Molecular and serological detection of *T. gondii* in sheep in Wasit province

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Abstract

The present study was conducted during the period from October 2013 to May 2014 in Wasit province to detect the infection of *T. gondii* in sheep using molecular and serological methods. Three hundred and five samples (300 blood samples and 5 placentas) were collected from suspected sheep. The serum samples were separated and examined by latex ″agglutination test″ to detect *Toxoplasmosis* serologically. Then 100 blood samples and 5 placental tissue samples were subjected to polymerase chain reaction (PCR) technique to detect the infection molecularly. The Serological results showed that 33.33% were positive (31.33% with chronic infection and 2% with acute infection). It was noted that the highest rate of infection was among the ages which is equal to or more than 3 years (≥3 years), reaching 45.55%, with significant difference (P≤0.05) between these ages. The present study indicated a lack of months effect on the distribution of parasite infection rates where these different months recorded relatively close rate ranged between 31.4% -35.97% with no significant difference (P<0.05). Regarding to polymerase chain reaction test, when a fragment of 399bp was amplified from B1 gene, the result showed that 4% of blood samples and 80% of placental tissue samples were positive to this test. In conclusion the *Toxoplasma* infection in sheep is relatively high in Wasit province.

Key words: *Toxoplasma gondii*, LAT, 2ME, B1gene, PCR.

الكشف الجزيئي والمصلي لداء المقوسات في الأغنام في محافظة واسط

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الخلاصة

أجريت الدراسة الحالية خلال الفترة من أكتوبر 2013 إلى مايو 2014 في محافظة واسط للكشف عن الإصابة بالمقوسة الكوندية في الأغنام باستخدام الطرق الجزيئية والمصلية. تم جمع ثلاث مئة وخمس عينات (300عينه دم و5عينات مشيمية) من الاغنام المشتبه بإصابتها بالطفيلي. تم فصل عينات المصل وفحصها بواسطة اختبار تلازن حبيبات للكشف عن الإصابة بالمقوسة الكوندية مصلياً ، ثم اخضع عدد من عينات الدم والأنسجة المشيمة (100عينه دم و5عينات مشيمية) للفحص بواسطة تقنية تفاعل سلسلة البلمرة والكشف عن الإصابة جزيئياً. أظهرت النتائج المصلية أن33.33٪ كانت موجبه للفحص عن داء المقوسات (31.33٪ للإصابة المزمنة و 2٪ للإصابة الحاده)، ولوحظت أن النسبة العالية للإصابة كانت بين الأعمار التي تساوي أو أكثر من 3 سنوات (≥3سنوات) مع وجود اختلاف معنوي (P≤0.05) بين هذه الأعمار. كما أشارت هذه الدراسة الى عدم وجود تأثير للأشهر على أنتشار نسبة الإصابة للطفيلي حيث سجلت الأشهر المختلفة معدل متقارب نسبياً تراوح بين 31.4٪ -35.97٪ مع عدم وجود فرق معنوي (P<0.05). فيما يتعلق باختبار تفاعل سلسلة البلمرة ، حيث تم تضخيم جزء (bp399) من جين B1 ، وأثبتت النتائج أن 4٪ من عينات الدم و 80٪ من عينات المشيمة كانت إيجابية لهذا الاختبار. نستنتج ان الإصابة بداء المقوسات في الاغنام عالي نسبياً في محافظة واسط.

الكلمات المفتاحية: المقوسة الكوندية ، اللاتكس و2ميركابتوأيثانول ، جين B1 ، تفاعل سلسلة البلمرة.

