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Original Article

The Prevalence of *Toxoplasma* Infection among Free-Ranging Chickens in Southern Iran Using IFA and Nested-PCR

Q Asgari, MH Motazedian, B Esmaeelzadeh, M Kalantari, GhR Hatam

Department of Parasitology and Mycology, Faculty of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

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Abstract

Background: As consumption of chicken meat may be as one of the sources of human infection, this study was undertaken to determine the prevalence of *T. gondii* in farm chickens (*Gallus gallus domesticus*) in Shiraz, southern Iran.

Methods: Two hundred and thirty one blood samples were collected from farm chickens by a cluster random sampling method and tested for toxoplasmosis by indirect fluorescent antibody technique (IFAT). The samples of the brain, heart, and liver of the chickens were tested by a Nested PCR method. The results were analyzed by SPSS software using Chi-Square test and a P value <0.05 was considered statically significant.

Results: Out of 58 seropositive chickens, 29 (1:16 in eight, 1:32 in 14, 1:64 in five and 1:128 in two birds) and out of seronegative chickens, three were enrolled in the study. The most infected tissue was liver (27 out of 29) and the lowest was the heart (16 out of 29) (α=0.05, P=0.002). None of the seronegative chickens was positive in PCR method. Only 2 out of 8 cases with a titer of 1:16 (as cut off point) were negative in PCR method whereas the remained were positive.

Conclusion: Based on cultural and food habits in our area, the meat and viscera of chicken may be important sources of infection in human when consuming semi-cooked meats. Considering the high prevalence of toxoplasmosis in chickens, standards in chicken breeding, education of environmental health personnel and standardization for preparation and handling techniques are required by Health and Veterinary organizations.

Key words: *Toxoplasmosis, Free-Ranging Chickens, PCR, IFAT, Iran*

Corresponding Author: Fax: + 98-711-2305291, E-mail: asgarig@sums.ac.ir

Introduction

Toxoplasma gondii is an obligate intracellular protozoan that infects humans and a wide range of mammalians and birds (1). The parasite is known to cause congenital disease and abortion both in humans and livestock (2-3). Maternal toxoplasmosis during early pregnancy of human may lead to death of fetus or may cause immunocompetence in humans. The disease has a severe form in immunocompromised patients such as 23% of HIV-positive patients that may develop toxoplasmic encephalitis (4). Human seropositivity was reported 51.8% in Iran with a 55% and 29% seroprevalence in northern and southern parts of the country, respectively (5-7).

The disease occurs mostly through ingestion of undercooked meat or by the oocysts excreted by infected cats (8). Infected chickens are considered as an important source of *T. gondii* worldwide (9). On the other hand, the rate of toxoplasmosis in free-ranging chickens as an intermediate host of *T. gondii* is one of the good indicators of environmental contamination due to use food habits of chicken (10). Clinical signs of toxoplasmosis in chickens include anorexia, emaciation, reduced egg production, ataxia, and blindness and even the mortality rate may be as high as 50% (11). The worldwide prevalence of anti-*T. gondii* antibodies in chicken was reported from nil to 40% by different methods and using different cut off points (9).

Furthermore, all of genetic types (I, II, III) of *T. gondii* isolates of patients that have been classified based on restriction fragment length polymorphism (12-13) were reported in free-range chickens (14-15). Since contaminated chickens may be the sources of human infection, this study was performed to determine the prevalence of *T. gondii* in free-ranging chickens (*Gallus gallus domesticus*) in the studied area using two methods of PCR and IFAT methods.

Materials and Methods

Study area

Fars Province is located in Southern Iran and has an area of about 53000 miles (8% of Iran's area) and is located at 27° 3' 42" longitude east (Fig. 1). It is mountainous with an average elevation of 5000 feet above sea level. The climate is quite dusty and dry, with warm summers, generally mild winters, and a great deal of sunshine throughout the year. Shiraz city is located in center part of the Fars Province (16).

Free-ranging chicken samples

In 2006, 231 blood samples were collected from farm chickens by a cluster random sampling method in sub-urban regions of Shiraz city in Fars Province. The chickens were 1-4 years old females used as a source of meat and egg in the region.

