Development and validation of an Enzyme Linked Immunosorbent Assay (ELISA) test for the diagnosis of toxoplasmosis in Sri Lanka

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(Index words: In-house ELISA, somatic antigen, toxoplasma, sensitivity, specificity)

Abstract

Introduction ELISA is the most widely used form of diagnosis for toxoplasmosis. Several commercial kits are currently used in Sri Lanka. However, these kits are not affordable in resource-limited settings.

Objectives Aim of this study was to develop a cost effective in-house ELISA for the detection of *Toxoplasma* antibody and to estimate the diagnostic accuracy compared to a commercial kit.

Methods Vero cell lines were inoculated with tachyzoites and harvested after 2-6 days and sonicated to obtain somatic antigen. The antigen was used as coating material in ELISA to detect antibodies against *T. gondii* in patient sera. Hundred and three patients' sera were analysed by in-house ELISA and kit ELISA. Optical density (OD) values were analysed statistically. *Toxoplasma* IgG avidity test was used to determine the chronic and acute phase of infection.

Results The optimum working dilutions for antigen was 0.846 μg/ml and for serum 1 in 100. The optimal cut-off values for the in-house ELISA within the range 0.85 to 0.98 at which the sensitivity was 95.3% and specificity was 98.3. The OD values of in-house ELISA were compared with OD values of kit ELISA and the results showed strong correlation between the two tests.

Conclusions The results of our study demonstrated that our in-house ELISA for detection of *T. gondii* antibody was as sensitive and specific as the commercial kit used in this study. Thus, the in-house ELISA is a useful, cost-effective tool for diagnostic and screening purposes.

Introduction

Toxoplasmosis is caused by an intracellular opportunistic protozoan, *Toxoplasma gondii*. Infection with this parasite both in humans and in animals is widespread in most countries globally. In healthy humans, *Toxoplasma* infection is asymptomatic or mild. For many years it was recognized as an infective agent that is responsible for congenital infection resulting in fetal death or abnormality, but in recent times it has also immerged as a major pathogen of humans with HIV infection and organ transplant [1, 2]. The life cycle of this parasite can only be completed in definitive hosts; cats and other feline. However, *T. gondi* infects a wide variety of warm blooded animals including humans as intermediate hosts. *T. gondi* is transmitted to humans via ingestion of food or water contaminated with oocysts shed in cat faeces, eating raw or undercooked meat containing tissue cysts in the flesh of intermediate hosts, transplacental (congenital) route or by blood transfusion or organ transplant.

Approximately one third of the world population has been exposed to this parasite. Based on seroprevalence, Sri Lanka, along with the neighboring countries India and Bangladesh are categorised in the intermediate prevalence group amongst the Asian countries [3]. The seroprevalence of healthy adults in Sri Lanka is 27.5% [4]. Clinical features associated with toxoplasmosis are varied and nonspecific. Hence, the diagnosis of toxoplasmosis based solely on the clinical findings is unreliable. Isolation and detection of *Toxoplasma* parasite in clinical specimens is very difficult. Therefore, the diagnosis of toxoplasmosis depends heavily on immunological tests, with detection of *Toxoplasma* specific IgG, IgM and IgA antibodies [5]. Further low avidity tests, which indicate recently formed immunoglobulin is also now available [6, 7]. In Sri Lanka diagnosis of toxoplasmosis is currently done using commercially available *Toxoplasma* IgG and/or IgM ELISA kits. A multitude of commercial kits are used in Sri Lanka to identify *T. gondii*. Many of these have not been evaluated in tropical countries and as such the interpretation and clinical implication of results are difficult. Further, these commercially available kits are costly. Thus, there is a need to develop cost effective in-house diagnostic tools to diagnose patients infected with *T. gondii* in Sri Lanka.

The aim of the study was to develop a cost effective in-house ELISA for the detection of *Toxoplasma* antibody

and to estimate the diagnostic accuracy compared to the commercial kit.

