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Comparison Between Nested-PCR and ELISA for the Detection of *Toxoplasma gondii* in Blood and Milk and its Genotyping in Lactating Goats and Aborted Women in Iraq

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ABSTRACT

The present study aimed to assess enzyme-linked immunosorbent assay (ELISA) and nested-polymerase chain reaction (n-PCR) methods based on *B1* gene for the detection of *Toxoplasma* (*T.*) *gondii* in the blood and milk of local Iraqi goats. The *SAG3* gene was also used to identify the genotyping of *T. gondii* in goats and aborted women in Iraq. A total of 240 (80 blood, 80 sera, 80 milk) lactating goats and 30 blood samples from aborted women were included in this study. A total of 17 (21.2%) infected goats were found in blood samples and 23 (28.7%) in milk samples when using n-PCR, while the numbers were 23 (28.7%) and 17 (21.2%) when using ELISA. Aborted women had an overall infection rate of 50% when using ELISA and 33% when using n-PCR. The degree of agreement between n-PCR in milk and blood was almost perfect (Kappa=0.801), with a sensitivity of 100 and a specificity of 90.5, while there was a slight degree of agreement (Kappa=0.14) between n-PCR and ELISA in blood, with 58.8 sensitivity and 74.6 specificity. The results of the comparison between n-PCR in blood and ELISA in milk showed positive samples of 17 (21.2%) for each, with 82.4 sensitivity and 22.2 specificity, and no agreement (Kappa=–0.046). Sequencing of the *SAG3* gene of *T. gondii* from goat and human isolates showed that the similarity ranged from 98.65–99.90% for genotypes I and III. In conclusion, n-PCR may be more accurate than ELISA for detecting *T. gondii* in blood and milk. In addition, the phylogenetic tree's evidence of a high degree of similarity between human and goat isolates provides further evidence that goats are an important reservoir of *T. gondii* and that public awareness is necessary.

Keywords: *Toxoplasma gondii*, *SAG3*, *B1* gene, ELISA, nPCR, woman, abortion, Iraq

INTRODUCTION

Toxoplasmosis is caused by the opportunistic intracellular protozoan *Toxoplasma* (*T.*) *gondii*, a member of the phylum Apicomplexa (1). Cats are the main host despite the parasite can infect any warm-blooded mammals; including humans, as a result, it is found across the world and may cause both acute and chronic toxoplasmosis (2). Around a third of the world's population is impacted by the parasite (3). The infection can be contracted by ingesting tissue cysts from infected intermediate hosts' meat or oocysts from cat feces through polluted water or food (4).

Goat and sheep toxoplasmosis is important because it causes significant economic and production losses and can be transmitted to humans (5). Because of their higher

activity and mobility, goats are more likely to come into contact with polluted sources, making them more susceptible to toxoplasmosis than sheep (6).

The acute phase in immunocompetent individuals causes transient low or mild symptoms that are unrecognized. *T. gondii* has been a life-threatening opportunistic infection that could result from the reactivation of silent infection or primary infection in immunocompromised people (7). Furthermore, congenital toxoplasmosis occurs when tachyzoites migrate via the placenta into the baby during a pregnant primary infection, which could lead to miscarriage, stillbirth, ocular, or neurologic illness, and neurocognitive defect in the newborns (7). Goats infected with toxoplasmosis are a major source of human infection due to the consumption of meat and milk from infected animals (8). Goats excrete tachyzoites in their milk (9, 10) and are resistant to processing in fresh cheese (11).

Many diagnostic methods for *T. gondii* were applied, such as serological detection by enzyme-linked immunosorbent assay (ELISA) and latex agglutination test (LAT), along with cell line culture, bioassays, and molecular techniques (12). Amplification-based assays, including polymerase chain reaction (PCR), nested PCR (n-PCR), and real-time PCR, have been widely developed for quick, definite, and accurate detection of toxoplasmosis among various detection techniques (13, 14). Among the sequences examined were SAG1, SAG2C, SAG2D, SAG2X, SRS52A, SAG3, MIC1, MICA2P, GRA5, GRA6, and GRA3 for HLA-A02 specific peptide binding, SAG1, SAG2C, GRA5, GRA6, and GRA7 for HLA-A03 specific peptide binding, and GRA3 and GRA7 for HLA-B07 specific peptide binding (15).

The current study aimed to compare serological and molecular methods for detecting *T. gondii* in blood and milk in humans and goats. Genetic analysis of the SAG3 gene was also used to identify the genetic sequences of *T. gondii*.

