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Comparison of Western blot and ELISA to detect the Antibody Titers in Human Toxoplasmosis

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Toxoplasmosis is a zoonotic parasitic disease which is caused by *Toxoplasma gondii* that infects all warm-blooded mammals including human beings. In the current study, it was explored to *Toxoplasma*-specific IgG titer while comparing quantitative ELISA and WB. Ten human sera samples labelled as 53, 217, 28, 277, 229, 07, 204, 275, 241 and 04, (known positive as a result of earlier testing in a commercial ELISA kit. were analysed through quantitative ELISA and WB. The WB analysis revealed antibody titers ranging from 800 to12800 with one sample (28) possessing a titer of 1:800, two samples (07, 241) at 1:1600, five samples (53, 277, 204, 275, 04) at 1:3200, one sample (217) at 1:6400 and one sample (229) at 1:12800. The same dilutions of these samples were subjected to our indigenous quantitative ELISA (coated with recombinant SAG1) to determine and antibody (IgG) titer that ranged from 200 to 1600 with two samples (28, 277) at a titer of 1:200, with four samples (53, 07, 204, 241) at a titer of 1:400, with three samples (217, 275, 04) at a titer of 1:800 and with one sample (229) at a titer of 1:1600. In our study, the titers of *T. gondii*-specific-IgG antibodies revealed in WB were found much higher as compared to those in ELISA but there is a high positive correlation (rs = 0.714) analysed by spearman's rho test.
Keywords: *T. gondii*, western blot, ELISA, titers, correlation.

INTRODUCTION

Toxoplasma gondii (Order: Eucoccidiorida, Family: Sarcocystidae) is a heteroxenous and zoonotic protozoan causing toxoplasmosis approximately in all homoeothermic vertebrates including humans and birds (Liu *et al.*, 2012). It is a cosmopolitan parasite that affects one third of human population, globally.(Hill and Dubey, 2002). It affects immunocompromised people in many countries and it is life-threatening for immune-suppressed people (Weiss and Dubey, 2009). It is transmitted through raw/undercooked meat containing tissue cysts of *T. gondii* or water contaminated with *T. gondii* oocysts (Jones and Dubey, 2010). Toxoplasmosis is an asymptomatic disease in healthy persons, and it causes abortion in pregnant female. It is diagnosed in humans through different serological diagnostic tools (Stroehle *et al.*, 2005). The serological tests/tools are used for identification of antibodies against *T. gondii* and these are considered initial and primary methods for diagnosis of toxoplasmosis (Montoya, 2002). Routinely, the identification of specific antibodies in serum is done through various serodiagnostic tests (Bhattacharyya *et al.*, 2013). ELISA, Immunochromatographic Test and LAT are employed for identification of toxoplasmosis (Ibrahim and Yen, 2011; Hassanein and Shehata, 2018; Onosakponome *et al.*, 2020). Western blot (WB) is more sensitive as compared to ELISA but WB is not considered as method of choice for identification of any disease in the laboratory, routinely and it is helpful for serodiagnosis in patients possessing short duration of disease and it can be used as a complement of ELISA (Karlsson *et al.*, 1989; Basso *et al.*, 2013; Persichetti *et al.*, 2017). This study has been designed to compare the antibody titers against *T. gondii* infection by WB and indigenous human ELISA kit (Rahman *et al.*, 2021).

MATERIALS AND METHODS

Isolation of human sera: Previously, a total of 400 sera were collected from humans and screened by our indigenous human ELISA kit named as Human Toxo IgG ELISA kit as described (Rahman *et al.*, 2021). All sera were collected from Diagnostic Laboratory, Mayo Hospital, Lahore. Ten human sera: 53, 217, 28, 277, 229, 07, 204, 275, 241 and 04 were selected based on positivity to find their titers and these were evaluated through ELISA kit.

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***Western blot analysis*:** The test human sera with different dilutions; 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 and 1:12800 were evaluated with WB analysis, respectively. Purified rSAG1 and standard protein marker were separated by SDS-PAGE (SDS–PAGE, BioRad, USA). Proteins were transferred to nitrocellulose membrane (Invitrogen, USA) and carried out in Immunoblot machine (Trans-Blot Turbo transfer system, Bio-Rad) at the rate of 25volts/1.3mA/7min. The membrane was washed with TBST buffer (3 times/15min). 5% skimmed milk-TBST was used for blocking and incubated for overnight (4°C). TBST buffer was used to wash the membrane 3 times (15min) and cut into strips. These strips were incubated with human sera at different dilutions 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600 and 1:51200 (2.5% skimmed milk-TBST/2 hrs.), respectively. Immunoblot was expressed through using AP-conjugated anti-human IgG (Thermo Fisher, USA) (1:500 dilution). The strips were washed with TBST followed by addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (Sigma-Aldrich, USA) for the detection of protein bands on the immunoblot. Reaction was stopped by addition of distilled water.

