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Human African trypanosomiasis: use of double centrifugation of cerebrospinal fluid to detect trypanosomes

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A double-centrifugation technique for the detection of trypanosomes in samples of cerebrospinal fluid is described and evaluated. The results of the analysis of samples of cerebrospinal fluid from 128 patients with Trypanosoma brucei gambiense *sleeping sickness from the Daloa area of Côte d'Ivoire, obtained using single centrifugation, are compared with those obtained using the new method. Double centrifugation is at least twice as sensitive as single centrifugation and results in an increase of 37% in the early detection of late-stage cases of the disease. The technique is easily implemented under field conditions.*

The normal progression of human African trypanosomiasis inevitably leads to the invasion of the central nervous system by trypanosomes and an increase in the protein level and white blood cell count in the cerebrospinal fluid (CSF) of patients. This phase of the disease has been defined classically as the second or meningo-encephalitic stage and its treatment requires use of a drug that crosses the blood–brain barrier. Unfortunately, such therapy is often associated with severe adverse side-effects, including encephalopathy.

The classical method of identifying trypanosomes in the CSF of sleeping sickness patients has been microscopic examination of the fluid sediment following centrifugation. Here, we describe a double-centrifugation technique that is considerably more sensitive than the classical method and also reduces microscope viewing time.

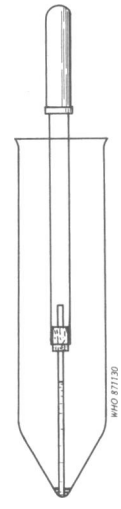

Fig. 1. Pipetting device to draw previously centrifuged cerebrospinal fluid (CSF) sediment into a capillary tube for further centrifugation.

MATERIALS AND METHODS

Approximately 6–8 ml of CSF from sleeping sickness patients was collected in conical centrifuge tubes. A few drops were used immediately to determine the cell count and the remainder centrifuged at 850 *g* for 10 minutes in a swinging bucket centrifuge. The supernatant was then decanted in one operation into another tube for use in other tests.

By means of an extended pipetting device (Fig. 1),[a] the centrifuged CSF sediment was drawn into a capillary tube (length 75 mm; internal diameter, 1.1–1.2 mm) that fitted into a standard microhaematocrit

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[a] Dade®. Supplied by American Scientific Products, McGraw Park, IL, USA.

centrifuge. Since the capillary tubes were flame-sealed over a Bunsen burner, they were not completely filled in order to avoid overheating the sample; also the end of the tube that had been used to draw the sediment was kept dry to avoid carbonizing CSF deposits during the sealing process. The sealed capillaries were then centrifuged in a microhaematocrit centrifuge at 13 000–15 000 *g* for 1 minute and stored vertically. Microscopic examination of the capillary sealed tip was carried out within 15 minutes of centrifuging, since trypanosomes rapidly become less mobile and more difficult to observe. For this purpose, the sealed end of the capillary tube was placed between a glass slide and a coverslip. Some filtered tap water was then added between the slide and the coverslip to reduce the light refraction. In order to view the trypanosomes microscopically, a magnification of ×300 was used with the following lens combination: a ×10 ocular, a ×1.5 multiplier, and a ×20 objective. Alternatively, a ×10 ocular and ×32 long-working-distance lens (7.5 mm) (total magnification: ×320) was found satisfactory. Trypanosomes were observed directly in the capillary tube. When a large number of white blood cells were present in the sample of CSF, the trypanosomes were found in close proximity to the sedimented cells, moving towards the supernatant.

The restricted microscope field that had to be viewed with the double-centrifugation technique shortened considerably the observation time compared with that in the single-centrifugation method, in which the sediment is transferred from the centrifuge tube to a glass slide with a coverslip and the whole surface of the sample scanned. However, a viewing chamber similar to that used in the miniature anion-exchange centrifugation technique (*2*), consisting of a coverslip permanently hinged to a glass slide, facilitated the examination of the capillary tubes used in the double-centrifugation method.

