Antibody response to *Toxoplasma gondii* in saliva samples from human immunodeficiency virus-infected patients

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ABSTRACT

Toxoplasma gondii is an important opportunistic infection among human immunodeficiency virus (HIV)-infected patients as it causes fatal encephalitis. In the present study, antibody response to *T. gondii* is assessed in saliva samples from 100 HIV-seropositive patients and 25 HIV-negative healthy controls by indirect enzyme-linked immunosorbent assay (ELISA). Sensitivity and specificity for detection of IgG and IgM in saliva is calculated using a positive antibody response in serum samples (from an earlier study) as the gold standard. IgG and IgM antibodies were found in 20% and 25% patients, respectively. One control subject showed the presence of IgM antibody. Sensitivity for IgG and IgM antibodies was 64% and 81.25%, respectively, while specificity was 94.67% and 85.71%, respectively. This study indicates that saliva samples can be used as an alternative to serum samples to detect anti-toxoplasma antibodies, particularly IgM, for the diagnosis of toxoplasma encephalitis in HIV/acquired immune deficiency syndrome patients.

KEY WORDS: Antibody. Saliva. Toxoplasma gondii.

Introduction

Toxoplasmosis is an important opportunistic infection among individuals infected with human immunodeficiency virus (HIV). At present, more than 40 million people worldwide are infected with HIV and 1400 people become infected each day. In India there are 3.82–4.58 million individuals with HIV infection.[1]

Patients with acquired immune deficiency syndrome (AIDS) are at high risk of developing toxoplasma encephalitis, as in 95% of cases it is due to reactivation of latent infection. However, it can also occur as a result of the acquisition of parasites from exogenous sources such as blood or transplanted organs.[2]

The incidence of toxoplasma encephalitis (TE) is directly related to the prevalence of HIV infection and anti-toxoplasma antibodies in the general population.[3] In India, the seroprevalence of toxoplasmosis is reported to be 1.5–22%,[4–6] and up to 57% in Uttar Pradesh.[7] Toxoplasma encephalitis occurs in nearly 50% of HIV patients with anti-toxoplasma antibodies; thus, early diagnosis of at-risk patients is imperative.[8]

Diagnosis of toxoplasmosis can be achieved by the demonstration of parasites in tissue samples, parasite isolation by culture techniques,[9] mouse inoculation,[10] serological methods such as antibody[8] or antigen detection[11] and molecular techniques such as the polymerase chain reaction (PCR).[12]

Although amplification of specific toxoplasma DNA sequences by a PCR technique may prove useful in the diagnosis of individuals co-infected with HIV and toxoplasmosis,[12,13] the problem of standardisation and non-availability in all hospitals has hampered its use in routine diagnosis. Moreover, the sensitivity of PCR has proved controversial.[13–18]

Furthermore, the demonstration of parasites in clinical samples requires expertise and facilities for parasite isolation are not available at most peripheral hospitals. Thus, the diagnosis of toxoplasmosis depends largely on serological evidence.

Serodiagnostic techniques for the detection of specific antibody responses are simple, non-invasive approaches that are useful in the diagnosis of toxoplasmosis in immunocompetent individuals. Reports on antibody response to *Toxoplasma gondii* in serum samples of HIV-infected individuals indicate that 12–67% show an IgG antibody response, while IgM antibodies are present in up to 16% patients.[19–22]

Interest in the use of oral fluids for the diagnosis of microbial disease in HIV cases[23] was initiated in 1986, and similar approaches were used subsequently for the diagnosis of parasitic diseases such as schistosomiasis,[24] neurocysticercosis,[25] amoebic liver disease,[26,27] trypanosomiasis[28] and intestinal helminthiasis.[29] Antibodies can be detected in oral fluids because they transudate from serum to the oral cavity through the crevicular fluid that is released via the gingival crevice.[30]

Few studies have been undertaken to evaluate the usefulness of saliva samples in the diagnosis of toxoplasmosis.[31,32] Moreover, these have been carried out largely in immunocompetent individuals; the one exception

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being the study of Amato Neto *et al.*,[33] who attempted *T. gondii* isolation in saliva samples from 26 AIDS patients. However, none of the samples was found to be positive.

Saliva collection is easy, non-invasive and can be used for large epidemiological surveys. A previous study[20] reported a higher prevalence of toxoplasma antibodies in sera of HIV-infected patients than in that from healthy subjects. Thus, the present study aims to detect IgG and IgM antibody responses to *T. gondii* in saliva samples from HIV-positive patients and to assess whether or not saliva samples can be used as an alternative to serum samples for the early diagnosis of toxoplasmosis in HIV patients.