MATERIALS AND METHODS

Ethics Statement

All procedures used in this study were reviewed and approved by The Scientific Committee of the Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, and the Ethics Committee of the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq in compliance with the ethical principles of animal welfare.

Animals and Sample Collection

The research was conducted at the Ruminant Research Station of the General Authority for Agricultural Research, Ministry of Agriculture, Baghdad, Iraq (location 1) and at AL-Dibuni Research Station for Research, Wasit, Iraq (location 2). Between 2021-2022, a total of 80 (40 from each location) adult lactating local breed goats were included in the study.

Samples collection

A total of 240 samples (80 blood, 80 sera, and 80 milk) from 80 goats were collected and subjected to the ELISA test and n-PCR. Eighty lactating goats (two tubes) Blood sample (10 mL) was drawn from the jugular vein using disposable needles and Vacutainer tubes (gel tubes) and then brought to the laboratory in a cooler box. Serum was extracted from blood samples by centrifugation at 2000 g for 10 min and kept at -20 °C in labeled Eppendorf tubes for ELISA. Blood samples for the molecular detection were also obtained from the jugular vein using a medical syringe with a capacity of 10 mL (Vacum Tube Needle) with EDTA, and the samples were kept at -20 °C till the DNA extraction.

Milk samples were taken from 80 lactating goats using plastic tubes with a capacity of 100 mL for ELISA IgG (Toxo-IgG) using ELISA Kit for Goat *Toxoplasma gondii* (SunLong Biotech Co., LTD, China), and the milk samples were also used for DNA extraction.

Nested PCR

For the molecular detection, the DNA was extracted from blood of goats and humans and milk from lactating goats using G-spin™ Total DNA Extraction Kit (iNtRON, Korea). The n-PCR approach was utilized to identify the presence of *T. gondii* DNA in circulating blood after using ELISA. Serological screening was applied to investigate the possibility of an accurate diagnosis in aborted women. This technique was performed for the direct detection of *T. gondii*, based on the *B1* gene, from animal blood, milk, and human blood samples. This technique was conducted according to the method described by Halleyantoro et al. (16). The primers were provided by Scientific Researchers. Co. Ltd, Iraq (Table 1).

PCR for *SAG3* Gene

The PCR technique was performed to detect the SAG3 gene in Toxoplasma gondii in blood samples of humans and goat. SAG3 was used for DNA sequencing for genotyping analysis. This method was carried out according to the method described by Vitale et al. (17). Related NCBI-Blast related *T. gondii* clonal lineages genotype I, II, and III isolates were submitted into NCBI GenBank and identified by accession numbers.

Table 1. Primer sequences used in the PCR and n-PCRor for amplifying the *B1* gene of *Toxoplasma gondii*

Primer		5'-3' sequence	Product size
*B1**	**F**	GGAACTGCATCCGTTCATGAG	160
	R	GGCGACCAATCTGCGAATACACC	
*B1***	**F**	TGCATAGGTTGCAGTCACTG	131
	R	TCTTTAAAGCGTTCGTGGTC	

*PCR primer (first), **Nested primer (second)

Statistical Analysis

A comparison was made between the results to obtain sensitivity, specificity, and kappa coefficient. The kappa coefficient classification included: poor agreement = less

than 0.00, slight agreement = 0.00–0.20, fair agreement = 0.21–040, moderate agreement = 0.41–0.60, substantial agreement = 0.61–0.80, and almost perfect agreement = 0.80–1.00 (18).

RESULTS AND DISCUSSION

Total Infection Rate

The prevalence of *T. gondii* was 21.2% and 28.7% by n-PCR in blood and milk, respectively (Table 2). The kappa was 0.801, which indicated a high agreement between the two tests with a sensitivity of 100 and specificity of 90.5. Similar results were reported by (19) in which the *T. gondii* DNA was detected in seven milk samples (28%) and five blood samples (20%) of sheep out of 25 samples. The high degree of kappa is in line with a study that included 127 adult lactating goats from 6 farms in Italy and reported that 13% of blood and milk samples were positive for n-PCR with a kappa coefficient of 1 (4).

