Fifteen Years of Toxoplasmosis Screening at the Institute of Tropical Medicine, a Diagnostic Reference Center in Venezuela

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ABSTRACT: Toxoplasmosis serodiagnosis was analyzed in the Instituto de Medicina Tropical in Caracas during 1999–2013. Subjects for toxoplasmosis diagnosis were grouped into five categories: pregnancy, human immunodeficiency virus (HIV)/acquired immune deficiency syndrome, ocular pathology, lymphadenopathy, and others. Testing for specific anti-*Toxoplasma* IgM/IgG antibodies, as well as IgG avidity, was performed. Analysis of 68,622 individuals resulted in an overall prevalence of 50.9%, with 50.9% in pregnant women, 63.8% in patients with eye lesions, 53.1% in HIV-positive individuals, 33.3% in patients with lymphadenopathy, and 49% in other individuals. *Toxoplasma gondii* infection in pregnant women aged ≤15 years was 32%, which increased to 64% in the middle age category. Diagnostic tests detected 54 recent infections in pregnant women and their follow-up showed no congenital infection. The present age-related and comorbidity prevalence data should be used to design control measures to prevent congenital transmission, coinfection in immunosuppressed patients, and eye lesions in children.

KEYWORDS: toxoplasmosis, IgG avidity, pregnancy, HIV, Venezuela

CITATION: Mauriello et al. Fifteen Years of Toxoplasmosis Screening at the Institute of Tropical Medicine, a Diagnostic Reference Center in Venezuela. *Human Parasitic Diseases* 2016:8 1–9 doi:10.4137/HPD.S36806.

TYPE: Original Research

RECEIVED: October 23, 2015. **RESUBMITTED:** November 29, 2015. **ACCEPTED FOR PUBLICATION:** Decemeber 2, 2015.

ACADEMIC EDITOR: Ashley Croft, Editor in Chief

PEER REVIEW: Five peer reviewers contributed to the peer review report. Reviewers' reports totaled 1161 words, excluding any confidential comments to the academic editor.

FUNDING: Financial support is provided by the IS of the "Instituto de Medicina Tropical" from its own income. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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Paper subject to independent expert blind peer review. All editorial decisions made by independent academic editor. Upon submission manuscript was subject to anti-plagiarism scanning. Prior to publication all authors have given signed confirmation of agreement to article publication and compliance with all applicable ethical and legal requirements, including the accuracy of author and contributor information, disclosure of competing interests and funding sources, compliance with ethical requirements relating to human and animal study participants, and compliance with any copyright requirements of third parties. This journal is a member of the Committee on Publication Ethics (COPE).

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Introduction

Toxoplasmosis is a parasitic zoonosis that is caused by the intracellular protozoan *Toxoplasma gondii*, which can invade any cell type and many species of mammals and birds.[1] At least one-third of the infected adult human population has specific antibodies against the parasite, which makes it one of the most common parasitic infections in rural and low-socioeconomic-level areas in warm and humid regions.[2,3] Generally, human toxoplasmosis infection is benign and goes unnoticed, or its clinical symptoms are flu-like fever, headache, sore throat, inflammation of lymph nodes, and muscle aches. Toxoplasmosis is a clear example of an infection that is not necessarily followed by a disease. However, severe clinical disease may occur when a primary infection is acquired during pregnancy or when the host immune system is compromised, as in transplant patients who undergo immunosuppressive therapies or in subjects infected with human immunodeficiency virus (HIV).[4] An intact immune system is needed to control the parasite that remains in tissues and is not associated with the pathology attributable to *T. gondii* infection in immunocompetent subjects. This balance between the natural immune response of the host[5] and the variable virulence of the three clonal lineages (types I, II, and III) present in geographic areas[6] can determine the course of infection.

There are many difficulties for toxoplasmosis diagnosis, beginning with clinical suspicion or identification, since toxoplasmosis can occur under different categories without following a typical symptom pattern. As a consequence, high seroprevalence in the general population and difficulty in isolating the parasite often result in a lack of parasitic confirmation despite clinical suspicion. Thus, the association of positive serology with suggestive clinical forms of the disease does not necessarily mean *T. gondii* causality.

In order to measure the attributable risk by *T. gondii* in Venezuela for primary infection in high-risk population groups, such as children, pregnant women, and patients with HIV, the present study analyzed 15 years (1999–2013) of screening *T. gondii* data from outpatients attending the Instituto de Medicina Tropical (IMT) of the Universidad Central de Venezuela. The scope of current *T. gondii* diagnosis and further recommendations are discussed.

Methodology

Personal data and medical history. Personal and clinical information was recorded from each applicant for *T. gondii* diagnosis at the IMT outpatient clinic. Patients were classified according to the motive for toxoplasmosis testing request: pregnancy, lymphadenopathy, ocular pathology, HIV/acquired

immune deficiency syndrome (AIDS), or others (ie, abortions). Patients attended the outpatient service voluntarily, or via physician's reference. Records and samples were also received from other health centers in the country. All subjects provided consent for a 5 mL blood sample obtained by venipuncture. Pregnant women with negative serology for toxoplasmosis were encouraged to repeat the test during the second and third trimesters. Results from only the first serum sample of patients were included in this study. Because this study comprised a retrospective analysis of patient records and samples obtained during the course of clinical care, the research was exempt from the requirement to seek ethics committee approval.