Methods

This retrospective descriptive study consisted of 103 participants which included 30 pregnant mothers referred for screening of toxoplasmosis, 40 patients with clinically suspected ocular toxoplasmosis referred by consultant ophthalmologists and 33 patients with clinically and histologically confirmed toxoplasmic lymphadenopathy referred by consultant physicians and consultant surgeons to the Department of Parasitology, Faculty of Medicine, University of Peradeniya from January 2010 to December 2013. Participants with a co-morbid diagnosis of toxocariasis were excluded from the study. From each patient, 2 ml of venous blood was collected into a plain bottle without anticoagulant via venepuncture. Blood was allowed to stand at room temperature (RT) for about 1 hour and kept at 4°C for 3 hours to yield the maximum volume of serum. Serum was separated by centrifugation (500 rpm for 5 minutes) and was stored in labeled sterile containers at -20°C for use in ELISA. Ethics approval was obtained from the Ethics Review Committee, Faculty of Medicine, University of Peradeniya.

Preparation of T. gondii somatic antigen: T. gondii, RH-88 tachyzoites (ATCC/BEI resources) were grown and maintained in African green monkey kidney cells (Vero cell line) according to an accepted method [8]. The Vero cells were seeded with tachyzoites (1×10^7) and incubated at 37°C, 5% CO_2, in DMEM medium (Sigma) containing glucose (4.5g/l), glutamine (292 mg/l), sodium pyruate (110 mg/l), sodium bicarbonate (2.2 g/l), penicillin (100000 u/ml) and streptomycin (10 mg/ml) in T_{25} cell culture flasks. When the maximum number of tachyzoites were observed (2-6 days) they were harvested from the cell culture medium by centrifugation of medium at 4°C, 2000 rpm for 3 minutes. The supernatant was discarded and sediment containing tachyzoites were pooled and sonicated using ultrasonic sonicator (Labson 2000) at 20 kilocycles per second in pulses of 20 seconds each for a total of 10 minutes [9]. The sonicated material was subjected to cold centrifugation at 4°C, 800 g 20 minutes to remove cellular debris. The supernatant containing the *Toxoplasma* somatic antigen was obtained and the protein concentration of antigens was estimated by commercially available protein estimation kit (Quick Start Bradford protein assay kit, Bio-Rad). Antigen was stored in aliquots of 0.5 ml at -20°C until use.

Development of in-house ELISA: In-house ELISA was developed according to a method described already [10]. Optimum working dilutions of antigen (0.846 µg/ml), serum (1:100) and conjugate were determined by checker board titration. The microtitration plates were coated with 0.846

µg/ml *Toxoplasma* somatic antigens and incubated at 4°C overnight. Plates were then washed in washing buffer (PBS with 0.05% Tween 20), post coated with 100µl PBS containing 1% bovine serum albumin (BSA) and 2.5% sucrose and incubated at RT for one hour. The plates were washed five times with washing buffer. Subsequently 100 µl of diluted (1:100) serum samples were added to the test well in duplicates and incubated for one hour at RT. Known negative and positive sera were used as control in each plate. Following the incubation, plates were washed three times in washing buffer to remove unbound serum and 100 µl of horse radish peroxidase conjugated rabbit anti-human IgG (Sigma Chem Co.) at a dilution of 1:5000 was added to each well. Plates were incubated for 1 hour at RT. Subsequently 100µl of the substrate o-phenylenediamine dihydrochloride, 2 mg tablets in 3% hydrogen peroxide solution (Sigma-Aldrich, India) was added to each well. After incubating for 20 minutes at RT, 100 µl of 3M H_2SO_4 was added to each to stop the reaction. The optical density (OD) at 492 nm was measured with an automated ELISA reader. Cut off point of kit ELISA (OD ≥ 1) was considered as *Toxoplasma* antibodies positive for in-house ELISA. The entire test was performed by a trained technical officer who was blind to the results of the other tests.

Diagnosis by commercial kits: 103 serum samples were tested for the presence of *T.gondii* specific IgG using Toxoplasma IgG Kit (AccuDiag™ ELISA, USA). An OD value ≥ 1 was considered as *Toxoplasma* antibody positive. The IgG positive and negative samples confirmed by commercial kits were used to validate the in-house ELISA. Commercial kit was considered as the gold standard in this study. The entire test was performed by a trained technical officer who was blind to the results of the other tests.

Data analysis and Validation of in-house ELISA: The in-house ELISA test for detection of *T. gondii* infection was validated using serum from 103 participants whose *T. gondii* infection status was determined through kit ELISA test. The sensitivity and specificity of the in-house ELISA tests to detect *T. gondii* antibodies was calculated by comparing with the kit ELISA test considering it as the definitive test.

