

Pulmonary toxoplasmosis in HIV-infected patients: usefulness of polymerase chain reaction and cell culture

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ABSTRACT: Toxoplasmosis is a serious opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS). The lung is a major site of infection after the central nervous system. The aim of the study was to assess the polymerase chain reaction (PCR) and cell culture for the detection of *Toxoplasma gondii*.

One hundred and thirty two human immunodeficiency virus (HIV)-infected patients with respiratory manifestations, who underwent fibreoptic bronchoalveolar lavage, were investigated. Detection of *Toxoplasma gondii* was compared using three techniques: Giemsa staining; polymerase chain reaction with specific primers derived from the P30 gene; and culture on the MRC5 cell line.

Toxoplasma gondii was detected in the same four samples by all three techniques.

We conclude that PCR adds little to conventional (and cheaper) tools already used to diagnose pulmonary toxoplasmosis.

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Toxoplasmosis is a serious cause of morbidity and mortality in immunocompromised patients, especially those with the acquired immunodeficiency syndrome (AIDS). Toxoplasmosis generally results from reactivation of latent infection [1]. *Toxoplasma gondii* can induce systemic disease with multiple sites of infection, or can infect one organ preferentially. Pulmonary toxoplasmosis, estimated to account for 4% of all cases of pneumonia in AIDS patients [2], is the second [3] or third [4] most frequent form of toxoplasmosis, after toxoplasmic encephalitis.

The diagnosis of pulmonary toxoplasmosis is difficult; clinical and radiological abnormalities are not specific [3, 5], and serological tests are of little value in immunodeficient patients [3, 6]. Trophozoites must be detected for a firm diagnosis, but the few parasites in many bronchopulmonary samples may be overlooked on Giemsa-stained slides. Mouse inoculation is too lengthy for routine use. Major improvements in *Toxoplasma gondii* detection have been made with cell culture [7-10] and deoxyribonucleic acid (DNA) amplification techniques [11-16].

We assessed the polymerase chain reaction (PCR) for the detection of *Toxoplasma gondii* and its value in the diagnosis of toxoplasmosis in immunocompromised patients. We compared the results to those of cell culture and Giemsa staining, together with clinical and radiological findings.

Materials and methods

We studied in-patients and out-patients of two Paris hospitals (Saint-Antoine and Tenon).

Patients and samples

One hundred and thirty two human immunodeficiency virus (HIV)-infected patients with respiratory manifestations (fever, cough and dyspnoea) suggestive of pneumonia underwent fibreoptic bronchoalveolar lavage (BAL). None had received prophylaxis for toxoplasmosis. One hundred and twenty seven patients had <200 CD4⁺ cells- μ l⁻¹.

BAL was performed with slow infusion and aspiration of aliquots of sterile saline (1 BAL per patient). A total of 240 ml (4 × 60 ml) was infused. A mean of 163±20 ml (mean±SD) of the volume infused was recovered, containing 230±50 × 10³ cells-ml⁻¹ (macrophages 65±5%, lymphocytes 9±2%, neutrophil leucocytes 26±6%).

All patients were monitored for one year.

Giemsa staining

Conventional Giemsa staining was performed on BAL sediments.

Deoxyribonucleic acid amplification

DNA was extracted from frozen cell pellets with phenol-chloroform after proteinase K digestion [17]. A negative control (sterile distilled water) was included in each extraction procedure. The two synthetic oligonucleotide primers described by DUPOUY-CAMET and co-workers [11] were used to detect a 282 base-pair (bp) fragment (position 418 to 700) of the nonrepetitive gene coding

for the major surface antigen P30, which is conserved in the different strains of *T. gondii* [18].

Amplification was performed in a Perkin Elmer Cetus thermal cycler, with 2.5 units of Taq DNA polymerase (Cetus) in a final reaction volume of 100 µl, with 10× Cetus buffer (Cetus), 500 µM deoxynucleoside triphosphate (Pharmacia), 0.7 µM each oligonucleotide and 1 µg of DNA. Samples were overlaid with paraffin oil. The amplification conditions were as follows: an initial cycle of denaturation (94°C, 5 min), annealing (55°C, 2 min) and extension (72°C, 2 min) was followed by 35 cycles consisting of 5 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C. The final extension step was extended by 5 min.

The amplification products (1/10 of the final volume) were run on a 2% agarose gel after ethidium bromide staining, with a size marker (123 bp Ladder, Gibco BRL). After ultra violet (UV) examination, the gel was transferred to a Hybond N+ nylon membrane (Amersham). Southern blots were analyzed with a 207 bp insert (position 454 to 661; ³²P-labelled probe). The membrane was incubated in prehybridization buffer, consisting of 5× Denhardt's solution, 5× standard sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS), 100 ng of salmon sperm DNA per ml and 16% formamide. It was then hybridized overnight at 42°C with the labelled probe. Blots were washed at room temperature for 5 min in 2× SSC, and at 42°C for 15 min in 2× SSC, 0.1% SDS, then autoradiographed with X-ray film at -80°C for 6 h.

The sensitivity of this PCR technique was first evaluated by testing serial dilutions of DNA from infected-mouse ascitic fluid. The sensitivity of hybridization was 100 fold higher than that of electrophoresis, detecting DNA equivalent to 0.1 parasite.

Each step of the procedure was carried out in a different room. A positive control (DNA extracted from mice ascites) and a negative control (sterile distilled water) were included in each experiment (in addition to the negative extraction control).

Cell culture method

We used the human embryonic fibroblast cell line MRC5 (bioMérieux, France), in the method described by DEROUIN and co-workers [19]. Briefly, after centrifugation, BAL pellets were resuspended in 4 ml of minimum essential medium [19], and 1 ml was placed on coverslip cell cultures in 24-well plates (Nunc, Roskilde, Denmark). Two replicate cultures were set up for each specimen. Plates were incubated at 37°C in a moist atmosphere with 5% CO₂. Immunofluorescence and Giemsa staining were performed after 3 days of culture.

