PARASITES IN THE TROPIC - A NEW PARADIGM SHIFT

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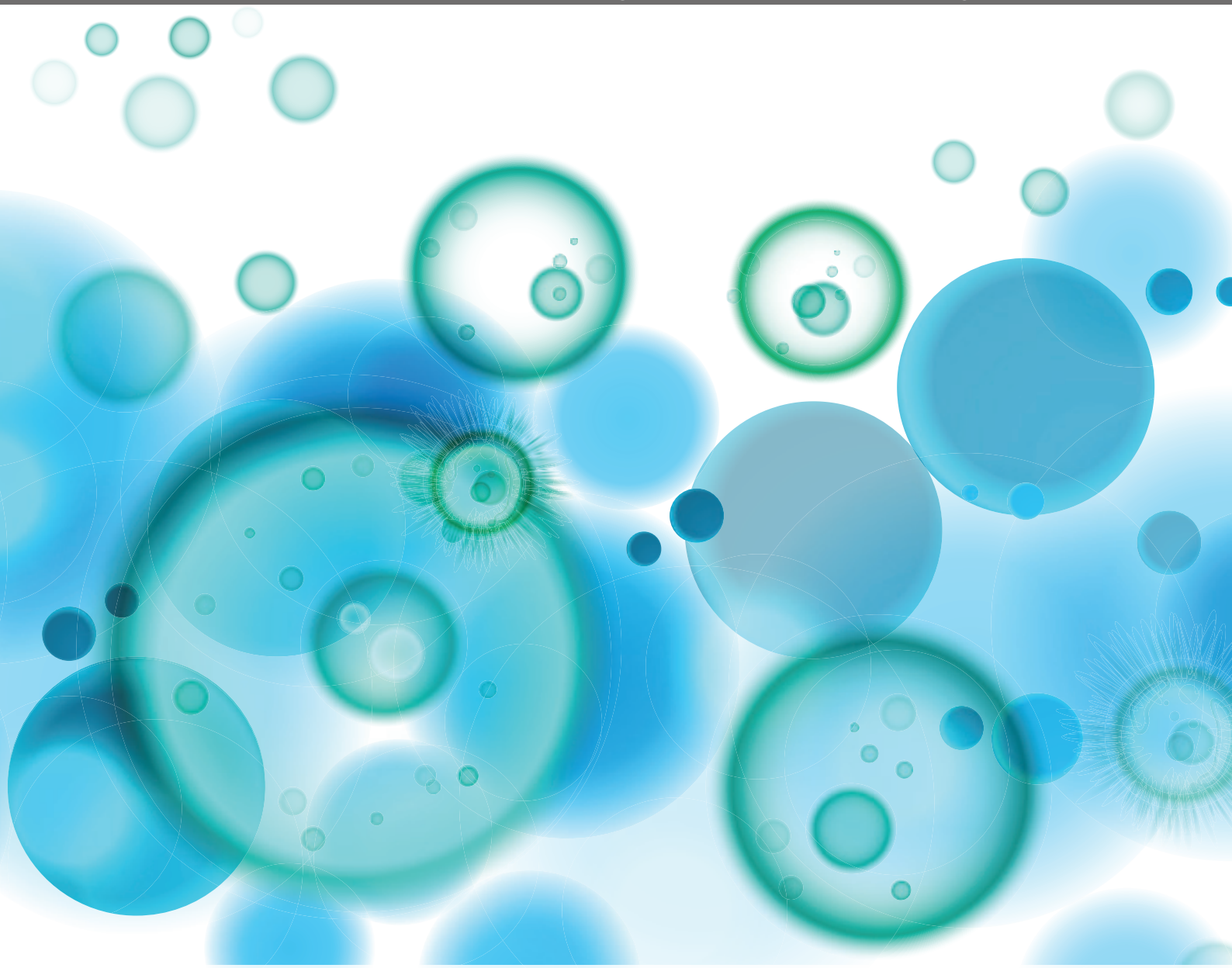

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PARASITES IN THE TROPIC - A NEW PARADIGM SHIFT

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The highlight of this eBook is to bring new insights into parasites in the tropic. To achieve that, much has been discussed about risk assessment, infection rates, disease burden, hormones and mechanism of immune response, genetic expression and susceptibility as well as, therapeutic modalities. Authors raised hypothesis, discuss concepts, and show open questions. The remaining important issues to resolve questions within parasites in the tropic – a new paradigm shift are briefly discussed below.

T. gondii, feline as the definitive host, is regarded as one of the most important parasites in the tropic. Human, as an accidental host, is the only species who still drinks raw milk or milk products particularly from animal sources. Based on the first paper, the author simplifies on how safe to drink milk to prevent the transmission of T. gondii by the insistence on heat treated milk before consumption. It is interesting to explore how hormone plays its role in Toxoplasma infection. Based on the second paper, the authors elucidated from thirty studies from humans, animals and cell cultures. Of these, it was shown that Toxoplasma infection was controlled by the presence of hormones found in different animal models. However, it is still premature to conclude which hormone that has a significant relationship with Toxoplasma infection.

It estimates that one-third of the world population infected with T. gondii but the majority are asymptomatic. Based on the third paper, it demonstrated that people having low prevalent of Toxoplasma infection by having close contact with animals. This study will enhance positive attitudes for more people to be committed towards helping animals. For more than three decades, T. gondii has since been identified as one of the most important opportunistic parasitic pathogens in immunocompromised. Seroprevalence of chronic toxoplasmosis was detected in at least one-third of HIV-infected individuals in the regional hospital of southern Thailand, as reported from the fourth paper. Thailand has successfully formulated anti-retroviral therapy for HIV/AIDS patients and as a result reported a rare incidence of AIDS-related cerebral toxoplasmosis (CT) in this setting. Based on the fifth paper, the authors demonstrated low IL-10 (Th2 response) and IFN-γ (Th1 response) as well as high TNF-α were produced in ocular and cerebral toxoplasmosis in AIDS patients. This might be due to South American strains and/or the genetic susceptibility of the host.

Due to high genetic diversity of T. gondii in Brazil, the sixth paper demonstrated that Calomys callosus survived chronically infected by T. gondii clonal type II strain and reinfected by Brazilian strains. However, congenital toxoplasmosis occurred leading to damaging effects of the developing fetus. The seventh paper conducted a questionnaire-based study on knowledge and practice on Toxoplasma infection among pregnant women from Malaysia, Philippines and Thailand. It clearly demonstrated that health education, a core value, is the cheapest and the best option to envisage the preventive strategies of feto-maternal toxoplasmosis from this region.

For treatment modality of congenital toxoplasmosis, a novel experimental therapeutic synergism of diclazuril plus atovaquone combination shows a promising outcome with no toxicity in treating this condition, as demonstrated in the eighth paper. However, it warrants for future trials to prove its properties against T. gondii in different clinical scenarios of human toxoplasmosis for more effective therapeutic regimens. In the ninth paper, the author discussed the pathogenesis of maternal and congenital toxoplasmosis, the current treatment in clinical practice, and the experimental treatment approaches for promising future trials. Overall, this protozoan represents the most extraordinary example of parasite in the tropic and beyond scientific imagination. Hence, there are still many challenges ahead and waiting for more explorations on T. gondii, the parasite that never dies.

Based on the findings from the tenth paper, it is interesting to identify common gene targets between Glossina p. gambiensis and Glossina m. morsitans that might shed some lights as a suitable candidate for controlling both acute and chronic forms of sleeping sickness. This therefore requires further investigations using proteomic analysis to ascertain the corresponding genes and its proteins as well as functional role that may help the search for more novel therapeutic agents.

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EDITORIAL
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Editorial: Parasites in the Tropic—A New Paradigm Shift

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The Editorial on the Research Topic

Parasites in the Tropic—A New Paradigm Shift

The highlight of this eBook is to provide new insights into parasites from the tropics. To achieve that, much has been discussed regarding risk assessment, infection rates, disease burden, hormones and mechanism of immune response, genetic expression, and susceptibility as well as therapeutic modalities. The authors raised various hypotheses, discussed a number of concepts, and listed currently unanswered questions. The remaining important issues to resolve within the parasites from the tropics context—a new paradigm shift—are briefly covered in the following.

Since 1900, *Toxoplasma gondii* (*T. gondii*) has continuously transcended from its initial mysterious, scientific discovery to its more advanced comprehension in modern times. *T. gondii*, with felines as the definitive host, is regarded as one of the most important parasites in the tropics. Humans, as an accidental host, are the only species who still drink raw milk or milk products particularly from animal sources. The author of the first paper simplified how safe it was to drink milk in order to prevent the transmission of *T. gondii* by the insistence on heat-treating milk before consumption. It is interesting to explore how hormones play a role in *Toxoplasma* infection. Based on the systemic review from the second paper, the authors made determinations from 30 studies on humans, animals, and cell cultures. Of these, it was demonstrated that *Toxoplasma* infection was controlled by the presence of hormones found in a number of animal models. However, it is still premature to conclude which hormone has a significant relationship with *Toxoplasma* infection.

It is estimated that one-third of the world population is infected with *T. gondii* but the majority of immunocompetent persons are asymptomatic. Based on the third paper, it demonstrated that people with low prevalence of *Toxoplasma* infection do so by having close contact with animals. Also, this study leads to enhancing positive attitudes toward helping animals. For more than three decades, *T. gondii* has been considered one of the most significant opportunistic parasitic pathogens in the immunocompromised. Seroprevalence of chronic toxoplasmosis was detected in at least one-third of HIV-infected individuals in a regional hospital of southern Thailand as reported in the fourth paper. Chronic toxoplasmosis is generally an asymptomatic condition; however, 95% of cerebral toxoplasmosis (CT) cases occur as a result of secondary reactivation of chronic toxoplasmosis in AIDS patients. Thailand has successfully formulated an anti-retroviral therapy for HIV/AIDS patients and, consequently, there is a minimal incidence of AIDS-related CT in this hospital.

Clinically, CT can be a life-threatening condition and is the most common clinical disease in the immunocompromised. With this, eye involvement is the most typical extracerebral toxoplasmosis but has the best prognosis. Based on the fifth paper, the authors demonstrated that low IL-10 (Th2 response) and IFN-γ (Th1 response) concentrations were observed in OT and CT/AIDS patients. However, large amounts of TNF-α are also produced in such patients, suggesting a pronounced inflammatory response is triggered by *T. gondii*. The nature of the infecting South American strains

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and/or the genetic susceptibility of the host may be reasons for this type of immune response and aids in the understanding of the control mechanisms of the immune system with respect to *T. gondii*.

A great prevalence of toxoplasmosis has been reported in South America, particularly Brazil. Based on the high genetic diversity of *T. gondii* in this region, the sixth paper uniquely exhibited that *Calomys callosus* survived chronic infection by *T. gondii* clonal type II strain and was reinfected by Brazilian strains. However, congenital toxoplasmosis occurred as a consequence of the acquired immune deficiency of the host. This could be because of the reactivation of the *T. gondii* ME-49 strain and strong pro-inflammatory immune responses, including Th1 cytokines and the antibody isotype during pregnancy. Therefore, vertical transmission of *T. gondii* takes place, leading to damage of the developing fetus.

T. gondii is one of the TORCH agents and plays a tragic role in congenital toxoplasmosis. Yet, acute *Toxoplasma* infection in a pregnant woman and congenital complications relating to morbidity and mortality in the newborn are reported at a very low rate in Southeast Asia. Supporting this, the seventh paper conducted a questionnaire-based study on knowledge of and practice for *Toxoplasma* infection among pregnant women from three Southeast Asian nations, namely Malaysia, Philippines, and Thailand. It clearly demonstrated that health education, a core value, is the cheapest and the best option for preventive strategies to eliminate feto-maternal toxoplasmosis from this part of the world.

In terms of treatment modality, the current therapies are ineffective for congenital toxoplasmosis, chronic disease, or severe side effects that may result in serious complications. A novel experimental therapeutic synergism of diclazuril plus atovaquone combination therapy appears to elicit promising outcomes with no toxicity when treating congenital toxoplasmosis, as was demonstrated in the eighth paper. However, future trials are warranted to establish the regimen's properties against *T. gondii* in different clinical scenarios of human toxoplasmosis in order to hone it into a more effective option. In the ninth paper, the author discussed the pathogenesis of maternal and congenital toxoplasmosis, the current treatment methodologies in clinical practice and promising experimental treatment approaches. Overall, this protozoan represents the most extraordinary example of a tropical parasite, one that sparks scientific imagination. However, there are still many challenges ahead and more exploration of *T. gondii*, the parasite that never dies, is needed.

Among parasitic diseases in Africa, one might also tend to think of a classic example of a neglected tropical disease (NTD), and human African trypanosomiasis (HAT), or sleeping sickness, comes to mind. HAT is caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*; the former causes the acute form and the latter evokes the chronic type. Based on the findings from the tenth paper, it is interesting to that the common gene targets between *Glossina p. gambiensis* and *Glossina m. morsitans* have been identified and might shed light on suitable therapeutic candidates for controlling both the acute and chronic forms. Thus, continued investigation, particularly using proteomic analysis to ascertain the corresponding genes and proteins, as well as the functional roles they play, may help the search for more efficacious agents.

AUTHOR CONTRIBUTIONS

VN: Data collection from the published articles and writing of this manuscript. YLL, SY, and HP contribute to the writing of the manuscript with comments. All authors have read and approved the final version of the manuscript.

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GENERAL COMMENTARY
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Commentary on: "Detection of *Toxoplasma gondii* in raw caprine, ovine, buffalo, bovine, and camel milk using cell cultivation, cat bioassay, capture ELISA, and PCR methods in Iran"

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A commentary on

Detection of *Toxoplasma gondii* in raw caprine, ovine, buffalo, bovine, and camel milk using cell cultivation, cat bioassay, capture ELISA, and PCR methods in Iran
Dehkordi, F. S., Borujeni, M. R., Rahimi, E., and Abdizadeh, R. (2013). Foodborne Pathog. Dis. 10, 120–125. doi: 10.1089/fpd.2012.1311

Comments on "detection of *Toxoplasma gondii* in raw caprine, ovine, buffalo, bovine, and camel milk using cell cultivation, cat bioassay, capture ELISA, and PCR methods in Iran."
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As it is known, toxoplamsosis occurs mainly by foodborne transmission: ingestion of raw or undercooked meat; unwashed fruit/vegetables, unhygienic water, or contaminated milk. Gaps in the concerning risk assessment of *Toxoplasma gondii* (T. gondii) by milk consumption are noted.

Recently, a paper launched a debate about milk contamination (Dehkordi et al., 2013). The authors reported the detection of the toxoplasmic DNA in milk of different naturally infected herds (ovine, caprine) including low sensitive hosts to the parasite (bovine, camels, buffalo). American scientists (Dubey and Jones, 2014) commented the findings pointing out some technical gaps about the sensitivity of employed methods (e.g., high rate of parasite detection by bioassay and cell culture), rising thus interrogations and seeking independent reproduction of data to affirm the reported conclusion.

Risk assessment studies associating *T. gondii* infections and milk consumption showed wide divergence. While some papers reported positive correlations between drinking milk and humans infection in Poland (Paul, 1998), USA (Jones et al., 2009), Mexico (Alvarado-Esquivel et al., 2010); other studies stated non-significant influence of milk or milk products consumption among pregnant women in Jordan (Nimri et al., 2004) and high risked populations in Kyrgyzstan (Minbaeva et al., 2013).

Essentially, outbreaks of human toxoplasmosis were reported mainly by the ingestion of raw (unpasteurized) goat milk with the contamination of 10 members from 24 individuals composed family. The authors stated serological evidence of acute *T. gondii* infection (One subject with

retinochoroiditis and the other 9 persons had asymptomatic infections). All 10 seropositive persons had recently consumed raw goat's milk from the family herd as compared with no consumption of raw milk by the 14 persons with negative results. No dietary item or other risk factors were as strongly associated with positive serological test results as raw milk consumption (Sacks et al., 1982). Studies worldwide attempted to analyze the risk factor of raw goat milk consumption among different populations: in Brazil when targeting pregnant women (Avelino et al., 2003; Barbosa et al., 2009; Moura et al., 2013); in Saudi Arabia targeting the same population (Almushait et al., 2014); in USA questioning a global population (Jones et al., 2009); in Mexico focusing on agricultural workers (Alvarado-Esquivel et al., 2013) and in Ethiopia investigating women population in general (Gebremedhin et al., 2013).

It has often been thought that the risk of acquiring an infection with *T. gondii* by drinking goat's milk is high and cow's milk, if any, is minimal. However, it was shown that only 1.6% of the control patients in USA specified they drank unpasteurized goat's milk in the past 12 months (Jones et al., 2009). Consequently consumption of unpasteurized goat's milk is retained relatively uncommon in the United States. Lately it was found that 14.1% of the seropositive pregnant women in Brazil had the habit of consuming raw cow's or/and goat's milk (Moura et al., 2013). When risk factors analyzed, unpasteurized/raw cow's milk consumption wasn't associated with toxoplasmic infection among agricultural workers in Mexico (Alvarado-Esquivel et al., 2013) as well in Brazil when targeting immune-competent population (Bahia-Oliveira et al., 2003). However other studies reported significant association between milk/dairy consumption and toxoplasmosis occurrence: in Brazil (Heukelbach et al., 2007; Santos et al., 2009; Silva et al., 2014); in Mexico (Alvarado-Esquivel et al., 2010); in Egypt (Elsheikha et al., 2009) and in Iran (Fouladvand et al., 2010).

Positive correlation was also retained with the consumption of other animals' milk. Indeed, risk factors analysis reported the consumption of raw buffalo dairy products in British Columbia (Proctor and Banerjee, 1994) and in Egypt among blood donors (Elsheikha et al., 2009). Sheep milk involvement in toxoplasmosis occurrence was also investigated as potential risk factors in Ethiopia (Gebremedhin et al., 2013) when targeting women population as well as in Brazil (Barbosa et al., 2009) when targeting pregnant females.

Few other studies investigated the detection of natural infection of the parasite within less consumed milk from camel and donkeys. In Iran, scientists reported a rate of camel milk contamination of 3.12% by culture bioassays (Dehkordi et al., 2013). In the frame of seroepidemiological investigation within camels, Ethiopian team revealed that 100% of the animal owners had consumed raw camel milk (Gebremedhin et al., 2014). Concerning *T. gondii* prevalence in the milk matrix of donkeys, only three studies investigated its analysis. Scientists in Egypt were able to detect the toxoplasmic antibodies in 46.3% of milk samples (Haridy et al., 2010). In Europe, Italian studies using molecular tools reported the detection of *T. gondii* DNA in 66.66% (Mancianti et al., 2014) and in 22.22% (Martini et al., 2014) of analyzed donkeys' milk samples.

The toxoplasmic transmission was attributed both to tachyzoites in the milk and to suckling. Toxoplasmosis was described in breast fed child whose mother had recently acquired toxoplasmosis (Azab et al., 1992; Bonametti et al., 1997). Moreover, mammary glands can be contaminated from environment and suckling calf-camel can acquire toxoplasmosis from milk/nipples of their infected mother. The toxoplasmic contamination is worsened due to smaller concentration of proteolytic enzymes that counter the parasite infection in the intestine of children and suckling animals (Ishag et al., 2006). It was experimentally confirmed that tachyzoites survived in the milk for three to 7 days at 4°C (Spišák et al., 2010) and in homemade fresh cheese for a period of 10 days (Hiramoto et al., 2001), proving consequently that raw milk can serve as a source of *T.gondii* infection.

Proponents of raw milk claim that unpasteurized milk/dairy products are more nutritive than pasteurized ones, even if it is stated elsewhere with equal nutritional values (Claeys et al., 2013). It was reported that many Americans consume products labeled as "organic" or from food cooperatives (Dubey and Jones, 2014). In Europe and according to the regional legislations; raw milk from any species can be sold immediately after milking by the producer or a local milk seller to the consumer, without any thermal treatment except refrigeration between 0 and 4°C (Mancianti et al., 2014).

Given that, the increase of organic milk demand, and in the light of these recent observations, there is great concern regarding whether it is safe to consume raw milk. Deeper analysis should be investigated and special awareness should be taken by consumers with the insistence on of heat treatment of the milk before consumption.

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The role of hormones on *Toxoplasma gondii* infection: a systematic review

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Background: *Toxoplasma gondii* is the causal agent of toxoplasmosis in which one third of the world's population has been infected. In pregnant women, it may cause abortion and severe damage to the fetal central nervous system. During pregnancy, the prevalence of toxoplasmosis increases throughout the second and third quarter of gestation, simultaneously progesterone and 17β-estradiol also increase. Thus, it has been suggested that these hormones can aggravate or reduce parasite reproduction. The aim of this study was reviewing the relationship between hormones and infection caused by *T. gondii* in several experimental animal models and humans, focused mainly on: (a) congenital transmission, (b) parasite reproduction, (c) strain virulence, (d) levels of hormone in host induced by *T. gondii* infection, and (e) participation of hormone receptors in *T. gondii* infection. Are the hormones specific modulators of *T. gondii* infection? A systematic review methodology was used to consult several databases (Pub Med, Lilacs, Medline, Science direct, Scielo, Ebsco, Sprinker, Wiley, and Google Scholar) dated from September, 2013 to March, 2014.

Results: Thirty studies were included; eight studies in humans and 22 in animals and cell cultures. In the human studies, the most studied hormones were testosterone, progesterone, prolactin, and 17β-estradiol. Type I (RH and BK) and Type II (Prugniaud, SC, ME49, T45, P78, and T38) were the most frequent experimental strains.

Conclusions: Thirty-five years have passed since the first studies regarding *T. gondii* infection and its relationship with hormones. This systematic review suggests that hormones modulate *T. gondii* infection in different animal models. However, given that data were not comparable, further studies are required to determine the mechanism of hormone action in the *T. gondii* infectious process.

Keywords: *Toxoplasma* infection, steroids hormones, no steroid hormones, toxoplasmosis, *Toxoplasma*

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is the causal agent of toxoplasmosis and one third of the world population has been affected by this parasite (el-On and Peiser, 2003). In immunocompetent adults, 80% of the cases can be asymptomatic. On the other hand, in immunocompromised patients, *T. gondii* is an opportunistic parasite that has been held responsible for mortal encephalitis (Cabrera-Muñoz et al., 2010).

Congenital transmission of *T. gondii* causes severe consequences in which the degree of damage depends on the time when the mother is infected (Speroff et al., 1999). Infection during early pregnancy can result in apoptosis of placental cells and fetal resorption (Senegas et al., 2009). When pregnant females infected during latter stage of pregnancy and inflammatory responses are low, congenital transmission is likely to occur (Roberts et al., 2001; Pfaff et al., 2008). The transmission frequency of *T. gondii* is high (80%) at end of pregnancy.

PREGNANCY AND *T. gondii* INFECTION

During pregnancy, maternal hormones alter the immune responses of the mother in the presence of fetal antigens. The increases in the susceptibility to infection and a diminished pro-inflammatory response have critical anti-parasitic properties that cause an unfavorable development of toxoplasmosis (Craig et al., 2001; Roberts et al., 2001; Prigione et al., 2006; Dionne et al., 2012). In the second and third trimester of gestation, there is a significant increase of 17β-estradiol and progesterone levels and it is during this period, when the prevalence of *Toxoplasma* infection increases (Montoya and Remington, 2008; Al-warid and Al-qadhi, 2012).

17β-ESTRADIOL AND *T. gondii* INFECTION

17β-estradiol (E2) is synthetized mainly in the ovary, breast, endometrial tissue, and brain. E2 plays a vital role in the menstrual cycle and human reproduction. In the nervous system, the estrogens are neuroprotective (Duenas et al., 1996; Arevalo

et al., 2010). It has been reported that the administration of pharmacological doses of 17β-estradiol increases the susceptibility to *Toxoplasma* infection (Pung and Luster, 1986).

PROGESTERONE

Progesterone is present in the ovary and corpus luteum where it is primarily involved in the second phase of the menstrual cycle and reproductive processes of women. Progesterone is synthetized in breast, endometrial, and brain too (Speroff et al., 1999). In cells infected with tachyzoites of *T. gondii*, progesterone did not regulate the replication of parasites (Gay-Andrieu et al., 2002). Progesterone levels are reduced during pregnancy in sheep after infection by *T. gondii* (Aiumalamai et al., 1990; Fredriksson et al., 1990).

TESTOSTERONE LEVELS REGULATION BY *T. gondii* INFECTION IN HUMAN BEINGS AND MICE

Testosterone and their derivatives (dihydrotestosterone and dehydroepiandrosterone) are androgens produced mainly in male gonads, adrenal glands and the brain. Testosterone can act directly as a ligand of androgen receptors (AR) found in several target tissues. Androgens stimulate the development of the secondary sexual characters in males, participate in human reproduction and maturation of human fetal testes (O'Shaughnessy and Fowler, 2014). In the brain, it is considered as a neuroprotective hormone (Kurth et al., 2014). IgG anti-*Toxoplasma* antibodies were significantly correlated to testosterone (Shirbazou et al., 2011), and results are different accord type strain (Kaňková et al., 2011). *T. gondii* produces high testosterone levels in infected animals and mRNA expression of luteinizing hormone receptor (LHR) (Oktenli et al., 2004; Abdoli et al., 2012; Lim et al., 2013).

THYROXINE (T4) AND *T. gondii* INFECTION

Studies in Nylar female mice infected with *T. gondii*, exhibited hypogonadotrophic hypogonadism secondary to hypothalamic dysfunction (Stahl et al., 1985, 1994). These mice infected with *T. gondii* Cornell strain, present atrophy in the thymus, ovaries, and uterus, cessation of cycling, anovulation, and decline of serum thyroxine (T4) levels (Stahl et al., 1985).

CORTICOSTEROIDS EFFECT ON *T. gondii*

Cortisol is a glucocorticoid hormone secreted by the adrenal cortex. It works through a signal transduction pathway that initiates by hormone linkage to specific cell receptors. Proteins synthesized by the glucocorticoid response inhibit or stimulate the specific tissue (Gardner et al., 2011). Cortisone increased the amount of tachyzoites, cysts and cystozoite, as the breakage of cysts released a higher resistant antigen-cystozoite in mice brains infected with *T. gondii* (Hulínská et al., 1990).

ANTI-PARASITIC EFFECT OF PROLACTIN ON *T. gondii* INFECTION

PRL is capable of inhibiting multiplication of *Toxoplasma* in murine microglial cell cultures (Benedetto et al., 2001). PRL significantly restricted intracellular growth of *Toxoplasma* in mice and human cell lines (Dzitko et al., 2010, 2012). Moreover, it been documented that women with hyperprolactinemia showed lower *T. gondii* prevalence (Dzitko et al., 2008). It has been reported that serum human prolactin (shPRL) has the capacity to bind to live RH tachyzoites (type I) and ME49 (type II) strains in a specific way (Dzitko et al., 2013).

The aim of this study was to review the relationship between hormones and infection by *T. gondii* in several experimental animal models and humans. Focusing the information on: (a) congenital transmission, (b) parasite reproduction, (c) strain virulence, (d) levels of hormone in host induced by *T. gondii* infection, (e) participation of hormone receptors in *T. gondii* infection.

MATERIALS AND METHODS

DATABASE SEARCH

Reports from September 2013 to February 2014 were obtained from a total of nine databases (Pub Med, Lilacs, Medline, Science direct, Scielo, Ebsco, Sprinker, Wiley, Google Scholar). Mesh terms were "*Toxoplasma* or toxoplasmosis or *Toxoplasma gondii*" combined with progesterone, 17β-estradiol, testosterone, cortisol, cortisone, aldosterone, 11-desoxicorticosterone, dihydrotestosterone, dehydroepiandrosterone, and non-steroid hormones; growth hormone, prolactin, parathyroid hormone, corticotrophin, insulin, glucagon, luteinizing hormone, thyroid stimulating hormone, human chorionic gonadotropin, antidiuretic hormone, oxytocin, melanocyte stimulating hormone, somatostatin, thyrotropin-releasing hormone, gonadotropin-releasing hormone, noradrenaline, adrenaline, melatonin, thyroxine, and triiodothyronine. *Toxoplasma* and hormones and strain *Toxoplasma*. The criteria used for including data were: the full text of papers written in English (reviews and case reports not considered), studies performed on humans, animals, and in cell cultures.

DATA COLLECTION METHODS

Two reviewers (GRML and GMAF) carefully studied all selected studies. The full text of selected original articles were obtained and reviewed. Inclusion criteria for this analysis were explicit data of all independent variables and at least one dependent variable; data collection and criteria eligibility were established for determining the frequency or proportion of each study. The independent variables were *T. gondii* strain, hormones, study design, stage of infection and developmental stage of the parasite, post infection evaluation time, age, host, and technical analysis. Dependent variables were increased or decreased of infection and number of parasites. Reference lists of full-text publications were examined for identifying studies not originally selected **Figure 1**.

From 30 articles meeting inclusion criteria, all results were captured on an Excel database. A number of studies presented frequency distribution of dependent variables; in these cases, the sum of the products of each value by frequency was included for comparison in the database. Some articles presented ranges, mean plus standard deviation; these articles were included in the database using the median.

RESULTS

One thousand two hundred and seventy eight articles potentially related to *T. gondii* or hormones were found. However, only 45 were selected and of these, 30 met the inclusion criteria for this

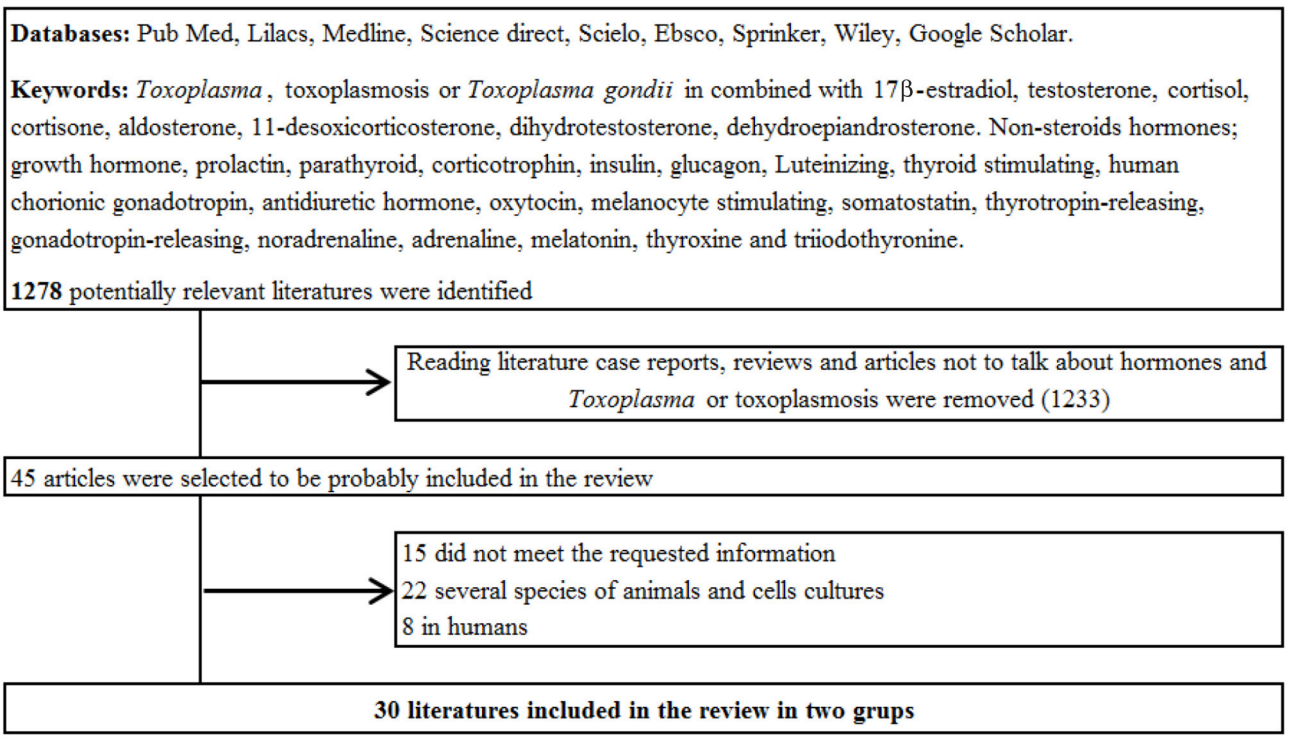

FIGURE 1 | This figure shows the flow of the search obtained, data collection methods, and database search.

systematic review. The analysis was divided into three categories: (A) humans in **Table 1**, (B) several species of animals in **Table 2**, and (C) Cell cultures in **Table 3** and studies conducted in the time period that this research included **Figure 2**.

HUMANS

Eight articles were performed with different hormones on humans, from 17 to 40 years old: Testosterone ($n = 5$) (Oktenli et al., 2004; Hodková et al., 2007; Flegr et al., 2008a,b; Shirbazou et al., 2011), 17β-estradiol and progesterone, dehydroepiandrosterone (DHEA), prolactin, and cortisol and testosterone ($n = 1$) (Dzitko et al., 2008; Al-warid and Al-qadhi, 2012; de la Torre et al., 2012). These studies used Radioimmunoassay (RIA) or Enzyme-linked ImmunoSorbent assay (ELISA) in 8 studies combined with other analytic methods (**Table 1**).

ANIMALS

Fifteen articles evaluated the hormone effect in *T. gondii* infection using different animal models: murine model ($n = 12$); in guinea-pigs (1) (Kittas and Henry, 1979), in mice (8) (Kittas and Henry, 1980; Pung and Luster, 1986; Hulínská et al., 1990; Stahl and Kaneda, 1998a,b; Liesenfeld et al., 2001; Kaňková et al., 2011; Puvanesuaran et al., 2012), and rats (3) (Abdoli et al., 2012; Lim et al., 2013; Mitra et al., 2013). Two from ewes (2) (Aiumalamai et al., 1990; Fredriksson et al., 1990) and one for goats (1) (Engeland et al., 1996) (**Table 2**).

Progesterone and testosterone were the most studied hormones ($n = 4$), estradiol ($n = 3$), corticosterone and thyroxine ($n = 2$) and cortisone, adrenaline, and prednisolone ($n = 1$). Eight *T. gondii* strains were also analyzed: two Type I (eight RH and four BK) and six Type II (two PRU, ME49 and SC and one T45, P78, T38) and two not specified (**Table 2**).

The most frequent parasite stage of development studied was the tachyzoite ($n = 11$), followed by cyst ($n = 8$), ooquiste ($n = 2$), and bradizoite ($n = 1$). The number of parasites used for each experiment depended on the stage of parasite development and the host. In the murine model, tachyzoites from 1×10^4 to 1×10^7 were used (Benedetto et al., 2001; Abdoli et al., 2012; Dzitko et al., 2013). The number of cysts used in different rodent species was from 8 to 100 (Stahl and Kaneda, 1998b; Liesenfeld et al., 2001). In an experiment with goats, 1250 bradyzoytes were used (Engeland et al., 1996) and in another study with sheep infected with ooquistes, the number of ooquistes was not indicated (Aiumalamai et al., 1990) (**Table 2**).

The post-infection time in each experiment was different, according to each species and parasite stage of development. In guinea pigs, 42 days (Kittas and Henry, 1979); mice, 4 to 60 days (Kittas and Henry, 1980; Pung and Luster, 1986; Hulínská et al., 1990; Stahl and Kaneda, 1998a,b; Liesenfeld et al., 2001; Kaňková et al., 2011; Puvanesuaran et al., 2012); in rats, 10 to 56 days (Abdoli et al., 2012; Lim et al., 2013; Mitra et al., 2013), in a goat, 54 to 73 days (Engeland et al., 1996) and in ewes 90.5 days (Aiumalamai et al., 1990; Fredriksson et al., 1990) (**Table 2**).

Concerning the route of infection, 15 studies were carried out, four subcutaneous (Kittas and Henry, 1979, 1980; Pung and Luster, 1986; Engeland et al., 1996) and six more by peritoneal administration (Hulínská et al., 1990; Stahl and Kaneda, 1998a,b; Abdoli et al., 2012; Lim et al., 2013; Mitra et al.,

Table 1 | Effect of hormones on *Toxoplasma gondii* infection in humans.

References		Age of the host (years)	Sex[a]	Analysis technique[b]	Hormones[c]	Diagnostic/group[d]	*N*	Results[e]			*p*
1	Oktenli et al., 2004	17–18	NS	ELISA	Testosterone			*T. gondii* antibodies			
						Control	20	IgM:0.53 ± 0.13	IgG:0.43 ± 1.08	–	
						Normal testosterone levels	31	**IgM:3.88 ± 1.14***	**IgG:4.95 ± 0.91***	↑	**<0.001**
						Low testosterone levels	9	**IgM:4.00 ± 1.03***	**IgG:4.50 ± 1.08***	↑	**<0.001**
				QUIL				Total Testosterone (TT) nM/L ± SD			
						Control	20	17.11 ± 1.01		–	
						Normal testosterone levels	31	17.29 ± 1.38		–	
						Low testosterone levels	9	**4.57 ± 0.56***		↓	**<0.001**
2	Hodková et al., 2007	21–24	NS	ELISA	Testosterone			*T. gondii* antibodies			
							89	Positive: 18		–	
								Negative: 71		–	
				Dom S				Dominance score			
						Infected	18	**0.184***		↑	=0.051
						Uninfected	71	−0.57*			
								Masculinity score			
				Mas S		Infected	18	0.17		↑	0.17
						Uninfected	71	−0.03			
3	Flegr et al., 2008a			RIA	Testosterone			Testosterone levels ng/mL			
		21.03	W				174	**0.230***		**–**	**<0.0001**
		20.91	M				91	0.387*		**–**	
				ELISA				*T. gondii* antibodies			
			W				174	Positive: 29		–	
			M				91	**Negative: 23**		↑	**<0.001**
4	Dzitko et al., 2008			ELISA	Prolactin			Seropositive *anti-Toxoplasma* antibodies			
		NS	W			Control	205	93			
			M			Control	76	39			
			W			Hyperprolactinaemia	168	**57***		↓	**=0.025**
			M			Hyperprolactinaemia	66	31			

(Continued)

Table 1 | Continued

	References	Age of the host (years)	Sex[a]	Analysis technique[b]	Hormones[c]	Diagnostic/group[d]	*N*	Results[e]			*p*
			W			Hypoprolactinaemia	32	10			
			M			Hypoprolactinaemia	9	3			
5	Flegr et al., 2008b			RIA	Testosterone			Testosterone levels nM/L			
		21.05	W				135	**0.23**		–	**<0.001**
		20.94	M				106	0.41		–	
								Digit radio 2D:4D			
			W	ELISA			194	Right: 0.315	Left: 0.587		
			M				106	**Right: 0.167**	**Left: 0.002***	–	**<0.01**
6	Shirbazou et al., 2011			ELISA				Seropositive *T. gondii* antibodies			
		NS	W				73	24		–	
		NS	M				107	39		–	
								Cortisol levels in blood			
		NS	W		Cortisol	Uninfected	12			–	
		NS	M			Uninfected	19			–	
		NS	W			Infected	24	**t: 5.774***			**<0.0001**
		NS	M			Infected	39			–	
								Testosterone levels in blood			
		NS	W		Testosterone	Uninfected	12			–	
		NS	M			Uninfected	19			–	
		NS	W			Infected	24	**t: 2.491***			**=0.002**
		NS	M			Infected	39			–	
7	Al-warid and Al-qadhi, 2012			ELISA				Anti-*Toxoplasma* antibodies			
		19–40	W			Uninfected	9	(–) IgG	(–) IgM	–	
						Acute	10	(–) IgG	(+) IgM	–	
						Sub-acute	9	(+) IgG	(–) IgM	–	
						Chronic	13	(+) IgG	(+) IgM	–	
								Progesterone levels ng/dL ± SD			
			W	ELISA	Progesterone (P4)	Uninfected	9	18.3 ± 9.84			
						Infected	32	11.19 ± 9.76			

(Continued)

Table 1 | Continued

References		Age of the host (years)	Sex[a]	Analysis technique[b]	Hormones[c]	Diagnostic/group[d]	*N*	Results[e]		*p*
								P4 levels ng/dL ± SD		
						Acute	10	5.35 ± 7.15	–	
						Sub-acute	9	15 ± 9.01	–	
						Chronic	13	14.62 ± 10.38	–	
								Estradiol levels pg/dL ± SD		
			W	ELISA	17β-estradiol (E2)	Uninfected	9	53.61 ± 76.24	–	
						Infectadas	32	88.19 ± 101.10	–	
								E2 levels pg/dL ± SD		
						Acute	10	70.66 ± 51.08	–	
						Sub-acute	9	92.51 ± 78.70	–	
						Chronic	13	108.02 ± 138.67	–	
8	de la Torre et al., 2012	20–29		ELISA	DHEAS			Seropositive *T. gondii* antibodies		
							82	42		
				IL				DHEAS levels ug/dL		
			W			Active RC by *T. gondii*	26	58	–	
			M					206		
			W			RS of RC by *T. gondii*	19	95*		=0.12
			M					199*		=0.79
			W			Positive of *T. gondii w* OL	16	113		
			M					177		
			W			Negative assay for *T. gondii*	21	122*		=0.3
			M					161*		=0.87

*[a]M, Men; W, Woman; NS, Not Specified. [b]ELISA, Enzyme-Linked ImmunoSorbent Assay; (QUIL), Chemiluminescence; Dom S, Dominance Score; Mas S, Masculinity Score; RIA, Radioimmunoassay; IL, Immunoluminimetric. [c]DHEAS, Dehydroepiandrosterone Sulphated; E2, 17β-estradiol; P4, Progesterone. [d]RS, Retinal Scars; RC, Retinochoroiditis; w OL, Without Ocular Lesions. [e]↑, Increased infection; ↓, Decrement infection; ↑, Increased hormone; ↓, Decrement hormone; * and bold, Statistically Significant. NS, Not specified.*

Table 2 | Effect of *Toxoplasma gondii* infection on hormones in animals.

