

## Anti-*Toxoplasma gondii* secretory IgA in tears of patients with ocular toxoplasmosis: immunodiagnostic validation by ELISA

Maria Isabel Lynch<sup>1,3/+</sup>, Elizabeth Malagueño<sup>2</sup>, Luiz Felipe Lynch<sup>1</sup>, Silvana Ferreira<sup>2,3</sup>, Raphael Stheling<sup>1,4</sup>, Fernando Oréfice<sup>1,4</sup>

<sup>1</sup>Centro de Ciências da Saúde <sup>2</sup>Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Recife, PE, Brasil

<sup>3</sup>Faculdade de Ciências Médicas, Universidade de Pernambuco, Recife, PE, Brasil <sup>4</sup>Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

*Toxoplasma gondii* causes posterior uveitis and the specific diagnosis is based on clinical criteria. The presence of anti-*T. gondii* secretory IgA (sIgA) antibodies in patients' tears has been reported and an association was found between ocular toxoplasmosis and the anti-*T. gondii* sIgA isotype in Brazilian patients. The purpose of this study was to provide an objective validation of the published ELISA test for determining the presence of anti-*T. gondii* sIgA in the tears of individuals with ocular toxoplasmosis. Tears from 156 patients with active posterior uveitis were analyzed; 82 of them presented characteristics of ocular toxoplasmosis (standard lesion) and 74 patients presented uveitis due to other aetiologies. Cases of active posterior uveitis were considered standard when a new inflammatory focus satellite to old retinochoroidal scars was observed. The determination of anti-*T. gondii* sIgA was made using an ELISA test with crude tachyzoite antigenic extracts. Tears were collected without previous stimulation. Detection of sIgA showed 65.9% sensitivity (95% CI = 54.5-74.4), 71.6% specificity (95% CI = 59.8-81.2), a positive predictive value of 72% (95% CI = 60.3-81.5) and a negative predictive value of 65.4% (95% CI = 54.0-75.4). sIgA reactivity was higher in the tears of patients with active posterior uveitis due to *T. gondii* ( $p < 0.05$ ). The test is useful for differentiating active posterior uveitis due to toxoplasmosis from uveitis caused by other diseases.

Key words: ocular toxoplasmosis - lachrymal sIgA - toxoplasmosis immunodiagnostic

Toxoplasmosis is a systemic illness produced by *Toxoplasma gondii*, an intracellular parasite of worldwide distribution (Tonelli 2000). The ocular form is responsible for 50-80% of posterior uveitis in Brazil (Oréfice & Garcia 2005).

The diagnosis of posterior uveitis due to toxoplasmosis is mainly based on clinical criteria. Patients with an active retinochoroidal lesion adjacent to a characteristic healed lesion, e.g., scars delimited with pigmented edges, with one or more foci, can be characterised as having a gold standard lesion (Lynch et al. 2004, 2008, Garweg 2005). Some authors have shown that the acquired form of the disease presents an aggressive clinical picture and an absence of old scars (Montoya & Remington 1996, Holland 2003).

Serological tests are important tools for the diagnosis of systemic toxoplasmosis. IgM, IgA, IgE and IgG antibodies of low avidity are produced in response to recent infections. The subsequent transition profile shows increased IgG avidity, gradual reduction of IgM, IgA and IgE isotypes. IgM may remain positive at low levels. High-avidity IgG is produced during the chronic infection period.

In ocular toxoplasmosis, there is no correlation between specific antibody levels and the symptomatology of the patient (Oréfice & Garcia 2005). Low IgG levels and absence of reactive IgM are common. Although levels of specific IgG in serum compared with IgG levels in the aqueous humour, as well as PCR analysis, have been useful for diagnosis, they show low sensitivities (Garweg et al. 2000). The determination of specific IgA subtypes in the serum and aqueous humor contributes to the diagnosis of ocular disease (Katina et al. 1992, Garweg et al. 2000). Secretory IgA (sIgA) is the dominant antibody in tears and together with lysozyme and lymphocytes in the lachrymal film forms the first ocular defensive barrier. Lynch et al. (2004) have found that there was strong association between the levels of sIgA in individuals presenting the clinical picture consistent with gold standard ocular toxoplasmosis. The test showed 84% sensitivity and 78% specificity, compared to individuals without ocular disease or symptomatology of toxoplasmosis.

