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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Evaluation of Excreted/Secreted Antigens Derived from Peritoneal of *Toxoplasma* Infected Small Mice to Detect IgG Against *Toxoplasma*

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Abstract: The present study, evaluated, using the components of peritoneal fluid of infected mice (as another source of E/SA), for the detection of *Toxoplasma* specific IgG in human serum. Peritoneal fluids of mice infected by intraperitoneal (IP) inoculation of *Toxoplasma* tachyzoites were collected after 3 days and centrifuged at 750 x g for 15 min then the supernatant was precipitated with ammonium sulphate solution (40% saturated) and used as components containing E/SA. Forty nine uninfected (without anti-*Toxoplasma* antibodies) and thirty two positive (with IgG to *Toxoplasma*) human serum samples were selected (all sera were first tested by standard method for detection of IgG antibodies anti-*T. gondii*) then the sera were tested by ELISA using E/SA. The cut-off point with 95% confidences was found to be 0.78. Moreover, sensitivity and specificity of the method were determined to be 84 and 92%, respectively. The present results indicate that peritoneal exudates from mice infected with *T. gondii* may be used as a source of antigenic material for the detection of *Toxoplasma*-specific IgG and may be valuable for the development of new tools in the serodiagnosis of toxoplasmosis.

Key words: Toxoplasmosis, excreted/secreted antigens, ELISA, *Toxoplasma*

INTRODUCTION

Toxoplasmosis caused by the apicomplexan parasite *Toxoplasma gondii*, is generally clinically asymptomatic in healthy individuals but may cause sever complications in pregnant women and immunocompromised patients. This parasite infects human by one of two acquired or congenital mechanisms and etiological could be divided to chronic (past exposure) and acute (recent exposure) phase.

An of time diagnosis of toxoplasmosis is important for treatment and reduction of the symptoms of disease in adults and fetuses (John *et al.*, 2006).

Diagnosis of infection can be established by the isolation of *Toxoplasma gondii* from blood or bodies fluid, demonstration of the parasite in tissues, detection of specific nucleic acid sequences with DNA probes (Aubert *et al.*, 2000). Due to demand to specific appliances, elongation of processes and risk for pathogens transmission to the staffs, these methods are not applied in clinical labs, therefore, laboratory diagnosis of *Toxoplasma* infection is usually based on the detection of specific antibodies in the serum sample of infected patients. Enzyme linked immunosorbent assay (E/SA) is one of the easiest tests to perform and many serological

kits for detection of *Toxoplasma gondii*-specific immunoglobulin by used this method are commercially available. Most of these commercial kits use a native parasite antigen produced from tachyzoites (Pitekiewicz *et al.*, 2004). More problems in serological tests are related the antigens that used in these tests. Several studies have been established to identify some *Toxoplasma* components as antigens for valuable diagnostic serological test. Many studies reported that *Toxoplasma gondii* excreted/secreted antigens (ELISA) appear to be suitable marker for toxoplasmosis serodiagnosis (Dalimi *et al.*, 2003; Ahn *et al.*, 1994; Eui and Ho, 2001).

Most of these studies have used E/SA obtained from supernatant of *Toxoplasma* call culture or by incubating tachyzoites in cell free media (RPMI-1640). The present study, evaluated using the components of peritoneal fluid of infected mice (as another source of E/SA) for the detection of *Toxoplasma* specific IgG in human serum.

MATERIAL AND METHODS

Serum sample: Serum samples from child bring age women were submitted to Rafsanjan university of medical sciences clinical labs for detection of anti-*Toxoplasma*

anti-bodies between September 2005-December 2006 were tested by ELISA IgG kit (toxog IgG, human western Germany) for detection of anti-*Toxoplasma* IgG and results were confirmed by sabin-fildman dye test (Gulie, 1996), of these 32 positive samples and 40 negative were selected.

Preparation of excreted/secreted antigens: Peritoneal fluid mice infected 3 days earlier with tachyzoites of *Toxoplasma* (RH strain) by intraperitoneal inoculation was taken and centrifuged (750 x g for 15 min). Then supernatant was discarded and precipitation with ammonium sulphate solution (40% saturated). After incubation at 4°C overnight was centrifuged (3000 x g for 30 m) sediment dialyzed against 0.05 M Tris buffer for 24 h (the dialyzed buffer was replaced every sixth) finally after concentration was stored at -20°C (Yamamoto *et al.*, 1998).

