

A SEROLOGICAL STUDY OF CYSTICERCOSIS IN PATIENTS WITH HIV

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SUMMARY

Neurocysticercosis (NCC) has attained the importance of one of the most common cause of focal brain lesions in patients infected with HIV (human immunodeficiency virus). Adequate data regarding the rate of this co-infection is lacking. Therefore, the present study was carried out to determine the prevalence of cysticercosis among HIV patients residing in Puducherry or its neighboring districts of Tamil Nadu State, India. A total of one hundred blood samples were collected from HIV seropositive cases visiting JIPMER hospital, Puducherry, between June 2007 and May 2008. Enzyme immuno-transfer blot (EITB) and enzyme linked immunosorbent assay (ELISA) were used to demonstrate anti-*T. solium* larval stage antibodies and Co-agglutination (Co-A) test was used to detect *T. solium* larval stage antigens in sera. Two HIV seropositive cases were found positive for anti-*T. solium* larval stage antibody by EITB and four were positive by ELISA. Only one sample was positive by both EITB and ELISA. No serum sample was found positive for *T. solium* larval stage antigen by Co-A test. The overall seropositivity detected by all the methods was 5% in this study group. The accurate clinical diagnosis of NCC in HIV is difficult due to deranged immunological parameters in the HIV infected patients. The results of this study provides important data on the prevalence of cysticercosis in HIV positive patients in Puducherry and neighboring areas which was previously unknown. This study will also increase awareness among physicians and public health agencies about *T. solium* cysticercosis in the selected group.

KEYWORDS: Cysticercosis; Enzyme immuno-transfer blot; Enzyme linked immunosorbent assay; Co-agglutination test; HIV positive patients.

INTRODUCTION

Cysticercosis, a disease caused by the larval stage of cestode parasite *Taenia solium* is widely prevalent in human and porcine hosts in many developing countries of Latin America, Africa and Asia⁷. Approximately 50 million people are infected with *T. solium* and about 50,000 deaths occur due to this disease worldwide every year⁴. Human cysticercosis results from the ingestion of food, water and vegetables contaminated with *T. solium* eggs or even by autoinfection in case of persons with intestinal taeniasis. The central nervous system (CNS) is the most common site of severe symptomatic infection where the condition is referred to as NCC²³.

Due to the increased frequency of HIV infection, cysticercosis has become one of the most frequent opportunistic infections and the most common cause of focal brain lesions in patients infected with HIV^{10,11}. Furthermore, cysticercosis is endemic in many regions of the world and more common in pig eating countries with low socioeconomic status and poor sanitation³. HIV infection is a serious problem in the present day world wide. A high rate of infection is found in many regions of the world, including India¹⁷. One of the major health problems among

HIV seropositive patients is superimposed infections due to the defect in immunity¹⁵. The increasing frequency of HIV infection in endemic areas of cysticercosis may increase this co-infection. Only few cases of cysticercosis in HIV - infected patients have been reported, and the influence of HIV infection on the natural history of cysticercosis is yet to be defined. We therefore conducted this study specifically to determine the seroprevalence of cysticercosis in HIV seropositive cases residing in Puducherry or its neighboring districts of Tamil Nadu State for specific *T. solium* larval stage antigens and anti-*T. solium* larval stage antibodies using immunological techniques developed and evaluated in this laboratory.

MATERIALS AND METHODS

Study group: A total of one hundred serum samples from HIV seropositive cases were analyzed. This study was carried out in the Out Patient Department and Inpatient wards of Medicine and Neurology in Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Puducherry, India. Serum samples collected from the patients were screened for antibodies against HIV by ELISA and confirmed by Western blot.

Controls: Sera collected from known positive NCC cases (confirmed by MRI, EITB and ELISA) were used as positive control (n = 16 definitive diagnosis group, as per the diagnostic criteria for the diagnosis of NCC by DEL BRUTTO *et al.* in 2001)⁷. For negative control, sera collected from normal healthy individuals were used (n = 25). Sera collected from patients with hydatidosis were used to detect the cross reactivity (n = 15). For Co-A test, 10 µL of total soluble protein antigen of *T. solium* larval stage was added to 90 µL of phosphate buffered saline (PBS) pH 7.2 (1:10) which was further diluted to 1:1280 using PBS pH 7.2. This was taken as positive control. Informed consent was obtained from all subjects in this group.

