



Diagnosis of Toxoplasmosis in HIV/AIDS patients with Immunoblotting

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Abstract

Toxoplasmosis is a common parasitic illness affecting humans. Reactivation of latent infection in HIV infected patients can cause encephalitis. Diagnosing the disease rests on serological methods. ELISA for IgG antibodies remains positive in most HIV-infected patients with toxoplasmosis while immunoblotting can predict the development of encephalitis. Immunodiagnosis by urine sample is easy and has been successfully tried in other parasitoses. So this study aimed at detection of antitoxoplasma antibodies by immunoblotting in sera and urine of HIV infected patients. In patients tested positive for antitoxoplasma IgG, the 97 kDa and 53 kDa antigenic bands were the most reactive in serum and urine respectively. These antigens can be purified and used for immunodiagnosis by ELISA and similar procedures to increase the specificity of diagnosis.

Keywords: Toxoplasmosis, HIV, Immunoblotting.

Introduction

Toxoplasmosis, caused by *Toxoplasma gondii*, is one the commonest parasitic infections of humans and other warm-blooded animals¹. The agent requires two hosts for completing its life cycle. Cats are the usual definitive hosts and mice and rats are intermediate hosts². Infection is acquired by feco-oral route, organ transplant or vertically. Clinical features range from mild febrile illness and lymphadenopathy in the immunocompetent host to encephalitis in the immunosuppressed host. i.e. the HIV infected patient. Congenital infection manifests commonly as chorioretinitis, hydrocephalus and intracerebral calcification².

Diagnosis rests on direct demonstration of the parasite, immunological methods and imaging. While imaging modalities like CT or MRI suffer from lack of specificity³, direct diagnosis is hazardous to the worker and often not conclusive of acute infection. Hence immunological methods are the mainstay for achieving correct diagnosis. Among these serological tests IHA has a poor sensitivity and is more labour-intensive⁴. IFA has low specificity. Sabin-Feldman dye test titres do not correlate with the severity of the illness⁵. ELISA (both conventional and avidity) can be used for diagnosis and prognosis. ELISA is about 88% sensitive and 97% specific for clinching a diagnosis of toxoplasmosis⁶.

Diagnosing *Toxoplasma* encephalitis (TE) in HIV-infected patients is difficult in that CT/MRI features closely mimic CNS lymphoma and IgM ELISA is often poorly sensitive. PCR (polymerase chain reaction) from peripheral blood is variable in sensitivity⁷. However, IgG levels remain elevated in the majority of patients with toxoplasmosis and AIDS. Immunoblot assay has been found to be useful in a handful of studies⁸.

Immunoblot assay can also predict independently the development of TE in HIV-infected patients with toxoplasmosis, as reported by some studies⁹.

Antigen or antibody detection in urine has been attempted for diagnosing several parasitic infections^{10,11} and appears to be useful in those areas where venepuncture is not possible due to lack of trained staff. Immunoblotting in urine is an untreaded territory. Keeping in mind all these points, the present study was undertaken to compare and evaluate IgG antibody response to *T. gondii* by Western Blotting in serum and urine samples of HIV infected patients.

Material and Methods

Subjects: One hundred (100) HIV infected patients were randomly selected from the immunodeficiency clinic of PGIMER, Chandigarh. Fifty (50) HIV negative healthy subjects were chosen as healthy controls and 30 patients with cysticercosis, amebiasis and malaria were selected as other disease controls. Five (5) ml of serum and 10 ml of urine were collected from each subject (patient or control).

Western Blotting: Antigen Preparation: The *Toxoplasma* antigen was prepared from RH strain of *T. gondii*. Tachyzoites were harvested from the peritoneal cavity of 5 Swiss Albino mice infected 3 days earlier by intraperitoneal route (1x 10⁴ tachyzoites in 0.5 ml). The harvested tachyzoites were suspended in PBS (Phosphate Buffer Saline, Ph 7.2) and thereafter subjected to differential centrifugation. In this process, firstly the peritoneal exudates were centrifuged at 65g for 10 minutes and the supernatant containing tachyzoites were isolated. The sediment containing mainly tachyzoites and a few leucocytes was again centrifuged at low speed to free

tachyzoites from leucocytes. Both the supernatants were mixed and centrifuged at 440g for 20 minutes. The resulting pellet was purified tachyzoites. Finally the pellet containing the purified tachyzoites was suspended in 300 µl of distilled water, kept overnight at 4°C and subjected to sonication at about 20 kilocycles per second in pulses for 10 minutes, in ice. Subsequently the sonicated material was subjected to cold centrifugation at 4°C at about 800g for about 20 minutes to remove cellular debris. The protein concentration of the final supernatant was determined by Biuret method. This antigen was used for serodiagnosis by Western Blotting.