A new ELISA method for ophthalmic cysticercosis diagnosis from the lachrymal sIgA anti *Taenia solium* meta-cestode ES antigen reported a specificity and a sensitivity of 92.3% and 50%, respectively; these are better parameters than the detection of IgG in serum (Sahu et al. 2008).

The purpose of this study was to provide an objective validation (phase III) for the ELISA-specific determination of anti-*T. gondii* sIgA in the tears of individuals with ocular toxoplasmosis.

### PATIENTS AND METHODS

Two groups of immunocompetent patients older than 10 years of age with active posterior uveitis were studied:

+ Corresponding author: malynch@nlink.com.br

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The active posterior uveitis presumably due to toxoplasmosis group (APUPT) consisted of 82 individuals and the active posterior uveitis due to other aetiologies group (APUOE) had 74 individuals. The APUPT group included individuals characterised by the presence of ocular inflammation, e.g., cells in the vitreous humor, active retinochoroiditis, as well as retinal vasculitis and anterior segment inflammation to varying degrees. Almost every patient (81) had evidence of previous retinochoroidal scarring. Only one patient did not show the above characteristics but had anti-*T. gondii* IgM and, therefore, was included in the APUPT group. Individuals in the APUOE group had active posterior uveitis due to other aetiologies that were serologically and clinically confirmed.

Patients in both groups presented inflammation of the right and/or left eye. When both eyes were affected, the eye with more severe inflammation at the time of sampling was considered as the affected eye. The healthy eye was considered as the unaffected eye. The disease was diagnosed through bio-microscopic examination and indirect binocular ophthalmoscopy at the Departments of Ophthalmology at The Federal University of Pernambuco and Federal University of Minas Gerais Hospitals. Patients were selected from April 2003-December 2006.

**Ethics** - Informed written consent was obtained from all the participants or their parents/guardians. This study was approved by the ethical committee for research on humans at the Federal University of Pernambuco, under the protocol 044/2004-CEP/CCS.

**Sampling of tears** - Tears from both eyes were collected from participants using sterilised filter paper strips (filter paper grade 237 Toyo Roshi®, Japan, 4). The sample was collected by introducing the edge of the strip into the outside third of the inferior eyelid, without previous stimulation. The strips were identified for each eye, coded and stored at -23°C.

**Serum samples** - Samples for anti-*T. gondii*-specific IgG and IgM determinations were collected from each patient, coded and kept at -23°C until indirect immunofluorescence was carried out.

**Crude *T. gondii* extract preparation** - This was done according to Lynch et al. 2004. RH strain tachyzoites were collected from the mouse peritoneal cavity on the 3rd day after infection with 0.01 M PBS (pH 7.2), pelleted at 800 g, sieved through a filter with a 3-µm pore size and washed twice. The protocol of extraction followed the antigen preparation described by Meek et al. 2000.

**sIgA determination** - Tears were eluted from weighed filter paper strips with 0.01 M PBS pH 7.2 under full mixing in order to obtain a 1:40 dilution. sIgA was determined by the ELISA capture method as described by Lynch et al. (2004). Briefly, 5.1 µg/mL of *T. gondii* crude antigen were diluted in 50 mM carbonate bicarbonate buffer (pH 9.0), immobilised in microplate wells for 3 h and then blocked with skimmed milk for 3 h at 18°C. Fifty µL of tears were incubated for 1 h at 37°C. Incubation with the rabbit anti-secretory piece (Sigma; 1:700) at 37°C for 1 h was followed by a similar incubation with a 1:800 dilution of a goat anti-rabbit, peroxidase-

conjugated secondary antibody, to detect the presence of specific sIgA. H<sub>2</sub>O<sub>2</sub> (0.05%) and OPD (0.032mg/mL citrate buffer) were used as substrate. The washing buffer was 0.05% PBS-Tween-20 (0.06 M; pH 7.2). The optical density of the reaction was read at 492 nm.