The Immunology Section (IS) of the IMT has used an enzyme-linked immunosorbent assay (ELISA) since 1994 for the detection of both IgG and IgM to *T. gondii*. Due to the persistence of anti-*Toxoplasma* IgM for months or even years after acute infection,[7,8] positive results may not imply a recent infection,[9] thereby requiring a second test to orientate the time of infection. Additionally, at the IMT, the intradermal test (IDT) was used between 1999 and 2002, which was substituted with the measurement of anti-*Toxoplasma* IgG antibodies avidity between 2003 and 2013. This test is based on the disruptive power of the antigen–antibody complex by a destabilizing hydrogen-bonding agent such as urea; it was first introduced for serodiagnosis of rubella in 1988.[10] It has been shown to be very useful in dating *Toxoplasma* infection, especially in pregnant women with a suspected recent infection.[11]

Antigens. A tachyzoite suspension was prepared from a pool of exudates from intraperitoneal mouse infection with Venezuelan parasite isolates (Neria, J and PJR, arbitrary names given to the parasite isolates). The supernatant of washed and centrifuged parasites was used as the soluble antigen according to Jacobs and Lunde,[12] which was modified by Maekelt et al for all diagnostic tests.[13]

Intradermal test. A *Toxoplasma* lysate was obtained from the peritoneal exudates of infected mice and was preserved in a 0.25% phenol solution for intradermal application and evaluation at 48 hours.[14]

ELISA-IgG and ELISA-IgM anti-*T. gondii*. The IgG ELISA was performed according to the modified method of Voller.[15] Polystyrene Maxi Sorp plates (Nunc) coated with the *Toxoplasma* antigen (2 µg protein/well) were used. Fifty microliters of 1/50 diluted serum sample with 5% (w/v) skimmed milk was added in duplicate and incubated at 37°C for 40 minutes. The plates were washed three times with phosphate-buffered saline (PBS)–0.05% (v/v) Tween-20 (PBST), followed by addition of 50 µL of 1/500 antihuman IgG conjugated to alkaline phosphatase and incubation at 37°C for an additional 40 minutes. The same washing procedure was repeated. Fifty microliters of the substrate *p*-nitrophenyl phosphate (pNPP) diluted in diethanolamine buffer, pH 9.6 (1 mg pNPP mL^{-1}), was added and incubated at 37°C for 15 minutes. To stop the reaction, 50 µL of NaOH (1 N) was added and the results were read at 405 nm on a TECAN plate reader Spectra Classic. The cutoff was set at 0.230 optical density (OD), measured as the average OD (+ 2 standard deviation) of 100 sera from patients negative for polyvalent-IgG ELISA, ELISA IgG, and IgM to *T. gondii*. The same technique was used for the detection of IgM by coating the plates with 5 µg of protein/well and using an anti-IgM alkaline phosphatase conjugate. Based on the IDT described, the sensitivity and specificity of the ELISA using the soluble antigen of *Toxoplasma* were found to be 97% and 100%, respectively.[16]

ELISA-IgG avidity. IgG avidity was measured using the modified methods.[13,17] Plates, antigen, and conjugates are the same as described earlier. Sera were placed in replicate *T. gondii* antigen-coated wells. After incubation at 37°C for 30 minutes, one well was washed with PBST for the conventional ELISA and the other was washed three times for 5 minutes with urea (6 M) dissolved in PBST. Both wells were washed with PBST, and the subsequent steps are the same as those described earlier. Results were interpreted as standardized by the IS-IMT: low-avidity antibodies (<30%) are related to the early stages of infection, high-avidity antibodies (>50%) are correlated with the chronic phase, and the cases that fall between 30% and 50% should be followed up.[13,18]

Results

Toxoplasmosis prevalence. A total of 73,112 requests for toxoplasmosis immunodiagnostics were received from January 1999 through December 2013 in the IS-IMT, of which 68,622 had complete data for prevalence analysis. Most data loss occurred for the "others" group due to: (1) samples sent from other centers with no age or motive for testing, (2) women with multiple abortions who did not give their age, or (3) volunteer patients who did not wish to declare the motive for toxoplasmosis diagnosis. Specific anti-*Toxoplasma* IgG antibodies resulted positive in 34,990 individuals, constituting 50.9% of all patients (Fig. 1). The number of examinees was 1,62-fold higher in 2013 as compared to that in 1999, while the seroprevalence declined from 59,8% in 1999 to 42% in 2013 (Fig. 2). The average prevalence in the first five years