Introduction

Toxoplasmosis is a cosmopolitan zoonotic disease caused by the protozoan parasite called *Toxoplasma gondii*, an obligate intracellular parasite capable of infected all warm-blood animals, including mammals, birds and humans (1). The parasite is mainly transmitted by food contaminated with oocyst dispersed by cats and other felines,

definitive hosts, uncooked meat containing tissue cysts or unpasteurized milk containing tachyzoite stage, and transplacentally (2,3). Most *T. gondii* infections in sheep occur through the ingestion of oocysts, a stage of the parasite, which is very stable and can survive in favorable conditions in the environment for over 12 months, contaminating pasture, feeds and drinking water (4). Abortions in sheep due to *T. gondii* are associated with a primary infection during the first or second trimester of gestation, while primary infection in the later stages of pregnancy leads to the birth of congenitally infected lambs, which are relatively rare (5, 6). Infected animals develop an effective immune response, which will protect against disease in subsequent pregnancies (4). The diagnosis of *Toxoplasma* infection is conventionally made by the direct demonstration or isolation of the parasite from biopsy or autopsy material, but such techniques are unsuitable for use large-scale surveys. Therefore, recourse has been made to immunoserological tests for specific host antibody, and a variety of tests have been described (7). Burg *et al*., (8) is the first who reported detection of *T. gondii* DNA from a single tachyzoite using the B1 gene in a polymerase chain reaction (PCR). Several subsequent PCR tests have been developed using different gene targets. Overall, this technique has proven very useful in the diagnosis of clinical toxoplasmosis. Due to the importance of toxoplasmosis, so a zoonotic disease and it's close relationship to the health of society, therefore the study was conducted in Wasit province and its aims to detection of *Toxoplasma gondii* infection in sheep in Wasit province by serological and PCR methods and mention the effect of some factors, like age and environmental conditions on the rate of infection.

Materials and methods

Blood and tissue samples collection:

Five ml of blood was collected from 300 aborted ewes aged < 1yr-≥3yr from different regions of Wasit province, during the period from October 2013 to May 2014.Serum were separated from the blood and stored at -20°C until use for LAT. The rest blood sample (2ml) was placed in EDTA tube. Also five (5) placental tissue samples were collected from aborted ewes. Both blood and placental tissue were kept at -20°C until using for DNA extraction.

1-Serological testing

Latex Agglutination Test (LAT) kit (Hannover, Germany) Toxo-latex was used to determine the infection with *T. gondii* serologically. Positive results agglutination of the latex particles suspension will occur within 4-6 minutes. 2-Mercaptoethanol was used with above test to detect the acute (IgM) or chronic (IgG) phase where mercaptoethanol inactivates the IgM antibodies while leaving the IgG antibodies' intact (9, 10).

2-Polymerase chain reaction (PCR)

Using the primers for amplification of B1 gene in *Toxoplasma gondii* were designed by using NCBI- GenBank Data base and primer3plus online and provided by Bioneer Company from South Korea (table1). The PCR technique was performed according to method described by (11, 12, 13, 14), where the DNA extraction was performed according to the manufacturer's, instructions (Bioneer, Korea) briefly, 50mg of placenta tissue samples was placed in1.5 ml micro centrifuge tube, then 200µl tissue lysis buffer and 20µl of Proteinase K were added and homogenize by micro pestle and incubated in 60°C for 1 hr. Regarding to the blood, 20µl proteinase K was added to a sterile 1.5 ml tube, and 200µl blood sample was added and mixed by vortex. After that, 200µl of binding buffer (GC) was added to each tube and mixed then all tubes were incubated at 60°C for 10 minutes.100µl of isopropanol was added to mixture and mixed well by pipetting, and then briefly spin down to get the drops clinging under the lid. The lysate was carefully transferred into Binding column (GD) (filter column) that fitted in a 2 ml collection tube, and then closed the tubes and centrifuged at 8000 rpm for 1 minute. Lysate was discarded and then 500µl Washing buffer 1 (W1) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute. Washing buffer 1 and then 500µl Washing buffer 2 (W2) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute.

Table (1): The primers used to detect the B1 gene in *T. gondii*.