Table 2. Comparison between infection rates of *Toxoplasma gondii* in local lactating goats using n-PCR in milk and blood

Test	Blood n-PCR		
Milk n-PCR	Negative	Positive	Total n (%)
Negative	57	0	57 (71.3)
Positive	6	17	23 (28.7)
Total n (%)	63 (78.7)	17 (21.2%)	80
Weighted kappa			0.801
Sensitivity			100
Specificity			90.5

Out of 30 blood samples in aborted women, 15 (50%) and 10 (33.4%) were positive for ELISA and n-PCR, respectively. The high prevalence found by ELISA is consistent with the findings of (20), who used ELISA to analyze blood samples from women with a history of abortion and found that 148 (30.83%) were positive. In comparison, n-PCR detected 30 (20.27%) of the 148 positive samples. The high prevalence of aborted women in the present study may indicates that *T. gondii* infection could be contributed to abortion.

Table 3 illustrates that blood ELISA detected 23 (28.7%), while blood n-PCR detected only 17 (21.2%). The kappa coefficient of 0.14. indicated a slight agreement between the two tests, with a sensitivity of 58.82 and a specificity of 74.60. Higher prevalence was detected by serological assays (51.76%) compared with molecular diagnosis (17.65%) of the *T. gondii* in the blood of sheep, as reported by (21). The difference in prevalence detected by the n-PCR assay compared to ELISA in the blood may be due to *T. gondii* being more tenacious in tissues than in blood after infection. Consequently, the umbilical cord, heart, placenta, and brain are appropriate samples for PCR analysis (22, 23). As well as in processed meat in sheep using PCR (24). Camossi et al. (5) and Silva et al. (25) reported that the serology results sometimes differ from the PCR results.

Table 3. Comparison between infection rates of *Toxoplasma gondii* in local Lactating goats using ELISA and n-PCR in blood

Test	Blood n-PCR		
Serum ELISA	Negative	Positive	Total n (%)
Negative	47	10	57 (71.3)
Positive	16	7	23 (28.7)
Total n (%)	63 (78.7)	17 (21.2%)	80
Weighted kappa			0.140
Sensitivity			58.8
Specificity			74.6

Table 4. Comparison between infection rates of *Toxoplasma gondii* in local lactating goats using ELISA in milk and n-PCR in blood

Test	Blood n-PCR		
Milk ELISA	Negative	Positive	Total n (%)
Negative	49	14	63 (78.7)
Positive	14	3	17 (21.2)
Total n (%)	63 (78.7)	17 (21.2)	80
Weighted kappa			-0.046
Sensitivity			82.4
Specificity			22.2

Despite the positive cases of ELISA on milk and n-PCR on blood, the kappa coefficient (-0.046) indicated no agreement between tests with a sensitivity of 82.35 and specificity of 22.2 (Table 4). The results of this study are comparable to those of (26), who reported that the anti-*T. gondii* antibodies were present in the sheep's 41.5% (114/275). However, there was no association between milk parasite excretion and IgG presence. A low kappa value presented no agreement between seroprevalence and the prevalence of parasites in milk. The animals tested positive for milk in the PCR but negative in the serology might be in the early stages of the disease, with inadequate antibodies to be identified in the serology. Thus, the presence of *T. gondii* DNA in goat milk does not imply that the parasite is still alive (10). Because IgGs are detectable within 1-2 weeks of infection and reach their peak between 1-2 months later, they are present for the rest of one's life at levels that progressively drop (27). The results disagreed with those obtained by (28) in Northwest Tunisia, where ELISA reported a rate of 31.2%, while a rate of 7.8% by n-PCR. According to (29), the low incidence of *T. gondii* observed by qPCR is due to the parasite being limited to organs and tissues rather than being dispersed in circulation. The n-PCR test in blood has the advantage of identification of recent and active toxoplasmosis (30).

Nested PCR Product Analysis

The samples were obtained from aborted women blood and the milk of goats. Lanes 1-15 showed some *T. gondii* at 131 bp. Nested PCR product and N represent negative control samples based on the B1 gene (Figures 1 and 2).

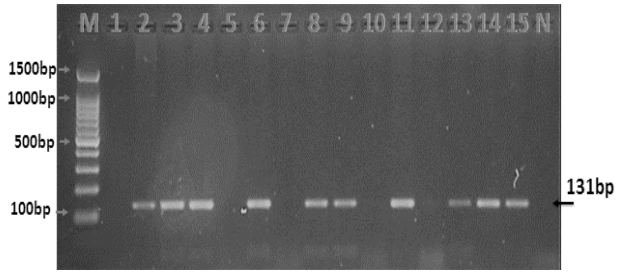

Figure 1. Agarose gel (1%) electrophoresis image shows the nested-PCR product analysis of the B1 gene of *Toxoplasma gondii* from aborted women blood samples. M: marker (1500-100 bp), Lanes (1-15)