Materials and methods

Subjects and samples

One hundred HIV-seropositive individuals attending Nehru Hospital, PGIMER, Chandigarh, India, between February and December 2001 were included in the study, all of whom gave advised consent. Twenty-five normal healthy individuals who were seronegative for HIV by an enzyme-linked immunosorbent assay (ELISA) technique acted as the control group.

Samples (1 mL) of unstimulated saliva were collected from all subjects. Immediately after collection, protease inhibitors, aprotinin (Sigma)[29] and pepstatin A (Sigma) were added at a final concentration for each of 2 µg/mL saliva. Samples were kept on ice until centrifuged at 2500 rpm for 15 min at 4°C.[34] Undiluted saliva and serum samples were stored at –20°C.

Antigen preparation

The RH strain of *T. gondii* maintained in Swiss albino mice was used to prepare the antigen.[35] Tachyzoites harvested from the peritoneal cavity of mice infected three days earlier were washed (x2) in 10 mmol/L phosphate-buffered saline (PBS, pH 7.2). Following centrifugation, the pellet was resuspended in distilled water. The parasites were disrupted by sonication for 1 min in an ultrasonic disintegrator (SONIPREP) and then centrifuged at 10,000 x*g* for 60 min. The supernatant was used as a soluble antigen. Protein content was estimated[36] and antigen was stored in aliquots at –20°C. This antigen was used to coat the wells of an ELISA microtitre plate.

Enzyme-linked immunosorbent assay

An ELISA technique to detect anti-toxoplasma IgG and IgM antibodies was carried out following the method of Voller *et al.*,[37] but with slight modification. Briefly, the optimum dilutions of antigen and saliva samples were determined by a checkerboard titration method. Each well of a microtitre plate was coated by overnight incubation at 4°C with 100 µL antigen (predetermined optimum dilution: 1 µg/well). After washing (x3) with PBS–Tween (PBS–T), the wells were blocked with 2% bovine serum albumin and the plate incubated at 37°C for 1 h. This was followed by washing (x3) in PBS–T and the addition of 100 µL undiluted saliva to each well. The plate was incubated at 37°C for 1 h, washed in PBS–T and then 100 µL horseradish peroxidase (HRP)-labelled antihuman IgG (1 in 4500 dilution; Dakopatts, Denmark) or HRP-labelled antihuman IgM (I in 1000 dilution; Dakopatts) was added to each well. The plate was incubated at 37°C for 1 h, washed in PBS–T and then 100 µL substrate solution comprising orthophenylene diamine (OPD) and hydrogen peroxide was added to each well. All plates were kept at room temperature for 15 min and the enzyme–substrate reaction was stopped with 1 mol/L sulphuric acid. Absorbance (*A*) was read in an automated ELISA reader (Eurogenetics A4, Tessenderle, Belgium) at 492 nm.

Each sample was assayed in duplicate and the mean *A* value calculated. Positive controls were saliva samples from toxoplasmosis patients with a high serum antibody titre (>1:1600). Negative controls were saliva samples from normal healthy individuals with undetectable serum IgG and IgM antibody responses to *T. gondii*. Five positive and five negative samples were included in each plate, as were substrate and buffer blanks.

Statistical analysis

Cut-off *A* value for a positive result was determined by adding two standard deviations (2SD) to the mean *A* value of five negative control samples. Samples with an *A* value greater or equal to the cut-off *A* value were regarded as positive. Results were compared between patient and control groups and evaluated by the χ^2 test.

Table 1. Anti-toxoplasma antibody profile in saliva samples of HIV-seropositive patients.

	Number positive (%)			
	IgG+/IgM+	IgG+/IgM-	IgG-/IgM+	IgG-/IgM-
Patients (*n*=100)	8 (8)	12 (12)	17 (17)	63 (63)
Controls (*n*=25)	0 (0)	0 (0)	1 (4)	24 (96)

Table 2. Concordance of toxoplasma IgG antibody in serum and saliva samples in HIV-seropositive patients (*n*=100).

IgG in saliva	IgG in serum		Total
	Positive	Negative	
Positive	16	4	20
Negative	9	71	80
Total	25	75	100

Antibody detection in saliva: sensitivity 64.0%, specificity 94.67%.
Positive predictive value (PPV) 30.0%,
Negative predictive value (NPV) 88.75%.
False positive 36.0%, false negative 5.33%.