Primer		Sequence	qPCR product size	GenBank Code no.
T. plasma gondii B1 gene	F	5¯GAACCACCAAAAATCGGAGA¯3	399bp	179871.1 AF
	R	3¯GATCCTTTTGCACGGTTGTT¯5		

Washing buffer 2 was discarded and then the tubes were centrifuged once more at 12000 rpm for 1 minute to completely remove ethanol. After that, Binding column (GD) filter column that containing genomic DNA was transferred to sterile 1.5ml micro centrifuge tube, and then added 50µl of Elution buffer and left stand the tubes for 5 minutes at room temperature until the buffer is completely absorbed into the glass filter of Binding column tube. Finally, all tubes were centrifuged at 8000 rpm for 1 minute to elute DNA, the DNA extract was stored in freezer (-20°C) until using. PCR reaction was prepared by using AccuPower PCR PreMix Kit (Bioneer, Korea) and this reaction was done according to company instructions. Then, all the PCR tubes transferred into PCR thermo cycler which adjusted to conditions: Initial denaturation one cycle at 95 ˚C for 5 min. then 30 cycles included denaturation 95 ˚C for 30 sec., annealing 58 ˚C for 30 sec. and extension at 72 for 40 sec. and finally final extension at 72 ˚C for 5 min. The PCR products were analyzed by agarose gel electrophoresis used 1% agarose (15). The sample was considered to be positive for *T. gondii* DNA if the band of 399bp is observed on agarose gel, and compared with the *T. gondii* DNA positive control.

Statistical analysis

The results of present study were analyzed by SPSS program (version) software 2010, using Chi-square test and P values of $p \leq 0.05$ were considered to record statistical significance (16).

Results

Detection of Toxoplasmosis

A. Serological examination (LAT test).

1-Incidence of Toxoplasmosis according to LAT with 2-Mercaptoethanol test.

One hundred (100) (33.33%) serum samples out of 300 were given positive results by LAT examination. Ninety four 94 (31.33%) of them were chronic cases (IgG positive), and 6 (2%) were acute cases (IgM positive) when examined by 2-Mercaptoethanol, with a significant differences at ($p< 0.05$) (Table 2).

Table (2): LAT and 2-Mercaptoethanol positive cases according to IgG and IgM test

LAT and 2-Mercapto-ethanol test	Examined sample	Positive No.	Percentage (%)
IgG	300	94	31.33 a
IgM		6	2 b
Total	300	100	33.33

•Different letters refers to the significant differences at (p< 0.05).

2-Incidence of Toxoplasmosis cases according to age.

Among the four groups of age, the results showed that the majority of positive cases (45.55%) were seen within the (≥ 3 year groups), with a significant differences at ($p< 0.05$) (Table 3).

3-Incidence of Toxoplasmosis according to months of year.

The percentages of incidence of Toxoplasmosis among the months of year were ranged between 31.4-35.97%, and there were no significance ($P< 0.05$) within those percentages (Table 4).

Table (3): LAT positive cases according to age

Age group	Examined No.	Positive No.	Percentage (%)
≤1yr	40	5	12.5a
1-2yr	70	16	22.857 ab
2-3yr	100	38	38bc
≥ 3yr	90	41	45.55 c
Total	300	100	33.33

•Similar letters refers to the non-significant differences among ages while different letters refers to the significant differences at (P< 0.05).

B. Molecular examination.

Out of 100 blood samples taken from serological positive cases, and examined by

conventional PCR (4) (4%) were given positive result, in other hand from 5 placental tissues which collected from aborted Toxoplasmosis sheep (4) (80%) were given positive PCR results (Table5).

Table (4): LAT positive cases according to months of year

Month	Examined No.	Positive No.	Percentage (%)
October	35	11	31.4a
November	30	10	33.33a
December	40	13	32.5a
January	35	12	34.28a
February	38	12	31.57a
March	39	14	35.97a
April	40	13	32.5a
May	43	15	34.88a
Total	300	100	33.33

***Similar letters refers to the non-significant differences at (P< 0.05).**

Table (5): PCR positive cases of Toxoplasmosis

Samples	Examined No.	Positive No.	Percentage %
Blood	100	4	4a
Placenta	5	4	80b

***Different letters refers to the significant differences at (p< 0.05).**

PCR positive Toxoplasmosis according to the IgG and IgM test.

Out of 94 IgG positive blood samples only one (1) was revealed positive result by PCR, while all of these 3 IgM positive blood samples gave positive PCR result. In other hand among the 5 placental tissue samples which collected from ewes with positive IgG (4) samples gave positive results with PCR (Table 6, Figure 1).

