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 parasite known to infect many species of warm-blooded animals including birds (1, 2, 3, and 4). Humans become infected by ingesting infected meat or by ingesting food and water contaminated with the resistant form of Toxoplasma (oocyst) excreted by cats (5). Ostriches (Struthio camelus) are large birds that have been imported into many developed countries where they are raised on game farms as nontraditional livestock. Meat from ostriches is considered highly palatable and low in fats. Many authors (5, 6) reported ostriches infection by T. gondii. The most widely used serological test for diagnosis of toxoplasmosis is the ELISA (IgM and IgG) (2). Most authors consider modified agglutination test (MAT) to be the most sensitive and suitable technique for this kind of study (7, 8, 9). Moreover, the polymerase chain reaction (PCR) allows detection of parasite DNA; it is highly specific and sensitive and very useful together with serological tests to differentiate the chronic, acute or reactivated infections (10, 11). PCR is also an important diagnostic method when the immune system is compromised or when antibody titers have no reach threshold levels of detection. In ostriches little is known of T. gondii infection in farmed and wild ratites 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 had about 1500 birds. Ostrich flocks were 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 grow 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, and diarrhea 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 was 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 venous blood was drawn from the brachial vein using a 5 ml size disposable syringe then transferred to 10 ml disposable sterile serum tube. The blood samples were left to clot then centrifugated at 3000 rpm for 5 minutes to separate the serum. Serum samples were transferred to 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 follow: 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 (100 µl) 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) incorporating mercaptoethanol and formalin-fixed whole tachyzoites used to detects only IgG antibodies because the mercaptoethanol used in the test destroys IgM-like substances that interfere with the specificity of the test. 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 based on statistically validated studies in pigs (7,8).

II. 2. 3 PCR technique

Detection of T. gondii infections in the blood and tissue samples was determined by nested PCR amplification targeting the B1 gene (13). Primary PCR amplification was done using B1F1 primers (5’-CCGTTGGTTCCGCT CCTTC-3’) and B1R1 (5’-GCAAACCAG CGGCAG CTGTCT-3’) at an annealing temperature of 55°C. In the second reaction, the primers B1F2 (5’-CGGCC TCCTTCGTCC GTCGT-3’) and B1R2 (5’-GTGGGGG CGGACCTCTCTTG-3’) were used at an annealing temperature of 60°C. First step PCR was 9 minutes at 95°C, followed by 35 cycles, each cycle consisting of 60 seconds at 94°C, 30 seconds at the annealing temperature for each primer pair, and 60 seconds at 72°C. The last cycle was for 8 minutes at 72°C. PCR products were analyzed by 1.5% agarose gel electrophoresis. The PCR amplification is predicted to yield a product of 213 bp for the presence of T. gondii.

III. Results

In the current study diagnosis of T.gondii infection in ostriches relied on the clinic signs, immunological studies and molecular studies. The clinical signs were non-specific including anorexia, emaciation, diarrhea and deaths. 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 applied 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).

Cases were categorized as acute when were only PCR positive or when the bird showed positive titers of IgM + PCR. Chronic cases were considered when birds were only IgG positive, IgG + PCR positive, IgM + IgG positive, or IgM + IgG + PCR 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%)
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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 corresponding to the expected size of the B1 gene sequence 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, diarrhoea and deaths. Little is known on the clinical signs of toxoplasmosis in ratites in general 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 consider MAT test to be the most sensitive (8, 9, 12). Of the 120 bird sera tested by MAT technique, 15 birds (12.5%) was found positive for antibodies of T. gondii, while 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 detected by the 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 seroprevalence of T. gondii in domestic birds varies within countries due to the methods used for testing, the number of examined animals, the type and hygiene of animal breeding (5, 16). In general the seroprevalence of T. gondii was low in this study, this may be attributed to the geographical location of the farm which decrease the possibility of infection, moreover, the high level of biosecurity applied in addition to very low density of cats in and around the farm.

Although serological testing has been the major diagnostic technique of toxoplasmosis it has many limitations because false positive results have been reported by other investigators (17, 18), in addition to the false negativity that may occur during the active phase of T. gondii infections (19). The need of application of more sensitive techniques like PCR and its uses for tissular studies is necessary in order to demonstrate the actual presence of the parasite in ostriches and obtain more data on prevalence, phases and frequencies of the parasite. In this study PCR assay was applied on the blood of ostriches to detect T. gondii DNA by using B1 gene as a target for amplification which is highly specific for T. gondii and is well conserved among all the tested strains (13). The cases were categorized depending on the result of blood-PCR combined with serological test as acute cases were considered when only PCR were 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 ostrich 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 research 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.

Reference

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

<table>
<thead>
<tr>
<th>Tests</th>
<th>No. of Positive birds</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM (ELISA)</td>
<td>5</td>
<td>4.2</td>
</tr>
<tr>
<td>IgG (ELISA)</td>
<td>11</td>
<td>9.2</td>
</tr>
<tr>
<td>IgG (MAT)</td>
<td>15</td>
<td>12.5</td>
</tr>
<tr>
<td>PCR</td>
<td>9</td>
<td>7.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Tests</th>
<th>No. of positive birds</th>
<th>%</th>
<th>Phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG only</td>
<td>8</td>
<td>6.7</td>
<td>Chronic</td>
</tr>
<tr>
<td>IgG + IgG</td>
<td>1</td>
<td>0.8</td>
<td>Chronic</td>
</tr>
<tr>
<td>IgG +PCR</td>
<td>2</td>
<td>1.7</td>
<td>Chronic</td>
</tr>
<tr>
<td>IgM+IgG+PCR</td>
<td>4</td>
<td>3.3</td>
<td>Chronic</td>
</tr>
<tr>
<td>PCR only</td>
<td>3</td>
<td>2.5</td>
<td>acute</td>
</tr>
</tbody>
</table>
Table 3: Distribution of Toxoplasma in the tissue of dead ostriches using PCR

<table>
<thead>
<tr>
<th>Examined birds</th>
<th>Heart</th>
<th>Brain</th>
<th>Leg Muscles</th>
<th>Breast Muscles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st bird</td>
<td>+ve</td>
<td>+ve</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2nd bird</td>
<td>ND</td>
<td>+ve</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3rd bird</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>ND</td>
</tr>
<tr>
<td>4th bird</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>ND</td>
</tr>
<tr>
<td>5th bird</td>
<td>ND</td>
<td>+ve</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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.

Graph 1: Types and frequency of Toxoplasma gondii according to the tests applied.