**IgG and IgM determination** - The indirect immunofluorescence method described by Camargo (1964) was performed with tachyzoites of the RH strain as antigens. A positive reaction with serum (dilution, 1:16) was considered as reactive serum and the titer was determined by the fluorescence staining around the parasites' edges. The reaction was carried out in 102 samples of serum, from 52 APUPT and 50 APUOE patients.

**Statistical analysis** - The Epi Info™ program version 6.04 (1997), the software SPSS 13 for Windows and the Evidence-Based Medicine Calculator, version 1.2, of BMJe in the Graphpad InStat, version 3.05 for Windows were used for statistical analyses. To compare sIgA reactivity between the groups, confidence intervals were determined and for the interpretation of results, a *t* test was carried out. Statistical significance considered when *p* < 0.05. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratio were calculated according to standard methods.

## RESULTS

Of the 156 patients, 82 (52.57%) were carriers of APUPT, 81 had recurrent ocular toxoplasmosis with scars and satellite lesions and one had a typical inflammatory lesion without scars and was considered to have acquired toxoplasmosis. The remaining 74 (47.43%) patients had uveitis from other aetiologies (APUOE).

In terms of gender, 79 patients (50.64%) were male and 77 (49.35%) were female. In relation to ages, 71 patients (45.5%) were between 10-30, 67 (43.30%) were between 31-50 and 18 (11.2%) were above 50 years.

Patients' origins showed that 85 (54.48%) were from the metropolitan region of Recife, only 20 (12.82%) came from other cities in state of Pernambuco and 51 (32.7%) were from Belo Horizonte (state of Minas Gerais).

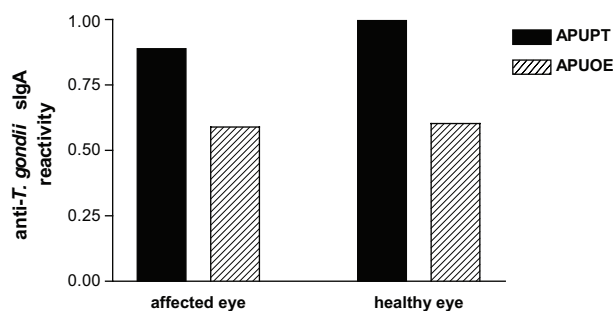
The IgG titers of anti-*T. gondii* from the 102 analysed samples showed that 50 (96.2%) patients with APUPT had an IgG > 1:16, whereas 34 (68%) with APUOE tested positive at this dilution. Two patients with toxocariasis showed IgG titers of 1:64. There was only one positive case of IgM among the APUPT carriers (Table I) and that patient had IgG and IgM titers of 1:320 and 1:32, respectively.

Anti-*T. gondii* sIgA levels in the tears of each group were measured and analysed using the arithmetic mean of sIgA reactivity for each eye. Among the APUPT patients, the average reactivity was 0.988 for the affected eyes and 0.996 for the unaffected eyes. The APUOE group showed lower average levels of sIgA, 0.589 for the affected eye and 0.603 for the unaffected eye; however, these differences were not significant. When comparing sIgA levels in the APUOE and APUPT groups, the difference between both groups was statistically significant, with values of *p* = 0.004 for the affected eye and *p* = 0.005 for the healthy eye (Figure).

TABLE I

Sera anti-*Toxoplasma gondii* IgG e IgM antibodies in patients with active posterior uveitis presumably due to toxoplasmosis group (APUPT) and active posterior uveitis due to other aetiologies group (APUOE)

Sera antibodies anti- <i>T. gondii</i>	Uveitis patients					
	APUPT		APUOE		Total	
	n	%	n	%	n	%
IgG						
> 1/16	50	96.2	34	68.0	84	82.4
Negative	2	3.8	16	32.0	18	17.6
IgM						
> 1/16	1	1.9	0	0.0	1	0.8
Negative	51	98.1	50	100	101	99.2
Total	52	100	50	100	-	-



Tears secretory IgA (sIgA) anti-*Toxoplasma gondii* mean reactivity in the affected and healthy eyes of active posterior uveitis presumably due to toxoplasmosis group (APUPT) and active posterior uveitis due to other aetiologies group (APUOE).

