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Re-shedding Trials of *Toxoplasma gondii* Oocysts from Experimentally Infected Kittens Reference to Strain Types I, II and III; Zoonotic and Histopathological Confirm

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Abstract

Cats are the only definitive host of *Toxoplasma gondii* (*T. gondii*) that shed millions of un-sporulated oocysts. The current study aimed at evaluating the serological and histopathological potential ratio of *T. gondii* oocysts shedding and re-shedding kittens. Blood and fecal samples collected from fifty-seven un-weaned kittens from various districts of Cairo Governorate, Egypt. All kittens serologically screened for *T. gondii* using the Latex agglutination test (LAT), and fecal materials inspected daily for oocysts. Only proven seronegative un-shedding kittens employed in the three experimental re-shedding studies using the three *T. gondii* strain types II, III, and I with the third trial focusing on the ability of re-shedding with corticosteroid therapy. One kitten corresponding to each type sacrificed for histo-pathological assay and IgM/IgG serum antibodies recorded along the trials course using ELISA. The overall sero-negative percentage was 59.7%, with 35.1 and 24.6% in shedding and un-shedding ones respectively. The shedding number of oocysts /100 mg/feces scored (7, 5 and 3); (30, 15 and 7); (20, 10 and 5) corresponding and sequence to the three *T. gondii* strain types I, II and III respectively. In addition, the average IgM/IgG ELISA titers recorded significance variations sequence to the three strain types. Corticosteroids therapy stimulates re-shedding ability for the third time. The findings concluded that kittens shed oocysts at a high rate; regard shedding and re-shedding qualities as a critical component in developing oocyst-borne human and animal toxoplasmosis effects. Strong protection against vaccinated cats are required, as is close interaction between cats and farm animals.

KEYWORDS
Toxoplasma gondii strains, Kittens, Shedding and re-shedding, Histopathology, ELISA.

INTRODUCTION

The parasitic zoonosis, *Toxoplasma gondii* (*T. gondii*), an obligate intracellular protozoan parasite capable of infecting all warm-blooded species, including humans, is the cause of toxoplasmosis. In immune-compromised people, it is one of the most opportunistic pathogens (Shaapan *et al*., 2021b). Abortion, miscarriage and incomplete pregnancy with neonatal deaths in both human and animals has been linked to economic and health problems because of the widespread nature of toxoplasmosis infection (NA *et al*., 2018). Cats are the only known definitive host of this parasite, which may reproduce sexually, excrete, and shed millions of un-sporulated, environmentally resistant oocysts. The subsequent soil sporulation and dusting that follow significantly infect humans and all intermediate animal hosts, contaminating surrounding foods and water (Moharm and Shaapan, 2020). Humans are susceptible to becoming infected by eating meat that contains tissue cysts (bradyzoites) in it that is raw or undercooked (Barakat *et al*., 2012), eating food or drinking water that has been contaminated with cat feces that has sporulated oocysts within it and ingesting unpasteurized milk that contains the quickly developing, tachyzoites stage (Elaadli *et al*., 2023). Therefore, avoiding contact with infectious stages, using biosecurity measures to assure the safety of both food and water, adopting good sanitation practices, and using vaccinations to reduce oocyst shedding and re-shedding by definitive feline hosts are the key control points to prevent oocysts-borne human toxoplasmosis (Smith *et al*., 2021).

Only the feline species that serves as its only host does *Toxoplasma gondii* have a sexual intestinal cycle. The gametogny that sheds and re-sheds the oocysts that are resistant to environmental factors from kittens' excrement has the potential to undergo genetic mutation (Gil *et al*., 2023). Sporozoites from feces always discharged within two weeks without sporulating. A few days later, soil sporulation takes place, beginning the most common human infectious tissue cysts stage because to intimate contact between man and meat-producing animals (Shaapan *et al*., 2021a). After the first oocysts excretion, cats usually develop immunity to the re-excretion of oocysts when challenged with homologous or heterologous strains of *T. gondii*. The number of oocysts eliminated during the second infection was lower than the first (Ahn *et al*., 2019). The environmental contamination by oocysts from re-infected adult cats is only 30% lower than from kittens, so, the excretion of *T. gondii* oocysts was higher in experimentally re-infected cats throughout the years, especially when a heterologous strain was used (Zulpo *et al*., 2018). The majority of kitten exposure is from *T. gondii* carrier prey, and chronic stage bradyzoites exposed kittens shed oocysts more frequently than acute-stage tachyzoites or oocysts-exposed kittens (Dubey and Jones, 2008). Within two weeks, one kitten might shed and re-

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shed up to one billion oocysts. Chronically infected kittens may shed oocysts again as a result of senility, immunosuppression, or treatment with anti-inflammatory corticosteroids (Elfadaly *et al*., 2015).

