

Serological assays and PCR for detection of *Toxoplasma gondii* infection in an ostrich farm at Ismailia Province, Egypt

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Abstract One hundred and twenty serum samples from ostriches (*Struthio camelus*) in a commercial farm in Egypt were tested for anti-*Toxoplasma* antibodies using ELISA test (IgM and IgG) and Modified agglutination test (MAT). Five out of 120 birds (4.2%) were found positive to IgM enzyme immunoassay and 11 birds (9.2%) were IgG enzyme immunoassay positive. Modified agglutination test detected IgG in 15 birds (12.5%). PCR amplification of *Toxoplasma gondii* DNA in the blood of birds was recorded in 9 birds (7.5%). The Cases were categorized into acute and chronic cases depending on the result of blood-PCR and serological test where the positive cases for IgG were the most frequent (6.7%). The tissues of five dead birds tested by PCR assay showed the amplification of DNA fragment of *T. gondii* from the heart and brain of two birds, from only the brain of another two birds and from the heart, brain and leg muscles of one bird. The present study concluded that, the chronic cases are the most prevalent among ostriches population and the specific serological tests as MAT alone or with ELISA can be used as a highly sensitive screening test followed by PCR as a confirmatory test for diagnosis of toxoplasmosis and for detection of primary acute and chronic toxoplasmosis in ostriches.

Key word: *Toxoplasma*, Ostriches, ELISA, MAT, PCR, Egypt.

I. Introduction

Toxoplasma gondii is a single-celled protozoa that infect several species of warm-blooded animals including birds (1, 2, 3, and 4). Humans can get infection by ingesting of infected meat or ingestion of contaminated food and water with the resistant oocyst form of *Toxoplasma* excreted by cats (5). Ostriches (*Struthio camelus*) are large birds that are raised on game farms as nontraditional livestock and have been imported into several developed countries. Meat of ostriches is highly palatable and of low fats. Many authors reported ostriches infection by *T. gondii* (5, 6). The most widely used serological test for diagnosis of toxoplasmosis is the ELISA (IgM and IgG) (2). Modified agglutination test (MAT) is considered to be the most sensitive and suitable technique for toxoplasmosis (7, 8, 9). Moreover, the polymerase chain reaction (PCR) allows detection of parasite DNA; it is highly sensitive, specific and is very useful together with serological tests to differentiate the acute, chronic or reactivated infections (10, 11). PCR is also an important diagnostic technique in case of compromised immune system or when antibody titers have not reach threshold levels of detection. In ostriches little is known of *T. gondii* infection in either farmed or wild raptives in Egypt. SO we conducted the present study to detect the infection in the commercial breeding facilities of ostriches by using of two serological tests, ELISA (IgM and IgG) and MAT test, in addition to molecular methods.

II. Materials and Methods

II. 1 Farm design

The present study was carried out in an ostrich farm located at El-Kassaseen, Ismailia province, Egypt. It has about 1500 birds. Ostrich flocks in the farm are divided into groups according to age as follow: 1- 10 days old chicks; 10 - 60 days old, 2-6 months old; 6-12 months old, and over 2 years. The ostrich chicks from day 1 to 10 days old were kept in rearing unit I (environmentally controlled with rubber mat floor). After that, they transferred to rearing unit II & III (run/pen) with concrete floor until they grew to 2 months and over 2 months to 6 months old respectively. Later on, they were transferred to the grower yard, where ostriches stayed up to 12 months old (when they were ready to be slaughtered) or until 24 months old (when they could replace discarded breeders). The ostriches' drinking water comes from tap water (surface water, Ismailia canal). All ostrich feeds (starter feed (22%), grower feed (16%) and breeder feed (20-22% protein) were obtained from feed processing company (FPCo) at the 10th of Ramdan city. The ratio of chopped green fodder to feed is maintained at 2:1 for both grower and breeder flocks. The ostrich farm is located 500 meters far from many cultivated lands with fruits and large animal farms (beef calves and dairy buffalos).

II.2 Ostriches examination

The clinical signs observed in the farm were anorexia, emaciation, diarrhea, eye affection and death of 5 birds. The dead ostriches were examined at necropsy; tissue samples (brain, heart, breast muscles and leg muscles) were collected from the dead birds and were frozen at -20 °C for molecular analysis. A total of 120 blood samples collected from live birds for serodiagnosis and molecular analysis.

II. 2. 1 Serum samples

Three ml of blood was collected from the brachial vein using a disposable syringe of 5 ml size, then transferred to 10 ml sterile serum tube. The blood samples were left to clot and then centrifuged at 3000 rpm for 5 minutes to get the serum. Serum samples were relocated in eppendorf tubes and stored at 4 - 8 °C for 24 hrs.

