1224, Vivantis, Malaysia) distinguished type II strains and digestion of the 5′ end amplicons using Sau3AI enzyme (RV1192, Vivantis, Malaysia) identified type III strains.

RESULTS

Enzyme-linked immunosorbent assay (ELISA): The seroprevalence of toxoplasmosis in she-goats was 51.6, 13.3 and 3.2% in Giza, El Beheira and Kafr El-Sheikh governorates of Egypt, respectively. While, in ewes was 34.0, 26.3 and 21.4% in Qalioubia, Kafr EL-Sheikh and Bani Sweif, respectively. 16 out of 32 clinically affected she-goats (50%) and 8 out of 14 clinically affected ewes (57.1%) were seropositive for toxoplasmosis (Table 2).

Concerning the reproductive performance, 24 Toxoplasma seropositive she-goats and ewes were clinically suffered from repeated abortion, abortion at 3 months of gestation, weak neonate, death after one week, stillbirth and infertility in the percentage of 16.7, 29.2, 8.3, 4.2, 8.3 and 33.3%, respectively.

Thirty-three and 16.6% of slaughtered she-goats and ewes from Cairo and Giza abattoirs were seropositive for toxoplasmosis,respectively, as shown in Table 2. It was noticed that prevalence of infection with toxoplasmosis is associated with age where ewes and she-goats more than 2 years had a higher rate of infection compared to other ages, Table 3.

Viability Test: The percentage values of microscopic examination were 26.4% of she-goats and 66.6% of ewes and positive mice viability values were 17.6% in she-goats and 53.3% in ewes Fig. 1 and Table 4.

Table 2: Prevalence of toxoplasmosis using ELISA in live and slaughtered ewes and she-goats

Animals	No. of ewes	No. (%) of positive ELISA ewes	No. of she-goats	No. (%) of positive ELISA she-goats	Total (%)
Live					
Clinically affected	14	8 (57.1%)	32	16 (50%)	24 (52.2%)
Apparently healthy	124	29 (23.4%)	180	29 (16.1%)	58 (19.1%)
Total	138	37 (26.8%)	212	45 (21.2%)	82 (23.4%)
Slaughtered	180	30 (16.6%)	206	68 (33%)	98 (25.3%)
Overall total	318	67 (21%)	418	113 (27%)	180 (24.4%)

Table 3: Prevalence of toxoplasmosis using ELISA in live ewes and she-goats according to age

Age (Year)	No. of ewes	No. of positive ELISA ewes (%)	No. of she-goats	No. of positive ELISA she-goats (%)
<1	22	2 (9%)	40	1 (2.5%)
1-2	38	7 (18.4%)	60	9 (15%)
>2	78	28 (35.8%)	112	35 (31.2%)
Total	138	37 (26.8%)	212	45 (21.2%)

Table 4: Viability test of positive ELISA slaughtered ewes and she-goats.

Species	No. of tissue collected from positive ELISA animals	Viability test	
		No. of positive Microscopically examined digested tissue (%)	No. of positive mice (%)
Ewe	30	20 (66.6%)	16 (53.3%)
She-goat	68	18 (26.4%)	12 (17.6%)
Total	98	38 (38.8)	28 (28.6%)

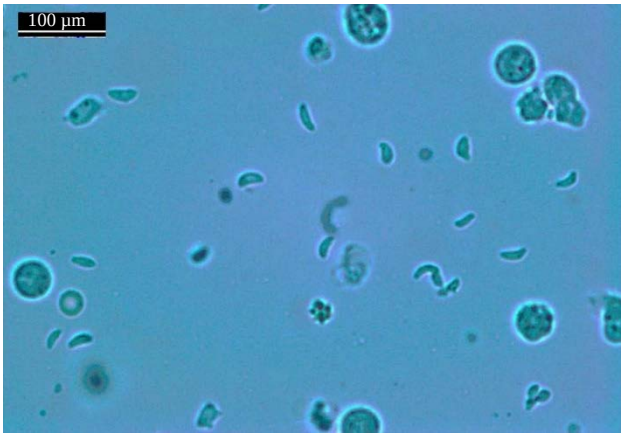

Fig. 1: Stages of tachyzoites represented by a slandered shape, elongated tachyzoites with flat shape and aggregations of many tachyzoites

