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RESEARCH ARTICLE

Immuno-Molecular Diagnosis of *Toxoplasma gondii* in Blood and Meat of Indigenous Chickens in Some Rural Regions of Iraq

Amal Hassan Abd Al-Shabbani

Clinical Laboratory Sciences, College of Pharmacy, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.

Abstract

Herein the object was to detect the prevalence rate of the parasite *Toxoplasma gondii* (*T. gondii*) in blood and meat samples of indigenous chickens. Total of 92 chickens of both sexes of more than one year old were selected from some rural areas of Iraq during a period of February to August / 2017 and tested by using indirect-ELISA and PCR techniques. Overall results showed that 19.57% and 14.13% of sera and meat samples respectively were seropositive for anti-*T. gondii*-antibodies. In meat samples, the sero-prevalence was 7.61% and 14.13% in tissues of skeletal and cardiac muscles respectively. Regarding PCR technique, the total results showed that 5.44% and 1.09% of blood clots and meat extracts respectively were positive for DNA reaction of *T. gondii*. In addition, although the PCR results were found to be positive in cardiac muscles (1.09%) only, no significant differences had been detected regarding the results of skeletal muscles that lack positive cases. According to the diagnostic assays, significant differences in positive results were reported between indirect-ELISA and PCR technique among the tested samples of sera and meat as well as skeletal and cardiac muscles.

Keywords: *Toxoplasma gondii, Chickens, Blood, Meat, Serology, Molecular, Iraq.*

Introduction

T. gondii is an obligatory intra-cellular protozoon of human and virtually most warm-blood animals, which classified as a singular coccidian species in *Toxoplasma* genus that belongs to *Apicomplexa* phylum and results in toxoplasmosis [1, 2]. Since first described in a small rodent (*Ctenoductyalus gondii*) in 1908, it's recognized progressively to be having an important food-borne widespread zoonosis globally [3]. Cats are recognized as the only final host to excrete the parasite resistant stage (oocysts) with their feces [4].

Human infection can be acquired due to ingestion of products contaminated with oocysts or tissue-cysts persist throughout under-cocked and row meat of field livestock [5]. Although, tissue-cysts represented final stages of life-cycle, about 30-60% of infections are resulted by consuming meat products [6].In most livestock, *Toxoplasma* cysts have high affinity to be located predominantly in heart, skeletal muscles, eye, and central nervous system; with lesser extent in visceral organs like kidney, liver, and lungs [7]. As well as, the parasite can be persisted at all host's age to increase the risk of infection when human consume meat products that are contaminated [8]. As chickens, birds are playing a great influence in *T. gondii* epidemiology acting as a reservoir for transmission of the parasite, not only for human, but also for cats that in turned shed oocysts into their surrounding environments [9]. Among all domestic animals, small ruminants are the main animals that showing clinical symptoms during acute or chronic infections with *T. gondii*, associated particularly with reproductive problems [10].

Many serological techniques were applied to evaluate *Toxoplasma* infection in poultry involving ELISA, indirect immunofluorescence antibody test (IFAT) and modified agglutination test (MAT) [11]. In a previous study performed by [12] on meat juice, a high degree of compatibility and agreement has been shown between the results of MAT and ELISA. However, ELISA is preferred in most studies due to the high sensitivity and specificity, simplicity, and accuracy to read the results as it is automatic [13]. Nowadays, PCR technique is used in most cases to detect the sequences of *T.*

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gondii tachyzoites, especially because it demonstrates high specificity and sensitivity in detection DNA of *T. gondii* in blood, and tissues [14] in experimentally and naturally infected animals [15].For first time in Iraq, the goals here was to assess spread of specific *T. gondii* among blood and meat of cardiac and skeletal muscles of indigenous chickens resident in some rural regions by using two of a commercially available and reliable diagnostic techniques; ELISA and PCR tests.

Materials and Methods

Study Samples

During a period of February to August 2017, an overall 92 of indigenous chickens, from both sexes with >1 year old, were sampled from some rural areas of Iraq. The samples of blood collected directly from the vein of wing by an EDTA-anticoagulant vacuum-tube that transported to laboratory and centrifuged at 4000 rpm for10 minutes. Sera were obtained and saved into numbered 0.5ml eppendorf microtubes for serological assays, whereas, clots kept into their tubes for later DNA isolation, and both samples were kapt at-20°C [16].