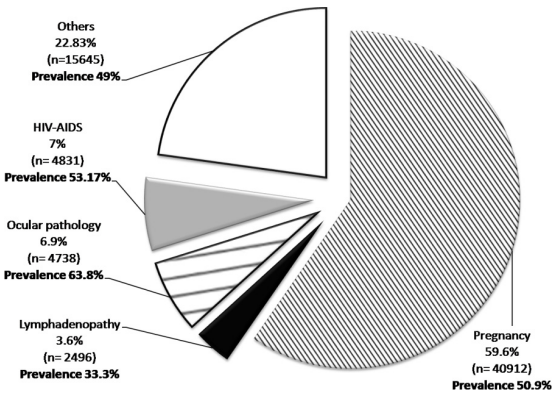
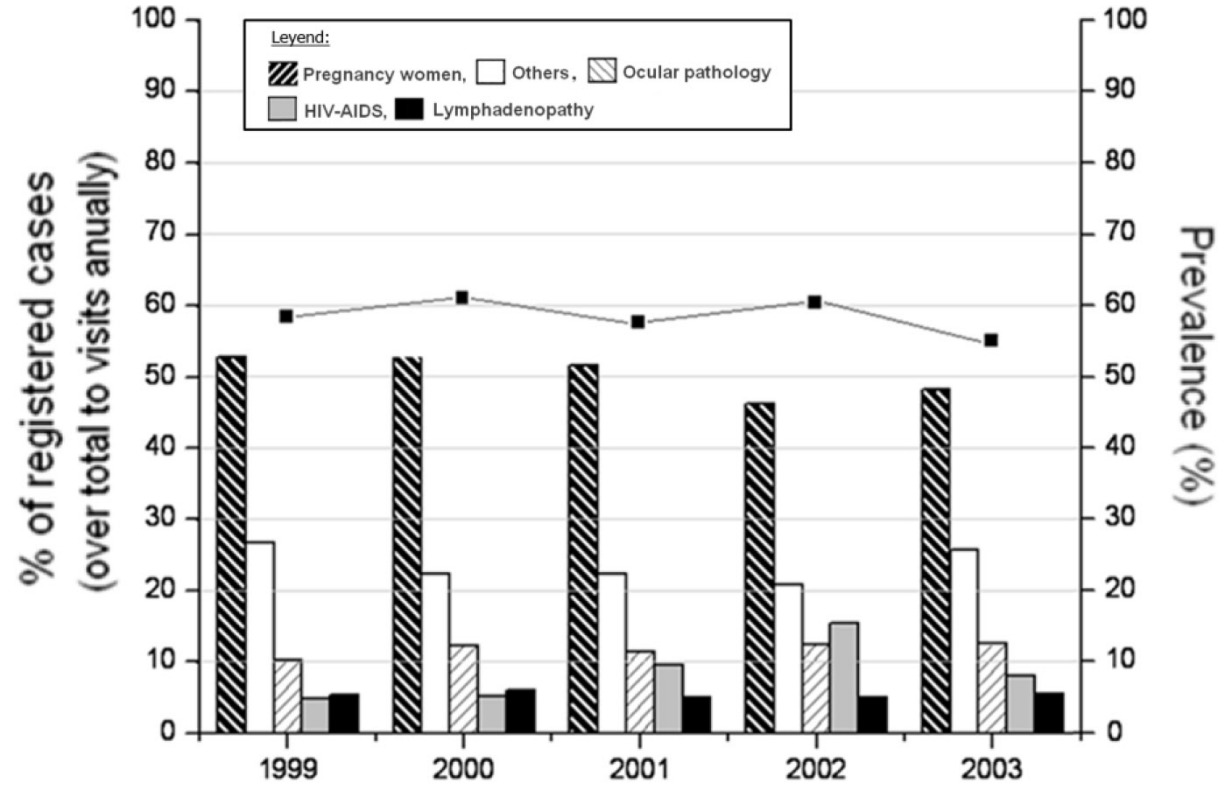
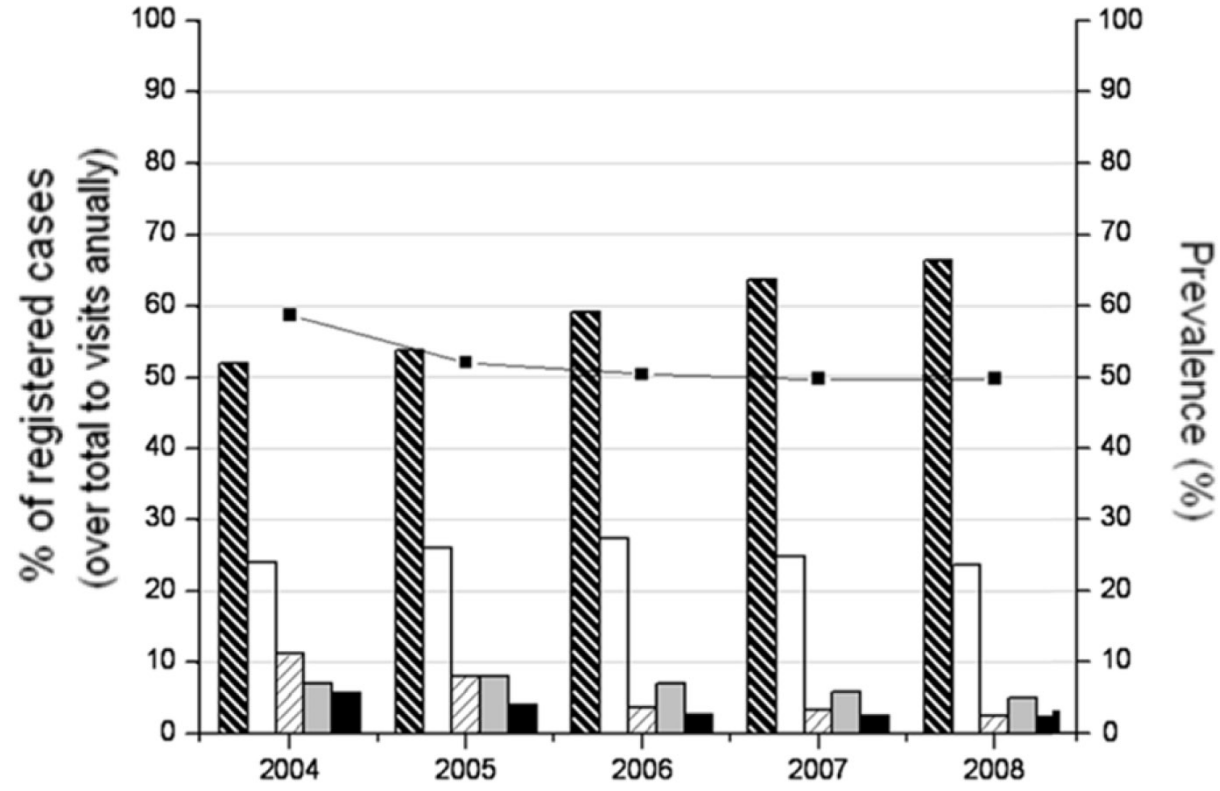


