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Original Article

Frequency of *Toxoplasma gondii* in HIV Positive Patients from West of Iran by ELISA and PCR

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Abstract

Background: *Toxoplasma gondii*, the obligate intracellular parasite is life threatening in AIDS patients. Diagnosis of toxoplasmosis is based on serological methods especially increasing of IgM and IgG titers, but finding of parasite or its components (antigenemia) may be beneficial method in order to detection of acute toxoplasmosis in immunocompromised patients.

Methods: Ninety-four serum samples from HIV positive patients were collected from Sanandaj, Kordistan west of Iran. These patients were lived in Sanandaj of whom 26 were prisoners infected with HIV virus in prison. *Toxoplasma gondii* antibodies were determined by IgG ELISA. *T. gondii* antigen was identified by capture-ELISA. PCR was performed on samples with *T. gondii* antigenemia. CD4+ T cells counts had been determined by flowcytometry and were obtained from records of each patient.

Results: Among the examined HIV seropositive individuals, 19.1% (18/94) and 5.3% (5/94) were positive for *Toxoplasma*-IgG and antigenemia, respectively. Besides, one of the samples was positively detected by PCR method. Mean age of participants was 37.9 ± 9.5 year. Prevalence of IgG antibody and antgenemia was higher in age group of 40-50 years old. The Mean of CD4+ T cells counts of participants (total of HIV+ patients, IgG positive patients and patients with antigenemia) was 699.2 ± 345.2, 655.1 ± 237.9 and 620.2 ± 215.1 respectively.

Conclusion: Capture-ELISA and PCR could confirm the *T. gondii* acute infection in HIV positive patients. For precise diagnosis of acute toxoplasmosis in HIV positive patient, performance of more studies based on more sensitive types of PCR is suggested.

Introduction

Toxoplasmosis is caused by the obligate intracellular coccidian parasite, *Toxoplasma gondii*. *T. gondii* can lead to serious diseases in immunocompromised patients such as HIV positive patients, transplant recipient and patients with cancer (1, 2). In most cases, central nervous system involvement can lead to encephalitis, which is one of the most important reasons of death among patients with HIV (1, 3).

Encephalitis occurs in the later stages of human immunodeficiency virus (HIV) infection due to reactivation of tissue cysts that remained latent after the primary infection which cause focal and life-threatening lesions in brain of HIV/AIDS patients (4,5). Rapid detection of toxoplasmosis in HIV-positive individuals can be effective in prevention of cerebral toxoplasmosis and other complications (6-8).Toxoplasmosis is usually diagnosed by serological methods but in HIV- infected patients serology is not reliable. Because of severe immune system dysfunction in these patients, a significant rise in IgG levels occurs in only 30% of patients, and only 20% of patients demonstrate a change in IgM titers during active toxoplasmosis (9, 10). On the other hand, definitive diagnosis of cerebral toxoplasmosis in immunocompromised patients using brain biopsy has high costs and complications (11). Thus detection of antigen for diagnosis of acute toxoplasmosis is a great interest in these patients; this can be achieved by biological tests (intraperitoneal inoculation to laboratory animals or inoculation to cell cultures in vitro) and DNA detection (12).

In recent years, to improve and accelerate the diagnostic procedures, studies using variety of PCR methods and detection of *T. gondii* DNA in cerebrospinal fluid (CSF), peripheral blood and serum had considerable progress (13-15).

Based on data collected from medical schools and health services at the beginning of 2013, 26,125 people infected with HIV/AIDS in Iran have been identified.Of these 89.8% were men and 10.2% were women (16).

The present study was performed for detection of anti- *T.gondii* IgG antibody and *T.gondii* antigenemia in serum samples from HIV/AIDS patients from Sanadaj, Kordistan west of Iran. Then PCR was performed for sera with positive *T.gondii* antigenemia.

Material and Methods

Sampling

This cross-sectional study was performed on HIV positive patients referred to Counseling Center of Behavioral Diseases in Sanandaj City, Kordistan from March to November 2011. Ninety-four serum samples from HIV positive patients were collected. These patients were lived in Sanandaj of whom 26 were prisoners with HIV virus in prison. Sera were kept frozen at -20 °C until use.

