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Detection of *Toxoplasma gondii* in AIDS Patients by the Polymerase Chain Reaction

Summary: In recent years, toxoplasmosis has become one of the most frequent and life-threatening opportunistic infections in AIDS patients. Despite strict clinical follow-up and repeated biological examinations, its diagnosis remains difficult to establish in the context of immunodeficiency because of the poor predictive value of serology. The aim of the study was to compare standard methods of diagnosis with the polymerase chain reaction (PCR), in an attempt to investigate the potential usefulness of PCR in the diagnosis of toxoplasmosis. Twelve biological samples (cerebrospinal fluid, bronchoalveolar lavage fluid, one brain biopsy and one liver biopsy) from 11 unselected AIDS patients were tested by PCR. The results showed good correlation (for eight out of 11 patients) between classical methods and PCR, and confirm the value of bronchoalveolar lavage for the diagnosis of toxoplasmosis in AIDS patients. The pathophysiological significance of the presence of *Toxoplasma* in samples tested is discussed.

Zusammenfassung: *Nachweis von Toxoplasma gondii unter Anwendung der Polymerasekettenreaktion bei AIDS-Kranken.* Bei AIDS-Kranken stellt die Toxoplasmose eine der häufigsten lebensbedrohlichen Infektionen dar. Wegen des geringen prädiktiven Wertes der Serologie ist die Diagnose im Zustand der Abwehrschwäche trotz konsequenter klinischer Kontrollen und wiederholter Laboruntersuchungen schwierig. Die vorliegende Studie wurde durchgeführt, um die Methoden der konventionellen Diagnostik mit der Polymerasekettenreaktion (PCR) zu vergleichen und den Nutzen der PCR in der Toxoplasmose-Diagnostik zu beurteilen. Die PCR wurde bei 11 unausgewählten AIDS-Kranken eingesetzt; für die Untersuchungen standen 12 Proben (Liquor cerebrospinalis, bronchoalveoläre Lavage-Flüssigkeit, eine Hirnbiopsie und eine Leberbiopsie) zur Verfügung. Die Ergebnisse zeigten bei acht der 11 Patienten eine gute Korrelation zwischen den klassischen Nachweismethoden und der PCR. Sie bestätigen den Wert der Bronchoalveolar-Lavage für den Nachweis der Toxoplasmose bei AIDS-Kranken. Die pathophysiologische Bedeutung der Toxoplasma-Organismen in den Proben wird diskutiert.

Introduction

The coccidian protozoan *Toxoplasma gondii* is the etiological agent of toxoplasmosis, an infection that occurs worldwide and is historically known for the congenital malformations it causes. In AIDS patients, reactivation of latent toxoplasmic cysts can result in fatal encephalitis as well as disseminated forms of toxoplasmosis. Twenty to 30% of AIDS patients with positive toxoplasma serology will develop a cerebral toxoplasmosis [1]. In these cases, the diagnosis is supported mainly by clinical evidence, e. g. central neurological signs, epilepsy, and computed tomography (CT) scan abnormalities. The diagnosis of disseminated forms (such as lung localization) is, however, more difficult, due to problems with interpretation of serological tests and the absence of specific clinical signs. Direct parasitological diagnosis is necessary, and time-consuming techniques such as *in vitro* and *in vivo* culture are appropriate. More recently, PCR has been used to diagnose congenital toxoplasmosis [2,3] and *Toxoplasma* infection in immunocompromized patients [4]. We are currently investigating the respective contributions of the classic techniques and PCR for the diagnosis of toxoplasmosis in AIDS patients.

Patients and Methods

Patients: The patient population consisted of 11 unselected AIDS patients clinically surveyed in the Department of Infectious Diseases at the Albert Michallon Hospital in Grenoble, France. For some of these patients, clinical and radiological features and the evolution of illness under specific treatment were not sufficient to make a clear diagnosis. Thus samples were taken for biological examination.

Samples: Four cerebrospinal fluid (CSF) samples, six bronchoalveolar lavage (BAL) specimens, one liver biopsy and one brain biopsy were submitted to the Department of Parasitology-Mycology for routine examination and *in vitro* culture of *T. gondii*. At the same time, corresponding sera were tested by classical serological techniques (indirect immunofluorescence assay, enzyme linked immunosorbent assay, immunosorbent agglutination assay). PCR assay was then carried out. For safety reasons, all samples from AIDS patients were incubated at 60°C for 2 h in order to inactivate HIV before PCR treatment.

Preparation of samples for PCR: For CSF and BAL specimens, 10–20 µl of the previously inactivated sample was added to one volume of a lysis buffer (KCl 50 mM, TRIS-HCl pH 8.3 10 mM,

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$MgCl_2$ 2.5 mM, gelatin 0.1 mg/ml, Nonidet p40 0.45%, Tween 20 0.45%) [5] and proteinase K, to achieve a 100 µg/ml final concentration. Lysis was performed at 50°C for 2 h and proteinase K was inactivated by incubation at 95°C for 10 min. Ten microliters of this crude lysate were used directly for PCR assays. For the two biopsies, tissue fragments were overlayered with the lysis buffer in an Eppendorf tube, and then finely ground under sterile conditions. Proteinase K was added to give the resulting suspension a 200 µg/ml final concentration. This suspension was then incubated for 2 h at 50°C, and centrifuged for 10 min. at 10,000 x g, to eliminate the non-soluble residues. Ten microliters of the clarified supernatant were used directly for amplification.

