

## Detection of *Toxoplasma gondii* Antibodies in various Poultry Meat Samples using Enzyme Linked Immuno Sorbent Assay and its Confirmation by Polymerase Chain Reaction

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This study was conducted to determine the presence of *Toxoplasma gondii* in poultry meat samples in Iran. A total of 55 chickens, 30 partridge, 50 quail, 28 ostrich and 25 turkey raw meat samples were collected from retail outlets in Isfahan, Iran. All samples were homogenized and then an indirect ELISA technique was performed for detection of antibodies against *T. gondii*. Finally, all positive and suspected samples which were diagnosed by ELISA methods were analyzed using PCR assay. From a total of 188 meat samples, 28 (15.42%), 20 (10.63%) and 140 (74.46%) samples were positive, suspected and negative using ELISA method, respectively. From a total of 48 positive and suspected ELISA results, 38 (79.16%) were positive and 10 (20.83%) were negative for presence of *T. gondii* using PCR method. Turkey had the highest while quail had the lowest prevalence of *T. gondii* in poultry meat samples. From a total of 38 positive samples, 5 (13.15%), 20 (52.63%), 8 (21.05%) and 3 (7.89%) samples were positive from samples which were collected in spring, summer, autumn and winter seasons, respectively. Statistical analyses were significant between the prevalence of *T. gondii* in various seasons and poultry meats.

**Key words:** *Toxoplasma gondii*, poultry meat, indirect ELISA, PCR, Iran.

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Poultry meat is raised as a complete food especially for adolescents. Its high value for proteins, minerals, fats and vitamins is undeniable. In a day, millions of people use from the poultry meat and its products. Therefore, hygienic quality of poultry meat has a high importance in public health but sometimes it will be changed and several infections and illness are occurred. Despite all accuracy in meat inspection, it can be contaminated with some microorganisms which are asymptomatic and microscopic. Toxoplasmosis is probably the

most widespread zoonotic disease in the world. Disease is caused by an intracellular protozoan parasite called *Toxoplasma gondii* (*T. gondii*) which can infect all mammals and birds species throughout the world. Based on the geographic location, 15 to 85% of the human population is asymptotically infected with *T. gondii*<sup>1</sup>. It is estimated that more than 60 million people in the US carry the *Toxoplasma* parasite, with 30 to 50% of the US population having antibodies to *T. gondii* and at least 30% of people in the world are infected with the organism<sup>2</sup>, with a higher prevalence of infection in France, 60 to 90% of the population are serologically positive<sup>3</sup>. In addition, *T. gondii* being the third leading cause of foodborne death in the United States<sup>4</sup>. The

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contaminated meat contain tachyzoites and several studies showed that consumption of raw infected meat can cause toxoplasmosis in human<sup>5-12</sup>.

The *T. gondii* infection in humans may occur vertically by tachyzoites that are passed to the fetus via the placenta, or horizontal transmission which may involve three life-cycle stages i.e. ingesting sporulated oocysts from cats or ingesting tissue cysts in raw or under cooked meat or tachyzoites in blood products or primary offal (viscera) of many different animals, tissue transplants, and unpasteurized milk<sup>6</sup>. The *T. gondii* tachyzoites appear ovoid, 2–6 µm long, with nuclei that are moderately basophilic and located centrally or towards the posterior end. Felines are considering as definitive hosts of *T. gondii*. They are infected by eating the contaminated tissues of infected intermediate hosts. Poultry are considering as one of the most important intermediate host for *T. gondii*. The *T. gondii* infection is very important in birds and especially in free-living birds. Free-living birds are known as the best indicator for soil contamination with *T. gondii*'s oocytes. Free-living birds make their own foods from the earth and carcasses of infected birds are the good source of infection for cats. Besides, consumption of contaminated poultry meat is the source of infection for human and many species of animals. Ironically, the clinical signs of toxoplasmosis are rarely seen in poultry. Previous studies showed that freezing of contaminated foods cannot be effective for eliminate or even reduce the levels of *T. gondii* infection<sup>10,13,14</sup>.

