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# Stage determination in sleeping sickness: comparison of two cell counting and two parasite detection techniques

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**Abstract**

OBJECTIVES Diagnosis of the neurological stage of human African trypanosomiasis is performed by examination of cerebrospinal fluid (CSF) for the presence of trypanosomes and numbers of white blood cells (WBC). Both CSF parameters are also used to assess treatment outcome during follow-up. In view of the importance of CSF examination, and the practical problems encountered with it, we compared the sensitivity of two trypanosome concentration techniques and the repeatability of two cell counting methods, as well as occurrence of systematic differences between them.

METHODS Patients were recruited at Dipumba hospital, in Mbuji-Mayi in the Democratic Republic of the Congo. In 94 CSF samples, trypanosome detection was performed with modified single centrifugation (MSC) and double centrifugation (DC). On 189 CSF samples with $\leq 30$ cells/$\mu$l, cell counting was performed in duplicate in a Fuchs–Rosenthal counting chamber and in a disposable Uriglass counting chamber.

RESULTS Modified single centrifugation detected trypanosomes in significantly ($P < 0.0001$) more patients (85) than DC (46). Cell counts did not differ systematically in the two methods. Variability in the differences between duplicate cell counts was significantly higher ($P = 0.002$) in Uriglass (SD of differences 2.03) than in Fuchs–Rosenthal (SD of differences 1.62).

CONCLUSIONS For analysis of CSF in the context of sleeping sickness stage determination and follow-up after treatment, we strongly recommend the MSC for parasite detection and the application of disposable counting chambers. When the first cell count is $\leq 20$ cells/$\mu$l, we recommend repeating the counting procedure on the same CSF specimen and taking the average of both countings.

**keywords** sleeping sickness, cerebrospinal fluid, *Trypanosoma brucei gambiense*, human African trypanosomiasis

## Introduction

Human African trypanosomiasis or sleeping sickness is caused by *Trypanosoma brucei (T.b.) gambiense* or *T.b. rhodesiense*. The parasites are transmitted by tsetse flies (*Glossina sp.*). Once injected with the saliva of an infected blood-sucking tsetse fly, the trypanosomes invade the peripheral tissues and organs of their mammalian host via the lymph and blood vessels (haemolymphatic stage). Eventually, they cross the blood–brain barrier to invade the central nervous system (meningo-encephalitic stage). Because of the selective permeability of the blood–brain barrier and the higher toxicity of drugs to treat the meningo-encephalitic stage, it is imperative to assess the disease stage before stage-specific treatment can be started (World Health Organization 1998).

In the absence of exclusive clinical signs, nor specific blood parameters, stage determination is performed by examination of cerebrospinal fluid (CSF), obtained by lumbar puncture. The CSF is examined essentially for the presence of trypanosomes and the numbers of white blood cells (WBC) per microlitre ($\mu$l). When trypanosomes are present or when cell count $\geq 5$ WBC/$\mu$l, a patient is considered to be in the meningo-encephalitic stage and should be treated accordingly (World Health Organization 1998). Both CSF parameters are also used to assess treatment outcome during follow-up (Lejon & Büscher 2005).

Important practical problems arise with regard to the determination of these parameters. For the CSF WBC


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count, the cut-off value of 5 WBC/$\mu$l is near the detection limit of most cell counting chambers, resulting in limited accuracy and repeatability when WBC counts are normal or close to normal. Also, the correct operating conditions for the cell counting chamber are often not followed, resulting in non-reliable cell counts. The most frequently observed deviations are incorrect filling of the cell counting chamber and the use of ordinary microscope coverslips when the original coverslips are broken. By using disposable cell counting chambers such as the Uriglass (A. Menarini Diagnostics, Firenze, Italy) or the KOVA Glasstic slide (Hycor Biomedical, CA, USA), these manipulation errors can be avoided at a cost of less than 0.20 € per test.