Table 3. Concordance of toxoplasma IgM antibody in serum and saliva samples in HIV-positive patients (*n*=100).

IgM in saliva	IgM in serum		Total
	Positive	Negative	
Positive	13	12	25
Negative	3	72	75
Total	16	84	100

Antibody detection in saliva: sensitivity 81.25%, specificity 85.71%.
PPV 52.0%, NPV 96.0%, false positive 14.28%,
false negative 18.75%.

Results

Sensitivity and specificity for saliva testing was calculated against the results obtained by serology in the same patients. Cut-off *A* value for positivity of IgG and IgM antibodies in saliva samples was 0.194 and 0.258, respectively.

In 100 HIV positive patients, anti-toxoplasma IgG and IgM antibody response was detected in 20% and 25% patients, respectively. All control subjects were negative for IgG antibodies but one subject showed the presence of IgM antibody; however, the *A* value of this saliva sample was equal to the cut-off value (0.258). The difference in positive response between the patient and control groups was statistically significant ($P<0.05$) for both IgG and IgM antibodies.

The full anti-toxoplasma antibody profile can be seen in Table 1.

Discussion

Detection of antibodies may help to assess the risk of developing cerebral toxoplasmosis in HIV-positive patient, as this complication occurs in 25–50% of individuals who have anti-toxoplasma antibodies.[20] Although studies have been undertaken[19–22] on the seroprevalence of toxoplasmosis in HIV/AIDS patients, the use of saliva samples as an alternative means of diagnosis has not been investigated.

Antibody detection in saliva samples has been used in many parasitic diseases. Its advantages include easier, non-invasive sampling and the fact that it can also be used for large epidemiological surveys. Hajeer *et al*.[31] analysed saliva samples from 27 patients with recent toxoplasmosis for specific IgG and IgM antibodies to *T. gondii* and found that 13/27 saliva samples were positive for IgG antibodies by direct agglutination test and 8/27 were positive for IgM by immunosorbent agglutination assay. The authors suggest that IgG and IgM in saliva are potentially useful markers of acute toxoplasmosis.

Loyola *et al*.[32] tested serum and saliva from 60 individuals for IgG and IgA antitoxoplasma antibodies. They detected IgG antibodies in 43 (71.7%) serum samples and in 12 (20%) saliva samples, and also detected IgA antibodies in 18 (30%) serum samples and 12 (20%) saliva samples. The authors conclude that the detection of salivary IgA reflects the serum level of this isotype, while salivary IgG does not.

However, only one study has used saliva samples to diagnose toxoplasmosis in AIDS patients.[33] In the study of 26 patients, including six with computed tomography imaging evidence suggesting cerebral abscess caused by *T. gondii*, the authors attempted to isolate parasites in saliva in patients with AIDS. They were unable to detect parasites in saliva using a mouse inoculation model, but did not attempt anti-toxoplasma antibody detection in saliva.

In the present study, anti-toxoplasma IgG and IgM antibody response was seen in 25% and 20% patients in saliva. In an earlier study by the same group, serum samples from the same subjects were analysed for the presence of IgG and IgM antibodies. The sensitivity and specificity for detection of these antibodies in saliva were calculated using serology as the gold standard (Tables 2 and 3).

The higher positivity for IgG in serum compared to saliva may be due to the fact that IgG antibodies are a transudate from serum to the oral cavity. The higher level of IgM positivity in saliva samples compared to serum samples can be attributed to the fact that IgM is a secretory immunoglobulin.[38] It is also possible that circulating antigen formed complexes with the high levels of circulating IgM, resulting in the detection of less free IgM.

Serum samples from the patients included in the present study were analysed previously for the presence of antigen. Of the 12 patients with IgM antibodies in their saliva but not in serum, antigenaemia was demonstrated in five, indicating acute infection in these patients. However. the possibility of a false-positive reaction cannot be ruled out in the remaining seven patients. In the present study, 37% of patients showed IgG or IgM in serum and/or saliva samples. These patients may be at high risk of developing toxoplasma encephalitis and should be considered for prophylactic anti-toxoplasma therapy.

The results of this study suggest that saliva samples can be used as an alternative to serum samples for detection of anti-toxoplasma antibodies in situations where serum collection is not possible or may be difficult, as in the case of large epidemiological studies. However, further studies involving larger number of subjects need to be carried out to confirm the present observations. □

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