Figure 1. Prevalence of toxoplasmosis during the period 1999–2013 diagnosed at the Immunology Section of the "Instituto de Medicina Tropical, Universidad Central de Venezuela" according to the consultation motive in 68,622 individuals.



Total requests	3048	3043	3210	2819	3359
Positives	1824	1869	1903	1688	1897
Prevelance (%)	59.8	61.4	59.2	60.4	56.4



4038	5073	5930	5889	6545
2310	2592	2966	2960	3254
57.7	51	50	50	49.7

Figure 2. (*Continued*)

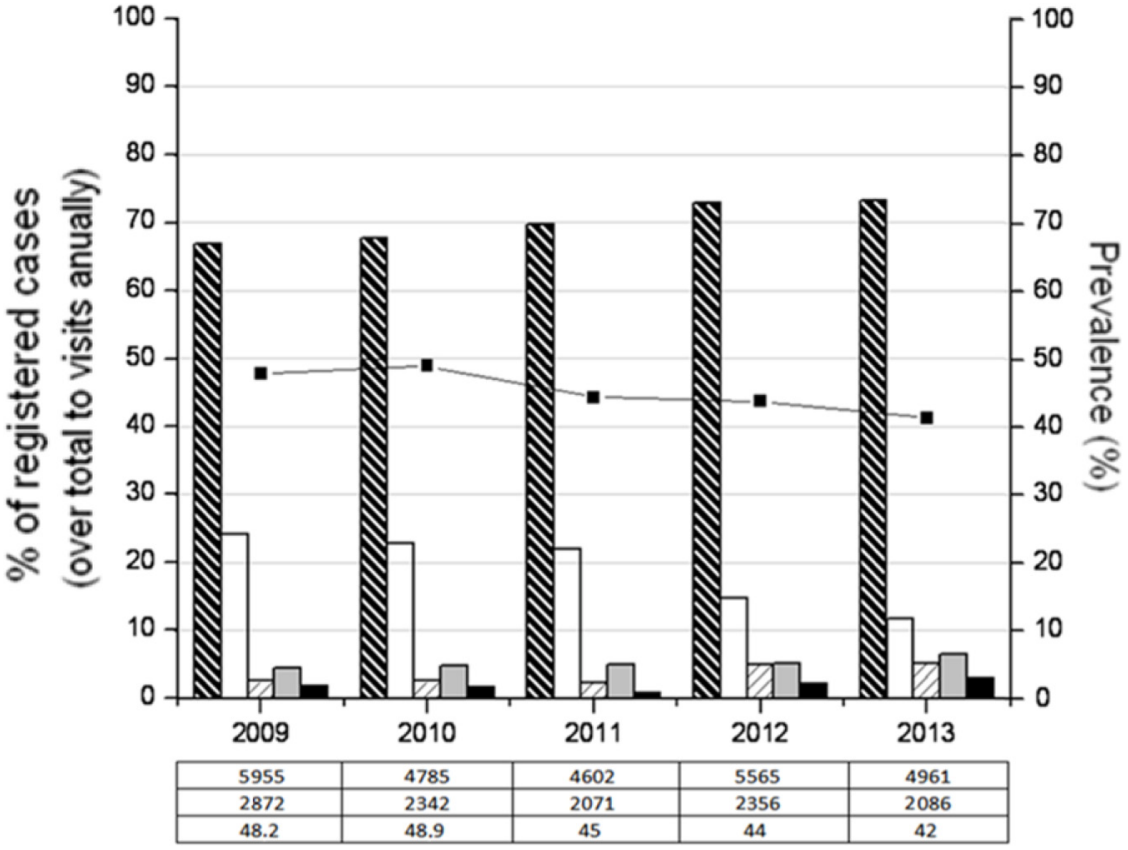


Figure 2. Annual distribution of consultation applications, their motives, and toxoplasmosis prevalence during the period 1999–2013 (Immunology Section of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela).

