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Research Article
Toxoplasma gondii Infection in Cattle Egret (*Bubulcus ibis*): First Report from Shebin El-Kom, Menoufia Governorate, Egypt

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Abstract

Background and Objective: *Toxoplasma gondii* (*T. gondii*) is capable of infecting a broad range of intermediate hosts. Cattle egret (*Bubulcus ibis*) is a species of herons that is common in Egypt. This work aimed to study the prevalence of *T. gondii* in cattle egret which is an efficient tool of investigating environmental contamination with *T. gondii*. **Materials and Methods:** Serum, heart and brain tissues of 51 cattle egrets were collected from Shebin El-Kom, Menoufia, Egypt and tested using Modified Agglutination Test (MAT) and mice bioassay. **Results:** There was a detection rate of 13.7% (7/51) in these birds using MAT. By intraperitoneal injection of mice with heart and brain tissues digest of MAT positive birds, the parasite was isolated from two *T. gondii* sero-positive birds (28.6%). The mice bioassay was confirmed by MAT, Hematoxylin and Eosin (HAND E) staining of the brain of the infected mice, also by Polymerase Chain Reaction (PCR) and sequencing. **Conclusion:** This is the first report of *T. gondii* infection in cattle egret from Egypt and more studies are needed on Egyptian wildlife to understand the sylvatic life cycle of the parasite.

Key words: Herons, cattle egret, *Toxoplasma gondii*, MAT, PCR, Menoufia Governorate

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Toxoplasmosis is a global zoonotic disease caused by the intracellular protozoan parasite, *Toxoplasma gondii* (*T. gondii*) *that* can infect all warm-blooded creatures, including humans and birds[1]. The parasite has a complex life cycle consisting of a sexual cycle in the intestine of its feline definitive host followed by an asexual one in its intermediate hosts[2]. Infection of the intermediate host can occur through one of three primary modes of transmission which are ingestion of tissue cysts in raw or undercooked meat, ingestion of oocysts shed by the definitive host into the environment or congenitally from infected mother to her fetus[3]. The course of *T. gondii* infection is profoundly affected by the person's immunological competence. Toxoplasmosis is usually asymptomatic in immune-competent individuals. However, it can be deadly in immune-compromised patients, such as HIV patients, cancer patients and organ transplant recipients[4,5].

Birds are important intermediate hosts for *T. gondii* and have a significant epidemiological value[6]. They are an important source of infection for fields via predation cycle and they also represent an efficient tool to monitor environmental contamination with *T. gondii* oocysts according to their feeding habits[7,8]. Cattle egret (*Bubulcus ibis*) is a cosmopolitan species of heron (family *Ardeidae*). It is found in the tropics, subtropics and warm temperate zones. It is widely spread in Egypt; in Nile Delta and Valley. The cattle egret is popular for its role in the biocontrol of cattle parasites and land pests because it feeds on a wide range of prey, particularly insects and moths as well as spiders, frogs, fish and earth-worms[9].

Even though *T. gondii* has been isolated from different types of birds, the role of a wild one in the epidemiology of *T. gondii* stays obscure, particularly in Egypt. So, this study aimed to elucidate the role of the cattle egret as an intermediate host for *T. gondii* and to estimate the parasite prevalence in cattle egret as an important indirect way to detect the extent of environmental contamination in Egypt.

MATERIALS AND METHODS

Study subjects and the bird's collection: The current study was conducted in the Menoufia governorate, Shebin El-Kom, Egypt (Fig. 1) in the period between October, 2019 and January, 2020. Fifty-one cattle egrets were collected from five different areas in Shebin El-Kom city and their age and sex were recorded (Fig. 2). Cattle egrets were captured and euthanized according to the international ethical guidelines after approval of the institutional ethical committee of Menoufia Faculty of Medicine, Menoufia University, Egypt.