***Commercial kit-based ELISA*:** *Toxoplasma gondii* IgG ELISA Kit (Invitrogen, Abnova, USA) was used to screen the human sera. The diluted sera were dispensed in the wells of rSAG1 coated plate. The unattached antibodies were eliminated through washing solution. HRP-conjugate was poured in 96-well plate and adhered to antibodies of sera. The complex of antigen-antibody was expressed by addition of Tetramethylbenzidine (TMB) solution. Then, the reaction was stopped by addition of distilled water. Subsequently, results were correlated with controls and calibrators. The test sera samples were added into wells. The calibrators, negative and positive controls were dispensed in duplicates with 1:40 dilutions. The plate was incubated (37°C/30min.) followed by washing (5 times). 100µl HRP-conjugate was dispensed in 96-well plate and it was incubated followed by washing. 100µl TMB was dispensed and incubated (37°C/15min.). 100 µl 1N HCl was added as a stop solution. OD was measured at 450nm through ELISA reader (ELX-800, BioTek, USA) within 15min. after the addition of stop solution.

Human Toxo IgG ELISA kit: The recombinant protein of *T. gondii* (rSAG1) was coated on 96-well plate (JET BioFil, China) at the rate of 0.125µg/ml in 50mM Na_2CO_3 followed by overnight incubation (4°C). It was washed (5 times) with 0.05% Tween-20 in 1X PBS) at the rate of 300µl per well. The blocking was done with 4% BSA in 1X PBS (200µl per well) followed by incubation (37°C/2hrs.) and washing (5 times) with the washing buffer. The test sera samples with different dilutions 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400 were screened through our indigenous human ELISA kit (Rahman *et al.*, 2021), respectively. One column of wells of the plate was used as negative control while another column was kept as blank. Plate was incubated followed by washing, Alkaline Phosphatase (AP)-anti-human antibodies (Thermo Fisher, USA) were added (1:5000 dilution) at the rate of 100µl per well and was incubated. After washing, p-nitrophenyl phosphate (Thermo Scientific, USA) was dispensed at the rate of 100µl per well followed by incubation. 1M NaOH at the rate of 100µl/well was added to stop the reaction and OD was measured (405nm) by ELISA reader (ELX-800, BioTek, USA).

Statistical analysis: Data were statistically analysed by spearman's rho test (Hamilton *et al.*, 2000). A test that is used to compare rank order was employed instead of values of variables for calculating the correlation. Under monotonic relationship between two variables, one variable has inverse association with respective to other (Woolson and Clarke, 2011).

RESULTS

***Titers of anti-T. gondii antibodies analysed by WB*:** Ten human sera showed bands at 35kDa with different titers of antibody in WB analysis. Serum 229 at 1:12800 dilution, sera 217 and 275 at 1:6400, sera 53, 277, 07, 204 and 04 at 1:3200, serum 241 at 1:1600 and subsequently, serum 28 at 1:800 dilution showed bands in WB analysis.

***NCM by WB analysis*:** WB analysis revealed antibody titers ranging from 800 to12800 with one sample (28) possessing a titer of 1:800, two samples (07, 241) at 1:1600, five samples (53, 277, 204, 275, 04) at 1:3200, one sample (217) at 1:6400 and one sample (229) at 1:12800 had presence of bands on NCM (Fig 1).

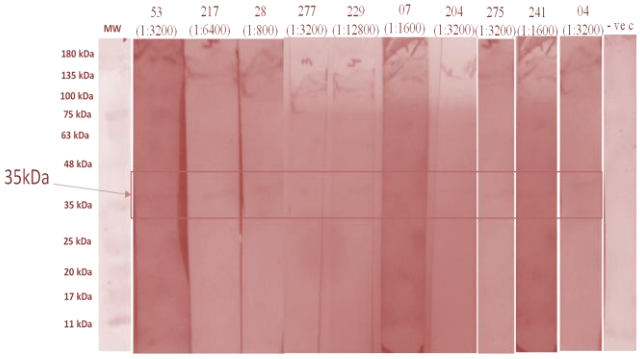


Figure 1. WB analysis of the antibody titers of sera: 53, 217, 28, 277, 229, 07, 204, 275, 241 and 04 using AP conjugated anti-human IgG (Thermo Fisher, USA). Lane 1 shows protein marker (Bio-Helix Cat # PM007-0500), Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10 show positive human sera 53, 217, 28, 277, 229, 07, 204, 275, 241 and 04 with titers of 1:3200, 1:6400, 1:800, 1:3200, 1:12800, 1:1600, 1;3200, 1:3200 1:1600 and 1:3200, respectively. Last lane of -ve control has no band.