	References	Type of study	Type of host	Age of the host (weeks)	Way of infection[a]	Stage parasite	Strain[b]	Number of parasites	Days post-infection	Analysis technique[c]	Hormones[d]	Group[e]	*N*	Results[f]	*p*
1	Kittas and Henry, 1979	*In vivo*	Guinea-pigs	NS	SC	Cysts	Bk	50	42	Number of *Toixoplasma* cysts ± SD					
										HIS	17β-estradiol (E2)	Control F:	8	88.75 ± 21.60	
												Control M:	8	**82.50 ± 21.1***	**<0.001**
												Gdt F:	8	63.00 ± 16.5	↓
												Gdt M:	8	65.25 ± 10.8	↓
												Gdt + Hex F:	8	200.25 ± 16.00	↑
												Gdt + Hex M:	8	184.00 ± 36.80	↑
2	Kittas and Henry, 1980	*In vivo*	Mice	11	SC	Cysts	Bk	30	42	Number of *Toxoplasma* cysts ± SD					
										HIS	17β-estradiol (E2)	Control F:	8	222 ± 42	
												Control M:	8	220 ± 23	
												Gdt F:	8	**189 ± 22***	↓ **<0.001**
												Gdt M:	8	**178 ± 24***	↓ **<0.001**
												Gdt + Hex F:	8	**598 ± 64***	↑ **<0.001**
												Gdt + Hex M:	8	**599 ± 45***	↑ **<0.001**
3	Pung and Luster, 1986	*In vivo*	Mice (B6C3F1)	8–10	SC	Cysts	T45	30	35	Number of *Toxoplasma* cysts ± SD					
										RIA	Control		6	982 ± 194	
											DES		6	**2244 ± 66***	↑ **<0.05**
											17β-estradiol		6	**1934 ± 198***	↑ **<0.05**
											5α-Dihydrotesti osterone		6	792 ± 164	↓
											Progesterone		6	1012 ± 172	↑
											Zeralanol		6	1463 ± 190	↑
											a-Dienestrol		6	**2405 ± 227***	↑ **<0.05**
											Corticosterone		6	**1954 ± 314***	↑ **<0.05**
										Effect of Tamoxifen, number of cysts ± SD					
										RIA	17β-estradiol (E2)	Control	6	1115 ± 112	
												Tamoxifen	6	975 ± 124	↓
												17β-estradiol	6	**2220 ± 182***	↑ **<0.05**
												Tamoxifen + E2	6	1027 ± 167	↓
4	Fredriksson et al., 1990	*In vivo*	Ewes (Scottish blackface)	NS	Oral	Oocysts	RH	2000	90.5	Progesterone levels (nM/L)					
										RIA	Progesterone (P4)	Control	3	10–20	
												Infected	13	10	↓ NS
												Vaccinated	15	10	↓ NS

(Continued)

Table 2 | Continued

References	Type of study	Type of host	Age of the host (weeks)	Way of infection[a]	Stage parasite	Strain[b]	Number of parasites	Days post-infection	Analysis technique[c]	Hormones[d]	Group[e]	*N*	Results[f]	*p*
5 Aiumalamai et al., 1990	*In vivo*	Ewes (Swedish Peltsheep)	52–104	NS	Oocysts	NS	NS	90.5	Progesterone levels (nM/L)					
									RIA	Progesterone (P4)		7	Day 5: 6–8 **Days 10 a 15: 19-**	↑ **<0.05**
6 Hulínská et al., 1990	*In vivo*	Mice (H VUFB)	4–5	IP	Cysts	P78	10		Number of tachyzoites and stozoites					
								5–14	HIS y MIC	Cortisone	Group 1	20	10–14 days	↑ –
								12–47			Group 2	20		
7 Engeland et al., 1996	*In vivo*	Goat (Norwegian)	NS	SC	Bradyzoites	NS	1250	54–73	Progesterone levels					
									ELISA y SF	Progesterone (P4)	Control	6		
											Infected	5		
8 Stahl and Kaneda, 1998a	*In vivo*	Mice (Nya: NYLAR)	NS	IP	Cysts	CS	8	3 and 4	T4 levels (Mean)					
									RIA	Thyroxine (T4)	Control	10	7.5	
											Infected	10	**3**	↓ **<0.01**
9 Stahl and Kaneda, 1998a	*In vivo*	Mice (Nya: NYLAR)	12	IP	Cysts	CS	8	4	Subnormal T4 response to a 1 |ig bolus or TRH (Mean)					
									RIA	Thyroxine (T4)	Control	8	11	
											Infected	8	**3**	↓ **<0.01**
10 Liesenfeld et al., 2001	*In vivo*	Mice (C57BL/6)	8–10	Oral	Cysts	ME 49	100	7	Number of parasitophorous vacuoles					
									NS	Testosterone	Control		657 ± 399	
											Testosteron		**426 ± 282**	↓ **=0.0141**
11 Kaňková et al., 2011	*In vivo*	Mice (BALB/c and C57 Black)	5–6	Oral	Cysts	T38	10	60	Differences in serum testosterone levels					
									RIA	Testosterone	M. *Toxo* infected	12	***Z* = −2.32**	↓ **=0.005**
											M. Controls	20		
											F. *Toxo* infected	12	***Z* = −2.76**	↓ **=0.020**
											F. Controls	20		
12 Abdoli et al., 2012	*In vivo*	Rats (Wistar)	NS	IP	Tachyzoites	RH	1×10^7		Effect of *T. gondii* infection on Serum Testosterone (ST)					
								10	ELISA	Testosterone	Uninfected	5	0.6 ± 0.01	
								10			Infected	3	**0.55 ± 0.02***	↓ **<0.05**
									Effect of *T.gondii* infection on IntratesticularTestosteron (ITT)					
								10			Uninfected	5	4.07 ± 0.02	
								10			Infected	3	**3.89 ± 0.05***	↓ **<0.05**

(Continued)

Table 2 | Continued

References	Type of study	Type of host	Age of the host (weeks)	Way of infection[a]	Stage parasite	Strain[b]	Number of parasites	Days post-infection	Analysis technique[c]	Hormones[d]	Group[e]	*N*	Results[f]	*p*
13 Puvanesuaran et al., 2012	*In vivo*	Mice (Swiss)	3	Oral	Tachyzoites	RH	1×10^4	4			Number of tachyzoites (Mean)			
									MIC	Prednisolone	Control	3	1.48×10^7	
											235 mg/kg	3	2.75×10^7	↑ **<0.05**
											470 mg/kg	3	2.92×10^7	↑ **<0.05**
											705 mg/kg	3	3.21×10^7	↑ **<0.05**
14 Lim et al., 2013	*In vivo*	Rats (Wistar)	7	IP	Tachyzoites	PRU	5×10^6	42–56			% Increase of Testosterona levels			
									ELISA	Testosterone		54	60%	↑ =0.057
15 Mitra et al., 2013	*In vivo*	Rats	6.5	IP	Tachyzoites	PRU	10×10^6	42–56			Circuling levels of corticosterone			
									ELISA	Corticosterone		126	**64%**	↓ **<0.05**

[a]*SC, Subcutaneously; IP, Intraperitoneally; NA, Not Applicable.* [b]*Type of strain: BK, Beverley; PRU, Prugniaud; CS, Cornell; RH, ME49, T45, P78, T38.* [c]*HIS, Histological; RIA, Radioimmunoassay; MIC, Microscopical; SF, Sabin and Feldman; ELISA, Enzyme-Linked ImmunoSorbent Assay.* [d]*E2, 17β-estradiol; P4, Progesterone; T4, Thyroxine; DES, Diethylstilbestrol; ST, Serum Testosterone; ITT, Intra testicular testosterone; TRH, Thyrotropin-Releasing Hormone.* [e]*M, Male; F, Female; Gdt, Gonadectomy; Hex, Hexoestrol.* [f]↑, *Increased infection;* ↓, *Decrement infection;* ↑, *Increased hormone;* ↓, *Decrement hormone;* * *and bold, Statistically Significant. NS, Not specified; SD, Standard deviation.*

2013). In four studies, oral administration was used for infection (Fredriksson et al., 1990; Liesenfeld et al., 2001; Kaňková et al., 2011; Puvanesuaran et al., 2012) and one was not specified (Aiumalamai et al., 1990) (**Table 2**).

CELL CULTURES

Seven studies were designed in cell lines; two in RAW 264.7 mouse cell lines (Gay-Andrieu et al., 2002; Gets and Monroy, 2005), one, in bone marrow stem cells (Jones et al., 2008) one in microglial cell cultures (Benedetto et al., 2001) and three with prolactin in Murine L929, Human Hs27, HeLa, and Peritoneal Blood Mononuclear cells (PBMC) (Dzitko et al., 2010, 2012, 2013; Abdoli et al., 2012) (**Table 3**).

Concerning non-steroid hormones, prolactin and thyroxine hormone have been studied. In this study, other non-steroid hormones such as growth hormone, parathyroid, corticotrophin, insulin and glucagon, luteinizing and follicle hormone, thyroid stimulating, human chorionic gonadotropin, antidiuretic, oxytocin, melanocyte stimulating, somatostatin, thyrotropin-releasing hormone, gonadotropin-releasing hormone, noradrenaline, adrenaline, melatonin, and triiodothyronine were not associated to *Toxoplasma* infection.

The laboratory analysis methods used were: Radioimmunoassay (RIA) (Pung and Luster, 1986; Aiumalamai et al., 1990; Kaňková et al., 2011). Enzyme-Linked Immunosorbent Assay (ELISA) (Engeland et al., 1996; Abdoli et al., 2012; Dzitko et al., 2012, 2013; Lim et al., 2013). A Morphological Method, (MM), Indirect Immunofluorescence (IFI), Flow Cytometry Analysis (CF) (Gay-Andrieu et al., 2002), Microscopy (Hulínská et al., 1990; Gay-Andrieu et al., 2002), in three histological studies (Kittas and Henry, 1979, 1980; Hulínská et al., 1990) and in two methods. Sabin and Feldman (SF) (Engeland et al., 1996) Inverse Reaction of Polymerase Chain and ELISA (Lim et al., 2013).

DISCUSSION

Congenital toxoplasmosis is one of the most significant burdens of *T. gondii* infection in humans. Both the maternal–fetal transmission and hormonal levels during pregnancy are poorly understood and yet, may play an important role during the course of the disease. In pregnant women with acute toxoplasmosis, low levels of progesterone and low levels of estrogens can induce severe infection caused by *T. gondii* (Al-warid and Al-qadhi, 2012). The changes in endocrine phenomena occurring during pregnancy, as well as the size and maturity of the placenta and the embryonic/fetal immune response definitely affect the ability to be protected from invasion or to fight infection (Ortiz-Alegría et al., 2010).

In pregnant women with toxoplasmosis, low levels of progesterone and estrogen can induce severe infection. Nevertheless, the mechanism is unknown (Al-warid and Al-qadhi, 2012). Current studies show that there weren't any statistically significant differences in progesterone levels between infected and uninfected women with *T. gondii*, although higher progesterone levels were observed in uninfected women compared to low level in infected women. Moreover, estrogen levels in both chronic and uninfected women did not exhibit significant differences, although

Table 3 | Effect of *Toxoplasma gondii* infection on hormones in cell cultures.

References		Type of Study	Type of cell culture[a]	Stage parasite	Strain[b]	Number of parasites	Days post-infection	Analysis technique[c]	Hormone[d]	Group	N	Results[e]		p
1	Benedetto et al., 2001	*In vitro*	MGC (C57BL/6)	Tachyzoites	RH	1×10^4	20 h	Intracellular replicaton of *T. gondii (Mean ± SD)*						
								ELISA	Prolactin (PRL)	Control		7.4 ± 1.0		
										PRL + rTNF-a		6.1 ± 1.0	↓	<0.05
2	Gay-Andrieu et al., 2002	*In vitro*	RAW 264.7	Tachyzoites	RH	3.3×10^6	3–20 h	*Toxoplasma gondii* replication						
								IF, FC y MIC	Progesterone			No significant differences		<0.05
3	Gets and Monroy, 2005	*In vitro*	RAW 264.7	Tachyzoites	RH	5×10^5	18–24	Percentage of infected macrophages						
								MIC	Adrenaline	Control				
										Adrenaline *a*		**5.55***	↑	<0.05
										Adrenaline p		**10***	↑	<0.05
4	Jones et al., 2008	*In vitro*	BmSCs	Tachyzoites	RH	2×10^6	1	Effect on LPS-induces killing on *T. gondii*						
								NS	Progesterone	Control		No significant differences		<0.05
										Infected				
5	Dzitko et al., 2010	*In vitro*		Tachyzoites	BK	2×10^5		Influence of rhPRL en la intensidad de multiplication de *T.gondii*						
			L929				6	MTT	Prolactine			No significant differences		
			Hs27							1000.0 (ng/mL)	18	**8.90 ± 3.46***	↓	**<0.01**
			HeLa									(No Sig. Diff.)		
								Inhibition of the proliferation rate (%) of *T. gondii*						
			L929				0 (min)			2.0–100.0 (ng/m L)	12	No significant differences		
							30			20.0 (ng/mL)	12	**19.87 ± 4.28***	↑	**<0.05**
										100.0 (ng/mL)	12	**23.66 ± 10.99***	↑	**<0.05**
							60			20.0 (ng/mL)	12	**19.66 ± 5.73***	↑	**<0.01**
										100.0 (ng/mL)	12	**25.53 ± 3.19***	↑	**<0.01**
							180			20.0 (ng/mL)	12	**26.76 ± 3.02***	↑	**<0.01**
			Hs27				0 (min)			100.0 (ng/mL)	12	**27.00 ± 2.50***	↑	**<0.01**
										2.0–100.0 (ng/m L)	12	No significant differences		
							30			20.0 (ng/mL)	12	**20.81 ± 4.21***	↑	**<0.01**
										100.0 (ng/mL)	12	**21.93 ± 5.48***	↑	**<0.01**
							60			20.0 (ng/mL)	12	**19.05 ± 2.63***	↑	**<0.01**
										100.0 (ng/mL)	12	**23.01 ± 5.93***	↑	**<0.01**
							180			20.0 (ng/mL)	12	**21.14 ± 5.62***	↑	**<0.01**
										100.0 (ng/mL)	12	**36.15 ± 11.53***	↑	**<0.01**

(Continued)

Table 3 | Continued

References		Type of Study	Type of cell culture[a]	Stage parasite	Strain[b]	Number of parasites	Days post-infection	Analysis technique[c]	Hormone[d]	Group	*N*	Results[e]		*p*
			HeLa				0 (min)			2.0–100.0 (ng/mL)	12	No significant differences		
							30			20.0 (ng/mL)	12	**23.05 ± 4.97***	↑	**<0.01**
										100.0 (ng/mL)	12	**31.74 ± 5.79***	↑	**<0.01**
							60			20.0 (ng/mL)	12	**27.71 ± 7.42***	↑	**<0.01**
										100.0 (ng/mL)	12	**31.71 ± 7.06***	↑	**<0.01**
							180			20.0 (ng/mL)	12	**29.64 ± 6.23***	↑	**<0.01**
										100.0 (ng/mL)	12	**32.12 ± 3.53***	↑	**<0.01**
6	Dzitko et al., 2012	*In vitro*	PBMC	Tachyzoites	BK	2.5 × 10 5	3	% of *T. gondii* proliferation						
								ELISA	rhPRL	0 (ng/mL)		76.35 ± 10.1		
										100.0 (ng/mL)		81.01 ± 11.6		
									sPRL	0 (ng/mL)		49.8 ± 4.6		
										100.0 (ng/mL)		**59.6 ± 3.1***	↓	**<0.01**
7	Dzitko et al., 2013	*In vitro*	L929	Tachyzoites		1 × 10 7		% increse of prolactine Levels						
					RH		30 (min)	ELISA	shPRL			10.1		
							90 (min)				NS	52.4	↑	=0.056
					ME49		30 (min)					16		
							90 (min)				NS	46.2	↑	=0.056

*[a]MGC, Microglial cell cultures; RAW 264.7, Murine Macrophage cell line; BmSCs, Bone marrow Stem Cells; L929, Mouse fibroblasts cell line; Hs27, Human foreskin fibroblast; HeLa, Human epithelial cells; PBMC, Peripheral Blood Mononuclear Cells. [b]Type of strain: Beverley (BK), RH, ME49. [c]MIC, Microscopical; IF, Immunofluorescence; FC, Flow Cytometry; ELISA, Enzyme-Linked ImmunoSorbent Assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. [d]PRL, Prolactin; rhPRL, Recombinant Human Prolactin; sPLR, Serum Prolactin; shPRL, Sheep Prolactin. [e]↑, Increased infection; ↓, Decrement infection; ↑, Increased hormone; ↓, Decrement hormone; * and bold, Statistically Significant. NS, Not specified; SD, Standard deviation.*

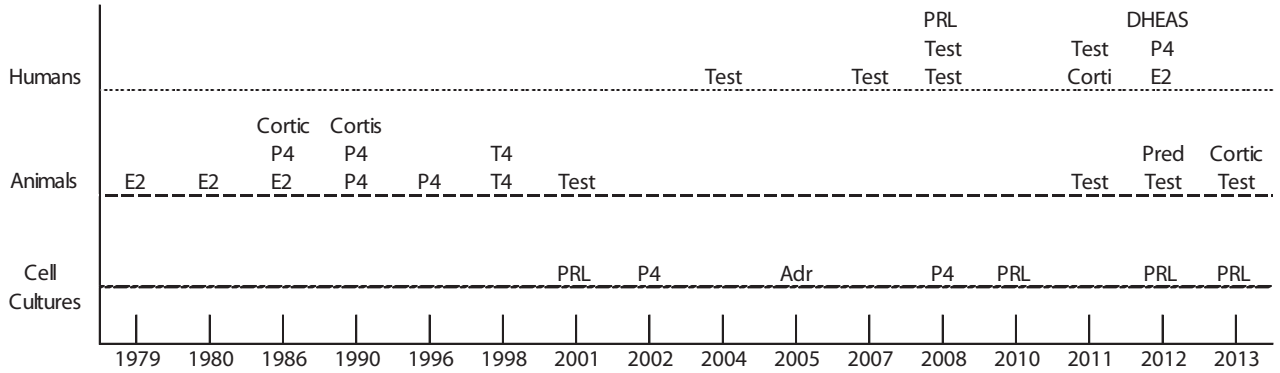

FIGURE 2 | Show studies development in human, animals and cells cultures with different hormones during a time period since 1979–2013. E2, 17β-estradiol; Cortic, Corticosterone; Cortis, Cortisone; Corti, Cortisol; P4, Progesterone; T4, Thyroxine; Test, Testosterone; PRL, Prolactin; Adr, Adrenaline; DHEAS, Dehydroepiandrosterone; Pred, Prednisolone.

infected women had a higher level, compared to uninfected women.

The study of 17β-estradiol in *T. gondii* infection began in 1979, when hexoestrol was administered to mice and increased the number of *T. gondii* cysts in muscle (Kittas and Henry, 1979). At the same time, the susceptibility to *T. gondii* infection increased in mice when pharmacological estrogen concentrations were used (Pung and Luster, 1986). Nevertheless, 35 years have passed since these experiments were performed and no further studies regarding 17β-estradiol mechanism in *T. gondii* infection have been reported.

Progesterone levels are reduced during pregnancy in sheep after infection by *T. gondii* (Aiumalamai et al., 1990; Fredriksson et al., 1990). This hormonal change could be contributing to the susceptibility to *T. gondii* infection in sheep.

In RAW 264.7 cells infected with tachyzoites of *T. gondii*, progesterone did not regulate the replication of parasites (Gay-Andrieu et al., 2002). However, bone marrow stem cells activated with Lippolysaccharide (LPS) and treated with progesterone, while infected with *T. gondii* tachyzoites, cells exhibited a significant reduction in parasite death compared to activated controls (Jones et al., 2008). These results suggest that progesterone can modulate the survival of parasites *in vitro*.

The results of this study showed that steroid hormones are the most studied toxoplasmosis interaction. However, the information has a great heterogeneity and is not comparable, due to their different experimental designs. For example, the progesterone has been studied in mice (Pung and Luster, 1986), sheep (Aiumalamai et al., 1990), goats (Engeland et al., 1996), and bone marrow stem cells cultures (Jones et al., 2008). Furthermore, in these experiments, different strains and parasite stage of development were used. Moreover, no study has shown how steroid hormones regulate *T. gondii* infection.

The first observation of *T. gondii* infection and its association with testosterone in humans shows that acute infection by this parasite produced temporary hypogondatrophic gonadal insufficiency (Oktenli et al., 2004). On the other hand, there are several human studies analyzing different genders, using portrait pictures of 89 male students, of which 18 were *Toxoplasma* infected, and 109 female students. When statistically corrected for age, men with latent toxoplasmosis were perceived as more dominant ($p = 0.009$) and masculine ($p = 0.052$). These results suggest that the higher level of testosterone could be responsible for at least some of the toxoplasmosis-associated shifts in human and animal behavior (Hodková et al., 2007). In 2008, Flegr showed that the relationship between age, gender and 2D:4D ratio in hands sharply increased with *Toxoplasma* infection. Infected males had higher testosterone levels, while infected females had lower levels, than *Toxoplasma*-free males and females, respectively. *Toxoplasma*-infected males had a lower left hand 2D:4D ratio than *Toxoplasma*-free males. These results suggest that the relationship between 2D:4D ratio is particularly strong for the left hand and 2D:4D dimorphism will probably be higher in countries with a high prevalence of toxoplasmosis (Flegr et al., 2008b). These results indicate that sexual hormones and gender are key factors determining susceptibility to *Toxoplasma* infection.

Significantly, lower levels of testosterone in male and female mice with latent toxoplasmosis (strain T38 of *T. gondii*) were compared to uninfected controls (Kaňková et al., 2011). On the other hand, Liesenfeld in 2001 described the effect of sexual steroids and gender in the susceptibility to infection by *T. gondii* in mice. Death occurred in female mice before males, and mortality in females was associated to an increase in the number of tachyzoites. Female mice testosterone treatment reduced the number of parasites and pathology.

5α-Dihydrotestosterone reduced the number of cysts in mice infected with *T. gondii* cysts strain T45. Mice treated with corticosterone increased twice the number of cysts of *T. gondii* (Pung and Luster, 1986; Hulínská et al., 1990). These results showed that corticosterone could exacerbate the infection process.

The prevalence of *T. gondii* infection was analyzed in women with hyper and hypoprolactinemia, with a significant increase in this last group (Dzitko et al., 2008). In other studies using peripheral blood mononuclear cells (PBMC) of patients with hyperprolactinemia revealed that exogenous recombinant human prolactin (rhPRL), as well as autologous shPRL from inactivated serum, significantly restricted intracellular growth of *Toxoplasma* in these cultures (Dzitko et al., 2012). PRL may be one of

the potential humoral factors implicated in the limitation of *T. gondii* invasion. A physiological increase in PRL concentration during pregnancy may significantly reduce the risk of *T. gondii* proliferating in the expecting mother (Dzitko et al., 2012).

rhPRL reduced *T. gondii* replication in human cells (Hs27 y HeLa) and murine cells (L929), (Dzitko et al., 2010, 2013). Afterwards in another experimental study, the replication of parasites was reduced in L929 cells treated with prolactin. These results indicate that the inhibition of replication of *T. gondii* was caused by a limited capacity of the parasites to penetrate host cells, as demonstrated by the reduced number of infected cells. On the other hand, PRL stimulates T cell proliferation (Clevenger et al., 1992) and the release of various protective cytokines as TNF-α which control efficiently the course of *T. gondii* infection (Benedetto et al., 2001). The possible PRL action could be bidirectional, namely PRL may limit the proliferation of *Toxoplasma* via surface host cell receptors (Dzitko et al., 2013) leading to the release of protective type-1 cytokines, such as interleukin 12 (IL-12) and IFN-c (Matalka, 2003), and by inhibiting their penetration ability (Dzitko et al., 2010, 2013).

In the last 35 years, researchers worldwide have made a great effort to advance in the field of knowledge on how the hormones are involved in *T. gondii* infection, however, a major number of studies and the use of modern molecular methods are required to define the mechanistic role of hormones in the regulation of toxoplasmosis.

IMPLICATIONS FOR RESEARCH

A crucial factor is the difference in experimental models to study of *T. gondii* infections and hormones. As well, type's strains and the number limited studies to comparative analysis.

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Seroepidemiology of toxoplasmosis among people having close contact with animals

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A cross-sectional study was conducted to determine the seroepidemiology of *Toxoplasma* infection and its risk association among people having close contact with animals. A total of 312 blood samples were collected from veterinary personnel (veterinarian, technicians, and students) and pet owners from veterinary clinics and hospitals in the area of Klang Valley, Malaysia. About 4 cc of blood samples drawn from agreed participants were processed for measurement of anti-*Toxoplasma* IgG and IgM antibodies as well as avidity test of *Toxoplasma* IgG by ELISA I, II, and III kits. Meanwhile, the demographic profiles and possible risk factors of these participants were also recorded in the standardized data collection sheets. Overall seroprevalence of toxoplasmosis was observed in 62 (19.9%) participants being 7 (18.4%) in veterinarians, 15 (33.3%) in veterinary technicians, 29 (14.9%) in veterinary students, and 11 (31.4%) in pet owners. Of 19.9% *Toxoplasma* seropositive samples, 18.3% was positive for IgG antibody, 1.0% for IgM antibody, and 0.6% for both IgG and IgM antibodies. Of three different IgG avidity ELISA kits, ELISA III showed high avidity in all five seropositive samples (IgM and IgG/IgM antibodies) indicating chronic *Toxoplasma* infection which is consistent with no evidence of clinical toxoplasmosis diagnosed during the time of this study. Univariate analysis showed that age group, gender, study population, gardening, task performance, and working duration were significantly associated with *Toxoplasma* seropositivity. Further analysis by multivariate analysis using logistic regression showed that age group of $\geq$30 years old (OR = 0.34, 95% CI = 0.18–0.63, $p = 0.001$) and working or study duration of >10 years having close contact with animals (OR = 5.07, 95% CI = 1.80–14.24, $p = 0.002$) were identified as significant risks for *Toxoplasma* infection. Based on the results obtained, a comprehensive *Toxoplasma* screening and health surveillance program on toxoplasmosis should be implemented among people having close contact with animals in general and confirmed *Toxoplasma* seronegative individuals in particular to prevent seroconversion.

Keywords: anti-*Toxoplasma* antibodies, IgG avidity, prevalence, risk factors, toxoplasmosis, people with animal-contact

INTRODUCTION

Toxoplasma gondii (*T. gondii*), an obligate intracellular protozoan parasite (a zoonotic pathogen) is capable of causing both the infection rate that affects approximately one-third of human populations worldwide and the disease burden of clinical toxoplasmosis in human. *Toxoplasma* infection can be transmitted via several routes in different host species (1). Many species of warm blooded animals can be infected including human and it was recognized by the National Institutes of Health, Bethesda, MD, USA as a category B priority pathogen (2). Consuming undercooked contaminated meat with tissue cysts, ingestion of *T. gondii* oocysts from water, soil, or cat litter and congenital infection through placenta will lead to toxoplasmosis (3–5). Majority of infected individuals are symptoms free (6). *T. gondii* poses a greater risk especially found among pregnant women and immunocompromised individuals. Small percentage of infected newborns develop mild to severe clinical manifestations such as lymphadenopathy, fever and malaise in mild infection, ocular disease and mental illness in moderate manifestation, and severe cases among infected pregnant women will lead to stillbirth, abortion, or live birth children with central nervous system impairment or impaired vision (5). Besides, infected newborns with more virulent types of *T. gondii* may lead to severe and even fatal diseases with pulmonary and multi-visceral involvement (5).

To date, numerous studies have suggested preventive strategies of toxoplasmosis in people having close contact with animals (4, 7, 8), which is due to their high risk behaviors. Unfortunately, scanty data were reported on toxoplasmosis among these people worldwide (9–11). In Malaysia, the seroprevalence of toxoplasmosis in general healthy population increased from 16 to 30% (12). Furthermore, most studies on toxoplasmosis have been mainly conducted in healthy persons, pregnant women, indigenous communities, and HIV-positive patients (12, 13). To the best of our knowledge, this is the first documented data ever reported

on toxoplasmosis among animal handlers in Malaysia. In addition, a current situation on epidemiology of toxoplasmosis in animal handlers is crucial and timely to be investigated, so that suggested preventive strategies can be achieved pragmatically in implementation. This study was therefore conducted to determine the seroprevalence of *Toxoplasma* infection among people having close contact with animals and their risk factors in acquiring *Toxoplasma* infection.

MATERIALS AND METHODS

STUDY SITE AND POPULATION

This prospective cross-sectional study was conducted from October 2013 to April 2014. A total of 312 participants were from Faculty of Veterinary Medicine, University Putra Malaysia, Selangor and various private veterinary clinics in the Klang valley (**Figure 1**) were recruited. The inclusion criteria of this study were (1) immunocompetents who have close contacts with animals which include veterinarians (38), veterinary technicians (45), veterinary students (194), and pet owners (35) and (2) age of more than 15 years. All eligible participants gave informed consent before the commencement of this study. All the participants' information related to socio-demographic such as their age, education level, occupation, and plausible risk-factors exposure associated with toxoplasmosis (presence of own cats at home, presence of stray cats at home, drinking untreated water, and having contact with soils) prior to 3 months before this study were recorded in the formatted questionnaire forms. An operational definition was used for the risk factors. Presence of own cats at home was defined as a person who is the owner of at least one cat or has close contact with cats while feeding and playing in the house. Presence of stray cats at home was defined as a person having a close proximity with stray cats roaming in the house compound. Drinking untreated water was defined as a person who consumes "untreated water," e.g., water from a pipe, tap, or rain. Contact with soil (gardening) was defined as person who has a direct exposure to soil while gardening or any kind of outdoor activities.

ETHICAL CONSIDERATION

This study was approved by the ethical review committee of University of Malaya Medical Centre (UMMC), MEC Ref. No. 1024.6 in accordance with the Helsinky Declaration for the inclusion of human subjects in research. The purpose and procedures of this study were explained to all the participants. Informed consents were obtained from agreed participants prior to samples and data collection.

SERUM SAMPLE COLLECTION

Approximately 5 mL venous blood was drawn, sera were processed and were kept at −20°C until further testing.

DETECTION OF ANTI-*TOXOPLASMA* ANTIBODIES

The collected serum was screened primarily for anti-*Toxoplasma* IgG and IgM antibodies by using standard ELISA commercial kit (IgG-NovaLisa, Dietzenbach, Germany) in accordance with the manufacturer's instruction. A positive sample for the anti-*Toxoplasma* IgG and/or anti-*Toxoplasma* IgM antibody was also tested for its avidity using three standard ELISA commercial kits, namely, ELISA-I, II, and III for comparison and according to its manufacturer instruction. The interpreted results for ELISA-I was that avidity of >40% suggest chronic/past infection and of <40% suggest acute/recent infection, for ELISA-II was that avidity of <15% (low avidity), indicates acute or primary infection and avidity between 15 and 30% (borderline activity) indicates possibility of primary infection during the last 6 months is possible and >30% (high avidity) excludes primary infection within last

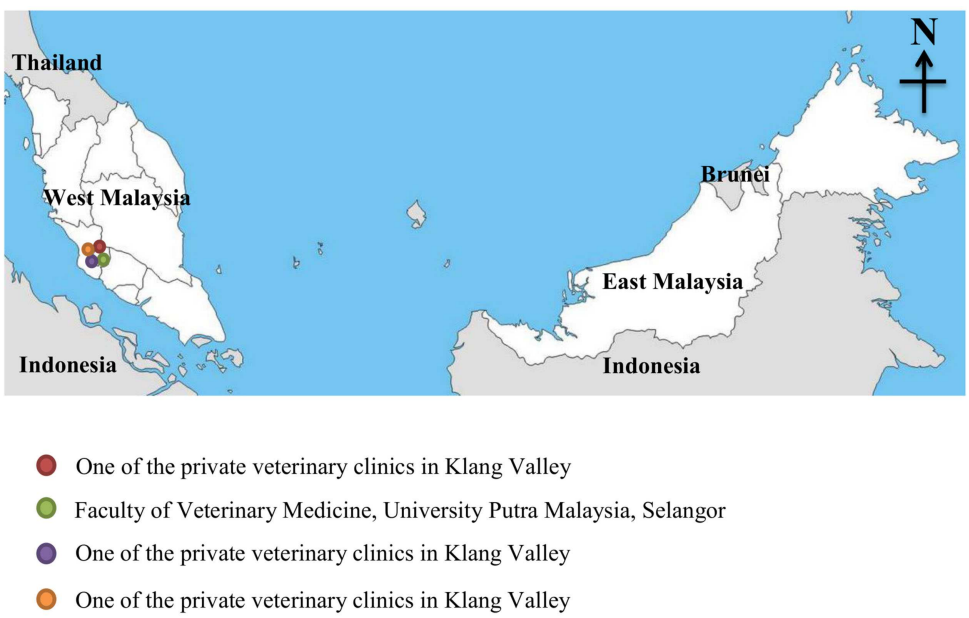

FIGURE 1 | Study sites in Klang Valley, Malaysia.

3 months and ELISA-III's avidity of <50% (low avidity) indicates primary infection.

STATISTICAL ANALYSIS

The data collected in the questionnaires and the serology results were analyzed by using statistical software SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). The qualitative variables were estimated and presented as frequencies and percentages. Univariate analyses and the χ^2 test were used to investigate the association between *Toxoplasma* seropositivity as a dependent variable and possible demographic and risk factors as independent variables; $p < 0.05$ was regarded as being statistically significant. However, to retain all possible significant association, variables that showed an association with $p \leq 0.20$ were used to apply to a multivariate logistic regression model (stepwise forward). Each dependent factor was modeled as dichotomous variables.

RESULTS

During this study period, a total of 312 people were recruited as studied subjects. The age range was 17–64 with a mean 27 ± 9.08 years. Majority of the subjects were in the age group of 21 and 30 years (228; 73.1%), female (234; 75%), Malay (139; 44.6%), veterinary students (194; 62.2%), and city dwellers (300; 96.2%).

The overall seroprevalence of toxoplasmosis in this study was 62 (19.9%) in which 57 (18.3%) samples were positive for IgG, 3 (1.0%) samples were positive for IgM, and 2 (0.7%) samples were positive for both IgG and IgM antibodies (**Table 1**). The positive IgM antibodies and samples with both positive for IgM and IgG antibodies were further tested for IgG avidity measurement using three standard commercial ELISA kits (I, II, and III) for comparison to differentiate between recent and past infections. Of five *Toxoplasma* seropositive samples, one sample was recently acquired and four other samples were past infections as detected from ELISA-I, all five samples were regarded as recently acquired infection, as demonstrated by ELISA-II, while ELISA-III showed past infection from all five seropositive samples (data were not shown). At the end of this study, there was, however, no clinical evidence of toxoplasmosis diagnosed in the *Toxoplasma* seropositive with low avidity.

Univariate analysis in relation to socio-demographic profiles showed that age group, gender, and study population were significantly associated with *Toxoplasma* seropositivity ($p < 0.05$) (**Table 2**). The results of this study further showed that the highest prevalence of *Toxoplasma* infection was found among veterinary technicians (33.3%) followed by pet owners (31.4%), veterinarians (18.4%), and veterinary students (14.9%). Interestingly, gardening (33; 26.6%) was significantly associated with *Toxoplasma* infection found among these subjects (**Table 3**). In addition, working duration and task performance were significantly associated with *Toxoplasma* seropositivity found among veterinary personnel (**Table 4**).

Further analysis by multivariate logistic regression showed that age group ≥30 years (OR = 0.34, 95% CI 0.18–0.63) contributes to high *Toxoplasma* seropositivity in the study population and working duration of more than 10 years (OR = 5.07, 95% CI 1.80–14.25) was identified as significant predictors of *Toxoplasma* infection among veterinary personnel (data were not shown).

Table 1 | Seroprevalence of toxoplasmosis among survey population as assessed by the ELISA test.

ELISA test	*Toxoplasma* seropositivity (62, 19.9%)		
	IgG+ve	IgM+ve	IgG+ve and IgM+ve
Positive	57 (18.3%)	3 (1.0%)	2 (0.6%)
Negative	255 (81.7%)	307 (99.0%)	310 (99.4%)
Total	312	310	312

Table 2 | Seroprevalence of *Toxoplasma* infection by the demographic characteristics.