In vitro cultures: In vitro cultures were performed using MCR5 embryonic fibroblasts (bioMérieux, Marcy l'Etoile, France). Briefly, after centrifugation (10 min. at 3,000 x g) (CSF, BAL samples) or grinding (biopsies), samples were inoculated into the culture in MEM medium (bioMérieux). The presence or absence of *Toxoplasma* was revealed after six days of culture by immunofluorescence. To date, this is the most sensitive and the least time consuming technique available for detecting the presence of living *T. gondii* tachyzoites in clinical samples [6,7].

PCR methods: Ten microliters of each sample were mixed in a 50 µl final volume (KCl 50 mM, TRIS-HCl pH 8.3 8.5 mM, $MgCl_2$ 1.5 mM, gelatin 0.1%) with each dNTP at 800 µM and each primer at 0.5 µM. Samples were overlayered with 50 µl mineral oil, and then incubated for 7 min. at 95°C. The PCR cycles began after addition of 1.25 units of Taq polymerase per tube. After 30 cycles (1 min. at 94°C, 2 min. at 55°C, 3 min. at 72°C), the amplified products were analysed by gel electrophoresis, transferred onto nylon membranes by Southern-blotting, and then hybridized with a specific radio-labelled oligonucleotide. The two PCR primers corresponded to nucleotides 91–112 and 264–286 of the $TGR1_E$ sequence, and the oligonucleotide probe corresponded to positions 192–214 [8]. This probe has revealed all the strains previously studied.

Results

The PCR test results for the 11 AIDS patients are presented in Figure 1; a comparison of clinical and biological results is shown in Table 1. Serology for *Toxoplasma* was considered negative when no specific IgG or IgM was detected by any of the techniques employed.

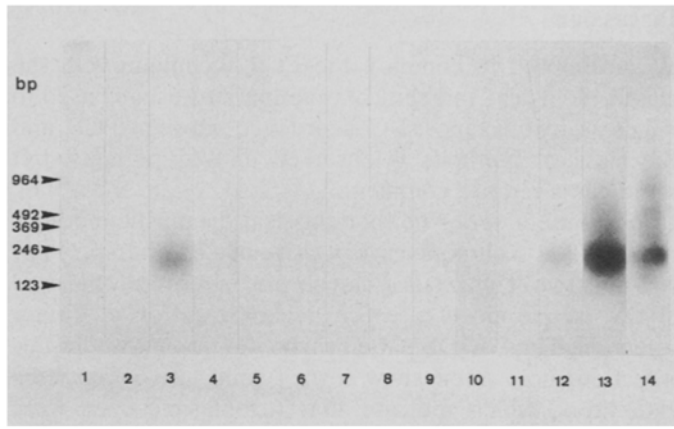

Figure 1: Results of PCR for detection of *T. gondii* in samples from 11 HIV-infected patients. Biological samples were submitted to PCR with primers corresponding to nucleotides 91–112 and 264–286 of the repeated toxoplasmic sequence $TGRI_E$. The amplified products were then separated on agarose gel, transferred onto nylon membranes by Southern-blotting, and hybridized with a ^{32}P probe corresponding to nucleotides 192–294 of $TGR1_E$. A negative control (1) was made by replacing the biological sample with water. A positive control (14) was made with 0.1 pg of toxoplasmic DNA (RH strain). Biological samples were CSF from patient no. 1 (2), BAL fluid from no. 2 (3), CSF from no. 3 (4), BAL fluid from no. 4 (5), a brain biopsy from no. 5 (6), CSF from no. 5 (7), BAL fluid from no. 6 (8), BAL fluid from no. 7 (9), CSF from no. 8 (10), BAL fluid from no. 9 (11), BAL fluid from no. 10 (12), and a liver biopsy from no. 11 (13).

None of the positive serological results represented a primary toxoplasmic infection. For patient nos. 3 and 10, the diagnosis was established on the basis of clinical signs, radiological parameters, and the evolution of illness under specific treatment (pyrimethamine + sulfadiazine). For patient no. 5, an additional *in vitro* culture of a brain biopsy was necessary to establish the definitive diagnosis of cerebral toxoplasmosis, because failure of the specific treatment had made the diagnosis uncertain. For patient no. 11, diagnosis was based on culture of a liver biopsy *in vitro*.

Table 1: Comparison of results of serology, culture, and PCR in biological samples from 11 HIV-infected patients.

