Molecular detection and seroprevalence of *Toxoplasmosis* in free range local chickens (*Gallus domesticus*) in Duhok province, Iraq

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(Received April 29, 2019; Accepted June 24, 2019; Available online 12 May 2020)

Abstract

Toxoplasmosis is a cosmopolitan zoonotic parasitic disease of mammals and birds; human infection occurs through consumption of raw or undercooked meat. Little was known about the infection rate of *T. gondii* among free range local chickens (*Gallus domesticus*) in Duhok province. Therefore, the present study was carried out to determine the infection rate in Duhok province by using ELISA (IgG) and conventional PCR. A total of 368 blood samples were collected from free range local chickens distributed in five different areas of Duhok province during the period from November 2016 to March 2017. The collected blood samples were from different sexes (hens and cocks) and from different age groups (less than 6 months and older than 6 months). The data found that the total infection rate was (84 / 368) 22.8% by ELSIA. The presence of the infection was confirmed by PCR and DNA sequencing. In this study, there were differences from area to area in the infection rates, the highest rate was reported in Semel district at 33.7% which was significantly ($p<0.05$) higher than 21.7%, 18.3%, 18.0% and 17.6% were reported in Akre, Shekhan, Amedi and Bardarash, respectively. However, no significant difference was found between age and sex groups, the results showed that hens and older chickens reported higher infection rates than cocks and younger age. The high prevalent of toxoplasmosis among free range chickens in Duhok province, it is highly recommended to follow strict hygienic measurements in order to minimize the role of chickens in transmission of infection to human.

Keywords: Chickens; PCR; ELISA; *Toxoplasma gondii*, Duhok
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الكشف الجزيئي والمصلي للخمج بداء القطط في الدجاج المحلي بالتربية المفتوحة (*Gallus domesticus*) في محافظة دهوك، العراق

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الخلاصة

داء المقوسات الكونديه مرض طفيلي مشترك واسع الانتشار في اللبائن والطيور، يصاب الإنسان عن طريق تناول اللحوم النية أو غير المطبوخة جيدا. هناك معلومات قليله حو نسبة الخمج بالمقوسات الكونديه بين الدجاج المحلي بالتربية المفتوح (كالاس دوميستيكس) في محافظة دهوك. لذلك أجريت الدراسة الحالية للتعرف على نسبة الخمج بالطفيلي في محافظة دهوك عن طريق فحص كشف الممتز للمستضد وفحص البلمرة المتسلسل. تم جمع ٣٦٨ عينة دم من ٥ مناطق مختلفة في محافظة دهوك من تشرين الثاني ٢٠١٦ الى اذار ٢٠١٧. تم جمع عينات الدم من كلا الجنسين (الدجاج والديكة) ومن مختلف المجاميع العمرية (اقل من ٦ شهور وأكبر من ٦ شهور). المعلومات وجدت أن نسبة الخمج الكلية كانت ٢٢,٨% (٨٤/ ٣٦٨) بالفحص الممتز للمستضد. ان وجود الخمج تم تأكيده بفحص البلمرة المتسلسل وتسلسل حامض الدنا. وجد في هذه الدراسة اختلافات في نسبة الخمج من منطقه الى أخرى وسجلت اعلى نسبة خمج في منطقة سيميل ٣٣,٧% وكانت معنويا اعلى من ٢١,٧، ١٨,٣، ١٨,٠، ١٧,٦% التي سجلت في عقره، الشيخان، العمادية، وبردرش على التوالي، بالإضافة لذلك لا يوجد فرق معنوي بين مجموعات العمر والجنس، وبينت النتائج بان الدجاج والأعمار الكبيرة اعلى نسب خمج من الديكة والأعمار

الصغيرة. واوضحت المعلومات بان الخمج بالمقوسات الكونديه مرتفعة بين الدجاج المحلي بالتربية المفتوحة في محافظة دهوك ولذلك ينصح بشكل كبير المتابعة بصرامة المعايير الصحية في سبيل تقليل دور الدجاج المحلي بالتربية المفتوحة لنقل الخمج للإنسان.

