SEROLOGICAL AND MOLECULAR DIAGNOSIS

Evaluation of different techniques in the diagnosis of *Toxoplasma* encephalitis

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This study evaluated the detection of antibodies, circulating antigens and parasite DNA by polymerase chain reaction (PCR) in the diagnosis of toxoplasma encephalitis. The detection of antibody classes and IgG avidity were not useful diagnostically. The detection of circulating antigens by the ELISA system described was not sufficiently sensitive. The detection of DNA by PCR was the most useful test especially in untreated patients, with a sensitivity of 62% overall, 81% in untreated patients and only 20% in treated patients. The use of non-isotopic probes makes the use of this technique feasible in routine diagnostic parasitology laboratories.

Introduction

Toxoplasmosis is a parasitic disease that affects millions of people worldwide, although it is asymptomatic in the majority of cases. Nevertheless, it is of clinical significance when primary infection occurs or when there is reactivation of infection in immunosuppressed patients, e.g., AIDS patients.

The incidence of toxoplasma encephalitis is directly related to the prevalence of anti-toxoplasma antibodies in the population and the prevalence of HIV infection. The prevalence among HIV-positive patients in the USA varies from 15% to 40% [1] but increases to 96% in certain areas of Africa and Europe [2]. Approximately 45% of all HIV-positive individuals with toxoplasma antibodies develop toxoplasma encephalitis [3]. Therefore, in areas where antibodies against *Toxoplasma gondii* are prevalent, toxoplasma encephalitis may arise in 25–50% of HIV-positive patients [1]. However, the widespread use of co-trimoxazole prophylaxis against *Pneumocystis carinii* has reduced the incidence of cerebral toxoplasmosis significantly.

This study evaluated the diagnostic usefulness of detecting antibody classes, circulating antigens and parasite DNA by polymerase chain reaction (PCR) in the diagnosis of toxoplasmosis in HIV-positive patients.

Materials and methods

Patient groups

Group 1 comprised 70 asymptomatic and immunocompetent adult patients with previous *T. gondii* infection, i.e., evidence of antibodies detected by micro-agglutination (bioMérieux, Marcy L'Etoile, France) but without specific IgM (ISAGA, bioMérieux).

Group 2 comprised 70 immunocompetent patients with no specific antibodies to *T. gondii* detectable by the above techniques.

Group 3 comprised 63 HIV-positive patients with central nervous system pathology other than toxoplasma encephalitis (infectious and non-infectious processes).

Group 4 comprised 16 HIV-positive patients with brain lesions compatible with toxoplasma encephalitis. Toxoplasma encephalitis was considered likely when certain features were seen on CT scan and when there was a positive response to specific chemotherapy. Positive CT findings are multiple low-density areas without contrast and nodular enhancement or ring-like enhancement with contrast [4]. Patients in this group were divided into two subgroups as described below.

Group 4a contained five patients who were receiving anti-toxoplasma therapy when cerebrospinal fluid (CSF) was obtained.

Group 4b contained 11 patients who had not previously received specific treatment at the time CSF was obtained and who were not taking any anti-toxoplasma drugs as treatment or prophylaxis for *P. carinii* or *Mycobacterium avium*.

Specimen methodology

Serum and CSF samples from each patient were stored at -70°C until processed. Serum was assessed for circulating antibodies and antigens. CSF was assessed for antigens and toxoplasma DNA by PCR.

IgG, IgM and IgA were detected and assayed according to methods described previously [5–8]. Sera were diluted 1 in 250 to detect IgG, and for the detection of IgM and IgA, they were diluted to 1 in 100. Controls and cut-off points were provided by Sorin Biomedica (Saluggia, Italy). IgG avidity was assessed with a *T. gondii* IgG avidity kit (Menarini, Barcelona, Spain) as described previously [9, 10].