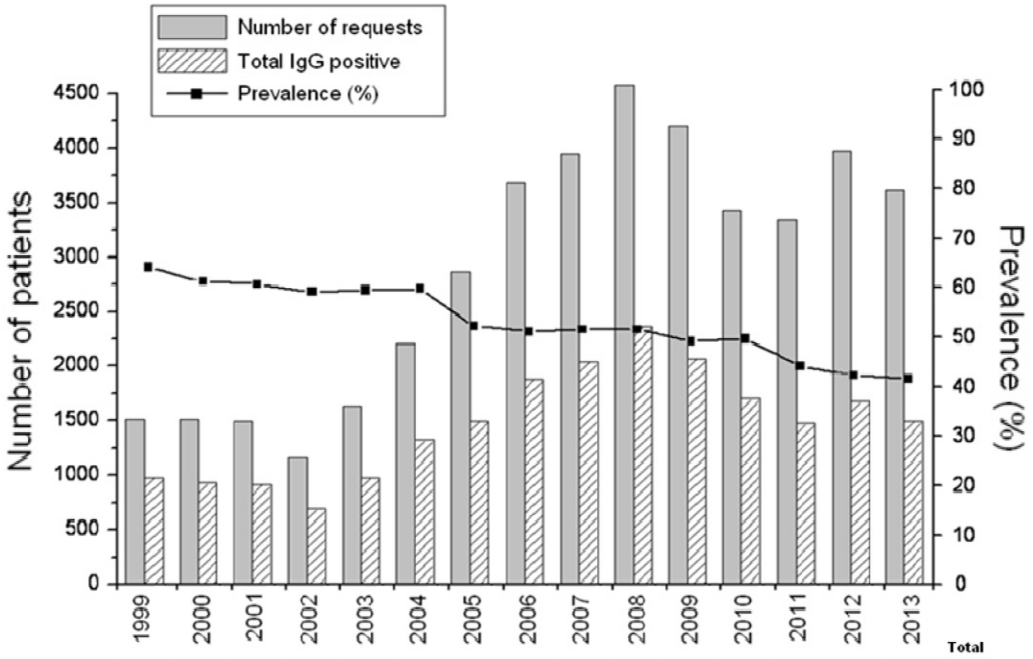
was 59,4%, which progressively decreased to 45.6% in the last five years (2009–2013).

Motive for *Toxoplasma* testing, its annual frequency, and age distribution. The most frequent motive for consultation was toxoplasmosis screening for pregnant women (59.6%), followed by the "others" group (22.8%), HIV/AIDS patients (7%), ocular pathology patients (6.9%), and lymphadenopathy patients (3.6%). The seroprevalence rates in each of these groups were 50.9% in pregnant women, 63.8% in ocular pathology patients, 53.1% in HIV/AIDS patients, and 33.3% in patients with lymphadenopathy (Fig. 1). The annual prevalence and its distribution according to the consultation motive are shown in Figure 2. Pregnancy was the most frequent motive for a diagnostic request each year over the period. Figure 3 and 4 show the annual prevalence and its age distribution, respectively. The prevalence was 32% in pregnant women below 15 years of age (*n*: 310), which increased with age to 64% in women aged ≥41 years. Most of the examined women were aged between 16 and 35 years (Fig. 4).

The results for other diagnostic consultation motives according to age group are summarized in Table 1. Screening for toxoplasmosis in HIV/AIDS patients has increased in recent years, with a corresponding decline in prevalence. A total of 4,831 patients were tested for this motive and specific IgG was found in 2,569 patients (53.17%); 22 children younger than 10 years old (0.85%) were coinfected with HIV and *Toxoplasma*. In 2,496 individuals with a localized or generalized lymph node syndrome, 831 were IgG positive for *T. gondii* (33.3%), of which 284 were children below 10 years of age (34.2%). The highest seroprevalence was measured in the category with ocular pathology. A total of 4,738 persons consulted with ocular pathology and 3,026 of them (63.8%) had anti-*Toxoplasma* antibodies; 7.7% of the positive cases were children aged below 10 years.

IgM, IgG, and avidity of the IgG results. From 1999 through 2002, when toxoplasmosis infection diagnosis in the IS was performed using ELISA IgG/IgM and IDT, out of 8,726 pregnant women, 5,446 (62.4%) were positive for IgG. Specific IgM was positive in 35 (0.64%) of those having negative IDT.

Since April 2003, routine IgG avidity test was performed in 17,182 pregnant women. An avidity of <30% was observed in 19 (0.11%) women, indicating recent infection. Only 14 patients had positive IgM (seven women aged 21–25 years, four women aged 26–30 years, and three women aged 31–35 years). All patients with recent infection received specific antiparasitic treatment, and no case of congenital infection was detected during monitoring of healthy newborns for one year, based on the follow-up with both IgG and IgM. Intermediate IgG avidity (between 31% and 49%) was seen in 149 pregnant women (0.86%) and the remaining



	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Total
Applicants request	1432	1433	1420	1102	1549	2094	2716	3488	3742	4343	3981	3251	3164	3770	3427	40912
IgG positive	924	881	865	654	925	1255	1421	1784	1932	2242	1957	1613	1397	1588	1420	20858
Prevalence (%)	64.5	61.5	60.9	59.3	59.7	59.9	52.3	51.1	51.6	51.6	49.2	49.6	44.2	42.1	41.4	50.9

Figure 3. Annual distribution of diagnostic requests for toxoplasmosis and prevalence in pregnant women during the period 1999–2013 (Immunology Section of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela).

