Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up

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Stage determination of human African trypanosomiasis is based on the detection of parasites and measurements of biological changes in the cerebrospinal fluid (CSF) (concentration of white blood cells > 5 cells per mm^3 and increased total protein levels). The patient is treated accordingly. Demonstration of the absence or presence of trypanosomes by the double centrifugation technique is still the only test available to clinicians for assessing treatment success. In this study, however, we evaluate the polymerase chain reaction (PCR) as a tool for assessing the disease stage of trypanosomiasis and for determining whether treatment has been successful. All 15 study patients considered to be in the advanced stage of the disease were PCR positive; however, trypanosomes were demonstrated by double centrifugation in only 11 patients. Of the five remaining patients, who were considered to be in the early stage, PCR and double centrifugation were negative. Following treatment, 13 of the 15 second-stage patients were found to be negative for the disease in at least two samples by PCR and double centrifugation. Two others were still positive by PCR immediately and one month after the treatment. Trypanosome DNA detection using PCR suggested that the two positive patients were not cured but that their possible relapse could not be identified by a search for parasites using the double centrifugation technique. Further evaluation of the PCR method is required, in particular to determine whether PCR assays could be used in studies on patients who fail to respond to melarsoprol, as observed in several foci.

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Human African trypanosomiasis evolves from the haematolymphatic phase (first or early stage) to the meningoencephalitic stage (second or advanced stage). Although neurological signs and symptoms do occur during the first stage, they become more pronounced and more frequent during the second stage (*1*). The stage determination of the disease is currently based on measurements of the biological changes occurring in the cerebrospinal fluid (CSF) and the presence of parasites. The generally accepted criteria for CSF biological alteration are the same as those currently used in neurology: concentration of white blood cells >5 cells per mm^3 and increased total protein levels (>37 mg per 100 ml in a dye-binding protein assay). In human African trypanosomiasis, an increased cell count and/or protein level, with or without the presence of trypanosomes, consequently leads to the conclusion that the disease is at an advanced stage, and the patient will be treated accordingly. The presence of trypanosomes alone without any CSF alteration is considered by some workers to be insufficient indication for central nervous system involvement and second-stage diagnosis (*2*). Consequently, they have suggested various techniques for identifying changes in CSF that would allow the diagnosis of second stage of the disease, such as the presence of antitrypanosomal-specific antibodies determined by immunofluorescence (*3*), autoantibodies directed against neurofilament proteins (*4*), trypanosome-specific antibody (*5*), and elevated trypanosome-specific and nonspecific IgM levels (*6*). It should be noted that IgM levels can now be determined through a recently developed latex agglutination test (Latex/IgM) which combines stability, sensitivity and simplicity (*7*), and which has demonstrated its many advantages for application in the field.

It has been shown that, following treatment, it is usually several months before the number of cells and total protein level in CSF return to normal. Demonstration of the absence or presence of trypanosomes by the double centrifugation technique (*8*) is still the only means available to clinicians for assessing treatment success. However, despite its good sensitivity, double centrifugation is hampered by the fluctuation of parasite numbers in CSF. Given the efficiency of the polymerase chain reaction (PCR) in detecting the presence of trypanosomes in whole blood (*9*), we report in the present article on the use of this technique as a tool for diagnosing disease stage and for determining treatment success or failure.

CSF samples were obtained from 20 patients from Côte d'Ivoire. For each patient, samples had been taken prior to treatment, at the end of the

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treatment, and at 1, 3, 6 and 12 months after treatment. Samples were aliquoted. One aliquot was used immediately to test for the presence of parasites by double centrifugation, cell count and determination of the protein level. Another aliquot of each sample was frozen in Nunc (Nalge Nunc International, Rochester, NY, USA) and stored in liquid nitrogen for further studies.

Aliquots were not available from all samples for this study. CSF double centrifugation, cell counts, determinations of protein levels and PCR could only be performed for each patient on samples collected before treatment and on at least two samples collected after treatment.

Each frozen 1.5-ml aliquot of CSF was thawed at room temperature and 500 μl was transferred to a 0.6-ml Eppendorf conical tube and centrifuged at 13 000 *g* for 20 min at 4 °C. By means of a pipette, 450 μl of the supernatant was gently removed and discarded. The pellet was resuspended in the remaining liquid and vortexed for 5 min. Subsequently, 100 μl of 5% Chelex solution in sterile purified water (Chelex® 100 Resin, Bio-Rad Laboratories, CA, USA) was added to each tube. The tubes were then vortexed for 1 min, centrifuged at 13 000 *g* for 1 min and incubated at 56 °C for 1 hour. This was followed by a second incubation at 95 °C for 30 min. After incubation, the tubes were again centrifuged for 5 min at 13 000 *g*. The supernatant, now containing single strands of DNA, was used directly or after storage at 4 °C for no longer than 2 days.