In addition to asymptomatic infection of *T. gondii* in poultry meat, its complications including abortion and congenital disorders in animals and human and finally the high resistance of this parasite to hard conditions like freezing, the high seroprevalence rate of toxoplasmosis has been report frequently. Study in La plata showed that from a total number of 29 chicken meat samples, 19 samples (65.51%) were infected with *T. gondii* using Modified Agglutination Test (MAT)<sup>15</sup>. The higher seroprevalence rate of *T. gondii* in poultry meat samples has been reported from Amazon (66%)<sup>16</sup>, Guatemala (74%)<sup>17</sup> and Nicaragua (85.7%)<sup>18</sup>.

Poultry meat is one of the most popular dishes in Iran but the epidemiology and prevalence of this parasite is essentially unknown in chicken,

partridge, quail, ostrich and turkey meat in Iran. Therefore, this present study was carried out in order to study the prevalence of *T. gondii* in chicken, partridge, quail, ostrich and turkey raw meat samples in Iran.

## MATERIALS AND METHODS

### *Samples collection and preparation*

In this study, which was conducted from March 2011 to March 2012 (in various seasons of the year), a total of 55 chicken, 30 partridge, 50 quail, 28 ostrich and 25 turkey raw meat samples were collected randomly from retail outlets in Isfahan, Iran. Before collecting poultry muscle meat samples, the external surfaces were disinfected with 70% alcohol to minimize surface contamination. Separate 10g breast muscle samples were collected using sterile scissors and tissue forceps. Samples were collected under sterile hygienic conditions and were immediately transported to the Food Microbiology Laboratory, Islamic Azad University, Shahrekord Branch, Iran at 4°C in a cooler with ice packs. Meat samples were homogenized in 3 ml of sterile phosphate-buffered saline (PBS) (pH 7.2) by using a mortar and pestle. The poultry which their meat samples collected for this study were clinically healthy and the meat samples showed normal physical characteristics. All meat samples were kept at –20 °C until processing.

### **Indirect ELISA test**

An indirect ELISA was carried out for the detection of IgG antibodies to *T. gondii* as described by Mineo *et al.* (1980)<sup>19</sup>, with some modifications. Briefly, 96 well ELISA kit (POURQUIER Company) previously coated with  $1 \times 10^5$  *Toxoplasma* tachyzoites/well was washed three times in PBS containing 0.1% Tween 20 (PBS-T). Diluted meat samples were added (50 µl/well) and incubated at 37°C for 45 min. Positive and negative meat controls previously determined by conventional serological tests (i.e., IHA and IFAT) were included on each plate. Washings in PBS-T were made between the steps of the reaction. Further, peroxidase-labeled rabbit IgG anti-goat IgG (prepared as described by Wilson and Nakane 1978) was added (100 µl/well) at 1:3,000 dilution in PBS-T for 1 h at 37°C. Next, enzyme substrate consisting of 0.03% hydrogen peroxide and orthophenylenediamine (OPD - Merck, Germany) in

0.1M citrate- phosphate buffer, pH 5.0 was added (50 µl/ well) and incubated for 10-15 min at room temperature. The reaction was stopped by adding (25 µl/well) 2N H<sub>2</sub>SO<sub>4</sub> and the absorbance was determined in ELISA reader (Molecular Devices, Sunnyvale, CA) at 492 nm. This test is valuable when:

$$OD_{pc>0.350} \cdot OD_{pc} / ON_{NC>\frac{3}{5}}$$

For each meat samples the SP value was determined as follow:

$$S/P = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}}$$

Finally, the results of ELISA test were achieved according to below points:

S/P ≤ 40%: Negative sample; 40% ≤ S/P ≤ 50%: Suspected samples; S/P ≥ 50%: Positive sample