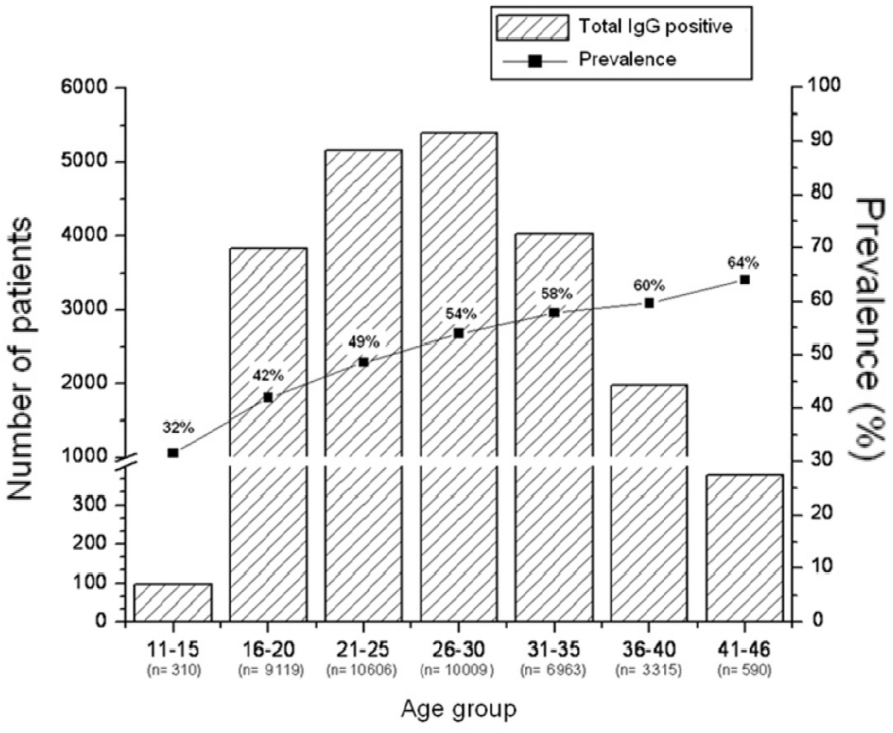


Figure 4. Prevalence of toxoplasmosis by age group in pregnant women during the period 1999–2013 (Immunology Section of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela).

Table 1. Prevalence of IgG anti-*T. gondii* in patients according to consultation motives other than pregnancy and age group during the period 1999–2013 (Immunology Section of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela).

REASON FOR CONSULTATION (TOTAL NUMBER OF PATIENTS)	AGE GROUP OF REACTIVE PERSONS	
	≤10 YEARS n (%)	≥11 YEARS n (%)
Lymphadenopathy (n = 2496)	284 (34.18%)	547 (65.82%)
Ocular symptoms (n = 4738)	229 (7.57%)	2797 (92.43%)
HIV—AIDS (n = 4831)	22 (0.85%)	2547 (99.1%)

17,014 (99%) had avidity values above 50%, the latter cases interpreted as chronic *Toxoplasma* infections (Table 2). In the latter two groups, IgM was negative.

Discussion

The diagnosis of toxoplasmosis is usually suspected based on epidemiological information and clinical symptoms, but in clinical practice, it is confirmed by direct (parasitological) or indirect (immunological) tests. Using serology to measure specific *T. gondii* antibodies is the initial method for diagnosis. A test panel or combination including the measurement of IgM, IgG, IgA, IgE, and IgG avidity should be used to determine whether positive results are consistent with a recently acquired or a chronic infection.[18,19] However, there are clinical conditions in which these techniques and others such as Western blotting (WB)[19,20] and polymerase chain reaction (PCR)[21,22] should be run in specific body fluids, such as amniotic fluid in high-risk pregnancies,[21,22] cerebrospinal fluid in cerebral toxoplasmosis,[23] and aqueous humor for ocular disease.[20] The combination of techniques and sample type can lead to a more reliable diagnosis to estimate the time course of infection with *T. gondii*. Traditional serology is routine for screening laboratories. In reference laboratories, confirmatory tests such as WB and DNA detection methods should also be available for special cases. The IS-IMT is currently incorporating PCR (targeting a 529-bp repeat element for the diagnosis of toxoplasmosis) routinely for cases with positive IgM or low IgG avidity of anti-*Toxoplasma* antibodies.

Table 2. Avidity of IgG anti-*T. gondii* in pregnant women according to the age group during the period 2003–2013 (Immunology Section of the Instituto de Medicina Tropical, Universidad Central de Venezuela, Caracas, Venezuela).

AGE RANGE	AVIDITY OF IgG		
	<30%	31%–49%	>50%
0–14	0	2	100
15–20	2	26	3155
21–25	7*	26	4097
26–30	5**	42	4341
31–35	4***	25	3351
36–40	0	24	1649
41–45	1	4	321
Total	19	149	17014

Notes: Patients with positive IgM associated: *7 patients; **4 patients; ***3 patients.