Naturally, infected domestic kittens and wild felids have been shown have mixed infections with more than one *T. gondii* strain. Higher virulent varieties, however, typically hid the biological characteristics of less virulent ones (Elfadaly *et al*., 2017b). *Toxoplasma gondii* isolates are becoming more varied and abundant, and recent molecular techniques have revealed the complexity of its global population structure. The 16 haplo-groups found within the six ancient groups up to this point include predominant clonal lineages (such type I, II, or III) strains, as well as a greater number of clusters that reflect recombination events (Dardé *et al*., 2020). Oocysts not just confined to the ground; they e also been observed in aquaculture and in marine animals as a source of water and food-borne zoonosis (Burgess *et al*., 2018). All intermediate and final hosts including humans, herbivores, omnivores, and carnivores where the protozoan survives and infects through any of the three infectious stages; tachyzoites, bradyzoites, and oocysts were affected by the additional intestinal cycle (Shaapan *et al*., 2010). Acute tachyzoites, however, multiplied quickly in blood cells. Bradyzoites (tissue cysts) can reactivate to the acute tachyzoite stage sequence to latent opportunistic infection while chronic stags reside in somatic cells for the respite of the host (Hassan *et al*., 2016). Also, latency possibly ranks to blood transfusion or organs transplantation and human horizontal infection arises result to oocysts contaminated food or water or due to tissue cysts within insufficient cooked meat (Abd El Wahab *et al*., 2018). Consumption of oocysts-contaminated vegetables has been linked to epidemics of acute toxoplasmosis (Elfadaly *et al*., 2017a) or through contaminated fish or marine animals, Human vertical transmission, on the other hand, often occurs by trans-placental diffusion, breastfeeding an infant from a carrier mother, or experiencing a latent opportunistic relapse (Sharma *et al*., 2018).

Toxoplasma gondii strain Types include Type I (RH-Tachyzoites) strain is a highly virulent strain for mice and disseminated with rapid death of 100% of mice even with one tachyzoite (LD1-100), difficult to form tissue cyst, shed none or very few atypical oocysts at 7-10 day post infection (DPI) and rarely isolated (mutation strain) (Elfadaly *et al*., 2012).

Type II (ME49-Bradyzoites) strain is a moderately mice virulent strain, highly produce tissue cysts, shed huge typical oocysts at 3-21 DPI and are the most commonly isolated from aborted and AIDS human cases (Hassanain *et al*., 2013a). Type III (Prugniaud-oocysts) strain is a mildly virulent for mice, moderately produce tissue cysts, shed moderate typical oocysts at 3-10 DPI and mainly isolated from meat producing and wild animals (Toaleb *et al*., 2014).

According to Silva *et al*. (2021), up to one-third of the world's population are carriers of *T. gondii*. In order to solve the unexplained nature of the high toxoplasmosis seroprevalence in humans and animals, recent ecological research on risk factors has been updated. Consequently, the main objective of the current study was to update the Egyptian ecological data concerning the percent of both *T. gondii* seropositivity along with oocysts shedding and re-shedding properties in kittens, in relation to histopathological confirm. Also, to evaluate the re-shedding of *T. gondii* oocysts in experimentally infected kittens with three strain types I, II and III in correction with shedding and re-shedding onset, pre-patent period and oocysts characters, all considered an excellent bio-indicators reflect the degree of zoonotic bio-hazard via kitten harboring *T. gondii* virulent strains.

MATERIALS AND METHODS

Ethical approval

Animal care and the experimental study procedures approved and carried out in accor-dance with animal ethics committee guidelines of the National Research Centre (NRC) Egypt under protocol number: 19-139.

Kittens' population

A total number of 57 kittens were collected from different regions from Cairo Governorate, Egypt. The collected newly born kittens of about 25 days old were adapted for handling, housed in metal cages, and offered *T. gondii* free foods.