Introduction

Toxoplasmosis is a ubiquitous zoonotic disease caused by an obligatory intracellular protozoan parasite, *Toxoplasma gondii* (1,2). It has been reported that *T. gondii* infects all warm-blooded animals including birds (3). Detection of *T. gondii* in chickens is considered as an important indicator for the heavy contamination of the environment with the oocyst, the infective stage of the parasite shed within the feces of the definitive host, namely cat (4). The infection of chickens with T. gondii occurs through ingestion of contaminated food and water containing the infective oocyst (5). Studies found a strong relationship between the prevalence of toxoplasmosis among human and consumption of undercooked or raw meat of different sources (6-10). Infected chickens with *T. gondii* have important roles in the prevalence of toxoplasmosis among human and other animals (11). Increasing infection rates of *T. gondii* have been reported in free range local chickens (12). Different birds such as chickens, ducks, turkey and pigeons have been reported to be infected with *T. gondii* and are regarded as important sources for human infection (13,14). Studies demonstrated that different epidemiological risk factors such as age, sex, geographical distribution and the feeding pattern have significant impact on the infection rates of *T. gondii* in human and animals (9,15,16).

It has been found that the infection rate of *T. gondii* to be higher in hens than in cocks in a study done on free range chicken in Thika Region of Kenya (3), besides, the same study showed a higher infection rate in older chicken than that of younger age. Furthermore, differences have been reported from area to area in infection rate of *T. gondii*; higher infection rate of the parasite was found in free range chicken in china when compared to the scales farms, the conventional chickens (17). However, *T. gondii* infection has reported in house kept chickens in Sulaimania province (18).

To our best knowledge the infection rate of *T. gondii* in free range local chicken by using ELISA and PCR has not been investigated before in any province of Kurdistan Region - Iraq Therefore, the present study was set to determine the infection rate of *T. gondii* in free range local chickens in Duhok province represented by five different districts and to understand to what extend the disease is distributed in these areas among chickens.

Materials and methods

Blood samples

A total of 368 blood samples were randomly collected from free range local chickens (*Gallus domesticus*) of different districts (Shekhan, Bardarash, Akre, Semel and Amedi) from Duhok Province-Iraq during November 2016-March 2017. Both gender and age groups were considered in this study.

ELISA

To determine the infection rate of *T. gondii* in free range local chickens in Duhok province-Iraq, blood samples were collected via wing vein into plane tubes. The collected blood samples were divided into two parts, first left at room temperature for 30 minutes to form clot and subsequently centrifuged for sera isolation and the second were collected in EDTA tubes and kept at -20ºC for DNA isolation. The collected sera were decanted into micro tubes and stored at-20ºC until tested by ELISA. A competitive ELISA was used to detect IgG antibodies using commercial ELISA kit (ELISA, ID Vet Innovative Diagnostic, France). The optical densities of the reactions were determined according to the manufacturer's instructions by measuring the absorbance at 450 nm using a micro plate reader (Bio-Tek- USA). The results are expressed as (S/P %) percentage of the mean absorbance calculated according to the following formula:

$$S/P\% = (OD\ sample-ODNC)/(ODPC-ODNC)\times 100.$$

Depending on manufacturer's recommendation sera were regarded negative when S/P% < 50% and positive when S/P% ≥ 50%.

DNA isolation

The whole blood samples were collected in EDTA tubes and kept at -20ºC as previous described. Total DNA was extracted from blood samples using a commercial kit (GeNet Bio, Korea) following the manufacturer's instructions. The extracted DNA samples were stored at -20 ºC till used.

Molecular characterization and identification of *T. gondii* by PCR

PCR was performed by amplification of the *B1* gene for detection of *T. gondii* in blood samples. The primers, targeting the *B1* gene, were used according to (19), the forward TOX4 (CG CT GC AG GG AG GA AG AC GA AA GT TG) and the reverse TOX5 (CG CT GC AG AC AC AG TG CA TC TG GA TT). The PCR reactions were done in a final volume of 25µl. The reaction mixture contained 12.5 ul, 2X HS Prime Taq Premix mastermix (G-7100, GeNet Bio, Korea). 1 µl of 10 pmol of each forward and reverse primers, 2 µl DNA template (50-300ng/ µl), and 8.5 ul RNase free water to a total volume of 25 ul DNA. The cycler condition

of PCR was set up according to Homan *et al.* (19) as detailed in table 1.

Finally, 10µl of PCR products were electrophoresed on 1% agarose gel with 85 Volts for 45 minutes and visualized under UV.