Fig. 1: Area of sample collection in Egypt
The blue boundaries show the Menoufia governorate and the red mark is on Shebin El Kom city

Fig. 2: A photo of a cat and a cattle egret feeding together on a dumpster
The picture was taken by the authors from one of the areas of samples collection

The collected birds were anesthetized with ether and killed by heart puncture. The collected blood from the heart of each bird was centrifuged at 3000 rpm for 5 min for serum preparation. The separated serum was kept at -20°C till tested for the presence of anti-*T. gondii* antibodies by Modified Agglutination Test (MAT). Also, the brain and heart of each bird were collected in a sterile plastic bag and kept at 4°C until the accomplishing of the serological examination.

Serological examination using MAT: MAT was performed as described previously by Dubey and Desmonts[10]. In brief, serum was two-folded diluted starting from 1:25 to 1:1600 using Phosphate Buffered Saline (PBS). Then, it was mixed with *T. gondii* antigen mixture which was generously provided by Dr. Chunlei Su, Department of Microbiology, University of Tennessee, United States of America. Sera with MAT titers of 1:25 or higher were considered positive. Positive and negative controls were included in each plate.

Bioassay in mice: Brain and heart from each seropositive cattle egret were pooled and digested in pepsin and bioassayed in mice, as described by El-Nawawi *et al.*[11]. Specific-pathogen free Swiss albino mice (each weighing 20-25 g) were inoculated intra-peritoneal with 1 mL of tissue homogenate for each mouse. Mice were daily inspected for the presence of any signs of disease. Mice were killed 47 days after infection and serum were tested for *Toxoplasma* antibodies by MAT, the brains of all inoculated mice were collected. Half of the brain was fixed in 10% buffered formalin, sectioned, stained with Hematoxylin and Eosin and examined with an optical microscope for *T. gondii* parasite detection. The other half was frozen for DNA extraction.

Detection of *T. gondii* by the Polymerase Chain Reaction (PCR)

DNA extraction from the mice brain tissues: Half of the brain tissue from positive mice by bioassay were subjected to DNA extraction. The tissues were homogenized in a homogenizer, in 10 mL of TE buffer (200 mM NaCl, 20 mM Tris, 50 mM EDTA, pH 8.0), for 3 min. The homogenized samples were transferred to microtubes and were further processed using QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

PCR reactions: Detection of *T. gondii* DNA extracted from the brain of the infected mice was carried out using the conserved repetitive B1 gene[12]. The forward primer sequence was 5'-GGA ACT GCA TCC GTT CAT GAG-3' and the reverse one was 5'-TCT TTA AAG CGT TCG TGG TC-3'. PCR reaction was set up in a final volume of 25 µL which included 12.5 µL master mix (DreamTaq Green PCR Master Mix (2X) Thermo-Scientific, USA), 1 µL of each primer, 5.5 µL distilled water and 5 µL extracted DNA. The cycling conditions were denaturation at 94°C for 5 min, followed by 45 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min for each cycle. The final cycle was followed by an extension step at 72°C for 7 min. The PCR products (5 µL from each reaction) were separated on a 2% agarose gel and visualized by staining with ethidium bromide. The reaction is expected to yield a product of 193 bp. A cell

lysate of RH strain of *T. gondii* was used as a positive control, which was provided by Zoonotic Diseases Department, National Research Centre, Egypt. A negative control using only nuclease-free water as DNA template was also included.

DNA sequencing: The amplicon was purified by QIA Quick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. It was sequenced in both directions by using Applied Biosystems™ 3500 xLGenetic Analyzer (Fisher Scientific, USA) by the primers employed in the PCR. The basic local alignment search tool (BLAST) of the US National Center of biotechnology information (NCBI) was used to identify most similar sequences to the obtained one.

Statistical analysis: Data were analyzed by SPSS statistical package version 23 (SPSS Inc. Released 2015. IBM SPSS statistics for windows, version 23.0, Armnok, NY: IBM Corp). The variables were expressed in number (No) and percentage (%). Chi-square test (χ^2) was used to study the association between qualitative variables. Whenever any of the expected cells were less than five, Fischer's exact test was used.