Characteristics	Total *N* = 312	*Toxoplasma* seropositivity *n* (%)	*p*-value
Age			
Range 17–64 years with a mean of 27 ± 9.08 years			
Age group			0.000
≤ 20	22 (7.1)	3 (13.6)	
21–30	228 (73.1)	36 (15.8)	
31–40	33 (10.6)	9 (27.3)	
≥41	29 (9.29)	14 (48.3)	
Gender			0.014
Male	78 (25)	23 (29.5)	
Female	234 (75)	39 (16.7)	
Race			0.053
Malay	139 (44.6)	32 (23.0)	
Chinese	115 (36.9)	14 (12.2)	
Indian	33 (10.6)	10 (30.3)	
Aborigine	4 (1.3)	1 (25)	
Foreigner	21 (6.7)	5 (23.8)	
Study population			0.011
Veterinarian	38 (12.2)	7 (18.4)	
Veterinary technician	45 (14.4)	15 (33.3)	
Veterinary student	194 (62.2)	29 (14.9)	
Pet owner	35 (11.2)	11 (31.4)	
Primary residency			0.233
Village	12 (3.8)	4 (33.3)	
City	300 (96.2)	58 (19.3)	

$p < 0.05$, significant association as potential risk factors; N, number examined; n, number of positive sample.

DISCUSSION

WHY TOXOPLASMOSIS IS IMPORTANT

The present study showed the overall seroprevalence of toxoplasmosis among people having close contact with animals was 19.9% and this infection rate did not appear to be very high. Since this is the first study of its kind conducted in Malaysia, it is therefore no previous study can be compared. However, the numbers of respondents provided us substantial interpretation about the current prevalence of toxoplasmosis among these people in Malaysia to signify this conclusive remark. In the literature, the first report on *Toxoplasma* infection among general healthy population in

Table 3 | Seroprevalence of *Toxoplasma* infection by plausible risk factors among people having close contact with animals.

Variables	Total *N* = 312	*Toxoplasma* seropositivity *n* (%)	*p*-value
Close contacts with cats			0.173
Yes	142	33 (78.6)	
No	170	29 (17.1)	
Water supply at home			0.531
River and mountain pipe	27	6 (22.2)	
Government pipe water	283	55 (19.4)	
Private pipe water	2	1 (50)	
Clean water resources			0.144
Yes	207	46 (22.2)	
No	105	16 (15.2)	
Eating with bare hands			0.379
Yes	233	49 (21.0)	
No	79	13 (16.5)	
Tasting foods while cooking or seasoning			0.438
Yes	251	49 (19.5)	
No	61	13 (21.3)	
Cleaning cooking utensils			0.054
Yes	300	57 (19.0)	
No	12	5 (41.7)	
Always gardening			0.015
Yes	124	33 (26.6)	
No	188	29 (15.4)	

$p < 0.05$, significant association as potential risk factors; N, number examined; n, number of positive sample.

Table 4 | Seroprevalence of *Toxoplasma* infection by other plausible risk factors in working or study area for veterinary personnel.

Activities/variables	Total *N* = 277	*Toxoplasma* seropositivity *n* (%)	*p*-value
Working duration			0.004
≤1 years	43	6 (14.0)	
2–10 years	218	37 (17.0)	
11–20 years	6	2 (33.3)	
≥21 years	10	6 (60)	
Task performance			0.019
Working field	83	22 (26.5)	
Study field	194	29 (14.9)	
Cleaning cat excrement			0.286
Yes	217	42 (19.4)	
No	60	9 (15)	
Wearing gloves			0.433
Yes	222	40 (18.0)	
No	55	11 (20)	
Washing hands			0.665
Yes	275	51 (18.5)	
No	2	0 (0)	

$p < 0.05$, significant association as potential risk factors; N, number examined; n, number of positive sample.

Malaysia was quite low (13.9%) and it has been increasing over the years ranging from 16 to 30% (14), 28.1% (15), and 40.8% (16). However, higher prevalence in the healthy population could be the pet owners and the result may not be comparable with this present study. It is interesting to note that our finding is shown within the same range when compared with (19.9 vs. 14.2%) a rare but similar previous study done in Canada (11). Our study further pointed out that veterinary technicians had the highest *Toxoplasma* infection rate (33.3%). This similar result is shown in the earliest study of toxoplasmosis among veterinary members in the United States (9). In contrary, a previous study from Canada showed that veterinarians had the highest *Toxoplasma* infection rate (16.4%) compared to other groups (11). Based on the results obtained, primary screening of *Toxoplasma* infection should be particularly initiated in high seropositive individuals like veterinary technicians and pet owners. This program should also include women with unknown *Toxoplasma* serostatus to identify primary infection and *Toxoplasma* seronegative individuals for seroconversion. This could help to reduce the incidence in this high risk group of toxoplasmosis.

WHEN IgG AVIDITY DOES ITS ROLE

In the present study, the infection rate of anti-*Toxoplasma* IgM antibodies was 1.0% suggesting a recently acquired *Toxoplasma* infection. Negative results for IgM antibodies strongly exclude the recent infection, while positive result for IgM test is difficult to interpret (10). Hence, the positive result of IgM antibodies was further analyzed using IgG avidity test to help differentiate between past and recent infections (17). Of this, all three seropositive samples for anti-*Toxoplasma* IgM antibodies showed high avidities indicating past infection.

A positive result for only anti-*Toxoplasma* IgG antibodies in this study was 18.3% indicating past or chronic infection. Positive result for both IgG and IgM antibodies in this study was 0.6% indicates either a recent infection or false positive test result (10). Therefore, IgG avidity (brand I, II, and III) measurement, a confirmatory test, was subsequently performed, which is to assist, in determining the time of infection (18, 19). Of this, ELISA-III showed the most accurate result on five seropositive samples indicating chronic or past infection followed by ELISA-II and -I. Supporting to this finding, there was no clinically confirmed case of toxoplasmosis diagnosed during the time of this study. This therefore suggests the following: more than one avidity tests should be performed in a single serum sample, a second blood sample (if no avidity test available) is required to be tested after 2–4 weeks of infection to confirm a recent infection or it can be considered as a false positive with supporting information of whether there is an evidence of clinical toxoplasmosis.

WHAT BACKGROUND TELLS ITS STORY

Based on demographic data, the seroprevalence of toxoplasmosis was higher in males (28.8%) than females (15.9%). This could be normally explained by the fact that males have a higher tendency to be involved in sports activities or other activities at work or outdoor alike that expose them to soil and also they are not

really careful about hand hygiene, which leads to increase risk of acquiring the infection (20). We also found that *Toxoplasma* seropositivity increases with age (21), as also seen the higher infection rate (43.5%) among the older age group (≥41 years old) in this study. This finding is in agreement with previous studies even though studies were conducted in different target groups of population (21, 22). As a multi-racial and -cultural country like Malaysia, it is interesting to note that the highest prevalent rate was found among Indians ethnic (29.0%), which is not surprising because there are outnumber of Indians working closely with animals found in Malaysia compared to other ethnics. This finding is, however, contrary to the fact that the highest seroprevalence of *Toxoplasma* infection has so far been documented among Malay ethnic, which is due to their close contacts with cats (9, 23). Supporting our finding, a previous study in Gombak District, Selangor showed similar result (24). However, ethnic group was not significantly associated with *Toxoplasma* infection in this study.

HOW RISK FACTOR AFFECTS THE TRANSMISSION OF *T. GONDII*

Our univariate analysis showed that gardening was identified as one of significant risk factors in this study ($p = 0.015$). The participants who frequently do gardening were highly infected (26.6%) compared with the ones who spent less time (15.4%). This could be explained due to the fact that the buried sporulated oocysts of cats might be contaminating the soil the soil and sand and the oocysts remain infectious for about several months and can last beyond 1 year (25). Oocysts have a buoyancy characteristic that may become infectious after raining since oocysts will float on the upper layer of the soil (26). Therefore, it is very important to avoid any materials or foods that come into close contacts with unforeseen contaminated soil. The analysis further showed that the task performance in working field was significantly associated with *Toxoplasma* seropositivity ($p = 0.019$). Of this, veterinarians and veterinary technicians had the higher *Toxoplasma* infection (26.5%) compared to veterinary students (14.9%). This finding was not surprising since the daily task like animal surgery and cleaning the cat excrement are most probably increasing the chance of *Toxoplasma* transmission if the necessary precaution was taken lightly. However, a previous study in Canada demonstrated that cleaning the cat excrement was not the significant factor contributing to *Toxoplasma* infection among veterinary personnel (11). Based on this finding, other unidentified risks associated with *Toxoplasma* infection should be further investigated before any conclusion could be made. Of note, working duration was also significantly associated with *Toxoplasma* infection ($p = 0.004$) where the longest working duration of ≥21 years had the highest prevalent rate (60%). This might be due to the higher exposure with the animals especially cats since they have a lot of experience in handling and having close contact with cats.

After multivariate logistic regression model was applied, it was interesting to find that only age group of ≥30 years old and working duration of >10 years were identified as significant risks for *Toxoplasma* infection. This could be explained due to the fact that primary behavioral practices should be advised among people with increasing age, which is more prone to disease transmission of *T. gondii*. Also, individual with increasing age may have other co-infections that may lower his immune system, which can increase the susceptibility to *Toxoplasma* infection. The longer working duration contributed to the risk factor of *Toxoplasma* acquisition since they had a daily routine of handling with animals for long hours. This suggests that they might have a higher chance of close contact with cats and more likely expose to sporulated oocysts in cat's feces, which can increase the risk of *Toxoplasma* transmission (11).

CONCLUSION

This preliminary study shows the high prevalence of chronic toxoplasmosis in both veterinary personnel and pet owners. Age group of ≥30 years old in overall studied populations and working duration of >10 years among veterinary personnel significantly contributed to *Toxoplasma* infection. Hence, basic personal hygiene and management in working and study areas among veterinary personnel should be taken into consideration to minimize the probability of exposure to *Toxoplasma* infection. Future similar study is recommended periodically and also to investigate other unidentified risk factors to eliminate the infection rate and to eradicate this parasite from the region.

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AUTHOR CONTRIBUTIONS

VN and TTC designed the study. NAA, TTC, RSKS, and VN carried out the study. GJBM contributed most on manuscript writing. RSKS, YALL, TTC, HA, and VN helped in manuscript writing and editing. RSKS, YALL, TTC, and VN provided opinions and suggestions about this manuscript. All authors read and approved the final version of the manuscript.

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Toxoplasma gondii – Prevalence and Risk Factors in HIV-infected Patients from Songklanagarind Hospital, Southern Thailand

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Toxoplasmosis is one of the most common opportunistic parasitic diseases in patients living with HIV/AIDS. This study aimed to determine the seroprevalence of *Toxoplasma* infection in HIV-infected patients and to identify associated risk factors in *Toxoplasma* seropositive patients. This study was conducted at a regional public hospital in Hat Yai, southern Thailand during October 2009 to June 2010. Blood samples were collected from 300 HIV-infected patients. Each subject also answered a socio-demographic and risk factors associated with *Toxoplasma* infection. The prevalence of anti-*Toxoplasma* IgG antibodies in HIV-infected patients was 109 (36.3%), of which 83 (76.2%) had past infection and 26 (23.9%) had recently acquired *Toxoplasma* infection as indicated by their IgG avidity. Multivariate analysis using logistic regression showed that gender difference (adjusted OR = 1.69, 95% CI = 1.05–2.72) was the only factor associated with *Toxoplasma* infection. From the results obtained, these HIV-infected patients could be at high risk of developing clinical evidence of severe toxoplasmosis. Therefore, it is necessary to introduce primary behavioral practices to prevent *Toxoplasma* infection among HIV-infected patients.

Keywords: HIV, IgG avidity, seroprevalence, risk factors, toxoplasmosis

INTRODUCTION

Toxoplasmosis is a clinical and/or pathological evidence of a disease caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite. *Toxoplasma* infection affects about one-third of the world population but the majority of infected individuals are asymptomatic (Montoya and Liesenfeld, 2004). In people who are living with HIV/AIDS, there is an increased risk of reactivation of latent *Toxoplasma* infection in several organs, particularly in the brain leading toxoplasmic encephalitis (TE) that further complicates the course of AIDS (Sukthana, 2006). Globally, the number of patients who died from AIDS has been declining over the years due to the introduction of highly active antiretroviral therapy (HAART). Toxoplasmosis, one of the HIV co-infections, has, however, contributed to the burden of medical care costs (Suwanagool et al., 1997) and to those patients who are repeatedly admitted to government hospitals

(Solomon et al., 2006). Till date, approximately 10% of people living with HIV/AIDS are from the Southern part of Thailand (Ministry of Public Health [MOPH], 2011). To the best of our knowledge, there is no epidemiological surveillance reported on the opportunistic infections in HIV-infected patients including toxoplasmosis in this region. Furthermore, IgG avidity testing, a qualitative method, is the first ever serodiagnostic method to be introduced in differentiating chronic from recently acquired *Toxoplasma* infection in these patients. This method definitely helps in better understanding the status of *Toxoplasma* infection and its proper management in HIV patients. It is, therefore, relevant to conduct an epidemiological study of toxoplasmosis by determining the seroprevalence, the association with risk factors and the measurement of IgG avidity from *Toxoplasma* seropositive patients.

MATERIALS AND METHODS

Study Population

This cross-sectional study was carried out at Songklanagarind hospital, Hat Yai, Songkhla province, Thailand with the approval from the ethical committee of the Faculty of Medicine, Prince of Songkla University, Thailand (EC 53-080-14-1-2). The study included 300 HIV-infected patients, who attended the outpatient clinic and/or admitted in the ward at the Department of Internal Medicine during October 2009 to June 2010 and their informed consents were obtained prior to this study. The study subjects were randomly selected from those HIV-infected patients in any age group who had their anti-HIV antibody status examined by screening using the ELISA test and confirmed by the western blot technique. The information on related socio-demographic such as age, sex, and occupation as well as risk factors associated with *Toxoplasma* infection, such as close contact with cats, consumption of uncooked meats, a history of receiving blood transfusion, and some clinical backgrounds such as receiving primary chemoprophylaxis and/or receiving HAART. A CD4 cell count was obtained from their hospital recorded.

Serum Samples

Approximately 5 mL of venous blood sample was drawn from the participating HIV patients by a venipuncture into a sterile test tube. The sera were obtained after separation by centrifugation at 2500 × *g* for 5 min, and subsequently kept at −20°C until use.

Detection of anti-*Toxoplasma* IgG antibodies

The serostatus of *Toxoplasma* infection was screened using a standard ELISA commercial kit (IgG-NovaLisa^{TM}, Dietzenbach, Germany) in accordance with the manufacturer's instructions.

Measurement of IgG avidity

A positive sample for anti-*Toxoplasma* IgG antibody was also tested for its avidity using a standard ELISA commercial kit (IgG-NovaLisa^{TM}, Dietzenbach, Germany); high avidity (>40%) indicated a past infection while a low avidity (<40%) indicated a recently acquired infection.

Statistical Analysis

Data obtained from both the questionnaire and laboratory tests were entered and analyzed using the statistical software SPSS version 10 (SPSS Inc., Chicago, IL, USA). The data with quantitative variables were expressed as a mean (±SD) and range, whereas, qualitative variables were estimated and presented as frequencies and percentages. The Chi-square (χ^2) test or Fisher exact probability test was chosen to determine the association between possible risk factors and disease transmission. Multivariate analysis adjusted by multiple logistic regressions was used to determine significant differences between demographics or confounding risk factors associated with *Toxoplasma* infection among study subjects. The *p*-value of ≤0.05 was regarded as statistically significant.

RESULTS

Demographic Profile of Study Subjects

Table 1 shows the age range of these HIV-infected patients was 21–78 with a mean of 40.4 ± 8.4 years. Majority of these patients were in the age group of 20–39 years (150, 50%), in addition, they were predominantly married (196, 65.3%), and were laborers (174, 58%). About half of these patients had completed secondary school level of education (153, 51%).

Seroprevalence of Toxoplasmosis in HIV/AIDS Patients and in Association with Demographic Characteristics

The seroprevalence of *Toxoplasma* infection in these patients was 109 (36.3%). Measurement of IgG avidity among *Toxoplasma* seropositive patients showed 83 (76.2%) had high avidity indicates past infection while 26 (23.9%) patients had low avidity indicates recently acquired *Toxoplasma* infection.

Using univariate analysis, this study identified that gender and a history of having cerebral toxoplasmosis were statistically significant factors associated with *Toxoplasma* seropositivity ($p < 0.05$). The data further showed that majority of male patients were significantly found in the age group of 40–59 years, and they make their living through their labor works. It is also shown that these male patients had not received high education, stayed outside the main city and eating of uncooked meat; however, there was no significant association (**Table 2**). Further analysis using multivariate logistic regression showed that gender (male) plays a significant role in *Toxoplasma* seropositivity with adjusted odds ratio of 1.69 (95% CI = 1.05–2.72; **Table 3**).

The Prevalence of TE in Patients with AIDS

The past clinical history of these patients showed that 10/300 (3.3%) of HIV/AIDS patients, aged between 28 and 52 years, 5 males, and 5 females, were diagnosed with TE prior to this

TABLE 1 | Univariate analysis of plausible demographic characteristics, clinical profiles, and other possible risk factors associated with *Toxoplasma* seropositive HIV-infected patients.

Demographic characteristics		Number (%)	Number IgG positive (%)	*p*-Value
Age group (years)	20–39	150 (50)	46 (30.7)	0.125[a]
	40–59	143 (47.7)	60 (42)	
	≥60	7 (2.3)	3 (42.9)	
Sex	Male	157 (52.3)	66 (42)	0.031[a]
	Female	143 (47.7)	43 (30.1)	
Marital status	Single	104 (34.7)	42 (40.4)	0.288[a]
	Married	196 (65.3)	67 (34.2)	
Education	Primary	75 (25)	27 (36)	0.870[a]
	Secondary	153 (51)	54 (35.3)	
	Tertiary	72 (24)	28 (38.9)	
Occupation	Laborer	174 (58)	62 (35.6)	0.728[a]
	Non-laborer	59 (19.7)	24 (40.7)	
	Other[b]	67 (22.3)	23 (34.3)	
Present address	Songkhla	149 (49.7)	49 (46.2%)	0.378[a]
	Outside	151 (50.3)	57 (53.8)	
CD4 (cells/cumm)	<200	52 (17.3)	22 (42.3)	0.111[a]
	200–499	134 (44.7)	54 (40.3)	
	≥500	114 (38)	33 (29)	
History of receiving chemoprophylaxis[c]	Yes	63 (21)	24 (38.1)	0.744[a]
	No	237 (79)	85 (35.9)	
History of receiving highly active antiretroviral therapy (HAART)	Yes	285 (95)	100 (35.1)	0.051[a]
	No	15 (5)	9 (60)	
History of toxoplasmic encephalitis (TE)	Yes	10 (3.3)	7 (70)	0.024[d]
	No	290 (96.7)	102 (35.2)	
History of contact with cats	Yes	191 (63.7)	65 (34)	0.272[a]
	No	109 (36.3)	44 (40.4)	
History of eating uncooked meat	Yes	58 (19.3)	20 (34.5)	0.744[a]
	No	242 (80.7)	89 (36.8)	
History of blood transfusion	Yes	4 (1.3)	3 (75)	0.106[d]
	No	296 (98.7)	106 (35.8)	

[a]*p-value was evaluated by* χ^2 *test.*
[b]*Other includes retiree, unemployed, housewives and students.*
[c]*Co-trimoxazole*
[d]*p-value was analyzed by Fisher exact probability test.*

study. Our study confirmed statistically significant ($p = 0.024$) sero-evidence of anti-*Toxoplasma* (IgG) antibodies in seven cases (data were not shown).

Other Co-opportunistic Infections in HIV/AIDS Patients

During the time of this study, there was no new diagnosis of TE reported from our patients. However, patients with *Toxoplasma* seropositivity and other concurrent opportunistic diseases were found as follow: 24/72 patients with tuberculosis (TB) had *Toxoplasma* seropositivity and 18 of these TB patients subsequently developed immune reconstitution inflammatory syndrome (IRIS-TB). *Toxoplasma* seropositivity was also found in: 15 patients with herpes virus infections (herpes simplex (HS) and herpes zoster (HZ) viruses), 2 patients with cytomegalovirus (CMV) infections, 2 patients with salmonellosis, 3 patients with histoplasmosis, 5 patients with cryptococcal meningitis, 6 patients with penicillosis, and 4 patients with non-tuberculous meningitis (NTM). Overall, there was no fatal case reported at the end of this study.

DISCUSSION

Approximately half of HIV-infected patients are co-infected with *T. gondii* (Shimelis et al., 2009; Daryani et al., 2011). In Thailand, previous studies of the prevalence of *Toxoplasma* infection among HIV-infected patients found the prevalence ranging from 22.4 to 53.7% (Wongkamchai et al., 1995; Chintana et al., 1998; Sukthana et al., 2000; Nissapatorn et al., 2001; Wanachiwanawin et al., 2001). In our study, 36.3% of subjects were infected with *Toxoplasma*. This is higher than a study among immunocompetent pregnant women (28.3%) in a study conducted in the same location at the same time (Nissapatorn et al., 2011). Differences in the sensitivity of the ELISA test kit may account for the differences in the prevalence of *Toxoplasma* infection (Chemoh et al., 2013).

TABLE 2 | Comparison of plausible demographic characteristics, clinical profiles, and other possible risk factors between male and female HIV-infected patients.

Demographic characteristics		Number (%)		*p*-Value
		Male	Female	
Age group (years)	20–39	60 (38.2)	90 (62.9)	0.000[a]
	40–59	93 (59.2)	50 (35)	
	≥60	4 (2.6)	3 (2.1)	
Marital status	Single	47 (29.9)	57 (39.9)	0.092[a]
	Married	110 (70.1)	86 (60.1)	
Education	Primary	33 (21)	42 (29.4)	0.248[a]
	Secondary	84 (53.5)	69 (48.2)	
	Tertiary	40 (25.5)	32 (22.4)	
Occupation	Laborer	103 (65.6)	71 (49.6)	0.000[a]
	Non-laborer	40 (25.5)	19 (13.3)	
	Other[b]	14 (8.9)	53 (37.1)	
Present address	Songkhla	76 (51)	73 (49)	0.648[a]
	Outside	81 (53.6)	70 (46.4)	
History of receiving chemoprophylaxis	Yes	40 (25.5)	23 (16.1)	0.064[a]
	No	117 (74.5)	120 (83.9)	
Seroprevalence of *Toxoplasma* infection	Positive	66 (42)	40 (28)	0.011[a]
	Negative	91 (58)	103 (72)	
History of contact with cats	Yes	102 (65)	89 (62.2)	0.711[a]
	No	55 (35)	54 (37.8)	
History of eating uncooked meat	Yes	37 (23.6)	21 (14.7)	0.072[a]
	No	120 (76.4)	122 (85.3)	
History of blood transfusion	Yes	1 (0.6)	3 (2.1)	0.262[c]
	No	156 (99.4)	140 (97.9)	

[a]*p-Value was evaluated by* χ^2 *test.*
[b]*Other includes retiree, unemployed, housewives and students.*
[c]*p-Value was analyzed by Fisher exact probability test.*

IgG avidity testing was developed to avoid the need of conducting confirmatory tests with a second serum sample to determine, if there is a recently acquired infection (Pour Abolghasem et al., 2011). A positive *Toxoplasma* IgG test with a low avidity suggests a recently acquired infection (Liesenfeld et al., 2001; Reis et al., 2006). Based on IgG avidity testing, 8.7% of our subjects had a newly acquired *Toxoplasma* infection and these patients were closely monitored. However, none developed clinical toxoplasmosis during the study.

Using multivariate analysis, male gender was found to be the only significant risk factor for *Toxoplasma* infection. This is consistent with other studies (Nissapatorn et al., 2007; Akanmu et al., 2010). Males were found to be more susceptible to acquire several infections due to their sex steroid hormones that decrease immune responses and influence disease resistance genes and their behaviors (Klein, 2000). These may be the reasons, why the seropositivity of male HIV/AIDS patients to *Toxoplasma* infection was significantly higher than the female HIV/AIDS patients (Roberts et al., 2001). Previously identified risk factors, such as close contact with cats, consumption of uncooked meats and history of receiving a blood transfusion were not significantly associated with *Toxoplasma* infection in our study.

Reactivation of latent *Toxoplasma* infection is common in immunocompromised hosts (Dahnert, 2003) making HIV patients at higher risk for clinical toxoplasmosis. TE is the most common neurological condition (42%) in HIV-infected patients (Ramirez-Crescencio and Velasquez-Perez, 2011). Ten subjects (3.3%) in our study had a previous history of TE as diagnosed by a combination of clinical TE: headaches, seizure, focal neurological deficits, histology and response of therapy, of these 70% (7) were seropositive for *Toxoplasma* infection. This finding is similar to another study who found some of the patients with confirmed TE were not positive for *Toxoplasma* antibodies (Skiest et al., 2000).

TABLE 3 | Multivariate logistic regression analysis of risk factors associated with *Toxoplasma* seropositivity.

Demographic characteristics[a]	Adjusted OR (95% CI)[b]	*p*-Value
Gender	1.69 (1.05–2.72)	0.042
History of receiving HAART	0.36 (0.13–1.04)	0.095
History of TE	4.30 (1.09–16.99)	0.075

[a]*Only risk factors with a* $p \leq 0.10$ *on univariate analysis were included in multivariate logistic regression analysis.*
[b]*OR, odds ratio; CI, confidence interval.*

CONCLUSION

Latent toxoplasmosis is still prevalent in our study population. Gender was the only significant risk factor for *Toxoplasma* infection in our study. Although the

number of HIV infected patients in Thailand has decreased nationwide (Ministry of Public Health [MOPH], 2011), we recommend newly infected HIV patients be warned about this opportunistic parasitic disease during the counseling period since both newly acquired and chronic *Toxoplasma* infections in patients with AIDS are occasionally developed clinical toxoplasmosis (Centers for Disease Control and Prevention [CDC], 2009).

AUTHOR CONTRIBUTIONS

VN, NS, and PS designed the study. WC, HA, TH, NS, and BC carried out the experiment. WC, NS, and VN helped in manuscript writing and editing. VN, NS, and PS provided opinions and suggestions about this manuscript. All authors read and approved the final version of the manuscript.

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Cerebral and ocular toxoplasmosis related with IFN-γ, TNF-α, and IL-10 levels

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This study analyzed the synthesis of Interferon gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-α), and Interleukin 10 (IL-10) in chronically infected patients which developed the symptomatic disease as cerebral or ocular toxoplasmosis. Blood from 61 individuals were divided into four groups: Cerebral toxoplasmosis/AIDS patients (CT/AIDS group) ($n = 15$), ocular toxoplasmosis patients (OT group) ($n = 23$), chronic toxoplasmosis individuals (CHR group) ($n = 13$) and healthy individuals (HI group) ($n = 10$). OT, CHR, and HI groups were human immunodeficiency virus (HIV) seronegative. The diagnosis was made by laboratorial (PCR and ELISA) and clinical subjects. For cytokine determination, peripheral blood mononuclear cells (PBMC) of each patient were isolated and stimulated *in vitro* with *T. gondii* antigen. IFN-γ, TNF-α, and IL-10 activities were determined by ELISA. Patients from CT/AIDS and OT groups had low levels of IFN-γ when were compared with those from CHR group. These data suggest the low resistance to develop ocular lesions by the low ability to produce IFN-γ against the parasite. The same patients, which developed ocular or cerebral toxoplasmosis had higher TNF-α levels than CHR individuals. High TNF-α synthesis contribute to the inflammatory response and damage of the choroid and retina in OT patients and in AIDS patients caused a high inflammatory response as the TNF-α synthesis is not affected since monocytes are the major source this cytokine in response to soluble *T. gondii* antigens. IL-10 levels were almost similar in CT/AIDS and OT patients but low when compared with CHR individuals. The deviation to Th2 immune response including the production of anti-inflammatory cytokines, such as IL-10 may promote the parasite's survival causing the tissue immune destruction. IL-10 production in *T. gondii*-infected brains may support the persistence of parasites as down-regulating the intracerebral immune response. All these indicate that OT and CT/AIDS patients produced low levels of IL-10 (Th2 response) and IFN-γ (Th1 response). They produced high TNF-α suggesting a high inflammatory response triggered by the parasite.

Keywords: *Toxoplasma gondii*, cerebral toxoplasmosis and AIDS, ocular toxoplasmosis, cytokines, immune response

INTRODUCTION

Lifelong infection with the obligate intracellular protozoan *Toxoplasma gondii* affects one-third of the human population globally (Weiss and Dubey, 2009). Although toxoplasmosis is asymptomatic in the majority of cases, *T. gondii* infection can have serious consequences in immunocompromised individuals and in cases of congenital infection. In the latter case, ocular infection, posterior retinochoroiditis, is the most frequent clinical manifestation of congenital and acquired toxoplasmosis (Delair et al., 2011; Olariu et al., 2011; Dubey et al., 2012).

The severity and prevalence of the disease vary greatly and is believed to be affected by the status of the host immune system (Garweg and Candolfi, 2009), the genotype of infective parasite strains (Pereira-Chioccola et al., 2009; Dubey et al., 2012), and the host genetic background (Mack et al., 1999; Sullivan and Jeffers, 2012).

While the physiopathological mechanisms that underlie retinal damage in ocular toxoplasmosis are yet not fully understood, the immune response might directly affect the pathogenesis of toxoplasmic retinochoroiditis and some cytokines have been shown to be fundamental to either control or block a protective response against *T. gondii* in experimental models (Talabani et al., 2010). Its importance is even greater in Brazil, where the prevalence and severity of ocular disease is higher than those reported in other regions of the world (Gilbert et al., 2008; Ferreira et al., 2014). In addition, reactivation of the latent infection occurs in

approximately 30% of immunocompromised patients (Sarciron and Guerardi, 2000; Robert-Gangneux and Dardé, 2012).

Cerebral toxoplasmosis is the most common AIDS-related opportunistic infection of the central nervous system and the most common cause of focal deficits in human immunodeficiency virus (HIV)-positive patients (Pereira-Chioccola et al., 2009; Vidal and Oliveira, 2013). It has been suggested that the neurological involvement of HIV leads to an imbalance of the immune response and, as consequence, the reactivation of the latent infection occurs (Lin and Bowman, 1992). However, the understanding of the physiopathology of cerebral toxoplasmosis remains incomplete.

In immunocompetent individuals, the immune system controls multiplication of the parasites and stops their dissemination. In addition, it favors the transformation of tachyzoites into bradyzoites and eventually, the occurrence of tissue cysts. Cytokines have been shown to play an important role in the pathogenesis of toxoplasmosis (Costa-Silva et al., 2012a; Ghasemi et al., 2012; Sullivan and Jeffers, 2012).

Although occur alterations in levels of antibodies and cytokines during the reactivation of *T. gondii* infection (Beaman et al., 1992; Meira et al., 2008, 2011; Hoti and Tandon, 2011), the role and function of cytokines, in cellular mediation, in the humoral response, as well as their impaired action in AIDS patients remain today only partially understood. This study was aimed to analyze the synthesis of Interferon gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-α), and Interleukin 10 (IL-10) in chronically infected patients which developed the symptomatic disease as cerebral or ocular toxoplasmosis.

MATERIALS AND METHODS

PATIENTS AND SAMPLES

This case-control study was conducted for 17 months (June 2011 to November 2012). We analyzed blood samples from 61 individuals divided into four groups. Two groups were formed of chronically patients infected, who developed the symptomatic disease: The CT/AIDS (cerebral toxoplasmosis and AIDS) group was composed of clinical samples from 15 patients with cerebral toxoplasmosis and AIDS (53% male and 47% female) aged between 23 and 64 years old. The OT (ocular toxoplasmosis) group was composed of clinical samples from 23 patients with ocular toxoplasmosis (57% male and 43% female) aged between 15 and 76 years old. The other two groups, CHR (chronic toxoplasmosis individuals) and HI (healthy individuals) were established as controls. Clinical samples from 13 chronic individuals for toxoplasmosis (44% male and 56% female) aged between 25 and 59 years old composed the CHR group. Samples from 10 healthy individuals, seronegative for *T. gondii*, (*Toxoplasma* uninfected group) (47% male and 53% female) aged between 28 and 38 years old composed the HI group. The OT, CHR, and HI groups were seronegative for HIV.

The 15 HIV-infected patients were admitted and treated at the Emilio Ribas Institute of Infectious Diseases, a tertiary teaching hospital in São Paulo, Brazil. The clinical diagnosis of cerebral toxoplasmosis in HIV-infected patients was based on: (1) progressive neurological deficits; (2) contrast-enhancing mass lesion(s) on computed tomography and/or magnetic resonance imaging; and (3) successful clinical and radiological response to antiparasitic treatment within 2 weeks (Portegies et al., 2004; Vidal and Oliveira, 2013).

OT patients were admitted and treated at the Ophthalmology Outpatient Clinics from Fundação Faculdade Regional de Medicina—Hospital de Base, a tertiary teaching hospital in São José do Rio Preto, São Paulo, Brazil. They were clinically diagnosed as having toxoplasmic retinochoroiditis, and the clinical evaluation was performed under fundoscopic examination using indirect binocular ophthalmoscopy, 20D lens (Binocular Ophthalmoscope ID 10, Topcon Corporation, USA). The clinical diagnosis of ocular toxoplasmosis was based on retina visualization and the description of the characteristic, the site and the size of exudative lesions or scars (Mattos et al., 2011; Ferreira et al., 2014). In order to determine the minimally invasive laboratory diagnosis, as defined before (Colombo et al., 2005; Mattos et al., 2011), blood samples from Group CT/AIDS and OT patients were collected before or until the third day of the antiparasitic therapy for toxoplasmosis.

Ethical considerations

All patients provided written informed consent and the institutional review boards of Ethics Committees of the all Institutions approved this study.

T. gondii and antigen

T. gondii lysate antigen (TLA) was prepared as described before (Meira et al., 2008; Costa-Silva et al., 2012b). Tachyzoites (RH strain) from Vero cell cultures in serum-free medium were purified by filtration. Then, parasites were washed, suspended in phosphate-buffered saline (PBS) and lysed using glass beads by vortex for 8 cycles for 4 min with 2-min intervals. Parasite extract was centrifuged (3000 g) and dissolved in 0.3 M NaCl, and the protein concentration was determined at 280 nm by spectrometry in a NanoDrop ND100 (Thermo Scientific). TLA was used for determination of humoral and cellular response by ELISA. Tachyzoites were further used for DNA extraction.

SEROLOGICAL DIAGNOSIS

ELISA were performed with microtiter polystyrene plates (flat bottom, low binding, Corning Incorporated, USA) as previously described (Meira et al., 2008; Mattos et al., 2011). Briefly, each well was incubated overnight at 4 C with TLA at a concentration of 1 μg/ml. The free binding sites were blocked by treating the wells with 5% skim milk-PBS and after 60 min, 50 μl each of serum sample (1:200 in 5% skim milk-PBS) were incubated for 60 min at 37°C. After washes with PBS-Tween 20, the wells were incubated for more 60 min at 37°C with a horseradish peroxidase-conjugated goat anti-human IgG (Sigma) and the substrate solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 0.05% *o*-phenylenediamine, 0.1% H_2O_2) was added to each well. Color development was stopped by adding 50 μl of 4 N H_2SO_4. Absorbance values were measured in an ELISA Labsystens-Multiscan MS Plate Reader (Minneapolis, Minnesota, USA) with a 492-nm filter. The optical density (O.D.) cutoff was 0.148 at 492-nm wavelength as previously described (Meira et al., 2008). The absorbance values were subtracted from the background,

and the arithmetic mean was calculated. O.D. results were transformed in ELISA-relative values (RV) that represent the ratio of the absorbance of each serum sample at an optical density of 492 nm to the cutoff value (serum O.D/cutoff O.D.). Values greater than 1.0 were considered reactive. Low RV was considered to be between 1 and 5; and high RV, above 6.

MOLECULAR DIAGNOSIS

The DNA of blood samples was extracted by QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. DNA pellets were dissolved in ultra-pure water. As a positive control, DNA was extracted from tachyzoite pellets using the same kit. DNA purity was determined by the ratio of O. D. at 260 and 280 nm in a NanoDrop ND100 (Thermo Scientific). The amplifications were carried out with a kit purchased from Promega (Go Taq Green Master Mix). The PCR mix (12.5 μL) was composed of 1 unit of Taq DNA polymerase, 10 mM Tris-HCl, pH 8.5; 50 mM KCl; 1.5 mM $MgCl_2$; and 200 mM of each dNTP. The reactions included The PCR mix, 5 μL of each DNA template and 25 pmol of each primer to a final volume of 25 μL. The primer pairs used were B22/B23, which amplified a 115-bp sequence from a specific repetitive region of B1 gene from *T. gondii* as target (Burg et al., 1989). To control the course of extraction and check for PCR inhibitors, all samples were assayed using β1/β2 (Mesquita et al., 2010), which amplified a 140 bp fragment of the human β-globulin gene. Each amplification run contained two negative controls (ultra-pure water and a negative DNA for toxoplasmosis) and one positive (DNA extracted from tachyzoite pellets). The thermal cycles and PCR products analyses were made exactly as described before (Colombo et al., 2005; Mesquita et al., 2010).

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND CYTOKINE ASSAYS

About 10 ml of blood containing sodium heparin as anticoagulant collected from all patients and an equal volume of PBS (pH 7.2) was added to the blood and the peripheral blood mononuclear cells (PBMC) separated over Histopaque 1077 as per protocol described by Sigma-Aldrich. After isolation, cells were washed two times with PBS by centrifugation at 1000 *g* for 10 min and resuspended in RPMI-1640 medium (Sigma) containing L-glutamine, 2 g/L sodium bicarbonate, 10% heat-inactivated fetal calf serum and 50 mg/ml of streptomycin. The viability of the cells used in the experiments was always higher than 85%, as measured by trypan blue exclusion (Sigma). The cells were then plated at a density of 1×10^6 cells per well into 48-well culture plates in a final volume of 500 μL.

PBMC were then stimulated with 1 μg/mL of TLA. Alternatively, as control for IFN-γ, TNF-α, and IL-10 experiments, cells were stimulated with 2 μg/mL of phytohemagglutinin (PHA). Culture medium was used in all experiments as negative control wells. PBMC cultures were maintained at 37°C in a 5% CO_2 atmosphere and supernatants were collected after 24–48 h of stimulation. The supernatant was analyzed after 24 h for TNF-α and IL-10, and 48 h for IFN-γ activity.

The cytokine concentrations were determined using the Ready-Set-Go ELISA kit according to the manufacturer's instructions (Ebioscience, San Diego, California). The limit of sensitivity of the assays was 2 pg/mL for IL-10 and 4 pg/mL for IFN-γ and TNF-α.

DATA ANALYSIS

The concentration of each cytokine, per group of patients was determined by median (in pg/mL). The statistical analyses were made by the non-parametric *Mann–Whitney* test for comparison between different groups in the GraphPad Prism 5.0 software. p-value < 0.05 was considered to be statistically significant.