Although the overall toxoplasmosis prevalence in the IMT varied by 20% over the period, the average 15-year prevalence was 50.9%, suggesting that half of the catchment population has specific IgG anti-*T. gondii* antibodies. Consequently, the association of positive IgG serology with this causal pathogen for recent infections is inadequate, unless it is complemented by direct evidence of the parasite (parasite isolation) or parasite DNA, or serology for indicators of recent infection, IgM, and IgG avidity. The prevalence presented herein is biased due to the fact that it is performed in a toxoplasmosis diagnosis reference center (50.9%); nonetheless, the prevalence reported by us in prenatal outpatients of a general hospital was 38%,[18] which is also high. Seroprevalence was similar within the "other" group (n = 15,645), among women with a recent or recurrent abortion, individuals with other conditions, and those who wanted diagnosis without any apparent clinical reason. It is possible that this group included subjects with HIV who did not wish to be identified and therefore did not provide full details.

The overall prevalence of toxoplasmosis in Venezuelan pregnant women referred to the IMT was 50.98% (20858/40912, Fig 3), which should motivate obligatory regulation of screening in women of childbearing age, preferably before pregnancy, to assess risk and appropriate public health strategies. Seronegative women should be screened monthly during pregnancy, as done in France.[24] There was a good correlation between IgM and IgG avidity for the 19 pregnant women with recent infection. Clinician management of the intermediate avidity group requires the evaluation of positive IgM, patient's symptoms, and epidemiologic risk for appropriate medical treatment.

Infection in 32% of pregnant women aged <15 years indicates *T. gondii* exposure since childhood. Early pregnancy does represent not only a social problem but also the risk for acute toxoplasmosis during pregnancy, in most cases undesirable. However, none of the pregnant women with recent toxoplasmosis (IgM positive and IgG avidity of <30%) belonged to the group younger than 15 years old. *T. gondii* hyperseropositivity (97.2%) was reported among 465 women for premarriage medical examination from Iran.[25] Early exposure to *T. gondii* during childhood benefits women since they will have acquired immunity that reduces the risk of acute infection

after fertility initiates and during pregnancy, thereby decreasing the probability of infection of the newborn. This may be the explanation for the low frequency of congenital cases. In another study conducted by us, in outpatients at the "Hospital Universitario de Caracas" (HUC) in 2010, 10 recent toxoplasmosis infections were diagnosed.[18] Consequently, during the 11-year period (since 2003 when the avidity of IgG was established at the IMT until December 2013), 29 pregnant women with recent infection were diagnosed at the IMT, 19 in the present study and 10 out of 678 pregnant women in the HUC in 2010. Active infection with *T. gondii* can severely compromise the gestation product, causing prematurity, foetopathy, irreversible brain damage, and neonatal death;[2–4] thus, screening during pregnancy is very important and still better before a woman conceives.[25] A study in France reported congenital infection in 513 newborns of 2,048 deliveries (25%) despite monthly monitoring of pregnant women for the detection of anti-*Toxoplasma* antibodies, which has been mandatory since 1995. The authors also highlight the importance of monitoring postnatal children, because 22% of the treated infants had eye injuries in the follow-up period of three years. One-third of them were diagnosed positive in the first year of age.[24]

Determination of IgM and IgG avidity in serum signals the need for early diagnosis of primary infections in pregnant women. The combination of three diagnostic tests such as IgM, IgG, and IgG avidity is offered only in Venezuela by the IMT. The assay of IgG avidity was first introduced for the serodiagnosis of rubella[10] and then adapted to the diagnosis of toxoplasmosis.[11,26–29] If a molecular test (PCR) is incorporated into this testing panel, then the probability of detecting the presence of the parasite will increase. From the two commonly used genomic repeats of *T. gondii* RE (repeated element 529 pb, based on nested PCR assay) has resulted to be more sensitive than B1 genomic target.[30]

In a previous study at the IS, from 1994 to 1998, 42 (1.3%) among 3,059 pregnant women with negative IDT were positive for *Toxoplasma* IgM, and the prevalence of primary infections was 13 per 1,000 pregnant women.[31] During the period 1999–2002, 35 (1.1%) among 3,280 pregnant women with negative IDT were positive for *Toxoplasma* IgM, and the prevalence of primary infections was 11 per 1,000 pregnant women. Thereafter, IDT was not applied, and a comparative study between the value of the IDT and IgG avidity could not be conducted.