Indirect fluorescent antibody test

The cut-off of IFAT for *T. gondii* was considered 1:16 dilution (17). The sera were diluted 1:16 in PBS (0.1 M phosphate, 0.33 M NaCl, pH: 7.2) for preliminary screening and the positive samples were two folds serially diluted up to 1:512 to obtain the real titer of IgG antibody. RH strain tachyzoites of *T. gondii* were used as antigen (Pasteur Institute, Tehran, Iran), fixed on wells of immunofluorescent slides. Ten micro-liters of each diluted serum was transferred into wells of the slides and incubated in a humidified chamber at 37ºC for 30 min. Slides were washed in PBS (two times, 7 min), dried, and were incubated for 30 min at 37 ºC with rabbit anti-chicken IgG conjugate (Bethyl Co.) diluted 1:200 and Evans

Blue solution diluted 1:10000. Slides were washed and air-dried. A drop of glycerol buffer was added and each slide was covered with a cover- slip. The samples were observed under the immunofluorescent microscope (Zeiss HBO 50). The fluorescein-stained tachyzoites were considered positive.

Organ Sampling

Twenty nine seropositive and 3 seronegative chickens were provided. Samples of brain, heart, and liver were collected from each chicken, and kept at -20 ºC.

DNA Extraction

One gram of the organs was homogenated and was diluted by double distilled water (1:10). DNA of each organ was extracted by adding proteinase K to 5 ml of each sample. Then lysis buffer was added (50 ml of Tris-HCl, pH=7.6; 1 mM of EDTA, pH=8.0; 1% Tween 20, 8.5 ml of proteinase K solution, 19 mg/ml) and incubated for 24 h at 37°C. The lysate was then extracted twice with phenol/chloroform/Isoamyl before the DNA was precipitated with absolute ethanol and resuspended in 100 µL of double distilled water and stored at 4°C.

Nested-Polymerase Chain Reaction

Nested primer sets were used for amplifying fragments of the B1 gene. The outer primers were from bases 171 to 190 (5′-CCG TTG GTT CCG CCT CCT TC-3′) and from bases 602 to 583 (5′-GCA AAA CAG CGG CAG CGT CT-3′) producing an amplified product of 432 bp. Inner primers were from bases 180 to 196 (5′-CCG CCT CCT TCG TCC GTC GT-3′) and from bases 392 to 372 (5′-GTG GGG GCG GAC CTC TCT TG-3′) producing an amplified product of 213 bp (18). The first 50 mL PCR reaction mixture contained outer primers at a final concentration of 50 pmol each, 20 mmol/L dNTPs and 1.25 U recombinant taq DNA polymerase in 1×PCR reaction buffer (50 mmol/L KCl and 10 mmol/L tris-HCl, 1.5 mmol/L Mg_2Cl, 0.1% triton×100; Sinagen Co., Iran). PCR amplification was performed for 2 min at 94°C for one cycle, followed by 30 cycles using denaturation at 94°C for 1 min , annealing for 2 min at 57°C and extension for 3 min at 72°C. The nested PCR reaction was performed using 5 µL of the first PCR reaction in a mixture containing the inner primers at final concentration of 50 pmol each, 20 mmol/L dNTPs, 1.25 U recombinant taq DNA polymerase in 1×PCR reaction buffer. Amplification was carried out at 94°C for 2 min (one cycle), then followed by 35 cycles each for denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. The run was terminated with a final extension at 72°C for 10 min. The amplification products were detected by gel electrophoresis using 3% agarose gel in 1×tris-borate-EDTA buffer. DNA bands were visualized using 0.5% ethidium bromide in the presence of ultraviolet light.

Analysis of Data

The results were analyzed by SPSS software using Chi-Square test.

Results

Antibodies to *T. gondii* were found in 58 (24.5%) of 231 chickens with titers of 1:16 in 29, 1:32 in 22, 1:64 in 5, 1:128 in 1 and 1:256 in 1 bird.

The liver, brain, and heart of 3 seronegative chickens and 29 chickens with titers of 1:16 in eight, 1:32 in 14, 1:64 in five and 1:128 in two birds were tested by nested PCR (Table 1). None of three seronegative chickens was positive in PCR method (Fig. 2).

Only two out of 8 cases with titer 1:16 as cut off point of IFAT were negative in PCR method whereas the rest of them were positive. The highest possibility of parasite existence in tissues was liver (27 0ut of 29) while the lowest was observed in 16 out of 29 chicken hearts (α=0.05, P=0.002).

