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Specific IgM, IgG and IgG1 directed against *Toxoplasma gondii* detected by flow cytometry and their potential as serologic tools to support clinical indirect fundoscopic presumed diagnosis of ocular disease

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ABSTRACT

In the present study we evaluated the anti-*Toxoplasma gondii* immunoglobulin profiles of a group of 118 individuals living in an endemic area. The aim of the study was to select biomarkers to support the ophthalmological diagnosis of retinal/retinochoroidal scars presumably caused by *T. gondii* infection. Overall anti-*T. gondii* reactivity of the IgM, IgG, IgA, IgE and IgG subclasses was investigated by flow cytometry-based anti-fixed tachyzoite antibodies (FC-AFTA) in four groups of subjects, referred to as: i) TOXO(L) – seropositive patients with retinal/retinochoroidal scars presumably caused by *T. gondii* infection; these patients were further subdivided according to morphological aspects of their ocular scar lesions as A, B or C; ii) TOXO(NL) – seropositive patients without ocular scar lesions; iii) NEG(L) – *T. gondii* seronegative patients presenting retinal lesions; and iv) NEG(NL) – *T. gondii* seronegative without retinal lesions (negative controls). Our data demonstrated that anti-*T. gondii* IgG profiles were able to discriminate the mean reactivity of TOXO(L) from all other clinical groups. Analysis of anti-*T. gondii* immunoglobulin profiles revealed that IgM and IgG were good biomarkers capable of discriminating between individual reactivity in patients with retinal/retinochoroidal scars presumably caused by *T. gondii* infection [TOXO(L)] from those caused by other clinical conditions. Furthermore, anti-*T. gondii* IgG1 reactivity was able to discriminate TOXO(L) from all other clinical groups. In conclusion, the pre-selected IgM, IgG and IgG1 anti-*T. gondii* antibody subclasses were able to segregate both TOXO(L) and the other subgroups, including the scar lesion group types (A, B, C), from other clinical conditions. These results suggest the applicability of this technique in the clinical laboratory to detect putative biomarker for diagnosis of ocular lesions in *T. gondii*-infected patients. Studies in other areas implementing the methods described in the present study would be of value and enable evaluation of a system for classification of presumed ocular toxoplasmosis scar lesions. This classification would make comparative studies on ocular toxoplasmosis conducted in different regions around the world possible.

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1. Introduction

Ocular toxoplasmosis is thought to be the most common potentially severe symptomatic manifestation of *Toxoplasma gondii* infection worldwide. The condition is associated with congenital, acute, and postnatally acquired *T. gondii* (Holland, 2003, 2004; Gilbert et al., 2008). Population-based studies in Brazil involving subjects with ages ranging from 10 years to older than 50 years from different regions of the country have reported prevalences of retinochoroiditis that vary from 2.6% to 17.7% (Glasner et al., 1992; de Abreu et al., 1998; Garcia et al., 1999; Bahia-Oliveira et al., 2001; Portela et al., 2004). Prevalence is based on the observation of retinal/retinochoroidal scar lesions via indirect fundoscopic examinations because, in epidemiologic surveys, it is rare to find patients with active toxoplasmic lesions. The intrinsic multifactorial nature of toxoplasmosis has an impact on the differential ocular prevalence even in the face of similar seroprevalences. Furthermore, the subjective criteria inherent in the observational diagnosis process using indirect funduscopy are important factors contributing to the differential prevalence. We and others have adopted a set of consensus criteria for characterizing retinal/retinochoroidal scar lesions presumably caused by *T. gondii* infection in the context of population based studies in Brazil (Aleixo et al., 2009; Bahia-Oliveira et al., 2009; Peixoto-Rangel et al., 2009; Dutra et al., 2013; Peixe et al., 2014). The set of criteria is based on the morphological aspects of the scars, including the pigmentation and degree of retinal tissue damage. Individually, three types of scar lesions are proposed: class A scar lesions that present well-marked boundaries, are usually surrounded by a pigmented halo and have extensive destruction of the retina and choroid; class B scar lesions that are characterized by a surrounding hypopigmented halo and a smaller degree of tissue destruction in comparison to class A; and class C scar lesions that are basically areas of retinal pigment epithelium hyperplasia or atrophy with a smaller degree of tissue destruction compared to classes A and B. The class A scar lesion is recognized by ophthalmologists as being typically representative of *T. gondii* infection. However, class B scar lesions have been recently described as *T. gondii* putative lesions (Aleixo et al., 2009; Bahia-Oliveira et al., 2009; Peixoto-Rangel et al., 2009; Dutra et al., 2013; Peixe et al., 2014). Previously, they had been described as atypical lesions presumably caused by *T. gondii* infection in the context of epidemiological surveys for toxoplasmosis (Silveira et al., 2001; Bahia-Oliveira et al., 2012). Similarly, class C lesions have recently been reported by different authors from Brazil and are also considered to be presumably caused by *T. gondii* infection (Aleixo et al., 2009; Peixoto-Rangel et al., 2009; Bahia-Oliveira et al., 2012; Dutra et al., 2013).

In the present study, we propose the potential utilization of immunoglobulin (IgM, total IgG and IgG1) levels in the patient's sera detected by flow cytometry as biomarkers to support indirect clinical fundoscopic diagnosis of ocular toxoplasmosis. Our findings will enable better estimation of the prevalence of ocular toxoplasmosis in epidemiological surveys and will be valuable for retrospective and prospective studies.

2. Materials and methods

2.1. Study population

The present study enrolled 118 individuals living in Campos dos Goytacazes, Rio de Janeiro, Brazil. All subjects submitted to *T. gondii* serological tests. IgM and IgG were assayed using the VIDAS TOXO kit (BioMérieux, RJ, Brazil) and specific IgA was tested with the Platelia Toxo kit (BioRad, SP, Brazil). All individuals were examined with binocular indirect ophthalmoscopy (Welch-Allyn, SP, Brazil) by two independent ophthalmologists. Retinal scars were photographed with a retinographer (Zeiss-Visucam).

