DIAGNOSIS OF TOXOPLASMOSIS IN SHEEP USING SEROLOGICAL (ELISA) AND MOLECULAR TECHNIQUE IN DUHOK GOVERNORATE - KURDISTAN REGION

Farhad **B**uzo **M**ikaeel, **J**assim **A**bdo and **L**okman **T**aib **O**mer
Duhok Research Center, Faculty of veterinary Medicine, University of Duhok, Kurdistan Region – Iraq.
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Abstract:

Toxoplasmosis is an important zoonotic diseases in human and animals. The disease caused by the protozoan *Toxoplasma gondii.* It is an economically important disease of livestock, especially sheep and goats. The present work aimed to diagnose toxoplasmosis in sheep using two methods, serological (ELISA) and molecular tools (PCR) and comparing the serological data with the molecular results to determine the sensitivity and the specifity of the molecular tools.

The study was carried out at Duhok Research Center, University of Duhok. Ninety sex whole blood samples were collected from in aborted ewes in Duhok governorate during the period September 2013-September 2014.The samples were collected from different localities including Aqra, Dohuk district, Shikhan and Zakho The serological tests showed that22(22.91 %) of the samples were positive from examined sheep serum by ELISA, while 23(23.93%) by using PCR assay. PCR was performed on all DNA of sheep blood samples to amplify B1 gene as a target sequence.

Good correlation between the results of PCR and ELISA were detected, there's no statistically significant difference, It can be concluded that ELISA combined with the PCR technique is a recommended tool for accurate diagnosis of Toxoplasmosis but PCR is more specific for detection of *T. gondii* with sensitivity of 95.45 % and specificity of 97.29%.When taking the ELISA as a reference test. The results of PCR assay showed it's important in the diagnosis of the carrier infected cases more than ELISA techniques.

Keyword: *Toxoplasmosis, Sheep, ELISA, PCR.*

1. Introduction

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, *Toxoplasma gondii* infection of sheep and goat poses a risk to public health, as well as economic losses due to reproductive failure (Dubey and Towel, 1986; Dubey and kirkbride, 1990; Edward and Dubey, 2013). The parasite was first described in 1954 (Buxton et al., 2007). Ovine toxoplasmosis has a worldwide distribution especially in temperate sheep rearing countries where the climatic conditions favor oocyst survival (Buxton and Rodger, 2008). Toxoplasmosis is a major cause of reproductive failure associated with abortion in sheep and goat. In sheep, abortion or prenatal mortality of lambs occur when ewes suffer a primary infection during pregnancy (Tenter *et al.,* 2001).

Toxoplasmosis is diagnosed mainly by direct smear, Immunohistochemistry, serology testing and PCR (Mazumder *et al.,* 1988; Ramadan *et al.,* 2007; Dubey, 2009, 2010)

Various ELISA methods using crude, fractionated, or recombinant antigens have been used to detect *T. gondii* antibodies in ovine sera. Compared ELISA based on crude and recombinant antigens found to have varying degrees of specificity in naturally and experimentally- infected sheep (Caballero-Ortega *et al.,* 2008).

The ELISA assay for *T. gondii* antibodies has been adapted for use in most domestic animals including sheep and goat (Dubey, 2008; Dubey, 2009). There is specific ELISA assays for both IgM and IgG subtypes. These ELISA assays are ideally suited to screen large numbers of samples and looking at the IgM/IgG ratio (Denmark and Chessum, 1978). Serological analysis using IFAT and ELISA has been widely employed in order to detect herds infected by toxoplasma, including swine and sheep flocks (Van der Puije *et al.,* 2000).