RESULTS

The first step was to establish the clinical and laboratory diagnosis of the patients from the both groups. As described in **Table 1**, the 15 patients from CT/AIDS group developed focal cerebral toxoplasmosis as they had CD4+ lymphocytes counts less than 100 cells/μl of blood. The values ranged from 4 to 84 cells/μl of blood. In addition, they had positive PCR for *T. gondii* in blood, high ELISA-RV in 67% of the patients and low, in 33% of them. Out of the 23 patients from OT group, the clinical and fundoscopic

Table 1 | Patients with cerebral toxoplasmosis or ocular toxoplasmosis: clinical and laboratory diagnosis as well as cytokines levels after PBMC stimulation with *T. gondii* TLA antigen.

	CT Patients[a] ($n = 15$)	OT patients[a] ($n = 23$)
Data of the sample collection (m/y)[b]	10/11–3/12	5/12–8/12
Median age (range)	43 (23–64)	63 (15–76)
Gender	8M – 7F	13M – 10F
Clinical diagnosis[c]	focal CT (100%)	Active RtC (14%)[d] Active and RtC scar (2%) RtC scar (84%)
HIV serology	Pos (100%)	ND[e]
$CD4^+$ lym counts[f] (range)	4–84	ND
Positive PCR in blood (for *T. gondii* DNA)	100% (15/15)	48% (11/23)
Anti-*T. gondii* antibodies ELISA (RV)[g]	Pos (12.8–3.0)	Pos (5.7–1.0)
[h]IFN-γ—median (range)	71.47 (0–633.24)	155.30 (27.35–2630.29)
[h]TNF-α—median (range)	2564.01 (21.12–11253.62)	2352.00 (401.03–11453.71)
[h]IL10—median (range)	3011.50 (17.87–726.38)	580.60 (39.15–2656.17)

[a] *CT (cerebral toxoplasmosis), OT (ocular toxoplasmosis).*

[b] *Date (month/year) of the collection.*

[c] *Diagnosis was defined by clinical, images and laboratory data as described in Materials and Methods Section.*

[d] *Active and retinochoroiditis (RtC) scars in the right and left eyes, respectively.*

[e] *Non-determined (ND).*

[f] *Number of CD4+ T lymphocytes/μl of blood.*

[g] *ELISA are expressed in relative values (RV), which represent the ratio of the absorbance of each serum sample at an optical density of 492 nm to the cutoff value (serum O.D./cutoff O.D.). Values greater than 1.0 were considered reactive.*

[h] *IFN-γ, TNF-α, and IL10 are expressed in in pg/mL. Pos, positive.*

examination determined that 2 patients had active lesions, 2 had both, active lesions and scars; and 19, retinochoroiditis scars. PCR was positive in 48% the patients. Low ELISA-RV was shown in 96% (**Table 1**).

The first part of the determinations of the cytokines was conducted in order to set up the conditions for PBMC cultures. Different TLA and PHA concentrations to stimulation of PBMC were tested (1, 2, 5, and 10 μg/mL) and the supernatants were tested after 24, 48 and 72 h of stimulation for each cytokine. After evaluation of these parameters, PBMC from CT/AIDS and OT patients; CHR and HI individuals were stimulated *in vitro* with 1 μg/mL of TLA as antigen. In parallel, as controls, the same cells were stimulated with 2 μg/mL of PHA and in the absence of the antigen. There was no proliferation in PBMC cultures in the absence of *T. gondii* antigen or PHA (data not shown). Next, IFN-γ, TNF-α, and IL-10 levels were evaluated by ELISA in the supernatant of PBMC collected from the four groups of patients. Median results of IFN-γ, TNF-α, and IL-10 levels after PBMC stimulation with TLA, per group of patient are shown in **Table 1**. Additionally, the median results of the each cytokine stimulated with PHA (as positive control) and TLA, per group, were analyzed and they were shown in details in **Figure 1**.

The IFN-γ data are shown in **Figure 1A**. PBMC from CT/AIDS and OT patients stimulated with PHA produced 840.60 pg/mL and 2205.00 pg/mL of IFN-γ, respectively. Differently, PBMC from CHR and HI individuals produced 4534.09 pg/mL and 3339.34 pg/mL, respectively. The differences between the results of CT/AIDS and CHR groups were statistically significant at $p < 0.005$.

PBMC from CHR individuals stimulated with TLA produced high IFN-γ amounts (1198.91 pg/mL). Nevertheless, PBMC from patients with CT/AIDS and OT produced low IFN-γ amounts (71.47 pg/mL and 155.30 pg/mL, respectively). PBMC from HI individuals produced 90.59 pg/mL of IFN-γ. The differences between results from CHR and CT/AIDS, CHR and HI individuals were statistically significant at $p < 0.005$, and between CHR and OT patients at $p < 0.0005$.

The TNF-α results are shown in **Figure 1B**. PBMC from individuals of all groups were able to produce large amounts of TNF-α using PHA as stimulus. CHR and HI individuals as well as CT/AIDS and OT patients, produced 4326.93 pg/mL, 2834.21 pg/mL, 8480.00 pg/mL and 9827.76 pg/mL of TNF-α, respectively. The differences between OT and HI were statistically significant at $p < 0.05$. Lower TNF-α level (992.71 pg/mL) produced from cells collected from CHR were shown when compared with CT/AIDS and OT patients (2564.54 pg/mL and 2352.52 pg/mL, respectively).

PBMC of HI individuals stimulated with TLA produced 329.60 pg/mL of TNF-α. The differences between the CHR and OT were statistically significant at $p < 0.005$ and between CT/AIDS and HI at $p < 0.005$, OT and HI at $p < 0.005$ and CHR and HI at $p < 0.05$.

The results of IL-10 levels are shown in **Figure 1C**. PBMC of CHR and HI individuals stimulated with PHA produced 3939.00 pg/mL and 5858.09 pg/mL while CT/AIDS and OT patients were able to produce 207.21 pg/mL and 2246.87 pg/mL, respectively. The differences between the CT/AIDS, OT and CHR were statistically significant at $p < 0.05$ and between CT/AIDS and HI at $p < 0.005$. In OT and HI groups the differences was at $p < 0.05$.

No differences in IL-10 levels were observed between PBMC collected from CHR individuals and CT/AIDS or OT patients (466.80 pg/mL, 311.50 pg/mL and 580.60 pg/mL, respectively). However, HI individuals produced high levels (3619.10 pg/mL). The differences between HI and CT/AIDS, HI and OT or HI and CHR were statistically significant at $p < 0.0005$.

DISCUSSION

This study evaluated the cellular response in PBMC samples from patients with cerebral toxoplasmosis and AIDS and patients with ocular toxoplasmosis using a TLA (Meira et al., 2013).

The clinical and laboratorial characteristics from the 38 chronically infected patients studied confirmed previous studies (Colombo et al., 2005; Mattos et al., 2011; Meira et al., 2013). The 15 patients from CT/AIDS group developed focal cerebral toxoplasmosis since they had low CD4+ lymphocytes counts. All patients had tachyzoites in blood since they had positive PCR. The *T. gondii* antigens produced high antibody production determined by ELISA-RV. The other group, which was composed of 23 patients with active lesions or retinochoroiditis scars, was a quite different. PCR results were positive in almost half of the patients but they had low ELISA-RV. No differences in IL-10 levels were observed between PBMC collected from CHR individuals.

The quality of the cells of all groups of individuals was in good conditions as showed in the PBMC stimulation with PHA. The importance of IFN-γ to resistance against *T. gondii* infection has been demonstrated, as this cytokine is responsible for regulation of *T. gondii* load and distribution in the eyes; essential mediator of the immune response to control *T. gondii* in the brain and to maintain the latency of chronic infection (Carruthers and Suzuki, 2007; Gaddi and Yap, 2007; Goldszmid et al., 2012; Nijhawan et al., 2013). However, patients develop cerebral toxoplasmosis after reactivation of latent infection during immunosuppression. In this study these patients (CT/AIDS group) had low levels of IFN-γ when were compared with those from CHR group (chronic infected individuals). The patients with ocular toxoplasmosis also had low levels of IFN-γ and confirm other studies that also shown IFN-γ levels were elevated in asymptomatic individuals compared with patients with acquired toxoplasmosis (Yamamoto et al., 2000; de-la-Torre et al., 2013). These data suggest that resistance or not to develop ocular lesions is associated with the ability to produce IFN-γ against the parasite. These data, yet, can be correlated with recent reported studies that severe ocular infections in South America due to highly variable *T. gondii* strains and characterized by a completely different immune response pattern and much higher ocular parasite loads (de-la-Torre et al., 2013, 2014; Pfaff et al., 2014). The high levels of this cytokine found in CHR individuals can be explained by its release by parasite-specific T lymphocytes, which are required to prevent cyst reactivation during chronic infection (Sarciron and Guerardi, 2000).

On the other hand, the same patients, which developed ocular or cerebral toxoplasmosis had higher TNF-α levels than CHR individuals. This proinflammatory cytokine is directly involved in the regulation of tachyzoite growth.

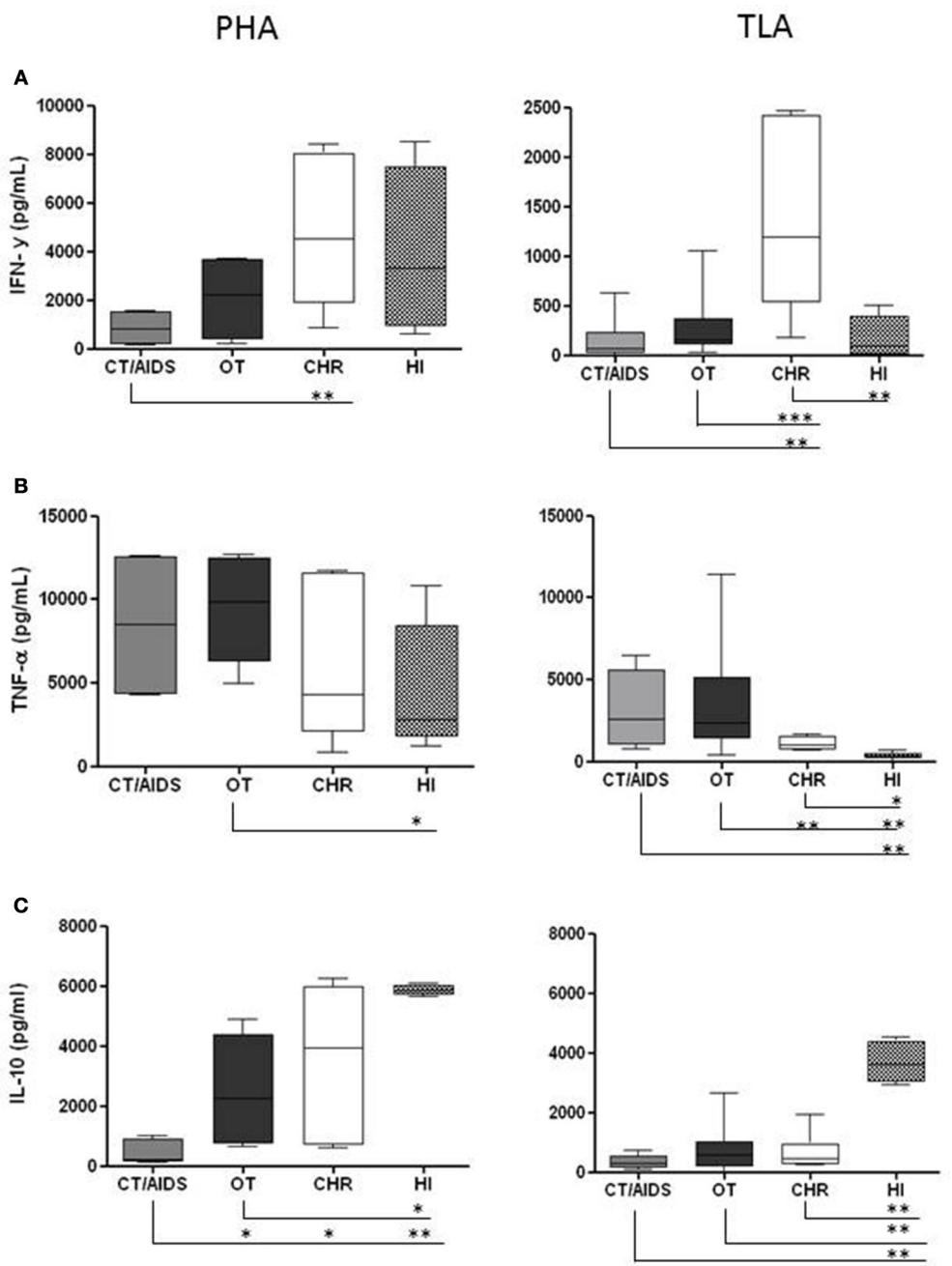

FIGURE 1 | Cytokine production from PBMC after PHA and TLA stimulation *in vitro*. PBMC (1×10^6/well) from cerebral toxoplasmosis/AIDS patients (gray columns), ocular toxoplasmosis (black columns), chronic (white columns), and healthy individuals (dotted columns) stimulated with 2 and 1 μg/mL of PHA and TLA, respectively. Supernatants were collected after 48 h for IFN-γ **(A)** and 24 h for TNF-α **(B)** and IL-10 **(C)**. The cytokine levels were determined by ELISA. The horizontal line indicates the median, bars the 25 and 75% percentiles, and vertical lines the 10 and 90% percentiles. Comparison of reactivity between groups by *Mann–Whitney* test (95% confidence interval) at $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

In addition, the balance between IFN-γ and TNF-α is the key mediators in triggering effector functions against *T. gondii* during both acute and chronic stages of infection (Denkers and Gazzinelli, 1998).

PBMC of OT patients also produced high TNF-α level after stimulus with TLA. These data are in agreement with other studies when suggested the susceptibility of OT patients with the production of TNF-α that contribute to the inflammatory response and damage of the choroid and retina (Wallace and Stanford, 2008; Talabani et al., 2010).

In AIDS patients, the TNF-α synthesis is not affected since monocytes are the major source this cytokine in response to

soluble *T. gondii* antigens (Sarciron and Guerardi, 2000). These findings are in agreement with our data, as we found high levels TNF-α in CT/AIDS patients suggesting a high inflammatory response.

IL-10 levels in response to TLA were almost similar in CT/AIDS and OT patients but low when compared with CHR individuals. Similar results have been shown by others (Vallochi et al., 2002; de-la-Torre et al., 2014) and support the idea that IL-10 seems to be central to the induction of the permissive state seen in the eyes of South American OT patients (de-la-Torre et al., 2014). These data, also, could be explained by the deviation to Th2 immune response including the production of anti-inflammatory cytokines, such as IL-10, TGF-β, and IL-4 favor the parasite's survival. This strategy is required to maintain immune equilibrium in the eye preventing the tissue immune destruction (Neyer et al., 1997; Gaddi and Yap, 2007). IL-10 production in *T. gondii*-infected brains may support the persistence of parasites as down-regulating the intracerebral immune response. The ratio of IFN-γ/IL-10 response in the CHR and HI groups is related to the immunoregulatory effect as a mixed Th1/Th2 profile since these individuals have no impairment of lymphocytes and IFN-γ, which is a key cytokine for the control of infection (Neyer et al., 1997; Kumar et al., 1998). Similar data have been shown in chronic infected mice (Costa-Silva et al., 2012a).

All these data together indicate that OT and CT/AIDS patients produced low IL-10 (Th2 response) and IFN-γ (Th1 response). In addition, they produced high TNF-α suggesting a high inflammatory response triggered by the parasite. IFN-γ levels found in these patients may be due a global decrease of CD4+ T cells in CT/AIDS and a possible central tolerance to parasite antigens in those patients with ocular toxoplasmosis. These particular characteristics in Brazilian patients may be due to the nature of the infecting South American strains that have shown more severe those from other regions (Ajzenberg et al., 2004; Ferreira et al., 2011) or to the genetic susceptibility of the host or by both combined events.

The monitoring of the immune response to antigens involved in the different clinical forms of infection can provide valuable information that can help in understanding the mechanisms of immune system control over parasites.

AUTHOR CONTRIBUTIONS

Vera Lucia Pereira-Chioccola and Cristina da Silva Meira designed the study and experiments; performed the data analysis, interpreted the data and wrote the manuscript. Cristina da Silva Meira, Thais Alves Costa-Silva, Ricardo Gava and Gabriela Motoie performed the laboratorial experiments (PCR, ELISA, Isolation of PBMC and cytokine assays). Cinara de Cássia Brandão de Mattos, Luiz Carlos de Mattos, José Ernesto Vidal and Gabriela Motoie revised critically the manuscript. Cinara de Cássia Brandão de Mattos and Luiz Carlos de Mattos interviewed the patients with ocular toxoplasmosis and collected the epidemiological data.

FAMERP Toxoplasma Research Group (Fábio Batista Frederico, Amanda Pires Barbosa, Plínio Pereira Martins Neto, Gildásio Castello Almeida Jr., and Mariana Previato) performed the inclusion of patients with ocular toxoplasmosis, sample collection, and develop the ophthalmological clinical evaluation and clinical analyses. *IIER Toxoplasma Research Group* (José Ernesto Vidal, Daniel Soares de Sousa Dantas, Tatiana Pimentel de Andrade Batista, Maria Jose Oliveira Kassab, Munir Bazzi, Daniel Paffili Prestes, Vanessa Levien Strelow, Adriana Weinfeld Massaia, Daniele Audi, Mariana Martins Lago, and Carlos Henrique Valente Moreira) performed the inclusion of patients with cerebral toxoplasmosis, sample collection, clinical diagnosis, acquisition data and follow-up of the patients.

All authors revised the manuscript, approved the final version submitted, published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Calomys callosus chronically infected by *Toxoplasma gondii* clonal type II strain and reinfected by Brazilian strains is not able to prevent vertical transmission

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Considering that *Toxoplasma gondii* has shown high genetic diversity in Brazil, the aim of this study was to determine whether *Calomys callosus* chronically infected by the ME-49 strain might be susceptible to reinfection by these Brazilian strains, including vertical transmission of the parasite. Survival curves were analyzed in non-pregnant females chronically infected with ME-49 and reinfected with the TgChBrUD1 or TgChBrUD2 strain, and vertical transmission was analyzed after reinfection of pregnant females with these same strains. On the 19th day of pregnancy (dop), placentas, uteri, fetuses, liver, spleen, and lung were processed for detection of the parasite. Blood samples were collected for humoral and cellular immune response analyses. All non-pregnant females survived after reinfection and no changes were observed in body weight and morbidity scores. In pregnant females, parasites were detected in the placentas of ME-49 chronically infected females and reinfected females, but were only detected in the fetuses of reinfected females. TgChBrUD2 reinfected females showed more impaired pregnancy outcomes, presenting higher numbers of animals with fetal loss and a higher resorption rate, in parallel with higher levels of pro-inflammatory cytokines and IgG2a subclass antibodies. Vertical transmission resulting from chronic infection of immunocompetent *C. callosus* is considered a rare event, being attributed instead to either reactivation or reinfection. That is, the pregnancy may be responsible for reactivation of the latent infection or the reinfection may promote *T. gondii* vertical transmission. Our results clearly demonstrate that, during pregnancy, protection against *T. gondii* can be breached after reinfection with parasites belonging to different genotypes, particularly when non-clonal strains are involved in this process and in this case the reinfection promoted vertical transmission of both type II and Brazilian *T. gondii* strains.

Keywords: ***Toxoplasma gondii*, congenital toxoplasmosis, parasite genotypes, Brazilian strains, reinfection**

INTRODUCTION

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan parasite belonging to the eukaryotic phylum Apicomplexa that can infect all warm-blooded animals (Sullivan and Jeffers, 2012). This parasite has a complex life cycle consisting of a sexual cycle in its feline definitive host and an asexual cycle in its intermediate host. Intermediate hosts, including humans, can be infected after the ingestion of raw or undercooked meat containing tissue cysts or after consumption of food or water contaminated with oocysts (Schlüter et al., 2014). *T. gondii* infects up to a third of the world's population and the infection is generally asymptomatic among humans and animals with normal immunity, but it can cause a high level of morbidity and mortality in immunocompromised individuals (Schlüter et al., 2014). Moreover, *T. gondii* can also be transmitted from the mother to the fetus, resulting in abortion or fetal abnormalities (Carlier et al., 2012; Adams Waldorf and McAdams, 2013).

In congenital toxoplasmosis, transmission to the fetus occurs predominantly in women who acquire primary infection during pregnancy (Remington et al., 2011; Carlier et al., 2012). In immunocompetent mothers who have been infected with *T. gondii* before conception, it has been preconized that immune mechanisms prevent transmission of the infection to their fetuses (Bojar and Szymanska, 2010). However, acquired immunity due to *T. gondii* infection does not fully protect against severe consequences to the child, caused either by reactivation of a latent infection in pregnant women with immunocompromised status or by reinfection, especially if the parasite strain is non-clonal

(Silveira et al., 2003; Elbez-Rubinstein et al., 2009). Both cellular and humoral components of the immune response play critical roles in resistance against *T. gondii* infection. Marked immunological modifications occur during pregnancy, which promote maternal tolerance to paternal alloantigens, leading to the successful implantation of the placenta and ensuring survival of the developing fetus. Increased hormone concentrations, i.e., progesterone, inhibit IL-12, TNF-α, and NO production by macrophages, increase IL-10 production by dendritic cells (DCs) and thereby dampen the development of strong Th1 cell responses. Consequently, pregnant women may be more susceptible to infection with *T. gondii* (Yarovinsky, 2014).

Toxoplasma gondii strains are genetically diverse. The genotype of the parasite has been implicated in disease severity (Elbez-Rubinstein et al., 2009; Wujcicka et al., 2014). *T. gondii* has a highly clonal genetic structure, with three major genetic types, I, II, and III, predominantly observed in North America and Europe (Howe and Sibley, 1995). On the other hand, an entirely distinct genotype pattern has been demonstrated in Central and South America, where an abundance of different strain types have been found (Pena et al., 2008; Khan et al., 2009; Su et al., 2012; Shwab et al., 2014). The type I strains, mostly found in South America, are highly virulent with a lethal dose 100 of ∼1 parasite (Khan et al., 2009), whereas type II and III strains are less virulent, with lethal doses 50 of $\sim 10^3$ and 10^5 parasites, respectively (Saeij et al., 2006). Also, in South America both congenital and ocular toxoplasmosis are more prevalent compared to Europe and more often associated with severe symptoms. It has been proposed that this could be associated with non-clonal strains (not type I, II, or III), mainly those isolated from South America (Gilbert et al., 2008).

More recently, analysis of isolates from domestic animals in Brazil revealed over a 100 restriction fragment length polymorphism (RFLP) genotypes, with four of these isolates being considered common clonal lineages, designated types BrI, BrII, BrIII, and BrIV (Pena et al., 2008; Dubey et al., 2012). Analysis of mortality rates in infected mice indicated that Type BrI is highly virulent, Type BrIII is non-virulent, and Type BrII lineages are intermediately virulent (Pena et al., 2008). Two parasite strains were recently obtained from chickens in Uberlândia city, Minas Gerais, Brazil and they were named TgChBrUD1 and TgChBrUD2. The TgChBrUD1 strain exhibited ToxoDB PCR–RFLP genotype #11 (also known as type BrII) and the TgChBrUD2 strain exhibited ToxoDB PCR–RFLP genotype #6 (also known as type BrI and Africa 1).

The placenta is the primary interface between the fetus and mother and plays an important role in maintaining fetal development and growth by facilitating the transfer of substrates and participating in modulating the maternal immune response to prevent immunological rejection of the conceptus. In addition, the placenta produces hormones that alter maternal physiology during pregnancy and forms a barrier against the maternal immune system (Watson and Cross, 2005). During pregnancy, the important protective role of the placenta against maternal–fetal *T. gondii* transmission has been reported (Robert-Gangneux et al., 2011). Although the gross architecture of the human and mouse placentas differ somewhat in their details, their overall structures and the molecular mechanisms underlying placental development are thought to be quite similar. As a result, the mouse is increasingly used as a model for studying the essential elements of placental development (Watson and Cross, 2005).

Calomys callosus (Rodentia, Cricetidae), a characteristic rodent in central Brazil, has been described as a useful experimental model to study congenital toxoplasmosis (Ferro et al., 2002; Barbosa et al., 2007; Franco et al., 2011). Congenital toxoplasmosis studies have shown that *C. callosus* is resistant to *T. gondii* strain ME-49 and vertical transmission occurs only during the acute phase of infection (Ferro et al., 2002; Barbosa et al., 2007). A previous study in this model showed that after 60 days of infection (doi) vertical transmission is not observed (Barbosa et al., 2007). In addition, our recent study showed that congenital toxoplasmosis does not occur in females chronically infected with the moderately virulent ME-49 clonal strain and reinfected with the highly virulent *T. gondii* RH clonal strain (Franco et al., 2011). Also, we observed that *C. callosus* is susceptible to infection by *T. gondii* TgChBrUD1 or TgChBrUD2 strains, since these animals died during the acute phase and weight loss and several clinical signs were observed after infection (Franco et al., 2014).

Considering that a primary *T. gondii* infection in *C. callosus* can provide protective immunity against reinfection with the highly virulent RH strain and that reinfection with non-clonal strains can promote vertical transmission, the present study aimed to verify if *C. callosus* chronically infected with the ME-49 strain may be susceptible to reinfection by the *T. gondii* TgChBrUD1 or TgChBrUD2 strains and if such reinfection may cause vertical transmission of the parasite. Moreover, we investigated the *T. gondii* genotype in placentas and fetuses to verify the effect of the reinfection on the reactivation of a latent infection and immune protection after reinfection.

MATERIALS AND METHODS

ANIMALS

Calomys callosus were kept under standard conditions on a 12-h light, 12-h dark cycle in a temperature-controlled room (25 ± 2°C) with food and water *ad libitum* in the Animal Experimentation Center, Federal University of Uberlândia, Brazil. Animal experiments and procedures were conducted according to local institutional guidelines for ethics in animal experimentation and approved by the Ethical Committee for Animal Experimentation (Protocol: CEUA/UFU 049/11).

PARASITE STRAINS

Cysts of the ME-49 strain were obtained from brains of *C. callosus* infected 30–45 days earlier with 20 cysts via the oral route as previously described (Barbosa et al., 2007). Briefly, brains were removed, homogenized, washed in sterile phosphate-buffered saline (PBS; pH 7.2) at 1000 × *g* for 10 min and cysts were counted under light microscopy for further experimental infection and strain maintenance. Tachyzoites of TgChBrUD1 and TgChBrUD2 strains were obtained initially from peritoneal exudates of previously infected Swiss mice and then maintained by serial passages in human fibroblast (HFF) cells. The cell culture-derived parasites were stained with 0.4% Trypan blue and counted in a hemocytometric chamber to determine the concentrations of

viable parasites, which were to be used in experimental infection protocols.

EXPERIMENTAL ANIMALS AND INFECTIONS

In the first set of experiments, *C. callosus* virgin females ($n = 15$), aged 2–3 months, were perorally infected with 20 cysts of *T. gondii* ME-49 strain. The females were randomly divided into three groups of five animals. After 60 doi, the reinfection was performed with an intraperitoneal inoculum of 100 tachyzoites of RH strain or TgChBrUD1 or TgChBrUD2 strains. Successful primary infection was determined by detection of specific antibodies in serum samples taken 5 days before reinfection. The females were monitored to evaluate body weight change, morbidity, and mortality for 25 days. Morbidity was assessed based on the clinical parameters as previously described (Bartley et al., 2006) with modifications: sleek/glossy coat, bright and active (score 0); hunched, starry stiff coat (score 1), reluctance to move (score 2).

In a second set of experiments, *C. callosus* virgin females ($n = 28$), aged 2–3 months, were randomly divided into four groups, as follows: Group 1 (Non-infected pregnant females), Group 2 (ME-49 chronically infected pregnant females), Group 3 (ME-49 chronically infected and TgChBrUD1 reinfected pregnant females), and Group 4 (ME-49 chronically infected and TgChBrUD2 reinfected pregnant females). Females of group 2, 3, and 4 were perorally infected with 20 cysts of ME-49 strain and after 60 doi, were mated with males and checked daily for the presence of a vaginal plug. The presence of a vaginal plug was considered as the first dop. The reinfection was carried out by an intraperitoneal inoculum of 100 tachyzoites of TgChBrUD1 or TgChBrUD2 strain for group 3 and 4, respectively at first dop. Blood samples were collected from all animals on the 55 doi before mating and reinfection for analysis of antibodies to *T. gondii*. The pregnant females were monitored to evaluate mortality and morbidity until the 19th dop, when animals were euthanized. The uteri were examined and the implantation sites were quantified. Normal and absorbed implantation sites were identified by visual observation. An implantation site with a shrunken placenta and a dissolved or discolored brown embryo was defined as an reabsorption site (Kusakabe et al., 2008). The fetal loss rate was calculated as the resorption sites by the total number of implantation sites (resorption plus normal implantation sites), as described previously (Joachim et al., 2001; Zenclussen et al., 2002). The uterus was collected only from reinfected females that presented resorption sites and no normal fetuses. Placentas, uteri and fetuses were collected for immunohistochemical, quantitative real-time PCR (qPCR) and RFLP–PCR assays. Placentas and fetuses were used for mouse bioassays. Different placentas and fetuses from the same female were used in these assays. In addition, liver, spleen and lung from pregnant females were collected for qPCR and RFLP–PCR assays. Blood samples were collected to determine the levels of IgG, IgG1, and IgG2a isotypes as well as the serum levels of Th1 and Th2 cytokines.

IMMUNOHISTOCHEMICAL ASSAYS

For immunolocalization of the parasites in the tissue samples, formalin-fixed samples were dehydrated and embedded in paraffin. Tissue sections measuring 4 μm in thickness were placed on glass slides and processed, as previously described (Ferro et al., 2002). Briefly, samples were first incubated with 5% acetic acid to block endogenous alkaline phosphatase and then with 2% normal goat serum to block non-specific binding sites. Next, samples were incubated at 4°C overnight with mouse anti-*T. gondii* polyclonal serum (1:100), which was produced by our laboratory by infecting Swiss mice with ME-49 strain, and then with biotinylated goat anti-mouse IgG (1:600) (Sigma-Aldrich, St. Louis, MO, USA). The reaction was amplified by avidin-biotin-alkaline phosphatase system (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, CA, USA) and developed with fast red-naphthol (Sigma). Samples were counterstained with Harris's hematoxylin and examined under light microscopy.

MOUSE BIOASSAY

Detection of *T. gondii* was evaluated by mouse bioassay as described elsewhere (Freyre et al., 2006; Barbosa et al., 2007). Placenta and fetal tissues (liver and brain) were homogenized in PBS and separately inoculated in Swiss mice by intraperitoneal route, in duplicate. Blood samples were collected at 35 days after inoculation and analyzed for seroconversion by ELISA and brains were collected for qPCR and RFLP–PCR assays.

DNA EXTRACTION AND QUANTIFICATION OF *T. gondii* BY QUANTITATIVE REAL-TIME PCR

Total DNA was extracted from 20 mg of each tissues using Wizard® Genomic DNA Purification Kit (Promega Co., Madison, WI, USA), according to the manufacturer's instructions. The number of parasites in tissues was determined by qPCR of extracted DNA using a TaqMan probe targeting the ITS1 sequence (GenBank Accession# AY143141). The primers for PCR amplification were ITS1-Fx: AGCGAAGGGGCTCAATTTCT and ITS1-Rx: TGAAATAACGGTGTGGGAAA, which amplified a 117 bp sequence. The ITS1 probe was 6-FAM/CGTGTCTCTGTTGGGATACTGATTTCCAGG/BHQ-1, with the 5′ end labeled with FAM and the 3′ end labeled with Black Hole Quencher-1 (BHQ-1; Eurofins Scientific, Longmont, CO, USA; Hill et al., 2012). The qPCR reaction had a total volume of 23 μl containing 17.74 μl of H_2O, 2.5 μl of 10 × PCR buffer + $MgCl_2$, 2.0 μl of 2.5 mM dNTP, 0.13 μl of 50 mM ITS1-Fx and ITS1-Rx primers, 0.2 μl of 50 mM ITS1 probe and 0.3 μl of 5 U μl Faststart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN, USA). Two microliters of purified DNA was added as template and PCR reaction mix was transferred to 96 wells plate. The reaction was carried out using a iQ™ 5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following conditions: 94°C for 60 s, then 45 cycles of 92°C for 15 s, 52°C for 30 s, and 72°C for 40 s. The threshold cycle (Ct) value for each sample was compared to the standard control (10^3 to 10^7 parasites/ml) and the relative parasite concentration was analyzed.

MULTILOCUS RFLP–PCR GENOTYPING OF *T. gondii*

The genotyping of strains was determined by RFLP–PCR as described previously (Su et al., 2010). Briefly, multiplex PCR was carried out using a set of mixed external primers in a single reaction. The pre-amplification step consisted of 95°C for

4 min, followed by 30-cycle PCR at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. A volume of 3 μl of the products served as template DNA for nested PCR with internal primers for each marker. The nested PCR amplification step consisted of 4 min at 95°C, followed by 35 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 90 s. The nested PCR products were digested using the appropriate restriction endonucleases (Su et al., 2010). The restriction fragments were resolved in 2.5% agarose gel and observed under ultraviolet light. Images were digitally photographed for the interpretation of genotyping data. A negative control, without DNA, was included in each reaction mixture and GT1 (type I), PTG (type II), CTG (type III), TgCgCa1, MAS, TgCatBr5, TgCatBr1, and TgCatBr2 strains were used as positive controls.

T. gondii-SOLUBLE ANTIGEN

Toxoplasma gondii-soluble tachyzoite antigen (STAg) was obtained as previously described (Mineo et al., 1980). Briefly, infected mouse peritoneal exudates (RH strain) were washed twice in PBS at 720 *g* for 10 min at 4°C. Parasite suspensions were adjusted to 1×10^8 tachyzoites/mL, treated with protease inhibitors and then lysed by five freeze thaw cycles and further by sonication (six 60-Hz cycles for 1 min each) on ice. After centrifugation (10,000 *g*, 30 min, 4°C), supernatants were collected and filtered through a 0.2-μm membrane (Corning Incorporated Costar, New York, NY, USA). The protein concentration was determined by using the Lowry method (Lowry et al., 1951) and STAg aliquots were stored at −80°C.

MEASUREMENT OF *T. gondii*-SPECIFIC TOTAL IgG, IgG1, AND IgG2a ISOTYPES

An indirect ELISA to detect serum IgG antibodies to *T. gondii* was carried out as previously described (Barbosa et al., 2007) in order to confirm preconceptional seroconversion of female *C. callosus*, as well as the seroconversion of Swiss mice inoculated in bioassay experiments as an indicator of *T. gondii* infection. In addition, IgG1 and IgG2a antibodies to *T. gondii* were carried out to evaluate the humoral immune response profile in pregnant reinfected *C. callosus* females. For detection of specific total IgG, low-binding polystyrene microtiter plates (Kartell SPA, Noviglio, Milan, Italy) were coated overnight at 4°C with *T. gondii* soluble antigen (10 μg/ml) in carbonate buffer 0.06 M (pH 9.6). After washing with PBS plus 0.05% Tween-20 (PBST), plates were incubated with serum samples (1:64) in 5% non-fat milk (PBS-TM) for 1 h at 37°C and subsequently with peroxidase-labeled goat anti-mouse IgG (1:1000, Sigma) in PBS-TM for 1 h at 37°C. For detection of specific IgG1 and IgG2a, high-binding polystyrene microtiter plates (Corning Incorporated Costar) were coated overnight at 4°C with *T. gondii* soluble antigen (10 μg/ml). Plates were blocked with 5% skim milk in PBS plus 0.05% Tween 20 (PBS-T) for 1 h. Serum samples were diluted 1:32 in 1% skim milk-PBS-T and incubated for 1 h at 37°C. IgG subclasses were detected with secondary goat anti-mouse IgG1 or anti-mouse IgG2a antibodies (1:1000, Sigma). After new washes, the reactions were developed with 0.03% hydrogen peroxide and 1 mg/ml o-phenylenediamine (OPD). The reaction was stopped with 2N H_2SO_4 and optical density (OD) was measured at 492 nm using a plate reader (Titertek Multiskan Plus, Flow Laboratories, Geneva, Switzerland). Results were expressed as ELISA index (EI) as follows: EI = OD sample/cut-off, where cut-off was established as mean OD values of negative control sera plus three standard deviations based on screening tests performed with negative and positive control sera. EI > 1.2 values were considered positive results.

IFN-γ, TNF-α, IL-10, AND TGF-β MEASUREMENTS IN SERUM SAMPLES

The concentrations of cytokines were measured by sandwich ELISA. The IL-10 and TNF-α (OpTEIA, BD Bioscience, San Diego, CA, USA) and IFN-γ and TGF-β (Duoset R&D Systems, Minneapolis, MN, USA) cytokines were assayed according to instructions from the manufacturers. The concentrations of cytokines in serum samples from pregnant reinfected *C. callosus* females were calculated from a standard curve of each murine recombinant cytokine. The limit of detection in the ELISAs was 31.3 pg/ml (IFN-γ), 15.6 pg/ml (TNF-α), 31.3 pg/ml (IL-10), and 15.6 pg/ml (TGF-β). Intra-assay and inter-assay coefficients of variation were below 20% and 10%, respectively.

STATISTICAL ANALYSIS

The data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± S.D. of experimental groups. The Kaplan–Meier method was applied to estimate the percentage of *C. callosus* females surviving (survival rate) after reinfection and survival curves were compared using the Log-rank test. Comparisons of the differences among cytokines and parasitism among various experimental groups were performed by Mann–Whitney or Kruskal–Wallis tests with Dunn multiple comparison post-test. The antibody production comparison before and after reinfection, for each groups, was analyzed by the Mann–Whitney, whereas comparisons between groups were analyzed by Kruskal–Wallis. Differences were considered statistically significant when $P < 0.05$.

RESULTS

FEMALES CHRONICALLY INFECTED WITH ME-49 ARE ABLE TO SURVIVE AFTER REINFECTION WITH HIGHLY INFECTIVE PARASITE STRAINS

The infection outcomes in females chronically infected with ME-49 and reinfected with the TgChBrUD1 or TgChBrUD2 strains were investigated and the RH strain was used as control. It was observed that all females chronically infected with ME-49 survived after reinfection with RH, TgChBrUD1 or TgChBrUD2. In addition, no significant difference in body weight changes and morbidity scores was detected (data not shown).

REINFECTION WITH BRAZILIAN STRAINS IS HARMFUL TO PREGNANCY AND REINFECTION WITH TgChBrUD2 RESULTED IN MORE FREQUENT IMPAIRED PREGNANCY OUTCOMES COMPARED WITH TgChBrUD1

The pregnancy in *C. callosus* females chronically infected with ME-49 was investigated after reinfection with *T. gondii* Brazilian strains on the first day of gestation. It was observed that all females from groups 1, 2, and 3 and five out of seven females from group 4 survived until the 19th dop (**Table 1**; **Figure 1A**). Comparison between groups showed survival rates significantly lower in group

Table 1 | Survival and pregnancy outcome for non-infected *C. callosus* females, females chronically infected with ME-49 and females chronically infected with ME-49 and reinfected in the first day of pregnancy by TgChBrUD1 (UD1) or TgChBrUD2 (UD2) strains of *Toxoplasma gondii*.