The clinical status of retinal/retinochoroidal scar lesions was defined according to previous reports (Bahia-Oliveira et al., 2012; Dutra et al., 2013; Peixe et al., 2014). The scar lesions were classified according to their morphological aspects and referred to as: class "A" presenting well-marked boundaries, usually surrounded by a pigmented halo and extensive destruction of the retina and choroid; class "B" characterized by a surrounding hypopigmented halo and a smaller degree of tissue destruction in comparison to class "A"; and class "C" showing areas of retinal pigment epithelium hyperplasia or atrophy with a smaller degree of tissue destruction compared to classes "A" and "B" (Peixoto-Rangel et al., 2009; Bahia-Oliveira et al., 2012; Peixe et al., 2014).

Individuals presenting multiple scar lesions were grouped based on the most severe scar lesion; for example, individuals with multiple scar lesion types A/B, A/B/C or A/C were classified as belonging to group A, while individuals with scar lesion type B/C were classified as belonging to group B.

The subjects were categorized into four groups based on serological profile and ophthalmoscopic records as follows:

- 1 TOXO(L): Patients with positive serology and retinal/retinochoroidal scars presumably caused by *T. gondii* infection (n = 53);
- 2 TOXO(NL): Patients with positive serology without retinal/retinochoroidal scars presumably caused by *T. gondii* infection (n = 39);
- 3 NEG(L): Individuals with negative serology and retinal/retinochoroidal scars (n = 5);
- 4 NEG(NL): Individuals with negative serology with no ocular lesions (n = 21).

The detailed clinical and demographical status of the study population is presented in Table 1.

This study was approved by the Ethical Committee of the Oswaldo Cruz Foundation (protocol # 347/06) and the National Commission for Ethics in Research, Ministry of Health (protocol # 013/2007).

2.2. IgG depletion from serum samples

IgG corresponds to up to 75% of all immunoglobulins in adult sera (Bradley, 1974). As a consequence, IgG will bind to the majority of epitopes, leaving few binding sites for other immunoglobulin classes. For greater accuracy in measuring IgM, IgA and IgE levels, an aliquot of each individual serum sample was pre-treated with Protein A agarose for IgG through adsorption (Sigma Chemical Co., St. Louis, MO, USA). Briefly, 100 µL of Protein A agarose was added to 100 µL of serum. The

Table 1
Study population.

<i>T. gondii</i> serology ^a	Clinical and demographic parameters				
	Clinical groups	n	Scar lesion types ^b	Gender (male/female)	Mean age (range)
Seropositive	TOXO(L)	17	A ^c	9/8	35 (14–51)
		17	B ^d	4/13	28 (06–61)
		19	C ^e	4/15	30 (14–56)
Seronegative	TOXO(NL)	39	–	13/2	32 (09–61)
	NEG(L)	5	B ^f and C ^g	4/1	39 (18–72)
	NEG(NL)	21	–	14/7	31 (15–56)
Total		118			

^a *T. gondii* serology was performed by commercial ELISA.

^b Clinical profile was determined by fundoscopic examination.

^c Toxoplasmosis patients with a single type A scar lesion or with multiple scar lesion types AB, ABC or AC.

^d Toxoplasmosis patients with type B or type BC scar lesions.

^e Toxoplasmosis patients with type C scar lesions.

^f Individuals with negative *T. gondii* serology with type B or BC scar lesions.

^g Individuals with negative *T. gondii* serology with type C scar lesions.

tubes were gently mixed for 1 h. After 30 min of incubation, the supernatant was carefully collected. The adsorbed sera were frozen at -20°C until use.

2.3. Preparation of fixed *T. gondii* tachyzoites and immunofluorescence by flow cytometry

T. gondii tachyzoites were prepared as previously described by Silva-dos-Santos et al. (2012). Briefly, *T. gondii* tachyzoites (RH strain) were grown intraperitoneally in BALB/c mice for 48 to 72 h. The peritoneal exudate was harvested by washing the peritoneal cavity with sterile 0.01 M phosphate-buffered saline at pH 7.2 (PBS), transferred to 50 mL polypropylene tubes, centrifuged at 4°C at $1000 \times g$ for 10 min, and the supernatant was discarded. The parasite suspension was washed three times by centrifugation at $1000 \times g$ for 10 min with PBS. The final parasite pellet was immediately resuspended in equal volumes of PBS and FACS-fix solution (per liter, 10 g paraformaldehyde, 10.2 g sodium cacodylate, and 6.65 g sodium chloride, pH 7.2; Sigma Chemical Co., St. Louis, MO, USA) and incubated at 4°C for 24 h. The parasites were again washed, resuspended in PBS, adjusted to 5.0×10^6 tachyzoites/mL, and stored at 4°C until used in flow cytometry-based immunofluorescence assays.

The detection of fixed *T. gondii* tachyzoite antibodies (IgM, IgA, IgE, IgG and IgG subclasses) by flow cytometry was performed as described by Silva-dos-Santos et al. (2012). Briefly, $50 \mu\text{L}$ (2.5×10^5) of pre-fixed tachyzoites was mixed with $50 \mu\text{L}$ of individual serum samples using serial four-fold dilutions (1/50 for IgG₂ and IgG₃; 1/100 for IgA, IgE, IgM and IgG₄; 1/1000 for IgG; and 1/4000 for IgG₁) in PBS supplemented with 3% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA). Next, the samples were incubated at 37°C for 30 min. After incubation, the parasites were washed twice with $200 \mu\text{L}$ of PBS–3% FBS (4°C , at $1000 \times g$ for 10 min) by centrifugation and reincubated in the dark at 37°C for 30 min in the presence of $50 \mu\text{L}$ of previously diluted fluorescein isothiocyanate (FITC)-conjugated anti-human IgM (1/200), IgA (1/200) or IgE (1/100) purchased from Southern Biotech (Birmingham, AL, USA) or biotin-conjugated anti-human IgG subclass antibodies including anti-IgG1 (1/20,000), IgG2 (1/1000), IgG3 (1/1000) and IgG4 (1/1000) purchased from Sigma Chemical Co. (St. Louis, MO, USA). For the biotin-conjugated

antibodies, an additional incubation step at 37°C for 30 min with $20 \mu\text{L}$ of diluted phycoerythrin conjugated-streptavidin was required (SAPE; 1/400) (Southern Biotech, Birmingham, AL, USA). FITC or SAPE-labeled parasites were washed and fixed with $200 \mu\text{L}$ FACS fix solution and maintained for at least 30 min (and up to 24 h) at 4°C in the dark before flow cytometric analysis.