According to DEROUIN and co-workers [19], inoculation with three parasites is required for culture positivity. This sensitivity is comparable to that of animal inoculation, which has the drawback of taking 4–6 weeks [20].

Results

Giemsa staining. Four BAL specimens were positive. In one of the four samples, a single trophozoite was

observed after a second examination (performed because of PCR and cell culture positivity).

PCR technique. As previously described [11, 12, 16], we observed no cross-amplification with other pathogens frequently found in such patients, such as *Pneumocystis carinii*, *Candida albicans* and *Cryptococcus neoformans*. The same four BAL specimens were positive by PCR. Each positive PCR result was checked once.

Cell culture. The four BAL specimens positive in PCR and Giemsa staining were also positive in cell culture. All other samples were negative. Thus, four of the 132 BAL specimens were positive using all three techniques. No cases of disagreement between the techniques occurred.

The four corresponding patients had CD4+ cell counts of <50 cells·µl⁻¹. Three patients had diffuse interstitial infiltrates on chest radiograph films. All had serological evidence of past exposure to *T. gondii*; only one had serological modifications, with a tenfold rise in the immunoglobulin G (IgG) titre but no detectable immunoglobulin M or A (IgM or IgA) antibodies. Two patients also had neurological signs (clinical and radiological). Two of the four patients died in less than 72 h, whilst the other two improved on specific therapy.

The remaining patients with negative results were infected by other micro-organisms (*Pneumocystis carinii*, *Candida* spp., viruses or bacteria) or had no identified aetiology.

Discussion

Various authors have obtained very good sensitivity with PCR (in the order of a single parasite), especially using purified DNA [11–14], with a 10–100 fold increase after hybridization. The sensitivity falls, but remains good, when cellular specimens are used [12, 13, 15], due to lower efficiency of amplification when DNA is contaminated by cellular debris [13]. CRISTINA *et al.* [13] amplified an original repeat sequence TGR1E with a sensitivity of 0.2 parasites after hybridization. ROTH *et al.* [21], using three primer systems, amplified a fragment of the B1 gene and a fragment of the P30 gene, with a detection limit of 10 parasites after the gel electrophoresis.

The prevalence of pulmonary toxoplasmosis in this study was 2.2%. In the few published PCR studies of BAL; CRISTINA *et al.* [13] found results comparable to ours, with three positive cases among 87 BAL specimens from AIDS patients; whilst BRETAGNE *et al.* [22] and ROTH, *et al.* [21] reported higher prevalences (respectively, 6 out of 42 and 3 out of 26 specimens from AIDS patients).

The clinical and radiological features of the four positive patients were nonspecific. It has been reported [3, 5] that these features are similar to those in other lung infections, such as *Pneumocystis carinii* pneumonia. Only one of the four patients showed an increase in specific IgG titres close to the time of onset. According to POMEROY and FILICE [3] and DEROUIN and co-workers [6],

toxoplasma serological results in AIDS patients are particularly difficult to interpret.

PCR was positive following gel electrophoresis in all four cases, and these results were confirmed by hybridization. We did not use uracil-D-glycosylase (UDG), in order to avoid contamination by amplified products from previous PCR reactions [22]. We used rigorous experimental conditions, as reported by KWOK and HIGUCHI [23]. The case in which a Giemsa-stained slide was positive only on re-examination underlines the difficulty of direct examination: the parasites are small (4–8 µm) and often scarce, and can escape close observation [24]. However, the low incidence of pulmonary toxoplasmosis and the small proportion of false-negative results obtained with standard staining do not warrant routine use of PCR.

The pathogenesis of toxoplasmic pneumonia is unclear. Dormant cysts appear to be rare in the lungs after primary infection, given the large number of negative BAL procedures (most were performed in patients with anti-*T. gondii* antibodies); in fact, little is known about the frequency of cysts in other organs than the brain. The current hypothesis is that parasites originate from a distant site and spread to the lungs *via* the bloodstream [5]. However, one of the four patients had both trophozoites and cysts in the BAL specimen, suggesting that pulmonary toxoplasmosis may occur by rupture of local cysts.

Finally, PCR and cell culture are interesting procedures for *Toxoplasma gondii* detection in BAL fluid, and were diagnostic in one of our patients. However, we consider that these methods are only complementary to Giemsa staining, the most rapid method, which also allows the detection of other common pathogens, such as *Pneumocystis carinii*. With regard to the detection of *Toxoplasma gondii* in other body fluids (blood, cerebrospinal fluid (CSF), *etc.*) in which Giemsa staining and direct examination are impossible, interesting results have been reported by LEBECH *et al.* [25], who obtained two cases of PCR positivity with CSF from two patients with cerebral toxoplasmosis. DUPOUY-CAMET *et al.* [26] obtained nine positive PCR results on blood samples from 14 patients with presumptive cerebral toxoplasmosis; however, recently the same authors [27] reported contradictory results and concluded that PCR has poor sensitivity for cerebral toxoplasmosis. In a parallel study (personal results), we found no case of PCR positivity when analysing blood from 10 patients with cerebral toxoplasmosis.

With the widespread utilization of trimethoprim-sulphamethoxazole as primary PCP prophylaxis in AIDS patients, which is also effective against *Toxoplasma gondii*, there has been a fall in the prevalence of pulmonary toxoplasmosis (only 4 cases out of 132 patients in this study), and the clinical utility of these newer methods is, thus, unclear.

In conclusion, PCR adds little to conventional and less expensive tools already in use to diagnosis pulmonary toxoplasmosis.

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