Experimental design

All the naturally exposed kittens (n.= 57) at beginning of the experiment, screened for *T. gondii* using the LAT and fecal materials inspected daily for oocysts shedding. While the only confirmed seronegative un-shedding kittens, (n.= 14) used for the three experimental re-shedding trials, two months intervals between each trial. At the first res-heeding trail, two kittens' still uninfected controls while 12 kittens were infected as each four kittens with the three *T. gondii* strain types I, II and III. One kitten corresponding to each type sacrificed for serological and histopathological assays. The remaining 9 kittens were exposed to the second re-shedding trial as each three kittens were infected with *T. gondii* strain types and one kitten corresponding to each type was sacrificed. At the third re-shedding trial, the last remaining 6 kittens were infected with the three *T. gondii*, 2 for each strain types along with corticosteroids therapy and at the end of experiment the six cats were humanity sacrificed for the serological and histopathological assays (Fig. 1).

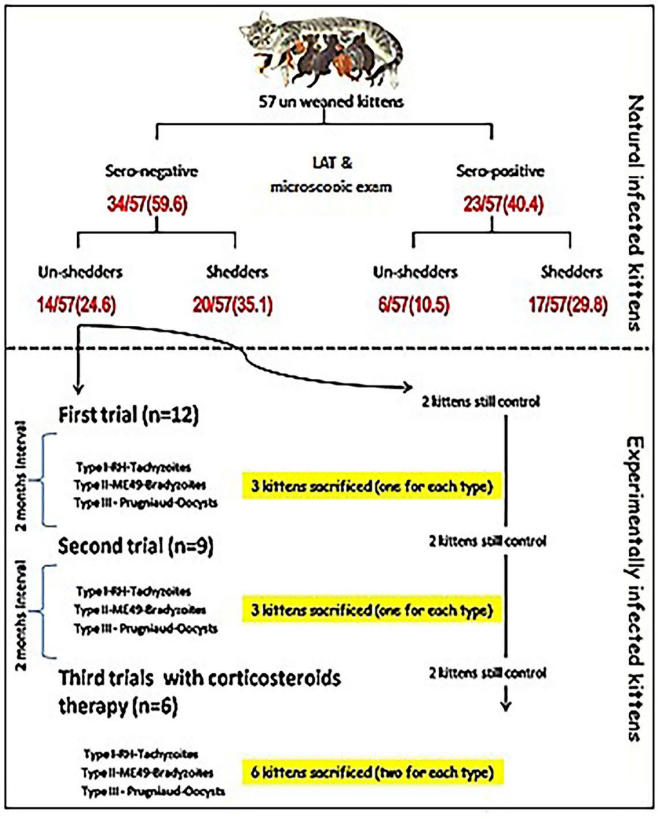

Fig. 1. Experimental design of kittens for the three experimental re-shedding trials with *T. gondii* strain.

Fecal samples inspection

T. gondii oocysts were isolated from kitten's fecal debris using a concentrated salt and/or sugar flotation technique and centrifuged for 5 minutes at 1800 r.p.m. before examined under a (x600) microscopic field. After 3 days, a 2.5% cesium chloride (CsCl) solution at room temperature used to promote sporulation of fresh oocysts (Hassanain *et al*., 2013b). The sporulated oocysts distinguished from the un-sporulated oocysts by the presence of two sporocysts, each containing four sporozoites. *T. gondii* oocysts should differentiated from Isospora oocysts, which may shed in cat feces in the same sample (Shaapan *et al*., 2015a).

Serological assays

Latex agglutination test (LAT): the LAT used as a screening test for anti *T. gondii* IgG in all kitten serum. The test carried out according to the kit manufacturer's instructions (Toxo Latex Kit, CamTech Medical, UK) and the procedures described by Abdalhamed *et al*. (2019), in brief, one drop of each negative and positive control sera was built-in for each try, sera samples mixed well, and the mixture slowly rotated for 6 minutes. When agglutination is found at dilutions of 1:64 or above, it is termed positive.