Designing of E/SA-ELISA

Optimum dilution of conjugate: Briefly, serially dilution of serum (containing anti *Toxoplasma* IgG was tested by ELISA IgG Kit and confirmed by dye test) was placed in each rows of 96 wells ELISA plate. (The 8 wells labeled be letters A-H was referred to as rows), incubated at room temperature for 2 h, washed three times with PBS-Tween 20, blocked for 1 h at 37°C with blocking solution (BSA+PBS) and washed indicated above. Then serially dilution of conjugate (Rabbit-anti human was labeled with peroxidase) was prepared in each of column ELISA plate (the 12 wells labeled be 1-12 were referred to as column). After incubated at 37°C for 1 h was washed with washing solution and 50 µL substrate added to each well. The optical densities (OD) at 492 nm were measured with an automatic ELISA reader.

Optimum dilution of serum: E/SA was diluted 1/100 in blocking buffer and distributed (50 µL per well) in ELISA plate. Incubated and washed as describe in previous step (optimum dilution of conjugate). The positive and negative serum samples (containing low, medium and high level anti-*Toxoplasma* IgG were tested and approved as previously) were selected and serially dilution duplicate from each sample prepared in tow columns of plate. After incubation at 37°C for 1 h was washed and 50 µL conjugate (dilution 1/500 in blocking solution) added in each well, incubated and washed as indicated above than 50 µL substrate (OPD) per well were added. After incubation step 15 min in room temperature, 50 µL arrest solution was added, Optical Density (OD) at 492 nm were read with an automatic ELISA reader.

Optimum of antigens (E/SA): Serially dilution of E/SA was placed in each rows of ELISA plate, incubated and washed as describe in previous stage (optimum dilution of conjugate). Then serially dilution of serum was prepared in each of plate columns. After incubator at 37°C for 1 h was washed with washing solution, 50 µL Conjugate (dilution 1/500 in blocking solution) added in each well, incubated at 37°C for 1 h, washed indicated above, than 50 µL substrate (orthophenglenediaino+hedrogenpleoxidase)per well added. After a final incubation step of 15 min in room temperature, 50 µL of arrest solution was added. The Optical Density (OD) at 492 nm were read with an automatic ELISA reader.

E/SA-IgG-ELISA with positive and negative sera: Each well of microliter plate was coated with 50 µL E/SA diluted in carbonate buffer, incubated for 2 h in room temperature. Then plate was washed three times with PBS and blocked for 1 h at 37°C with blocking solution (BSA+PBS). The plate was washed as indicated above and 50 µL of test (positive, negative or control) serum sample diluted 1/200 in blocking buffer was applied to each well (duplicate wells were use for each sample), incubated and washed as described above than 50 µL substrate (OPD) per well added. After incubation for 15 min in room temperature 50 µL arrest solution was added and optical density (OD) at 492 nm were read with on automatic ELISA reader.

RESULTS

Designing of E/SA-ELISA: Supernatant sediment of peritoneal fluid of small mice was infected with *Toxoplasma gondii* tachyzoites (RH strain) by use solution of ammonium sulphate saturation (40%) was selected as E/SA.

According to plateau-height (where being no decrease in OD or color when the antigens or antibodies are decreased on plate). Plate background (where the residual level of the color or OD independent of the dilution of conjugate, antibody or antigen) and end point (last dilution showing OD or color above the plate background) of chessboard titration of antigens, antibody and conjugate, the optimal dilution was determined for conjugate and antigen 1/500, 1/100, respectively and for antibody (serum) as note which recommended by manufacturer.

Evaluation of E/SA-ELISA for detection of IgG: According to the results of serological tests (commercial ELISA IgG kit and dye-test) 32 serum samples containing anti-*Toxoplasma* IgG only (positive) and 40 negative for

Table 1: Results (OD) obtained by ELISA (commercial kit) for negative serums

No.	1 (OD)	2 (OD)	3 (OD)	4 (OD)	5 (OD)
A	0.216	0.217	0.289	0.261	0.208
B	0.269	0.307	0.248	0.703	0.498
C	0.250	0.797	0.172	0.277	0.202
D	0.390	0.958	0.189	0.248	0.171
E	0.278	0.238	0.329	0.274	1.084
F	0.235	0.212	0.270	0.711	0.218
G	0.211	0.630	0.006	0.174	0.210
H	0.180	0.204	0.754	0.284	0.244