RESULTS

Detection anti-toxoplasma antibodies using MAT: The presence of *T. gondii* infection in cattle egret from Shebin El-Kom, Menoufia, Egypt for the first time was documented. Fourteen male birds and 32 female birds were collected. Five birds were undifferentiated neither males nor females. The samples included 5 juvenile birds and 46 adults. Antibodies to *T. gondii* were found in 7 out of 51 (13.7%) birds. The MAT titers were 1/50 in 4, 1/100 in 2 and 1/200 in 1 cattle egret. None of the tested juvenile birds were positive by MAT, while all 7 seropositive birds were adults (15.2%). Three male birds out of 14 (21.4%) and four female birds out of 32 (12.5%) were MAT positive. There was no significant difference between males and females or adults and juveniles in seropositivity to *T. gondii* ($p>0.05$) (Table 1).

Mice bioassay: Seroconversion of mice occurred in 6 mice inoculated with homogenate from positive birds. *Toxoplasma gondii* cysts were found in 2 out of 7 (28.6%) seropositive cattle egrets by mice bioassay. The *T. gondii* infective stages; tissue cysts and free tachyzoites were detected in the mice brains by H and E staining (Fig. 3a-b) when euthanized.

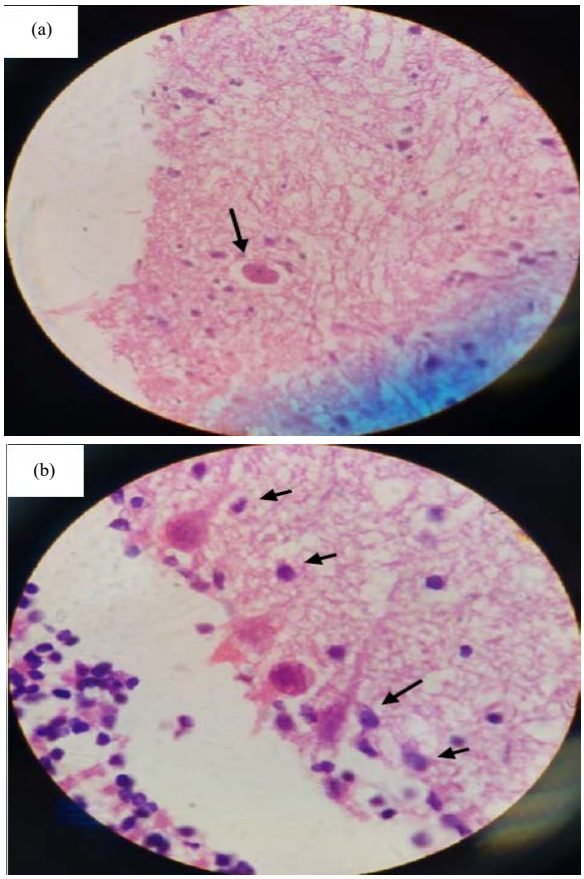

Fig. 3(a-b): H and E stained mouse brain infested with *T. gondii*, (a) Tissue cyst and (b) Free tachyzoites (X 400) (Black arrow)

Table 1: Seroprevalence of *T. gondii* infection in cattle egrets as evaluated by MAT test

	Tested birds		Positive birds by MAT	
Variables	Number	Percentage	Number	Percentage
Total	51		7	13.7
Age				
Juvenile	5	9.8	0	0.0
Adult	46	90.2	7	15.2
Gender				
Undifferentiated	5	9.8	0	0.0
Male	14	27.5	3	21.4
Female	32	62.7	4	12.5

PCR and DNA sequencing results: PCR of B1 gene (193 bp) confirmed the presence of DNA of the parasite in only one sample from the two seropositive cattle egrets (Fig. 4). This bird had a titer of 1:200. Sequences were confirmed as *T. gondii* DNA by blasting them on Genbank and they showed 84.85% identity with the AF 179871 sequence of *T. gondii* and type III strain VEG (accession number LN714499) in GenBank.