Enzyme linked immunoabsorbent assay (ELISA): the kittens, which represented to the three are-shedding trials exposed to ELISA. Commercial Toxoplasma IgM/IgG ELISA kit (Atlas Medical Company, UK) was performed following the manufacturer's instructions with the procedure described by Alanazi *et al*. (2018) at 450 nm optical density and using automated ELISA reader (BIOTEK, INC. ELx, 800UV USA). Concentration of antigen, serum and conjugates dilutions assessed by checkerboard titration. Cut-off value was intended by negative mean OD values +2SD (0.154 IgM / 0.148 IgG) (Hassan *et al*., 2012).

Histopathological assay

T. gondii experimental kittens intestine were exposed to histopathological assay, tissue specimens of about 0.5 cm, fixed in 10 % neutral buffered formal saline solution, sectioned and further cutting at 5 um and staining with haematoxylin and eosin (H & E) used for detection of *T. gondii* parasites. The acute or chronic *T. gondii* related lesions with the pathological abnormalities then investigated under an optical microscope at power magnifications ranging from x 400 to x 1500 (Abd El Wahab *et al*., 2022).

Corticosteroids therapy

Two types of corticosteroids, Sluo-cortif as rapid onset corticosteroids in combination with dexamethazone sodium phosphate as maintenance long acting corticosteroids used to stimulate the *T. gondii* oocysts re-shedding ability at the third time of infection. Sluo-cortif used at a dose 50 mg/kg, daily I.M injection for seven successive days. While dexamethazone sodium phosphate was used at a dose of 6 mg/kg I.M injection, twice weekly for two weeks (Elfadaly *et al*., 2015).

RESULTS

T. gondii infection in kittens by LAT and microscopic examination

The un-weaned naturally infected kittens screened for anti-*T. gondii* antibodies using LAT and for *T. gondii* oocysts shedding by daily fecal materials inspection. The overall sero-positive percent of kittens was 40.3% where, 29.8 and 10.5 % of them were shedding and un-shedding for *T. gondii* oocysts, respectively. While the overall sero-negative percent was 59.7 %, where, 35.1 and 24.6 % in shedding and un-shedding, respectively. The total shedding percent 64.9% verses to 35.1 % un-shedding (Table 1).

Table 1. *T. gondii* screening in kittens using the LAT and fecal microscopic inspection.

Un-weaned kittens	Number (%)	Sero-negative Number (%)	Sero-positive Number (%)
Shedders	37 (64.9)	20 (35.1)	17 (29.8)
Un-shedders	20 (35.1)	14 (24.6)	6 (10.5)
Total (%)	57	34 (59.7)	23 (40.3)

IgM / IgG seropositive kittens' reference to oocysts shedding characters

Oocysts shedding characters in the three experimental re-shedding trials study using the three different *T. gondii* strain types recorded pre-patent periods of (7, 3 and 12); (10, 13 and 17) and (12, 15 and 20) day post infection. The shedding periods were (15, 10 and 7); (18, 7 and 5); (21, 10 and 3) days. While the

Table 2. ELISA IgM / IgG seropositive kittens reference to oocysts shedding characters

T. gondii strain Types	Average IgM / IgG ELISA titer				Oocysts shedding Characters		
	1week		2 weeks				
	IgM	IgG	IgM	IgG	PPP	No/100 mg	SP
The first re-shedding trial (n=12)							
Type I strain (RH-Tachyzoites)	0.72	0.39	0.58	1.49	7DPI	7 oocysts with free sporozoites	15D
Type II strain (ME49-Bradyzoites)	0.56	0.53	0.38	1.41	10 DPI	30 oocysts typical and dense	18D
Type III strain (Prugniaud-oocysts)	0.23	0. 285	0.43	1.27	12DPI	20 oocysts mild avirulent	21D
The second re-shedding trial (n=9)							
Type I strain (RH-Tachyzoites)	0.36	1.62	0.35	1.64	3DPI	5 oocysts with free sporozoites	10D
Type II strain (ME49-Bradyzoites)	0.29	1.33	0.25	1.35	13 DPI	15 typical oocysts	7D
Type III strain (Prugniaud-oocysts)	0.42	0.99	0.41	1.38	15DPI	10 oocysts mild avirulent	10D
The third re-shedding trial (n=6)							
Type I strain (RH-Tachyzoites)	0.34	1.58	0.27	1.75	12DPI	3 oocysts with free sporozoites	7D
Type II strain (ME49-Bradyzoites)	0.27	1.36	0.23	1.31	17 DPI	7 typical oocysts	5D
Type III strain (Prugniaud-oocysts)	0.51	1.25	0.33	1.33	20DPI	5 oocysts mild avirulent	3D