All statistical calculations were performed using SPSS version 19. A receiver-operating characteristic curve (ROC curve) for the 'in-house ELISA' test was obtained. The OD values obtained in the in-house ELISA was compared with OD values from the kit ELISA and the Pearson correlation coefficient was calculated.

Toxoplasma IgG avidity testing: Avidity assay was performed according to the method described by 'focus diagnostics'. Avidity was assessed by setting up triplicate sets of the routine *Toxoplasma* ELISA for *T. gondi* specific IgG. However, after initial incubation with antibody one set was washed with 6 M urea for 8 minutes and the other set was washed with wash buffer lacking urea. The avidity

index (AI) is calculated using the formula given below. AI values <0.20 indicate low avidity, values of 0.20-0.25 indicate intermediate avidity, and values > 0.25 indicate high avidity [11].

Avidity index = $\dfrac{\text{Absorbance with urea}}{\text{Absorbance without urea}}$

Results

The age of the participants ranged between 6 to 56 years with a mean age of 32 years. The study population consisted of 62 females and 41 males. The common symptoms presented by ocular patients were inflammation, pain, redness, sensitivity to light and blurred vision.

Of the 103 samples the kit ELISA identified 43 cases to be seropositive and 60 cases to be seronegative. The in-house ELISA test detected *Toxoplasma* antibodies in 42 cases and the other 61 were seronegative. The sensitivity of the in-house ELISA was 95.3% and the specificity was 98.3% (Table 1). The positive predictive value was 97.6% and negative predictive values was 96.7%.

Table 1. Diagnostic 2✕2 table demonstrating the computation of sensitivity, specificity and predictive values

	Kit ELISA		
	Positive	Negative	
In-House ELISA Positive	41	1	Positive predictive value=0.976
In-House ELISA Negative	2	59	Negative predictive value=0.967
	Sensitivity=0.953	Specificity=0.983	

The area under the ROC curve for the in-house ELISA test was 0.97 with a standard error of 0.02 (Figure 1). The OD values of all the 103 samples diagnosed by the in-House ELISA matched with the result of the kit ELISA although the same OD values were not achieved. However, three of the samples were wrongly diagnosed by the in-house ELISA; two samples as false negative and one sample as false positive. Figure 2 graphically presents the correlation between the kit ELISA and the in-house ELISA. A highly significant positive correlation between the OD values of kit ELISA and in-house ELISA was observed (Pearson= 0.834, p<0.0001). Hence, the in-house ELISA test can be considered as an excellent test to diagnose toxoplasmosis.

All 43 seropositive samples tested with the *Toxoplasma* IgG avidity test yielded a result of high avidity cases showing that the infection was in the chronic phase.
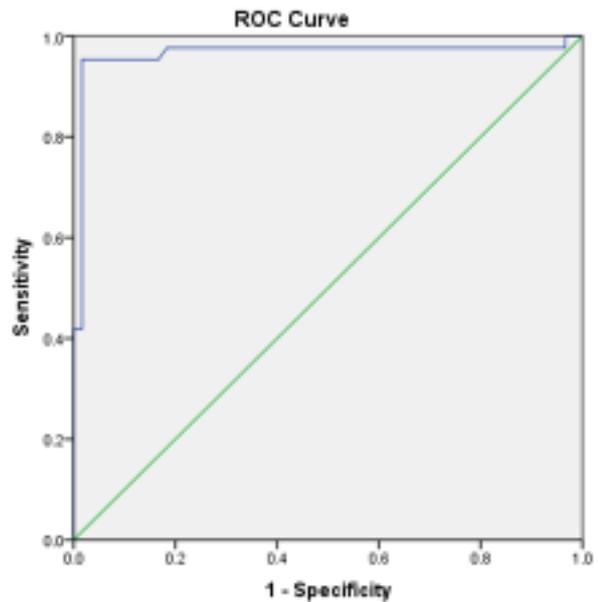

