Molecular Detection to *Toxoplasma gondii* in Serum Sheep Samples

Balkes Fadel Hade

Department of Parasitology, College of Veterinary, University of Baghdad, Baghdad, Iraq

Abstract: *Toxoplasma gondii* an obligate intracellular protozoan is one of the most common parasites that infect warm blooded animals including man. Polymerase chain reaction was applied for the detection DNA of the pathogenic protozoan *T .gondii* based on 35-fold-repetitive gene (the B1 gene) as a target. Blood samples from (66) sheep were taken to extracted serum from it, the result indicated that (51) serum were positive by enzyme linked immune-sorbent assay) ELIZA) and (15) serum apparently healthy as control group, same serum samples were taken to extract DNA from it and to detect the B1 gene if present. The B1 gene was present and conserved in all *T . gondii* strains and to detect this gene from purified DNA samples, a two-stage of PCR) nested) was conducted employing oligonucleotide specific primers for detected this gene. Results indict that (39) sheep are infected with the parasite. This combination of specificity which detected of the B1 gene based on PCR which is regarded a very useful method for diagnosis of toxoplasmosis in sheep.

Key word: *Toxoplasma gondii*, sheep, serum, PCR**.**

الكشف الجزيئي عن طفيلي التوكسوبلازما (داء القطط) في عينات مصل الاغنام

بلقيس فاضل هادي الشمري

فرع الطفيليات، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة: المقوسات القندية (التوكسوبلازما) هو طفيلي يعيش مجبر داخل الخلايا وهو من الطفيليات الدموية (الأوالي) الذي يصيب الأنسان والحيوان وهو المسبب لداء القطط المتميز بأحداث الأسقاط المتكرر. حاولت هذه الدراسة ان تسلط الضوء على الطرق المصلية المعتمدة على قياس كمية الأضداد في مصل الأغنام ومقارنة النتائج بالفحوصات الجزيئية المعتمدة على تطبيق سلسلة تفاعل البلمرة واستخدام الجين كهدف لوجود الطفيلي التوكسوبلازما على اساس تكرار الجين 35 مرة داخل المادة الوراثية للطفيلي. شملت هذه الدراسة (66) رأس غنم كعينات للاجراء الفحص المصلي) عليها لكشف وجود الاصابة بالطفيلي اذ اكدت النتائج ان (51) رأس غنم كانت موجبة الاصابة بداء القطط في حين ان (15) راس غنم كانت سليمة من الاصابة (استخدمت كعينة سيطرة). تم اجراء الفحص الجزيئي لنفس العينة بالاعتماد على طريقة سلسلة تفاعل البلمرة للكشف عن وجود الجين الخاص بالطفيلي في عينة المصل، اذ اظهرت النتائج ان (39) راس غنم مصابة بالطفيلي من اصل (51) رأس غنم أكدت اصابتها بداء القطط بواسطة الفحوصات المصلية، بينما أكدت النتائج ان (4) من عينة السيطرة (15) كانت مصابة بالطفيلي لم يتمكن الفحص المصلي كشف الاصابة فيها.

Introduction

Toxoplasma gondii an obligate intracellular protozoan, is one of the most common parasites that infects warm blooded animals including man, It belongs to the phylum Apicomplexa, which including *Plasmodium* spp. (which cause malaria),*Eimeria* spp. (which cause avian coccidiosis) (1). *Toxoplasma gondii*, represent a major source of human and animal disease Its infection found in 30%-50% in worldwide (2). The infection being clinically asymptomatic because of effective protective immunity involving extra cellular antibody and intracellular T-cell factors; however, this parasitic disease can cause life threatening conditions in congenital toxoplasmosis as this disease can be transmitted to animals and represents a serious health risk for people living in close contact with infected animals and the infection is especially dangerous for animals with suppressed immune systems and pregnant animals which lead to abortion with single or repeated miscarriage (3,4,5).

Cats are the only animal in which sexual reproduction of the organism occurs, and for this reason cats are the only domestic animal which has the potential to shed *T. gondii* eggs. When disease does occur, it may develop following primary infection character by changes in lymph nodes which showed reactive follicular hyperplasia with irregular clusters of macrophages with eosinophilic cytoplasm while latent infection associated with distribution of cyst in skeletal muscle

heart, liver, spleen, kidney, pancreas or brain due to an inadequate immune response (6). *T. gondii* can also cause serious damage to different tissue organs of its infected host depending on the site where it forms its cyst (7). Infection with *T. gondii* is a major cause of fetal death since *T. gondii* can be transmitted to the fetus through the placenta from an infected animal or at vaginal delivery (8).