Elisa: At first Elisa was carried out in serum and urine samples of the patients and controls and data were recorded. The IgG positive samples were further analysed by immunoblotting.

Immunoblotting: The proteins in the crude antigen were separated by SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) using the method described by Stroehle *et al*¹². At first the soluble crude *Toxoplasma* antigen was loaded on a vertical SDS-PAGE gel (10%) using premixed electrophoresis buffer (Tris-Glycine-SDS). Following this electrophoresis was carried out at 140 volts and 0.04 ampere constant current for 1 hour with a calibrated, prestained molecular weight standard (page ruler, broad range, prestained protein ladder, Plus II, 10-250 kDa MW, MBI Fermentas). After separation of the antigenic fractions, they were blotted onto nitrocellulose membrane using a semi-dry apparatus under electric current (60 Volts and 0.3 ampere) for 1 hour and 10 minutes. Then the membranes were washed 3 times for 5 minutes each in distilled water, dried between filter papers and stored at 4°C till use. Before use, papers were cut into strips, dipped in methanol, and washed in 0.1% PBST. They were then blocked with 3% BSA in PBST overnight at room temperature.

After washing, samples (serum diluted 1 in 100 in 1% BSA and undiluted urine) were loaded in each trough in a volume of 2 ml and incubated for 2.5 hours at room temperature. Then after 3 washings in PBST with 1% BSA, strips were incubated for 2.5 hours at room temperature with goat anti-human IgG HRP-labelled conjugate, diluted 1 in 4000 in 1% BSA in PBST. Next to this, after 2 washings in PBST and one washing in PBS, bound antibodies were detected using H₂O₂ as substrate and DAB as chromogen¹².

Results and Discussion

Thirty eight percent patients were asymptomatic. The rest had various coexisting illnesses and symptoms like pulmonary tuberculosis, chronic or persistent diarrhea, oral thrush etc.

Age: The mean age of the HIV infected patients was 32 years (range 24 to 64 years). The mean age of the subjects in the disease control group was 19 years (range 14 to 23 years) and in the healthy control group it was 29 years (range 24 to 32 years).

Sex: Among HIV infected patients 68% were males and 32% were females. In the healthy control group 70% were males and 30% were females, whereas in the disease control group 71% were males and 29% were females.

Immunoblotting: SDS-PAGE of *T. gondii* antigen after Coomassie Brilliant Blue staining of the gel showed a number of polypeptide bands of following molecular weights (kDa) as detected by Gel Doc syste, shown in figure 1.

190, 175, 130, 116, 110, 103, 97, 93, 84, 80, 73, 64, 58, 53, 47, 43, 36 and 24 kDa

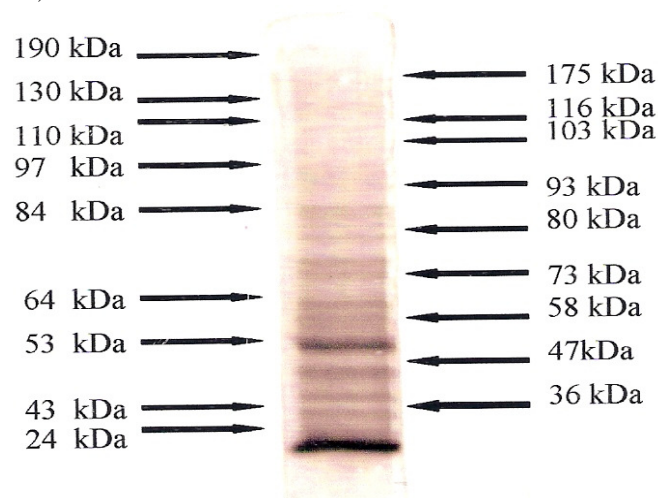


Figure-1
Coomassie blue staining of resolved antigenic bands

Thereafter Immunoblotting revealed the following:

Serum Samples: Group IA (HIV positive patients antitoxoplasma IgG positive by ELISA): Total number of patients in this group was 42. Soluble *Toxoplasma* antigen resolved into 18 bands on SDS PAGE. However, 11 components out of these reacted with patients' sera of this group. Most of the patients' sera reacted with bands of molecular weights of 64 kDa, 73 kDa, 80 kDa and 97 kDa.