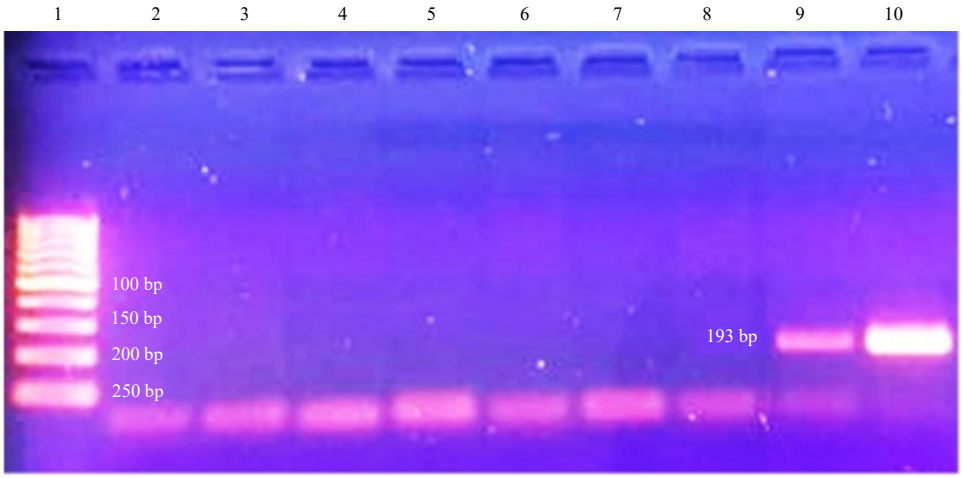

Fig. 4: Detection of Toxoplasma genome by PCR using Toxoplasma gene B1 primers
Lane 1: 100 bp DNA ladder, Lanes 2-8: Negative specimens, Lane 9: Positive isolate (193 bp) and Lane 10: Reference RH strain

DISCUSSION

Many studies have been done to detect *T. gondii* infection in animals from Egypt, but little is available on the parasite prevalence in birds especially wild ones[13]. In the current study *T. gondii* seroprevalence in 13.7% in Cattle egret was detected from Egypt using MAT. Regarding *T. gondii* prevalence in birds in Egypt, the most studies have been related to birds that are intended for human consumption, for example, chickens[14,15], domestic turkeys and ducks[16-18] which have shown varied prevalence rate of the parasite antibodies. Few reports detected seroprevalence of anti-*T. gondii* antibodies in wild birds living in Egypt. The free-range chicken showed an infection rate of 40.4% using MAT[19] and 69.5% when applying ELISA[20]. While *T. gondii* antibodies were detected in 29.8% of Egyptian native quails on using MAT and in 25.5% with latex agglutination test[21]. Diverse host species may display inconstancy in susceptibility to parasitic infection with regards to resistance (variability in the propensity to become infected) and intolerance (variability in the harm caused by a given level of infection)[22]. Detection of the *T. gondii* in Cattle egret, herein, indicates that this wild bird serves as an intermediate host for the parasite. Consequently, this could reflect the environmental contamination with the parasite due to Cattle egret ground-feeding and opportunistic feeding habits[23]. They also, like most birds, are resistant to develop symptoms when infected with *T. gondii* whether naturally or experimentally and this makes them an excellent reservoir for the parasite[24]. Also, Cattle egret has a longer life span compared to rodents and smaller birds[25].

Egrets may get the infection via ingestion of food contaminated with oocysts or directly from the infected soil, also, they eat insects that carry the parasite oocyst[26]. Egrets in Egypt are frequently seen feeding on garbage and big dumpsters. Stray cats are also common in Egypt and cats are seen wandering in streets where humans and birds live. Stray cats play an important role in the continuity of the parasite life cycle in Egypt especially in rural areas where stray cats may get access to domestic animal areas and human houses. These cats live outdoors and feed on garbage, food remaining including undercooked or raw meat, small rodents and birds as well as dirty food contaminated with soil and possibly *T. gondii* oocysts followed by shedding millions of oocysts to the environment[27,28]. Male and female egrets in the current study have the same feeding habits and the obtained results had no significant difference between them regarding. *T. gondii* seropositivity. Although there was no significant difference between adults and juveniles in acquiring *T. gondii* infection, all the positive birds were adults. These non-significant results could be attributed to the small sample size and longer life span of the adults compared to juveniles. Humans in Menoufia governorate have revealed high prevalence rates of *T. gondii* specific antibodies recording 67.5, 52.2 and 36.84% by El Deeb *et al.*[29], Nassef *et al.*[30] and Ibrahim *et al.*[31] respectively. Humans serve as an intermediate host for the parasite and are exposed as other birds and animals. The presence of such prevalence in birds, humans and animals indicates the widespread of *T. gondii* in this area. Humans are omnivorous and they can get the infection through several ways either through ingestion of oocyst in contaminated food or water or ingesting tissue cysts in

undercooked or raw meat. They also can get the infection vertically[32], which makes them highly exposed to the infection and increase their susceptibility to it.