Preparation of the *T. solium* larval stage antigens: The *T. solium* larval stage was collected from the tissue of naturally infected pig, which were dissected free from surrounding tissue into sterile PBS pH 7.2 containing 40 mg each of kanamycin, nalidixic acid and ampicillin with protease inhibitor, phenylmethylsulphonyl fluoride (PMSF) (0.006%) at room temperature. Whole cysts after removing the cyst fluid were taken to prepare the complete somatic homogenate antigen as per the method described by SREENIVASAMURTHY *et al.*, 1999²⁷. Ten grams of ruptured whole cysts were homogenized manually in glass tissue homogenizer with PBS (pH 7.2) containing PMSF (0.1 mM) at 4 °C. The homogenate was further subjected to sonication, eight times at 12kHz with 30 seconds cooling interval on an ice bath. The homogenate was centrifuged at 4 °C for 30 minutes at 15,000 rpm in refrigerated centrifuge. The supernatant was used as the *T. solium* larval stage somatic antigen²⁷. The protein analysis of complete somatic homogenate was carried out by standard procedure described by LOWRY *et al.*, 1951¹⁶.

ANTIBODY DETECTION

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was indicated for analyzing the protein profile of the antigen, by characterizing & separating proteins based on their molecular weight. Briefly, the metacestode antigen was subjected to SDS-PAGE in 10% gel for resolving the antigenic peptides using a submerged gel electrophoresis apparatus (*Bangalore Genei, India*) using standard protocol²⁴. The antigen preparations were separated under non-reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gels on 10 X 8 cm (1-mm thick) mini-gels (*Bangalore Genei, India*). The gels were stained with Coomassie Brilliant Blue and molecular weight was determined using standard molecular weight markers (*Bangalore Genei, India*)³⁰.

Enzyme immuno transfer blot: The glycoprotein antigen used in EITB was prepared by using metacestodes obtained from naturally infected pig to analyze the antibody response. For immunoblotting analysis, the separated antigens mentioned above were electrophoretically transferred to a 0.22-µm-pore-size nitrocellulose membrane (NCM) (*Pierce, Rockford*) in a mini-Transblot cell (*Pharmacia Biotech, USA*). The free binding sites were blocked by treatment with 3% bovine serum albumin (BSA) in PBS for two h and washed in PBS containing 0.1% Tween 20. Analysis was carried out with human sera diluted 1:100 in PBS (pH 7.2) containing 0.1% Tween 20 (PBST)³⁰. Horseradish peroxidase-conjugated goat antibody to human IgG diluted 1:1000 (*Bangalore Genei*), was used as secondary antibody. Antibody reactivity was visualized with a substrate solution containing diaminobenzidine

(*Pierce, Perbio*) (6 mg/mL in PBS containing one mL of 30% hydrogen peroxide (*Sigma*)².

Enzyme linked immunosorbent assay: ELISA was performed as per the procedure described by standard method of Crowther with few modifications⁶. Briefly, one µg protein per 100 µL concentration of the antigen was prepared in antigen coating buffer (PBS pH 7.2) as per the method described earlier and used for coating plates (NUNC) followed by blocking non-specific binding sites by 2% BSA in PBS (pH 7.2). Optimum dilution (1:50) of test serum sample and known *T. solium* larval stage antibody-positive serum samples were prepared in sample dilution buffer, added in duplicate in the wells, and goat anti-human-IgG-HRP conjugated secondary antibody (*Bangalore Genei, India*) was added to each well for detection. Substrate solution prepared freshly by adding six mg of OPD in 10 mL of PBS pH 7.2 and 10 µL of H₂O₂ (30%) was added just before to use. The reaction was stopped by adding 50 µL of 2N H₂SO₄ in each well to avoid over reaction and development of optimum colour⁹. The absorbance was taken at 492 nm in an ELISA reader (BIO RAD). Various controls were used for validity of the assay such as antigen blank, antibody blank, negative control serum, and known positive control serum. Serum sample which gave OD₄₉₂ value of more than *cut-off* (mean of 50 negative serum samples + 2 SD) was considered as positive. The cut-off used in the present study was 0.157.

ANTIGEN DETECTION

Co-agglutination test: Co-A test to detect *T. solium* larval stage antigen in the serum was carried out as per the method described by us before²⁰. In this test Cowan's strain of *Staphylococcus aureus* rich in Protein A (SAPA) cells, sensitized with polyclonal antibodies (hyperimmune serum against *T. solium* larval stage) were used to detect specific *T. solium* larval stage antigen in the serum. The hyperimmune serum was used at the concentration of 100 µL/10 mL of SAPA cells suspension (1%) as the optimal sensitizing dose (OSD). Hyperimmune serum against *T. solium* larval stage whole cyst antigen (total soluble protein antigen) was raised in white New Zealand rabbit in divided doses with Freund's complete adjuvant in the primary dose and with incomplete adjuvant in the booster doses. The IgG fraction from the immune serum was purified by precipitation with cold saturated ammonium sulphate by the method of GOTTSTEIN¹⁴.