Table 1. Distribution of the results of analysis of samples of cerebrospinal fluid (CSF) from 128 study patients with sleeping sickness

	Cells[a]	Protein[b]	Double centrifugation[c]	No. of patients
	−ve	−ve	−ve	72
	+ve	+ve	+ve	26
	−ve	−ve	+ve	20
	−ve	+ve	+ve	4
	−ve	+ve	−ve	4
	+ve	+ve	−ve	1
	+ve	−ve	+ve	1
Total	28 +ve	35 +ve	51 +ve	128

[a] +ve indicates the presence of >5 white blood cells per mm^3 in CSF.
[b] +ve indicates a level >37 mg/dl in CSF.
[c] +ve indicates the detection of trypanosomes in CSF.

Table 2. Comparison of the results of double and single centrifugation of cerebrospinal fluid from the 128 study patients with sleeping sickness.

	Double centrifugation		
Single centrifugation	No. positive	No. negative	Total
No. positive	23	0	23
No. negative	28	77	105
Total	51	77	128

Samples of CSF from 128 African patients who were hospitalized in the Daloa Research Centre clinic, Côte d'Ivoire, with parasitologically confirmed *T.b. gambiense* sleeping sickness were examined (Table 1). Both single- and double-centrifugation techniques were used. In order to optimize the sensitivity of the single-centrifugation method, the whole sample of CSF was centrifuged and the necessary amount of sediment deposited on a glass slide and covered with a coverslip for viewing microscopically at ×400 magnification. The remaining fraction of sediment was then used in the double-centrifugation method.

The total protein level in the CSF was determined using a dye-binding protein assay (*3*) and cells were counted in a Nageotte chamber. Results for these two tests were considered normal if the total protein level was $\leqslant 37$ mg/dl and total number of white blood cells was $\leqslant 5/mm^3$ (*4*).

RESULTS

Trypanosomes were observed in 51 samples of CSF with the double-centrifugation technique and only in 23 samples with single centrifugation. It should be noted that all the samples that were positive with the single-centrifugation technique were also positive with the double-centrifugation method, while 77 samples of CSF were negative in both methods (Table 2).

Samples of CSF from 26 patients exhibited trypanosomes and were positive for number of cells and protein level, while an elevated protein level occurred in 35, and an increased number of cells in 28 samples

(Table 1). Among 27 samples of CSF that were positive for both number of cells and protein level, 26 were positive with the double-centrifugation technique and one (No. 101) that had a protein level of 39.91 mg/dl and 6 cells/mm^3 was negative.

Upon double centrifugation, 30 samples of CSF were positive for trypanosomes and exhibited increased protein levels, 21 were positive for trypanosomes but had normal protein levels, while five samples were negative for trypanosomes and had an elevated protein level (Table 1). Similarly, after double centrifugation, 27 samples of CSF were positive for trypanosomes and number of cells; 24 samples were positive for trypanosomes but the number of cells was normal; and one (patient No. 101) was negative for trypanosomes but had an increased number of cells (Table 1).

Also, 31 samples of CSF exhibited raised protein levels and/or number of cells and were positive for trypanosomes after double centrifugation; 20 samples were positive for trypanosomes but negative for both protein and number of cells; and five samples were positive for either protein and/or cells but negative for trypanosomes (Table 1). Also, among the 20 samples that were positive for trypanosomes after double centrifugation but negative for protein level and number of cells, in only three could trypanosomes be found using the single-centrifugation technique.

DISCUSSION

The choice of treatment for sleeping sickness is determined by the stage of the disease, for which three criteria for CSF are generally used (*4*)—one of which is the presence of trypanosomes. For the 128 patients tested, double centrifugation was over twice as sensitive as single centrifugation in identifying *T.b. gambiense* parasites in CSF. The two other criteria used are protein level and number of white blood cells, values for which are selected arbitrarily in the lack of any other suitable and measurable quantities; and this probably accounts for the use of different upper limits in various countries for the number of cells in CSF. An upper limit of 39 mg/dl for protein in CSF has been conservatively recommended (determined by the dye-binding protein assay), while the limit for number of cells is 5/mm^3. In the present study, CSF analysis results have been interpreted accordingly.