Moreover, Molecular assays such as PCR make it possible to detect small quantities of target DNA and potentially provide an alternative sensitive diagnostic tool, specific molecular diagnostics of toxoplasmosis is generally based on the detection of a specific DNA sequence, using different assays and protocols, mostly from highly conserved regions such as the B1 gene repeated 35 times in the

genome, 529 bp repetitive element with about 200-300 copies in the genome, ITS-1 (internal transcribed spacer) that exists in 110 copies and 18S rDNA gene sequences(Jones *et al.,* 2000; Habibi *et al.,* 2012; Moazeni Jula *et al.,* 2013; Tavassoli *et al.,* 2013). In general, this technique has been proven as a useful method in diagnosis of clinical toxoplasmosis (Dubey, 2008), it is highly specific and sensitive and very useful together with serological tests to differentiate the chronic, acute or reactivated infections (Neil and Lappin, 1991; Switaj *et al* 2005)

Early diagnosis of infection is of great consequence for reducing the severity of the disease and the risk of congenital toxoplasmosis (Behbehani and Al-Karmi, 1980; Edward and Dubey, 2013).

Few serological studies have been conducted on the prevalence of *T. gondii* antibody in farm animals in Kurdistan Region but no molecular study for detection of parasite DNA have been used. Therefore, the aims of the present study were to estimate the prevalence of the *T. gondii* antibodies in aborted ewe's serum by using serological tests (ELISA) and to compared with the detection of parasite DNA using molecular tools (PCR) in order to determine the sensitivity and specifity of diagnostic techniques for the diagnosis of Toxoplasmosis in sheep Duhok Governorate,

2. Material and method:

The study was carried out in the Duhok Research Center /Faculty of veterinary medicine, university of Duhok. The sheep of this study were of local breeding (Karadi), only female aborted ewes were involved in this work.

2.1 Sampling:

Ninety sex whole blood samples were collected from aborted ewes in Duhok governorate during the period September 2013 to September 2014. The samples were collected from different localities including Aqra, Duhok district, Shikhan and Zakho.

The blood samples were collected from the jugular veins of the sheep, each sample was divided in to the two parts, one left few minutes to obtain serum for serological tests, the other part of the blood samples was collected in sterile tubes with EDTA for PCR investigation. The collected sera and blood samples were coded and preserved at -20 °C until used.

2.2 Serological assay:

Sheep IgG antibodies against *T. gondii* were tested using an enzyme-linked immunosorbent assay (ELISA). The indirect Elisa (ID. VET. Innovative diagnostics, France) was performed using commercial kit. Optical densities (OD) were read at 450 nm. The results were expressed as the percentage of the mean absorbance values of sample (S) to the mean absorbance value of the positive (P) control sample provided with the diagnostic kit. The resultant SP ratio was expressed as a percentage (S/P %). According to the manufacturer's recommendation, sera with S/P% $\leq$ 40% should be regarded as negative, between 40 and 50% as doubtful, between 50% $\leq$ and < 200% as positive, and $\geq$ 200% as strong positive

2.3 Polymerasechain reaction (PCR):

2.3.1 DNA extraction

Genomic DNA was extracted from blood samples using a commercially available kit, QIAamp DNA, blood and tissue kit (Qiagen, Hilden, Germany). DNA extraction and purification protocol was recommended by the manufacturer.

Amplification was conducted in a total volume of 25 µl. The reaction mixture contained 12.5 ul, 2X ready PCR mix (KAPA2G, PCR kit, KAPA Biosystems; USA) which consisted of 1.25 ul Taq-Pol, 75 mM Tris-HCL (pH 8.8), 1.5 mM MgCl2, and 0.2 mM of each dNTP. The reaction mixture contained 12.5 ul master mix, 10 pmol of each forward and reverse primers (Table 1), 1 ug DNA template, and 8.5 ul RNase free water to a total volume of 25 ul.

2.3.2 Primer selection

The primer pairs used in the PCR experiment were TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (CGCTGCAGACACAGTGCATCTGGATT), as described by (Homan *et al.,* 2000)

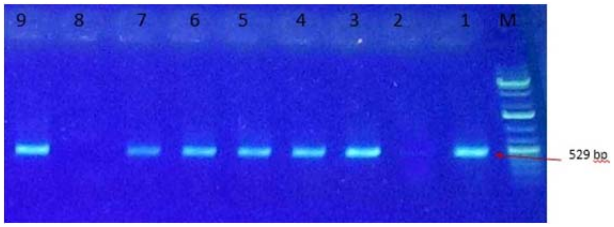
2.3.3 PCR technique

The amplification was carried out in a thermal cycler (Ependrof, Germany) according to the following program: an initial denaturation step at 95°C for 7 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing, primer at 55°C for 1 min, extension step at 72°C for 45 sec. and a final extension step at 72°C for 5 min. Amplified PCR products were the PCR products examined by electrophoresis in 1.5%

agarose gel, stained with ethidium bromide solution, visualized under UV transilluminator and photographed