PPP: pre-patent period; SP: shedding period; DPI: day post-infection.

number of shedder oocysts per 100 mg of kittens' feces scored (7, 5 and 3); (30, 15 and 7); (20, 10 and 5), corresponding and sequence to the three *T. gondii* strain types I, II and III, respectively (Fig. 2). However, the average IgM/ IgG ELISA antibodies titers recorded significance variations sequence to the three trials and the strain types I, II and III as gradual decrease in the IgM titer, in contrast gradual increase in IgG antibodies titer from first to second and third trail of experimental infection. In addition, the corticosteroids therapy stimulates re-shedding ability for the third *T. gondii* infection trial (Table 2).

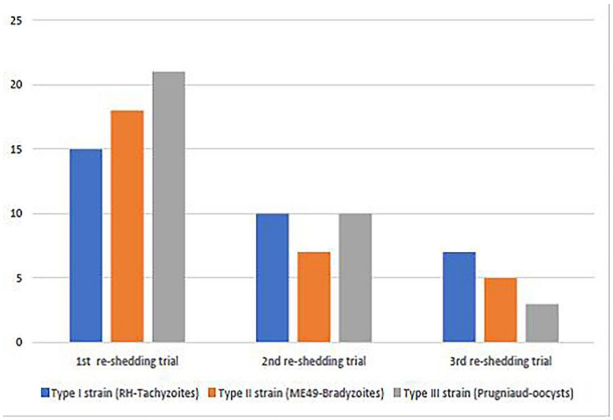

Fig. 2. Shedding periods of *T. gondii* oocysts corresponding to infection with types I, II and III strains.

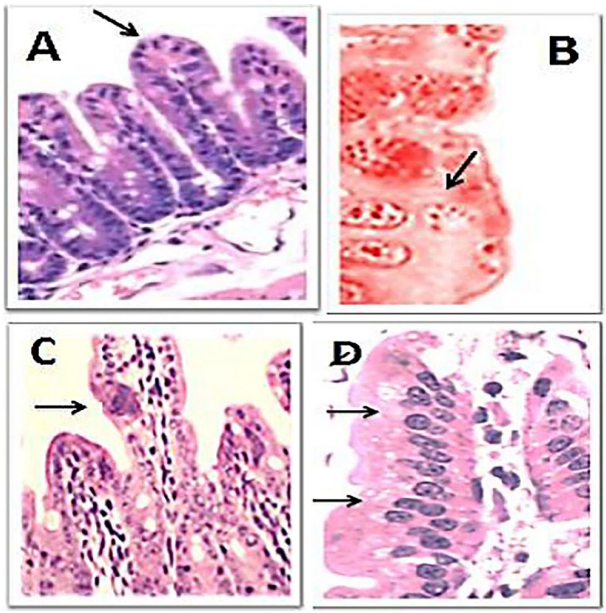

Fig. 3. Kitten's intestine infected with *T. gonddi*; H&E. (A): submucosa heavy parasitized with merozoites and shizonts [arrow] x400. (B): infiltration of the different enterocytes and microgamonts x1000. (C): merozoites liberated in lamina propria [arrow] x600. (D): lamina propria and villi infiltrated with microgamonts [arrow] x1000.

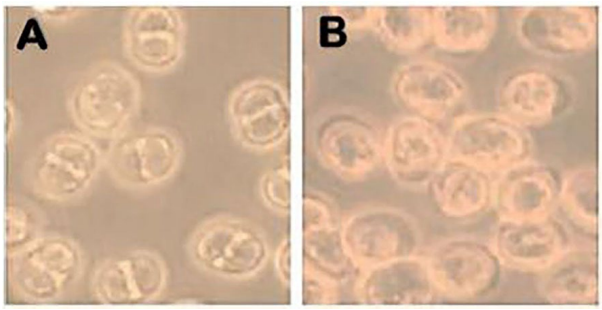

Fig. 4. *T. gonddi* oocysts in CsCl solution of kitten's fecal sample; sporulated (A) and un-sporulated oocysts (B), x 1500.