Circulating antigens were detected by an ELISA sandwich technique with two polyclonal antibodies. Hyperimmune antibodies were obtained by immunising rabbits and guinea-pigs with a sonicated preparation of tachyzoites from a *T. gondii* RH strain that had previously been purified by a lectin procedure (Sigma) [11]. The hyperimmune antibodies were purified by an ammonium sulphate and caprylic acid process [12]. Rabbit hyperimmune antibodies ($2\text{ }\mu\text{g}$) were fixed on to an ELISA slide. Samples ($100\text{ }\mu\text{l}$) were added and incubated at 37°C for 24 h and the presence of circulating antigens was detected by adding $2\text{ }\mu\text{g}$ of guinea-pig hyperimmune antibody and incubation for 2 h at 37°C . Anti-guinea-pig antibodies marked with peroxidase (Sigma) were then added and tetramethylbenzidine-dihydrochloride (TMB) and hydrogen peroxide were used in developing. Readings were taken at 450 nm. The cut-off point used was the mean + 2SD of a group of serum samples taken from patients without *T. gondii* antibodies [13–15].

DNA was extracted for PCR by two procedures. In the first (A) CSF was centrifuged at $13\,000\text{ }g$ for 15 min and the sediment was resuspended in $80\text{ }\mu\text{l}$ of buffer – 0.1 M Tris-HCl, pH 8, 0.01 M EDTA, pH 8 – and proteinase K (Boehringer Mannheim, Germany) $800\text{ }\mu\text{g}$ diluted in sterile distilled water $20\text{ }\mu\text{l}$. After pre-incubation for 3 h at 56°C , this mixture was incubated for 10 min at 100°C .

In method (B), after amplification of the DNA extracted by the above procedure, the rest of the sample was subjected to a purification process consisting of extraction with phenol chloroform and subsequent DNA precipitation with isopropanol.

Fragments of the B1 gene, which is repeated 35 times within the *T. gondii* genome, ($5'$ -GGA ACT GCA TCC GTT CAT GAG and $5'$ -TCT TTA AAG CGT TCG TGG TC) were used as primer oligonucleotides and amplification was by 40 cycles of 60 s at 94°C , 90 s at 55°C and 60 s at 72°C [16]. A non-isotopic $5'$ -GGC GAC CCA ATC TGC GAA TAC CAC C probe ($100\text{ }\mu\text{g}/\text{ml}$) was used, marked at the end with

digoxigenin for developing, which was in contact with the sample overnight. This was then developed with a chemiluminescent compound (Luminescent Detection Kit for Nucleid Acids; Boehringer Mannheim, Germany) [17], according to the manufacturer's instructions, and chemoluminescence was detected by exposure of X-ray film (Fig. 1).

After purifying the tachyzoites as described above, several suspensions with different numbers of tachyzoites were prepared and then subjected to PCR. The minimum number of tachyzoites that could be detected with this technique was calculated and the detection limit was set at 5 tachyzoites/sample for each DNA extraction technique.

Statistical methods

For both sensitivity and specificity, the confidence interval was calculated for a ratio; therefore the exact method was used, based on the Snedecor F or an approximation of the normal distribution of a binomial under appropriate conditions ($np > 5$ and $n[1 - p] > 5$).

Results

Antibody detection

IgG was detected in all the serum samples from patients with toxoplasma encephalitis. Thirty-four of the 63 patients in group 3 had IgG antibodies. IgM was not detected in any of the samples. IgA was found in six of 70 immunocompetent patients with previous toxoplasmosis (group 1), in two of 63 patients without toxoplasma encephalitis (group 3) and in three of 16 HIV-positive patients with toxoplasma encephalitis (group 4). The sensitivity and specificity of IgA detection in the diagnosis of toxoplasma encephalitis were 18% and 94%, respectively.

Fig. 1. Detection of chemoluminescence by X-ray film. Lanes A, B, C and D show DNA detection by PCR of clinical samples. The presence of parasite DNA in the sample is indicated by chemoluminescence. Lane E corresponds to the calculation of the detection limits of this technique.