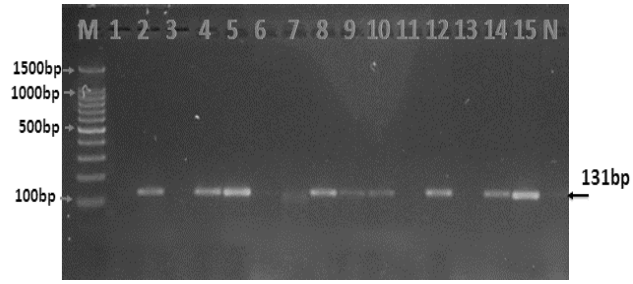

Figure 2. Agarose gel electrophoresis image shows the nested-PCR product analysis of the B1 gene of *Toxoplasma gondii* from milk samples. Where M: marker (1500-100bp), Lanes (1-15) Electrophoresis of a 180 bp fragment of E. coli O157:H7 stx1 gene as electrophoresed for 1 hour on ethidium bromide-stained agarose (2%) gel at 70 volt/cm2 and 1x TBE. Lane M: DNA ladder (100 bp). Amplification of PCR product was found

DNA Sequencing

The phylogenetic tree analysis showed that the local isolates of *T. gondii* in goats (Tg-IQ-Goat. 1, Tg-IQ-Goat. 2, and Tg-IQ-Goat. 4) and the *T. gondii* of human isolates (Tg-IQ-human. 3, Tg-IQ-human. 4, and Tg-IQ-human. 5) were close in the genetic relationship in genotype III (AF340229.1). Similarly, the *T. gondii* goats' isolates (Tg-IQ-Goat. 3 and Tg-IQ-Goat. 5) and the *T. gondii* human isolates (Tg-IQ-human. 1 and Tg-IQ-human. 2) were also found to be genetically close to genotype I (AF340227.1). Still, there was no similarity with *T. gondii* of genotype II (AF340228.1) at a total genetic change of 0.01, as shown in Figure 3. The homology sequence identity between *T.* III isolates showed genetic homology sequence identity ranging from (98.65-99.90%) (Table 5).

The present study's fin10dings regarding the prevalence of *T. gondii* in goats revealed the significance of controlling this disease. The high number of women in this study who had abortions shows that *T. gondii* infection is linked to abortion. The genetic homology sequence identity ranged from (98.65 to 99.90%) in goats and humans, indicating the parasites caused zoonotic disease and based on the current study's findings, the possibility of *Toxoplasma* transmission through the consumption of raw milk and an impact on public health.

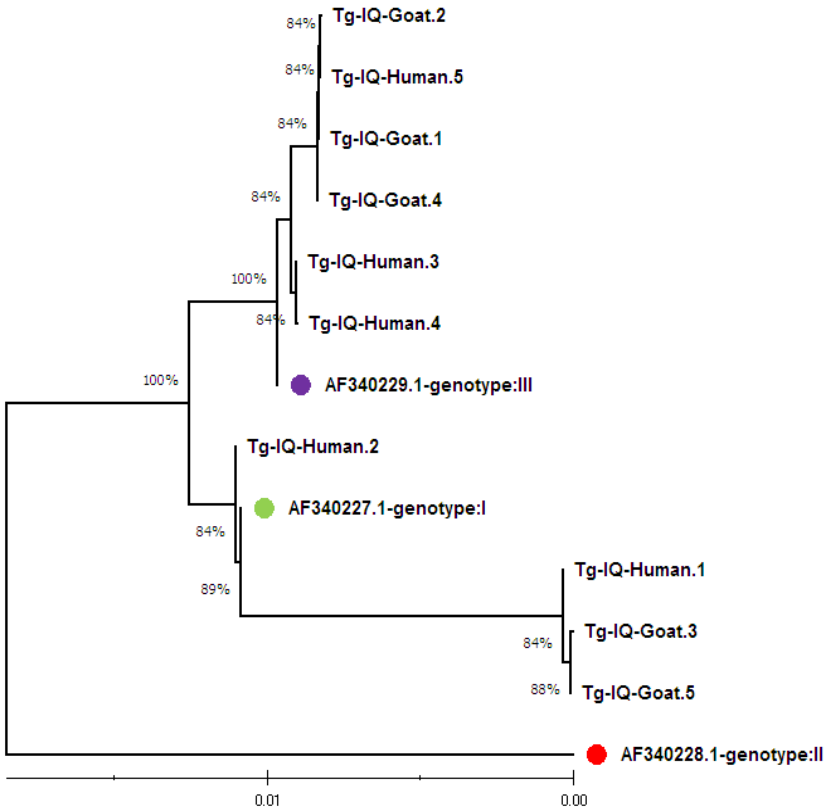

Figure 3. Phylogenetic tree analysis based on the complete sequence of surface antigen (SAG3) gene in T. gondii of local goats and human isolates used for genotyping analysis