The CD4+ T cells counts for each participant had been done by flowcytometry and was documented in records of each patient. So these data was calculated for later analysis

IgG-ELISA

Tachyzoites of *T. gondii,* RH strain (from School of Public Health, Tehran University of Medical Sciences) were harvested from mice peritoneum after 3 days of inoculation. Tachyzoites were washed, sonicated, centrifuged at 14000g for 1 hour; the supernatant was collected and the protein quantity was determined by method of Bradford (17).

Microtiter 96 well plates were coated with 100 µl containing 5 µg/ml of protein and kept at -20°C until use. For IgG ELISA, plates were washed with PBST (phosphate buffer saline, 5% tween), then blocked with blocking buffer (skimmed milk 2.5% in PBST). After incubation and washing, 100µl of diluted sera (1:200) in PBST were added to each well. Af-

ter incubation and washing, 100µl of anti- human IgG conjugated with HRP (horse radish peroxidase) (DAKO, Denmark) in dilution of 1:1000 with PBST was added to each well and afterward performed incubation and washing. Then, chromogenic substrate OPD (orthophenylen-diamidine) (Merck, Germany) was added to each well. Enzymatic activity was obvious after 15 minutes. Reaction was terminated by adding of 20% sulfuric acid. Optical density was recorded at 492nm with an automated ELISA reader (BIOTEC, LX800, USA).

Capture-ELISA

Rabbits were immunized with soluble antigen of *T.gondii,* RH strain and polyclonal antibody was obtained from sera of rabbits as previously described (18). Polyclonal rabbits antisera at a concentration of 30 µl/ml and diluted in coating buffer (PBS, PH: 7:2) were coated in microtiter plates. Plates were incubated overnight at 4˚C. After washing, blocking buffer (skimmed milk 2% in PBST) added to each well. After one hour of incubation, plates were washed 3 times with PBST. Afterward patient's sera with titers of 1:10 in PBST were added to each well. After incubation and washing, home- made rabbit anti- *T. gondii* antibodies conjugated with HRP, was diluted in PBST (1:10) and added to each well (6). Subsequently, chromogenic substrate (OPD) (Merck, Germany) was added and enzymatic activity was obvious after 15 minutes. The reaction was stopped by adding of sulfuric acid 20% and optical density was recorded at 492 nm with an automated ELISA reader (BIOTEC, LX800, USA).

For each ELISA and capture- ELISA methods the amount of cut-off was calculated. In each procedure 30 negative sera was tested by the method again and the cutoff was determined as the mean plus two times of the standard deviation of the absorbance readings obtained for the negative samples (X±2SD). The optical density more and less than cut off were considered as positive and negative respectively.

PCR Method

PCR was performed on samples with positive result in capture ELISA. The DNA was extracted from the sera with *T. gondii* antigenemia by PCR kit (QIA Gene amp DNA mini kit, Germany) according to the manufacturer's instructions. The amplification of B1 gene was carried out with two sets of primers (9):

B1ToxoF
5'GGAACTGCATCCGTTCATGAG3'

B1ToxoR 5'TCTTTAAAGCGTTCGTGGTC3'

For amplification, 25µl of mastermix (Ampliqon, Denmark), 2µl of primer F and R, 4 µl of extracted DNA and 17 µl of distilled water were mixed by shaker and then centrifuged at 1000 rpm for 20 seconds.

The reaction was carried out in a thermo cycler (PeQlab, England). After an initial denaturation at 95˚C for 10 min, 40 cycles were run, including denaturation (92˚C for 30 sec), annealing (55˚C for 50 sec), and extension (72˚C for 30 sec) and final extension at 72˚C for 7 min. PCR products and DNA ladder (Solis_Biodyne, Estonia) were electrophoresed in 1.5% agarose gel (Merck, Germany) and stained with ethidium bromide and DNA safestain. The amplicons of 200 bp were visualized under UV illumination. In each time a positive and negative control was tested. Positive control was contained DNA of tachyzoites of *T. gondii* RH strain and negative control was a serum with negative results for antigen and antibody to *T. gondii*.

Results

Mean age of participants was 37.9 ± 9.5 years. Prevalence of IgG anti-*Toxoplasma* antibodies and antgenemia were higher in age group of 40-50 years old. There was no statistical significant difference among age groups (Table 1).