Table (6): PCR positive Toxoplasmosis according to IgG and IgM test.

Samples	No. of Examined cases	IgG Positive	PCR Positive	%	IgM Positive	PCR Positive	%
Blood	100	94	1	1.06Aa	6	3	50Ab
Placental tissue	5	5	4	80Ba	0	0	0Bb

*** The capital letters refers to the vertical statistical reading while small letters refers to horizontal reading.**
*** Different letters refers to the significant differences at (p< 0.05).**

Fig. 1: Agarose gel electrophoresis image that show the PCR product analysis of B1 gene of *Toxoplasma gondii* at 399bp PCR product in aborted sheep blood samples and tissue placenta. Where L: Ladder 100bp, lane (1) positive IgG blood samples, lane (2-4) positive IgM blood samples, lane (5-8) positive IgG tissue samples and PC positive control.

Discussion

Toxoplasmosis is a zoonotic disease caused by *T. gondii* and has been known in many countries since 1908 (17). (18) Reported that the prevalence of toxoplasmosis varies among countries, depending on traditions, customs and the life styles of the inhabitants. *T. gondii* infection distributed worldwide, with prevalence rates ranging from 0% to 100% in different countries and even in areas of the same state (17, 19, and 20). There are several methods, including immunological and molecular techniques for detection *Toxoplasma* infection.

Detection of Toxoplasmosis in sheep

A. Serological test (LAT and 2-Mercaptoethanol Test).

Sheep are important in the epidemiology of *T. gondii* infection; asymptomatic sheep can serve as a source of infection for humans (21). Sheep are important to the economy of many countries because they are a source of food for humans. Sheep are commonly infected with the protozoan parasite, *T. gondii*. Infection with the parasite may cause early embryonic death, fetal death and mummification, abortion, stillbirth, and

neonatal death (22, 23). Infected sheep meat is a source of *T. gondii* infection for humans and carnivorous animals (24). The present study showed that, the rate of infection in sheep according to LAT was 33.33%. This results indicate detection of *Toxoplasma* among sheep in the study area, with a significant difference at ($P<0.05$). Among the infected cases, 31.3% was appear in IgG, while 2% was in IgM, by using 2-Mercaptoethanol test cause cleavage of disulfide bonds of IgM and loss of agglutinin activity. Thus, comparison of result obtained in the absence or presence of these agents is often used to distinguish IgM from IgG activity and to differentiate between acute and chronic infection (25). In sheep inoculated intravenously with tachyzoites, IgM was detected by one month and persisted for three months (26). The rate of infection of this study was nearly similar to the study done in Baghdad (27.9%) by (27), but it was lowest than the result 79.03% which recorded by (28) in Basra province, whereas higher than the rate of infection in studies of (29) when he recorded 24.46% positively in Nineveh. (30) Recorded rates of infection with *T. gondii* in sheep naturally aborted during the period 2006 to 2008 amounted to 25%, 18.6%, 16.06% and 12.71% in the provinces of Mysan, Basra, Muthanna and Thi-Qar respectively. Studies in neighboring countries recorded infection rates close to the results of this study, where (31) and (32) recorded 34.6% and 31% respectively in Turkey, also (33) referred to that the seroprevalence rate in sheep in Ardabil state in Iran was 30%. (34) Examined serum samples from sheep, goats and cows for *T. gondii* antibodies by the use of the LAT and indirect haemagglutination test and found the antibodies in 24.5% of sheep and 19.25% of goats. Many other rates of infections were recorded like 57% in Canada, 23% in Greece, 27.6% in Morocco, 53.65% in Poland and 49.9% in Sicily (35, 36, 37, 38, and 39). The differences in the identification of antibodies in sheep may regard the presence of cats close to the sheep and excrete the oocyst of parasite, which is characterized by extreme resistance to environmental conditions and retaining its susceptibility to infection for a long time (40, 41, and 42). (43) and (44) they pointed to there is high rate of infection in the warm and humid areas compared to the areas of cold and dry because of the viability of *T. gondii* oocysts in warm and damp climate, also, the difference in the techniques used in the investigation of infection (45), in addition to poor storage of feed used in animal feeding which provides moisture for the growth and proliferation of fungi, which is one of the inhibited immune factor and assistance on the infection with parasite (46). The current study referred to relationship between age of animal and the infection with toxoplasmosis, whereas the highest rate 45.55% of infection recorded among sheep with age group that equal or more than three years ($\geq$3 years) and decrease toward the small ages. The reason of that may be related to continuous exposure to the parasite in the environment as age progressing (47, 48, 49). Results of the present study were agreement with results reported by (44, 50, 51), and nearly to study of (52) in Scotland, this allowed the association between seroprevalence and age to be assessed to determine if an increased cumulative chance of environmental exposure to *T. gondii* will be reflected in increased seroprevalence in older animals. Seropositivity for *T. gondii* increased with age from 37.7% for sheep that were about 1 year old, to 73.8% for sheep that were older than 6 years. Many authors like (53) and (44), referred to that the age is an important factor. Older sheep have a higher prevalence of toxoplasmosis than younger sheep, also (24) reported to an increase in seropositivity to *T. gondii* with animal age. Further studies looking at development of specific antibodies in sheep, as an indicator of exposure to *T. gondii*, have shown that there is an increase in seroprevalence associated with age indicating that there is extensive environmental contamination with *T. gondii* oocysts and that most infections in sheep occur following birth (54, 55). The low prevalence of infection in young animals, his probably due to lower parasite exposure, suggesting that the chance of contact with sporulated oocysts increases with age (39, 56, 57), also (58) suggested that adult