II. 2. 2 Immunassay

Enzyme-linked immunosorbent assay (ELISA)

The presence of specific IgM and IgG antibodies to *T. gondii* in ostriches was determined by the enzyme-linked immunosorbent assay (ELISA) using a commercial Toxoplasma IgG and IgM enzyme immunoassay test kits (BioCheck, Inc) as follows: Ninety-six-well, polystyrene flat-bottomed microtitre plates coated with *T. gondii* antigen were used. Tested sera were diluted at 1:40 in PBS-T and added to the plates after which the plates were incubated at 37°C for 30 minutes. After incubation, the plates were washed and flicked five times with diluted wash buffer (1X). Enzyme conjugate (100 µl) was added to each well and mixed gently for 10 seconds and incubated at 37°C for 30 minutes. The enzyme conjugate then removed and the well were washed and flicked 5 times with diluted wash buffer (1X). Afterward, TMB reagent (100µl) was dispensed into each well, mixed gently for 10 seconds and incubated at 37°C for 15 minutes. The reaction was stopped by addition (1NHCl), where mixing occurred for 30 seconds. Plates were read by measuring optical density (OD) at 450nm within 15 minutes with a microwell reader. An OD of 0.901 was considered as the cut-off point.

Modified agglutination test

Modified agglutination test (MAT) including mercaptoethanol and formalin-fixed whole tachyzoites used to detect only IgG antibodies. The mercaptoethanol used in destroying IgM-like substances that interferes with the test specificity. All ostrich's sera were screened using dilutions of 1:25, 1:50, 1:100, and 1:500, using the modified agglutination test (MAT) as described by Dubey and Desmonts (12). A titer of 1:25 was considered indicative of *T. gondii* (7,8).

II. 2. 3 PCR technique

Detection of *T. gondii* infection in the blood and tissue samples was determined by nested PCR amplification for the B1 gene (13). Primary amplification had B1F1 primers (5'-CCGTTGGTTCCGCCT CCTTC-3') and B1R1 (5'-GCAAAACAG CGGCAG CGTCT-3') at annealing temperature of 55 °C. In the consequent reaction, the primers B1F2 (5'-CCGCC TCCTTCGTCC GTCGT-3') and B1R2 (5'- GTGGGG CCGACCTCTCTTG-3') were used at annealing temperature of 60°C. First step PCR was at 95°C for 9 minutes, followed by 35 cycles, each cycle was at 94°C for 60 seconds, 30 seconds at the annealing temperature for each pair of primer, and at 72 °C for 60 seconds. Last cycle was at 72°C for 8 minutes. The PCR products were investigated by 1.5% agarose gel electrophoresis. The PCR amplification yield a product of 213 bp for the *T. gondii*.

III. Results

In the current study diagnosis of *T.gondii* infection in ostriches relied on the clinical signs, immunological studies and molecular studies. The clinical signs were non-specific including anorexia, emaciation, diarrhea, eye affection and deaths (Fig.3). The serological tests show that out of 120 examined ostriches by ELISA, 5 bird (4.2%) were positive in the IgM enzyme immunoassay, Whilst 11 birds (9.2%) were IgG enzyme immunoassay positive (Table. 1). Modified agglutination test (MAT) detects only IgG in 15 birds (12.5%) (Table.1). All the positive sera for ELISA (IgG) were positive for MAT which showed high specificity for the detection of anti-Toxoplasma antibodies in comparison to ELISA technique. PCR assay on the blood of ostriches succeeded to detect *T.gondii* DNA by using B1 gene as a target for amplification in 9 birds (7.5%) (Fig. 1).

Birds were considered acutely infected when they had only PCR positive or when they had positive IgM + PCR titres. Chronic infection were encountered when birds had only IgG positive, IgG + IgM positive, PCR + IgG positive or PCR + IgM + IgG positive. In the current study positive IgG + PCR (1.7%) and positive IgM + IgG + PCR cases (3.3%), positive IgM + I gG (0.8%), positive PCR only (2.5%) and positive IgG (6.7%) were the most frequent cases (Table. 2). Graph (1) clears that the chronic infection was the most prevalent among examined ostriches.

In dead ostriches, amplification of 213 bp DNA fragment of the expected size of the sequence of B1 gene specific for *T. gondii* had been detected from the heart and brain of two birds, from only the brain of another two birds and from the heart, brain and leg muscles of one bird but no amplification was detected when the breast muscles were tested (Fig. 2 and Table. 3).

IV. Discussion

Diagnoses of *T.gondii* infection in ostriches relied on the clinic signs, immunological and molecular studies. The clinical signs were non-specific including anorexia, emaciation, diarrhea, eye affections and deaths. In general, little is well known about the clinical symptoms of toxoplasmosis in ratites and specifically in ostriches (14).

The applied immunological studies in our study were ELISA (IgM and IgG) and MAT (IgG). The number of positive serum samples was higher using MAT test than ELISA test. Concerning this; most authors considered MAT test as the most sensitive (8, 9, 12). Of the 120 bird sera tested by MAT technique, 15 birds (12.5%) were found positive for antibodies of *T. gondii*. ELISA test showed that the prevalence of IgM of *T.gondii* was 4.2% and that of IgG was 9.2%. In this respect, antibodies against *T. gondii* were previously detected by modified agglutination test in 1.17% of ostriches from Spain (15), in 2.9% of ostriches from Canada (5), in 14.36% of ostriches from Brazil (14) and in 55.9% of ostriches from Zimbabwe (6). The variance of the seroprevalence of *T. gondii* in domestic birds within countries may be attributed to the number of examined animals, the methods used for testing, the type of animal hygiene and breeding (5, 16). In general, the seroprevalence of *T. gondii* was low in this study, this may be due to the geographical location of the farm which decrease the possibility of infection, the high level of applied biosecurity, in addition to very low density of cats which act as a main infection source in and around the farm.