An internal control in which the parasites were incubated in the absence of human serum but in the presence of all secondary reagents was included to monitor unspecific binding. In all batches of experiments, positive and negative control sera were also included.

Flow cytometric measurements were performed on a Becton Dickinson FACSCalibur. The Cell-Quest software package was used for data acquisition, storage and analysis. Stained parasites were run in the cytometer and 5000 events per sample were acquired. The parasites were identified on the basis of their specific forward (FSC) and side (SSC) light scattering properties. Parasites were selected by gating on the FSC $\times$ SSC dot plot distribution. The relative FITC (FL-1) or SAPE (FL-2) fluorescence intensity of labeled parasites from each assay was determined as the percentage of positive fluorescent parasites (PPFPs) using a single histogram chart. A marker was set on the histogram distribution of the internal control at PPFP $< 2\%$ as the non-specific binding limit. This marker was maintained in each batch of tests to obtain the PPFP values along the titration curve of individual samples as proposed by Silva-dos-Santos et al. (2012).

2.4. Statistical analysis

Comparative analysis of immunoglobulin reactivity (PPFP) among clinical groups [TOXO(L), TOXO(NL), NEG(L) and NEG(NL)] as well as lesion type subgroups [A, B, C and TOXO(NL) + NEG(L) + NEG(NL)] were performed by Kruskal–Wallis followed by Dunn's test. Significance was considered at $p < 0.05$.

Receiver Operating Curve (ROC curve) analysis was performed to determine the best cut-off edge to segregate immunoglobulin reactivity between TOXO(L) and TOXO(NL) + NEG(L) + NEG(NL). Additionally, the ROC curve data provided the accuracy of each test with Area Under the Curve (AUC) measurements. Sensitivity = $[\text{true positives} / (\text{true positives} + \text{false negatives})] \times 100$,

specificity = [true negatives / (true negatives + false positives)] $\times$ 100, and likelihood ratio (LR) = [sensitivity / (1 – specificity)] were also calculated for each immunoglobulin reactivity pattern.

Categorical analysis of the ability of the immunoglobulins to segregate TOXO(L) and lesion type subgroups (A, B, C) from [TOXO(NL) + NEG(L) + NEG(NL)] were assessed using the Chi-square test or Fisher's Exact test.

GraphPad Prism 5.0 software was used for all statistical analyses and graphical representations.

3. Results

3.1. Anti-*T. gondii* IgG profile detected by flow cytometry (FC-AFTA) was able to discriminate the mean reactivity of TOXO(L) from all other clinical groups [TOXO(NL); NEG(L) and NEG(NL)]

The overall reactivity of anti-*T. gondii* immunoglobulins (IgM, IgG, IgA, IgE and IgG subclasses) detected using flow cytometry-based anti-fixed tachyzoite antibodies (FC-AFTA) are presented in Fig. 1. Data analysis demonstrated that all immunoglobulin patterns with the exception of IgE were able to discriminate the TOXO (L and/or NL) from the NEG (L and/or NL) clinical groups. IgG1 reactivity assessed at a serum dilution of 1/1000 was uniquely able to discriminate TOXO(L) from all other clinical groups [TOXO(NL); NEG(L) and NEG(NL)] (Fig. 1).

3.2. Similar anti-*T. gondii* immunoglobulin patterns were observed in all TOXO(L) subgroups regardless of ocular scar lesion type

The panoramic anti-*T. gondii* immunoglobulin profiles (IgM, IgG, IgA, IgE and IgG subclasses) of serum samples from TOXO(L) subgroups categorized according to lesion types (A, B and C) are presented in Fig. 2. Data analysis did not demonstrate any significant differences among the TOXO(L) subgroups, underscoring the similarities among anti-*T. gondii* immunoglobulin patterns displayed by all TOXO(L) subgroups regardless of ocular scar lesion type (Fig. 2).

3.3. Anti-*T. gondii* IgM and IgG are good biomarkers for discrimination of individual reactivity of patients with retinal/retinochoroidal scars presumably caused by *T. gondii* infection [TOXO(L)] from other clinical conditions [TOXO(NL) + NEG(L) + NEG(NL)]

The ability of anti-*T. gondii* immunoglobulins (IgM, IgG, IgA and IgE) to discriminate TOXO(L) from other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)] is shown in Fig. 3. ROC curve analysis was used to establish the best cut-off to segregate immunoglobulin reactivity between groups. PPFP values of 15%, 95%, 20% and 40% were selected as the edges for discrimination indices for IgM, IgG, IgA and IgE reactivities, respectively. The 1/100 serum dilution was selected to measure IgA and IgE, whereas 1/400 and 1/1000 were the best conditions for discriminatory analysis of IgM and IgG, respectively. IgM and IgG reactivities were the most promising