animals are more exposed to *T. gondii* transmission and that this increased exposure results in greater chances of becoming infected or reinfected, and this may be explained on the basis that older animals are less resistant to toxoplasmosis due low immunity (59). When study the effect of different months on the rate of infection the study found that there is no significant differences ($p<0.05$) among the percentages which recorded of each month that which ranged 31.4% - 35.97%, this may because depended of the presence of cats is a factor associated with infection, as shown by (39) and (60). The presence of cats is of fundamental importance in the epidemiology of *T. gondii* since, depending on the environmental conditions oocysts shed in the feces of cats can survive for months or even years (61). These oocysts can then become a main source of infection for sheep.

B. Molecular examination (PCR).

The present study showed that the rate of *T. gondii* infection was 7.6% according to the PCR technique, where out of 105 examined samples (100 blood, and 5 placenta samples) 4(4%) blood, and 4(80%) placenta were gave a positive result. This result was nearly similar to the result of (62) indicated *T. gondii* infection 11.1% in Sardinia/Italy diagnostics related to sheep abortions, whereas (63) found (38%) in meat samples were obtained from UK retail outlets gave the expected polymerase chain reaction products when amplified with primers specific for the species from which the meat originated, (64) demonstrated *T. gondii* infection in 87% of brain and 55% of muscle samples from congenitally infected sheep. (65) Detected the parasite more frequently in skeletal muscle than in any other tissue in experimentally infected sheep. (66) Found that *T. gondii* DNA is detected more frequently in ovine brain and heart than in skeletal muscle. Interestingly, the detected *T. gondii* in high frequency from the placental tissue, confirming previous studies (67, 68). (69) Mentioned that a negative PCR result does not exclude recent infections because the PCR sensitivity, in which a single trophozoite can be detected in a clinical sample, has potential problems for some types of specimens. Generally the low percentage of infection in this study when used PCR technique may be due to the cases of chronic phase, which related to the blood samples because the trophozoite absence from blood through chronic cases (70). The PCR technique can be a favorable method when using to examination of tissue samples in infected cases (71, 72). Whereas the differences among percentages that, recorded in the current study and other studies, may be attributed to the different origins of used samples (73). In conclusion, *Toxoplasma* infection in sheep is relatively high in Wasit provinces; also *Toxoplasma* infection among animals (sheep) is importance, because some of the infected animals play a distinct role as a source of human infection.

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