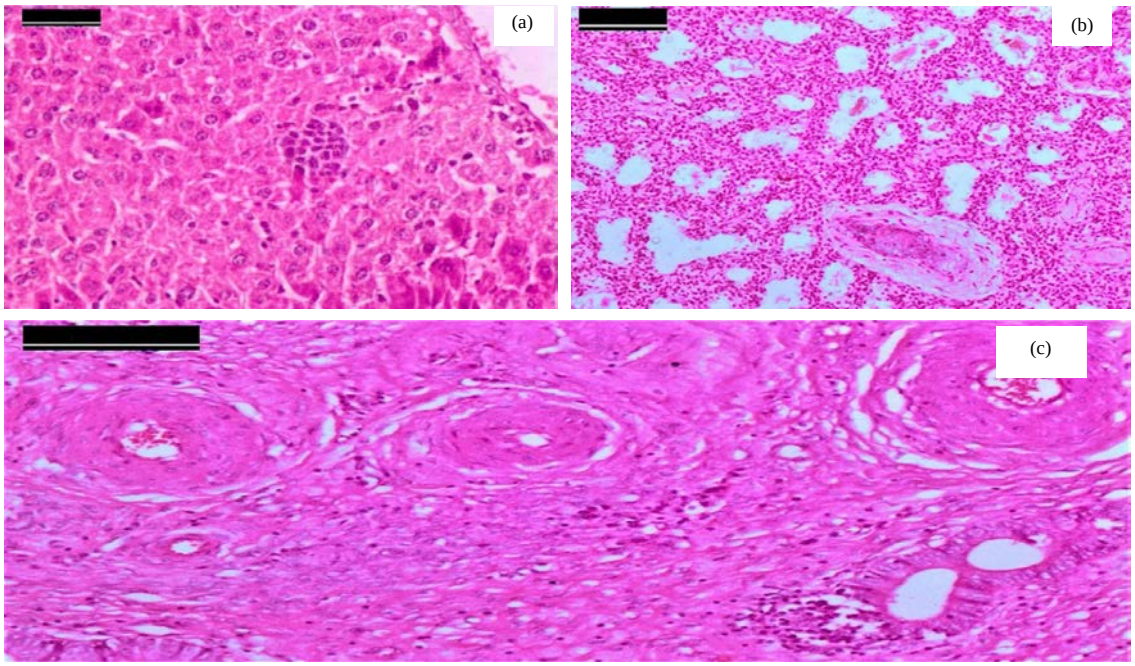

Fig. 2 (a-c): Histopathological picture of the liver, lung and uterus, (a) Liver of aborted goat fetus showing tissue cyst of toxoplasma bradyzoites (H and E, ×100), (b) Lung of aborted goat fetus showing perivascular fibrous connective tissue proliferation with severe thickening of the alveolar wall and diffuse inflammatory cells infiltration (H and E, ×100) and (c) Uterus of ewe showing congestion of uterine blood vessels with perivascular fibrous connective tissue proliferation and focal inflammatory cell infiltration (H and E, ×100)

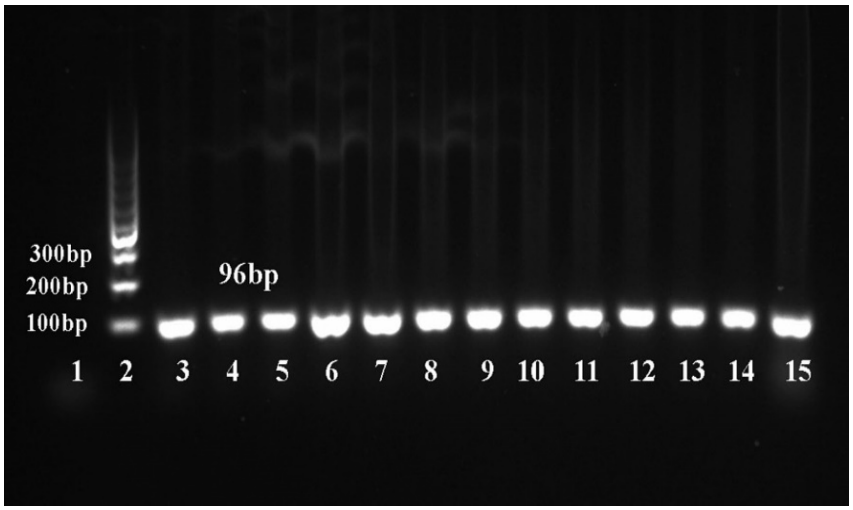

Fig. 3: PCR amplification of *T. gondii* B_1 gene, an agarose gel electrophoresis showing PCR amplification product of toxoplasma
Lane 1: Negative control, Lane 2: 100bp Ladder, Lane 3: Positive control and Lane 4-15: Representative toxoplasma positive samples