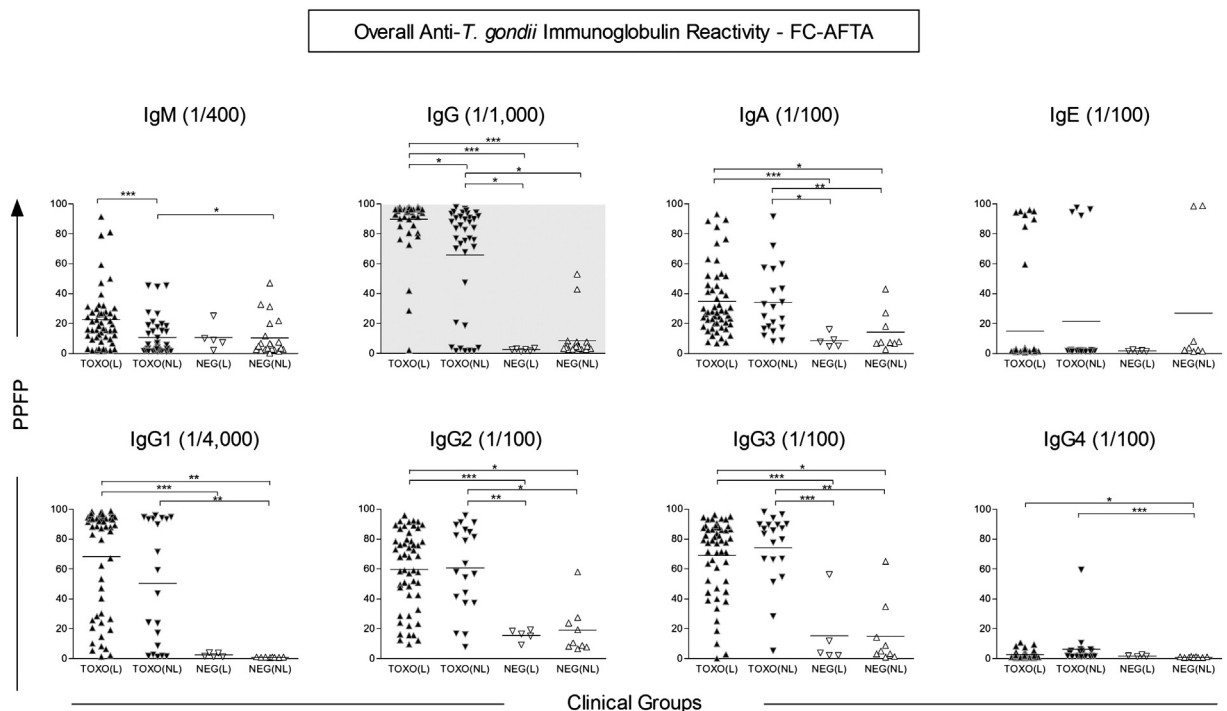


Fig. 1. Overall reactivity of anti-*T. gondii* immunoglobulins (IgM, IgG, IgA, IgE and IgG subclasses) detected by flow cytometry-based anti-fixed tachyzoite antibodies (FC-AFTA) in serum samples from TOXO(L) (Δ = 53), TOXO(NL) (∇ = 39), NEG(L) ($\diamond$ = 5) and NEG(NL) ($\square$ = 21). Data are expressed as the mean percentage of positive fluorescent parasites (PPFPs) and scattering of individual values for all immunoglobulins. Statistical significance was determined by Kruskal–Wallis followed by Dunn's test; significance was considered at $p < 0.05$, $p < 0.01$ or $p < 0.001$ as represented by *, **, and ***, respectively. Significant differences between groups are highlighted by connecting lines. IgG reactivity assessed at a 1/1000 serum dilution was uniquely able to discriminate TOXO(L) from all other clinical groups [TOXO(NL); NEG(L) and NEG(NL)]. This differential profile is underscored by a gray background.

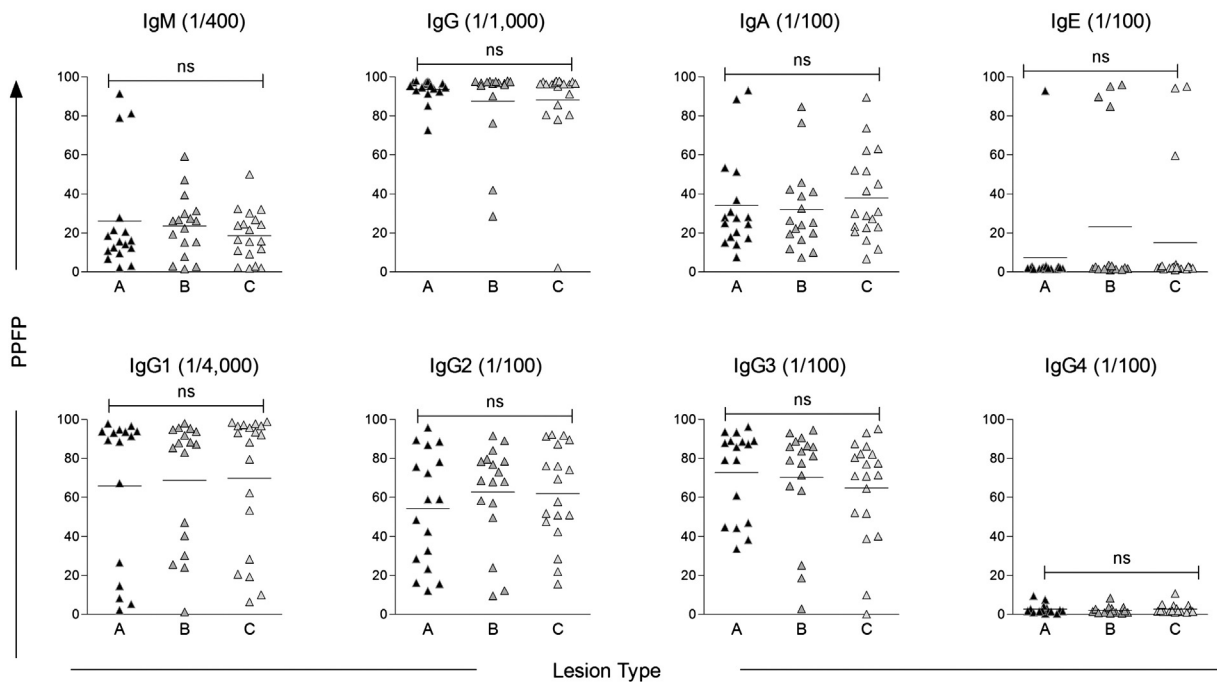
Anti-*T. gondii* Immunoglobulin Reactivity According to Lesion Type - FC-AFTA