Out of 82 patients with APUPT, 54 tested positive for sIgA. Of the APUOE group, 21 patients were positive. Therefore, the sensitivity of the method was 65.9% (95% CI = 54.5-75.4), the specificity was 71.6% (95% CI = 59.8-81.2), the PPV was 72% (95% CI = 60.3-81.5), the NPV of 65.4% (95% CI = 54-75.4) and the positive likelihood ratio was 2.33 and negative likelihood ratio was 0.49 (Table II).

The relationship between different aetiologies of uveitis injuries and the reactivity of anti-*T. gondii* sIgA were analysed in patients with APUOE (Table III). A higher frequency of false-positive results was observed in patients with uveitis caused by *Toxocara* spp, *Treponema pallidum* infection or rheumatic origin. Unfortunately, the number of samples was too small to carry out any meaningful study of association.

## DISCUSSION

It is clear that uveitis is an important cause of blindness worldwide. Of all the cases of posterior uveitis, 30-50% are attributable to *T. gondii* (Belfort et al.

1978, Jain et al. 1998, Gehlen et al. 1999). It has been established that toxoplasmic retinochoroiditis is the most common identifiable cause in different parts of the world (Holland 2003). Early and accurate diagnosis promotes adequate treatment, mitigating the visual impairments of the disease (Reis et al. 1998). Therefore, the identification of a reliable marker of active ocular toxoplasmosis is necessary, particularly when the clinical aspects are not well defined.

In this study, groups with active posterior uveitis were analysed, one group with clinically typical ocular toxoplasmosis and another group having posterior uveitis due to different causes.

The studied samples did not differentiate between genders. Consistent with previous reports (Schellini et al. 1993, Sebben et al. 1995, Lynch et al. 2004), APUPT tends to occur more frequently in younger individuals; in this study, 45.5% of cases were between 10-30 years. This is an important aspect, since the disease leads to long-term morbidity when scars appear in the macula (Lynch et al. 2008). This fact affects the quality of life of the individual and can deprive patients of their ability to work.

Here, the frequency of latent toxoplasmosis in the Brazilian population, demonstrated by Oréfice and Garcia (2005), was confirmed by the presence of IgG-positive sera in 82.4% of the individuals. IgM is considered a marker of systemic activity of the illness, although, although in the reactivated ocular form it produces mostly a negative result.

Except for one patient, all individuals of the APUPT group presented with the reactivated illness, showing retinochoroidal scars (Lynch et al. 2008) and IgM-negative sera. Only one patient had acquired ocular toxoplasmosis without scars; this patient tested positive for IgM, had an IgG dilution of 1:320 and was positive for sIgA in the tears.

The sensitivity and specificity of this test for lachrymal sIgA detection showed satisfactory results, compared with other tests used to determine the production of specific antibodies in secretions. That study, carried out by Meek et al. (2000) with *T. gondii* crude extracts, detected the presence of IgA antibodies against surface proteins of tachyzoites in the tears of healthy individuals. Detection of anti-*T. gondii* IgA in the saliva of healthy volunteers was described by Loyola et al. (1997). Additionally, Borges and Figueiredo (2004) reported IgA-positive results in Brazilian patients with neurological manifestations from other aetiologies.