Table 1: Toxoplasmosis in Shiraz free-ranging chickens by IFAT and PCR

Titer (IFAT)		N	Positive cases by PCR					
			Liver		Brain		Heart	
			N	%	N	%	N	%
Positive	<1:16	3	0	0	0	0	0	0
	1:16	8	6	75	5	62.5	1	12.5
	1:32	14	14	100	14	100	11	78.6
	1:64	5	5	100	4	80	2	40
	1:128	2	2	100	2	100	2	100
	Total	29	27	93.1	25	86.2	16	55.9

Fig. 1: The study area of Shiraz City, Fars Province, southwest Iran

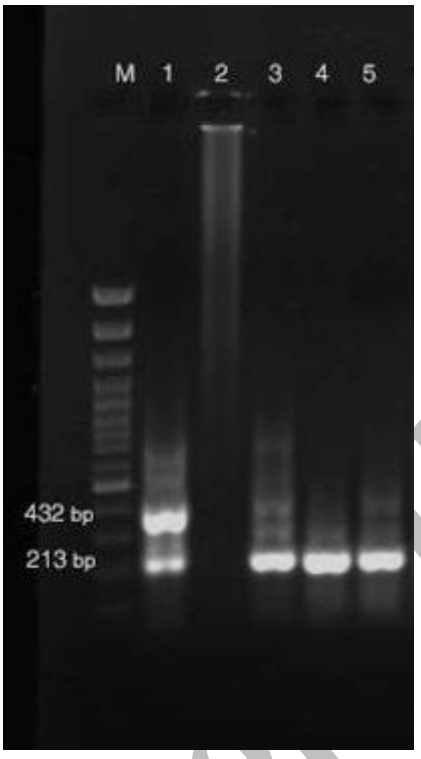

Fig. 2. The amplicons produced, in the Nested PCR based on the B1 gene primers, from tissues. Samples of chicken in Shiraz (lane 3-5). For reference, sample (lane 2) was seronegative and sample (lane 1) was from *Toxoplasma gondii* from Tehran University of Medical Sciences. The amplified product of 432 bp belonged to the first stage of nested PCR whereas the amplified product of 213 bp was following stage of test

Discussion

This study revealed a high seroprevalence of 24.5% in free-ranging chicken that was close to that of Dubey et al. (22) who found a 26% seroprevalence in chickens in Peru. The prevalence was markedly less than the values detected in the chickens from Chile (23) and Colombia (24) reporting a seroprevalence of 55.39% and 44.4%, respectively. The prevalence rate in these chickens was reported 17%, 36.3% and 40% in United State (25), Austria (21) and India (10), respectively. The prevalence in Middle East countries was reported 40.4% (26) and 46.9% (15).

The rate of the infection in Sub-urban areas from Tehran (27) and Shiraz (28) cities in Iran was reported 33% and 36.1% respectively whereas the prevalence rate of toxoplasmosis in free ranging chicken of Mazanderan Province as a humid area in Northern Iran was 51% (29, 30).

The cut-off of IFAT for T. gondii was considered 1:16 dilution (17). The sero-negative samples, which were also negative in PCR test, showed that the cut-off was candidly used in the past studies. A number of tissue samples from sero-positive were negative in

PCR showing probably the tissue cysts not been present in the part of the sample.

In this study, by using PCR method, the highest rate of infection was seen in liver of the seropositive chickens (93.1%) which is commonly used as a food source in the area. The liver is usually consumed undercooked; therefore, bradyzoites of parasite stay alive and may cause Toxoplasma infection. The consumption of semi-raw liver especially in pregnant women and children is conventional.

The sources of infection for humans, worldwide, vary greatly with culture, ethnic, geographical location and eating habit differences (9). Jacobs and Melton found T. gondii in ovaries, oviduct, and muscle of chicken by using inoculation into mice (19). Boch et al. isolated T. gondii from the brain and heart of hens in Germany (20). Thus, meat of chicken must be considered as a source of infection in human. Although the infection in ovary and oviduct is possible, chicken eggs must not be considered as a source of infection for human (21).

Furthermore, all major genotypes (I, II, III) of T. gondii isolates of patients that have been classified based on restriction fragment length polymorphism (12-13) were reported in free-range chickens (14-15). On the other hand, the rate of toxoplasmosis in free-ranging chicken is an important indicator of environmental contamination because of food habits (10).

Many factors such as management and hygienic standards in breeding, density of cats and environmental conditions are effective on the acquisition of T. gondii oocysts by animals (9). Humidity and temperate temperature favor the oocyst survival. Shiraz City is situated in Southern, Iran where has dry and sub-Saharan environment with an average annual rainfall not over 350 mm. However, other climatic characters such as temperature and altitude in these areas are different; for example, Southern parts are warmer than other parts. The majority of free chicken in these areas are raised for meat and egg production by people living in villages and sub-urban regions of Shiraz. Based on cultural and food habits in this area, meat and viscera of chicken may be important source of infection in human when consumed semi-raw materials.

Considering the above-mentioned findings, standards in chicken breeding, education of environmental health personnel and standardization for preparation and handing techniques are required to prevent human infection.

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