**DNA extraction and PCR**

DNA was extracted from positive and suspected meat samples using the QiagenQIAamp System (Qiagen, Valencia, CA), which specifically binds nucleic acids to a silica-gel membrane in a centrifuge compatible spin column. Contaminants and PCR inhibitors are washed through, and DNA is eluted using 70 °C water. A sample volume of 200 µL was used per extraction. Positive and suspected meat samples which were diagnosed using indirect ELISA were tested for *T. gondii* with PCR technique (20). A 193-base-pair product of the 35-fold repetitive B1 gene of the RH strain of *T. gondii* was amplified using the following primers. TX2 5'-3' (TCTTTAAAG CGTTCG TGG TC) and TX4 5'-3' (GGA ACT GCA TCC GTT CAT GAG) (Burg *et al.* 1989). The amplification reactions were performed using Amplitaq DNA polymerase, Gene Amp dNTPs (deoxynucleoside triphosphates) with dUTP, and AmpErase UNG (all from Perkin Elmer, Foster City CA). The PCR-amplified products were

examined by electrophoresis in a 1.5% agarose gel, stained with a 1% solution of ethidium bromide, and examined under UV illumination. In this study, *T. gondii* DNA and DNase free water were used as the positive and negative controls, respectively.

**Statistical analyses**

Differences in the seroprevalence of *T. gondii* in various poultries were analyzed using a Chi square test in SPSS for Windows (Release 18.0 standard version, SPSS Inc., Chicago, Illinois). The differences were considered statistically significant when P<0.05.

**RESULTS**

In this present study from a total of 188 poultry meat samples which were tested for presences of antibodies against *T. gondii* by ELISA method, 28 (15.42%), 20 (10.63%) and 140 (74.46%) samples were diagnosed as positive, suspected and negative samples, respectively (Table 1). All positive and suspected meat samples were tested using PCR assay. The results showed that from a total of 48 positive and suspected samples which were diagnosed by ELISA method, 38 samples (79.16%) were positive by PCR technique (Table 2). In the other hand, 38 out of 188 poultry meat samples (20.21%) were diagnosed as positive using ELISA and PCR techniques. Our results showed that all of the 28 positive samples which were diagnosed by ELISA method were confirmed as positive by PCR. In the other hand, from a total of 20 suspected samples, the PCR technique was able to detect the 10 samples as positive.

Results showed that turkey (36%) had the highest while quail (12%) had the lowest prevalence of *T. gondii* in poultry meat samples

**Table 1.** Distribution of *T. gondii* in various poultry meat samples using antibody detection ELISA test

Meat samples	No. of meat samples	No. of positive samples (%)	No. of suspected samples (%)	No. of negative samples (%)
Chicken	55	9(16.36)	8(14.54)	38(69.09)
Partridge	30	3(10)	3(10)	24(80)
Quail	50	5(10)	2(4)	43(86)
Ostrich	28	6(21.42)	-	22(78.57)
Turkey	25	5(20)	7(28)	13(52)
Total	188	28(15.42)	20(10.63)	140(74.46)

**Table 2.** Application of PCR in order to confirm the ELISA results.

Meat samples	No. of positive and suspected samples in ELISA test	No. of positive samples in PCR technique (%)	Total positive samples
Chicken	17	13(76.47)	13(23.63)
Partridge	6	4(66.66)	4(13.33)
Quail	7	6(85.71)	6(12)
Ostrich	6	6(100)	6(21.42)
Turkey	12	9(75)	9(36)
Total	48	38(79.16)	38(20.21)

**Table 3.** Seasonal distribution of *T. gondii* isolated from poultry meat samples.

Species	No. of positive samples with PCR	Spring (%)	Summer (%)	Autumn (%)	Winter (%)
Chicken	13	1(7.69)	7(53.84)	2(15.38)	1(7.69)
Partridge	4	1(25)	2(50)	1(25)	-
Quail	6	1(16.66)	3(50)	2(33.33)	-
Ostrich	6	1(16.66)	3(50)	1(16.66)	1(16.66)
Turkey	9	1(11.11)	5(55.55)	2(22.22)	1(11.11)
Total	38	5(13.15)	20(52.63)	8(21.05)	3(7.89)

(Table 2). Besides, from a total of 38 positive samples, 5 (13.15%), 20 (52.63%), 8 (21.05%) and 3 (7.89%) samples were positive in spring, summer, autumn and winter seasons (Table 3).