Avidity of IgG

High avidity antibodies (indicative of previous infection) were present in 67 of 70 subjects in group 1 (immunocompetent with previous infection), in 31 of 34 patients in group 3 (HIV-positive patients without toxoplasma encephalitis) and in the 16 patients in group 4 (HIV-positive patients with toxoplasma encephalitis). Low avidity IgG antibodies were found in patients without circulating antigens and without other evidence of toxoplasma encephalitis and because of this, together with the absence of IgM, these results were considered as false positives.

Antigen detection

The results from the detection of circulating antigens in serum and CSF samples are shown in Table 1. The sensitivity and specificity in serum samples were 12% and 93%, respectively. In CSF samples, the sensitivity was 6%, while the specificity was 100%.

PCR

The PCR results obtained in the detection of toxoplasma DNA are shown in Table 2. With extraction technique A, the overall sensitivity was 62%; for untreated subjects, sensitivity increased to 81%, while in treated subjects this fell to 20%. With extraction technique B, the overall sensitivity was 50%, reaching

Table 1. Circulating *Toxoplasma* antigen in serum and CSF

Patient group*	Number of patients tested	Number of positive results	
		CSF	Serum
1	70	0	5
2	70	0	3
3	63	0	6
4	16	1	2
4a	5	0	0
4b	11	1	2

*Patient groups: 1, immunocompetent patients with antibodies; 2, immunocompetent patients without antibodies; 3, HIV patients without *Toxoplasma* encephalitis; 4, HIV patients with *Toxoplasma* encephalitis; 4a, treated; 4b, untreated.

Table 2. Comparison of two DNA extraction techniques used in PCR to diagnose toxoplasmosis

Patient group*	Number of patients tested	Number of positive PCR results with	
		technique A†	technique B†
1	70	0	0
2	70	0	0
3	63	0	0
4	16	10	8
4a	5	1	1
4b	11	9	7

*Patient groups: 1, immunocompetent patients with antibodies; 2, immunocompetent patients without antibodies; 3, HIV patients without *Toxoplasma* encephalitis; 4, HIV patients with *Toxoplasma* encephalitis; 4a, treated; 4b, untreated.

†see *Materials and methods*.

Table 3. Sensitivity and specificity of the different diagnostic techniques

Technique	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Antibody				
Ig G (serum)	100	1	48.8	41–55
Ig M (serum)	0	0	100	100
Ig A (serum)	18	4, 45	94	89–98
Avidity IgG	0	0	94	89–99
Antigen				
Ag (serum)	12	1–42	93	89–96
Ag (CSF)	6	0–30	100	100
PCR (CSF)				
PCR (A)*	62	47–84	100	100
PCR (B)*	50	29–74	100	100

*A and B refer to extraction techniques.

63% in untreated subjects but falling to 20% in treated subjects. Specificity in all cases was 100%. Table 3 shows the sensitivity and specificity for the different techniques.

Discussion

Toxoplasma encephalitis poses a diagnostic problem that traditional serological methods rarely resolve. Therefore this study aimed to study different methods of diagnosis, including more recent approaches. The presence of IgG and the absence of IgM in the group of subjects with toxoplasma encephalitis parallels the results of Derouin *et al.*, who studied a group of 62 patients and detected IgG in 61 and IgM in only three [18]. Similarly, Zufferey *et al.* studied 47 HIV-positive patients with toxoplasmic reactivation and found IgM in only 6% [19].

The detection of low avidity antibodies correlates well with primary infections. Thus, Holliman *et al.* have stated that when infection has occurred within the last 3 months, low avidity antibodies may be detected [9]. In the present study, *T. gondii* infections in immunosuppressed subjects were considered most likely to represent previous infection in the majority of cases.