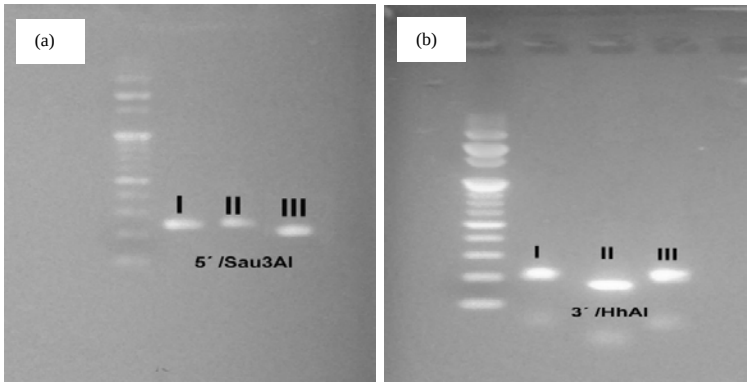

Fig. 4 (a-b): SAG2 nested PCR analysis, (a) Sau3AI restriction analysis of the 5′ amplification products from type I (RH), II and III strains and (b) HhaI restriction analysis of the 3′ amplification products from type I, II, and III strains

Histopathological examination: The histopathological picture of the liver of an aborted goat fetus showed a tissue cyst of toxoplasma bradyzoites (Fig. 2a). Some other liver samples showed degeneration of hepatic cells with diffuse inflammatory cells infiltration and congestion of hepatic blood vessels. The lung of the aborted goat fetus shows severe thickening of the alveolar wall with diffuse inflammatory cells infiltration and accumulation of inflammatory exudate in its lumen (Fig. 2b). The uterus of slaughtered ewes shows congestion of uterine blood vessels with perivascular fibrous connective tissue proliferation and focal inflammatory cell infiltration (Fig. 2c).

Polymerase chain reaction (PCR): PCR was applied as a confirmatory test on 28 *T. gondii* isolates and affirmed 16 ewes and 12 she-goats isolates as positive with PCR product (96 bp) as shown in Fig. 3.

Toxoplasma gondii DNA was detected in 12.5% (1/8) of sheep and 6.25% (1/16) of goat's milk samples.

Genotype analysis: Digestion of the 5′ amplification products of reference isolates with Sau3AI identified allele 3 (type III strains) from alleles 1 and 2 (type I and II strains) (Fig. 4a) and digestion of the 3′ amplification products of reference isolates with HhaI recognized allele 2 (type II strains) from alleles 1 and 3 (type I and III strains) (Fig. 4b).

In this investigation, using the PCR-RFLP technique, out of 16 ovine toxoplasma isolates, 14 (87.5%) samples were typed as type II and 2 (12.5%) as type III. Also, all the 12 caprine toxoplasma isolates (100%) were labelled as type II. RH isolate (type I) was used as a control as it is undigested by both restriction enzymes.

DISCUSSION

Using ELISA, the seroprevalence of toxoplasmosis in she-goats in different governorates of Egypt was 51.6, 13.3 and 3.2% in Giza, El-Beheira and Kafr El-Sheikh, respectively while in ewes, it was 34.0, 26.3 and 21.4% in Qalioubia, Kafr EL-Sheikh and Bani Sweif, respectively. Such seroprevalence variability may be due to production systems, herd or flock size, farm management, intensive farming, temperatures, mean rainfall and humidity maintaining higher soil moisture levels which allow survival of oocysts in the environment[12,13].

From our study, using ELISA, a toxoplasma seroprevalence of 26.8% was recorded in live ewes in different governorates of Egypt (Table 2). This was parallel to Sanad and Al-Ghabban[14] from Saudi Arabia (23.4%) but lower than Hassanain *et al.*[15] (61.4%) and Abd El-Razik *et al.*[5] (52.4%) in Egypt, Mor and Arslan[16] in Turkey (95.7%) and Romanelli *et al.*[17] in Brazil (51.5%). However, it was upper to that of Alazemi[18] in Kuwait (17.8%), Van Helden and Hammond-Aryee *et al.*[19] in South Africa (8%) and Gharbi *et al.*[20] in Tunisia (1.8%).

Regarding live she-goats, total seropositivity of 21.2% was noted (Table 2). This was parallel to that of Mahmoudvand *et al.*[21] in Iran (21.5%) and higher than that of Issa[22] in Iraq (13.04%) and Fengcai *et al.*[23] in China (17.6%) and lower than that of Younis *et al.*[12] (50.6%) and Abd El-Razik *et al.*[5] in Egypt (46.6%).

Hereby, the seroprevalence recorded in live ewes (26.8%) was found to be relatively higher than that in live she-goats (21.2%). The same was reported by Sharif *et al.*[24] in Iran and Abd El-Razik *et al.*[5] in Egypt.