The nuclear genome of *T .gondii* is about 87 kb, a mitochondrial genome 6 kb, and an episomal 35 kb plastid-like genome (9). The congruence of karyotype and genetic linkage mapping are reproduced exactly 14 nuclear chromosomes for *T. gondii* (10). The B1 gene is a 2214 base pair (bp) sequence on (IX) chromosome with unknown function that is repeated 35 times in the genome of *T. gondii* (8, 11).

The classical diagnosis of toxoplasmosis can be performed is based on detection of specific antibodies anti - *Toxoplasma* immunoglobulin, (IgM, IgG) (12). The polymerase chain reaction (PCR) has been used as an alternative to serology by amplification of *Toxoplasma* DNA sequences present in various clinical samples, such as tissues (13), aqueous humor (14), and human blood (15). This molecular technique provides important information on the pathobiology of *Toxoplasma* infection.

Aim of Study

Comparative diagnosis of toxoplasmosis by using serological method (ELISA test) and molecular

method (n PCR) in serum sheep samples, and their importance in the identification of infection.

Materials and Methods

Specimen Collection and Preparation

A total number of (66) normal and aborted sheep were included in the present study .One sample of 3 ml of venous blood were drawn from each subject of this study and was placed in a sterilized plain tube and left to stand for 30 minutes at room temperature to clot, then centrifuged at 2000 rpm for 10 minutes for serum collection which was aspirated by using micropipette and dispensed into sterile tube and stored in °20 -)C (until used for serological and molecular test.

Serological Kits

Toxoplasma-IgM 'Toxoplasma*-IgG .I d screen ‹France, for sheep .the manufacturer's instructions were followed for both kits.

Molecular Kit

DNA was extracted from serum samples using a commercial purification system (standard extraction method of genomic DNA using a commercial kit (QIAamp genomic DNA kit, Germany) following the manufacturer's instructions. The extracted DNA was kept in the deep freezer (-20°C) until used.

Amplification and Detection of *T. gondii* DNA by Nested PCR

Nested PCR was performed on all DNA samples to amplify a fragment from B1 gene, which is present in 35 copies and is conserved in the *T. gondii* genome. The primer used in the first round of the PCR (inner primer pair) are F1 (5-GGAACTGCATCCGTTCATGAG-3), and R1 (5- TCTTTAAAGCGTTCGTGGTC-3), which correspond to nucleotides 694-714 and 887-868, respectively. The primer used in the second round (outer primer pair) are F2 (5-TGCATAGGTTGCAGTCACTG-3), and R2 (5-GGCGACCAATGTG CGAATAGACC-3), which correspond to nucleotides 757-776 and 853-831, respectively. The PCR mixtures without DNA and with DNase-free water were used as negative controls to monitor for cross-contaminations. Statistical analysis which is used in the analysis of data was computer program SPSS version 9.

Results and Discussion

a) Serological Results:
Serological testing has been one of the major diagnostic techniques for *T. gondii* infection which based on the detection of specific IgM_IgG pattern antibodies.
1) The infected sheep and controls number (66) are divided in two groups according to(ELISA test) results as mention in table: (1)

Table (1): The prevalence of *T. gondii* antibodies in study groups using ELISA.

Type of Cases	ELISA positive		ELISA negative	
	NO.	%	NO.	%
Infected Sheep	51	% 100	0	% 00.0
Non Infected Sheep (control group)	0	% 00.0	15	% 100
Statistical analysis	Cal. $\chi^2 = 66.15$** ; Tab. $\chi^2 = 6.634$; $P \le 0.01$			

** Significant differences at level 0.01 ($P \le 0.01$)

2) The infected sheep and controls number (66) are divided in to four groups according to pattern of antibody and the results showed that among (66) samples of sheep serum who had been tested for specific IgG and IgM antibodies by using ELISA, 5 (9.8 %), 15 (29.4 %), 31 (60.8 %) of the infected sheep were IgM+–IgG⁻, IgM+–IgG+ and IgM⁻–IgG+, respectively. On the other hand, the results of the 15 sheep (control group) indicated that all of them (100 %) were negative for anti-T. gondii antibodies (IgG and IgM). In the current study the presence of class (IgM+) antibodies against toxoplasmosis was interpreted as diagnosis of the acute form of the disease, but the tendency of IgM to remain detectable for a long time has been demonstrated (16).