Group	Strain	Female survival *n*/*N* (%)	Females with normal fetuses *n*/*N*	Females with reabsorbed fetuses *n*/*N* (%)	Reabsorption sites/ implantation[a] *n*/*N* (%)[b]
1	Non-infected	7/7 (100)	7/7	3/7 (42.8)	3/28 (10.7)
2	ME-49	7/7 (100)	7/7	3/7 (42.8)	3/30 (10)
3	ME-49 + UD1	7/7 (100)	5/7	4/7 (57.1)	11/34 (32.3)
4	ME-49 + UD2	5/7 (71.2)	1/5	4/5 (80)	19/23 (82.6)

[a] *Normal and reabsorbed implantation sites were identified by visual observations. An implantation site with a shrunk placenta and a dissolved or discolored brown embryo was defined as a reabsorption site. The n/N represent the total amount of reabsorption sites/implantation for all females for each group.*

[b] *The fetal loss rate was calculated as the reabsorption sites to the total number of implantation sites (reabsorption plus normal implantation sites).*

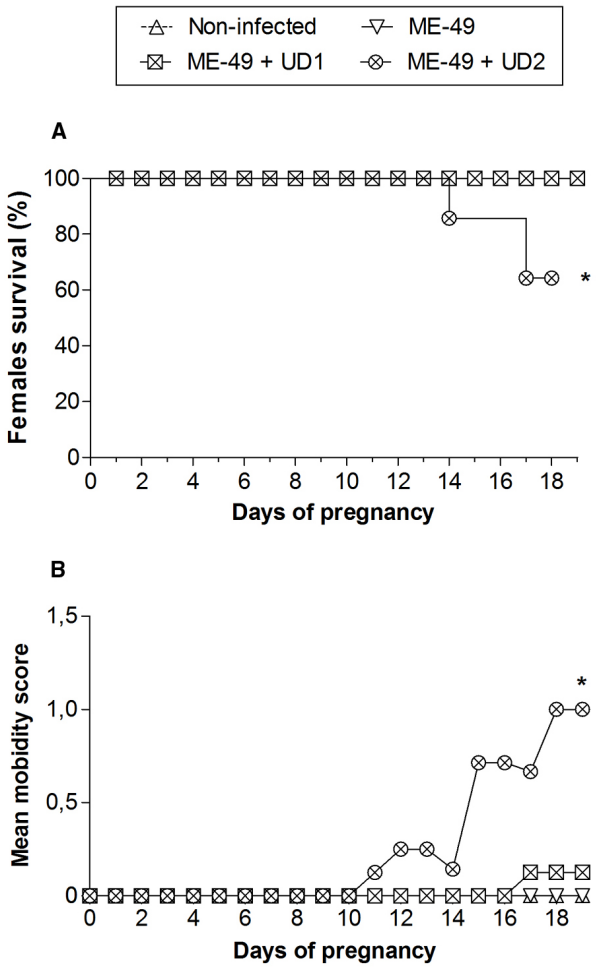

FIGURE 1 | (A) Survival curves and **(B)** morbidity of non-infected *C. callosus* females, females chronically infected with ME-49 and females chronically infected with ME-49 and reinfected in the first day of pregnancy by TgChBrUD1 (UD1) or TgChBrUD2 (UD2) strains of *Toxoplasma gondii*. Females (n = 7 for each group) were monitored until the 19th day of pregnancy. *P < 0.05. **(A)** Log-rank testand. **(B)** Wilcoxon signed rank test.

4 (**Figure 1A**). Females from groups 1, 2, and 3 showed no change in morbidity scores (**Figure 1B**). On the other hand, females from group 4 started to be hunched with starry stiff coats around the 11th dop and demonstrated reluctance to move on the 15th day, showing higher morbidity scores ($P < 0.05$; **Figure 1B**).

On the 19th dop, all females in group 1 presented normal fetuses and three out of seven females presented reabsorbed fetuses (42.8%). A total of 28 implantation sites with three reabsorption sites were observed, showing fetal loss of 10.7% (**Table 1**). In group 2, all females presented normal fetuses and three out of seven females presented reabsorbed fetuses (42.8%). A total of thirty implantation sites with three reabsorption sites were observed, showing fetal loss of 10% (**Table 1**). In group 3, five out of seven females presented normal fetuses and four females presented reabsorbed fetuses (57.1%). Considering the four females who had reabsorbed fetuses, two of them showed only the implantation sites with signs of necrosis. A total of 34 implantation sites with eleven reabsorption sites were observed, showing fetal loss of 32.3%. In group 4, only one out of five females presented normal fetuses and four females presented reabsorbed fetuses (80%). Considering the four females who had reabsorbed fetuses, all of them showed the implantation sites with signs of necrosis. A total of 23 implantation sites with 19 reabsorption sites were observed, showing fetal loss of 82.6% (**Table 1**).

Tissue parasitism was investigated by qPCR and immunohistochemistry in the uterus. All uteri from groups 3 and four presented parasites (**Table 2**) and TgChBrUD2 reinfected pregnant females had significantly higher uterine parasite loads in comparison to TgChBrUD1 reinfected pregnant females (**Figure 2A**). Immunohistochemical assays confirmed the presence of *T. gondii* in uterus (**Figures 2B,C**). The genotyping of parasites in two uteri from group 3 showed that one uterus presented the ME-49 strain and another one presented the TgChBrUD1 strain (**Table 3**). In group 4, the four uteri presented TgChBrUD2 strain parasites (**Table 3**).

THE ACQUIRED IMMUNE RESPONSE OF *Calomys callosus* FEMALES CHRONICALLY INFECTED WITH THE *T. gondii* ME-49 CLONAL STRAIN IS INSUFFICIENT TO PREVENT VERTICAL TRANSMISSION FOLLOWING REINFECTION WITH STRAINS FROM BRAZIL

Vertical transmission was evaluated by immunohistochemical assays, mouse bioassay and PCR. It was observed that females chronically infected with ME-49 did not transmit *T. gondii* vertically, while females chronically infected with ME-49 and reinfected with Brazilian strains were able to transmit vertically. Immunohistochemical results showed parasites in placenta

Table 2 | Results of PCR assay for *C. callosus* females chronically infected with ME-49 and females chronically infected with ME-49 and reinfected in the first day of pregnancy by TgChBrUD1 (UD1) or TgChBrUD2 (UD2) strains of *Toxoplasma gondii*. PCR was also carried out in brain tissues from mouse in the bioassay.

		Pregnant females				
Group	**Strains**	**Positive PCR result in tissues from pregnant animals *n*/*N***			**Positive PCR result in brain (mouse bioassay) *n*/*N***	
		Placentas	**Fetuses**	**Uterus**	**Placentas**	**Fetuses**
1	–	NI	NI	NI	NI	NI
2	ME-49	4/7	0/7	NI	NI	NI
3	ME-49 + UD1	5/5	1/5	2/2	3/5 (2†)	1/5 (1†)
4	ME-49 + UD2	1/1	0/1	4/4	0/1 (1†)	1/1

†*Animals died between 7 and 10 days after inoculum.*

NI, not investigated.

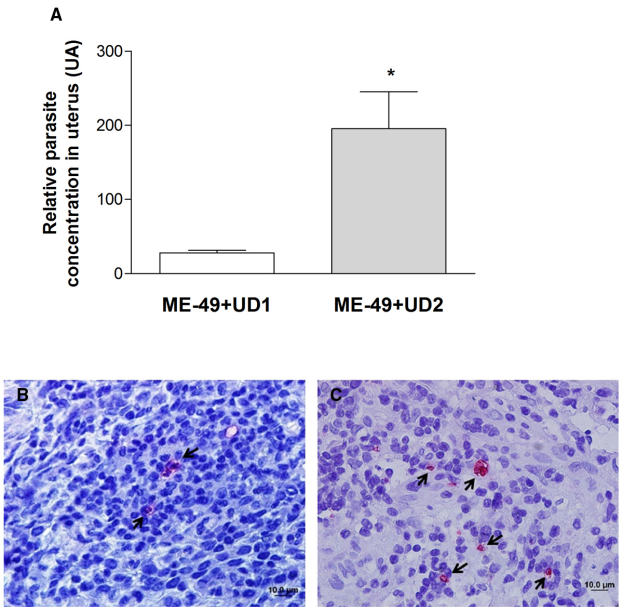

FIGURE 2 | (A) Comparative parasite burden in the uterus tissue from TgChBrUD1 (UD1; n = 2) and TgChBrUD2 (UD2) reinfected pregnant females (n = 4). *P < 0.05 (Mann–Whitney test). **(B)** Representative photomicrographs of uterus tissue from TgChBrUD1 and **(C)** TgChBrUD2 reinfected pregnant female. Arrows indicate parasites inside the parasitophorous vacuoles.

and fetus tissues from group 3 and placenta from group 4 (**Figures 3A–C**).

The ELISA to detect IgG antibodies was carried out in serum from survival Swiss mice after 30 days after infection. *T. gondii*-specific IgG antibodies were detected in mice that were inoculated with placentas and fetuses from group 3 (**Figure 3D**). *T. gondii*-specific IgG antibodies not were detected in mice that were inoculated with fetuses from group 4 (**Figure 3D**).

The qPCR showed that *T. gondii*-DNA was detected in placentas from group 2 ($n = 4$), group 3 ($n = 5$), and group 4 ($n = 1$; **Table 2**). *T. gondii*-DNA was detected in fetus tissues from group 3 ($n = 1$). On the other hand, we did not observe *T. gondii*-DNA in fetal tissues from groups 2 and 4 (**Table 2**). The qPCR from brains of mice used in mouse bioassays showed that *T. gondii*-DNA was detected in mice inoculated with placenta tissues from three females of group 3. The mice inoculated with placenta tissues from the other two females of group 3 and from one female of group 4 died between 7 and 10 days after inoculation (**Table 2**). In addition, *T. gondii*-DNA was detected in mice inoculated with fetal tissues from groups 3 and 4 (**Table 2**). The mice inoculated with fetal tissues from one female of group 3 died between 7 and 10 days after inoculation (**Table 2**).

Tissue parasitism was investigated and it was observed that ME-49 chronically infected females showed low parasite concentration in the placenta. On the other hand, all placentas from TgChBrUD1 and TgChBrUD2 reinfected females showed high parasite concentration, but with no significant difference (**Figure 4A**). When fetal tissues were analyzed, fetuses from TgChBrUD1 reinfected females showed low parasite concentration, while no parasite was detected for any fetuses from ME-49 chronically infected females and fetuses from TgChBrUD2 reinfected females (**Figure 4A**).

The genotyping of *T. gondii* was analyzed in qPCR positive samples. The ME-49 strain was observed in all placentas from group 2 (**Table 3**). In group 3, three females presented the ME-49 strain in the placenta, while two females and one female presented the TgChBrUD1 strain in the placenta and fetuses, respectively (**Table 3**). Group 4 presented the TgChBrUD2 strain in placentas (**Table 3**). In the brains from mice bioassays, the ME-49 strain was observed in placenta and fetus samples from TgChBrUD1 reinfected females (**Table 3**). The TgChBrUD2 strain was observed in fetus samples from TgChBrUD2 reinfected females (**Table 3**).

TgChBrUD2 REINFECTED FEMALES PRESENTED HIGHER PARASITE BURDEN IN THE LIVER, SPLEEN AND LUNG

The qPCR results showed that TgChBrUD2 reinfected females had significantly higher parasite concentrations in liver, spleen and lung in comparison to ME-49 chronically infected females (**Figures 4B–D**) and significantly higher parasite concentration in spleen when compared with TgChBrUD1 reinfected females (**Figure 4C**). Comparison between organs in different groups of animals showed no significant difference between the ME-49 chronically infected females and the TgChBrUD1 and TgChBrUD2 reinfected female groups (data not shown). The geno-

Table 3 | Genotyping of *Toxoplasma gondii* in tissues from placenta, fetus, uterus, liver, spleen, and lung from *C. callosus* females chronically infected with ME-49 and females chronically infected with ME-49 and reinfected in the first day of pregnancy by TgChBrUD1 (UD1) or TgChBrUD2 (UD2) strains of *Toxoplasma gondii*, as well as in the brain tissue from mouse bioassay. Data represent numbers of the samples and strain detected in each tissue.

		Genotyping							
Group	**Strain**	**Placenta**	**Fetus**	**Uterus**	**Liver**	**Spleen**	**Lung**	**Brains from mice bioassay**	
								Placenta	**Fetus**
1	–								
2	ME-49	(4) ME-49	x	x	(5) ME-49	(3) ME-49	(4) ME-49	NI	NI
3	ME-49 + UD1	(3) ME-49 (2) UD1	(1) UD1	(1) ME-49 (1) UD1	(4) UD1 (1) ME-49 and UD1	(1) ME-49 (4) UD1	(2) ME-49 (3) UD1	(2) ME-49	(1) ME-49
4	ME-49 + UD2	(1) UD2	x	(4) UD2	(5) UD2	(4) UD2	(4) UD2 (1) ME-49 and UD2	x	(1) UD2

x, absence of sample; NI, not investigated.

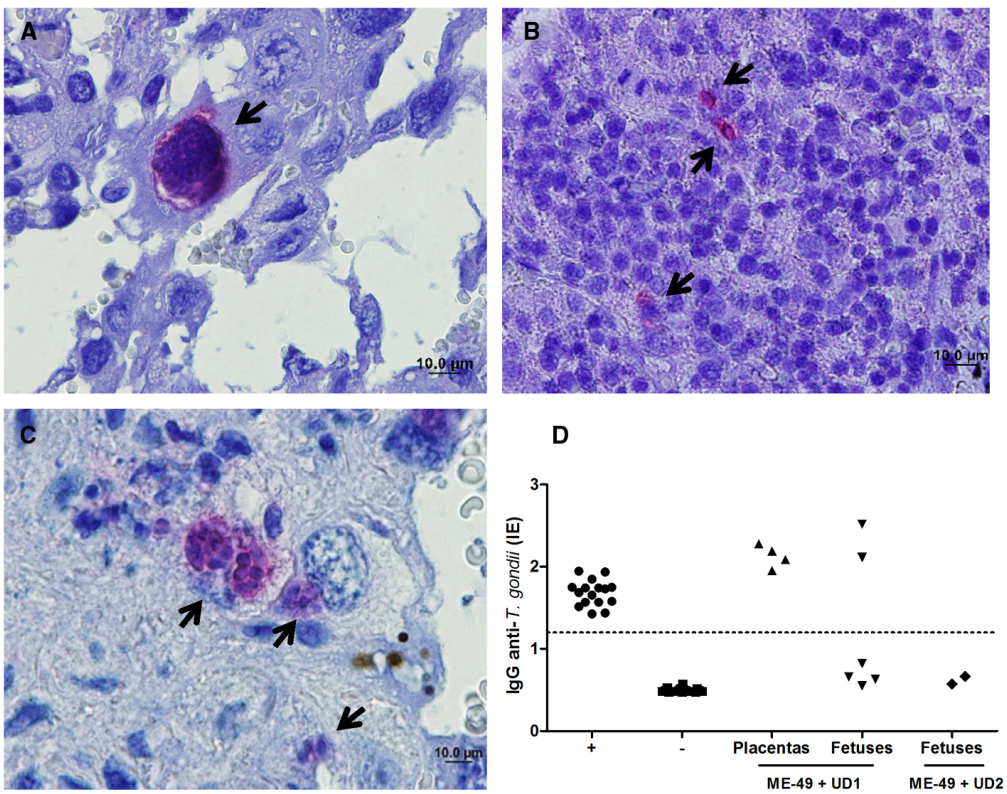

FIGURE 3 | Representative photomicrographs of (A) placenta and (B) fetus from TgChBrUD1 reinfected pregnant female and (C) placenta from TgChBrUD2 reinfected pregnant female. Arrows indicate parasites inside the parasitophorous vacuoles. **(D)** IgG analysis of survival Swiss mice inoculated with placentas (n = 4) and fetuses (n = 6) tissues (liver and brain) from TgChBrUD1 (UD1) reinfected female or fetuses (n = 2) from TgChBrUD2 (UD2) reinfected female. Antibody levels were measured by ELISA and expressed as an ELISA index (EI).

typing of parasites in group 2 showed the ME-49 strain in liver ($n = 5$), spleen ($n = 3$), and lung ($n = 4$) (**Table 3**). In group 3, four females presented the TgChBrUD1 strain in the liver, while one female presented a mixed infection. One female presented the ME-49 strain and four females presented the TgChBrUD1 strain in the spleen. When the lung was analyzed, two females presented the ME-49 strain and three females presented the TgChBrUD1 strain (**Table 3**). Group 4 presented the TgChBrUD2 strain in liver ($n = 5$) and spleen ($n = 4$). Four females presented the TgChBrUD2 strain in the lung, while one female presented a mixed infection (**Table 3**).

REINFECTED PREGNANT FEMALES SHOWED ELEVATED LEVELS OF IFN-γ, TNF-α, AND IL-10

To determine whether the infection with *T. gondii* may change the balance of Th1/Th2 type reactivity in reinfected females, the

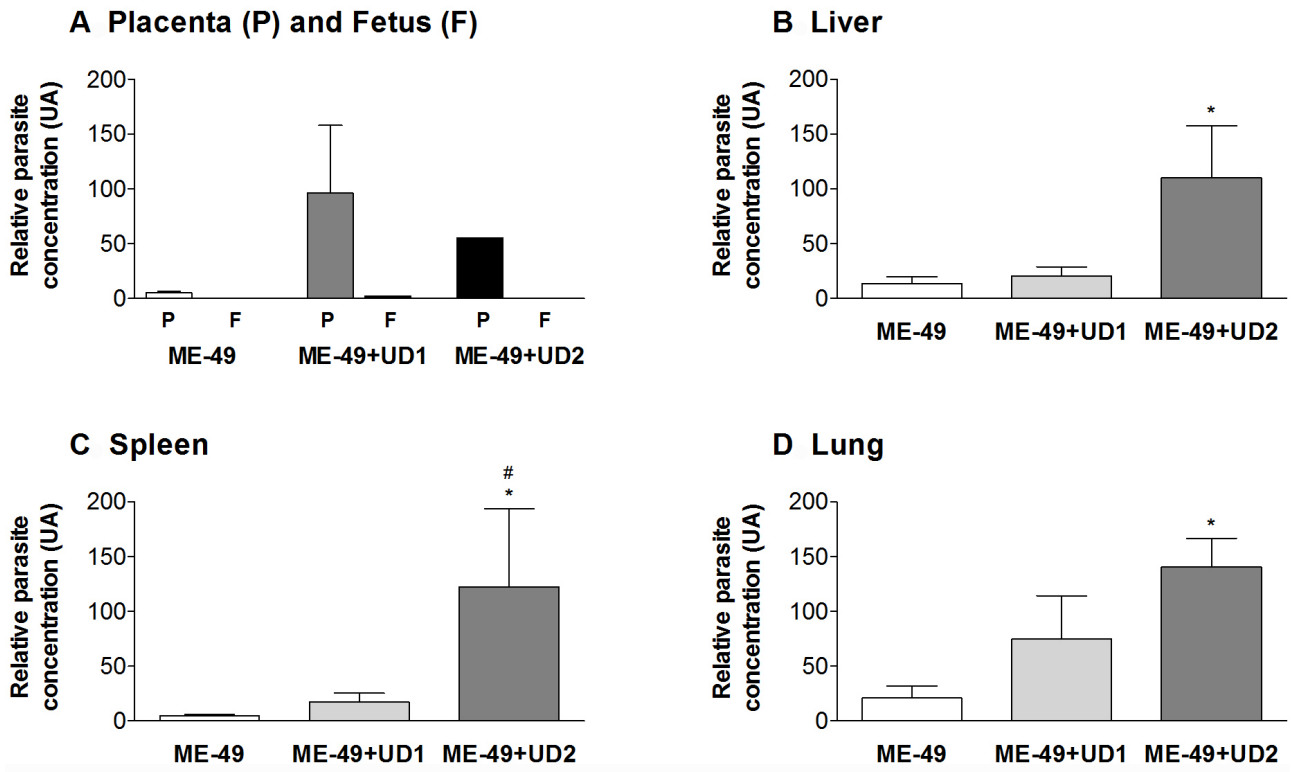

FIGURE 4 | Comparative parasite burden in the (A) placenta (P) and fetus (F), (B) liver, (C) spleen, and (D) lung tissues from ME-49 chronically infected pregnant females, TgChBrUD1 (UD1) and TgChBrUD2 (UD2) reinfected pregnant females (n = 7 for each group). *Comparison between ME-49 chronically infected pregnant females and TgChBrUD1 or TgChBrUD2 reinfected pregnant females (Kruskal–Wallis test, $P < 0.05$). #Comparison between TgChBrUD1 and TgChBrUD2 reinfected pregnant females (Mann–Whitney test, $P < 0.05$).

levels of IFN-γ, TNF-α, IL-10, and TGF-β1 in serum samples from groups were measured. The IFN-γ and TNF-α levels were higher in TgChBrUD1 and TgChBrUD2 reinfected females compared with non-infected and ME-49-chronically infected females ($P < 0.05$; **Figures 5A,B**). The IL-10 levels were higher in TgChBrUD1 and TgChBrUD2 reinfected females compared with non-infected and were higher in TgChBrUD1 reinfected females compared with ME-49-chronically infected females ($P < 0.05$; **Figure 5C**). No significant differences were found in IFN-γ, TNF-α, and IL-10 levels between non-infected and ME-49-chronically infected females or between TgChBrUD1 and TgChBrUD2 reinfected females (**Figures 5A–C**). When analyzing TGF-β1 in serum samples, low levels of the cytokine were observed in ME-49-chronically infected females, TgChBrUD1 and TgChBrUD2 reinfected females compared with non-infected females ($P < 0.05$; **Figure 5D**).

ME-49 CHRONICALLY INFECTED PREGNANT FEMALES AND TgChBrUD1 REINFECTED PREGNANT FEMALES DEVELOPED HIGH IgG2a/IgG1 RATIOS

In order to additionally verify whether reinfection interferes with the Th1 response induced by *T. gondii*, the specific IgG, IgG1, and IgG2a levels were measured by ELISA in *C. callosus* serum samples. Seroconversion was confirmed before pregnancy, on day 55 of infection with ME-49 strain (**Figure 6A**). The comparison between IgG levels before pregnancy and after pregnancy showed no significant differences in non-infected females and ME-49 chronically infected females, while TgChBrUD1 and TgChBrUD2 reinfected females showed higher IgG levels after pregnancy (**Figure 6A**). In addition, no significant differences were found in IgG levels in the serum from ME-49 chronically infected females compared with TgChBrUD1 or TgChBrUD2 reinfected females before or after pregnancy. Also, no significant differences were found when TgChBrUD1 and TgChBrUD2 reinfected females were compared (**Figure 6A**). The analysis of IgG1 and IgG2a showed low levels for all groups (data not shown). The ratio of IgG2a/IgG1 was lower in ME-49 chronically infected females and TgChBrUD1 reinfected females after pregnancy (**Figure 6B**). The comparison between IgG2a/IgG1 ratio before pregnancy and after pregnancy showed no significant differences in ME-49 chronically infected females and TgChBrUD2 reinfected females, while TgChBrUD1 reinfected females showed lower IgG2a/IgG1 ratios after pregnancy (**Figure 6B**).

DISCUSSION

Congenital toxoplasmosis resulting from chronically infected immunocompetent pregnant women is considered a rare event, being attributed to either reactivation or reinfection (Silveira et al., 2003; Elbez-Rubinstein et al., 2009; Remington et al., 2011).

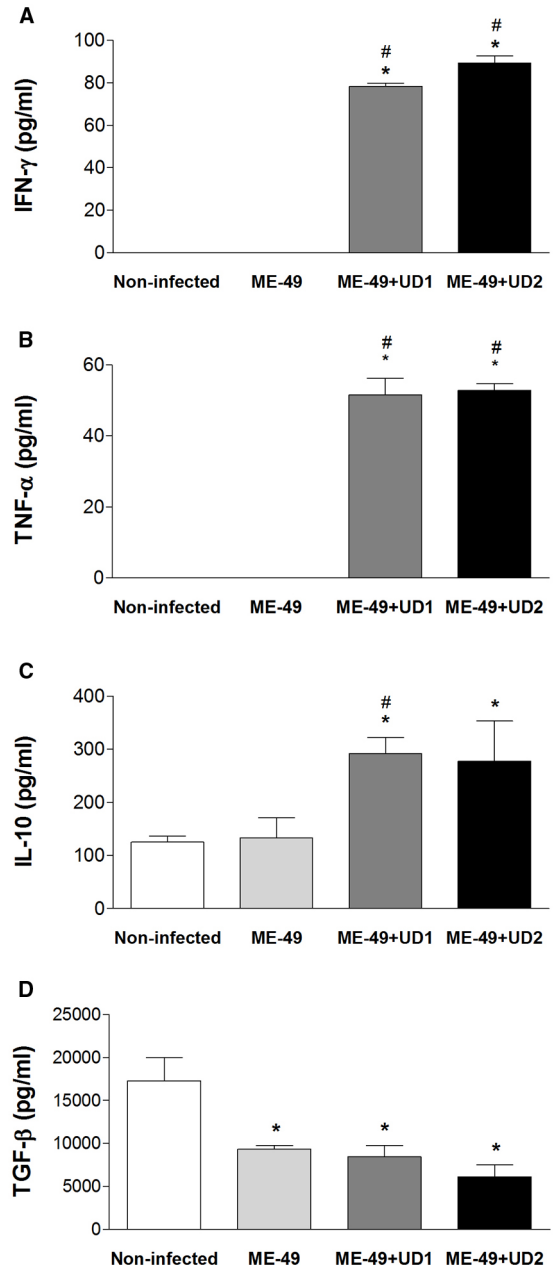

FIGURE 5 | Production of IFN-γ (A), TNF-α (B), IL-10 (C), and TGF-β (D) in serum samples from non-infected *C. callosus* females, females chronically infected with ME-49 and females chronically infected with ME-49 and reinfected in the first day of pregnancy by TgChBrUD1 (UD1) or TgChBrUD2 (UD2) strains of *Toxoplasma gondii* (n = 7 for each group). *Comparison between non-infected with ME-49 chronically infected pregnant females, TgChBrUD1 and TgChBrUD2 reinfected pregnant females (Kruskal–Wallis test $P < 0.05$). #Comparison between ME-49 chronically infected pregnant females with TgChBrUD1 and TgChBrUD2 reinfected pregnant females (Kruskal–Wallis test $P < 0.05$).

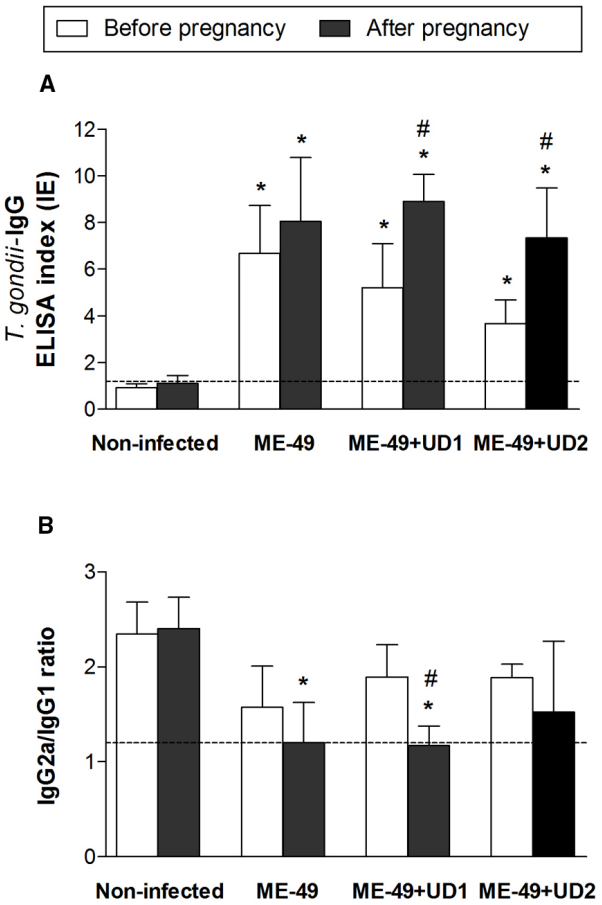

FIGURE 6 | Levels of *Toxoplasma gondii*-specific total IgG (A) and IgG2a/IgG1 ratio (B) in serum samples from TgChBrUD1 (UD1) and TgChBrUD2 (UD2) reinfected pregnant females (n = 7 for each group) compared with non-infected and ME-49 chronically infected females before and after pregnancy. Antibody levels were measured by ELISA and expressed as an ELISA index. *Comparison between non-infected or ME-49 chronically infected females with TgChBrUD1 and TgChBrUD2 reinfected females before and after pregnancy (Kruskal-Wallis test $P < 0.05$). #Comparison between TgChBrUD1 and TgChBrUD2 reinfected pregnant females before and after pregnancy (Kruskal-Wallis test $P < 0.05$).

Reinfection cases may be associated with exposure to a large number of parasites, to a more virulent strain or to a parasite of a different genotype (Elbez-Rubinstein et al., 2009; Valdes et al., 2011; Carlier et al., 2012), since the tachyzoites of virulent strains have a greater capacity for invading cells, being able to cross biological barriers and multiplying in particular intracellular compartments (Carlier et al., 2012).

Congenital transmission of *T. gondii* generally occurs only during the acute phase of infection in *C. callosus* (Ferro et al., 2002; Barbosa et al., 2007), similar to what is observed in pregnant women. Our previous studies showed that congenital toxoplasmosis does not occur in females chronically infected with the *T. gondii* ME-49 strain and reinfected with the same strain or reinfected with a virulent *T. gondii* RH strain (Ferro et al., 2002; Franco et al., 2011). In this study, our data demonstrated that *C. callosus* females chronically infected with the ME-49 strain survived after reinfection with the *T. gondii* TgChBrUD1 or TgChBrUD2 strains. Also, it is possible to observe vertical transmission of the parasite, when this host is reinfected with these Brazilian strains.

Previous studies from our group have shown that *C. callosus* is resistant to ME-49 and highly susceptible to infection by RH (Favoreto-Junior et al., 1998). Our recent study showed that *C. callosus* males and females are also highly susceptible to infection by TgChBrUD1 or TgChBrUD2, with mortality after 9 doi (Franco et al., 2014). Recently, a study using mice Swiss females showed that the prime-infection with the ME-49 strain conferred protection against reinfection with the virulent strains CH3 and EGS (Silva et al., 2012). In the present study, we observed that *C. callosus* females chronically infected with ME-49 survived for more than 21 days after TgChBrUD1 or TgChBrUD2 reinfection. Thus, this evidence suggests that primary infection in *C. callosus* elicits an acquired immune response capable of protecting immunocompetent animals against the virulent strain used in the reinfection.

The immunity acquired after primary infection by *T. gondii* has been considered to be efficient in preventing vertical transmission during reinfection (Abou-Bacar et al., 2004; Pfaff et al., 2007). However, cases of congenital toxoplasmosis have been reported in infants born to immunocompetent mothers who had been infected with the parasite before conception, suggesting the occurrence of maternal reinfection during pregnancy (Elbez-Rubinstein et al., 2009). Thus, the hypothesis that the primary *T. gondii* infection leads to life-long immunity and prevents vertical transmission during reinfection has been questioned by several authors using immunocompetent murine models (Ferro et al., 2002; Freyre et al., 2006; Pezerico et al., 2009; Franco et al., 2011). Some studies showed that complete protection against congenital toxoplasmosis is possible when strains belonging to the same genotype (Ferro et al., 2002) or clonal strains (Pezerico et al., 2009; Franco et al., 2011) are used for infection and reinfection. On the other hand, reinfection of chronically infected rats with heterologous and clonal *T. gondii* strains showed a considerable amount of parasites in fetal tissues (Freyre et al., 2006).

Recently, natural mixed infections, resulting from coincident or sequential exposure to parasites of different genotypes, have been observed in humans (Andrade et al., 2010; Carneiro et al., 2013), although it is still unclear whether the protection triggered by the primary infection is genotype-specific. In the present study, we observed vertical transmission of *T. gondii* in *C. callosus* females chronically infected with ME-49 and reinfected with TgChBrUD1 or TgChBrUD2. In addition, our data confirm that vertical transmission of *T. gondii* does not occur in *C. callosus* females chronically infected with ME-49 (Barbosa et al., 2007) despite infection of placental tissues, indicating reactivation of latent infection during gestation and demonstrating the role of the placental barrier in preventing vertical transmission.

Our data showed that when groups of reinfected animals were compared, TgChBrUD2 reinfected females were more susceptible during pregnancy. This group presented lower survival and the reinfection induced a higher morbidity score, showing that when pregnant, females are susceptible to reinfection by Brazilian strains. Moreover, TgChBrUD2 reinfected females presented a high number of pregnant animals with fetal reabsorption and a high fetal loss rate. When the parasitism was analyzed, TgChBrUD2 reinfected females showed higher parasite loads in uterus tissues compared to TgChBrUD1 reinfected females.

Congenital toxoplasmosis may occur due to either an exogenous (reinfection) or an endogenous origin (reactivation; Abou-Bacar et al., 2004; Pfaff et al., 2007). Besides evaluating the *T. gondii* DNA in tissues from reinfected females, we also analyzed the participation of the *T. gondii* genotype in the process of reinfection. The genotyping showed the ME-49 or TgChBrUD1 strains in uterus tissues from TgChBrUD1 reinfected females but only the TgChBrUD2 strain in uterus tissues from TgChBrUD2 reinfected females. When placentas and fetuses were analyzed, it was observed that TgChBrUD1 reinfected females presented parasites in both locations. Also, the only pregnant female reinfected with TgChBrUD2 presented parasites in both placenta and fetal tissue as well. Infection was confirmed by PCR of brain tissue from mouse bioassays. Our results showed ME-49 or TgChBrUD1 in placental tissues and TgChBrUD1 strain in fetal tissues from TgChBrUD1 reinfected females, while only TgChBrUD2 was found in placentas from TgChBrUD2 reinfected females. Therefore, reinfection with these Brazilian strains promoted vertical transmission of the *T. gondii* ME-49, TgChBrUD1 or TgChBrUD2 strains in *C. callosus* females.

The genotyping results for TgChBrUD2 reinfected females showed only one type of strain in tissue samples from reinfected females, though this does not exclude the possibility of the presence of ME-49, since a low DNA concentration can be undetectable by RFLP–PCR. It is also necessary to address divergent results observed in tissues from the same females in different assays. This may be explained by the fact that we used different fetuses from the same mother to evaluate vertical transmission in each of the assays (immunohistochemistry, mice bioassay, and PCR assay). For example, the fetuses from one female used in bioassays were not infected and, for this reason, the Swiss mice remained seronegative and survived until 30 days after infection. On the other hand, fetuses from different females of the same group were infected and were responsible for the death of Swiss mice around 7 days after infection or responsible for the PCR positive results in brains of these animals. We found the Swiss mice dead around 7 days after infection, suggesting that these animals died because of *T. gondii* acute infection. Therefore, it is important to take into account that these different results found in different approaches could also be explained by biological differences in terms of site of implantations in the uterus, resulting in particular microenvironments facilitating or not facilitating the infection by *T. gondii*.

The TgChBrUD1 and TgChBrUD2 strains belong to the non-clonal genotype profile (type BrII and type BrI, respectively) predominant in Brazil, while the ME-49 strain belongs to the clonal type II genotype, frequently found in Europe and in North America (Pena et al., 2008). These stark differences are likely to be due to the predominance of more virulent genotypes of the parasite in Brazil, which are rarely found in Europe. Thus, our results corroborate the authors' hypothesis that the immunity acquired against European strains may not protect against reinfection by strains of a different genotype (Freyre et al., 2006; Elbez-Rubinstein et al., 2009).

Because these strains are drawing on genes that are outside the domestic gene pool that contributes to types I, II, and III, the severity of infection could be explained, in part, by a poor adaptation of the immune system against these genotypes of *T. gondii*. Host control of *Toxoplasma* induces potent Th1-type immune responses with the production of the pro-inflammatory cytokine IL-12, which is produced by macrophages and DCs in response to Toll-like receptor (TLR) recognition of molecular structures broadly conserved across microbial species (Yarovinsky, 2014). IL-12 in turn activates NK and T cells to secrete IFN-γ, which plays a major role in restricting proliferation of tachyzoites during the acute stage of infection (Abou-Bacar et al., 2004; Melo et al., 2011; Suzuki et al., 2011). The latter activates effector mechanisms for intracellular elimination of *Toxoplasma*, including the activation of interferon-regulated GTPases, induction of reactive nitrogen intermediates, tryptophan degradation and autophagy in human cells (Melo et al., 2011; Gazzinelli et al., 2014).

Acute *T. gondii* infection during gestation may disrupt the maternal-fetal immunological balance in favor of anti-parasitic pro-inflammatory abortogenic cytokines, such as IFN-γ and TNF-α, which are reported to be potentially deleterious for conception (Shiono et al., 2007). In this study, *C. callosus* females chronically infected with ME-49 and reinfected with TgChBrUD1 or TgChBrUD2 showed high levels of IFN-γ, TNF-α, and IL-10 compared with ME-49 chronically infected females. On the other hand, low levels of TGF-β were observed in ME-49 chronically infected females and TgChBrUD1 and TgChBrUD2 reinfected females compared with non-infected females. These data suggest that the elevated inflammatory immune response induced by *T. gondii* could be involved in the higher number of reabsorbed fetuses and absorbed implantation sites observed in females reinfected with Brazilian strains. Moreover, even the strong pro-inflammatory immune response induced by the parasite was not sufficient to control the infection, being unable to prevent the vertical transmission and the high parasite concentration observed in placenta, liver, spleen and lung tissue of reinfected females.

Accordingly, a previous study showed high levels of IFN-γ when C57BL/6 mice were infected with RH strain in an early stage of pregnancy and this phenomenon was related to high reabsorption rates of implantation sites (Ge et al., 2008). The high levels of IFN-γ produced suggested that the Th1 type cellular immune response was mainly activated. On the other hand, an increase in IL-10 was also observed, suggesting that anti-inflammatory responses were activated.