For detecting *T. gondii* antibodies in birds, MAT and indirect fluorescent antibody tests are the most commonly used tests as they are considered the standard and most reliable tests[33]. In this study, MAT was used because it is an easy and cheap method and it has been validated in birds[34]. MAT does not require species-specific conjugates, thus making it suitable to use to evaluate the antibodies in numerous wild avian species[6]. It is also highly sensitive and specific and does not cross-react with related organisms[35]. Mice bioassay is one of the methods that directly detect *T. gondii* in tissues of animals and birds and indicate active infection[36], also, it amplifies the parasite number to facilitate its detection by other methods like PCR. Out of 7 birds that were positive by MAT, six birds were mice bioassay positive by MAT and two by H and E stained sections. Also it can use one mouse for each seropositive bird which might affect the results of the mice bioassay. The titer of antibodies to *Toxoplasma* is another factor that can play a part in successful isolation. Dubey *et al.*[34] recorded that the isolation frequency of *Toxoplasma* from the chicken heart using mouse bioassay usually improved with rising MAT antibody titers.

In the context of the speech, on experimental *Toxoplasma* infection to pigeons, the parasite was isolated by mice bioassay from tissues of 5 out of 12 (41.7%) birds infected experimentally with sporulated oocysts although the infection is verified by serology and nested PCR[33]. For detecting *Toxoplasma* DNA in the tissues of the mice, we used PCR of the B1 gene which is highly conserved in all *T. gondii* strains. Approximately 35 copies of this gene are found in *T. gondii* genome but absent in other mammalian cells[37]. Although seven birds were MAT positive and mice bioassay was successful in 6 of these birds, we were able to get positive PCR results from only one mouse out of the 6 positive mice by MAT. This may be a result of a limited sample size, random distribution of the parasite or low numbers of tissue cysts in mice[38]. It is believed that was what happened in our case. In a study done by Wyss *et al.*[39], they identified positive PCR samples in 9 (6%) brain and diaphragm tissues from 150 sheep, while ELISA was positive in 79 (52.67%) of these animals. The small size of the sample analyzed by PCR is also mentioned by these authors as an explanation of the different outcomes of PCR and serology. The positive *T. gondii* PCR sample was confirmed that by the sequencing of the B1 gene.

Based on reviewed literature and the results obtained from this study, *Toxoplasma gondii* affects most species of warm-blooded animals, including wild birds like cattle egrets (herons). These results proved the significant prevalence of toxoplasmosis in cattle egrets from menoufia Governorate, Egypt and pointed out to the importance of wild birds as intermediate host can transmit the zoonotic important *T. gondii* parasite to human. Therefore, this study give the attention to the importance of preserving the environmental hygiene from microbial contamination, especially the *T. gondii* infective stages, for the avoidance of transmission of infection to wild birds and thus to humans.

CONCLUSION

This study documented for the first time, in Egypt, that cattle egrets are the intermediate host for zoonotic important *T. gondii* parasite and it is a proof on contamination of the environment by *Toxoplasma* oocysts. This marks the importance of more research to be done on wildlife in Egypt to investigate these birds and animals as a sentinel for *T. gondii* infection.

SIGNIFICANCE STATEMENT

This study declared that cattle egrets from Menoufia, Egypt was for the first time can obtain the *T. gondii* infection from its surrounding contaminated environment with *Toxoplasma* oocysts and it would be considered as an important vector and intermediate host for zoonotic important *T. gondii* parasite. This study helps the researchers to apply more studies on wildlife in Egypt to investigate these birds and animals as a sentinel for *T. gondii* infection.

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