Results of (IgM⁻–IgG+) in many cases of present study were a sign that these sheep were infected at some time in their life and they are now immune to the disease. Further tests can help to determine when the infection occurred based on the levels of the antibodies whether rising or falling. However, when (IgG+) antibodies against T. gondii were present in the serum they indicating that toxoplasmosis cysts were already present in the tissues (17). The cases with positive (IgM+–IgG+) in infected sheep are difficult to interpret in the first serum samples without any previous results.,the definitive conclusion is coming from the study of a second serum samples three weeks afterward or using other anindected test.The cases without anti-Toxoplasma antibodies (IgM⁻_ IgG⁻) indicate that thes sheep has not been infected with T. gondii .the results was show in table (2).

Table (2): The distribution of T .gondii antibodies using ELISA in infected sheep and control

Pattern of Antibody	Infected No.(51)			Control No.(15)		
	No.	%	χ^2	No.	%	χ^2
IgM⁺ -IgG⁻	5	% 9.8	1.56ns.	0	00.0	/
IgM⁺ -IgG⁺	15	% 29.4	** 44.26	0	00.0	/
IgM⁻ -IgG⁺	31	% 60.8	** 17.26	0	00.0	/
IgM⁻ -IgG⁻	0	00.0	/	15	%100	**57.97
Total	51	100.0		15	100.0	

ns.: mean no-significant differences at level 0.05 (P>0.05).

** Significant differences at level 0.01 ($P \le 0.01$).

b) Molecular Results

Burg *et al*.(1989) first researcher who reported detection of *T. gondii* DNA from a single tachyzoite, using the B1 gene in PCR (8).

1) Positive and negative PCR analyses for infected sheep and control supjects.

The infected sheep and controls number (66) are divided in two groups according to (nPCR) analysis. The subjects under study were exhibited that, highly positive results for toxoplasmosis by using ELISA technique 51\51(100) compere with positive results of PCR 39/51 (76.5 %) to the same samples such augmentation was dependent on the fact that the diagnosis of toxoplasmosis is made indirectly by serological methods or directly by PCR, the latter method does not depend on an immune response or the titers of antibody. .the results was show in table (3).

Table (3): PCR Analysis of toxoplasmosis in infected sheep and controls.

PCR analysis Result	Infected Number(51)		Control Number(15)		Total	
	NO.	%	NO.	%	NO.	%
PCR +ve	39	% 76.5	4	% 26.7	43	% 65.15
PCR –ve	12	% 23.5	11	% 73.3	23	% 34.85
Total	51	% 100	15	100.0	66	% 100
Statistical analysis	Cal. $0.01 P \leq$ ؛ $6.634 = \chi^2$ Tab. ؛ $**12.62 = \chi^2$					

** Significant differences at level 0.01 (P≤ 0.01).

2) Pattern of Antibody Detection by nPCR to infected sheep and control (66 Total Number).

The fact that could explain higher positive result (% 9.8) 5 IgM -$^+$IgG $^-$ with ELISA to infected sheep compared with lower PCR results 2 (% 5.1)to the same cases are the probability to find residual IgM ؛$^+$or it may be due to false positive) IgM.(8)

In samples which carry the pattern IgM- $^+$IgG ($^+$nPCR) assay results showed the actually positive rate of that pattern which are (% 25.7) 10 only ؛that while minimize the diagnosis problem association with that pattern .nPCR results indicated that 27 (69.2%) of the samples were carry) IgM -$^-$IgG ($^+$ pattern her chronic infection was expected and possibility of vertical transmission in chronically infected pregnant sheep in cases of reactivation ؛ and that agree with (18) and ؛(19) This findings lead to the interpretation that chronic toxoplasmosis infection is able to promote positive PCR results when

the parasite is found circulating .So the results of PCR in this study indicate that there were a high relationship between IgM⁻- IgG⁺ pattern and occurrence of abortion, and this technique was a useful tool for identification and diagnosis of toxoplasmosis because PCR can detected the (193 bp) and (96 bp) fragments respectively as showed in figure (1) and (2).