4. Results

In the results of the diagnosis of *T.gondii* infection in sheep relied on the immunological (ELISA) and molecular tests, as presented in Table 1, ELISA detected anti- *T. gondii* antibodies in 22 out of 96 sheep (22.91%), whereas 23/ 96 (23.9%) of the samples were positive by PCR. Two of the samples were positive with PCR but they were negative with ELISA assay and one sample was seropositive but negative with PCR (Figure 1).



(Fig. 1). Gel agarose electrophoresis (1.5%) for *T. gondii* PCR products analysis M: 100 bp DNA size marker Lane 1 :(control positive) *T. gondii* B1 gene PCR products (529 bp) were amplified by external primers. Lanes 2 is negative control and 3, 4,5,6,9 is positive clinical samples for *T. gondii* B1 gene b, 8 is negative clinical samples.

Table 1. Infection rate of *T. gondii* in examined sheep using PCR, ELISA

	ELISA	PCR
Positive	22(22.91%),	23(23.9%)
Negative	74(77%)	73(76%)
Total	96	96

Compared with ELISA The sensitivity and specificity of PCR for detecting Toxoplasmosis measured according to use ELISA test as reference tools The sensitivity and specificity were 95.45% , 97.29 respectively (Table 2).

Based upon the Kappa statistical test, a good correlation 0.913(perfect agreement) between the results of ELISA and PCR. Strength of agreement based on ê was judged according to the following guidelines: <0.2=slight; 0.2–0.4=fair; 0.4– 0.6=moderate; 0.6–0.8=good; >0.8=very good, and 0.8-1= perfect agreement.

Table 2. Relative sensitivity and specificity of PCR in comparison with standard ELISA

		ELISA		
		Positive	Negative	
PCR	Positive	$21_{(a)}$	$2_{(b)}$	23
	Negative	$1_{(c)}$	$72_{(d)}$	73
		22	74	96

Sensitivity = a/ (a + c);95.45%
Specificity = d/ (b + d) 97.29
Kappa agreement *= 0.913
*When two measurements agree by chance only, kappa = 0. When the two measurements agree perfectly, kappa = 1

5. Discussion:

Sheep represent an important source of meat, milk and wool for humans in many countries, and toxoplasmosis causes great economic losses to sheep industry worldwide (Buxton *et al.*, 2007) The prevalence of *T. gondii* infection in sheep and goats may be due to sheep free range livestock associated with *T. gondii* infection due to contamination of environment with oocytes. The frequent presence of stray cats in a humid rainy climate favor the survival of oocytes which contributed to the Toxoplasma prevalence in Kurdistan. The primary goal of this study was to assess the risk of *T. gondii* infection to sheep in Kurdistan and to use different tools for the diagnosis of the parasite from aborted ewe. Out of 96 samples 22(22.91%) were positive by using ELISA while the 23(23.9%) samples were positive with B1 gen using PCR two.

Two samples were positive with PCR but negative with ELISA assay. The explanation is that there are cases of infection with *T. gondii* where serum levels of antibody are still not enough to trigger ELISA-sensitive reactions, ELISA is unable to detect the infection at early stages of infection. Again, in acute phase of infection *T. gondii* when death can happen in only few days, the disease progression is too fast to let immune system of animal to produce ELISA-detectable levels of antibody (Nguyen *et al.*, 1996).One sample was seropositive but PCR negative results can be explained by the fact that antibodies may be present in the absent of parasite or may be false positive reaction due to the cross-reactivity, since several studies suggested a closer relationship between *T. gondii*

sera and *N. caninum* (Nishikawa *et al.,* 2002).