Table 1: The cycler condition for B1 gene

The process	°C	Time	No. cycle
Denaturation	95	4 minutes	1
Denaturation	95	45 s	35
Annealing	55	45 s	35
Extension	72	45 s	35
Extension	72	5 minutes	1

Sequencing of a partial B1 gene fragment

The PCR products of five samples were selected randomly and sent for sequencing with the primers Toxo4 and Toxo5. The sequencing was performed at Macrogen Company, Korea. The sequences were analyzed, checked and aligned using BioEdit sequence alignment editor 7.0.0 (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA). The sequence was submitted to GenBank (Genbank accession MK704514). The similarity of the sequence with homologous sequences deposited in GenBank was calculated using the "BLAST" tool on the National Center for Biotechnology Information (NCBI) website.

Statistical analysis

The Chi squared test was used to determine the association between the variables and infection and a P value < 0.05 was considered statistically significant.

Results

PCR

For detection the presence of *T. gondii* in blood of free-range local chickens in Duhok province, the whole blood from ELISA seropositive chickens (only 35 seropositive randomly selected from 84 were tested for targeting *B1* gene by using conventional PCR. The results revealed that 5 out of 35 at rate 14.29% were positive and clearly showed amplicon size of 529 bp, (Figure 1).

The DNA sequence was aligned to NCBI and it was 100% identical and similar to (KX270388). Then the sequence was submitted to the GenBank, and was assigned an accession number "MK704514" (Figure 2).

ELISA

In total, the data of the present study found that 84 out of 368 tested sera from free range local chickens reacted positively for anti *T. gondii* IgG antibodies and revealed a seropositive rate at 22.8%. The infection rates of *T. gondii* were detected in different districts of Duhok province. The reported data revealed that the infection rates were district to district different, where the highest infection rate was reported in Semel at 31 (31.7%) followed by Akre 20 (21.7%), Shekhan 11 (18.3%), Amedi 9 (18%), and the lowest infection rate was in Bardarash at 13 (17.6%). Statistically, the reported rate in Semel was significantly P<0.05 higher than that were reported in the others districts, (Table 2).

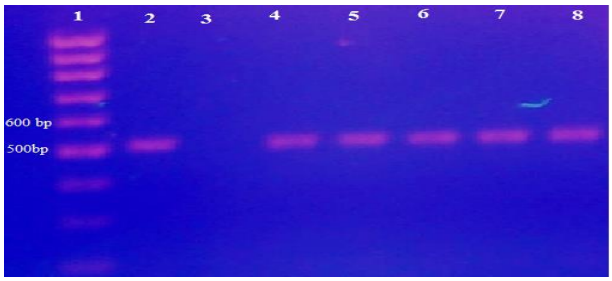

Figure 1: PCR products of *T. gondi* on 1% agarose. Lane 1 100 bp (GeNet Bio, Korea) ladder, lane 2 positive control (previously isolated strain from Dicla university, Diyarbakir/ Turkey), lane 3 negative control and lanes 4-8 tested samples animals.

Toxoplasma gondii glycerol-3-phosphate dehydrogenase (B1) gene, partial cds; mitochondrial

GenBank: MK704514.1

FASTA Graphics

Go to:

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REFERENCE   1  (bases 1 to 495)
  AUTHORS   Mikaeel,F.B. and Mohammed,A.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-MAR-2019) Pathology and Microbiology, University of
            Duhok, Chanden Street 1, Duhok, Duhok 42001, Iraq
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Figure 2: Accession number in the GenBank

The effect of sex and age of tested chickens on the infection rates of *T. gondii* was detected in this study. Despite no significant difference was found between age and sex groups, results showed that hens and older chickens

reported higher infection rates than cocks and younger age and the rates were 40 (47.6%) and 44 (52.4%), respectively. Regarding to age groups, the infection rates was higher in chickens older than six months 46 (54.8 %) compared to 38 (45.2 %) in younger chickens of less than six months old, (Table 3).

Table 2: Infection rates of *T. gondii* in free range local chickens in different districts in Duhok province, Iraq

Districts	Seropositivity No. (%)		p-value
	Positive	Negative	
Semel	31 (33.7%)	61 (66.3%)	0.05* Significant
Akre	20 (21.7%)	72 (78.3%)	
Shekhan	11 (18.3%)	49 (81.7%)	
Amedi	9 (18%)	41 (82%)	
Bardarash	13 (17.6%)	61 (82.4%)	

Table 3. Infection rates of *T. gondii* in free range local chickens in different location of Duhok province, Iraq based on sex and age of tested chickens

Chicken	Seropositivity No. (%)		p-value
	Positive	Negative	
Gender			0.542* Non Significant
Cocks	40 (47.6%)	146 (51.4%)	
Hens	44 (52.4%)	138 (48.6%)	
Age			0.976* Non Significant
< 6 months	38 (45.2%)	129 (45.4%)	
> 6 months	46 (54.8%)	155 (54.6%)	

Discussion

Toxoplasmosis is considered as important zoonotic diseases and increasing attention has been paid to understand the main sources for the human toxoplasmosis (20).