Table 1: Frequency of *Toxoplasma* -antigen and anti-*Toxoplasma* IgG antibodies based on age in HIV positive patients in Sanandaj, Kordistan, west of Iran, 2011

Age groups	No. (%)	ToxoIgG [No.(%)]	Toxo-Antigen [No. (%)]
18-28	19 (20.2)	3 (15.7)	0 (0.0)
29-39	25(26.5)	4 (16)	1 (4)
40-50	39 (41.4)	9 (23)	3 (7.6)
≥50	11(11.7)	2 (18.1)	1 (9.0)

Of the 94 participants, 89 were males (94.6%) and 5 were females (5.4%). Among these, 18 patients were IgG positive to *Toxoplasma* (19.1%) (Cut off= 0.533) (18 males and 0 female) and 5 patients (5.3%) had antigen of *Toxoplasma* (4 males and 1 female) (Cut off=0.9). Higher values from the cut off were considered as positive. In our study, one of samples was positively detected by PCR method. Moreover, this sample had the highest titer in IgG-ELISA and capture- ELISA.

Moreover, in electrophoresis of PCR products staining with ethidium bromide was better than DNA stain (Fig.1).

CD4+ T-cell count/µl had been done in all HIV+ positive patients. According to the records of each patient including total of HIV+, IgG positive and patients who had antigenemia, the mean of CD4+ T cells counts was 699.2 ± 345.2, 655.1 ± 237.9 and 620.2 ± 215.1 respectively. In addition, CD4+ T-cell count in 100 lymphocytes was ranged between 7 and 33, with a median count of 19.8± 6.3, 20.1±5.7 and 15.4± 5.5 cells in 100 lymphocytes, respectively.

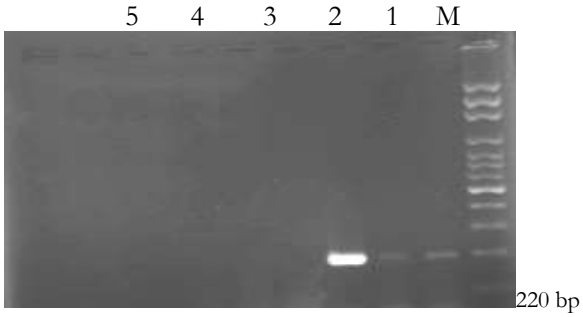

Fig.1: Result of PCR from HIV/AIDS positive sera with *T. gondii* antigenemia from Sanandaj, Kordistan, west of Iran in 2011
Lane M: marker (Solis – Biodyne)/ Lanes 1 and 2: HIV- positive serum with *T. gondii* antigenemia, stained with etidium bromid and DNA safe stain, respectively./ Lane 3: Positive control (DNA of tachyzoites of *T. gondii*)/ Lanes 4 and 5: negative control serum.

Approximately 33% of HIV positive patients had a CD4+ T lymphocyte count of ≤500 cells/µl (Table 2).

Table 2: Frequency of CD4+ T cells counts in HIV+/AIDS patients in Sanandaj, Kordistan, west of Iran, 2011

HIV+ patients (CD4+ T cells counts)	<200 No.(%)	200-499 No. (%)	≥500 No.(%)	Total No.(%)
Total of patients	4 (4.2)	27 (28.8)	63 (67)	94 (100)
T. gondii IgG positive patients	0 (0.0)	4 (22.2)	14 (77.8)	18 (100)
Patients with *T. gondii* antigenemia	0 (0.0)	1 (20)	4 (80)	5 (100)

Discussion

In the present study, from 94 tested serum samples of HIV/AIDS patients, 18 (19.1%) samples had anti *T. gondii* IgG antibody, five (5.3%) were positive for *T. gondii* antigenemia by capture-ELISA in which 1 sample had positive results with PCR.

There are few studies for detection of antigen in the serum of normal individuals and HIV patients by ELISA (19-21). Hassl et al. were able to recognize circulating antigen in

32 (16%) sera of 200 HIV patients by means of a three-layer enzyme-linked immunosorbent assay (21). For antigen detection, capture- ELISA methods is rapid and simple with high sensitivity and specificity in urine and serum samples (18). There is not any study based on antigen detection to diagnosis of acute toxoplasmosis in Iranian HIV positive patients, whereas seroprevalence rate of IgM antibodies to *T. gondii* have been reported between 0% and 9.7% in the country (22-25).