The test used here for anti-*T. gondii* sIgA antibodies showed that some patients with ankylosing spondylitis, rheumatoid arthritis and syphilis had sIgA in their tears. This may be due to the presence of the natural antibodies shown to exist in healthy individuals of endemic areas, probably in response to a conserved group of antigenic proteins of other parasites (Meek et al. 2002). Also, considering the regional prevalence of toxoplasmosis, it is possible that these patients may have circulating bradyzoite antigens that stimulated the mucosal immune system, including the eye-associated lymphoid tissue, of which the conjunctive and lachrymal glands are part (Meek et al. 2003, Knop & Knop 2005). The two patients

TABLE II

Evaluation of sensitivity, specificity, predictive values, probabilities of illness and confidence interval (CI) for the ELISA detection of secretory IgA (sIgA) anti-*Toxoplasma gondii* in tears

ELISA sIgA	Groups		Sensitivity CI (95%)	Specificity CI (95%)	Positive predictive value	Negative predictive value
	APUPT	APUOE				
Positive	54	21	65.9	71.6	72	65.4
Negative	28	53	54.5-75.4	59.8-81.2	60.3-81.5	54.0-75.4
Total	82	74				

APUOE: patients with active posterior uveitis due to other aetiologies; APUPT: patients with active posterior uveitis presumably due to toxoplasmosis group; likelihood ratio (+): 2.33; likelihood ratio (-): 0.49.

TABLE III

Secretory IgA (sIgA) to *Toxoplasma gondii* in tears of patients with posterior uveitis due to other etiologies (APUOE)

Uveitis etiology	anti- <i>T. gondii</i> sIgA n (%)
Infectious	
Hanseniasis	0/1 (0)
Syphilis	2/3 (66)
Tuberculosis	1/2(50)
Herpes zoster virus	0/4 (0)
Herpes simplex virus	1/2 (0)
Toxocariasis	2/2 (100)
Non infectious	
Choroiditis	2/7 (28.5)
Intermediate uveitis	1/7 (14.2)
Ankyloising spondylitis	2/4 (50)
Rheumatoid arthritis	3/5 (60)
Vasculitis	0/4 (0)
Traumatic	0/3 (0)
Postsurgical	0/2 (0)
Fuchs heterochromic ciclitis	0/2 (0)
Behcet's disease	1/1 (100)
Vogt-Koyanagi-Harada	0/2 (0)
Idiopathic	6/23 (26)
Total	21/74 (28.37)

with uveitis that was serologically attributed to toxocariasis had positive serum IgG reactions to anti-*T. gondii* (1:64) and IgA-positivity in the tears. Jones et al. (2008) have demonstrated that co-infection of these two parasites is common in the USA. We were unable to determine whether these IgG levels were immune responses to toxoplasmosis concomitant with *Toxocara* infection. Nevertheless, the average sIgA reactivity in this test showed greater values in the APUPT patients (0.988), compared to the APUOE group (0.589) ( $p < 0.05$ ). The unaffected eye also showed a positive reaction to sIgA. A previous study demonstrated that the sensitivity of the

ELISA test used here was 84% and the specificity was 78%, when comparing the sIgA reactivity of anti-*T. gondii* in the tears of APUPT patients to sIgA from clinically healthy individuals (Lynch et al. 2004).

In the eye, the local production of antibodies in the aqueous humour, compared to the serum antibody levels (Goldmann-Wittmer coefficient), can be determined within a sensitivity range of 41-80% (Desmont 1966, Bornard & de Gottrau 1997, Garweg et al. 2000, Fardeau et al. 2002) and with a specificity of ~90%, depending on the technique used for measuring the antibodies. Thus, using immunoblotting, the sensitivity for IgG detection was 50% with a specificity of 93%; for IgA detection, however, 35% of the patients tested positive and 20% of normal controls had IgA that was reactive to *T. gondii* (Garweg et al. 2004). The combination of ELISA and immunoblotting tests for IgG detection in the aqueous humour resulted in diagnosis of 83% of patients with toxoplasmosis (Villard et al. 2003). It has also been demonstrated that local production of antibodies continues for months after the inflammatory activity has stopped, even within the unaffected eye (Garweg 2005).

The method presented here identified the presence of sIgA with sensitivity (65.9%) and specificity (71.6%) in the tears of patients that manifested clinically defined, gold standard uveitis due to toxoplasmosis. These results are comparable with other tests described in the literature for the diagnosis of ocular toxoplasmosis. This test, however, is not invasive and it is easy to perform, innocuous and of low cost.

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