It has been reported that human become infected with *T. gondii* through ingestion of contaminated food and drinking water containing infective oocyst or consuming undercooked meat containing tissue cysts, tachyzoites and/or bradyzoites (20).

Due to the increasing in popularity of consuming free-range local chicken in Duhok province and due to the exacerbated human toxoplasmosis, namely in pregnant women; the present study was set to determine to what extend *T. gondii* is present among free range local chicken of different areas in Duhok province.

The current study for the first-time used ELISA anti *T. gondii* IgG antibodies to detect the infection rate of *T. gondii* in free range local chickens in Duhok province. The data revealed that the overall infection rate was 22.8%. It is hard to find a work done on *T. gondii* infection in free range local chickens in Iraq used ELISA and PCR to compare our data with. However, a higher infection rate at 60% of *T. gondii* in domestic chickens was reported by (18) in Sulaimani province. The differences between the two studies could be due the different techniques were used in these two studies, or could be due to the number of the tested samples, where in this study tested 368 by ELISA IgG compared to 65 sera were tested by (18) using latex agglutination test (LAT). The increasing infection rates of *T. gondii* in free range local chickens is believed to be due to the feeding pattern of the bird, where birds that feed from the ground directly are subjected to food contamination especially *T. gondii* oocyst, and can be regarded as important indicators for the environmental contamination and source for human infection (21). In addition, it has been reported that cats could enhance the chickens' toxoplasmosis through their defecation on grass where chicken fed on (3).

To confirm the ELISA results and the presence of *T. gondii* infection, PCR was done for some of ELISA seropositive samples that were randomly selected. The data found that *B1* gene targeting *T. gondii* DNA was only amplified in 14.29% of tested blood. The low infection rate by PCR compared to the ELISA results is suggested to be due to the absence of the parasite in the blood at the collection time and the parasite has already localized within the body as a tissue cyst, tachyzoites and/or bradyzoites.

This study found differences in the infection rates from area to area, where the highest rate was reported in Semel and the lowest rate was in Bardarash. The reported differences are assumed to be due to multi factorials such as variation in the densities of stray and domestic cats present within the area, number of free-range local chickens, and feeding pattern. Higher infection rates of *T. gondii* in free range chickens were reported in Kenya in areas where great densities of stray cats and overcrowded chickens per farms where found (3). Besides, evidence found that infection rate of *T. gondii* in free range chickens of different part of the world range from 2%-100% (21). The great variations was found as a result of differences in climate changes which has great impact on the lifespan of oocysts, or differences in the used diagnostic techniques, the kind of the samples were depended and seasonality (22-24).

The present study has also investigated the infection rate of *T. gondii* among different sex groups; the data found that the infection rates were lower in cocks than that of hens. These data are in line with (23,25).

It has been reported that the female hormones play an important role in the susceptibility of the animals to *T. gondii* through reducing their immune responses to the infection (26,27).

The infection rate of *T. gondii* among different age groups of free-range local chickens was also detected in this study; the reported data found that chickens of older age showed higher infection rates than younger chickens. This variation could be directly related to the exposure times

which increased with age; thus, the older animals have more chance to be infected than younger ages. It has been showed that *T. gondii* infection increased with age (28).

Conclusion

Taken together, the present work found high infection rate of *T. gondii* in free range local chickens in Duhok Province and this indicative for heavy environmental contamination with parasite. Because of the high prevalence of toxoplasmosis among free range chickens, it is highly recommended to follow strict hygienic measures in order to minimize the role of chickens in transmission of infection to human. Furthermore, more works are needed to do to examine chicken's meat and other birds such as quails, ducks, turkeys and partridges. For further future work on *T. gondii*, it is recommended to inoculate the pathogen to laboratory animal by using this positive chicken blood. As to confirm that an active circulating parasitic stages.

Acknowledgment

My thanks and appreciation to all stuff of Duhok Research Center and College of Veterinary Medicine for their help during research work.

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