Both cellular and humoral components of the immune system play a role in resistance against secondary infection. Reinfection is accompanied by an intense immune response, often manifested by the elevation of IgG levels and the appearance of IgM antibodies (Gavinet et al., 1997). In the present study, higher IgG levels were observed in serum from TgChBrUD1 and TgChBrUD2 reinfected pregnant females after pregnancy compared with IgG levels before pregnancy, confirming the ability of *T. gondii* to induce a humoral immune response after reinfection. The ME-49 chronically infected females and TgChBrUD1 reinfected females presented lower IgG2a/IgG1 ratio in pregnancy compared with IgG2a/IgG1 ratio before pregnancy, suggesting the establishment of a Th2 type immune response in pregnancy and reinfection. The Th2 pattern is characterized by a preferential production of complement-independent IgG1 antibodies, whereas Th1 responses are characterized by production of complement-dependent IgG2a antibodies. Consistent with these isotype profiles, antigen-specific $CD4^+$ Th2 type T helper cells produce IL-4 that supports switching to IgG1, while Th1 type T helper cells are characterized by the generation of IFN-γ that supports switching to IgG2a (Mosmann et al., 1997). Thus, our data indicate that the immune response developed during gestation and reinfection in TgChBrUD1 reinfected females could be associated with higher pro-inflammatory cytokines and IgG1 subtype profiles showing a predominance of mixed Th1/Th2 responses. On the other hand, TgChBrUD2 reinfected females presented higher pro-inflammatory cytokines and IgG2a subtype profiles, suggesting predominance of the Th1 response.

In conclusion, our results showed that *C. callosus* females chronically infected by a *T. gondii* classical type II clonal strain survive after reinfection with Brazilian strains, but the acquired immune response of this host is insufficient to prevent congenital toxoplasmosis. The pregnancy promoted *T. gondii* ME-49 strain reactivation and the reinfection caused vertical transmission of *T. gondii*. Also, pregnant females that have been reinfected by Brazilian strains developed strong pro-inflammatory immune responses including Th1 cytokines and antibody isotype, leading to damage for the developing fetuses.

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Knowledge and practice on *Toxoplasma* infection in pregnant women from Malaysia, Philippines, and Thailand

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Toxoplasma gondii, is one of the infectious agents of congenital TORCH infections, causes severe clinical outcomes in fetus and newborns. Nevertheless this life-threatening parasitic disease is preventable by simple preventive measures related to lifestyle during pregnancy. We aim to study on the knowledge about toxoplasmosis and practices that prevents this infection among the pregnant women. Total of 2598 pregnant women from Malaysia, Philippines, and Thailand were randomly surveyed to determine the knowledge and their practices on *Toxoplasma* infection. The questionnaire covered respondents' general information and knowledge on plausible risks factors, symptoms, timing of infection, prevention knowledge, and preventive behavior regarding *Toxoplasma* infection. Majority of these pregnant women were in their age group of 20–29 years (50.9%), completed secondary level of education (51.7%), in their second trimester of pregnancies (38.1%), non-parous (36.6%), and had no history of abortion (90.4%). Based on this survey, only 11% of these pregnant women had read, heard, or seen information regarding toxoplasmosis and 3.5% of them were aware of being tested for the infection. A small percentage of these pregnant women knew that *T. gondii* were shed in the feces of infected cats (19.4%) and sometimes found in the raw or undercooked meat (11.0%). There was 16.1% of responding women knew that toxoplasmosis is caused by an infection. Demographic profiles such as age group, level of education, pregnancy term, and number of children of the pregnant women showed significant association with their responses toward prevention knowledge and preventive behavior related questions ($P < 0.05$). Thus, it is suggested that health education on toxoplasmosis and primary behavioral practices should be consistently offered to reproductive age women in general and pregnant women in particular. This information could help to reduce vertical transmission of *Toxoplasma* infection during pregnancy.

Keywords: toxoplasmosis, knowledge, practice, pregnant women, Malaysia, Philippines, Thailand

INTRODUCTION

Toxoplasma gondii, an obligate intracellular coccidian protozoan, is the causative agent of Toxoplasmosis. *Toxoplasma* have three main forms in its entire life cycle, being; oocysts, tachyzoites, and bradyzoites. The definitive host of *Toxoplasma* is the felines, where oocysts are produced in the intestines and passed in feces. The oocyst is infective to humans and other intermediate host (mammals and other warm blooded animals). Once infected, *Toxoplasma* develops itself into tachyzoites (rapid multiplication of *Toxoplasma* in trophozoite form) or maintain dormant in bradyzoites (tissue cysts), depending on the host immune status (John and Petri, 2006). The infection in pregnant women may cause devastating effects in the fetus. If the infected tissue of an animal being consumed, it acts as a transmission mode of infection. Furthermore, this parasitic infection can be transmitted by ingestion of *Toxoplasma* oocysts contaminated fruits and vegetables or unclean water, through blood transfusion, and by receiving organ transplant (Pereira et al., 2010). Exposures to contaminated feces by cleaning cat's litter, gardening, or handling contaminated soils are some of the ways of this parasitic infection being transmitted.

Most countries in South America, Middle Eastern and other low-income countries reported high seropositive for *Toxoplasma* infection from both normal or immunocompromised host, e.g., 59% in Brazil (Ferezin et al., 2013), 84.7% in Congo (Doudou et al., 2014), 3.98% in China (Hua et al., 2013), 83.6% in Ethiopia

(Zemene et al., 2012), 75% in India (Chintapalli and Padmaja, 2013), 10.3% in Japan (Sakikawa et al., 2012), 11.8% in Taiwan (Chou et al., 2011) and 30.9% in Tanzania (Mwambe et al., 2013). Meanwhile, the recent seroprevalence rate of *Toxoplasma* infection in pregnant women was reported as 49% in Malaysia (Nissapatorn et al., 2003), 23.8% in the Philippines (Salibay et al., 2008) and 28.3% in Thailand (Nissapatorn et al., 2011).

Toxoplasma infection in immunocompetent persons is usually asymptomatic (Halonen and Weiss, 2013) however; infection in the immunodeficient patients and in fetus through their pregnant mother may lead up to severe and often fatal toxoplasmosis. The clinical signs in infected fetus will be intracranial calcifications, hydrocephalus, eye infection, seizures, miscarriage, or death (Paquet and Yudin, 2013). The annual incidence of congenital toxoplasmosis was estimated at 190,100 cases globally (Torgerson and Mastroiacovo, 2013). The clinical presentation was not or rarely shown in infected pregnant women, even during acute infection. Some of the pregnant women may present symptoms like malaise, low-grade fever and lymphadenopathy (Montoya and Remington, 2008). Anti-parasitic antibiotic therapy is the currently available treatment for *Toxoplasma* infected pregnant women (Paquet and Yudin, 2013). The pregnant women were given spiramycin antibiotic if the infection occurs during the first 18 weeks of gestation, meanwhile pyrimethamine, sulfadiazine and folic acid were given to pregnant women who acquired this parasitic infection after 18 weeks of gestation and onwards (Montoya and Remington, 2008). In the meantime, developments of vaccines are still being studied (Verma and Khanna, 2013).

As toxoplasmosis being one of the TORCH [acronym for a group of five infectious disease namely; Toxoplasmosis, Others (Hepatitis B), Rubella (German measles), Cytomegalovirus (CMV), Herpes Simplex Virus (HSV)] infectious agents in pregnant women, knowledge and practice regarding this disease, and preventive measures in lifestyle for this parasitic infection should be given to the pregnant women. To the best of our knowledge, questionnaire base study on toxoplasmosis has never been conducted in pregnant women from this part of the world. Therefore, this survey aimed to evaluate the level of knowledge and practices on toxoplasmosis among pregnant women who visiting the antenatal clinics (ANC) or hospitals from their respective countries namely Malaysia, Philippines, and Thailand. Southeast Asia is a region where its people share their similarities in term of geographical location, tradition, and culture in their ways of life. This study would definitely provide the new insight on toxoplasmosis in pregnant women.

MATERIALS AND METHODS

This survey was carried out from January, 2012 to June, 2013 among 3 neighboring countries namely Malaysia, Philippines, and Thailand in Southeast Asia. A total of 2598 being 756 pregnant women visiting ANC at the Department of Obstetrics and Gynecology, University of Malaya Medical Centre, Kuala Lumpur, Malaysia, 1063 pregnant women visiting ANC at the general hospitals and in private clinics and lying-in/maternity hospitals in Luzon and Mindanao, the Philippines and 779 pregnant women visiting ANC at the Songklanagarind and Hatyai hospital, Songkhla province and Pattani hospital, Pattini province, Thailand were recruited (**Figure 1**). Inclusion criteria are pregnant women in any gestational periods, in any age groups and given verbal consent to participate in this study.

Demographic information and questions related to general knowledge about toxoplasmosis, risks factors, symptoms, and timing of infection, prevention knowledge, and preventive behavior was adopted from a previous study (Jones et al., 2003) with some modifications. Prior to answering questionnaires, these pregnant women were briefed about toxoplasmosis in term of the definition, risk factors, clinical features, preventive measures by medical practitioners and professional nurses. All data collected were entered and analyzed using SPSS version 17 (IL, Chicago, USA). Data with qualitative variables were expressed as frequency and percentage. Statistical analysis was performed using chi-square test as appropriate. A P-value of <0.05 was regarded as statistically significant.

RESULTS

A total of 2598 pregnant women participated in this survey with 756, 1063, and 779 from Malaysia, Philippines and Thailand, respectively. Majority of Malaysian pregnant women were in age group of 20–29 years, married, graduates, in their third trimester, expecting their first born, and had no history of abortion. Filipino pregnant women were mostly in age group of 20–29 years, married, were in secondary school leavers, in their second trimester, having = two children, and had no history of abortion. Meanwhile, Thai pregnant women were mostly in age group of 20–29 years, married, had tertiary level of education, in second trimester, have no children earlier, and had no history of abortion as shown in **Table 1**.

In general, only 11% of these pregnant women had read, heard, or seen any information regarding toxoplasmosis and 3.5% of them were aware of being tested for the infection. A small percentage of these pregnant women knew that *Toxoplasma* were shed in the feces of infected cats (19.4%) and sometimes found in the raw or undercooked meat (11.0%). There was 16.1% of responding women knew that toxoplasmosis is caused by an infection, but 4.9% thought that it is caused by poison. Moreover, there was a low level of knowledge (<10%) about other risk factors such as consumption of undercooked meat, drinking untreated water and receiving blood transfusion. A high percentage of pregnant women indicated they do not eat undercooked meat and that they practice good hygienic measures such as washing their hands after handling raw meat, gardening, or changing cat litter as shown in **Table 2**.

Table 3 shows the association between demographic profiles of pregnant women from each country with their preventive knowledge and preventive behavior on toxoplasmosis (Raw data can be found in Tables 3A,F from Supplement 1). Malaysian pregnant women in the age group of >40 years significantly avoiding stray cats; who had tertiary education significant association with their responses in feeding their cat with dry or commercial food, avoiding stray cats, let someone else changing cat litter box daily, wash cooking utensils after each use, washing hands after gardening, changing cat litter and handling raw meat while who educated from primary education significantly reported to wash their hand

FIGURE 1 | Study sites in Malaysia, Philippines, and Thailand.

before eating and eat raw meat. Pregnant women who are in their first trimester significantly associated with avoiding stray cat and routinely wash hand before eating food while who are in their third trimester significantly practice in cooking meat well. Meanwhile, in Filipino pregnant women, only who graduate from primary level of education showed significant association with avoiding stray cats and who having ≥ two children significantly washing hands after handling raw meat. Thai pregnant women who in age group of 30–39 years indicated their significant association with prevention knowledge of washing fruits/vegetables before eating and clean cooking utensil after each use; age group <20 significantly associated with wash hand after changing cat litter, handling raw meat and before eating food; age group 20–29 years significantly eating raw meat. Married Thai pregnant women significantly associated with cooking meat well, washing fruits/vegetables before eating, cleaning cooking utensil after each use and washing hand before eating food while single pregnant women significantly eat raw meat. Thai pregnant women who were graduated from tertiary education or higher significantly associated with all questions ask when compare to other age group, including eating raw meat. Thai pregnant women who expecting their first child significantly associated with washing hand after changing cat litter, handling raw meat and before eating food. Only Thai pregnant women who had no history of abortion significantly associated with changed cat litter box daily.

DISCUSSION

The prevalence of *Toxoplasma* infection varies among the three regions studied, namely 49% in Malaysia (Nissapatorn et al., 2003), 23.8% in the Philippines (Salibay et al., 2008), and 28.3% in Thailand (Nissapatorn et al., 2011). However, the knowledge about toxoplasmosis and its related preventive knowledge and behaviors may reduce the infection rate and its disease burden in pregnant women.

In the respondents' answers on the general knowledge of toxoplasmosis, majority of these pregnant women have no knowledge or unsure about this parasitic infection. This finding indicates the importance of educating the pregnant women with the preventive measures which have been highlighted in previous studies (Elsheikha, 2008; Costa et al., 2012; Amin et al., 2013). Malaysian and Thai pregnant women appear to have better precaution or preventive practice toward *Toxoplasma* infection when compared to their Filipino counterparts. This could be due to Malaysian and Thai pregnant women have higher education (≥tertiary level of education) which confirms their knowledge about toxoplasmosis.

Most of our subjects were not sure about the risk factors, symptoms, and timing of toxoplasmosis. Nevertheless, they routinely practice primary preventive behaviors, particularly in good hygienic condition against *Toxoplasma* infection. This evidence is supported by most of our pregnant women (>83%) wash their hands after gardening, changing cat litter and handling raw

Table 1 | Demographic profiles of the pregnant women.

Factors	Sub-factor	Total (%)	Total (%)		
			Malaysia	Philippines	Thailand
Age group	<20	123 (4.7)	5 (0.66)	57 (5.36)	61 (7.83)
	20–29	1323 (50.9)	**375 (49.60)**	**558 (52.49)**	**390 (50.06)**
	30–39	1025 (39.5)	353 (46.69)	385 (36.22)	287 (36.84)
	≥40	127 (4.9)	23 (3.04)	63 (5.93)	41 (5.26)
Marital status	Single	173 (6.7)	3 (0.40)	73 (6.87)	97 (12.45)
	Married	2425 (93.3)	**753 (99.60)**	**990 (93.13)**	**682 (87.55)**
Education	≤Primary	169 (6.5)	18 (2.38)	45 (4.23)	106 (13.61)
	Secondary	1342 (51.7)	268 (35.45)	**770 (72.44)**	304 (39.02)
	≥Tertiary	1087 (41.8)	**470 (62.17)**	248 (23.33)	**369 (47.37)**
Trimester	1st	690 (26.6)	151 (19.97)	368 (34.62	171 (21.95)
	2nd	990 (38.1)	235 (31.08)	**388 (36.50)**	**367 (47.11)**
	3rd	918 (35.3)	**370 (48.94)**	307 (28.88)	241 (30.94)
No. of children	None	952 (36.6)	**319 (42.20)**	294 (27.66)	**339 (43.52)**
	1	768 (29.6)	245 (32.41)	234 (22.01)	289 (37.10)
	≥2	878 (33.8)	192 (25.40)	**535 (50.33)**	151 (19.38)
History of abortion	Yes	250 (9.6)	148 (19.58)	8 (0.75)	94 (12.07)
	No	2348 (90.4)	**608 (80.42)**	**1055 (99.25)**	**685 (87.93)**
Total		2598	756	1063	779

The bold values indicate the highest number of pregnant women for each category from Malaysia, Philippines and Thailand, respectively.

meat. *Toxoplasma gondii* poses a public health problem for both infection rate and disease burden that have been reported in pregnant women from different parts of the world. This parasite has been found in any given environment conditions that contaminating with cat's feces. Approximately 1.2 million metric tons of cat feces were being deposited in the environment annually with the oocysts burden measured in a community survey was 3 to 434 oocysts per square foot in USA (Torrey and Yolken, 2013) and there have been reports showing that having a close contact with cats and cleaning their litter may transmit the disease to pregnant women (Fakhfakh et al., 2013). Exposure to infected cats by these women could lead to severe outcomes to her carried fetus. Many studies reported *Toxoplasma* infection in animals for human consumption (Bangoura et al., 2013; Hill and Dubey, 2013; Kang et al., 2013; Lopes et al., 2013), 12.2% of pregnant women who consume raw meat especially Malaysian who were graduate from primary school and Thai's who were in age group 20–29 , single, graduate from tertiary education and in their first semester of pregnancy could be at risk for *Toxoplasma* infection.

Further analysis revealed that there were some significant association found between the demographic profiles and the responses from these pregnant women regarding their prevention knowledge and preventive behavior. Many of these pregnant women (>30%) know that by avoiding stray cats, allowing someone else to change the cat's litter box, making sure the cat's litter box is changed daily, cleaning cooking utensil after each use are preventive measures of toxoplasmosis. Most of them (>80%) routinely wash their hand after gardening, changing cat litter and after handling raw meat indicated their constantly practice basic personal hygiene during their pregnancy. Looking at pregnant women in the Philippines, it was found no significant association between demographic profiles and their knowledge on toxoplasmosis, except for their level of education and the number of children. Overall, level of education, marital status, trimester of pregnancy and the number of children play an important role in preventing *Toxoplasma* infection found in these pregnant women. History of abortion did not have any significant association except in Thai pregnant women who were changing cat's litter box daily.

This study highlights the level of knowledge and practice on awareness of *Toxoplasma* infection among the pregnant women from three Southeast Asian countries: Malaysia, Philippines, and Thailand. The findings of this study provide vital information in better understanding about the knowledge and practice of toxoplasmosis among pregnant women in this region. Therefore, it emphasizes the need for implementation of health education among this target group to further educate them on the preventive and control measures. We strongly believe that with adequate knowledge and awareness through health education on toxoplasmosis will remarkably eliminate the infection rate and subsequently eradicate its disease burden in these countries. The obtained data also coincide with the actual situation that the incidence of primary acute toxoplasmosis is very low and also clinical evidence of congenital toxoplasmosis is rarely

Table 2 | Pregnant women responses for knowledge and practice on Toxoplasmosis by country.

		Malaysia (*n*, %) (*N* = 756)			Philippines (*n*, %) (*N* = 1063)			Thailand (*n*, %) (*N* = 779)			Total (*n*, %) (*N* = 2598)		
		Yes	**No**	**Not sure**	**Yes**	**No**	**Not sure**	**Yes**	**No**	**Not sure**	**Yes**	**No**	**Not sure**
General information knowledge	K1	161	519	76	41	964	58	83	340	356	**285 (10.9)**	1823 (70.2)	490 (18.9)
	K2	14	706	36	49	979	35	27	396	356	**90 (3.5)**	2081 (80.1)	427 (16.4)
	K3	206	195	355	176	759	128	36	144	599	**418 (16.1)**	1098 (42.3)	1082 (41.6)
	K4	37	289	430	76	825	162	15	140	624	**128 (4.9)**	1254 (48.3)	1216 (46.8)
	K5	256	108	392	186	713	164	62	91	626	**504 (19.4)**	912 (35.1)	1182 (45.5)
	K6	74	182	500	156	682	225	57	84	638	**287 (11.0)**	948 (36.5)	1363 (52.5)
Risk factors	R1	367	81	308	104	584	375	65	46	668	**536 (20.6)**	711 (27.4)	1351 (52.0)
	R2	95	166	495	87	658	318	63	53	663	**245 (9.4)**	877 (33.8)	1476 (56.8)
	R3	59	210	487	42	654	367	23	91	665	**124 (4.8)**	955 (36.8)	1519 (58.5)
	R4	115	188	453	41	668	354	25	80	674	**181 (6.9)**	936 (36.0)	1481 (57.0)
	R5	182	156	418	38	667	358	24	78	677	**244 (9.4)**	901 (34.7)	1453 (55.9)
Symptoms and timing of infection	S1	339	69	348	121	651	291	43	79	657	**503 (19.4)**	799 (30.8)	1296 (49.8)
	S2	323	50	383	97	672	294	60	57	662	**480 (18.5)**	779 (29.9)	1339 (51.5)
	S3	168	58	530	36	589	438	48	53	678	**252 (9.7)**	700 (26.9)	1646 (63.4)
	S4	83	68	605	8	683	372	37	54	688	**128 (4.9)**	805 (30.9)	1665 (64.1)
	S5	75	192	489	1	618	444	27	74	678	**103 (4.0)**	884 (34.0)	1611 (62.0)
	S6	143	113	500	32	649	382	54	53	672	**229 (8.8)**	815 (31.4)	1554 (59.8)
	S7	53	132	571	0	621	442	26	75	678	**79 (3.0)**	828 (31.9)	1691 (65.0)
	S8	96	72	588	52	648	363	28	58	693	**176 (6.7)**	778 (29.9)	1644 (63.3)
	S9	104	62	590	84	620	359	25	65	689	**213 (8.2)**	747 (28.8)	1638 (63.0)
	S10	148	59	549	212	548	303	39	51	689	**399 (15.4)**	658 (25.3)	1541 (59.3)
Prevention knowledge	P1	281	96	379	179	585	299	264	109	406	**724 (27.9)**	790 (30.4)	1084 (41.7)
	P2	454	63	239	192	592	279	319	86	374	**965 (37.1)**	741 (28.5)	892 (34.3)
	P3	458	72	226	174	611	278	242	111	426	**874 (33.6)**	794 (30.6)	930 (35.8)
	P4	448	48	260	201	579	283	246	96	437	**895 (34.4)**	723 (27.8)	980 (37.7)
	P5	285	77	394	194	550	319	236	54	489	**715 (27.5)**	681 (26.2)	1202 (46.3)
	P6	323	85	348	170	569	324	240	51	488	**733 (28.2)**	705 (27.1)	1160 (44.6)
	P7	359	70	327	230	561	272	219	56	504	**808 (31.1)**	687 (26.4)	1103 (42.5)
Preventive behavior	B1	685	25	46	882	163	18	645	51	83	**2212 (85.1)**	239 (9.2)	147 (5.7)
	B2	638	38	80	584	412	67	574	71	134	**1796 (85.1)**	521 (20.0)	281 (10.8)
	B3	681	31	44	892	136	35	586	62	131	**2159 (83.1)**	229 (8.8)	210 (8.0)
	B4	32	0	724	1024	25	14	562	76	141	**1618 (62.3)**	101 (3.9)	879 (33.8)
	B5	68	645	43	155	899	9	94	535	150	**317 (12.2)**	2079 (80.0)	202 (7.8)

K1 to K6 are the knowledge on general information of toxoplasmosis. K1: Have you ever read, heard, or seen any information about toxoplasmosis. K2: Have you ever been tested for toxoplasmosis? K3: Is toxoplasmosis caused by an infection? K4: Is toxoplasmosis caused by a poison? K5: Is toxoplasmosis (T.gondii) shed in the feces of infected cats? K6: Is toxoplasmosis (T.gondii) sometimes found in raw or undercooked meat? R1 to R5 are the knowledge on risk factors of toxoplasmosis. R1: Can people get toxoplasmosis by changing cat litter? R2: Can people get toxoplasmosis by eating undercooked meat? R3: Can people get toxoplasmosis by receiving blood transfusion? R4: Can people get toxoplasmosis by drinking untreated water, e.g., rain, tap, or unboiled? R5: Can people get toxoplasmosis by gardening without gloves? S1 to S10 are the knowledge on symptoms and timing of infection of toxoplasmosis. S1: Can pregnant women develop serious complications after infection with toxoplasmosis (T.gondii)? S2: Can unborn and/or newborn children develop serious complications after infection with toxoplasmosis (T.gondii)? S3: Can toxoplasmosis in a pregnant women cause fever and feeling like you have the flu? S4: Can toxoplasmosis in a pregnant women cause swollen glands (lymph node)? S5: Can toxoplasmosis in a pregnant women cause no symptoms? S6: Toxoplasmosis (T.gondii) can only be passed from a pregnant woman to her fetus if she is newly infected during that pregnancy. S7: Toxoplasmosis (T.gondii) is rarely passed from a pregnant woman to her fetus if she is newly infected during that pregnancy. S8: A baby with toxoplasmosis may have no signs of illness at birth, but develop illness later. S9: A baby with toxoplasmosis may have vision problems. S10: A baby with toxoplasmosis may be treated with medicine. P1 to P7 are the preventive knowledge on ways to avoid toxoplasmosis. P1: Feeding your cat dry or commercial cat food and not letting it kill and eat rodents. P2: Avoiding stray cats. P3: Letting someone else change the cat's litter box. P4: Making sure the cat's litter box is changed daily. P5: Toxoplasmosis can be prevented by cooking meat well until no pink is seen and the juices run clear. P6: Toxoplasmosis can be prevented by thoroughly washing and/or peeling all fruits and vegetables before eating them. P7: Toxoplasmosis can be prevented by cleaning all cutting boards and utensils thoroughly after each use. B1 to B5 denotes the preventive behaviors of the pregnant women since becoming pregnant. B1: Do you routinely wash your hands after gardening? B2: Do you routinely wash your hands after changing cat litter? B3: Do you routinely wash your hands after handling raw meat? B4: Do you routinely wash your hands before eating food? B5: Do you eat raw meat?

The bold values indicate the total number of pregnant women who answered "Yes" for each questions in the questionnaire.

Table 3 | Association between demographic profiles of pregnant women from each country with their preventive knowledge and preventive behavior on Toxoplasmosis.

	Factors	P1	P2	P3	P4	P5	P6	P7	B1	B2	B3	B4	B5
Malaysia	Age group		*										
	Marital status												
	Education	*	**	**	**			*	*	*		**	
	Trimester		*			**						*	
	No. of children											*	
	History of abortion												
Philippines	Age group												
	Marital status												
	Education		*										
	Trimester												
	No. of children										**		
	History of abortion												
Thailand	Age group						*	*		*	**	*	*
	Marital status					*	**	**				**	**
	Education	*	**	**	**	**	**	*	**	**	**	**	**
	Trimester	**			*	**	*	*	**		*		**
	No. of children									**	**	*	
	History of abortion				*								

*Significant association were tested by chi-square, *p < 0.05; **p < 0.01. P1 to P7 are the preventive knowledge on ways to avoid toxoplasmosis. P1: Feeding your cat dry or commercial cat food and not letting it kill and eat rodents. P2: Avoiding stray cats. P3: Letting someone else change the cat's litter box. P4: Making sure the cat's litter box is changed daily. P5: Toxoplasmosis can be prevented by cooking meat well until no pink is seen and the juices run clear. P6: Toxoplasmosis can be prevented by thoroughly washing and/or peeling all fruits and vegetables before eating them. P7: Toxoplasmosis can be prevented by cleaning all cutting boards and utensils thoroughly after each use. B1 to B5 denotes the preventive behaviors of the pregnant women since becoming pregnant. B1: Do you routinely wash your hands after gardening? B2: Do you routinely wash your hands after changing cat litter? B3: Do you routinely wash your hands after handling raw meat? B4: Do you routinely wash your hands before eating food? B5: Do you eat raw meat?*

reported in this region. Our finding is supported by a previous study in Belgium showed that there was a significant decrease in the incidence of *Toxoplasma* seroconversion after the introduction of intensive counseling for pregnant women about toxoplasmosis (Gollub et al., 2008). Other previous studies also highlight the importance of health education among the pregnant women in order to reduce the seroprevalence of this disease hence minimizing the adverse effects of infection in the fetus or newborn (Fonseca et al., 2012; Amin et al., 2013; Pereboom et al., 2013). Unfortunately, it was found that some physicians, obstetrics, and medical staffs had lack of knowledge on this parasitic infection thus failed to provide sufficient information the pregnant women (Ziemba et al., 2010; Alvarado-Esquivel et al., 2011; da Silva et al., 2011). Therefore, medical personnel should be educated on the level of knowledge and practice of toxoplasmosis, subsequently, an appropriate health education could then be provided to pregnant women and the healthcare related staffs to better understand manifestation of this parasitic infection.

CONCLUSIONS

In this study, the knowledge and practices on toxoplasmosis among the pregnant women from Malaysia, Philippines, and Thailand were studied. Of the respondents, a substantial part did have knowledge about preventive practices to avoid toxoplasmosis during their pregnancy. Advising pregnant women about their healthy lifestyle and practices of good preventive measures to prevent this parasitic disease remain crucial. Awareness and education about this parasitic infection in pregnant woman would be helpful in preventing disease transmission and the incidence of clinical outcomes in their carried fetus or newborns. In accordance with this study, we have implemented healthcare education programme related to *Toxoplasma* infection in pregnant women by distribution of brochures in the study areas; Malaysia, Philippines and Thailand and in their respective national languages (Brochures included as Supplement 2).

AUTHOR CONTRIBUTIONS

Cristina C. Salibay, Nongyao Sawangjaroen, and Veeranoot Nissapatorn designed the study. Cristina C. Salibay, Julieta Z. Dungca, Mary Mae M. Cheung, Nongyao Sawangjaroen, and Si-Lay Khaing carried out the experiment, Hemah Andiappan contributed most on manuscript writing. Cristina C. Salibay, Hemah Andiappan, Nongyao Sawangjaroen, Veeranoot Nissapatorn, Waenurama Chemoh, and Ching Xiao Teng helped in manuscript writing and editing. Cristina C. Salibay, Julieta Z. Dungca, Mary Mae M. Cheung, Noor A. Mat Adenan, Nongyao Sawangjaroen, Si-Lay Khaing, Yee-Ling Lau and Veeranoot Nissapatorn provided opinions and suggestions about this manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Toxoplasmosis complications and novel therapeutic synergism combination of diclazuril plus atovaquone

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Toxoplasmosis is a major cause of foodborne disease, congenital complication, and morbidity. There is an urgent need for safe and effective therapies to encounter congenital and persisting toxoplasmosis. The hypothesis was: combination diclazuril plus atovaquone to exert a novel therapeutic synergy to prevent toxoplasmosis syndromes.

Methods: Pregnant dams were treated with diclazuril and atovaquone monotherapy or combination therapy and infected i.p with *Toxoplasma* tachyzoites.

Results: Infected dams developed severe toxoplasmosis associated syndrome with increases in the abdominal adiposity surrounding uteri, gansterointestinal and other internal organs and excessive weight gain. Numerous organisms along with infiltration of inflammatory cells were detected scattered into adipose tissues. Combination therapy ($p < 0.01$) and to a lesser extent diclazuril ($p < 0.05$) protected dams from inflammatory fat and excess weight gains. This was consistent with pancreatitis development in infected dams (versus normal $p < 0.05$) with infiltration of inflammatory cells, degeneration and necrosis of pancreatic cells followed by the degeneration and loss of islets. Combination and monotherapy protected dams from these inflammatory and pathological aspects of pancreatitis. Infected dams exhibited severe colitis, and colonic tissues significantly shortened in length. Brush border epithelial cells were replaced with infiltration of lymphocytes, granulocytes, and microabscess formations into cryptic microstructures. Combination therapy synergistically preserved colonic structure and normalized pathological damages ($p < 0.001$) and to a lesser degree diclazuril monotherapy protected dams from colitis ($p < 0.05$) and gastrointestinal toxoplasmosis. Other complications included severe splenitis ($p < 0.001$) and hepatitis ($p < 0.001$) which were normalized with combination therapy.

Conclusion: Combination diclazuril plus atovaquone was safe and with a novel therapeutic synergism protected dams and fetuses from toxoplasmosis.

Keywords: toxoplasmosis, combination, diclazuril, atovaquone, synergism, obesity, *Toxoplasma,* gastroenteritis

INTRODUCTION

Toxoplasmosis is a major cause of foodborne disease, hospitalization, and congenital complications related morbidity and mortality (Mead et al., 1999; Scallan et al., 2011; Hoffmann et al., 2012). *Toxoplasma* is categorized as class B human pathogen by the CDC and NIH. Toxoplasmosis, a cosmopolitan syndrome, is considered as "forgotten disease of vulnerable and poverty" which infects the many in rural (Hotez, 2008) as well as the urban areas. Congenital toxoplasmosis is due to transmission of *Toxoplasma* organisms from infected mom to the fetus and typically associated with pregnancy immunosuppression. Congenital toxoplasmosis causes severe complications in fetal and neonate to compromise a lifelong adverse consequences (Remington et al., 2004; Olariu et al., 2011; Kieffer and Wallon, 2013). *Toxoplasma* organisms are transmitted through consumption of contaminated meat, milk, dairy product with cysts forms. However, the main source of *Toxoplasma* infection is considered as vegetables, fruits, and water contaminated with oocysts from cat feces in the field, while over 93 million cats are kept as pets in the USA. These households may include immunocompetent as well as immunosuppressed, obese and/or diabetic and pregnant individuals, and at risk of developing toxoplasmosis (Esch and Petersen, 2013).

Toxoplasmosis, a global disease, and in excess of billion people are expected to have *Toxoplasma* infection. *Toxoplasma* is associated with anorexia or obesity as organisms alter and reside in inflamed adipose tissues (Carter, 2013). Excessive weight gain is reported in infected pregnant women compared with uninfected individuals (Kankova et al., 2010; Flegr, 2013), as well as

Abbreviations: BSA, bovine serum albumin; CDC, center for disease control; CNS, central nervous system; EPM, equine protozoal myeloencephalitis; FDA, Food and Drug Administration; H&E, hematoxylin eosin; IACUC, institutional animal care use committee; IBC, institutional biosaftey committee; IHC, immunohistochemical staining; i.p, intraperitoneally; MEM, minimum essential medium; NIH, national institute of health; PBS, phosphate buffer saline; *S. neurona, Sarcocystis neurona*; Tox, *Toxoplasma gondii.*

in a feto-maternal toxoplasmosis model (Oz and Tobin, 2012; Oz, 2014). *Toxoplasma* infected animals had increased weight gain and atrophy of myenteric neurons of the jejunum (Hermes-Uliana et al., 2011). Obesity has become a cosmopolitan syndrome and poorly understood pathogenesis with a potential link to toxoplasmosis. Other toxoplasmosis complications are gastroenteritis, pancreatitis, diabetes, retinochoroiditis, and encephalitis.

Current available therapy for congenital toxoplasmosis is spiramycin associated with pyrimethamine plus sulfadoxine combined therapy, to protect fetus from *Toxoplasma* organism transmission in actively infected moms. However, this approach is not always effective and the treatment has fetotoxic side effects (Habib, 2008; Berrebi et al., 2010; Cortina-Borja et al., 2010; Julliac et al., 2010). Pyrimethamine while used is a pregnancy classified C drug, which may cause bone marrow suppression in the mom and the newborn. In a clinical trial in France, 24% of sera positive women treated with spiramycin and pyrimethamine plus sulfadoxine combination delivered *Toxoplasma* infected infants (Bessieres et al., 2009). Spiramycin monotherapy can be effective only when administered during early stage of pregnancy and is principally a preventive measure (Julliac et al., 2010). More than half of patients treated with spiramycin retained *Toxoplasma* DNA in their blood and remained infected (Habib, 2008). Fifty-five percent of patients treated with combination of sulfadiazine + pyrimethamine plus folinic acid therapy have adverse effects (Capobiango et al., 2014). Meanwhile, the efficacy of azithromycin, clarithromycin, atovaquone, dapsone, and cotrimoxazole (trimethoprim-sulfamethoxazole), has not been clinically proven (Petersen and Schmidt, 2003). Considering the importance of complications and the worldwide epidemic, there is an urgent need for effective and nontoxic therapeutic modalities for congenital or persisting chronic toxoplasmosis.

Diclazuril and its related benzeneacetonitriles have been used in treatment and prevention of livestock and poultry coccidiosis (Assis et al., 2010) and *S. neurona* in EPM. Diclazuril is a safe and effective compound at therapeutic dose levels (Assis et al., 2010; Oz and Tobin, 2014). Diclazuril targets chloroplast derived chlorophyll a-D1 complex present in *Toxoplasma* and other Apicomplexans and not exists in mammalians cells (Hackstein et al., 1995).

Atovaquone is a FDA approved toxoplasmosis treatment but not in feto-maternal toxoplasmosis (Cortina-Borja et al., 2010; Oz and Tobin, 2012; Oz, 2014). Atovaquone is a safe and effective drug against plasmodial infections (Hudson et al., 1991), *Babesia microti,* causative of human babesiosis (Hughes and Oz, 1995; Oz and Westlund, 2012) and other opportunistic disease, *Pneumocystis* pneumonia (Oz et al., 1999).

Recently, the efficacy of diclazuril and atovaquone monotherapy were reported against inflammatory and infectious aspects of mild to moderate feto-maternal toxoplasmosis (Oz and Tobin, 2012, 2014; Oz, 2014). Therapeutic diclazuril plus atovaquone combination have not been previously reported against colitis, pancreatitis and some other inflammatory complications in toxoplasmosis. This investigation explores the efficacy of combination therapy with diclazuril plus atovaquone to exert a novel therapeutic synergism to protect against toxoplasmosis.

MATERIALS AND METHODS

ETHICS

This research was conducted according to the guidelines and approved by the IBC and the Care and Use of Laboratory Animal Care (IACUC) at University of Kentucky Medical Center.

Toxoplasma gondii PROPAGATION

Toxoplasma Type II isolates including ME-49 strain are reported predominant in human congenital Toxoplasmosis (Ajzenberg et al., 2002). For this investigation, *Toxoplasma* organisms from PTG strain (ME-49, ATCC50841) were originally cloned and propagated by Dr. Daniel Howe of the Maxwell H. Gluck Equine Research Center at the University of Kentucky (Howe et al., 1997; Oz and Tobin, 2012). Briefly, Tachyzoites were cultured by serial passage in bovine turbinate cells and maintained in MEMRS (HyClone Labs, Inc.) supplemented with 4% fetal clone III (HyClone, Labs, Inc.), Penicillin/streptomycin/fungizone (BioWhittaker, Inc.), and nonessential amino acids solution (HyClone, Labs, Inc.). Upon disruption of the host cell monolayer, extracellular tachyzoites were harvested and purified from host cell debris by filtration through 3.0 μm membranes. Tachyzoites were enumerated in a hemocytometer and suspended in PBS to the appropriate concentrations for inoculation. All inoculations were administered i.p. in 100 μL volume into dams within 1 h of harvesting to ensure viability.

CONGENITAL TOXOPLASMOSIS MODEL

Day 1 programmed pregnant (9 weeks old) CD1 mice were purchased from Charles River Lab Inc., Wilmington, MA, USA). Dams were housed individually in microisolator cages in a pathogen free environment and maintained at 22°C with a 12: 12 h light: dark cycle at the Maxwell H. Gluck Equine Research Center Laboratory Animal Facility. Animals were fed irradiated rodent chow and sterilized drinking water *ad libitum*. After 5 days acclimation, dams were weighed and ear punched for appropriate identification. They were assigned into 6–8 animals per group and injected i.p. with 100 μL PBS containing 0 or 600 tachyzoites with 0.5 mL insulin syringes. Control dams received 100 μL injection with PBS alone (Oz and Tobin, 2012). Animals were monitored daily three times for distress, pain, physical appearance, and vaginal discharge to detect abortion or early delivery (Oz and Tobin, 2012, 2014). The experiment was terminated on gestation day 16 before possible early or premature birth to study the fetal and maternal aspects of the disease.

SPECIMENS COLLECTION

Animals were euthanatized using CO_2 inhalation. Immediately their chests were opened and blood from heart collected in microtainers (BD Biosource, Rockville, MD, USA) for hematocrit evaluation. Sera were separated and stored at frozen –80°C. The splenic weight and length were recorded. Heart, liver, and uterus were excised and weighed. Colonic contents were removed and colonic length and weigh data measured and flash frozen in liquid nitrogen and stored at –80°C for future studies. Live fetuses were removed from uteri, counted, and weighed and their lengths measured using a digital caliper. All aspects of the investigation were performed according to the guidelines by Institutional Biosafety

Committee (IBC) and IACUC at University of Kentucky Medical Center.