In addition, heart and skeletal muscles were collected from slaughtered chickens within individually numbered containers, which first frosted for a whole day at -20°C, then thawed at 20°C for 12 hours for releasing of meat juices that leaked from the tissues. Then, the tubes of meat juice were centrifuged for 2 minutes at 3000 rpm (to remove debris), and then, the supernatants were stored, for serological analyses, at -20°C, whereas, the residual tissues were kept at same degree for DNA extraction [17, 18].

Serological Examination

ID Screen® ELISA test kit that is available commercially (*IDVET Innovative Diagnostics, France*), designated for indirect detecting of specific antibodies in ruminants, dogs as well as cats, was applied in this study on chickens with some adaptations. As described by [19], the conjugate of the test kit was replaced by a whole molecule anti-chicken IgG peroxidase conjugate (*Sigma Aldrich, Germany*), whereas, the samples and positive control were diluted at 1:15 and 1:40, respectively.

According to manufacturer instructions, ELISA records were recorded after adding stop solution at 450 nm of optical density (OD) by using a microplate spectrophotometer (*Bio Tek, USA*), and the obtained results were expressed according to the following formula: "[Sample/Positive percentage (S/P%) = $OD_{Samples} - OD_{Negative\ Controls} \times 100 / OD_{Positive\ Control} - OD_{Negative\ Control}$]. The positive cutoff was established as [5× mean ± standard deviation (SD) of mean of all tested samples], and the values were considered positives when S/P ≥ 0.62" [20].

Molecular Confirmation

First, tissues of skeletal and cardiac muscles were sectioned, homogenized, dipped in 70% ethanol alcohol to remove lipid as well as connective tissue, and then mingled with 50µL phosphate buffered saline. Then, 200 µL from each sample, clots and tissues, were used for DNA extraction that applied by a commercially DNAeasy blood and tissue kit (*Qiagen, Germany*).As described by [21], DNA was amplified at 529 base pair fragment specific for *T. gondii* DNA using the primers as follow: Tox4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and Tox5 (5'-CGC TGCAGACACAGTGCATCTGGATT-3').

The PCR reaction processed using of thermal cycler (*MJ-BIO RAD, USA*) following the manufacturer directions. The products from each PCR were then ran through electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and then visualized under an ultraviolet transluminator.

Statistical Analysis

All results were introduced for computer to be arranged and tabled by Microsoft Office Excel (*2007*), and analyzed with descriptive statistics and Chi-square (x^2) test through IBM/SPSS program (v^{23}). At a level of P≤0.05, the significant differences were measured between the total positive ELISA and PCR results of sera and tissues, as well as, within the positive results of both diagnostic tests [22].

Results

Of 92 indigenous chickens, the results for examination 92 of sera and meat juice samples from each one by an indirect-ELISA for IgG-antibodies anti- *T. gondii* were revealed, (Table/ 1).

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Table 1: Serological results of sera and meat juice by indirect-ELISA

	Sample	Total No.	Seropositives	Seronegatives
1	Sera	92	18 (19.57%) A	74 (80.43%)
2	Meat Juice	92	13 (14.13%) B	79 (85.87%)

Variation in large letters, vertically, refer to significant difference

Meat juice results of skeletal and cardiac muscles are shown, (Table 2).

Table 2: Serological results of meat juice by an indirect-ELISA

	Meat Juice Sample	Total No.	Seropositives	Seronegatives
1	Skeletal muscle	92	7 (7.61%) B	85 (92.39%)
2	Cardiac muscle	92	13 (14.13%) A	79 (85.87%)

Variation in large letters, vertically, refer to significant difference

PCR results of clots and meat crude that were positives for *T. gondii* DNA are shown in (Table 3).

Table 3: Molecular results of clots and meat crude samples by PCR

	Sample	Total No.	Seropositives	Seronegatives
1	Clot	92	5 (5.44%) A	87 (94.57%)
2	Meat Crude	92	1 (1.09%) B	91 (98.91%)

Variation in large letters, vertically, refer to significant difference

PCR results of meat crude seropositive samples are shown in (Table/ 4).

Table 4: Molecular results of meat crude samples by PCR

	Meat Crude Sample	Total No.	Seropositives	Seronegatives
1	Skeletal muscles	92	0 (0%) B	92 (100%)
2	Cardiac muscles	92	1 (1.09%) B	91 (98.91%)

Variation in large letters, vertically, refer to significant difference

PCR products for identification of represented positive samples with *T. gondii* DNA showed by electrophoresis as explained previously (Figure 1). Lane M: DNA marker (100-1500 bp); Lane P: positives control; Lane N: negatives control; Lanes 1 to 5: positive clot samples; and Lane 6: positive cardiac muscle.