Group IB (HIV positive patients, antitoxoplasma IgG negative): Total number of patients in this group was 58. Out of this 18 bands resolved on SDS-PAGE, 9 reacted with sera of this group. Most of the patients' sera reacted with polypeptides bands of molecular weights 64 kDa and 73 kDa. None reacted with the 97 kDa band.

Group II (Other Disease Controls): This group consisted of 30 patients, 10 each of malaria, cysticercosis and amebiasis. Most of the sera of the subjects in the malaria subgroup reacted with polypeptides of molecular weight 64 kDa and 84 kDa. Most of the sera in the cysticercosis subgroup reacted with polypeptides of molecular weight 53 and 43 kDa, while most of the sera in the amebiasis subgroup reacted with bands of molecular weight 80 and 103 kDa. None of the other disease

controls reacted with 24 kDa, 97 kDa, 116 kDa, 130 kDa, 135 kDa and 195 kDa antigenic bands.

Group III (Healthy Controls): Total number of subjects in this group was 50. Most of the patients' sera identified bands of molecular weights of 84, 64 and 103 kDa.

Thus it can be concluded that the most specific immunoreactive antigenic fraction of *T. gondii* in HIV infected patients in serum was the 97 kDa protein since it did not cross-react with sera of disease controls or healthy controls.

Urine: Group IA (HIV positive patients, serum antitoxoplasma IgG positive): Out of 18 bands resolved on SDS-PAGE, 10 components reacted with patients' urine of this group. The most immunoreactive bands of molecular weights 93 kDa, 64 kDa and 53 kDa.

Group IB (HIV positive patients, serum antitoxoplasma IgG negative,): Out of 18 bands resolved on SDS-PAGE, 10 components reacted with patients' urine of this group. The most immunoreactive bands were of molecular weights of 93 kDa, 84 kDa, 64 kDa and 80 kDa.

Group II (Other Disease Controls): Out of 18 *T. gondii* antigenic bands resolved on SDS-PAGE, In the malaria subgroup most of the urine samples of the subjects reacted with polypeptides of molecular weight 64 kDa and 84 kDa. Most of the urine samples in the cysticercosis subgroup reacted with polypeptides of molecular weight 58 and 43 kDa, while most of the samples in the amebiasis subgroup reacted with bands of molecular weight 80 and 103 kDa.

Group III (Healthy Controls): Total number of subjects in this group was 50. Most of the patients' urine identified bands of molecular weights of 84, 80, 64 and 103 kDa.

It was concluded that the most specific immunoreactive antigenic fraction of *T. gondii* in HIV infected patients in serum was the 53 kDa protein since it did not cross-react with sera of diseased controls or healthy controls.

Toxoplasmosis is one of the commonest parasitic illnesses of man affecting all ages. It is otherwise a self-limited infection in the immunocompetent host but can manifest as severe encephalitis and chorioretinitis in immunocompromised state, e.g. the HIV infected patient. Detection of parasite-specific antibodies is one of the pillars for achieving diagnosis but in HIV infected patients, IgM antibody is often not detectable. IgG antibody level, however, remains elevated in about 97% of HIV infected patients having toxoplasmosis^{13,14}. Immunoblotting has been shown to be a useful diagnostic procedure in mice models⁸. It has another advantage in that the most reactive antigenic bands can be eluted and used for ELISA to increase the specificity of diagnosing the stage of infection¹⁵. Urine sample has been reported to be useful for the diagnosis of

parasitic illnesses like hydatidosis and schistosomiasis^{10,11}, and has the greatest advantage of being easily obtainable and non-invasive, especially in peripheral settings where venepuncture is difficult to carry out. Immunoblotting in urine is an untreaded territory. So this study was planned to compare and evaluate IgG antitoxoplasma antibody detection by Immunoblotting in serum and urine samples of to detect the most reactive bands. Western Blotting showed that most antitoxoplasma IgG-positive sera reacted against the 97 kDa antigenic fraction of *Toxoplasma gondii*. This is important with regard to findings by other researchers who had found bands of molecular weight 27-32 kDa to be most reactive¹⁶. A study from Brazil quotes the most immunoreactive band in acute and chronic toxoplasmosis in HIV infected patients to be the 97 kDa antigenic band¹⁷. The most immunoreactive band in urine samples of the HIV infected patients was the 53 kDa antigenic band. These antigenic bands did not react with control samples or samples of other HIV positive patients who were negative for antitoxoplasma IgG antibodies in ELISA, and can hence be used as antigens in user-friendly test systems like ELISA to enhance the specificity of immunodiagnosis.

Conclusion

The purified proteins can be purified and used for immunodiagnosis of Toxoplasmosis.

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