Fig. 2. Overall reactivity of anti-*T. gondii* immunoglobulins (IgM, IgG, IgA, IgE and IgG subclasses) detected by flow cytometry-based anti-fixed tachyzoite antibodies (FC-AFTA) in serum samples from TOXO(L) subgroups categorized according to lesion types A ($\blacktriangle$ = 17), B ($\triangle$ = 17) and C ($\triangle$ = 19). Data are expressed as the mean percentage of positive fluorescent parasites (PPFPs) and scattering of individual values for all immunoglobulins. Statistical significance was determined using Kruskal–Wallis followed by Dunn’s test. No significant differences were found among the TOXO(L) subgroups.

biomarkers for discrimination between patients with retinal/retinchoroidal scars presumably caused by *T. gondii* infection [TOXO(L)] and those from other clinical conditions [TOXO(NL) + NEG(L) + NEG(NL)], with moderate performance indices for IgM (Se = 66.0%, Sp = 74.2%, AUC = 0.75 and LR+ = 2.6) and good performance indices for IgG (Se = 71.2%, Sp = 90.2%, AUC = 0.89 and LR+ = 7.2) (Fig. 3).

3.4. Anti-*T. gondii* IgG1 reactivity is an alternative biomarker able to discriminate TOXO(L) from all other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)]

The ability of anti-*T. gondii* IgG subclasses detected by flow cytometry-based anti-fixed tachyzoites antibodies (FC-AFTA) to discriminate TOXO(L) from other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)] is presented in Fig. 4. ROC curve analysis was used to establish the best cut-off to segregate IgG subclass reactivity between groups. PPFP values of 75%, 60%, 70% and 15% were used as edges to obtain the best indices of IgG1, IgG2, IgG3 and IgG4 reactivities. The 1/100 serum dilution was selected to measure IgG2, IgG3 and IgG4, whereas 1/4000 was selected for IgG1. Data analysis showed that IgG1 reactivity assessed at a 1/4000 serum dilution was best able to discriminate TOXO(L) from all other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)] with moderate performance indices (Se = 63.5%, Sp = 72.7%, AUC = 0.77 and LR+ = 2.4) (Fig. 4).

3.5. Pre-selected IgM, IgG and IgG1 anti-*T. gondii* reactivity segregated TOXO(L) subgroups (A, B, C) from other clinical conditions [TOXO(NL) + NEG(L) + NEG(NL)]

The ability of anti-*T. gondii* IgM, IgG and IgG1 to discriminate TOXO(L) subgroups categorized according to the type of ocular scar lesion (A, B and C) from other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)] is presented in Fig. 5. Data analysis of individual reactivity was carried out at pre-established serum dilutions (IgM = 1/100, IgG = 1/1000 and IgG1 = 1/4000) and pre-selected PPFP cut-off edges (IgM = 15%, IgG = 95% and IgG1 = 75%). The results revealed that all pre-selected immunoglobulins (IgM, IgG and IgG1) were able to discriminate the TOXO(L) subgroup reactivity patterns from all other clinical conditions [TOXO(NL) + NEG(L) + NEG(NL)] with moderate performance (IgM = 59 to 76% versus 74%, IgG = 68 to 75% versus 88% and IgG1 = 63 to 65% versus 76%, respectively) (Fig. 5).

4. Discussion

Flow cytometric approaches to detect antibodies against protozoan parasites have proven their value for serological diagnosis (Cozon et al., 1993, 1999; Cordeiro et al., 2001; Martins-Filho et al., 2002; Rocha et al., 2002, 2003; de Andrade et al., 2007; Lemos et al., 2007; Vitelli-Avelar et al., 2007; Andrade et al., 2009; Martins-Souza et al., 2009; Silva-dos-Santos et al., 2012). In fact, Fc-based assays capable of detecting