The differences in the prevalence percentage between sheep and goats perhaps as a result of the differences in exposure to *T. gondii* and the variations in feeding practices since sheep graze close to the ground than goats that prefer browsing[25,26].

Here, seropositivity of 33% slaughtered she-goats were recorded Table 2 which was parallel to Younis *et al.*[12] in Egypt (32%), Gebremedhin *et al.*[26] in Ethiopia (43%), but lower compared to that reported by Abd El-Razik *et al.*[5] in Egypt (39.2%) and García-Bocanegra *et al.*[27] in Bulgaria (59.8%).

Regarding slaughtered ewes in Egypt, a seroprevalence of 16.6% was noted in Table 2. This result was parallel to that of Gebremedhin *et al.*[26] in Ethiopia (20%) but higher than that of Derakhshan and Mousavi[28] in Iran (3.3%) and lower than that verified by Abd El-Razik *et al.*[5] in Egypt (47.1%). The differences in the prevalence percentage could be due to the diagnostic techniques used, cut-off value and sample size[29]. The high seroprevalence recorded in Egypt could be due to widespread stray cats, environmental pollution at each farm and extensive animal production systems as agreed by Abdel-Rahman *et al.*[30].

Hereby, it was noticed that the prevalence of toxoplasmosis is linked with age as a risk factor where ewes and she-goats more than 2 years had a higher rate of infection (31.2%) in comparison to other ages, as shown in Table 3. The older animals have more chance for frequent exposure to the infective stage of the parasite[31,32]. These results were considered by Issa[22] and Hossain *et al.*[33] whose recorded that older sheep and goats had a higher prevalence of toxoplasmosis than younger ones.

Currently, the history of clinically affected ewes and she-goats presented repeated abortion, abortion at 3 months of gestation, abortion at the late stage of gestation weak neonate, stillbirth and infertility in the percentage of 16.7, 29.2, 8.3, 4.2, 8.3 and 33.3% respectively. The abortion rate was higher than other reproductive diseases and this was agreed by AL-Hatami *et al.*[34] who recorded an abortion rate of 71.8 and 94.4% in sheep and goats, respectively.

The microscopic investigation was only accomplished on the digested tissue specimens of the ELISA positive slaughtered ewes and she-goats. The rate was 66.6% in ewes and 26.4% in she-goats (Table 4). This was upper to that of Abd El-Razik *et al.*[5] who confirmed a rate of 37.3 and 15% in tissue samples from diaphragm, heart and thigh muscles collected from slaughtered sheep and goats at abattoirs in Egypt.

Mouse bioassay is the gold standard method for *T. gondii* isolation as reported by Burrells *et al.*[35]. A viability test was just done on microscopically positive tissues containing bradyzoites like protozoa, where ewes and she-goats recorded 53.3 and 17.6%, respectively (Fig. 1) and (Table 4). These results agree with that of Gebremedhin *et al.*[36] who demonstrated 57.45% of *T. gondii* viable strains isolated from sheep. Here, the high level of isolation of tissue cysts reveals an extraordinary effect on public safety as it is an indication of the environmental contamination of feline oocysts and the open-air management system as reported by Gebremedhin *et al.*[36].

Regarding histopathological examination, the liver of the aborted goat fetus showed a tissue cyst of Toxoplasma bradyzoites (Fig. 2a). Some other liver samples showed degeneration of hepatic cells with diffuse inflammatory cells infiltration and congestion of hepatic blood vessels. In Egypt, Ahmed *et al.*[37] revealed irregular multiple necrotic foci scattered throughout the hepatic lobules, severe congestion of hepatic sinusoids and the portal areas were greatly infiltrated with lymphocytes. Also, Khanamir *et al.*[38] revealed that toxoplasmosis stages, especially tissue cyst, were presented and distributed through the liver.

Lung revealed severe thickening of the alveolar wall with diffuse inflammatory cells infiltration and accumulation of inflammatory exudate in its lumen (Fig. 2b). Moreover, the uterus of slaughtered ewes showed congestion of uterine blood vessels with perivascular fibrous connective tissue proliferation and focal inflammatory cell infiltration (Fig. 2c). Ahmed *et al.*[37] previously confirmed that the demonstration of the tissue parasites with the characteristic histopathological images in the placenta, liver, lung and brain of the aborted fetus is important for the diagnosis of ovine toxoplasmosis.

PCR assay is the main method for *T. gondii* DNA identification with greater sensitivity and precision than routine methods [6]. Among PCR measures for the identification of *T. gondii* DNA, the B_1 gene is the most commonly used[5].