## DISCUSSION

The results of this present study show that poultry meat samples are one of the main sources for *T. gondii*. Infection and illness can be occurred by consumption of raw infected poultry meat. Detailed inspection is not performed on poultry carcasses in Iraninan's slaughterhouses. However, infected poultries are usually asymptomatic. In the present study the ELISA test showed that 28 meat samples (15.42%) were positive for presence of *T. gondii* antibodies while 20 meats (10.63%) were diagnosed as suspected samples using this assay. But after PCR it was recognized that from a total of 48 positive and suspected meat samples, 38 samples (79.16%) were positive, too. Therefore, we recommended use of PCR test in order to confirm the results of serological methods like ELISA. In the same study<sup>17</sup> which was performed on heart, brain and muscles of 19 birds, all results were confirmed using PCR techniques (42.1% prevalence rate of *T. gondii*).

Velmurugan *et al.* (2008)<sup>21</sup> reported that 25% of poultry heart biopsies were infected by *T. gondii* and their results were approved using molecular techniques.

Despite all defects of serological tests, many studies have been suggested their efficiency<sup>22-24</sup>. Our results showed that the seroprevalence rate of *T. gondii* in poultry meat samples was 15.42% which was higher than Italy (13.7%)<sup>25</sup>, Kenya (13.3%)<sup>26</sup>, Mexico (6.2%)<sup>27</sup> and Nigeria (6.32%)<sup>21</sup> but entirely was lower than Illinois (100%)<sup>28</sup>, Guyana (65.8%)<sup>29</sup>, Ghana (64%)<sup>25</sup>, Chile (55.3%)<sup>30</sup> and Austria (36.3%)<sup>31</sup>. The same studies in Portugal<sup>31</sup>, Sri Lanka<sup>26</sup>, Uganda<sup>32</sup> and USA<sup>28</sup> have been reported that the prevalence of *T. gondii*'s cysts in brain and muscles of poultries were 1% to 100%.

It seems that the high differences between the prevalence of *T. gondii* in various countries showed that its prevalence is closely depending on geographic region. Our study for the first time showed that the highest prevalence of this parasite were in summer season (52.63%). Besides, information showed that the relative temperature of summer in this area of Iran was 42 °C in average while in autumn, winter and spring were 14 °C, 5 °C and 20 °C. Our results showed significant

differences ( $P < 0.05$ ) between relative temperature of summer with autumn, winter and spring seasons. Therefore, seasons and their relative humidity have an important role in prevalence of *T. gondii*.

The results of this present study showed that the turkey had the highest prevalence of *T. gondii* (36%), followed by chicken (23.62%), ostrich (21.42%), partridge (13.23%) and quail (12%). Statistical analysis showed significant differences ( $P < 0.05$ ) between the levels of contamination in turkey with quail and partridge meat and chicken with quail meat samples. The high prevalence of *T. gondii* in turkey herds showed the higher close contact of these herds with stray cats and rodents. In addition the various prevalence rate of *T. gondii* which were reported from various countries were depends on sorts of poultry's breeding, sorts of nutritious, ages of birds, amount of tested samples, sorts of tested tissues and finally laboratory tests. In our study, using from breeding birds samples is one of the most important reasons for low prevalence of *T. gondii* in poultry meat samples. Besides, Dubey (2010)<sup>33</sup> showed that the age of birds has a high effect on the prevalence of *T. gondii*. All of our samples were collected from young birds. Therefore, the low prevalence of *T. gondii* in poultry meat samples of our study can be justified. There are no significant differences between the ability of PCR and ELISA methods for detection of *T. gondii* in poultry meat samples.

Koethe et al. (2011)<sup>34</sup> showed that 20.2% of turkeys were infected with *T. gondii* using serological tests. Another study showed that 2.9% of ostrich were serologically infected with *T. gondii*<sup>35</sup>. The seroprevalence rate of *T. gondii* in pigeon<sup>36</sup> and quail<sup>37</sup> were reported 95% and 14.3%, respectively. The seroprevalence of *T. gondii* in chicken meat samples were reported from 10% to 100% in various studies<sup>25,37-39</sup>. Therefore, with these high incidences of *T. gondii* in poultry meat all around the world, accurate poultry meat inspection is essential.

### CONCLUSIONS

We recommended use of PCR in order to rapid and accurate detection of *T. gondii* in poultry meat samples. Observing the detailed meat inspection especially in the warmer seasons, scrutiny of suspect carcasses, observe the

sanitation principles in slaughterhouses and finally maintenance of meats in appropriate temperature are the important factors for eliminate the prevalence of *T. gondii* in poultry meat.

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