Detection of *T. gondii* DNA using PCR minimizes the problems which the researcher may face when using serological methods only and facilitates the diagnosis in complex cases. Compared to other countries, it has been found that *T. gondii* seroprevalence in Duhok was higher than that in Rahim Yar Khan (Punjab), Pakistan (11.2%) and India (3.8%) (Ramzan *et al.,* 2009; Sharma *et al.,* 2008), but lower than the prevalence rate of toxoplasmosis in Turkey and Iran which were (31%) for both (Taraneh *et al.,* 2006 and Sharif *et al.,* 2014 respectively). The differences could be related to differences in ecological and geographical factors such as temperature, rainfall or landscape differences. The study area had overall low temperatures and it generally thought that the prevalence and risk of *T. gondii* infection decrease with decreasing temperature because it affect the survival of oocytes in the environment such as pastures.

In Iraq, the prevalence of the *T. gondii* in sheep is higher in south than that of the north of the country (Ali Akber *et al.,* 2014) study showed that the province of Babylon is the highest in its seropositivity percentage in sheep 39.5%, followed by the province of Al-Anbar (34.4%) and the province of Nineveh (30%) as compared to the study of other Provinces. The present study showed that *T. gondii* seroprevalence in Duhok was lower than Babylon, Al-Anbar and Nineveh respectively. The differences could be due to the hot and humid environment in south, higher prevalence rate of toxoplasmosis in warm, moist areas compared to those with colder and dry is attributed to the longer viability of *T. gondii* oocytes in moist or humid environments The study area had overall low temperatures and it generally thought that the prevalence and risk of *T. gondii* infection decrease with decreasing temperature because it affect the survival of oocytes in the environment such as pastures. PCR provides a simple and safe method for accurate and early diagnosis of toxoplasmosis (Tavassoli *et al.,* 2009; Moazeni Jula *et al.,* 2013).

In General many study has been performed using the serological and moleculare diagnosis of *T. gondii* such as (Burg *et al.,* 1989; Al-Sanjary and Hussein, 2012; El-Madawy and Metawea 2013; Al-Abady *et al.,* 2014), all those studies agree with our conclusion that molecular (PCR) is more sensitive and specific tools than the serological tools for the detection of *T. gondii* parasite . The sensitivity and the specificity of PCR for detecting Toxoplasmosis was measured according to ELISA test as a reference test

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دەستنیشانکرنا نەساخیا کتکا ل ناڤ میهێن سپی دا ب ڕێکا سیرولوجی (ELISA) و گەردیلەیی (PCR) ل پارێزگەها دهوکێ/هەرێما کوردستانێ

پوختە:

نەساخیا کتکا (توکسوپلاسموسس) ئێکە ژ گرنگترین نەساخییێت ڤەگر دناڤبەرا مروڤ و ئاژەلاندا. نەساخیا ناڤبری ژ ئەگەرا توشبون ب مشەخورا (*Toxoplama gondii*)یە. د بواری ئابوری دا ئەڤ نەساخیە یا گرنگە ل ناڤ بزن و پەزادا. ئەڤ ڤەکولینە هاتیە ئەنجام دان ژبو دەستنیشانکرنا نەساخیا کتکا ب ڕێکێن سیرولوجی (ELISA) و ڕێکا گەردیلەیی (PCR) و هەروەسا بەراورد کرنا ئەنجامێت سیرولوجی و گەردیلەیی بو دیارکرنا هەستیاری و تایبەتمەندیا ڕێکا گەردیلەیی.

ئەڤ ڤەکولینە یا هاتیە ئەنجام دان ل سەنتەرێ دهوك یێ ڤەکولینێن ڤێتێرنەری، زانکویا دهوك. نوتو شەش نمونێن خینێ هاتینە وەرگرتن ل ناڤ میهێن بەرهاڤێتی ل پارێزگەها دهوکێ ل ٢٠١٣/١٠ تا ٢٠١٤/١٠. نمونە هاتنە کومکرن ل جهێن جودا جودا ل ئاکرێ، دهوك، شێخان و زاخو.