Utilizing PCR as a confirmatory test, all the 28 suspected isolates were affirmed as *T. gondii* (16 and 12 from slaughtered ewes and she-goat, respectively) with PCR product (96 bp) relying upon B_1 gene amplification (Fig. 3). where PCR results completely confirmed the results of the viability test and this was parallel to that of Abd El-Razik *et al.*[5]. This similarity may return to the direct extraction of genomic DNA from *T. gondii* isolates which was isolated from the peritoneal fluid of mice.

Regarding PCR on milk, There are several reports of detection of *T. gondii* DNA in the milk of small ruminants[5,39,40] which increases the probability of transmission of *T. gondii* through intake of raw milk and its non-pasteurized derivatives which are strong concerned a public health risk factor.

In this study, *T. gondii* DNA was found in milk samples obtained from positive ELISA live ewes and she-goats in a percentage of 12.5 and 6.25%, respectively. The obtained she-goats results (6.25%) were nearly similar to Bezerra *et al.* [41] in Brazil (6.05%) and Ahmed *et al.* [42] in Egypt (8%) but lower than Sadek *et al.*[39] in Egypt (22.73%) and higher than Tavassoli *et al.*[43] in Iran (1.07%).

On the other side, the obtained ewes PCR results (12.5%) were parallel to Sadek *et al.*[39] who reported 10.71% of Toxoplasma DNA detection in sheep milk samples in Egypt, higher than Fusco *et al.*[44] and Ahmed *et al.*[42] who reported 3.4% and 2% of sheep milk samples in Italy and Egypt, respectively.

Many serologically positive animals had negative milk PCR results i.e. didn't excrete the parasite in their milk as the elimination depends on the animal infection phase and their immunity[39,41,45]. Gazzonis *et al.*[46], found tachyzoites in the milk of infected goats during acute infection and become a potential cause of human infection.

Genotyping has the main role in studies on the population biology, epidemiological studies and in the identification of the source of infection of *T. gondii*[47]. From a clinical point of view, the possible association between genotyping and disease pattern may also be important in the creation of new methods for diagnosis, prophylaxis and control of the disease in humans and animals[47]. *T. gondii* has a particularly peculiar population structure classified according to their virulence effect on mice into three genotypes (types I, II and III)[47].

Recently, various techniques were developed for genotyping of *T. gondii*[48]. The approach for the judgment to identify the *T. gondii* isolates genotypes was multilocus PCR-RFLP due to its easiness, pronounced sensitivity and implementation[49].

In this investigation, using nested PCR amplification of the SAG2 locus applied here (5 SAG2 and 3 SAG2), followed by RFLP revealed that all 12 samples of she-goats and 14 samples of ewes 26 (92.8%) were keyed as type II while only 2 samples of ewes (7.1%) as type III utilizing PCR-RFLP examination. In the current study, type II strains were predominant (92.8%) and these results agreed with others[50-54] in Italy and Brazil, respectively and in Egypt agreed with that of Abd El-Razik *et al.*[5], Abdel-Hameed and Hassanein[55], Elfadaly *et al.* [56]. Type II superiority may be due to its fitness and improved ability to compete with else genotypes, along with its ability to form large numbers of cysts[56]. In small ruminants worldwide, the type III clonal lineage is sometimes found[5,57].

From our study, type II and III are generally the supreme genotypes that mostly circulate in Egypt among sheep and goats and this agreed with that of Abd El-Razik *et al.*[5] and Elfadaly *et al.*[56], This shows that the vast number of infected stray cats in farms with bad management situations are continuously exposed to sheep and goats to *T. gondii* infection due to significant ecological contamination with oocysts shed[30]. In Egypt, the prevention and control of toxoplasmosis can be sophisticated through governing the cats' population, thus preventing the spread of oocysts in the environment.

CONCLUSION

Our results confirmed the high prevalence of toxoplasmosis in small ruminants in Egypt. The presence of *T. gondii* in the milk of ewes and she-goats raises the possibility of the spread of infection. Molecular techniques proved to be sensitive and specific for early diagnosis of toxoplasmosis from different animal samples. Genetic characterization showed *T. gondii* genotype II as the most prevalent genotype with zoonotic importance in Egypt.

SIGNIFICANCE STATEMENT

This study ascertains the opportunity and effects of the PCR technique to fast and precise detection of toxoplasmosis in small ruminants. This study will help the researcher to understand the role of milk in the transportation of infection. Moreover, the obtained data showed that the circulating *T. gondii* genotypes in small ruminants are that of public health importance.

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