The test was performed as per the procedure described by SHARIFF & PARIJA (1993)²⁶. Sensitization of these cells was done by adding 0.1 mL of rabbit antisera to one mL of 10% Staphylococcal suspension and incubated for 30 minutes at 37 °C in water bath. These incubated cells were washed in PBS and re-suspended to a concentration of 2% in PBS 7.2 with 0.1% sodium azide. The test was performed on glass slides, which were divided with a glass marking pencil into two equal halves. 25 µL of the test serum sample was placed on each half of the slide. An equal volume (25 µL) of sensitized SAPA cell suspension was added to the serum on one half. The same volume (25 µL) of a 2% suspension of unsensitized SAPA cells was added to the serum on the half as a cell control. The slide was then rotated manually for two min and then observed. In a positive Co-A test, the addition of sensitized SAPA cells to the test serum resulted in the formation of large visible clumps of bacterial cells within two min; in a negative reaction, no visible clumping was observed. Agglutination with the sensitized cells and not with the

unsensitized cells was considered to be a positive result. It is a qualitative test, so there is no cut-off used.

RESULTS

SDS PAGE & enzyme immunotransfer blot: In the present study the high molecular weight antigenic peptides were observed to be reactive and specific to patient sera more frequently than the low molecular weight antigenic peptides when *T. solium* larval stage somatic antigens were used. A total of six major bands were detected in the *T. solium* larval stage antigen in the SDS-PAGE profile. The major antigenic peptides were found to be 100kDa, 75kDa, 67.74kDa, 51.6kDa, 47kDa, 43.5kDa. Only two antigenic peptides, i.e. the 75kDa, 67.74kDa, were demonstrated by the EITB in serum samples. Of 100 serum samples tested, two (2%) had anti-*T. solium* larval stage antibodies. The antibody to *T. solium* larval stage somatic antigen reacting with one or more of these peptides in the serum was considered positive by the test.

Enzyme linked immunosorbent assay: ELISA demonstrated reactive *T. solium* larval stage antibody titre in four (4%) serum samples collected from HIV seropositive cases. The cut-off OD₄₉₂ values of the ELISA using *T. solium* larval stage somatic antigen was used to detect IgG antibodies in the serum. The test detected antibodies in sera from known positive controls and did not show any antibodies in the sera of negative controls and patients with hydatid disease.

Co-agglutination test: The Co-A detected *T. solium* larval stage antigen in none of the serum samples out of total 100 HIV seropositive cases. The results were compared with that of EITB for detection of antibodies. Among these samples two (2%) were positive for anti-*T. solium* larval stage antibody by EITB and four (4%) were positive for antibodies by ELISA. Only one sample was found positive in both tests indicative of a low concordance between the two assays. Thus, overall five samples were positive for anti-*T. solium* larval stage antibodies. The antigen detection limit of the Co-A test using the particular batch of reagent preparation was found to be up to 0.27 µg²¹.

DISCUSSION

Relatively few cases of HIV and NCC co-infection have been reported, even in areas where there is high prevalence of both diseases individually^{5,25,28,29}. In a study conducted in Mexico only 1.1% of NCC has been reported in HIV positive cases, compared with 2.4% in control autopsies¹⁵. In South Africa, co-infections by HIV, *M. tuberculosis*, and *T. solium* are relatively common in densely populated area where the parasite is widely distributed¹⁸. NCC has been reported in HIV cases traveling to areas with high intensity of cysticercosis²⁹. In this report HIV test serology was positive by ELISA and Western blot and the CD4+ T-lymphocyte count was 473 cells/µL. SERPA *et al.* (2007) reported NCC as a fatal infection in a HIV infected Colombian immigrant at Spain¹⁰. An unusual form of epidural spinal racemose NCC revealed by compression of Cauda equina in a HIV infected man was reported in France. SERPA *et al.* (2007)²⁵ identified 27 cases of NCC-HIV co-infection in Houston. The most frequent presentation (61%) was with multiple parenchymal lesions⁸. Similarly, NCC and HIV co-infection are reported from different areas of the world including, Italy, Ecuador, Mexico and Zimbabwe^{10,11,25,28,29}. However, little is known about the association of HIV and the clinical course of cysticercosis. Concurrent

infection with *T. solium* and HIV would be expected to occur more frequently because of the increasing frequency of HIV infection in India where cysticercosis is endemic.