ئەنجامێن سیرولوجی دیاربی کو ٢٢(٢٢.٩١٪) ژ نمونان ئەرێنی بون ب ڕێکا ELISA، بەلێ ٢٣(٢٣.٩٣٪) ئەرێنی بون ب ڕێکا PCRێ. هەمی نمونێن خینا پەزی ب ڕێکا PCR هاتنە پشکنین بو دیتنا جینێ B1 وەکو زنجیرا ئارمانجکری.

پەیوەندیەکا باش دناڤ بەرا ئەنجامێن ELISA و PCR ێ دا هاتنە دیتن، چ جیاوازییێن بەرچاڤ د ناڤبەرا هەردو ڕێکادا نهاتنە دیتن. ئەم دێ گەهینە وێ ئەنجامێ کو ELISA دگەل PCR ێ پێکڤە دێ ڕێکێن باش بن بو دەستنیشانکرنەکا باش و دروست بو نەساخیا کتکا. PCR یا تایبەتمەندترە بو دیارکرنا *Toxoplama gondii*دگەل هەستیاریا ٩٥.٤٥٪ وتایبەتمەندیا ٩٧.٢٩٪. ئەنجامێن PCR ێ دیارکەت کو ڕێکا ناڤبری زورا گرنگە بو دەستنیشانکرنا میهێن هەلگر ب نەساخیێ پتر ژ ڕێکا ELISA.

تشخيص داء المقوسات في الأغنام باستخدام فحص المصلي (الاليزا) و التقنية الجزيئية في محافظة دهوك–إقليم كوردستان

الخلاصة:

داء المقوسات يعتبر من الأمراض المهمة المشتركة بين الحيوانات والإنسان. الإصابة بهذا المرض يتسبب عن طريق العدوى بطفيلي من الأوالي *Toxoplasma gondii*. يعتبر هذا المرض من الأمراض المهمة من الناحية الاقتصادية في الثروة الحيوانية خاصة في الأغنام والماعز. أجريت الدراسة الحالية لتشخيص المرض في الأغنام بواسطة فحص المصلي (ELISA) و التقنية الجزيئية (PCR) وكذلك مقارنة النتائج في كلا الفحصين لتحديد نسبة الحساسية والدقة التقنية الجزيئية.

أجريت الدراسة في مركز دهوك للبحوث البيطرية في جامعة دهوك. تم جمع ستة وتسعون عينة دم من النعاج التي أجهضت خلال فترة الحمل في محافظة دهوك من الفترة أيلول ٢٠١٣ ولغاية أيلول ٢٠١٤. تم جمع العينات من عدة مناطق في المحافظة وتشمل منطقة عقرة، مركز مدينة دهوك، منطقة شيخان و منطقة زاخو.

بينت الفحوصات المصلية باستخدام فحص الأليزا الاثنان وعشرون (٢٢.٩١٪) عينة نتائج ايجابية، في حين أظهرت الفحص الجزيئي باستخدام تقنية PCR نتائج ايجابية لثلاثة و عشرون عينة (٢٣.٩٣٪). الفحص الجزيئي باستخدام تقنية PCR أجريت على جميع العينات بعد استخلاص المادة الحمض النووي من الدم مباشرةَ لغرض تضخيم جين B1.

هذه الدراسة أعطيت علاقة جيدة بين نتائج فحص ELISA و PCR. لم تكن هنالك أي اختلاف في دلالة الإحصائية بين الفحصين. نستطيع أن نلخص من هذه الدراسة بأن استخدام فحص ELISA مع تقنيةPCR هي الطريقة الأمثل لتشخيص المرض بصورة دقيقة ولكن بالرغم من ذلك تبقى تقنية PCR أكثر دقةَ لتحري عن *T. gondii* مع نسبة الحساسية تصل إلى (٩٥.٤٥٪) ونسبة الدقة تصل إلى (٩٧.٢٩٪). عندما تأخذ فحص (ELISA) كفحص موصى به، نتائج تقنية (PCR) أظهرت أهميتها في التشخيص في حالات المزمنة أكثر من نتائج الفحص المصلي (ELISA).