DICLAZURIL AND ATOVAQUONE THERAPIES

To study safety and efficacy of diclazuril plus atovaquone against toxoplasmosis, dams were divided into groups of 18–24. Dams received regimens, diclazuril monotherapy, atovaquone monotherapy, diclazuril plus atovaquone combination therapy, or sham incorporated into daily diet (Oz et al., 2007; Oz and Tobin, 2012, 2014). The control group received sham treatment (inert talcum powder). Treatments were initiated on Day 5 of pregnancy and continued until day 16. On day 8 of pregnancy dams on treatments or sham control arms were further divided into three subgroups of 6–8 animals and were injected each with PBS alone, or PBS containing 600 tachyzoites and treatments were continued until dams were euthanized. Pregnant animals voluntarily consumed their diets with no significant changes in their appearance, food consumption, or weight loss/gain.

PATHOLOGICAL ASSESSMENTS

Hematoxylin eosin staining

A portion of examined tissues from each dam was placed into cassettes and fixed with 10% neutral PBS formalin. The specimens were dehydrated and embedded in paraffin, and tissue sections of 5 μm were stained by H&E for histopathological evaluation.

Giemsa staining

Giemsa is a delicate polychromatic stain that reveals the fine nuclear detail of *Toxoplasma* organisms (Oz and Tobin, 2012). Giemsa stain contains methylene blue azure basic (MBAB) dyes combined with eosin acidic dyes. The deparaffinized slide sections were stained with the polychromatic Giemsa (40 drops/50 mL distilled water) to stain nuclei of the *Toxoplasma* organisms and to permit differentiation among the cells. Then, the slides were depreciated in 1% glacial acetic acid, dehydrated in alcohol and xylene series, and mounted in synthetic resin on slides.

Immunohistochemical staining (IHC)

Anti-*Toxoplasma* antibody and IHC procedure were kindly provided by Dr. David S. Lindsay at University of West Virginal. Briefly, paraffin-embedded sections were cut, deparaffinized with xylene, rehydrated in alcohol baths, washed in PBS with 0.1% BSA, quenched endogenous peroxidase activity by incubating in 3% hydrogen peroxide in methanol for 30 min, and then blocked with rabbit serum (Dako number 1699), 30 min. The sections were incubated with polyclonal Rh anti-*Toxoplasma* antibody, diluted 1: 500 for 90 min, and developed with DAB-chromogen (Dako, Carpinteria, CA, USA) for about 5 min until signal developed and subsequently counterstained with hematoxylin then ammonia treated dehydrate stepwise through alcohol, clear with xylene (Oz and Tobin, 2012, 2014).

COLONIC TISSUES PREPARATION AND EVALUATION

Colonic tissues were flushed with PBS (pH 7.2) and a portion from proximal and distal colonic tissue was fixed in 10% neutral formalin for histological examinations. The remainder was flash-frozen in liquid nitrogen and stored at –80°C. The formalin fixed sections were processed and stained with H&E and slides evaluated by Ziess light microscopy. The severity of colitis as assessed with a histological semiquantitative grading score and performed in a blinded fashion. The scores were based on histopathological features with a numeric value (0: normal to 4: severe) assigned according to the tissue involvement that corresponded to either of the following criteria (Oz et al., 2007, 2010, 2013).

(Grade 0)—no detectable lesions, no inflammatory cells, and normal mucosal appearance.
(Grade 1)—focal inflammatory infiltrate in the mucosa.
(Grade 2)—mild multifocal inflammation with moderate expansion of the mucosa.
(Grade 3)—moderate multifocal inflammation with moderate expansion of the mucosa.
(Grade 4)—severe diffuse inflammation with crypt epithelium disruption and ulceration.

ADIPOSITY TISSUE PREPARATION AND STAINING

Portions of the abdominal adipose tissue from each dam were removed, placed in a cassette and fixed in the 10% buffered formalin and processed for histophathological slides staining with Giemsa, IHC, and H&E to study the structure and possible organisms.

HEPATIC TISSUE PREPARATION AND STAINING

A portion of the right lobe from liver tissues of each dam was placed in cassette and fixed with 10% neutral PBS formalin. The specimens were dehydrated and embedded in paraffin, and tissue sections of 5 μm were stained by H&E. Each slide was evaluated under Ziess light microscopy. Hepatic lesions were graded on a scale of 0–4 + based on degeneration, inflammation, and necrosis (Oz et al., 2006, 2011) as follows.

(Grade 0)—no detectable lesions, no degeneration, infiltration of inflammatory cells, and normal tissue appearance.
(Grade 1)—focal infiltration of inflammatory cells in the tissue and hepatocytes degeneration.
(Grade 2)—mild multifocal infiltration of inflammatory cells, and hepatocytes degeneration.
(Grade 3)—moderate multifocal infiltration of inflammatory cells and hepatocytes degeneration.
(Grade 4)—severe diffuse infiltration of inflammatory cells and necrosis.

PAIN RELATED BEHAVIORAL TEST

Assessment of Pain Related Mechanical Allodynia by Testing Abdominal Withdrawal Threshold. Abdominal withdrawal responses to mechanical stimuli were quantified with von Frey monofilaments (Semmes-Weinstein Anesthesiometer Kit) according to our previous publications with some modification (Oz and Tobin, 2012, 2014). Dams were placed into plastic enclosures on the custom-made screen meshed platform. The monofilament range used for this study included five different intensities corresponding to (hair diameter) gram force [(4.08) 1.0 g; (3.61) 0.4 g; (3.22) 0.166 g; (2.83) 0.07; (2.36) 0.02 g forces]. Testing for mechanical stimulation was performed on the first and the last days of treatment. A single trial consisted of five applications of the each filament used once every 6 s to allow dam to cease any response and return to an inactive position. Mean values of the

percentage of responses of the abdominal withdrawal to each filament (mean withdrawal/5 × 100) were used as % scores for this study. This behavioral test reflected basal level for reflex score and any possible sensory changes observed in the treated mice. A total of four dams were tested per each group.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM unless otherwise stated. Data were evaluated with ANOVA followed by appropriate *post hoc* test (Tukey compared all pairs) using GraphPad Instat version 3 for Windows (Graph-Pad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

RESULTS

In the preliminary trial, groups of naïve dams were treated with diclazuril monotherapy, atovaquone monotherapy, diclazuril plus atovaquone combination therapy, or inert talcum sham treatment. Dams consumed medicated diets with no detectable side effects such as changes in physical appearance, appetite, food consumption, and the rate of weight gain or fetotoxicity and abortion.

TOXOPLASMOSIS AND INFLAMMATORY ADIPOSITY

For the next investigation, groups of dams were treated with (a) diclazuril monotherapy, (b) atovaquone monotherapy (c) diclazuril plus atovaquone combination therapy, or (d) received sham treatment. Then each group was further subdivided and injected with sham, or a dose of 600 tachyzoites. Infected dams developed *Toxoplasma* infection (600 tachyzoites) versus uninfected normal controls received sham (PBS) injection. The infected-sham treated dams showed a progressive severe toxoplasmosis complications including anemia, hydrothorax, and ascities ($p < 0.05$). Combination therapy with diclazuril plus atovaquone and diclazuril monotherapy protected dams from anemia, hydrothorax, and ascites (**Figure 1A**). Normal-sham injected and sham-treated controls (control) gained body weight during pregnancy compared with excessive pathological weight gain due to accumulation of inflammatory adiposity in *Toxoplasma*-infected (Tox) sham treated dams ($p < 0.001$). Combination therapy with diclazuril plus atovaquone synergistically protected dams ($p < 0.01$) and to a lesser extent diclazuril monotherapy ($p < 0.05$) prevented pathological accumulation of adipose tissues and excess weight gain. In contrast, atovaquone monotherapy had no significant effect on the weight gain and accumulated adiposity (**Figure 1B**). Massive inflammatory adipose depot was detected in the abdominal cavity surrounding uteri and gastrointestinal and kidneys. The adipose tissues were shown to harbor numerous inflammatory cells in H&E stainings as well as *Toxoplasma* organisms as confirmed with Giemsa and IHC stainings (**Figure 2A**). Organisms were not detectable in dams with combination therapy.

Toxoplasma INDUCED SPLENITIS

Splenic tissues enlarged significantly and increased in weight and length in infected-sham treated dams. Enlarged splenic tissues from *Toxoplasma* infected dams showed significant infiltration of epithelioid cells and multinucleated giant cells with loss of germinal structure and caused a severe splenomegaly. *Toxoplasma* organisms were detected in IHC staining. Combination therapy diclazuril plus atovaquone synergistically prevented dams from severe splenitis and tissue damages ($p < 0.001$), **Figure 2B**, **Table 1**.

Toxoplasma INDUCED COLITIS

Colonic tissues from infected-sham treated dams were significantly shortened in length (10.4 ±0.2 vs. infected 8.7 ±0.6 cm, $p < 0.001$) but decreased in weight ($p < 0.01$), presumably through the mechanism of sloughing off of the brush boarder due to infection (**Figure 3A**). Colonic pathology manifested with shortening of crypts with numerous microabscess formations in the cryptic structures and infiltration of inflammatory

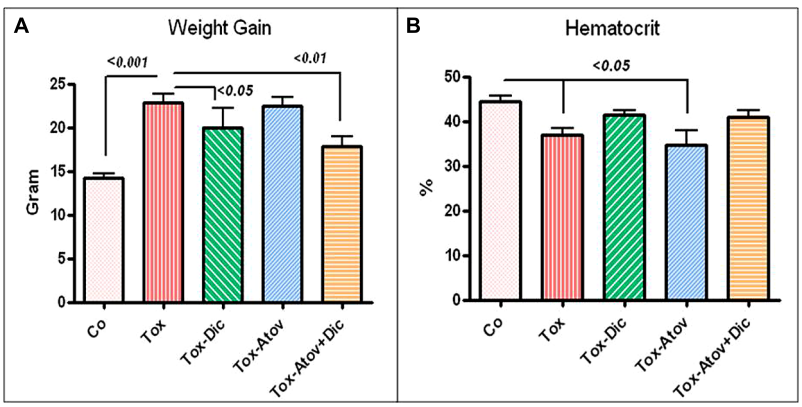

FIGURE 1 | (A) *Toxoplasma* infection caused significant anemia in sham treated dams (Tox). Combination diclazuril plus atovaquone therapy (Dic + Atov) and diclazuril monotherapy (Dic) protected dams but atovaquone (Atov) monotherapy had no effect. **(B)** Body weight gain during pregnancy in normal sham controls (Control) compared with excess pathological weight due to accumulation of inflammatory fat in *Toxoplasma* infected (Tox) sham treated dams ($p < 0.001$). Combination therapy with diclazuril plus atovaquone synergistically protected dams ($p < 0.01$) and to a lesser extent diclazuril monotherapy ($p < 0.05$) prevented pathological fat accumulation and excess weight gain. Atovaquone monotherapy had no significant effect ($n = 6$–8/group).

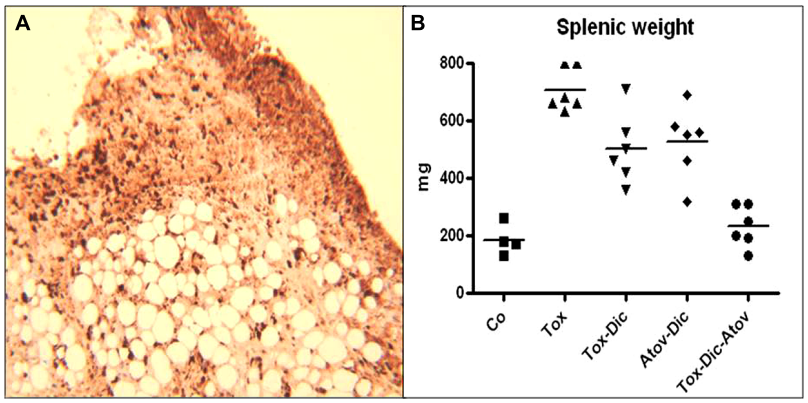

FIGURE 2 | (A) Demonstrates inflammatory fat depot located in abdominal cavity and harboring numerous organisms. IHC stained *Toxoplasma* organisms (dark brown). ($n = 6$ group). **(B)** Spelenic weight distribution in *Toxoplasma* (Tox) infected ($p < 0.001$) compared to diclazuril (Dic) monotherapy ($p < 0.01$), atovaquone (Atov), synergistic effect of diclazuirl plus atovaquone (Dic + Atov) combination therapy ($p < 0.001$) and normal sham controls (Co). ($n = 6$/group).

Table 1 | Efficacy of diclazuril and atovaquone monotherapy or combination treatment on toxoplasmosis.

Tissues	Control	Tox	Tox + Dic	Tox + Atov	Tox + Dic + Atov
Fetal weight	700 ± 40	530 ± 14[c]	650 ± 25[b]	710 ± 25	720 ± 20
Splenic length (mg)	2.28 ± 013	3.22 ± 0.2[c]	2.8 ± 0.18	3 ± 0.1[a]	2.3 ± 0.13[b]
Pain score*(%)	20 ± 6	43 ± 3[b]	40 ± 4[b]	25 ± 2.9	25 ± 2.8

Tissues from normal sham treated and PBS containing no tachyzoites injected controls (Control), infected-dams with Toxoplasma tachyzoites and treated with sham (Tox), compared with infected dams from diclazuril monotherapy (Tox + Dic), Atovaquone monotherapy (Tox + Atov), or combination diclazuril plus Atovaquone (Tox + Dic + Atov) therapy. Dams were monitored daily three times until day 16 of pregnancy before termination. Number 6–8/each group.

**Percent abdominal pain related behavioral response to von Frey stimuli with 0.166 GM force. Abdominal hypersensitivity significantly increased in infected dams (Tox). Combination therapy (Atov + Dic + Atov) and atovaquone monotherapy (Tox + Atov) similarly normalized pain induced behavioral modification in dams, but diclazuril monotherapy (Tox + Dic) had no effect. [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.*

cells, including lymphocytes, with scattered neutrophils detected in the mucosal architecture. Combination therapy synergistically prevented pathologic changes ($p < 0.001$) and to a lesser extent diclazuril monotherapy ($p < 0.05$) preserved the colonic length and weight and the integrity of the microstructure against inflammatory response (**Figure 3B**). In contrast, atovaquone monotherapy had no significant protective effect on colonic inflammation and necrotic/atrophic responses to the infection.

Toxoplasma INDUCED HEPATITIS

Hepatic structures of infected-sham treated dams enlarged twofold and increased in weight due to a substantial inflammatory response to the organisms ($p < 0.001$) **Figure 4A**. Pathological investigation demonstrated severe hepatitis with infiltration of inflammatory cells, multinucleated dysplastic hepatocytes, giant cell transformation, stellate cells activation and hepatic cells necrosis (pathological mean score of 3.5 from 4 most severe) **Figure 4B**. Combination therapy with diclazuril plus atovaquone exerted unique synergism and preserved hepatic appearance, weight and microstructure ($p < 0.001$) and to a lesser degree, diclazuril monotherapy ($P < 0.01$) and atovaquone monotherapy ($p < 0.05$) prevented *Toxoplasma* induced hepatitis (**Figures 4A,B**). Overall, these effects of combination therapy present an striking synergy between two structurally distinct compounds in protecting architecture from exaggerated inflammatory reaction.

Toxoplasma INDUCED PANCREATITIS

This was consistent with moderate to severe *Toxoplasma* induced pancreatitis in infected dams ($p < 0.05$) with infiltration of inflammatory cells, vacuolization, degeneration, and necrosis of pancreatic cells followed by the degeneration and loss of beta cells and islets (**Figure 5A**). Combination therapy with diclazuril plus atovaquone therapy and monotherapy protected dams from these inflammatory and pathological aspects of pancreatitis (**Figure 5B**) and gastrointestinal toxoplasmosis.

CONGENITAL TOXOPLASMOSIS

Infected dams had nested smaller fetuses ($p < 0.001$) and sporadic preterm labor or stillbirth. Combination therapy diclazuril plus atovaquone as well as monotherapy with atovaquone similarly and to a lesser extent diclazuril monotherapy ($p < 0.01$) protected nested fetuses from retardation and demise (**Table 1**). In addition, uteri considerably augmented owing to accumulation of inflammatory fat, influx of inflammatory cells in infected-sham treated dams and *Toxoplasma* organisms were detected in Giemsa stained and IHC slides (not shown). Combination therapy with

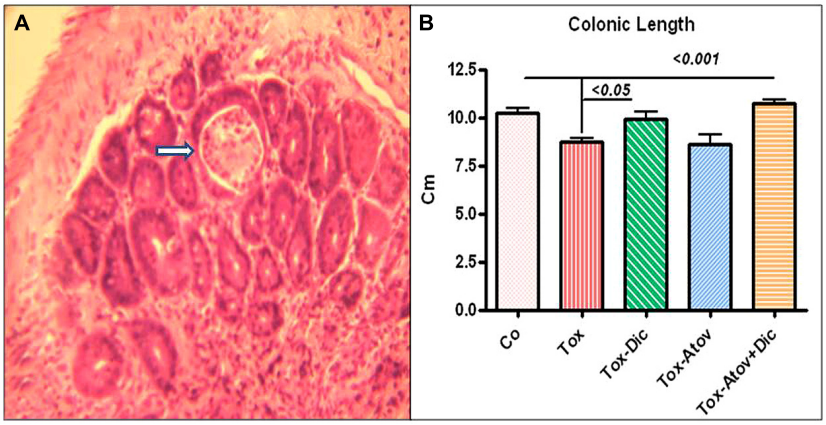

FIGURE 3 | (A) Colonic section stained with H&E from *Toxoplasma* infected sham treated dam (Tox) developed severe colitis with destruction of brush border, and loss of colonic epithelial cells, microabscess formation (open arrow) and infiltration of inflammatory cells into mucosa ($n = 6$ /group). **(B)** Colonic length shortened due to infiltration of inflammatory cells, and microabscess formation in infected sham treated dams ($p < 0.001$). Combination therapy with diclazuril plus atovaquone (Dic + Atov) preserved colonic structure and to a lesser extent Diclazuril (Dic) monotherapy improved the colitis ($p < 0.01$). ($n = 6$–8/group).

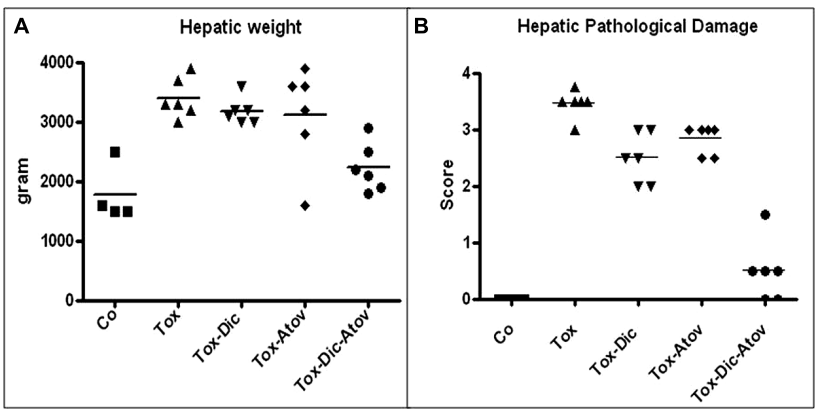

FIGURE 4 | (A) Hepatic weight distribution in *Toxoplasma* (Tox) infected ($p < 0.001$) compared to diclazuril (Dic) monotherapy ($p < 0.01$), atovaquone (Atov) monotherapy and combined diclazuirl plus atovaquone (Dic + Atov) therapy ($p < 0.001$) and normal sham controls (Co). **(B)** Hepatic pathological score distribution in *Toxoplasma* (Tox) infected dams ($p < 0.001$) compared to diclazuril (Dic) monotherapy ($p < 0.01$), atovaquone (Atov) monotherapy ($p < 0.05$), combination diclazuirl plus atovaquone (Dic + Atov) therapy ($p < 0.001$) and normal sham controls (Co). Pathological slides were stained with H&E. ($n = 6$–8/group).

diclazuril plus atovaquone improved the infectious inflammatory response and edema but with no significant changes in the uteri weight, presumably due to the increased number of healthy fetuses (not shown).

TOXOPLASMOSIS AND ABDOMINAL HYPERSENSITIVITY

Finally, pain related abdominal hypersensitivity significantly elevated in *Toxoplasma* infected-sham treated dams manifested with severe abdominal withdrawal and excess grooming in comparison to normal sham control dams ($p < 0.05$). Combination diclazuril plus atovaquone therapy and atovaquone monotherapy preserved the normal abdominal response to von Frey stimuli (**Table 1**). However, diclazuril monotherapy had no significant effect on the dams' response to the mechanical stimuli.

DISCUSSION

Toxoplasma is a leading cause of foodborne diseases, congenital complications, morbidity and mortality. Yet, toxoplasmosis is an underestimated syndrome and usually detected in autopsy or remains undetected due to the non-specific symptoms and lack of clinical awareness of healthcare individuals (Munir et al., 2000). *Toxoplasma* organisms are transmitted through consumption of undercooked meat, milk and dairy product contaminated with cysts forms. However, the predominant source of *Toxoplasma* infection is considered as vegetables, and fruits contaminated with oocysts from the cat feces in the field (Oz, 2014). In addition, contaminated water is reported as a major source for infection during pregnancy in rural area (Andiappan et al., 2014). Considering high number of cats (>93 million) residing in households in the USA, immunocompromised individuals, and expecting moms,

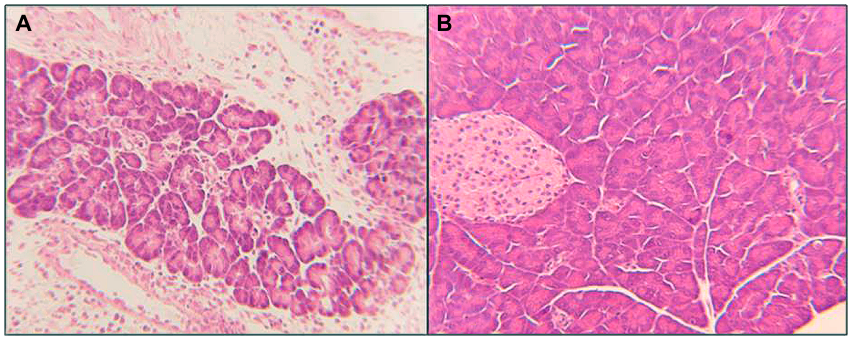

FIGURE 5 | Pancreatic section from *Toxoplasma* infected and treated dams stained with H&E. (A) Pancreatitis: demonstrates loss of microstructure, degeneration, and necrosis of pancreatic cells, degeneration and loss of islets, replaced with infiltration of inflammatory cells. **(B)** Combination diclazuirl plus atovaquone (Dic + Atov) therapy protected pancreatic architecture against inflammatory and infectious response, and preserved panc and beta cells, and islet's microstructure. (n = 6–8/group).

as well as the increasing obese and/or diabetic population are at a high risk of developing toxoplasmosis (Esch and Petersen, 2013). Therefore, awareness of healthcare communities as well as individuals is necessary to contain stray cats, and prevent pets from infection in order to protect the owners from imminent complications.

Toxoplasmosis is a "forgotten disease of vulnerable and poverty" which infects the many in rural (Hotez, 2008) as well as urban area. While, poverty persists, obesity has become a cosmopolitan complication with undetermined pathogenesis. This investigation reports accumulation of excessive infectious and inflammatory adiposity and pathological weight gain in *Toxoplasma* infected dams. *Toxoplasma* association with obesity was supported in a clinical trial with 999 psychiatric healthy normal subjects with exclusion of those with personality and serious mental disorders which have strong association with toxoplasmosis as well as obesity (Reeves et al., 2013). Individuals with positive anti-*Toxoplasma* antibodies had twice the odds to be obese compared to seronegative individuals. Further, obese individuals had significantly higher anti-*Toxoplasma* IgG titers compared to those who were not obese (Reeves et al., 2013). In contrast, no relation with obesity and anti-*Toxoplasma* IgG titers was reported in a trial with confounding factor of excluding individuals over 45 years of age when subjects mostly are prone to develop toxoplasmosis reactivation and obesity (Thjodleifsson et al., 2008). *Toxoplasma* may alter weight gain by reducing muscle lipoprotein lipase and modulating tissue lipoprotein lipase activity during chronic infection to promote triglyceride distribution in adipose tissue (Picard et al., 2002). From 1227 Mexican Americans tested for anti-*Toxoplasma,* 110 (9%) were found seropositive. In fact, this population commonly suffers from high rates of chronic inflammatory diseases, obesity and type-2 diabetes, further suggesting a correlation between toxoplasmosis and these chronic complications (Rubicz et al., 2011).

Toxoplasmosis may manifest with clinical symptoms of acute or recurrent abdominal pain and pancreatitis (Parenti et al., 1996). Chronic progressive pancreatitis may be associated with fat necrosis, obstruction of bile duct, focal hepatic necrosis, elevated amylase and lipase serum values, and abdominal fat. Similarly, in this study infected dams developed increased abdominal inflammatory pain related modifications and severe pancreatitis and hepatitis. There is an association of *Toxoplasma* infection with liver cirrhosis. While, severity of toxoplasmosis complications depend on the immune status of the patient and the strain. Acute *Toxoplasma* infection in mice with RH strain reveal a significant correlation between the increased number of hepatic stellate cells and the amount of *Toxoplasma* antigens, representing an active role for hepatic stellate cells in the pathogenesis of *Toxoplasma*-induced hepatitis (Atmaca et al., 2013). Moreover, the prevalence of anti-*Toxoplasma* IgG is significantly higher among the primary biliary cirrhosis patients (71%) compared with controls without cirrhosis (40%, $p < 0.0001$), whereas the infection burden is rare in healthy subjects (20% vs. 3%, respectively, $p < 0.0001$). It is predicted that *Toxoplasma* to increase the risk of primary biliary cirrhosis in patients (Shapira et al., 2012). Since, latent infection is fairly common, and once infected organisms reside for the lifelong; the *Toxoplasma* interventions with safe and effective regimens will have a great impact on health related concerns in vulnerable individuals.

Available treatments for toxoplasmosis, sulfasalazine, pyrimethamine, sulfadiazine, and spiramycin, have major side effects and not always effective. Seroconvert pregnant women are treated with spiramycin to reduce the risk of fetal placental transmission. However, spiramycin treated patients retain *Toxoplasma* DNA in peripheral blood and remain infected (Habib, 2008). In addition, spiramycin is effective only in early pregnancy and not after organisms penetrate the placenta and fetus (Julliac et al., 2010). In a 20 year prospective trial of infected moms treated with spiramycin alone or combined with pyrimethamine-sulfadoxine, 17% of newborns had established congenital toxoplasmosis and 26% developed chorioretinitis after birth (Berrebi et al., 2010). In another study the transmission rates of toxoplasmosis were 7% in the first, 24% second, and 59% in third trimesters, respectively, for infected mothers treated with combination spiramycin and pyrimethamine-sulfadoxine (Bessieres et al., 2009).

Because of these shortfalls, there is urgent need for more effective therapeutic modalities with no toxicity to encounter

congenital as well as recurrent toxoplasmosis. In this investigation combination of diclazuril plus atovaquone therapy synergistically protected dams and fetuses from severe complications of toxoplasmosis including gastrointestinal and the inflammatory adiposity accumulation.

Atovaquone (hydroxy-1,4-naphthoquinone) an standard of therapy against acute toxoplasmosis is not approved for congenital infection. Atovaquone suppresses mild gastrointestinal toxoplasmosis in pregnancy model (Oz and Tobin, 2012; Oz, 2014). However, atovaquone monotherapy is not effective against severe complications of colitis, hepatitis and splenits and inflammatory fatty deposits as shown here.

Additionally, diclazuril (4-chlorophenyl [2,6-dichloro-4-(4,5-dihydro-3H-3,5-dioxo-1,2,4-triazin-2-yl)pheny l] acetonitrile) is used in livestock to prevent coccidiosis and equine infection with *S. neurona*. Diclazuril is orally absorbed with steady-state concentrations in plasma and cerebrospinal fluid (CSF) to inhibit the proliferation of 95% of the organisms (Assis et al., 2010; Oz, 2014; Oz and Tobin, 2014). Dicalzuril exclusively binds and affects *Toxoplasma* organelle for photosynthetic reaction center (protochlorophyllide) containing a trace of chlorophyll (Hackstein et al., 1995). This herbicidal-binding site for diclazuril is highly specific for *Toxoplasma* and other Apicomplexans, providing an exceptional chemotherapeutic sensitivity. Therefore, diclazuril binds the chloroplast epitopes and interacts with the D1 protein, with no intervention with the mammalian cells. In addition, diclazuril downregulates expression of serine/threonine protein phosphatase and causes apoptosis of *Eimeria tenella* merozoites (Zhou et al., 2013). Serine/threonine protein phosphatase (EtRACK) has 98% homology with *Toxoplasma* with a predicted mechanism of action for diclazuril efficacy against toxoplasmosis. Diclazuril dose dependently protects against moderate feto-maternal gastrointestinal complications in model (Oz, 2014; Oz and Tobin, 2014). Yet, combination diclazuril plus atovaquone therapy has superior synergic effects against toxoplasmosis in comparison to diclazuril and atovaquone monotherapy. As such, combination therapy with a promising safety and efficacy proven in the most vulnerable group during "fetal maternal" stages can be applicable as a preventive measure in the endemic areas specifically in pregnancy as well as in pets. It is anticipated that the novel combination diclazuril plus atovaquone therapy to be as effective in maternal congenital as well as acute and chronic persistence CNS and ocular toxoplasmosis in patients.

CONCLUSION

Diclazuril plus atovaquone combination was safe with a novel therapeutic synergism protected dams from inflammatory and infectious colitis, pancreatitis, obesity and other pathological complications as well as preserved the fetuses against congenital toxoplasmosis. The future trials will prove the anti-toxoplasmonsis properties of diclazuril plus atovaquone combination in acute or chronic ocular, CNS and congenital toxoplasmosis in patients.

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Maternal and congenital toxoplasmosis, currently available and novel therapies in horizon

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Over one billion people worldwide are predicted to harbor *Toxoplasma* infection frequently with unknown lifelong health consequences. Toxoplasmosis is an important cause of foodborne, inflammatory illnesses, as well as congenital abnormalities. Ubiquitous *Toxoplasma* has a unique tropism for central nervous system with a mind-bugging effect and is transmitted sexually through semen. Currently available therapies are ineffective for persistent chronic disease and congenital toxoplasmosis or have severe side effects which may result in life-threatening complications. There is an urgent need for safe and effective therapies to eliminate or treat this cosmopolitan infectious and inflammatory disease. This investigation discusses pathogenesis of maternal and congenital toxoplasmosis, the currently available therapies in practice, and the experimental therapeutic modalities for promising future trials.

Keywords: fetal maternal, congenital toxoplasmosis, mind alteration, sexual transmission, atovaquone, diclazuril

INTRODUCTION

Over one billion people worldwide are predicted to harbor *Toxoplasma* infection frequently with unknown lifelong health consequences. Toxoplasmosis is one of the most important foodborne inflammatory illnesses, as well as congenital abnormalities (Hoffmann et al., 2012). *Toxoplasma* is classified as "Category B pathogen" which once infected, the organisms dwell in organs such as muscles and brain in cyst forms for the life of the patient/host to become reactivated. The organisms have a sexual stage in cat's intestinal epithelial cells which form resistant oocysts passed in feces and matured in dirt (**Figure 1**). Humans and other animals develop systemic infection in asexual form by ingestion of contaminated vegetable, fruits, water, or consumption of infected milk and undercooked sea food, poultry, and livestock. Tachyzoites infect nucleated host cells and utilize monocytes, macrophages, and dendritic cells as "Trojan Horse" (1) to escape the host immune defense (Elsheikha and Khan, 2010), (2) to bypass the blood–brain barrier (Bierly et al., 2008) and the placenta barricade, and (3) to spread and form systemic disease. *Toxoplasma* infests particularly rural and impoverish communities of women, African American, Hispanics, and Native Americans as a "frequently ignored disease of poverty" (Hotez, 2008). Toxoplasmosis is considered as the second major cause of foodborne death in the United States (Scallan et al., 2011). The *Toxoplasma* annual cost of illnesses is about $3 billion and the quality-adjusted life loss is equal to 11,000 years in the United States (Hoffmann et al., 2012). Toxoplasmosis in immune-intact individuals is generally symptomless and undetected or appears like flu syndrome and malaise. However, it can cause severe pathological consequences in immunocompromised patients, fetuses, and neonates and lead to demise and death (Dubey and Jones, 2008).

MATERNAL CONGENITAL TOXOPLASMOSIS

The importance of maternal and congenital transmission has long been recognized since 1939; when a neonate from New York developed toxoplasmosis (Wolf et al., 1939; Jones et al., 2001). During progression of pregnancy, maternal immune system confronts a dual predicament: the growing embryo, and the environmental toxins and pathogens threatening mom and fetus. In fact, successful pregnancy involves an elegant equilibrium in organizing the immune system at the fetal-maternal and uteri milieu resulting in tolerance (TH2) of the fetus (Norwitz et al., 2001; Muzzio et al., 2014) and defense (TH1) against the pathogenic agents. Women who have acute or reactivated toxoplasmosis during pregnancy can transplacentally transmit organisms to their fetus. As, tachyzoites bypass the placental blood barrier and invade the fetal organs to propagate and compromise the embryonic developmental process. About 50–80% of child-bearing Brazilian women and 50% of children have anti-*Toxoplasma* antibodies. Also, 5–23 neonates are found to be infected per 10,000 in Brazil (Dubey et al., 2012).

Congenital toxoplasmosis can manifest with severe complications, such as miscarriage, fetal developmental retardation, encephalitis, neurological, mental illnesses, visual, and auditory inflammatory disorders, cardiovascular abnormalities, and pains (Dunn et al., 1999; Gilbert and Gras, 2003; Gras et al., 2005; McLeod et al., 2006; Remington et al., 2006; Oz and Tobin, 2012). The severity of complications relies on the gestation period, as the early infection shows more severe outcomes (Dunn et al., 1999; Remington et al., 2006). While, fetuses infected in late gestation are born normal, may develop central nervous system (CNS) symptoms and retinochoroiditis later in life. Also, the new lesions may occur in untreated as well as treated children (Dunn et al., 1999).

A predominant source of infection in North America is contaminated food and water with oocysts passed in the cat's (definite host) feces (Boyer et al., 2011). Sera and surveys from 76 moms with congenital infected newborns were collected from four different epidemic areas and investigated by the National collaborative Chicago-based congenital toxoplasmosis. The data revealed 78%

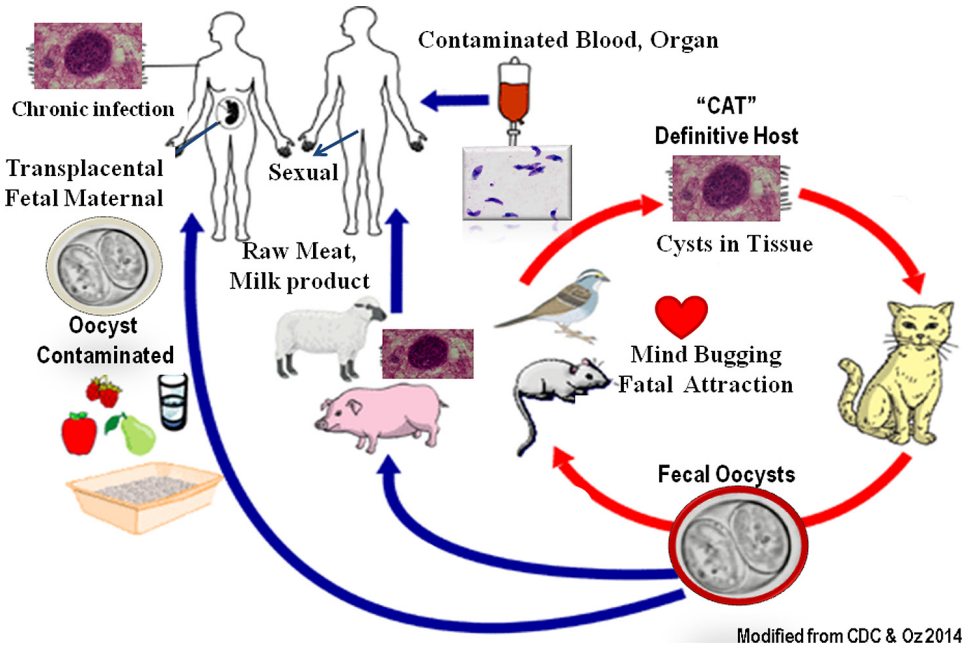

FIGURE 1 | Multiple sources of ubiquitous *Toxoplasma* for maternal congenital transmission.

of the moms acquired primary infection from oocysts form, while only 49% had direct contact with house cats. Hence, extensive educational hygienic programs, effective cats' infection prevention, and vaccination plans, along with serological testing of pregnant women and newborns, followed by the treatments are needed to prevent maternal congenital toxoplasmosis (Boyer et al., 2011).

MATERNAL REACTIVATION AND CONGENITAL TOXOPLASMOSIS

Toxoplasmosis reactivation is a major concern in pregnant, immunodeficient, blood transfusion, bone marrow, and organ transplant patients, when the protective cyst's wall ruptures and organisms reach the lymphatic and blood cells to activate and propagate the infection. Maternal congenital toxoplasmosis is instigated by the transplacental transmission of organisms in maternal infection (Buxton et al., 1991), as *Toxoplasma* organisms alter balance in immune milieu leading to inflammatory response. A low number of *Toxoplasma* organisms can induce an extensive inflammatory and immune reaction as shown in the murine model of fetomaternal toxoplasmosis (Oz and Tobin, 2012). Therefore, taming exaggerated inflammatory response in fetal–maternal toxoplasmosis is necessary to prevent severe tissue destruction and fatality during the pathogenic clearance.

According to Massachusetts Department of Health, about 1 case of congenital toxoplasmosis occurs for every 10,000 live births. It is estimated that from 4,000,000 live births each year in the United States, 400 have acquired congenital toxoplasmosis (Mead et al., 1999). This rate is extensively higher for other developing countries. For instance, retrospective trials from Argentina (2000–2011) reviled 18% (2206/12035) prevalence rate of anti-*Toxoplasma* antibody in pregnant women. Thirty eight per 10,000 of these moms had developed acute infection and 5.8% transplacentally infected their neonates (Carral et al., 2013).

TOXOPLASMA MIND-BUGGING SEXUAL ATTRACTION AND MENTAL DISORDERS

Recent investigations reveal that *Toxoplasma* provokes a brain and mind alteration with sexual arousal in rats seeking cat, while uninfected normal rats fear and avoid predator's urine odor with an immediate, innate survival defensive behavior (House et al., 2011; Knight, 2013). Therefore, the brain impaired and fearless infected rodents are eaten up by feline to fulfill the organism's sexual propagation in definitive host "cat". *Toxoplasma* manipulates the limbic brain neurons responsible for instinct defensive response and augments activity in adjacent limbic regions of sexual desire when exposed to cat's urine odor (House et al., 2011).