Figure 1. **ROC curve showing the sensitivity and specificity of In- House ELISA.**
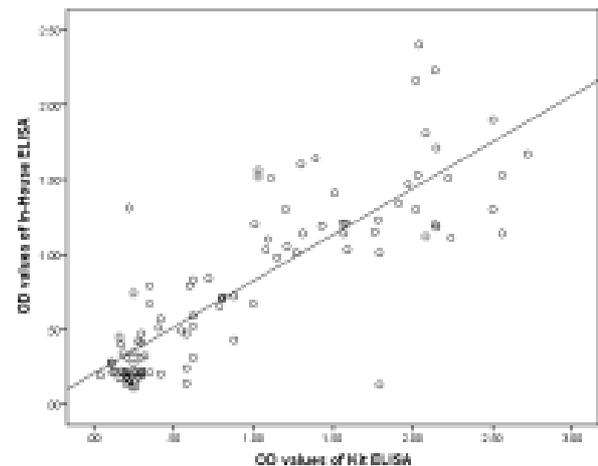

Figure 2. **Highly significant positive correlation was observed between the OD values of Kit ELISA and In-House ELISA [r (103) = 0.834, p <0.05].**

Discussion

The zoonotic protozoan, *T. gondii*, affects one third of the human population worldwide [12, 13]. It is described as a '*Silent threat*' in most of the Asian countries [14]. After entering the human, the parasite establishes itself by forming tissue cysts at various anatomical sites manifesting in a clinical spectrum of disorders that range from asymptomatic infections to severe infections involving multiple organs [15]. Most important clinical manifestations include ocular and neurological disorders.

On the higher end of the spectrum it is capable of causing schizophrenia and bipolar affective mood disorders which reduces the quality of life [16,17]. Toxoplasmosis is also regarded as a risk factor for epilepsy [18]. Transplacental transmission from mother to fetus may have serious consequences for the fetus when acute infection occurs during pregnancy. The neonate once infected by *T. gondii* may develop hydrocephalus, microcephaly, intracranial calcifications, retinochoroiditis, strabismus, blindness, epilepsy, psychomotor and mental retardation, petechiae due to thrombocytopenia and anemia [19, 20]. But most often the infection is asymptomatic and if not treated, the baby may develop retinochorditis or neurological disorders in childhood or adulthood [21, 22].

Currently, the diagnosis of toxoplasmosis in our country relies heavily on the commercially available ELISA kits. Thus, in response to this we have focused on developing an in-house ELISA to effectively diagnose toxoplasmosis. When compared with kit ELISA, the in-house ELISA was highly sensitive and specific. In commercial kit the undiluted patient sera was used but the in-house ELISA was sensitive to sera that was diluted 100 times. The pearson correlation coefficient was computed to compare the OD values of the in-house ELISA with the gold standard; an excellent highly positive correlation (r = 0.834) was obtained suggesting that our diagnostic tool is very similar to the commercial kit.

Another objective of this study was to establish a diagnostic method to distinguish between acute phase and chronic phase infection. It is of great importance to distinguish acute from chronic infection especially in the case of pregnant mothers to evaluate the transmission risk and decide on the course of treatment. IgM antibodies can depict recent infection, however in some cases IgM antibodies remain in the blood for almost a year, hence relying on IgM can provide false positive results. The use of IgG is effective since it appears in very low concentration during the first few weeks of infection and peaks only after 12 weeks to 6 months and remains throughout life [23]. The IgG antibody avidity test detects the IgG in the blood and it is very useful to identify a recent infection [24]. The IgG avidity index shifts from low to high within 5 months of infection. Patients with a low avidity index are in the acute phase while those with the high avidity index are in the chronic phase of the infection. Low avidity depicts a recent infection thus enabling the clinicians to plan the appropriate treatment for the patient or the neonate. In the present study all the IgG positive samples (n=43) had high avidity thus showing that the infection was chronic in all those cases.

Conclusions

The results of our in-house ELISA showed it was a reliable method for the detection of antibody against *T. gondii*. It had sensitivity and specificity comparable to commercial assay for *T. gondii* antibody detection. The

in-house ELISA could detect antibodies from patient serum at a dilution of 1 in 100 and was also cost effective. Thus, the in-house ELISA may be adopted for screening large number of samples in resource limited settings in Sri Lanka. The *Toxoplasma* avidity test ELISA can also be used to screen pregnant mothers for acute phase infection, thus helping them take necessary precautions to eliminate the parasite and making sure that the fetus is not prone to neurological or ocular defects.

Conflicts of interest

There are no conflicts of interest.

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