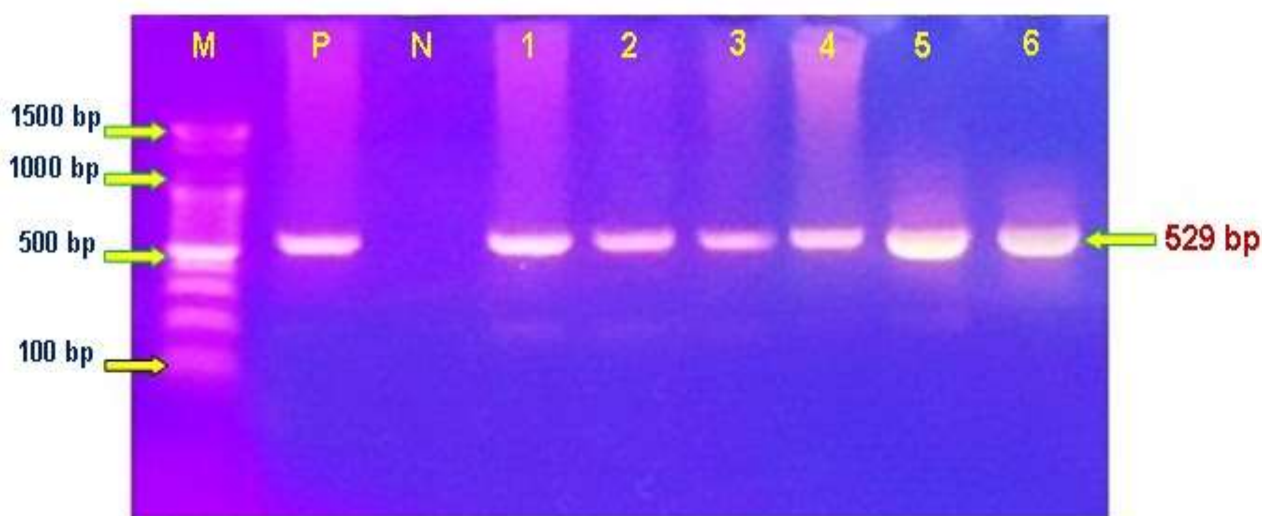

Figure 1: PCR products of positive samples by electrophoresis

Results of indirect-ELISA and PCR reported significant differences (P≤0.05) when compared, respectively, among sera and meat juice as shown in (Table 5); as well as among skeletal and cardiac muscles as in (Table 6).

Table 5: Positive results of sera and meat by ELISA and PCR

	Sample	Total No.	ELISA	PCR
1	Sera	92	18 (19.57%) a	5 (5.44%) b
2	Meat	92	13 (14.13%) a	1 (1.09%) b

Variation in small horizontal letters means significant differences at P≤0.05

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Table 6: Positive results of skeletal and cardiac muscles by ELISA and PCR

	Sample	Total No.	ELISA	PCR-
1	Skeletal muscles	92	7 (7.61%) [a]	0 (0%) [b]
2	Cardiac muscles	92	13 (14.13%) [a]	1 (1.09%) [b]

Different small horizontal letters means significant variations (P≤0.05)

Discussion

Till yet, this study is the pioneer to report the prevalence of *T. gondii* in blood and meat juice of indigenous chickens in some rural regions of Iraq. Two reliable diagnostic assays; indirect-ELISA and PCR tests, were applied on blood and meat of skeletal and cardiac muscles. Totally, the results of indirect-ELISA of meat juice samples were seropositive.

Our results supported by a previous study that showed that there was a strong positive correlation in specific antibody titers of serum and heart meat juice [23]. However, high levels of antibody in sera compared to meat juice might lead to conclude that these antibodies might have been originated from parasitized blood and not from heart or skeletal muscles. Also, the concentration of antibody in meat juice may be influenced by other factor called WHC (water-holding capacity) of muscles [24].

After slaughtering, the pH of muscles gets dropped reducing the WHC and increasing amounts of meat juice, which resulted in decreasing of antibody levels [25]. Nonetheless, post-mortem exchanges can be varied, greatly, among slaughters (animals, muscles, parts of muscles) depending on genetics, handling of animal pre-slaughters, processing of meats, and complications that occurred during serology of meat juice [18]. Among samples of meat juice, considerable ratios of skeletal and cardiac muscles appeared seropositives with specific antibodies against toxoplasmosis.