In patients with HIV, coexistence with toxoplasmosis in our study was 53.1%. Therefore, it is clear that all patients with HIV should include toxoplasmosis serology routinely, as it is the primary opportunistic pathogen with serious consequences for this high-risk group. It is important to note that 22 children below 10 years of age had both infections, which adds another evidence that the Venezuelan population is being infected at a young age. Being such a common parasitic infection in the general population and the most frequent as HIV opportunistic pathogen, PCR should be run at the reference laboratories to demonstrate the indirect presence of parasite, thereby increasing the possibility of diagnosis.[32–34] Moreover, loop-mediated isothermal amplification assay has shown better sensitivity and specificity than the conventional PCR based on the same genomic targets for the detection of *T. gondii* in children with leukemia, suggesting that it is an appropriate technique for routine molecular diagnosis.[35] In patients with eye lesions and lymphadenopathy syndrome, the association with toxoplasmosis was 63.8% and 33.3%, respectively, suggesting that it is more likely that *T. gondii* is associated with eye injuries than with lymphadenopathy. Lymphadenopathies are associated with diseases such as cytomegalovirus, lymphomas, and Epstein–Barr. Despite the bias of an outpatient cohort, the prevalence values reported here represent a warning to doctors and pediatricians for the need to rule out toxoplasmosis using the appropriate criteria. In children, uveitis and chorioretinitis may occur and acute toxoplasmosis may go unnoticed because the symptoms are not associated with this parasite. Most of the cases are detected in the chronic phase, with damage by the parasite widespread, especially in the retina; 34.1% of all lymphadenopathies and 7.5% of eye lesions were observed in children. Appropriate treatment and case management can be guided by immunodiagnostics. However, acute phase detection is an important diagnostic problem due to the consequences, mainly in ocular disease, congenital cases, and immunosuppressed patients. Knowing the estimated time since the result of the avidity test in pregnant women decreases medication use, provides a warning for the possibility of congenital infection, and reduces maternal anxiety.[11] Efforts should be channeled to make the diagnostic tools accessible in all centers.

The present study corroborates the high prevalence of toxoplasmosis in different human groups in Venezuela, as reported in previous studies: high prevalence in pregnant women (38%; 53.5%),[18,36] indigenous communities (49.7%),[37] children (54%),[36] and malnourished infants (10.9%).[38] In Maracaibo, 33% of women of childbearing age were positive (*n*: 100) and 18% were positive for IgM.[39] Certainly, toxoplasmosis is a worldwide zoonosis and a cosmopolitan human parasitosis, but strains from South America are phenotypically and genetically different from those in Europe and North America.[40] Regional seroprevalence of toxoplasmosis in countries of Latin America close to Venezuela is also high. In Costa Rica, 58% of 400 volunteers aged 20–40 years have specific antibodies and persons from low socioeconomic status had higher prevalence (67.1%).[41] *Toxoplasma* contact starts early in Ecuador, with a prevalence of 60.2% in 9-year-old children.[42] Two extensive reviews from Brazil and Colombia stated that in Brazil, up to 50% of elementary schoolchildren and 50–80% of women of childbearing age have *T. gondii* antibodies.[43] In Colombia, based on a large number of publications in several human risk groups (soldiers, pregnant women, dog owners, healthy, mental, ophthalmic, HIV patients, etc.),

the authors stated that the prevalence of toxoplasmosis ranges from 0% in some newborns to 93.75% in HIV patients.[44]

Screening for toxoplasmosis should be included not only in routine examination for those clinical symptoms suggestive of infection but also in risk groups such as pregnant women and their newborns, in order to implement early treatment and avoid serious outcomes. Moreover, even in pregnant women with negative testing, monthly serological screening should be mandatory, as done in France since 1995.[24]

The incorporation of molecular detection of parasites and other techniques for special fluids will provide additional diagnostic tools for the acute phase of toxoplasmosis. This effort by laboratories and reference centers must be matched by the availability of specific anti-*Toxoplasma* drugs such as pyrimethamine and sulfadiazine, which are sometimes scarce or inaccessible in Venezuela.

Conclusions

The high prevalence of toxoplasmosis in the Venezuelan population impedes attributing certain pathologies to the causative protozoan *T. gondii*, although high-risk groups and conditions such as pregnancy, childhood, ocular lesions, HIV/AIDS, and lymphadenopathic diseases require special consideration and care. Evaluation of parasite-specific IgM, IgG, and IgG avidity can provide guidance regarding recent or chronic infections. The overall prevalence of toxoplasmosis in Venezuelan pregnant women is 50.9%. Young age groups with specific antibodies suggest that Venezuelan children are infected early. Screening of toxoplasmosis should be mandatory monthly in seronegative pregnant women, and HIV/AIDS patients require screening to discard toxoplasmosis coinfection. Eye injuries are more associated with *T. gondii* than lymphadenopathy in children. In reference centers such as the IMT, other tests (PCR and immunoblotting) combined with evaluation of other fluids should be available for special cases.

Acknowledgments

The authors wish to acknowledge the contribution of Magdalena Soto, Carlos Losada, and Carolina Quiaro for their help in their specialties, and to the IMT outpatient coordinator Nahir Martínez and her team for their support in the reception of patients during the last three years. The authors are deeply grateful to Dr. Janine Ramsey Willoquet for her English corrections and improvements to the manuscript.

Author Contributions

Conceived and designed the experiments: BAN, LM, and ZD-B. Analyzed the data: BAN, LM, and AM-C. Wrote the first draft of the manuscript: BAN and LM. Contributed to the writing of the manuscript: BAN, LM, and AM-C. Agreed with the manuscript results and conclusions: BAN, LM, ZD-B, AM-C, and MA. Jointly developed the structure and arguments for the paper: BAN, LM, ZD-B, and AM-C. Made critical revisions and approved the final version: LM, ZD-B, MA, AM-C, and BAN. All authors reviewed and approved the final manuscript.

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