Although serological testing is considered the main diagnostic technique for toxoplasmosis, it has some limitations because of the false positive results which have been reported by other investigators (17, 18), in addition to the false negative results that may happen during the active phase of *T.gondii* (19). The need for more sensitive techniques as PCR assay and its uses for tissue studies is essential to demonstrate the actual infection in ostriches and to obtain more data on prevalence, frequencies and phases of the parasite. In this study PCR assay detected *T. gondii* DNA in the blood of ostriches by amplification of B1 gene specific to *T.gondii* and is conserved well among all of the tested strains (13). The infected cases were categorized depending on the result of blood-PCR combined with serological test as acute cases were considered when only PCR was positive or when the bird showed positive titers of IgM + PCR. Moreover chronic cases were that birds had only IgG positive, IgG + PCR positive, IgM + IgG positive, or IgM + IgG + PCR positive (20). In our study the chronic infection was the most prevalent as IGg positive cases was the most frequent (6.7%).

In different tissues of ostriches amplification of B1 gene of *T. gondii* was detected from 5 dead birds in brain, heart, breast and leg muscles. In this respect, no available literatures were found but the distribution *T. gondii* in the tissue of the heart, brain, and pectoral muscle of chicken was recorded (21).

V. Conclusion

The present study is the first time to adopt PCR and serology of *T. gondii* antibodies in ostriches in Egypt and suggests the probability of acquiring toxoplasmosis from ostriches as a food source where the chronic cases are the most prevalent among ostriches population. The specific serological tests as MAT alone or with ELISA can be used as a highly sensitive screening test followed by PCR as a confirmatory test for diagnosis of toxoplasmosis and for detection primary acute and chronic toxoplasmosis in ostriches. Because the ingestion of oocysts from cat feces is an important route of *Toxoplasma* infection, Future researches should be addressed to detect the method of ostrich infection by *T. gondii* which may be due to the access of cat to ostrich farm, contamination of birds food by cat feces during manufacturing, transportation and storage, water contamination or any other possible sources.

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Table 1: Incidence of *T. gondii* among examined ostriches using serological and PCR tests

Tests	No. of Positive birds/120	%
IgM (ELISA)	5	4.2
IgG (ELISA)	11	9.2
IgG (MAT)	15	12.5
PCR	9	7.5

Table 2: Phases and frequency of *T. gondii* infection in ostriches according to serological and PCR results

Tests	No. of positive birds/ 120	%	Phases
IgG only	8	6.7	Chronic
IgM + IgG	1	0.8	Chronic
IgG +PCR	2	1.7	Chronic
IgM+IgG+PCR	4	3.3	Chronic
PCR only	3	2.5	acute

Table. 3: Distribution of Toxoplasma in the tissue of dead ostriches using PCR

Examined birds	Heart	Brain	Leg Muscles	Breast Muscles
1 st bird	+ve	+ve	ND	ND
2 nd bird	ND	+ve	ND	ND
3 rd bird	+ve	+ve	+ve	ND
4 th bird	+ve	+ve	ND	ND
5 th bird	ND	+ve	ND	ND

ND: not detected



Fig. 1. Amplified product analyzed by 1.5% gel electrophoresis. Lane 1: negative blood sample for Toxoplasma, Lane : 2,3,5,6,7, 8 positive amplification. Lane 4 : positive control samples . Lane M molecular weight marker 100 bp



Fig. 2. Amplified product analyzed by 1.5% gel electrophoresis. Lane 1, 2, 8 positive amplification of brain, Lane 3 negative amplification from breast muscles, Lane 4, 5, 6 positive amplification from heart. Lane 7 Positive amplification from leg muscles, Lane M molecular weight marker 100 bp

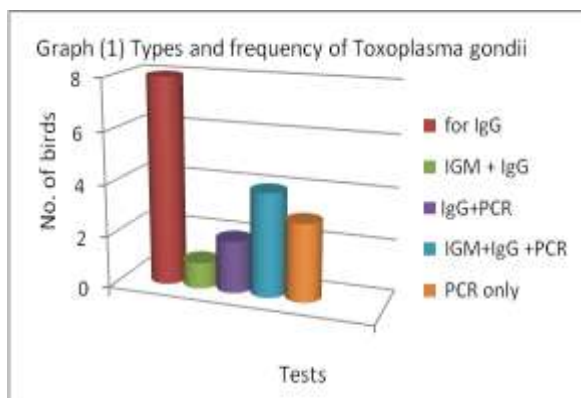


Fig. 3 Some ostriches showing eye affections (arrows)