These results are in compatible with others [18, 26] as the highest seropositive were reported in hearts, and this significant differences might be related to different sources of skeletal muscles. In chickens, a study [27] referred to that the best rates of *T. gondii* recovery can be obtained by collection of the hearts of chickens as the parasite is localized, mostly in muscles of the heart more than skeleton or brain.

In most Iraqi rural areas, the indigenous chickens are persisting usually in localities where a variety of animal species (cattle, sheep, goats, dogs and cats) are reared. At similar environment, the rates of contamination and transmission of infection were increased obviously [28]. Also, the quantity of *T. gondii* oocysts that shed by dogs or cats in nature is vague, and some species of animals can act as reservoirs for certain pathogens [29].

Globally, the frozen imported meats are not critical in epidemiology of *T. gondii* due to the fact that freezing eliminate this protozoan [6]. Whereas broiler chickens that reared in poultry industry for meat production typically take less than six weeks to reach slaughter size, and during this time they received a high controlling and therapeutic attention that reducing the risk of *T. gondii* contamination and/or transmission [29].

However, the majority of risks to consumers were believed to result from products of chickens that persist as freshly meats [30]. In United States, a study found that about 90% of health burdens are caused mainly by five pathogens that involved only one parasite, *T. gondii*; and 30-63% of human toxoplasmosis was related to meat-borne source [31]. The risk of infection from cooked meat in human society is lower since high temperature eliminate *T. gondii* cysts in tissues [29].

In a previous report, it was validated that the ELISA has 88.6% and 98% of sensitivity and specificity respectively, during examination of sera samples of infected pigs with *T. gondii* [32]. It was revealed that using tissue fluids instead of serum samples lower the sensitivity to 60%, and this could be added to the diluting impact of the enhancement solutions, and this might affect the detection of anti-*Toxoplasma* antibody [29].

PCR-dependent protocols are invented to identify *T. gondii* DNA in blood and meat with feasibility to be alternative for bioassay in detecting and genotyping of the parasite [33]. PCR is high sensitive, specific, cheap, and rapid test that can provide species-specific markers for detecting of *T. gondii* straight from biological samples [34]. The examination of blood and meat samples by

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PCR revealed positive samples with *T. gondii* DNA. Also, only mild ratio of cardiac muscles was found positives.

These results do not agree with [23] that stated that the cardium is one of the most tissues that are parasitized by *T. gondii*, especially in chronic infection in chickens. Experimentally, a study stated that the parasite might be found in tissues and blood of mice after acute infection [35]. Also, the study concluded that the parasite can be diagnosed by PCR in blood samples after two days of infection and in tissues after 15 days of post-infection [36].

While, others found very low DNA detection results in experimentally infected chickens which might refer to the short period of the parasite presence in this species [37].However, performing of PCR on blood can also be beneficial due to the less interfering materials that can be available [38]. Regarding of diagnostic test, the results of indirect-ELISA and PCR reported that some of sera and meat samples showed positive results. In meat tissues, whereas 7.61% of skeletal muscles were seropositives, 14.13% and 1.09% of heart muscles were positive by indirect-ELISA and PCR.

However, sensitivity of serum ELISA may reach 100% and meat juice-ELISA 76.9% [15]. Some studies showed that the experimentally infected animals were appeared positives by ELISA after 24 hours of infection, whereas by PCR they were diagnosed as positives 18 hours post-infection. This means that the amplification of B1 gene by PCR is more accurate than ELISA immunoblotting and cell culture [23]. Nonetheless, detection of *T. gondii* DNA in meat samples has shown to be less accurate than ELISA as using small samples of meat (50-100g) are for DNA extraction might contain few tissue cysts.

This may explain why big ratios of these samples were positives by ELISA and negatives by PCR [33]. In addition, the utilizing of blood as samples for detecting of *T. gondii* DNA, particularly during acute phase of infection, appeared to be better than serological ways because of the high sensitivity of PCR and the direct demonstration of the parasite [36].

In conclusion, the study demonstrated that there were significant differences among results of sera and meat samples, and cardiac and skeletal muscles. However, meat juice can be used in addition to blood samples in large-scale investigations of toxoplasmosis, and the concentration of antibodies in meat juice can depend on which muscle is used for the examination. Also, the usage of developed ELISA in slaughtered chickens would be beneficial due to the importance of chickens in *T. gondii* epidemiology mostly as a substantial factor in risk of zoonotic infection and food safety.

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