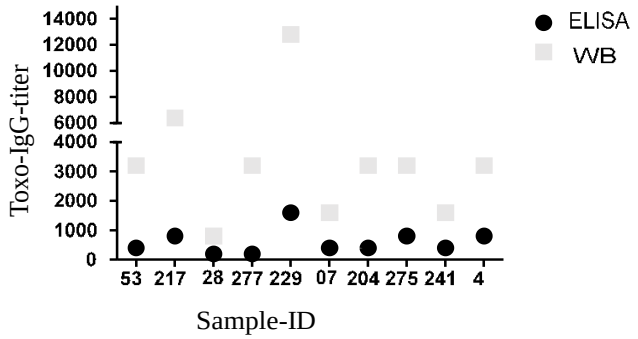
NCM of WB analysis expressed the bands of expected size in all the positive sera (53, 217, 28, 277, 229, 07, 204, 275, 241 and 04) with antibody titers of 1:3200, 1:6400, 1:800, 1:3200, 1:12800, 1:1600, 1:3200, 1:3200, 1:1600 and 1:3200, respectively. The size of positive bands was 35kDa. The antibody titers against *T. gondii* were high in WB analysis as compared to ELISA.

***Titers of anti-T. gondii antibodies analysed by ELISA*:** Ten human sera were positive in rSAG1 ELISA at different antibody titers while serum 03 was negative at all antibody titers. Serum 229 showed the highest titer 1:1600, sera: 275, 04 and 217 indicated 1:800, sera: 53, 07, 204 and 241 showed 1:400 and subsequently, sera: 28 and 277 expressed 1:200. The cut off value for our indigenous human ELISA kit was 0.398 and it was determined by ROC curve (Rahman *et al.*, 2021). According to cut off value antibody titers of sera 53 (1:400), 217 (1:800), 28 (1:200), 277 (1:200), 229 (1:1600), 07 (1:400), 204 (1:400), 275 (1:800), 241 (1:400) and 04 (1:800) were positive. ELISA can find the titers of antibodies from 1:10 to 1:1600 sera of infected humans with *T. gondii.*

***Comparison of Toxoplasma-IgG antibody titers in ELISA and WB*:** WB was found more powerful in determining *Toxoplasma* specific IgG titers in human sera. Titer detected in WB was always a multiple (4 to 16 times) of the corresponding titer detected in ELISA. 50% of the samples (5 out of 10; namely 28, 07, 275, 241, 04) depicted a WB titer that is 4x of the corresponding titer in ELISA, 40% (4 out of 10; namely 229, 53, 217, 204) showed a WB titer that is 8x of the ELISA titer where 10% (1 out of 10; namely 277) displayed a WB titer that was 16x of the ELISA titer (Table 1 and Fig 2).

Table 1. Comparison of *Toxoplasma*-IgG antibody titers in ELISA and WB.

Human sera known positive to *Toxoplasma*	Toxo IgG titer in ELISA	Toxo IgG titer in WB	Ratio (WB/ELISA)
53	400	3200	08
217	800	6400	08
28	200	800	04
277	200	3200	16
229	1600	12800	08
07	400	1600	04
204	400	3200	08
275	800	3200	04
241	400	1600	04
4	800	3200	04



The weighted average of WB titer is 2.42 (Table 2).