Patient No	Sample	Serology in blood	Culture *in vitro*	PCR	Diagnosis
1	Cerebrospinal fluid	−	−	−	NE
2	Bronchoalveolar lavage	+	ND	+	NE
3	Cerebrospinal fluid	+	−	−	CT
4	Bronchoalveolar lavage	+	−	−	NE
5	Brain biopsy	+	+	−	CT
5	Cerebrospinal fluid	+	−	−	CT
6	Bronchoalveolar lavage	−	ND	−	NE
7	Bronchoalveolar lavage	−	ND	−	NE
8	Cerebrospinal fluid	−	−	−	NE
9	Bronchoalveolar lavage	+	−	−	NE
10	Bronchoalveolar lavage	+	−	+	CT
11	Liver biopsy	+	+	+	HT

ND: not done, NE: no evidence for toxoplasmic infection at the time of sampling, CT: cerebral toxoplasmosis, HT: hepatic toxoplasmosis

Discussion

We attempted to compare the PCR technique with the classic biological and clinical examinations usually used to diagnose toxoplasmosis. Concordance between PCR and the classical methods is observed in 8/11 patients, but some cases require comment.

In our patient series neither classical diagnostic methods nor the PCR technique gave positive results when serology was negative. This result is not surprising: toxoplasmosis in AIDS is, in most cases, a reactivation of a former infection. Thus, AIDS patients who develop a toxoplasmic infection most often show low or mild anti-*Toxoplasma* IgG titers, which indicate that toxoplasmic cysts were previously present in the patient's tissues. Serological testing is therefore of little value for diagnosing toxoplasmosis in AIDS patients [9]. Toxoplasmosis can occur in patients with negative serology, but only in the very rare cases of primary toxoplasmic infection [10].

Of the seven patients for whom the diagnosis of toxoplasmosis was not confirmed, only in one case (patient no. 2) was the PCR result positive. Unfortunately, *in vitro* culture was not available to confirm or invalidate this result. Nevertheless, we speculate that tachyzoïtes were present in this patient's BAL specimen. Pneumopathy was observed in this patient and *Pneumocystis carinii* infection was suspected but not confirmed by the biological tests. Despite the lack of a confirmed diagnosis trimethoprim-sulfamethoxazole treatment was administered, which cured the pneumopathy. Since this treatment is known to act not only against *P. carinii* but also against *T. gondii*, the pneumopathy could have been caused by a toxoplasmic reactivation with a parasitic dissemination [11,12].

Of the four cases of proven toxoplasmosis, two were confirmed by PCR results. For patient no. 11 concordance was observed between the *in vitro* culture and PCR; the post-mortem biopsy, performed after acute parasitic hepatitis, revealed a great number of parasites. For patient no. 10 the PCR result was concordant with the presumed diagnosis although the culture was negative. The presence of toxoplasmic DNA when parasites are not detected in culture can be explained by the non viability of the tachyzoïtes. In fact, in addition to being given pyrimethamine-sulfadiazine for cerebral signs, patient no. 10 was treated with trimethoprim-sulfamethoxazole for proven *P. carinii* pneumopathy. Thus, PCR may be sensitive enough to detect remaining toxoplasmic DNA in BAL samples during severe anti-toxoplasmic treatment.

For patients no. 3 and 5, the three PCR tests were negative despite irrefutable clinical and radiological evidence of cerebral toxoplasmosis. In these two cases, CSF samples were negative by both PCR and *in vitro* culture. In fact, the value of CSF examination for the detection of cerebral toxoplasmosis has been discussed [13]. A brain biopsy was obtained from patient no. 5 because of a persisting lesion during pyrimethamine-sulfadiazine treatment. Six months later, cerebral lesions finally led to death by epilepsy. Microscopic examination of a cerebrospinal biopsy from this patient showed rare toxoplasmic cysts, and, for a few of them, local dissemination of tachyzoïtes. In this case, the *in vitro* culture was positive. The discordant results might be explained by the fractionation of the brain tissue, yielding heterogeneous samples with or without parasites, which would account for the difference between the results of *in vitro* culture and PCR tests. In addition, due to the presence of latent cysts in patients who were not infected recently, the PCR technique is unable to distinguish between a recent disease and a reactivation, because healthy people previously infected by *Toxoplasma* have cysts in their brain. In brain biopsy, only histological examination can differentiate between latent and acute infection [14].

The routine application of PCR requires a large investment in materials and reagents. Furthermore, many precautions must be observed to avoid contamination of the PCR test by exogenous DNA [15–17]. However, results are obtained with PCR more quickly than with *in vivo* culture. In addition, duration of PCR can be reduced from four to two days by using a nested PCR test [18–20].

In conclusion, a satisfactory concordance between classical diagnostic methods and PCR was observed in our study. The detection of *Toxoplasma* tachyzoïtes by PCR (such as in BAL samples or liver biopsy) confirm the parasite as the cause of clinical symptoms [21–23] since the parasite is not present in these samples in healthy individuals positive for *Toxoplasma*. The presence of tachyzoïtes in such samples provides evidence that toxoplasmosis in AIDS patients is due to parasitic dissemination with occurrence of parasitemia. Thus, PCR, together with the routine tests, may be a supplemental test in some especially difficult cases. We therefore propose that further investigations be carried out in order to determine the value of BAL and blood analysis for diagnosis of disseminated toxoplasmosis.

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