In this study, the finding revealed a *Toxoplasma*-IgG antibody seroprevalence rate of 19.1% in HIV positive individuals which was similar to the study of Davarpanah et al. (18.2%) in Shiraz (26) and significantly lower than other studies in Iran such as 41% in Qom, 38.01% in Mashhad, 49.7% in Tehran and 77% in north of Iran (22-26). Geographical location and other risk factors are effective on the infection with *T. gondii* (22, 27, 28). It is possible that differences in the environments, also cold and dry weather of this area explains the lower prevalence of *T. gondii* infection found in our study. The prevalence of *Toxoplasma* in west of Iran has been estimated from 12 to 18 percent which is consistent with our results (29).

Previous serological studies on HIV positive patients in many part of world exhibited that the prevalence varied depending on the geographical location: 62.1% in Marrakesh (27), 93% in Addis Ababa, Ethiopia (8), 5.4% in Japan (30), 44.8% in Kula Lumpur, Malaysia (31), 50% in Mexico (32), 40% in USA (33) and 36.7% in Spain (34).

Some researchers believe that high titer of IgG anti-*Toxoplasma* antibodies might be representative of active infection or a high-risk of its development, but others do not agree with this point (27).

AIDS patients with CD4+ T cells counts below than 200 cells/µl are at risk of *Toxoplasma* encephalitis (10, 35). It is estimated that approximately in one- third of HIV-positive patients, latent *Toxoplasma* infection can spread to advanced toxoplasmosis and encephalitis (36).

In our study, patients who were positive for IgG and antigen of *T. gondii* had CD4+ T cells count more than 200 cells/µl. In healthy people, the normal range of CD4+ T cells count in 100 lymphocytes is between 22 and 62, whereas the majority of patients in our study had a lower value.

In our study, five patients had active *T. gondii* infection according to capture –ELISA, which one of them had positive PCR result too. Molecular methods, especially types of PCR for rapid and accurate identification of toxoplasmosis with different biological samples are used in many laboratories (6, 7, 15, 37).

For diagnosis of cerebral toxoplasmosis using PCR, CSF samples had been applied which indicated different sensitivities ranging from 11% to 100%, but it has more specificity at about 96% to 100% (6, 13, 38). In addition, PCR methods using peripheral blood indicated different sensitivities from 16 to 86 percent (6, 11, 37). Moreover, serum and urine samples were used to detect acute toxoplasmosis in infected mice using PCR methods (15). Originally, *Toxoplasma* genes that are used in PCR methods were B1 and P30 genes (9, 39). B22 and B23 primers also have been used for detection of cerebral toxoplasmosis (6, 9). In acute *Toxoplasma* infection, parasitemia is detectable by PCR method from the second week to the 17th weeks since the beginning of the infection (40). Investigations showed a significant correlation between increasing of IgG antibody titers and DNA detecting by PCR method in HIV-positive patients (6), but positive serological results in patients do not necessarily correspond to acute and active phase of infection (41). In Brazil, 72 people of 128 AIDS patients without cerebral toxoplasmosis had *Toxoplasma*-specific IgG antibodies by ELISA and indirect immunofluorescence (IFA), whereas only three of the 128 patients were found to be PCR positive (6).

Haghpanah et al. evaluated different clinical stages of toxoplasmosis on immunocompetent

patients by application of serological and PCR methods. Result of PCR was negative for patients who were in chronic phase (reduction in IgM titer and increasing in IgG titer) and early acute phase (low titer of IgM and no IgG). Their study demonstrated which result of PCR was positive only in patients who were definitely in acute phase (high titers of IgM and low titers of IgG) (41). In our study, from five patients who had antigenemia, one was detected by PCR method; this patient had the highest antibody and antigenemia titers. The other four patients showed negative PCR results. This difference could be explained by different sensitivities of PCR, chronic or early phase of infection and DNA extraction from serum.

Doing quantitative serology methods and PCR using peripheral blood in HIV-positive patients can be effective in better management of disease and preventing of spreading of brain lesions (6, 28).

Conclusion

19.1% of HIV positive samples had antibody against *T. gondii*, so they need special attentions due to encephalitis. In addition, capture- ELISA could be used as screening method for *T. gondii* antigen detection in HIV positive patients and acute infection could be confirmed by PCR, although more studies according to different type of PCR methods are suggested.

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