Table 2. Weighted average of WB titers to ELISA titer

Titer ratio (WB/ELISA) (Assigned weightage, w)	known human sera (Data point, x)	WB weighted average of titer (Data point, x) (Assigned weightage, w)
04	5	20
08	4	32
16	1	16
WB weighted average of titer		2.42

WB analysis showed higher titers of antibodies against *T. gondii* as compared to ELISA. The correlation between WB and ELISA titers was found out by spearman s rho test. The value of spearman's rho (rs) differs between +1 and -1. The rs = -1 shows perfect disagreement and rs = +1 indicated perfect agreement between values of X and Y (Hamed, 2016). The value rs=0.90 to 1.00 shows very high positive correlation and rs = −0.90 to −1.00 indicates very high negative correlation, rs= 0.70 to 0.90 indicates high positive correlation and rs =−0.70 to −0.90 indicates high negative correlation, rs=0.50 to 0.70 expresses moderate positive correlation or rs =−0.50 to −0.70 expresses moderate negative correlation, rs=0.30 to 0.50 reveals low positive correlation and rs =−0.30 to −0.50 shows low negative correlation and rs=0.00 to 0.30 shows negligible positive correlation and rs =−0.00 to −0.30 shows negligible negative correlation (Mukaka, 2012). In this study, rs= 0.714 indicated high positive correlation between WB analysis and ELISA.

DISCUSSION

The sensitivity with respect to titers of these sera through WB was more as compared to ELISA for identification of toxoplasmosis. Zollner *et al* (2008) described that WB analysis had superior sensitivity and it was reached at 100% due to detection of bands for antigen 5 and hyaluronidase as compared to standard diagnostic techniques for allergy due to wasp venom (Zollner *et al.*, 2001). Urwijitaroon *et al* (1997) described that WB analysis was the most confirmatory test but it was expensive, time consuming and need the technical skill

for performing this test. (Urwijitaroon *et al.*, 1997). Maria *et al* (2022) described that this study employed Western blotting as a reference test and was established with ELISA and found that the tested cat sera which indicated the high sensitivity and specificity of Western blotting as compared to ELISA (Galván-Ramírez *et al.*, 2022). . Karlsson *et al* (1989) described that WB analysis was more sensitive than ELISA. The specificity was not improved by WB analysis. It was not a method of choice test for screening purpose for disease in the laboratory, routinely but it can be used a complementary with ELISA for serodiagnosis of disease (Karlsson *et al.*, 1989).Yi *et al* (2015) explained that the WB was employed as a reference test to validate the sensitivity and specificity of the ELISA in the detection of *T. gondii*–specific antibodies in mink (Gu *et al.*, 2015). (Ballam *et al.*, 2000). Alhajj *et al.* (2021) described that WB and ELISA were two different methods and these were based on the principle of immunodetection. ELISA was used to quantify a specific protein from the mixture of various proteins and used to find out the epitopes of the protein. WB was used to find out the size of protein (Alhajj and Farhana, 2021). Nami, Babak (2013) explained about the importance and higher sensitivity of WB. WB was a useful and effective method for detection and characterization of proteins in small amounts like a clock protein. It can detect a specific protein in the samples. The antibodies in the serum can be detected by binding the antibodies with specific protein in different ways. It is used to determine molecular weight and purity of protein. Nylon or Polyvinylidene difluoride is used as membrane in WB. The membrane has high binding capacity with protein and chemically stable. Some proteins only bind to Nylon. The immunogenic responses that are achieved from infectious agents (like bacteria, viruses) are difficult to isolate from patient samples. WB has ability to detect this response. Three common enzyme substrates are used, chemiluminescent and fluorescent create light, and it is detectable through scanner of X-rays. That is the reason which enables the WB high level of sensitivity and quicker processing time (Nami, 2013). Najafov *et al* (2017) described that WB was more sensitive method due to high affinities of antibodies with respective to their epitopes and its amplificatory nature. It can even detect picogram quantities of proteins (Najafov and Hoxhaj, 2017).

Conclusions: The sensitivity for serum analysis and titer of antibodies against *T. gondii* was high in WB analysis. In this study, the rs= 0.714 indicated high positive correlation between WB analysis and ELISA. It is concluded that WB is more sensitive for finding the titers of antibodies against *T. gondii* than titers of antibodies through ELISA.

Authors contribution: Conceptualization, M.I.R., H.A. and M.Z.S.; methodology, M.I.R., H.A. and S.-u.-R. formal analysis, M.I.R., H.A. and S.-u.-R.; investigation, S.-u.-R., U.U., M.I.R. and H.A.; resources, M.I.R. and H.A.; data curation, S.-u.-R. and U.U.; writing—original draft preparation, M.I.R., S.-u.-R., U.U. and H.A.; writing—review and editing, M.I.R., H.A. and M.Z.S.; visualization, M.I.R.; supervision, H.A.; project administration, M.I.R., H.A.; funding acquisition, M.I.R. and H.A. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

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