Table 2: Results obtained by E/SA-ELISA for positive serums

No.	1 (OD)	2 (OD)	3 (OD)	4 (OD)
A	0.871	1.524	1.262	0.875
B	0.855	2.254	0.960	0.257
C	2.056	0.590	1.248	0.881
D	0.890	0.496	0.747	1.205
E	1.005	2.349	0.554	0.977
F	0.850	1.296	1.260	1.373
G	0.790	1.268	0.880	0.850
H	1.001	0.908	1.288	0.798

anti-*Toxoplasma* anti-bodies (negative) were selected and tested by E/SA-ELISA as described in materials and methods. The mean and standard deviation of optical densities of negative sera were calculated 0.32 and 0.23, respectively and the mean value plus two SD was determined the cut off value (0.78).

Three negative serum samples scored above cut-off (Table 1) and positive serum samples 5 cases are lower than cut off (Table 2), resulting in sensitivity, specificity, positive predictive value and negative predictive value E/SA-ELISA test for detection of anti-*Toxoplasma* IgG were 84, 92, 90 and 88%, respectively.

DISCUSSION

Serologic test for detection of immunoglobulin IgG and IgM antibodies are commonly performed for diagnosis of acute acquired toxoplasmosis in the immunocompromised patient. A positive IgG titer in most cases is sufficient to establish that a patient has been infected with *Toxoplasma gondii*, because IgM antibodies may persist for ≥ 1 year following the acute infection (John *et al.*, 2006).

The detection of anti-*Toxoplasma* antibodies by ELISA method is commonly performed in many medical centers. The results of such tests are generally well accepted by clinicians because of their excellent sensitivities and specificities, the rapid availability of results and the relatively low costs of the tests, in additional reagents are commercially availability (Tanyuksel, 2004). Despite the fact that serological test give satisfactory results, the production of reliable antigens remained laborious and expensive (Laurence, 2000). A vast rang of molecular components of

Toxoplasma gondii has been evaluated as target antigens to determine the immunity, pathogenesis and diagnosis of toxoplasmosis. Some of these components the so-called excreted/secreted antigens (E/SA) are released from the dense granular of *Toxoplasma*, which form the majority of the circulating antigens in sera from host's with acute toxoplasmosis (on statute 90% circulating antigens). In additional E/SA are proteins expressed at the tachyzoites, sporozoite and encysted bradyzoite stages (Alessader *et al.*, 2002; Ahn, 1994).

Observation on the immune response to these components and resultant protection against re-infection in both human and murine infections may prove useful in the development of new strategies for active immunization against *Toxoplasma gondii* (Costa-Silva *et al.*, 2008; Dalimi *et al.*, 2003). Several reports have emphasized the value of detecting specific antibodies against excreted/secreted antigens of *Toxoplasma gondii* for diagnosing acute toxoplasmosis because they are one of the first target of the host immune response, therefore those components appear to be potential serological marker for toxoplasmosis serodiagnosis (Patricia and Ankonio *et al.*, 2008; Eui and Ho, 2001; Hafid *et al.*, 1992).

The method of preparation of the tachyzoite antigens has a significant effect on assay performance. The most previous studies have used E/SA that obtained incubation tachyzoite in cell free media such as RPMI, PBS. Production of large quantities of E/SA using these methods were difficult and expensive (Aubert, 2000; Dannemann *et al.*, 1990; Alessader *et al.*, 2002).

Yamamoto (1998) reported that peritoneal fluid of mice that infected with *Toxoplasma gondii* RH strain reacted with serum samples from toxoplasmosis patient and also showed that precipitation obtain with 30-40% saturated ammonium sulphate solution of these components contains most of the antigens of potential use in immunodiagnosis and few of the contaminant mouse proteins (Yamamoto, 1998). Present results reveal that there is excellent agreement between results obtained with the IgG-E/SA ELISA and sabin-feildman day test as the reference test for serological diagnosis of toxoplasmosis and commercial kit.

The present results indicate that peritoneal exudates from mice infected with *Toxoplasma* may be used as a source of antigenic material for detection of *Toxoplasma*-specific antibodies and may be valuable for the development of new tools in the serodiagnosis of toxoplasmosis by immuno-enzymatic assay. detection of IgM and IgA antibodies with purified E/SA is currently being evaluated.

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