Toxoplasmosis is a sexually communicable disease as organisms are transmitted by contaminated semen during natural mating. Also, artificial insemination with contaminated semen can infect animals with vertical transmission with 80% embryonic disruption (Arantes et al., 2009; Lopes et al., 2013; Wanderley et al., 2013). Indeed, there exists a potential sexual transmission route with infected semen during mating as well as artificial insemination with subsequent vertical transmission to the progeny in humans.

Toxoplasma has strong tropism for the CNS with adverse affect in the brain neuro-structural development and pathological as well as psycho-behavioral impairment and mental challenges (Bachmann et al., 2005; Brown et al., 2005; Wang et al., 2006). Maternal *Toxoplasma* infection has been related

with risk for schizophrenic events and autism with over 40 supporting investigations for the incidence of *Toxoplasma* infection among these patients (Flegr, 2013). *Toxoplasma* infection may evolve brain dopamine dysregulation (Torrey et al., 2007; Fekadu et al., 2010). Longitudinal and cross-sectional trials in seropositive females with chronic toxoplasmosis have shown high risk of self-harm, accidents and non-fatal suicidal aggression than in seronegative individuals (Pedersen et al., 2012; Zhang et al., 2012).

Pregnant women with latent infection have a higher risk of infants with genetic or developmental disorders such as premature and postnatal slow motor development due to infection provoked immunosuppression in moms. Some of these defects are related to malnutrition caused by diarrhea and gut disorders or directly congenital toxoplasmosis induced cognitive and developmental deficits (Kankova et al., 2012).

AUTOIMMUNE DISEASE AND TOXOPLASMOSIS

Ubiquitous *Toxoplasma* infection is indicated to provoke series of chronic inflammatory and autoimmune disorders. Immunosuppressants and monoclonal antibodies such as anti-TNF which are widely used in healthcare for the treatment of autoimmune diseases, and organ transplantation may result in acute toxoplasmosis in these patients. However, the nature of this interaction and mechanism between the development of acute toxoplasmosis and immunosuppressant therapies are still being investigated. In a clinical trial, sera of 1514 patients with 11 different autoimmune diseases from health centers in Europe and Latin America and 437 matched controls examined for the prevalence of anti-*Toxoplasma* antibodies, IgG and IgM and auto-antibodies (Shapira et al., 2012). Fourty-two percent of patients had anti-*Toxoplasma* antibody IgG, versus 29% of those without autoimmune complications ($p < 0.0001$). Anti-*Toxoplasma* antibody IgG was associated with anti-phospholipid syndrome, autoimmune thyroid diseases, systemic sclerosis, and rheumatoid arthritis ($p < 0.0001$). Anti-*Toxoplasma* antibody IgM was more prevalent in patients with anti-phospholipid syndrome ($p < 0.01$), systemic sclerosis ($p < 0.05$) and inflammatory bowel disease (IBD; $p < 0.05$) than in controls. These findings strongly support that *Toxoplasma* may contribute to the autoimmune disease pathogenesis (Shapira et al., 2012).

Crohn's disease and ulcerative colitis (IBD) are considered as autoimmune response of a leaky gut to microbiota, when toxins, like Gram-negative lipopolysaccharide (LPS), bypassing the inflamed epithelia by underlying dysregulated oral tolerance (Brandtzaeg, 2001; Oz et al., 2009, 2013). The use of biological blockers, like anti-TNF antibodies, and immunosuppressives in IBD patients increases the risk of opportunistic diseases (Oz and Ebersole, 2009). Crohn's patients are prone to intestinal abscess formation including *Toxoplasma* infection (Epple, 2009). Dams infected with *Toxoplasma* develop severe colonic inflammatory response with significant shortening in colonic length, infiltration of lymphocytes, and macrophages and microabscess formations in the cryptic microstructures resembling Crohn's pathogenesis (Oz and Tobin, 2012, 2014; Oz, 2014). Similar to colitis, *Toxoplasma*-induced ileitis elevates precarious gut microbial, LPS and lipopeptide contents (Erridge et al., 2010). Anti-*Toxoplasma* titer has been detected significantly higher in IBD patients than healthy controls; supporting the notion that toxoplasmosis to trigger IBD and specifically Crohn's disease pathogenesis in patients (Lidar et al., 2009). *Toxoplasma* infection causes an excessive Th1 systemic inflammation and promotes pro-atherogenesis (Portugal et al., 2009; Lige et al., 2013). Therefore, inflammatory response may act as a dual-edged sword. While, necessary for the host defense and recovery against pathogens, unleashed exaggerated chronic inflammation against *Toxoplasma* infection causes loss of function in organs as seen in the autoimmune syndrome (Carter, 2013).

DIAGNOSIS OF MATERNAL CONGENITAL TOXOPLASMOSIS

Maternal *Toxoplasma* infection as a serious risk factor for the fetus requires accurate and urgent diagnosis for possible prevention and treatments. Maternal congenital toxoplasmosis is commonly diagnosed with utilizing repeated serological tests to assess the types and the levels of anti-*Toxoplasma* antibodies. Pregnant moms are required to be tested in Austria, France, Italy, Portugal, and Uruguay for antibody detections, but a limited screening program is used in Belgium, Germany, and Switzerland. Congenital and neonatal screening for toxoplasmosis is performed in over two million women and their babies each year in Europe, North and South America with estimated cost of over 500 million dollars (Petersen and Schmidt, 2003). While, the United States does not require routine screening, it is recommended that infants with serious systemic complications to be tested for toxoplasmosis (Armstrong et al., 2004). In addition, seronegative pregnant women indicating no previous exposure to infection are at risk for the infection and recommended to be serology tested monthly until the labor.

Diagnosis of toxoplasmosis is based on the presence of IgM and IgG anti-*Toxoplasma* antibodies, and molecular techniques to detect organisms (Teixeira et al., 2013). Acute infection is associated with high levels of anti-*Toxoplasma* IgM antibody followed by a rise in IgG levels in 1–3 weeks. Detection of IgM or elevation of IgG anti-*Toxoplasma* antibodies suggests acute or reactivation with a possible transmission of infection to the fetus. An amniotic fluid test is required to confirm fetal health status and possible exposure to the maternal infection.

Sabin-Feldman dye test "the international gold standard" is a complement-lysis-based assay and relatively sensitive and specific for anti-*Toxoplasma* IgG antibody. The test is considered more reliable than available ELISA kits, but requires live organisms treated with each diluted serum analyzed under the microscope (Dando et al., 2001).

In infants with neurological disorders, anti-*Toxoplasma* IgM and IgA antibodies plus cerebrospinal fluid PCR to detect *Toxoplasma* DNA are considered to provide a high sensitivity for diagnosis of congenital toxoplasmosis (Olariu et al., 2014). CSF-PCR was positive in 47% of about 60 infants from infected moms, while 0% positive in uninfected healthy ones.

Additionally, western blot analysis is used to detect IgM and IgA (di Carlo et al., 2011) and RT-PCR for DNA in amniotic fluid with 98% sensitivity and 100% specificity (Teixeira et al., 2013).

α-FETOPROTEIN SERUM ANALYSIS

α-Fetoprotein, released by embryonic hepatic cells, is a biomarker to predict development and birth defects, and useful in prediction of fetoplacental health and growth progression. Altered levels of maternal α-fetoprotein are associated with pregnancy, hepatic complications, tumors, fetal demise, and resorption (Mizejewski, 2007) and may be found useful in prediction of immune responses and intrauterine death in toxoplasmosis (Kaur and Verma, 1995; Oz, 2014).

CURRENTLY AVAILABLE AND NOVEL ANTI-TOXOPLASMOSIS THERAPIES IN HORIZON

Toxoplasmosis is a forgotten disease with no safe and effective therapy available for chronic persistent or pernicious fetal–maternal infection. Spiramycin has been used in feto-maternal toxoplasmosis prevention and treatment in Canada, Latin America, and Europe for decades but is classified under "experimental therapy" in the United States. Spiramycin monotherapy is effective in early pregnancy as a preventive measure but not after fetal exposure to the infection. In a prospective cohort trial in Brazil, 58% of newborns from spiramycin-treated moms, in contrast to over 73% from untreated ones had congenital infection (Avelino et al., 2014). More than 50% of patients treated with spiramycin retained *Toxoplasma* DNA in peripheral blood and remained infected (Habib, 2008). In another clinical trial of the neonates from infected moms treated with spiramycin and pyrimethamine plus sulfadoxine in France, 24% of 257 children were diagnosed with congenital infection. Of these, 7% were predicted to be infected in the first, 24% in the second, and 59% in the third trimesters, respectively (Bessières et al., 2009). Other fetal–maternal treatments are azithromycin, clarithromycin, atovaquone, dapsone, and cotrimoxazole (trimethoprim–sulfamethoxazole), however, their efficacy has not been proven (Petersen and Schmidt, 2003).

ATOVAQUONE AND MATERNAL CONGENITAL

Atovaquone, a hydroxy-1,4-naphthoquinone and FDA approved, is fairly safe and effective treatment against tachyzoites and cyst forms of *Toxoplasma* and anti-*Plasmodial* (Hudson et al., 1991; Dunay et al., 2004). It is used in adults, yet not approved for fetal–maternal and children toxoplasmosis (Cortina-Borja et al., 2010). Atovaquone is anti-fungal *Pneumocystic* pneumonia and anti-*Babesia microti,* causative of human blood-borne babesiosis endemic in New England and North Eastern in the United States (Hughes and Oz, 1995; Oz and Westlund, 2012). Atovaquone acts by targeting mitochondrial respiration and binds to the ubiquinol oxidation on cytochrome bc1 complex to block and to collapse the membrane in the organisms (Srivastava and Vaidya, 1999; Freyre et al., 2008). Atovaquone has a half-life of 1.5–3 days and mainly binds to plasma proteins (99%) and is excreted into feces (94%) without being metabolized (Rolan et al., 1997). Atovaquone has been shown to protect against maternal congenital toxoplasmosis and inflammatory complications in murine model (Oz and Tobin, 2012). Atovaquone was superior than the standard of care with combined pyrimethamine plus sulfadiazine or pyrimethamine plus clindamycin therapies against brain inflammatory responses and the severity of infection in the mice (Dunay et al., 2004).

MATERNAL CONGENITAL TOXOPLASMOSIS AND DICLAZURIL

Diclazuril [4-chlorophenyl [2,6-dichloro-4-(4,5-dihydro-3*H*-3,5-dioxo-1,2,4-triazin-2-yl)phenyl acetonitrile] is related to herbicides and used to protect poultry and livestock against coccidiosis, induced gastroenteritis, morbidity, and mortality. *Toxoplasma and* coccidians are members of the phylogeny *Apicomplexan* with a highly conserved region of protochlorophyllide with traces of plant chloroplast epitope not present in humans and animals. Apicoplast is an extranuclear DNA organelle containing transcriptional and translational device in *Toxoplasma* with specific enzymes unwinding DNA. It is presumably originated from eukaryotic *Ciliate* ancestors and prokaryotic green alga in evolution (Köhler et al., 1997). Apicoplast has a unique sensitivity to herbicidal agents with a safe and attractive region for drug discovery and vaccine target in *Toxoplasma* metabolic pathway, absent in the humans and animals.

Diclazuril and its related compounds specifically invade and attach to chloroplast epitope and the D1 protein of the *Toxoplasma* apicoplast without interacting to damage the mammalian host organs (Hackstein et al., 1995). Additionally, diclazuril can downregulate expression of serine/threonine protein phosphatase in merozoites of *Eimeria* to induce apoptosis with possible mechanism of action against *Toxoplasma* organisms (Zhou et al., 2013; Oz, 2014).

Diclazuril is a non-toxic agent (Assis et al., 2010) with rapid absorption following oral administration to reach a constant level in plasma and cerebrospinal fluid. Recent studies have shown diclazuril to be well tolerated and effective in murine model for maternal and congenital toxoplasmosis (Oz and Tobin, 2014). While atovaquone protects against some aspects of gastrointestinal complications in experimental congenital toxoplasmosis in murine (Oz and Tobin, 2012), diclazuril was superior than atovaquone in improving anemia, colonic length, and hepatic complications against maternal toxoplasmosis (Oz and Tobin, 2014). In addition, diclazuril and atovaquone combination therapy is anticipated to exert a unique synergistic effect against toxoplasmosis. Diclazuril monotherapy or combination with atovaquone therapy may warrant clinical trials in maternal congenital as well as in ocular and chronic toxoplasmosis. Finally, diclazuril is anticipated to be used as a novel protective and preventive measure to eliminate the cycle of *Toxoplasma* infection in the definitive host, feline.

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Midgut expression of immune-related genes in *Glossina palpalis gambiensis* challenged with *Trypanosoma brucei gambiense*

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Tsetse flies from the subspecies *Glossina morsitans morsitans* and *Glossina palpalis gambiensis*, respectively, transmit *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. The former causes the acute form of sleeping sickness, and the latter provokes the chronic form. Although several articles have reported *G. m. morsitans* gene expression following trypanosome infection, no comparable investigation has been performed for *G. p. gambiensis*. This report presents results on the differential expression of immune-related genes in *G. p. gambiensis* challenged with *T. b. gambiense*. The aim was to characterize transcriptomic events occurring in the tsetse gut during the parasite establishment step, which is the crucial first step in the parasite development cycle within its vector. The selected genes were chosen from those previously shown to be highly expressed in *G. m. morsitans*, to allow further comparison of gene expression in both *Glossina* species. Using quantitative PCR, genes were amplified from the dissected midguts of trypanosome-stimulated, infected, non-infected, and self-cleared flies at three sampling timepoints (3, 10, and 20 days) after a bloodmeal. At the 3-day sampling point, transferrin transcripts were significantly up-regulated in trypanosome-challenged flies versus flies fed on non-infected mice. In self-cleared flies, serpin-2 and thioredoxin peroxidase-3 transcripts were significantly up-regulated 10 days after trypanosome challenge, whereas nitric oxide synthase and chitin-binding protein transcripts were up-regulated after 20 days. Although the expression levels of the other genes were highly variable, the expression of immune-related genes in *G. p. gambiensis* appears to be a time-dependent process. The possible biological significance of these findings is discussed, and the results are compared with previous reports for *G. m. morsitans*.

Keywords: ***Glossina palpalis gambiensis, Trypanosoma brucei gambiense*** **infection, midgut, immune gene expression**

INTRODUCTION

Tsetse flies (*Glossina* sp.) are responsible for the cyclical transmission of protozoan known as trypanosomes, which are the causative agents of Human African Trypanosomiasis (HAT; or sleeping sickness) and Animal African Trypanosomiasis (or nagana) throughout sub-Saharan Africa (Simarro et al., 2003). It is estimated that 60 million people in 36 African countries are at risk of HAT (WHO, 2006). Sleeping sickness is fatal if untreated (Holmes, 2013). No vaccine is available for the mammalian host, as the variant surface glycoprotein (VSG) coating the trypanosome plasma membrane makes the development of a vaccine unlikely. Furthermore, part of this VSG composition and structure periodically varies, which in turn causes periodic antigenic variations that allow the trypanosome to escape both injected and/or natural host-produced antibodies. Finally, this coat prevents antibodies from gaining access to invariant surface molecules (MacGregor et al., 2012). To complicate matters, chemotherapy treatments have major harmful side effects and are difficult to administer (Priotto et al., 2008), and the emergence of parasite resistance has decreased the efficacy of drug treatments (Baker et al., 2013).

The fly vector, which is strictly hematophagous, acquires the parasite during a bloodmeal on an infected host, whether human or animal. To be transmitted, trypanosomes must first establish in the midgut; then they migrate to the salivary glands, where they mature into an infective metacyclic form; they are finally secreted in the saliva during a bloodmeal (Hu and Aksoy, 2006).

In ideal laboratory conditions, 40% or more of challenged flies will eliminate their ingested trypanosomes (Lehane et al., 2003, 2008). For field flies, infection rates rarely exceed 10% of the population (Frézil and Cuisance, 1994). Only a small number of flies are able to transmit parasites to a host (Aksoy et al., 2003; Rio et al., 2004). Approximately 72 h following ingestion of the infected bloodmeal, a process of attrition leads to the complete elimination of the infection in a high proportion of flies, whereas parasites are established in the gut during a successful infection. Flies can be arranged into two groups following this

attrition process: those that are susceptible to infection when trypanosomes are detectable in the fly's gut, and those that are refractory (or self-cleared) when trypanosomes are undetectable (Gibson and Bailey, 2003). Differential expression of midgut effector molecules in different tsetse species or strains may account for the variability in susceptibility to trypanosomes (Haddow et al., 2005). Many other factors are involved in determining the success or failure of the infection and maturation processes (Maudlin and Welburn, 1994). These include fly sex, age, and nutritional status at the time of exposure to infectious trypanosomes (Welburn and Maudlin, 1992); antimicrobial peptides (AMPs; Hao et al., 2001; Hu and Aksoy, 2006); trypanosome-binding lectins (Maudlin and Welburn, 1988; Welburn et al., 1994); gut-associated EP protein (Chandra et al., 2004; Haines et al., 2005, 2010); and reactive oxygen species (ROS; Hao et al., 2003).

Since trypanosomes initiate their cycle within the host midgut, an improved understanding of the differential expression of immunity genes could provide opportunities to identify genes possibly involved in tsetse refractoriness, as well as those involved in active infections.

Previously, Lehane et al. (2003) reported a number of selected genes (including genes related to fly immunity) that exhibit altered expression patterns in response to trypanosome infection, during their establishment in the fly gut. These studies were conducted on insectary-maintained flies belonging to the subspecies *Glossina morsitans morsitans* (initially collected in Zimbabwe) and challenged with *Trypanosoma brucei brucei*. In east African countries, flies of the *morsitans* group transmit trypanosomes belonging to the subspecies *Trypanosoma brucei rhodesiense*, causing the acute form of sleeping sickness. Conversely, *Glossina palpalis gambiensis* (*palpalis* group) flies in West Africa transmit trypanosomes belonging to the subspecies *Trypanosoma brucei gambiense*, causing the chronic form of the disease (Hoare, 1972). We chose to investigate *G. p. gambiensis* challenged with *T. b. gambiense*, as this approach had previously not been utilized to examine this specific *Glossina*/trypanosome couple. Furthermore, our choice enables checking whether the responses of the two *Glossina* subspecies to their, respectively, transmitted trypanosome subspecies are comparable or not. We investigated the *G. p. gambiensis* response at the trypanosome invasion step, as it is determinant in whether the parasite will establish within the fly gut (i.e., flies susceptible to trypanosome infection) or if it will be eliminated (i.e., flies refractory to trypanosome infection, or self-cleared/self-cured flies). Finally, we investigated 12 immune genes selected from those previously reported to be highly over-expressed in *G. m. morsitans* challenged with *T. b. brucei* (Lehane et al., 2003).

MATERIALS AND METHODS

ETHICS STATEMENT

All experiments on animals were conducted according to internationally recognized guidelines. The experimental protocols were approved by the Ethics Committee on Animal Experiments and the Veterinary Department of the Centre International de Recherche Agronomique pour le Développement (CIRAD), Montpellier, France.

T. b. gambiense STRAIN AND FLY INFECTION

The *T. b. gambiense* isolate S7/2/2 used for fly infections was isolated in 2002 by rodent inoculation from a HAT patient detected in the sleeping sickness focus of Bonon, Côte d'Ivoire (Ravel et al., 2006).

Female *G. p. gambiensis* tsetse flies were collected from the CIRAD Baillarguet insectary. Following adult emergence, the population was maintained in a level 2 containment insectary at 23°C and 80% relative humidity (Geiger et al., 2005). This fly colony originated from Burkina Faso, where it was first collected 40 years ago.

A *T. b. gambiense* stabilate was thawed at room temperature and 0.2 ml was injected intraperitoneally into Balb/cj mice. To monitor infection, tail blood was examined using a phase-contrast microscope at 400× magnification. Teneral flies (less than 32 h old) were fed on the abdomens of infected mice (30 flies per mouse, on average); mice displayed parasitemia levels between 16 and 64 $\times$ 10^6 parasites/ml, as determined by the matching method (Herbert and Lumsden, 1976). Only flies that had ingested a large bloodmeal were retained for further studies. After 10-day and 20-day timepoints, anal drops were collected from flies that fed on infected mice, and their infection status was assessed. *T. b. gambiense* presence was determined by PCR of chelex-extracted anal drop DNA using the TBR1 and TBR2 primers (Moser et al., 1989). The presence of parasites in the anal drop was positive indication for midgut infection (i.e., susceptible flies). By contrast, the absence of the parasite indicated that these flies receiving an infected bloodmeal were refractory to infection. Anal drop analysis was selected in this study to determine fly contamination status since the whole midgut was later used for RNA extraction. The prevalence of midgut infection was less than 5% for 10-day flies and greater than 10% for 20-day flies, corresponding with recently recorded values from artificial infection experiments (Ravel et al., 2006; Hamidou Soumana et al., 2014). Using this procedure, flies were separated into infected and self-cured groups (i.e., flies that had ingested trypanosomes in their bloodmeal but had cleared the infection), and dissected according to the method described by Penchenier and Itard (1981). The 3-day group of flies received an infected bloodmeal and was dissected 3 days later; they were compared with 3-day flies fed on an uninfected bloodmeal, considered as control flies. Dissected tsetse fly midguts were collected (pool of seven fly guts per sample) in 400 µl of RNA later reagent and stored at −80°C until RNA extraction.

Sampling times were chosen according to a previously determined time course of susceptible fly infection by trypanosome (Van Den Abbeele et al., 1999; Ravel et al., 2003). The 3-day and 10-day sampling times were respectively, selected to target differentially expressed genes involved in early events associated with trypanosome entry into the midgut, and the establishment of infection. The 20-day time point was selected to target genes involved in events occurring relatively late in trypanosome infection, within its vector.

TOTAL RNA ISOLATION

Midguts were dissected from 3-day flies (fed on either an infected or a non-infected bloodmeal), as well as from infected and

self-cleared flies at 10 and 20 days after the infective bloodmeal. Each timepoint consisted of four biological replicates (seven pooled midguts). Total RNA was then extracted from each sample using Trizol reagent (Invitrogen), according to the manufacturer's specifications. RNA integrity was assessed after extraction using agarose gel electrophoresis. RNA quality and the absence of any DNA contamination were checked on an Agilent RNA 6000 Bioanalyzer and quantified using the Agilent RNA 6000 Nano kit (Agilent Technologies).

IMMUNITY-RELATED GENES AND QUANTITATIVE REAL-TIME PCR PRIMERS

Lehane et al. (2003) identified genes with putative immune-related functions in *G. m. morsitans* following *T. b. brucei* infection. Twelve highly up-regulated genes were chosen from this study to investigate their possible differential expression in either *G. p. gambiensis* refractory flies versus *T. b. gambiense* infected flies (10- and 20-day samples), or in trypanosome-stimulated flies versus control flies (3-day samples). Gene expression was measured by quantitative PCR using specific primer pairs (**Table 1**) designed with the PrimerBlast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Pairs of *G. p. gambiensis* tubulin beta-1 gene-specific primers were designed using the sequences from the *G. m. morsitans* tubulin beta-1 gene (GenBank accession number, DQ377071; Attardo et al., 2006).

cDNA SYNTHESIS AND QUANTITATIVE REAL-TIME PCR

Samples were treated with RNase-free TURBO DNase I (Ambion). First-strand cDNA was then synthesized from 5 μg of total RNA using random hexamers and SuperScript II Reverse-Transcriptase (Invitrogen), according to the manufacturer's instructions. Quantitative PCR was performed in triplicate using 2 μl of cDNA on an Mx3005P QPCR System (Agilent Technologies) and using the Brilliant II SYBR Green qPCR Kit (Agilent Technologies). The *G. p. gambiensis* housekeeping gene tubulin beta-1 was used as the reference gene to calculate the normalization of the relative quantification of expression. Cycle thresholds (Ct) for each reaction were obtained using the MxPRO QPCR Software (Agilent Technologies). PCR conditions were as follows: 94°C for 5 min (1 cycle); 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min (39 cycles); and 72°C for 10 min (1 cycle). The amplification efficiency was checked by the standard curve method, and melting curve analysis was performed to check PCR specificity. Relative quantification was calculated using the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen, 2001) and was determined for a given gene with respect to the calibrator.

STATISTICAL ANALYSIS

Quantitative PCR data was analyzed using the $2^{-\Delta\Delta C(t)}$ method. Data were then normalized against the *G. p. gambiensis* tubulin gene to determine the consecutive gene expression levels between infected and self-cleared flies, or stimulated and naive flies, for three timepoints post-infective bloodmeal (3, 10, and 20 days). A separate Kruskal–Wallis test (Hollander and Wolfe, 1973) was used for each immune gene, with R statistic software (version 2.15.0) for assessing differences in transcript expression levels. The transcription level of genes was expressed as the difference in Ct values and ΔCt values between infected and self-cleared flies.

RESULTS

Midgut transcript responses of *G. p. gambiensis* were assayed using quantitative PCR amplification of selected immune-related genes at 3, 10, and 20 days after *T. b. gambiense* challenge. Transcription analysis of the multiple timepoints following the challenge were used to access information about the temporal kinetics of gene regulation in the host midgut response against trypanosome establishment.

TRANSCRIPT VARIATION 3 DAYS AFTER FLY TRYPANOSOME CHALLENGE

Variation in transcript expression level was assessed at the early stage of infection by comparing 3-day flies that received an infective bloodmeal with 3-day flies fed on a non-infected bloodmeal.

Table 1 | Primers of immunity-related genes designed for quantitative PCR.

Gene symbol	Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)	Accession number
GMOY003306	EP protein	GCT-GAA-GTT-GGG-AAG-ACT-GC	AGC-TTG-CTC-GAA-AGC-TTG-AT	108	AY077716.1
GMOY010190	Transferrin	CAA-CGG-GCT-TGA-GTT-TAT-CA	GTC-CCG-AAT-TGG-AAT-GTG-TC	128	AF368908.3
GMOY001942	Chitin binding protein	TGG-TTT-TGC-CGA-TGT-TCA-TA	CAA-CCC-ATC-TCC-TCC-CAT-AA	108	DQ307192.1
GMOY002442	Serpin-1	AAG-GTG-ACC-CCG-TTG-ATG-TA	ACC-TGC-TAG-GTT-AGC-GTT-CG	123	JQ312066.1
GMOY005573	Sphinginase	TCC-GAT-ATT-CCC-AGC-GTT-AG	CAC-TTT-GAG-GTA-GCC-AAC-GAC	122	JQ308535.1
GMOY000597	Thioredoxin peroxidase-2	TAA-TTC-GTG-TGC-GGA-AGA-TG	TTG-GAA-ATG-ACT-GCC-TTG-GT	117	AY625506.1
GMOY003093	Nitric oxide synthase	GGC-TTT-TCT-TTG-GTT-GTC-GT	CGG-TGT-ATT-TGG-TTC-TCT-GGA	122	AY152725.1
GMOY002443	Serpin-2	AGA-GTC-CCG-AAG-ATT-TGC-AT	TAT-AAA-TTG-CGT-GGG-CAA-CA	137	JQ312067.1
Gmm0601	Thioredoxin peroxidase-3	TTG-CTG-TGG-TAG-GCA-AAG-AA	TTT-AAT-GCG-CTC-GCT-AAA-AGA	137	AY625502.1
GMOY003656	Serpin-4	TTC-TCC-CTT-TGC-TGT-GTG-GT	ACG-CCG-AAC-GTA-TAA-CTT-GC	122	JQ312069.1
GM-489	C1-Tetrahydrofolate synthase	TAA-TTC-CGG-TTT-CCG-TAT-TCA	CGG-CTT-CGT-GGT-AGC-TAT-GT	101	EZ422151.1
GMOY000148	*Glossina* tubulin	CCA-TTC-CCA-CGT-CTT-CAC-TT	GAC-CAT-GAC-GTG-GAT-CAC-AG	149	DQ377071

The infected bloodmeal induced a significant increase ($p < 0.05$) in transferrin transcripts 3 days after the trypanosome challenge (**Figure 1A**).

GENE EXPRESSION IN INFECTED VERSUS SELF-CLEARED FLIES

Comparison of infected and self-cleared flies showed that serpin-2 and thioredoxin peroxidase-3 were expressed significantly higher ($p < 0.05$) in self-cleared flies at 10 days post-challenge with the trypanosome (**Figures 1B,C**, respectively). At 20 days post-infected bloodmeal, nitric oxide synthase (NOS) and chitin-binding protein were significantly overexpressed in refractory tsetse flies versus infected flies ($p < 0.05$; **Figures 1D,E**, respectively). Most of the other selected genes displayed differences in gene expression between refractory and infected flies, although their recorded differences were not statistically significant.

EXPRESSION LEVEL IN SUSCEPTIBLE FLIES THROUGHOUT THE COURSE OF THE INFECTION

By comparing transcript levels at the three timepoints post-challenge with the parasite, we observed a decrease in the expression of chitin-binding protein transcripts along the progression of the infection ($p = 0.03$) for stimulated flies (3-days sampling timepoint) as compared to infected flies sampled 10 days post-infected bloodmeal uptake. Similar results were recorded for the chitin-binding protein transcript expression level, when comparing tsetse flies infected for 10 and 20 days ($p = 0.02$).

DISCUSSION

In the present study we investigated the expression profile of immune-related genes in *G. p. gambiensis* following a *T. b. gambiense* challenge. The expression level of selected genes was compared at three crucial time points of the infection process using quantitative PCR.

Twelve immune-related genes were selected on the basis of their high differential expression in the *G. m. morsitans*/*T. b. brucei* couple, as previously reported by Lehane et al. (2003). In the *G. p. gambiensis*/*T. b. gambiense* system, only 5 of these 12 genes displayed different expression profiles between trypanosome-challenged flies and control flies.

Nitric oxide is a signaling and immune effector molecule synthesized by the NOS (Bayne et al., 2001; Rivero, 2006). NOS production is induced in *Drosophila* midgut and hemocytes

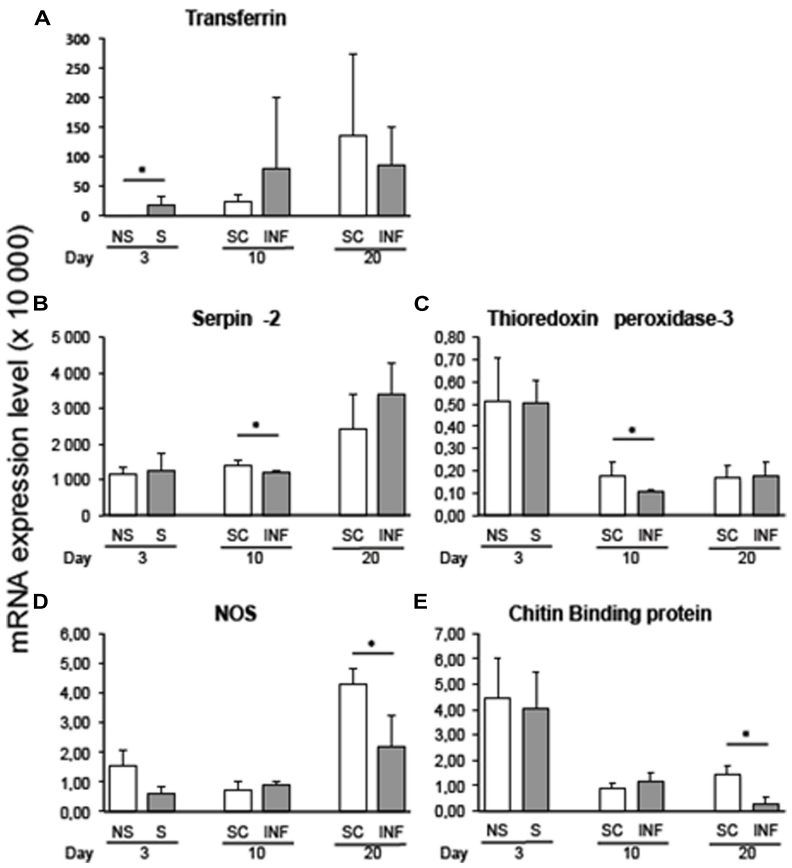

FIGURE 1 | qRT-PCR expression analysis of immune-related *Glossina palpalis gambiensis* genes at 3, 10, and 20 days post-challenge with *Trypanosoma brucei gambiense*, normalized against the *G. p. gambiensis* tubulin gene. (A) Transferrin; **(B)** Serpin-2; **(C)** Thioredoxin peroxidase-3; **(D)** NOS; **(E)** Chitin binding protein. The "*" represents significant difference between infected and self-cleared samples ($p < 0.05$).

challenged with bacteria or parasitoids; in mosquito it was described as a midgut-associated parasite antagonist that kills *Plasmodium* ookinetes (Peterson et al., 2007). Our results, showing a significant up-regulation of NOS transcripts in self-cleared flies at 20 days, are in agreement with those previous findings. Nitric oxide could be a part of the process leading to *T. b. gambiense* clearing in *G. p. gambiensis*. This has been demonstrated by injecting a specific NOS inhibitor into *Drosophila* body cavity prior to infection, which significantly increased parasite survival (Carton et al., 2009). However, Hao et al. (2003) showed NOS to be down-regulated by infection and not modulated by tsetse age. Its host immune response involvement may depend on the tsetse/trypanosome species couple.

Digestion of the bloodmeal can also generate ROS, which may cause damages such as enzyme inactivation, DNA degradation, and deterioration of the cellular membrane (Droge, 2002). No difference was found in the expression of thioredoxin peroxidase-2 between infected and self-cleared flies, while thioredoxin peroxidase-3 was significantly up-regulated in 10-day self-cleared flies. This enzyme may offer protection against ROS generated during the immune response (Lehane et al., 2003).

Among the three serpin (serine protease inhibitor) genes investigated, only serpin-2 was significantly over-expressed in self-cleared flies at 10 days post-challenge. The main molecular functions attributed to serpins range from the inhibition of blood coagulation to host inflammation and platelet aggregation, which are likely crucial for blood-feeding insects (Stark and James, 1995; Chmelar et al., 2011). Immune-related CLIP domain serine proteases and their inhibitors, the serpins were previously identified in *G. morsitans* (Mwangi et al., 2011). Serine proteases play an important role in the activation of the Toll or IMD pathways. Many serine proteases involved in the immune response exist in a fine balance with serine protease inhibitors to ensure that the impact of protease-activated cascades remains localized in time and space (Muta and Iwanaga, 1996; Jiang and Kanost, 2000). *Drosophila* serpin also plays a role in the regulation of Toll-mediated antifungal defense (Levashina et al., 1999; Ahmad et al., 2009). The large number of serpin transcripts found in the tsetse midgut may reflect the need to inactivate the complement and coagulation cascades of the bloodmeal, so as to protect the midgut epithelium and retain the meal in a physical state suitable for digestion. Thus, differences in serpin gene expression between the different groups of *G. p. gambiensis* flies fed on blood may reflect differences in their function according to the flies' status.

Surprisingly, no significant changes were found in *G. p. gambiensis* EP protein transcript levels at any stage of the *T. b. gambiense* infection. In *G. m. morsitans,* EP protein was strongly up-regulated following fly challenge with Gram-negative bacteria, as well as in response to trypanosome infection (Haines et al., 2005, 2010). Furthermore, tsetse EP protein may be involved in immune modulation, as RNAi knockdown increased susceptibility to trypanosome infection. Tsetse EP protein transcript levels are, however, dramatically reduced after 3 days of starvation (Haines et al., 2010). In our study, flies were starved for 3 days prior to dissection to remove any bloodmeal in the fly gut, which could explain the absence of variation in EP protein transcripts. Akoda et al. (2009), however, reported starvation to result in a significant reduction in non-induced baseline immune gene expression, but only after a longer starvation (4 days for newly emerged flies; 7 days for older flies).

In hematophagous insects, iron-binding protein is essential for sequestering iron, which overabundance can quickly lead to oxidative stress, a potentially destructive process for membranes, proteins, and nucleic acids. The transferrin gene expression level was significantly increased in 3-day stimulated flies versus control flies. This observation is in agreement with results reported on transferrin transcription in mosquito (Yoshiga et al., 1997) and in *Bombyx mori* (Yun et al., 1999). In tsetse flies and other insects, transferrin plays multiple physiological roles in immunity, iron metabolism, and reproduction, and displays tissue-dependent expression levels (Nichol et al., 2002). As shown in other insects, transferrin mRNA levels increase upon bacterial challenge in tsetse, suggesting that transferrin may play an additional role in immunity (Guz et al., 2007). In contrast, tsetse flies that had cleared the trypanosome did not show any difference in transferrin transcript levels when compared with infected flies at 10 and 20 days post-infected bloodmeal. Similar results were reported by Lehane et al. (2003) for *T. b. brucei* infected *G. m. morsitans* versus self-cleared flies. The parasite, competing in limited dietary iron environment, may modulate host gene expression.

Chitin-binding protein gene expression increased significantly in self-cleared flies 20 days after the infected bloodmeal. Chitin is the main constituent of the peritrophic membrane (PM), a physical barrier preventing trypanosome entry into the ecto-peritrophic space, and thus constitutes an obstacle to parasite establishment in the midgut. This chitin-binding gene is homologous to the *Drosophila* gene *chit*, which encodes a chitinase-like protein (Kawamura et al., 1999). In *Sodalis glossinidius*, the secondary symbiont of the tsetse fly that favors fly infection by trypanosomes, a homologous gene encodes a chitinase that was previously hypothesized to hydrolyze pupal chitin into glucosamine which inhibits the fly midgut lectin lethal to procyclic forms of the trypanosome (Welburn and Maudlin, 1999). Based on the trypanosome developmental cycle within the tsetse fly, one would expect the increase in chitinase gene transcripts to occur much earlier than the observed 20 days post-infected bloodmeal. In *Anopheles*, for example, chitin-binding protein and the enzyme involved in PM formation both displayed increased expression 3–24 h after the bloodmeal in all flies analyzed, independent of their infection status (Dimopoulos et al., 1998). This does, however, raise an additional question on the actual role of this protein in *Anopheles*.

The overall results on *G. p. gambiensis* immune-related genes shows their expression to be highly dependent either on the stage of trypanosome invasion and/or the status of the fly (i.e., susceptible or refractory to trypanosome infection). The midgut response represents part of the *Glossina* defense arsenal against trypanosomes. Nevertheless, important variability between individual tsetse fly responses to trypanosome infection was observed. This variability could be due to variation in the size of the infected bloodmeal, and in turn to the differences in the number of ingested parasites; it

could also be due to normal biological variability in the individual host's response. In addition, significant differences were noticed between the *G. p. gambiensis* gut immune-related response and that displayed by *G. m. morsitans* following infection of the gut with *T. b. brucei*. These results strongly encourage broader investigations aimed at evaluating and identifying the factors causing these differences between the *G. p. gambiensis* and *G. m. morsitans* responses. Improved understanding in this domain is expected to be particularly relevant to identify common gene targets that would be suitable for controlling both forms of sleeping sickness. Transcriptional analysis is expected to provide data that are at the basis of the physiological response(s) of an organism to any perturbation. The recorded data will, in turn, provide further research directions that could consist, in a next step, in a proteomic analysis to assess, whether or not, the expressed genes are really translated into the corresponding proteins, and, finally, which role they actually play.

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