PCR was performed according to the method described by Penchenier et al. (*9*) using *Trypanosoma brucei*-specific primers (*10*). The amplification conditions were as follows: 40 cycles with the denaturation step at 94 °C for 1 min, the annealing step at 56 °C for 1 min, and the polymerization step at 72 °C for 1 min. The final elongation was at 72 °C for 5 min. Samples of 10 μl of each reaction product were run in 2% agarose gel with 1x 40mM Tris-acetate, 1mM EDTA (TAE) buffer at 100 V for 30 min. The gel was then stained for 20 min with a 0.5 μg/ml solution of ethidium bromide before being visualized under ultraviolet light.

Table 1 shows the PCR, double centrifugation, protein level and cell count results for the pre-treatment samples taken from each patient. Of the 15 patients considered to have second-stage trypanosomiasis according to the number of cells and/or protein content, all were PCR positive; however, trypanosomes were demonstrated by double centrifugation in only 11 of these patients. For the five remaining patients, who were considered to be first stage, the PCR and double centrifugation results were negative. Among these, one (patient 2522) had a cell count of 24, and might be in an early-late stage of the disease, a result which supports the controversial value of 5 cells for the cut-off between the first and second stages (*11*). Furthermore, patients with up to 20 cells in their CSF have been successfully treated with pentamidine rather than melarsoprol (*12*).

For the post-treatment samples, 13 of the 15 patients considered to be in the second stage were found to be negative in at least two samples by PCR and double centrifugation. Two others (patients 2585 and 2552) were still PCR positive both immediately and 1 month after receiving treatment. For these two patients, the double centrifugation did not disclose the presence of parasites but their cell counts and protein levels were abnormal.

The detection of parasites either by double centrifugation or by parasite DNA detection using PCR seems to be the only reliable technique for assessing the efficacy of trypanosomiasis treatment, since cell number and protein level are slow to return to normal and remain high even 3 months after treatment has ended. Detection of trypanosome DNA using PCR in the two positive patients indicated that they had not been cured but that their potential relapse could not be identified using the double centrifugation technique since it is not sufficiently sensitive.

Occasionally, CSF samples with a normal cell count and protein level have been found to harbour trypanosomes (*13*). In such cases, the patients are considered to be in the first stage of the disease. Other occasional observations indicate a difference in CSF analysis depending on the site of the spinal puncture (suboccipital or lumbar) (*14*). PCR may be an appropriate method for studying such unusual cases.

Our study indicates that the PCR technique described could be useful for determining the stage of the disease in human African trypanosomiasis. It might also be useful for identifying early treatment

Table 1. **Results of PCR, double centrifugation, protein level determination and cell count for patients with human African trypanosomiasis prior to treatment**

Patient	PCR	Double centrifugation	Protein level (mg/100 ml)	Cell count	Stage
2501	+	+	70	872	2
2555	+	+	70	274	2
2565	+	+	71	1044	2
2585	+	+	53	122	2
2591	+	+	38	930	2
2511	+	+	60	728	2
2552	+	+	127	132	2
2589	+	+	60	278	2
2592	+	+	71	228	2
637	+	+	NP[a]	36	2
649	+	+	NP	30	2
2564	+	–	87	450	2
2567	+	–	45	260	2
2571	+	–	71	1152	2
2494	+	–	68	252	2
2520	–	–	19	0	1
2521	–	–	26	2	1
2522	–	–	29	24	1
2586	–	–	24	4	1
2543	–	–	34	0	1

[a] NP = not performed.

failures. Further evaluation of the method is required, in particular to determine whether PCR assays could be used in studies on patients who fail to respond to melarsoprol, as observed in several foci (e.g. Angola, Sudan, and Uganda). ■

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Résumé

Amplification génique (PCR) appliquée à la détection du stade et au suivi de la trypanosomiase africaine humaine