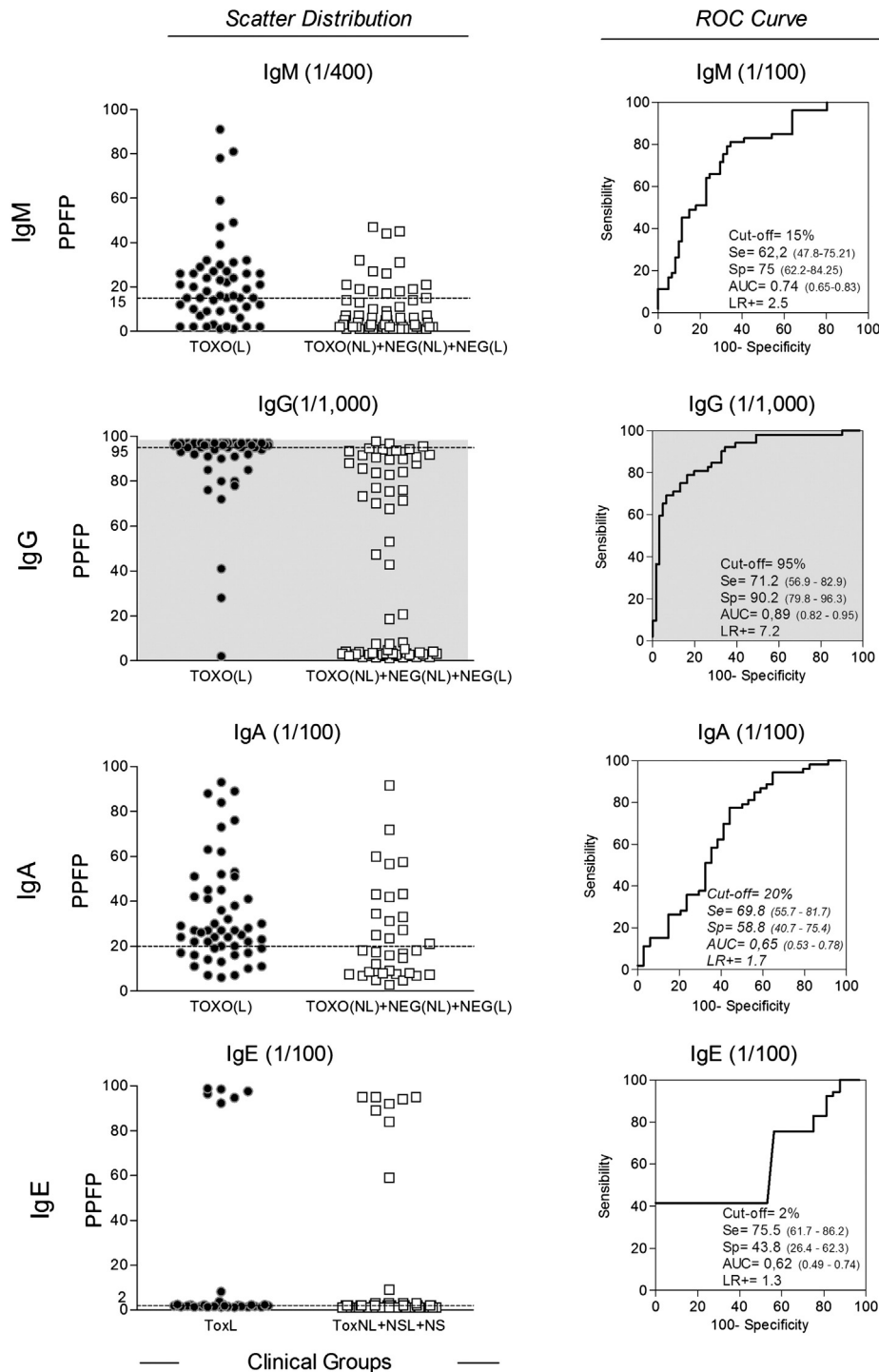
Performance of Anti-*T. gondii* IgM, IgG, IgA and IgE for TOXO Lesion Diagnosis - FC-AFTA

Fig. 3. Ability of anti-*T. gondii* immunoglobulins (IgM, IgG, IgA and IgE) detected by flow cytometry-based anti-fixed tachyzoite antibodies (FC-AFTA) to discriminate TOXO(L) (● = 53) from other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)] (□ = 65). Data are expressed as scattering distribution of percentage of positive fluorescent parasites (PPFPs) for all immunoglobulins. ROC curve analysis was used to establish the best cut-off edge to segregate immunoglobulin reactivity between groups. The cut-off edges and the performance indices (Se, Sp, AUC and LR+) are provided. IgM and IgG reactivities assessed at sera dilutions of 1/400 and 1/1000 were the best biomarkers able to discriminate TOXO(L) from all other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)], respectively. These differential profiles are underscored with a gray background.