In immunocompetent subjects, Jaqueti *et al.* detected IgA following the acute phase of the disease, but this did not correlate with acute *T. gondii* infection [20]. With regard to immunosuppressed patients, Zufferey *et al.* studied 47 HIV-positive patients with toxoplasma reactivation and found IgA in only 18% [19]. Patel *et al.* also assessed the IgA detection in HIV-positive patients and concluded that it was of limited value [21]. Pinon *et al.* studied HIV-positive patients with acute toxoplasmosis and found IgA in 38%. However, in HIV-positive patients without acute toxoplasmosis, IgA was detected in only 12% of cases [22]. In the present study, IgA was detected in some immunocompetent patients (6 of 70) without acute *T. gondii* infection. In the diagnosis of acute toxoplasma encephalitis this technique had a sensitivity of 18%

and a specificity of 96%, similar to those reported by other authors, and its clinical usefulness is therefore limited. As has been stated elsewhere [23, 24], the detection of IgG antibodies is of limited value in the diagnosis of toxoplasma reactivation in HIV-positive patients, but the presence of IgG may be important in predicting prognosis.

The technique used in the present study for the detection of circulating antigens demonstrated low sensitivity for both CSF and serum and had a low specificity with serum samples. Dannemann *et al.* did a prospective study of antigenaemia in 11 serum samples from HIV-positive subjects and did not detect antigens in any patients, although two subjects had acute toxoplasma encephalitis [25]. Hassl *et al.* studied the presence of circulating antigens in serum and CSF samples from patients with stage III and IV HIV infection and detected circulating antigens in 32 serum samples from 20 patients by ELISA; antigenaemia correlated well with serological results and clinical symptoms [26]. However, in that study, strict criteria were not used in the diagnosis of toxoplasma encephalitis. Candolfi *et al.* studied 115 serum samples from 19 transplant recipients with clinical and serological evidence of toxoplasma reactivation [27]. Antigens were detected in eight of these patients before or concurrent with a rise in IgG. They believed that elevated levels of IgG eliminated the presence of antigen in the serum by forming immune complexes and that this indicated that the period of antigenaemia was very short [27]. Letillois *et al.* used monoclonal antibodies to detect toxoplasma antigen and compared this with surface antigens in HIV-positive subjects; they found a sensitivity of 30% and specificity of 92% [28]. The detection of circulating antigens as a diagnostic approach presents difficulties because of the need for standardisation of methods and the timing of samples. Therefore, its usefulness as a diagnostic tool is limited.

The detection of parasite DNA by PCR has emerged as a useful diagnostic tool. However, variations in technique can affect results. The present study found differences between the two DNA extraction techniques used, so in order to increase sensitivity, both methods should be used. As regards the amplified sequence, the one described by Burg *et al.* [16] was chosen, i.e., the B1 gene which is repeated 35 times within the genome. Wastling *et al.* compared two sequences, one found in the gene that codes protein P30 of the tachyzoite and the other in gene B1. They concluded that use of the B1 sequence increased the sensitivity [29]. The detection limit of the present technique was five tachyzoites/ml of sample. This is similar to that reported by other authors. Burg *et al.* detected the presence of only one micro-organism when assessing parasites alone, while 10 tachyzoites were detected when a mixture of parasites and white blood cells in a ratio of one parasite: 10⁵ leucocytes

was assessed [16]. Gross *et al.* detected the presence of 10 parasites in 100 µl of sample with a great number of human cells [30].

Several authors have evaluated PCR as a diagnostic tool for toxoplasma encephalitis and in spite of the fact that they used different work protocols, the results are promising in all cases [31–34]. The detection of the amplified DNA fragment by chemiluminescent probes makes the procedure feasible in laboratories that do not have facilities for handling radioactive isotopes. Both techniques have similar sensitivity.

An important issue when evaluating PCR as a diagnostic tool for toxoplasma encephalitis is whether the patient is receiving specific treatment. As the results of the present study and those of other authors show [35], diagnostic sensitivity decreases when patients are receiving treatment and, furthermore, the results obtained by this technique may be affected by treatment for other infections such as *P. carinii* or *M. avium* (if treatment consists of drugs with anti-toxoplasma activity). Therefore, the PCR appears to be the most promising approach in the diagnosis of toxoplasma encephalitis. The use of non-radioactive probes, without increasing the time needed to carry out the test, makes it feasible in routine diagnostic laboratories. However, it is important to be aware of the limitations of this technique, including the need for standardisation and the possibility of false negative results, especially in samples from patients receiving specific antibiotic treatment.

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