Table 5. Homology sequence identity between *Toxoplasma gondii* isolated from local goats and humans isolates compared to *Toxoplasma gondii* genotypes deposited on NCBI BLAST

		Homology sequence identity		
***Toxoplasma gondii* isolates number**	**GenBank accession number**	**Genotype**	**GenBank accession number**	**Identity %**
Tg-IQ-Goat.1	OL792791	III	AF340229.1	99.90
Tg-IQ-Goat.2	OL792792	III	AF340229.1	99.٩٠
Tg-IQ-Goat.3	OL792793	I	AF340227.1	99.٣١
Tg-IQ-Goat.4	OL792794	III	AF340229.1	99.90
Tg-IQ-Goat.5	OL792795	I	AF340227.1	٩٨.٦٥
Tg-IQ-human.1	OL792796	I	AF340227.1	99.28
Tg-IQ-human.2	OL792797	I	AF340227.1	99.90
Tg-IQ-human.3	OL792798	III	AF340229.1	99.90
Tg-IQ-human.4	OL792799	III	AF340229.1	99.90
Tg-IQ-human.5	OL792800	III	AF340229.1	99.90

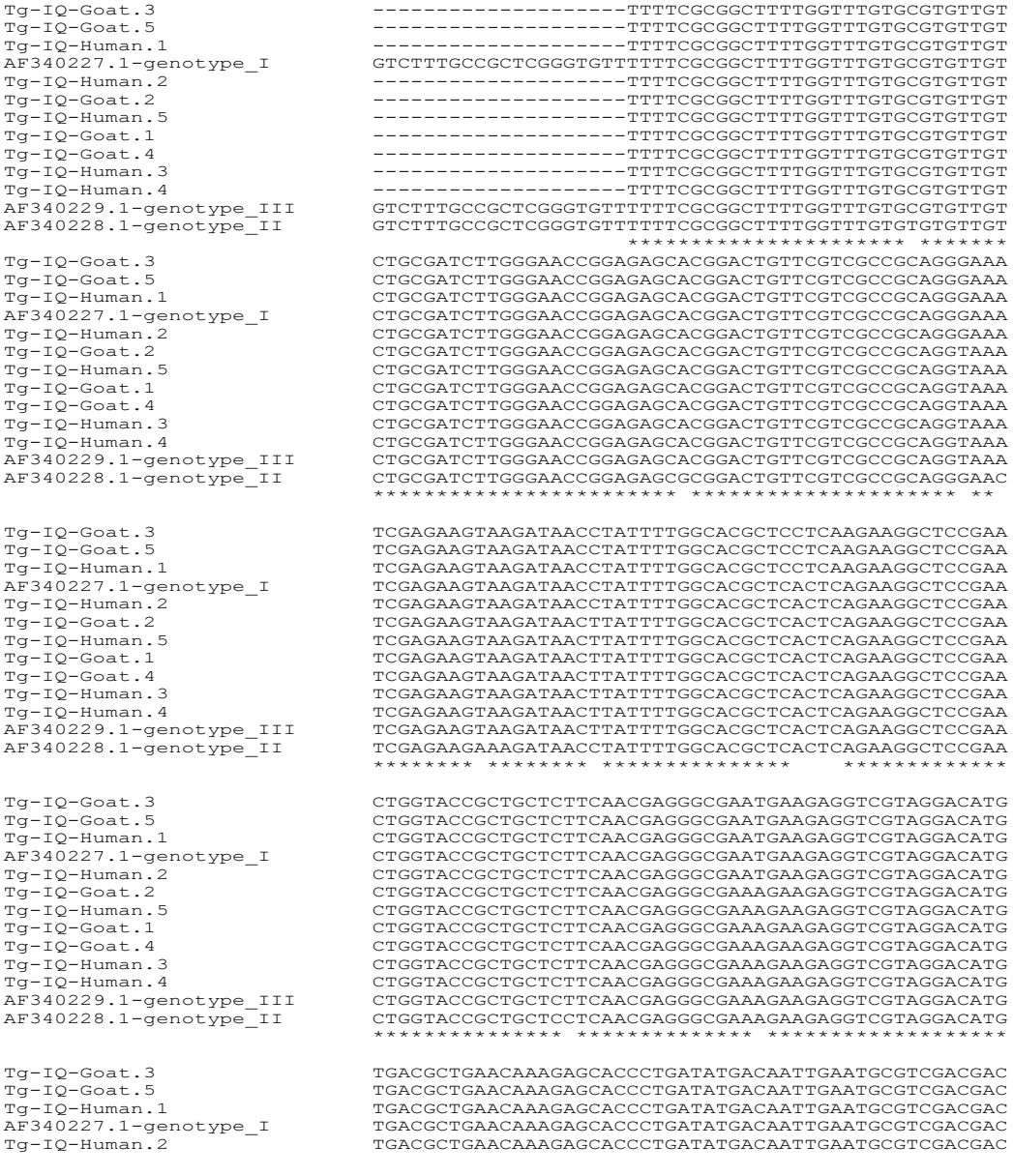

Figure 4. Multiple sequence alignment analysis of *Toxoplasma gondii* surface antigen (SAG3) gene complete sequence in local goats and human blood-positive samples with NCBI-GenBank *Toxoplasma gondii* genotypes isolates. The multiple alignment analysis was constructed using (The clustalW alignment tool. Online). That alignment analysis showed the nucleotide alignment similarity as (*) and substitution mutations in the SAG3 gene between isolates

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N/A.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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مقارنة بين ELISA و Nested PCR في الكشف عن *Toxoplasma gondii* في الدم والحليب وتحليل شجرة النشوء والتطور في الماعز والنساء المجهضة في العراق