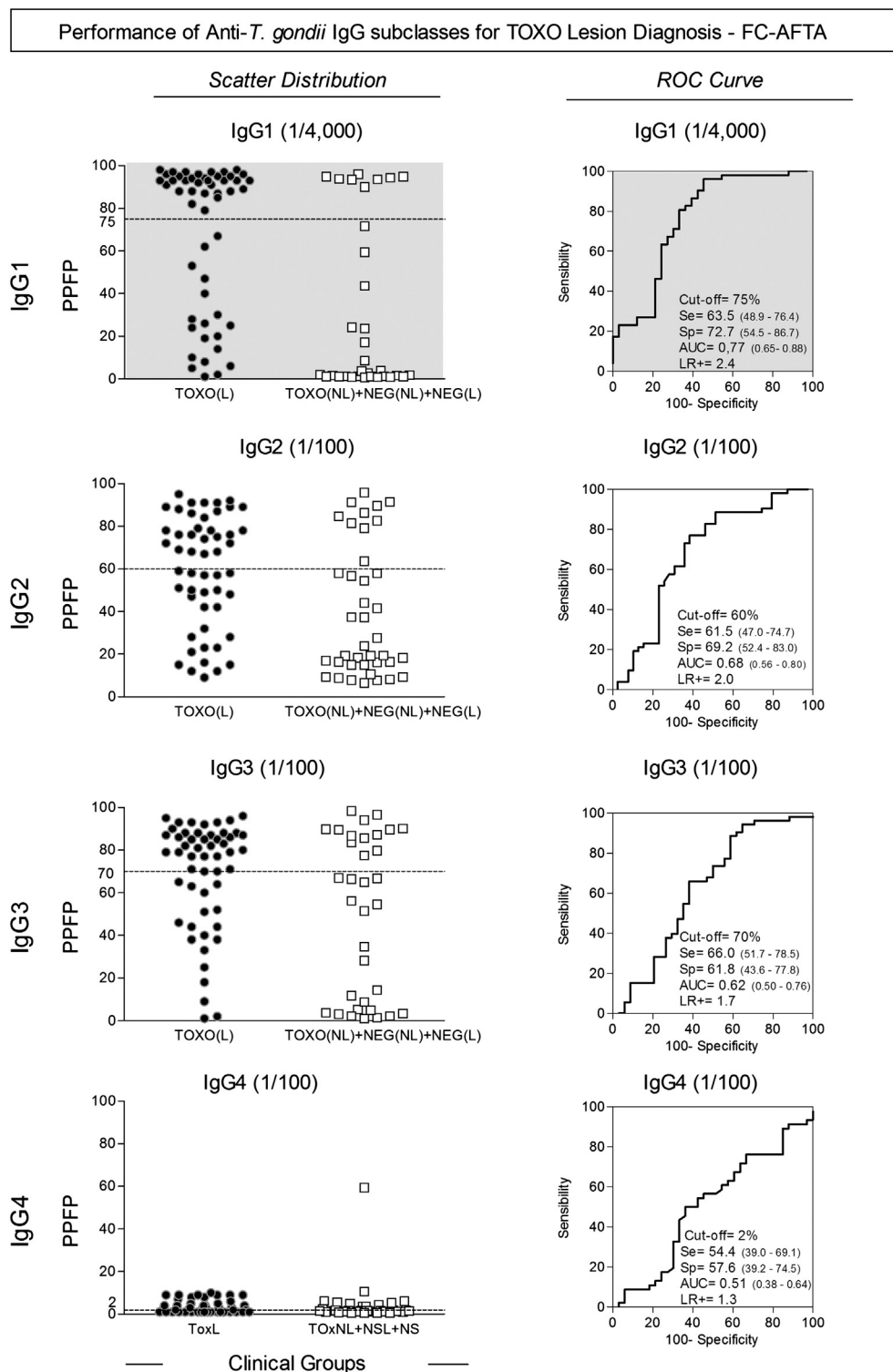
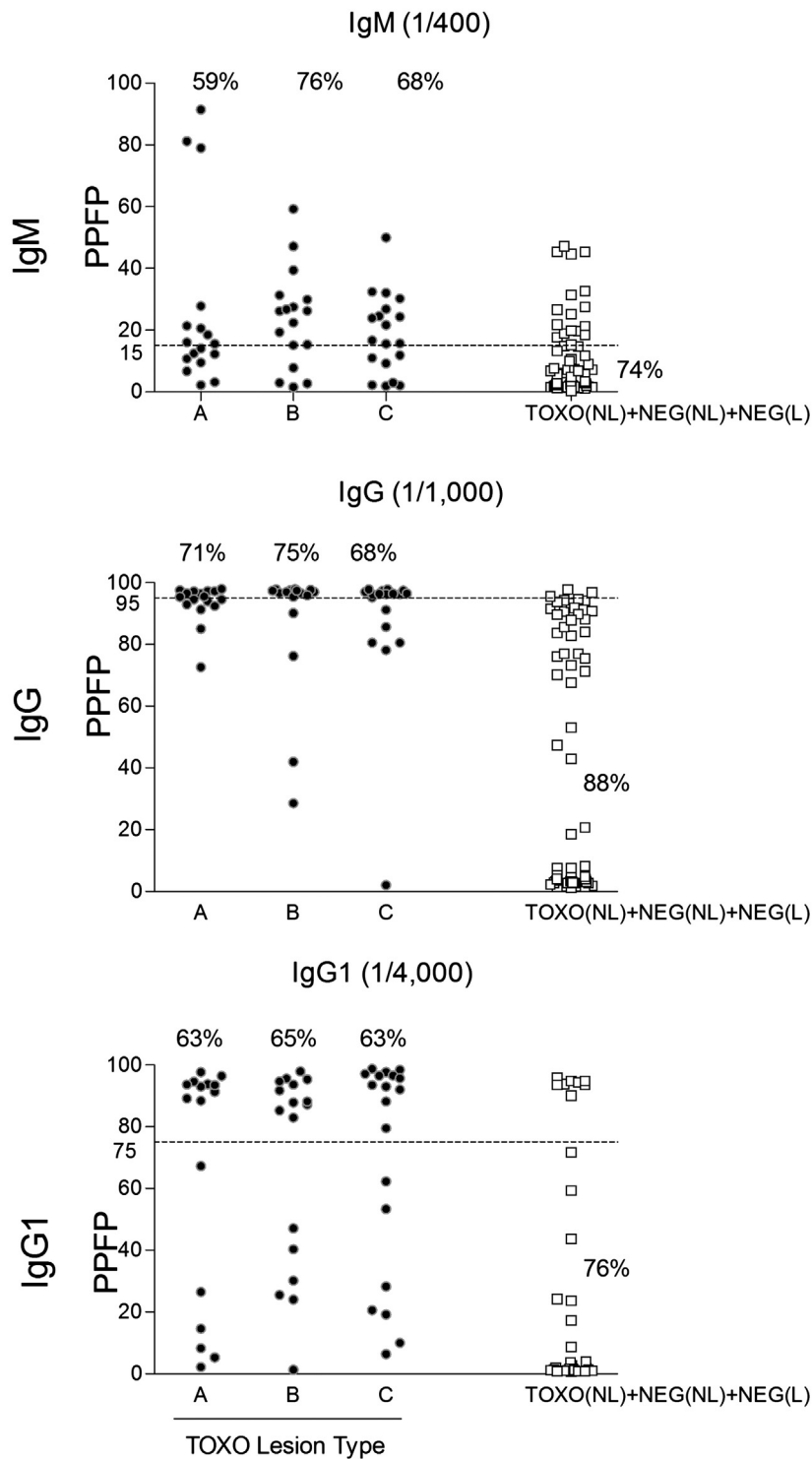


Fig. 4. Ability of anti-*T. gondii* IgG subclasses detected by flow cytometry-based anti-fixed tachyzoite antibodies (FC-AFTA) to discriminate TOXO(L) (● = 53) from other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)] (□ = 65). Data are expressed as scattering distribution of percentage of positive fluorescent parasites (PPFPs) for all immunoglobulins. ROC curve analysis was used to establish the best cut-off edge to segregate IgG subclass reactivity between groups. The cut-off edges and the performance indices (Se, Sp, AUC and LR+) are provided. IgG1 reactivity assessed at a 1/4000 serum dilution was the single biomarker able to discriminate TOXO(L) from all other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)]. This differential profile is underscored with a gray background.