Histopathological assay

The histopathological examination of kittens' intestine confirmed acute and chronic *T. gondii* associated lesions with the pathological alterations of kittens gastrointestinal tracts (GIT) the results insure oocysts re-shedding aptitude sequence to the virulent strains. Where, the parasite load was elevated with the sequence first and second propagation, the submucosa revealed heavy parasitization with merozoites and shizonts, infiltration of the different enterocytes stages of schizonts and the intestinal villus and lamina propria infiltrated with liberated merozoites and microgamonts (Fig. 3).

T. gonddi oocystss detection in kitten's fecal sample

The fecal materials of examined kittens inspected daily for detection of oocysts shedding using a concentrated salt and/or sugar flotation technique and purified through cesium chloride (CsCl) solution. The sporulated oocysts distinguished from the un-sporulated oocysts by the presence of two sporocysts, each containing four sporozoites (Fig. 4).

DISCUSSION

When it deals with environmental issues and human-animal toxoplasmosis, *T. gondii* environmentally resistant oocyst stage is essential to the parasite's survival in terms of both its widespread distribution and the large variety of intermediate hosts that it infects and it is essential for *T. gonddi* oocysts to spread through shedder and re-shedder kittens. (Shapiro *et al*., 2019). In the current study, the total shedding percent in the un-weaned naturally infected kittens 37 (64.9%), representing 20 (35.1%) and 17 (29.8%) of them were sero-negative and sero-positive for *T. gondii* antibodies, respectively. The extremely high shedding rates have an adverse effect on public health and zoonotic epidemiology and the characteristics of oocysts' biology may help to explain *T. gondii*'s was global spread and how it to be one of the most common infectious diseases affecting humans as well as animals (Shaapan *et al*., 2012). Oocysts were widely distributed across the soil in subsequent rains by the wind, earthworms, or arthropods (Abdel-Shafy *et al*., 2015). Therefore, exposure to soil contaminated with *T. gondii* oocysts is a substantial risk factor for human infection and is probably the most prevalent means of transmission to rodents, birds, and food-producing animals (Egorov *et al*., 2018). Additionally, by observing the proportion of seropositive individuals as well as the predisposition for oocysts shedding and re-shedding in kitten populations, the oocysts-shedding kittens was higher (35.1%) from the sero-negative group than (29.8%) from the sero-positive group. This was be demonstrated by the fact that the majority of kittens either came from mothers or were exposed to *T. gondii* infections early in the feeding cycle. Negative antibodies that were given to kittens don't seem to have stopped them from shedding oocysts (Al-Malki, 2021). Moreover, the young, un-weaned kittens may shed up to one billion more oocysts than adults may over the course of two weeks and seropositive un-shedders kittens might have finished their shedding cycle before collection (Valenzuela-Moreno *et al*., 2019).

Furthermore, a higher sero-positivity of 23 (40.3%) where, 17 (29.8) were shedders and 6 (10.5) were un-shedders kittens was obtained in the present study, indicating that Egyptian kittens consistently followed a semi-stray pattern and that indoor kittens were not fully street-limited. when eating the litter is an established habit and hunting is common (Crowley *et al*., 2019). Because of ignorant cat owners, indoor kittens that eat the viscera and remnants of chicken carcasses expose cats to tissue cysts, the most prevalent stage of cat disease and shedding (Hassanain *et al*., 2011). The current investigation identified a higher prevalence

of Toxoplasma infection in the stray or semi-domesticated cats. the obtained results are consistent with that indicated by Inpankaew *et al*. (2021), who determined the prevalence of *T. gondii* in semi-domesticated cats and pet cats was 11.5 and 1.5%, respectively; the semi-domesticated cats aged 1–5 years (14.9%) had a higher prevalence of infection than domesticated cats (1.3 %,) of the same age. Similar to this, (Vollaire *et al*., 2005) assessed the seropositive toxoplasmosis cases in the United States and found that there were considerable variations between client-owned cats and stray cats, ranging from 16.1% to 43.5%. (Haddadzadeh *et al*., 2006) also pointed out a significant contrary in their data, indicating that the overall infection rate was (63%) in stray cats and (36%) in domestic cats. In contrast, it is important to note that, according to the findings of different assessed studies, *T. gondii* seroprevalence did not differ between cats domiciled in urban and semi urban or rural areas. However, cats who consumed prey, raw meat, and/or unpasteurized milk had a statistically significant increased risk of contracting *T. gondii* compared to cats who did not consume any raw food (Brennan *et al*., 2019; Elfadaly *et al*., 2018). Consequently, recently, (Kokkinaki *et al*., 2023), study investigating risk factors for *T. gondii* seropositivity in different populations of cats in Greece, including client-owned cats, stray cats, and cats who lived in catteries and reported no appreciable differences between stray and client-owned cats