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الخلاصة

هدفت الدراسة الحالية إلى تقييم طريقة ELISA و Nested PCR (nPCR) للكشف عن *T. gondii* في دم وحليب الماعز المحلي العراقي ، وكذلك التعرف على النمط الجيني للماعز والنساء المجهضة في العراق. تضمنت الدراسة ما مجموعه ٢٤٠ عينه (٨٠ دم ٨٠ سيرم، حليب ٨٠) من انثى ٨٠ ماعز حلوب و ٣٠ دم من النساء المجهضات بااستخدام ال n-PCR و ELISA. أظهرت النتائج ان اجمالي الاصابة في الماعز بلغت ١٧ (٢١.٢٪) و ٢٣ (٢٨.٧٪) لـ n-PCR في الدم والحليب على التوالي وبالمقارنة ، كانت النتائج ٢٣ (٢٨.٧٪) في الدم و ١٧ (٢١.٢٪) في الحليب بواسطة ELISA وكان إجمالي الإصابة في النساء المجهضات باستخدام ELISA ١٥ (٥٠٪) و ١٠ (٣٣.٤٪) بواسطة nPCR. أشارت النتائج إلى توافق عالٍ وفقًا لمعامل كابا (٠.٨) ، مع حساسية ١٠٠ ونوعية ٩٠.٤٨. كما أوضحت النتائج أن ٢٣(٢٨.٧٪) من الماعز كانت موجبة لأختبار ELISA ، و ١٧ (٢١.٢٪) من الماعز كانت موجبة لأختبار nPCR. أشارمعامل كابا الى توافق طفيف بين الاختبارين ، مع حساسية (٥٨.٨٢) ونوعية (٧٤.٦). أظهرت نتائج المقارنة بين nPCR في الدم و ELISA في الحليب عينات موجبة ١٧(٢١.٢٪) لكل منهما ، مع حساسية (٨٢.٣٥) ونوعية (٢٢.٢) مع عدم توافق (كابا=-٠.٠٤). أظهرت نتائج تسلسل الجين (SAG3) لـ *T. gondii* بناءً على عزلات الماعز والبشر أن التشابه يتراوح بين ٩٨.٦٥-٩٩.٩٠ للنمط الجيني الأول والثالث. يمكن اعتبار استخدام nPCR للكشف*T. gondii* في الدم أكثر دقة من ELISA في الدم والحليب. بالإضافة إلى ذلك ، فإن دليل شجرة النشوء والتطور على وجود درجة عالية من التشابه بين عزلات الإنسان والماعز يوفر دليلا إضافيا على أن الماعز هي خزان مهم ل *T. gondii* وأن الوعي العام ضروري.

الكلمات المفتاحية: داء المقوسات ، النمط الجيني ، تفاعل البوليميريز المتسلسل المتداخل ، إلاليزا