In the present study EITB and ELISA were used to detect anti-*T. solium* larval stage antibody in patients with HIV. In most of the studies sensitivity and specificity of EITB reported was 98% and 100% respectively^{1,3,13}. Many of the investigators have found a varying sensitivity and specificity with ELISA test for detection of anti-*T. solium* larval stage antibody, using different antigenic preparations¹⁹. However, ELISA and EITB are the most frequently used tests in recent times. By using EITB and ELISA, 5% serum samples were positive for anti-*T. solium* larval stage antibody in HIV cases. The anti-*T. solium* larval stage antibody positive cases were from Villupuram, Cuddalore, Thiruvannamalai districts that are immediately neighboring to Puducherry where pig farming is commonly done.

Among the anti-*T. solium* larval stage antibody positive cases, none of them were presenting the symptoms of NCC or other form of cysticercosis. The presence of anti-*T. solium* larval stage antibodies in five cases indicates that the cases either have suffered from cysticercosis in past or having an infection with no overt clinical manifestations. In this study the seroprevalence of cysticercosis was less in HIV patients compared to non HIV infected individuals (6.1%)²². This apparently lower prevalence in this population may be due to absent or low immune response in patients with HIV or the cystic stages of the parasite may not evoke a strong detectable antibody response while they remain alive and intact.

T. solium larval stage antigen was detected by Co-A test in 57% of cysticercosis cases with hyperimmune sera raised to whole cyst antigen in the previous studies^{20,21,22}. An interesting finding was that none of the HIV seropositive cases were positive for *T. solium* larval stage antigen. The failure of Co-A in these cases to detect antigen could be either due to the absence of antigen in the serum, or antigen concentration that were below detection limit of the assay or as a result of degradation of antigen during storage of the serum at -20 °C.

Diagnosis of cysticercosis in HIV patients is very important, because tuberculous meningitis and toxoplasmosis are very common in this group which mimics NCC³². So a consideration of cysticercosis in this group of patients can help in early diagnosis. Detection of the antibodies in the serum to *T. solium* larval stage antigens is the most commonly used and convenient method for the laboratory diagnosis of cysticercosis³¹. As the immune response is lowered in HIV positive patients the sensitivity of antibody detection may be reduced in this group of patients. Symptomatic cysticercosis largely depends on the host inflammatory response³³. Decreased cell-mediated immunity characteristic of advanced HIV infection makes cysticercosis more likely to remain asymptomatic^{31,32}. In sero-diagnosis the initially reported high sensitivity and specificity of the EITB assay may be ambiguous to detect antibody in this study group. So the result of EITB assay may be more reliable in patients with AIDS, in combination with radiographic and clinical data.

CONCLUSION

This study provides important data on the sero-prevalence of *T. solium* cysticercosis in HIV positive patients in Puducherry and neighboring

areas which was previously unknown. This population is at risk for *T. solium* cysticercosis where the parasite affects the immune response of the host. We hope this study will increase awareness among physicians and public health agencies about *T. solium* cysticercosis in the selected group. As the incidence of HIV is increasing in endemic areas of cysticercosis, simultaneous diagnosis of both diseases is an event to be expected.

RESUMO

Estudo sorológico da cisticercose em pacientes com HIV

Neurocisticercose (NCC) tem alcançado a importância de uma das mais comuns causas de lesões focais no cérebro em pacientes infectados pelo HIV (vírus da imunodeficiência adquirida). Dados adequados relativos à frequência desta co-infecção estão faltando. Portanto, o presente estudo foi realizado para determinar a prevalência da cisticercose entre pacientes com HIV residindo em Puducherry ou distritos vizinhos do Estado de Tamil Nadu, Índia. Um total de cem amostras foram coletadas de casos soropositivos do Hospital JIPMER, Puducherry, entre junho de 2007 e maio de 2008. "Enzyme immunotransfer blot" (EITB) e ELISA foram utilizados para demonstrar anticorpos contra a fase larval do *T. solium*. Testes de co-aglutinação (Co-a) foram usados para demonstrar antígenos da fase larval do *T. solium* no soro. Dois casos HIV soropositivos foram positivos para anticorpos contra a fase larval do *T. solium* por EITB e quatro foram positivos por ELISA. Somente uma amostra foi positiva por ambos EITB e ELISA. Nenhuma amostra de soro foi positiva para antígeno da fase larval do *T. solium* pelo teste Co-a. A soropositividade total detectada por todos os métodos foi 5% neste grupo de estudo. O diagnóstico clínico exato de NCC em HIV é difícil devido aos desordenados parâmetros imunológicos nos pacientes infectados pelo HIV. Os resultados deste estudo fornecem dados importantes sobre a prevalência da cisticercose em pacientes HIV positivos em Puducherry e áreas vizinhas que eram previamente desconhecidas. Este estudo também aumentará a atenção dos médicos e agências de saúde pública sobre a cisticercose por *T. solium* em grupo selecionado.

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Received: 26 May 2008

Accepted: 18 June 2009