Performance of Anti-*T. gondii* IgM, IgG and IgG1 for TOXO Lesion Types Diagnosis - FC-AFTA

Scatter Distribution - Sensitivity and Specificity Indexes



antibodies on the surface of protozoan parasites have been proposed to be a serological criteria for a cure (Martins-Filho et al., 2002; Lemos et al., 2007; Martins-Souza et al., 2009) as well as for discrimination among different clinical groups of patients with diseases caused by *Trypanosoma cruzi* (Cordeiro et al., 2001; Vitelli-Avelar et al., 2007). In toxoplasmosis, the use of FC-based assays was first described to detect anti-fixed tachyzoite immunoglobulins (Cozon et al., 1993, 1999). Recently, a flow cytometric-based algorithm based on IgM levels and IgG avidity was proposed as a new serological approach to discriminate between acute and chronic toxoplasmosis with 100% of sensibility and sensitivity (Silva-dos-Santos et al., 2012). In this study, we described a new application of FC-based immunofluorescence assays in toxoplasmosis that contribute to the presumed diagnosis of ocular disease.

The lack of a classification system or a consensus proposal to describe the diversity of scar lesions presumably caused by *T. gondii* infection influences the prevalence reported in various population based studies in Brazil (Bahia-Oliveira et al., 2012). The ocular prevalence of toxoplasmosis reported from population surveys is based on indirect ophthalmoscopic diagnosis that is intrinsically based on assumptions and is dependent on the observation of retinal/retinochoroidal scars by ophthalmologists in association with classical serologic determinations. Such studies are limited because patients in general do not present for ocular puncture for aqueous humor collection (paracentesis), which is an invasive procedure mainly if used for population based studies. Nonetheless, even in the face of an active lesion considered typically representative of a *T. gondii* ocular lesion the sensitivity and specificity of PCR of aqueous humors are far from satisfactory, especially given the problems associated with ocular puncture.

In our hands, the isotypes IgA, IgE, and IgG subclasses IgG2, IgG3 and IgG4 did not possess a strong ability to discriminate between the TOXO(L) group and the other groups; however, the very low levels of IgE and IgG4 against *T. gondii* tachyzoites support the Th1 molecular signature that has been reported for human toxoplasmosis (Bahia-Oliveira et al., 2009, 2012; Garweg and Candolfi, 2009). In contrast, IgM, total IgG and IgG1 sensitivity and specificity for FC-AFTA ranged from 66 to 74.2 and from 71 to 90.2, respectively. Such values are comparable to those reported in studies measuring aqueous humor samples from patients with presumed ocular toxoplasmosis, suggesting that they have the potential to be utilized as biomarkers for ocular toxoplasmosis (Brézin et al., 1991; Garweg et al., 1996; Robert et al., 1996; Mattos et al., 2011; Papadia et al., 2011; Sugita et al., 2011; Bourdin et al., 2014). Comparisons between studies using aqueous humors to evaluate parasite DNA or antibodies specific to *T. gondii* show discrepancies and inconsistencies in terms of sensitivity values (Brézin et al., 1991; Aouizerate et al., 1993; Garweg et al., 1996; Robert et al., 1996; Verbraak et al., 1996). A well conducted study by Bou et al. (1999) investigating aqueous humor

samples collected from patients with typical active ocular toxoplasmosis reported sensitivity and specificity values of 53.3% and 83%, respectively. The authors evaluated peripheral blood samples and aqueous humor samples from the same patients by PCR and observed that among all patients with ocular toxoplasmosis positive results for aqueous humor samples were accompanied by respective positive results for blood samples. Because blood and aqueous humor samples were always coincident in terms of positive and negative results for PCR, the authors suggested that ocular toxoplasmosis should not be considered a local event and recommended that peripheral blood PCR be used to discriminate between ocular toxoplasmosis and other ocular diseases to avoid the problems associated with ocular puncture (Bou et al., 1999).

The significance of defining molecular parameters (biomarkers) associated with non-invasive procedures as alternative approaches to support the estimation of clinical diagnosis of ocular toxoplasmosis by indirect funduscopy is of importance mainly for epidemiological studies. The proposed methodology provides opportunities for retrospective and prospective studies on ocular toxoplasmosis, with the possibility of adding important pieces of knowledge concerning the clinical presentation of ocular toxoplasmosis. Beyond a use as potential biomarkers to support clinical fundoscopic diagnosis of presumable ocular toxoplasmosis, FC-AFTA IgM, total IgG and IgG1 evaluation might also be used to create an algorithm for a universally adopted system of retinal/retinochoroidal *T. gondii* scar lesion classification. Studies in other areas that implement the methods described in this study will contribute to better evaluation and possibly propose a system to classify presumable ocular toxoplasmosis scar lesions. These types of systems may permit followup to evaluate the evolution of scar lesions (described here as type B and type C) that we and others have proposed to be associated with *T. gondii* infection (Aleixo et al., 2009; Bahia-Oliveira et al., 2009, 2012; Dutra et al., 2013; Peixe et al., 2014). They also may potentially motivate adoption of a consensus agreement among ophthalmologists to establish a system for the classification of presumed ocular toxoplasmosis scar lesions, making it possible under some pre-defined parameters to conduct comparative studies on ocular toxoplasmosis in different regions around the world.

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Fig. 5. Ability of anti-*T. gondii* IgM, IgG and IgG1 detected by flow cytometry-based anti-fixed tachyzoite antibodies (FC-AFTA) to discriminate TOXO(L) subgroups categorized according to the type of ocular scar lesion (●) A (n = 17), B (n = 17) and C (n = 19), from other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)] (□ = 65). Data are expressed as scattering distribution of percentage of positive fluorescent parasites (PPFPs) for all immunoglobulins at pre-established sera dilutions (IgM = 1/100, IgG = 1/1000 and IgG1 = 1/4000). The pre-selected PPFP cut-off edges (IgM = 15%, IgG = 95% and IgG1 = 75%) are represented by dashed lines. All pre-selected immunoglobulin reactivities (IgM, IgG and IgG1) were able to segregate TOXO(L) subgroups from all other clinical groups [TOXO(NL) + NEG(L) + NEG(NL)]. Categorical analysis of the ability of immunoglobulin reactivity to segregate TOXO(L) subgroups (A, B, C) from [TOXO(NL) + NEG(L) + NEG(NL)] was carried out by Chi-square or Fisher's Exact tests.

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