In this study Toxoplasma DNA was detected in 4\15 (26.7 %) with seronegative serology as mentioned in tabel (4). One possible explanation is that those supjects are not able to produce specific antibodies, representing a state of immunodeficiency or sometimes they may tested early in the course of the disease before their body had a chance to produce antibodies ,Or alternatively, it could correspond to a very recent infection at the time of serological, leading to an insufficient production of immunoglobulin at level detected by serology.This finding indicate that the individuals that test negative by ELISA but they are actually positive PCR have a recent infection or apparent parasitemia or active toxoplasmosis, which is likely to be clinically significant and they are at risk of transmitting the infection to their fetuses and couse abortion.

Table (4): The pattern of T. gondii antibodies and PCR result in infected sheep and control

Pattern of antibody	PCR - positive					
	Infected no. (39)			Control no. (4)		
	No.	%	χ^2	No.	%	χ^2
IgM⁺ - IgG⁻	2	5.1 %	0.6 ns.	0	00.0	/
IgM⁺ - IgG⁺	10	25.7 %	3.455 ns.	0	00.0	/
IgM⁻ - IgG⁺	27	69.2 %	13.4**	0	00.0	/
IgM⁻ - IgG⁻	0	00.0	/	4	26.7 %	14.67 **

ns.: no-significant differences at 0.05

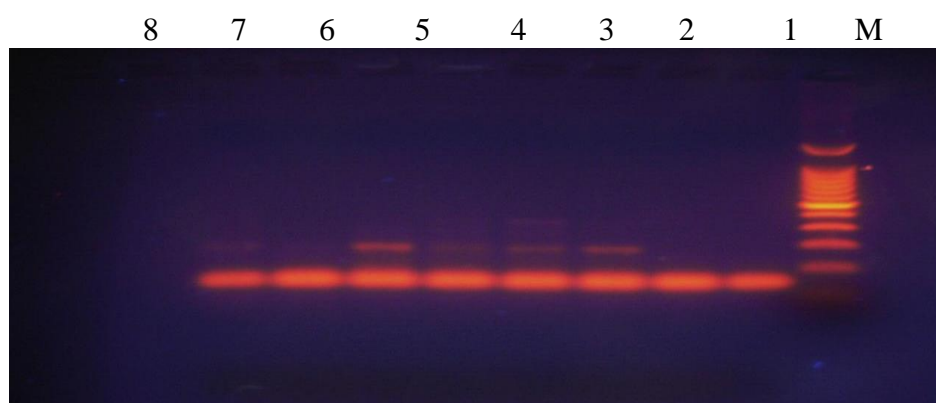** Significant differences at 0.01 (P≤ 0.01).

**Figure (1): First run of n PCR (amplification of 193bp fragment B1 gene of T. gondii)
Lane-M: molecular marker (100bp). Lanes 1 negative control.
Lanes 2, 7are negative samples. Lanes3, 4,5,6,8 are positive samples .Running
condition Agarose gel5،(%1.5) volt/cm ،for 2 hrs ،stained with ethidium bromide.**
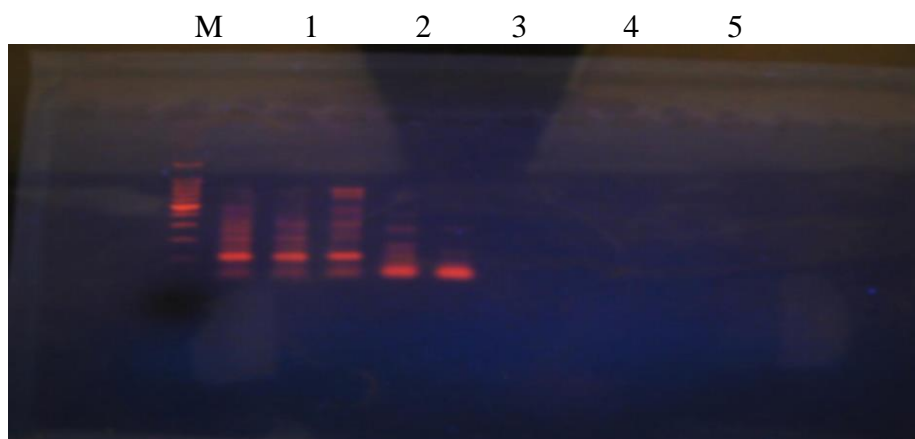
Figure (2): Second run of nPCR (amplification of 96bp fragment of B1 gene T. gondii)
    Lane-M is molecular marker (100 bp). Lanes 1,2,3 positive samples, Lanes 4 negative sample. Lanes 5 negative control.Agarose gel (1.5%), 5 volt/cm for 2 hrs. stained with ethidium bromide.

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