In Egypt, there is a direct interaction between food animals and human toxoplasmosis and the human toxoplasmosis possibly occur by either direct contact with cats, ingesting of poorly cooked meat or the two sources (Zeedan *et al*., 2022). Furthermore, the *T. gondii* virulent strains identified by the mutton bioassay showed that not all sero-positive sheep are associated with zoonotic biohazard, indicating that feeding uncooked mutton is bad health habit as a source of human toxoplasmosis (Hassanain *et al*., 2011). In the same way, the high incidence of cattle and camel toxoplasmosis, which are significant human infective sources, has a detrimental effect on public health. In the same way, cattle and camel toxoplasmosis are major infective sources for human, and their high prevalence has a negative impact on the public health (Toaleb *et al*., 2013). The primary risk factors for sheep and women seropositive samples were also analyzed, relatively higher seroprevalence of *T. gondii*, 62.6%, recorded in sheep than 57.3% in aborted women (Elaadli *et al*., 2023). The wastage nourished Egyptian lambs, which established harboring *T. gondii* virulent strains revealed abundant (26%) (Al-Kappany *et al*., 2018) or higher seo-prevalence (47, 5 %) rate of infection (Elfadaly *et al*., 2017a). Furthermore, up to 57.1% of ready-to-eat Egyptian meat meals confirmed positive for *T. gondii* by PCR (Shaapan *et al*., 2015b). This explains the zoonotic hazards, which caused directly by T. gonsii oocysts contaminating and increasing the amount of food, water, and vegetables, or indirectly by meat-producing animals that are carrying tissue cysts (Marín-García *et al*., 2022).

The present study confirm varied average IgM/IgG antibodies titer in the three re-shedding trials reference to the infection by the three *T. gondii* strain types I, II and II . So, average IgM/IgG ELISA antibodies titer probability significant biomarkers for the onset of shedding and re-shedding periods (Valenzuela-Moreno *et al*., 2019). Additionally, the high sero-positivity in unwanted kittens is a bio-indicator of how *T. gondii* strain types I, II, and III shine their role in the ecology of toxoplasmosis through repeatedly exciting oocyst shedder and re-shedder kittens (Elfadaly *et al*., 2017b). Type-I (RH) is the most highly virulent human strain, both in the current investigation and according to (LD50 & LD100). It is difficult to isolate, though. This is because it believed that this strain frequently causes host mutations, especially in immunocompromised individuals (Hassanain *et al*., 2018). Type II (ME49) strain produced large, typical oocysts and was moderately pathogenic against mice where, type II was unquestionably the most common strain in Egyptian mutton and the most common strain in toxoplasmosis in humans (Dardé *et al*., 2020). Type III (Prugniaud strain) was mildly virulent for mice, shed a moderate typical oocysts, which is the strain infect the vast majority of animals and humans stock isolates (Elfadaly, 2023). Histopathological assay revealed acute and chronic *T. gondii* associated lesions with the pathological alterations of kitten's gastrointestinal tract. The findings ensure that oocysts can shed once more in a way that is suitable for virulent strains and the parasite load increased because of the first and second propagation sequences (Dubey and Jones, 2008).

CONCLUSION

As a key bio-indicator of the severity of Egypt's oocysts-borne toxoplasmosis, the current study's findings revealed that the ovulation and oocysts shedding rates in kittens are consistently high on zoonotic perception and public health. In addition, this data reached the conclusion that socioeconomic factors such as cat housing status, feeding habits, and sex differences significantly affect how common cats are in various countries. Egypt is an endemic country with a high rate of oocysts deposition; Egyptian cats always live in a semi-stray pattern. Therefore, must increases oocysts-borne toxoplasmosis protection for humans and animals in order to eradicate and reestablish features, in addition to immunizing indoor kittens and routinely screening pregnant women, we accepted Egyptian rigorous preventive events to stop cats from connecting with farm animals, increase awareness of feline breeders, and warn of the dangers of improperly cooked meat.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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