It has been suggested that the central nervous system is invaded by trypanosomes soon after a patient becomes infected, that the primary and later stages of the disease have a tendency to overlap, and that the presence of trypanosomes probably precedes the increase in protein level and number of white blood cells in the CSF (*1*). The presence of trypanosomes in CSF in the absence of biological alterations might therefore be a precocious sign of the advanced stage of the disease, although trypanosomes cultivated *in vitro* do not survive in CSF alone but require also the presence of serum (L. Jenni, personal communication, 1986). This indicates the inability of the parasite to survive in a nutritionally poor environment such as that provided by normal CSF. Thus only a continuous leakage of trypanosomes from blood into CSF would provoke a sufficient deterioration of the central nervous system to alter the composition of the CSF and eventually provide an environment that could support trypanosomal growth. The elimination of parasites from blood before any alteration of the CSF has occurred might therefore suffice to cure the infected patient. Further research to confirm these hypotheses is needed and this could be carried out with the double-centrifugation technique, which can detect trypanosomes in a large number of patients who have no raised protein level or increased number of cells in their CSF.

An elevated protein level and/or an increased number of white blood cells in the absence of trypanosomes was found in only five samples of CSF, for which detailed analysis results are provided in Table 3. It should be noted that none of these samples had a protein level above 60 mg/dl or a white blood cell count above 6/mm^3 in CSF. An attempt was therefore made to correlate the presence of trypanosomes with the number of cells and protein level in the CSF. For example, when 3, 5, or 6 cells per mm^3 were used as the upper limit along with a protein level of 37 mg/dl, five "late stage" patients remained negative for the presence of parasites in CSF; however, when the protein threshold level was increased to 40 mg/dl the total number of "late stage" patients dropped to three and finally to zero when 60 mg/dl and 6 cells/mm^3 were used as the upper limits, respectively. The significance of increasing the threshold for protein level and number of cells to the

Table 3. Results of the analysis of five samples of cerebrospinal fluid that were negative for the presence of trypanosomes

Patient	No. of cells (per mm^3)	Protein level (mg/dl)
101	6	39.91
205	3	38.26
204	1	57.32
130	0	41.22
152	0	37.83

last-mentioned values remains to be shown in relation to the success of treatment with drugs that do not cross the blood–brain barrier. Similarly, the presence of trypanosomes in CSF without a raised protein level and an increase in the number of white blood cells in CSF requires further investigation.

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RÉSUMÉ

TRYPANOSOMIASE AFRICAINE: UTILISATION DE LA DOUBLE CENTRIFUGATION DU LIQUIDE CÉPHALORACHIDIEN POUR LA MISE EN ÉVIDENCE DES TRYPANOSOMES CHEZ LES MALADES

Une technique de double centrifugation, pour la mise en évidence des trypanosomes dans le liquide céphalorachidien (LCR) a été mise au point et évaluée; elle consiste en deux centrifugations successives du LCR. A la suite d'une première centrifugation dans un tube conique à 850 *g* pendant 10 minutes, le surnageant est décanté et le culot prélevé dans un tube capillaire (microhématocrite) avec une pipette au corps allongé. Un soin particulier est apporté au remplissage du capillaire afin d'en conserver approximativement 1 cm vide et sec pour un scellement à la flamme. Le capillaire scellé est alors placé dans une centrifugeuse à microhématocrite et centrifugé à 3825–5100 *g* pendant 1 minute. La conservation du capillaire se fait en position verticale (culot vers le bas) jusqu'à l'examen au microscope à un grossissement total de 300 ou 320×, qui doit avoir lieu dans les quinze minutes qui suivent la dernière centrifugation, avant que les trypanosomes ne commencent à perdre leurs mouvements caractéristiques.

La double centrifugation (DC) a été mise en œuvre sur le LCR de 128 trypanosomés à *T.b. gambiense* de la région de Daloa en Côte d'Ivoire et comparée à la simple centrifugation. Il s'est avéré que la DC est au moins deux fois plus sensible que la simple centrifugation. De plus, la DC a permis de montrer que le parasite était souvent présent dans le LCR alors que la protéinorachie (P) et la cytorachie (C) classaient le patient en première période ($P <= 37$ mg/dl; $C <= 5/mm^3$). En conclusion, une réévaluation des taux limites des deux paramètres P et C à 40 mg/dl et à 6 /mm^3 a été suggérée pour qu'ils concordent, avec la présence du parasite, au deuxième stade de la maladie. Mais une étude devrait être entreprise pour établir la valeur des nouveaux paramètres envisagés par rapport aux différents schémas de traitement de la maladie du sommeil.

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