La détermination du stade de la trypanosomiase africaine humaine est actuellement fondée sur la recherche du parasite et la mesure des modifications biologiques du liquide céphalorachidien (LCR) : une concentration des leucocytes >5 par µl et une augmentation de la protéinorachie (>37 mg/100 ml avec la méthode colorimétrique). Une augmentation de la cellularité et/ou de la concentration en protéines, avec ou sans présence de trypanosomes, débouche sur un diagnostic de stade avancé (deuxième stade), et le patient sera traité comme il se doit. La mise en évidence de l'absence ou de la présence de trypanosomes par double centrifugation demeure le seul moyen dont dispose le clinicien pour juger de la réussite du traitement. Compte tenu de l'efficacité de la PCR pour détecter la présence de trypanosomes dans le sang total, nous avons étudié cette technique en tant qu'instrument permettant d'évaluer le stade de la maladie et de déterminer le succès ou l'échec du traitement. On a obtenu des prélèvements de LCR pour 20 patients de Côte d'Ivoire. Sur 15 patients considérés comme ayant une trypanosomiase de deuxième stade en fonction de leurs cellulorachie et protéinorachie, tous étaient PCR positifs; toutefois, la double centrifugation n'a montré la présence de trypanosomes que chez 11 de ces patients. Les résultats de la PCR et de la double centrifugation pour les 5 patients restants, considérés comme en étant au premier stade, étaient négatifs. Un de ces patients avait 24 cellules par µl et pouvait se trouver à un stade soit précoce soit tardif de la maladie, ce qui vient à l'appui du chiffre contesté de 5 cellules par µl comme étant le seuil entre le premier et le deuxième stade. Des prélèvements postérieurs au traitement effectués chez 13 des 15 malades présumés au deuxième stade ont été trouvés négatifs au moins à deux occasions, à la fois par la PCR et par la double centrifugation. Les deux autres sont demeurés positifs pour la PCR, immédiatement après le traitement et un mois suivant la fin de ce dernier. La recherche du parasite soit par double centrifugation soit par la PCR pour mettre en évidence l'ADN du parasite semble être la seule technique fiable, car le nombre des cellules comme la concentration en protéines ne reviennent à la normale que lentement et demeurent élevés même trois mois après le traitement. Les résultats de la PCR donnent à penser que les deux patients positifs n'étaient pas guéris, mais que leur rechute éventuelle n'a pu être identifiée par une recherche du parasite à l'aide de la double centrifugation, cette technique n'étant pas suffisamment sensible. Il faut évaluer plus avant la PCR, notamment pour déterminer si elle peut être utilisée dans les études sur les patients qui ne répondent pas au mélarsoprol ainsi qu'on a pu l'observer dans plusieurs foyers.

Resumen

Empleo de la reacción en cadena de la polimerasa para la determinación de la fase de la tripanosomiasis africana humana y su seguimiento

La determinación de la fase de la tripanosomiasis africana humana se basa actualmente en la detección de los parásitos y en la medición de los cambios biológicos del líquido cefalorraquídeo (LCR): concentración de leucocitos superior a 5 células por µl y aumento de las proteínas totales (>37 mg/100 ml usando un método colorimétrico). Así pues, un aumento del número de células y/o de la concentración de proteínas, con o sin presencia de tripanosomas, permitirá diagnosticar una fase avanzada (segunda fase) de la enfermedad y tratar al paciente en consecuencia. La demostración de la ausencia o presencia de tripanosomas mediante la técnica de doble centrifugación es todavía el único medio de que dispone el personal clínico para evaluar el éxito del tratamiento. En vista de la eficiencia de la reacción en cadena de la polimerasa (RCP) como medio de detección de la presencia de tripanosomas en sangre entera, hemos evaluado el uso de esta técnica para determinar la fase de la enfermedad y el éxito o el fracaso del tratamiento. Se obtuvieron muestras de líquido cefalorraquídeo de 20 pacientes de Côte d'Ivoire. Los 15 pacientes a quienes se consideraba en la segunda fase de tripanosomiasis, según el recuento leucocitario y el nivel de proteínas en su LCR, eran todos RCP-positivos. En cambio, la doble centrifugación sólo mostró tripanosomas en 11 de esos pacientes. Los resultados

de la RCP y de la doble centrifugación para los cinco pacientes restantes, a quienes se consideraba en la primera fase, fueron negativos. Uno de esos pacientes tenía 24 células por µl y podía hallarse tanto en una fase temprana como en una fase avanzada de la enfermedad, lo que respaldaría la consideración del controvertido valor de 5 células por µl como el límite entre las fases primera y segunda. Las muestras posteriores al tratamiento de 13 de los 15 pacientes a quienes se consideraba en la segunda fase fueron negativas por lo menos en dos ocasiones tanto en la RCP como en la doble centrifugación. Los otros dos seguían siendo RCP-positivos inmediatamente después del tratamiento y un mes más tarde. La detección de los parásitos, ya sea por doble centrifugación o empleando la RCP para identificar el ADN del parásito, parece la única técnica fiable, ya que tanto el recuento leucocitario como los niveles de proteínas tardan en volver a la normalidad y siguen siendo altos incluso tres meses después del tratamiento. Los resultados de la RCP sugieren que los dos pacientes positivos no estaban curados, pero no se hubiese podido prever su eventual recaída buscando los parásitos mediante la técnica de doble centrifugación, ya que ésta no es lo bastante sensible. Es necesario evaluar mejor el método de la RCP, en particular para determinar si puede emplearse en los estudios de pacientes que no responden al melarsoprol, como se ha observado en varios focos.

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