The finding of trypanosomes in CSF allows the immediate classification of a patient in the meningo-encephalitic stage or is the absolute proof of treatment failure. Given the small volume of CSF examined during cell counting, the sensitivity of trypanosome detection in the cell counting chamber is low. Therefore, concentration of the parasites by centrifugation of a larger volume of CSF is recommended. In double centrifugation (DC), several millilitres of CSF are centrifuged in a simple table centrifuge where after the supernatant is discarded. The CSF sediment is then taken up in capillary tubes that are sealed at one end. After centrifugation in a haematocrit centrifuge, the tubes are mounted on a microscope slide to search for the trypanosomes among the WBC in the sediment (Cattand *et al.* 1988). Double centrifugation is laborious and time-consuming, while trypanosomes in CSF are fragile and quickly die (Pentreath *et al.* 1992). In addition, flame sealing the capillary may instantly kill trypanosomes when performed incorrectly. In the modified single centrifugation (MSC), several millilitres of CSF are centrifuged in a sealed glass Pasteur pipette or a special plastic collector tube (Miézan *et al.* 2000; Büscher *et al.* 2009) after which the tube is mounted on a microscope slide using a special viewing chamber, and trypanosomes are directly searched for in the sediment. Limiting the number of manipulations and avoiding the heating step should increase chances of finding trypanosomes in CSF, if present.

Our objective was to compare the sensitivity of the MSC with that of the DC. We further compared a classical cell counting chamber (Fuchs–Rosenthal) with a disposable counting chamber (Uriglass, Menarini) in terms of repeatability and systematic differences in cell counts.

## Methods

### Ethics statement

The study protocol was approved by the Ethics Committee of the Ministry of Health, Kinshasa, DRC and the Ethics Committee of the University of Antwerp, Belgium. All participants provided written informed consent. For participants younger than 18 years, written informed consent was obtained from a parent.

### Study design and site

This prospective observational study was conducted in the Kasai Oriental province in the Democratic Republic of the Congo (DRC) where sleeping sickness is endemic. In 2006, the prevalence of the disease was estimated at 0.75% by the Programme National de Lutte contre la Trypanosomiase Humaine Africaine (PNLTHA) but decreased to 0.47% in 2008 (Kande Betu-Ku-Mesu 2009). Tests were carried out by skilled and trained technicians with extensive experience in diagnostic trials.

### Patients

Patients were recruited at Dipumba hospital, in the city of Mbuji-Mayi (DRC) from March to October 2008. Inclusion criteria for the comparison of MSC and DC were HAT patient with confirmed presence of trypanosomes in lymph nodes or blood and more than 11 years old. Inclusion criteria for the comparison of Fuchs–Rosenthal and Uriglass were HAT patient with CSF cell count <30 WBC/$\mu$l in Kova Glasstic counting chamber (Hycor) and more than 11 years old. Patients with macroscopically visible haemorrhagic CSF were excluded. From patients included in the study, 5 ml of CSF were collected by lumbar puncture. Before further manipulations, CSF was mixed gently.

### Modified Single Centrifugation and Double Centrifugation

For MSC, a flame-sealed glass Pasteur pipette of 9 mm diameter was filled with 2 ml of CSF. For DC, 2 ml of CSF was dispensed in a polypropylene centrifugation tube of 13 ml. Both tubes were centrifuged at 1200 g for 10 min. The sealed Pasteur pipette was mounted in a viewing chamber with the tip submerged in clean water and covered with a coverslip to minimise light diffraction, followed by immediate examination under a microscope at 10 × 10 magnification. From the 13-ml tube, the supernatant was discarded and the sediment was resuspended and taken up in two capillary tubes that were subsequently flame sealed with a Bunsen burner. Both capillary tubes were centrifuged at 13 000 g for 10 min and mounted in a viewing chamber with

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the sealed end submerged in clean water and covered with a coverslip for immediate examination at 10 × 10 magnification. For both techniques, presence of trypanosomes was scored as positive or negative.

### Fuchs–Rosenthal and Uriglass

Two counting chambers of a Uriglass slide and two counting chambers of a Fuchs–Rosenthal slide were filled. The counting chambers were left for 5 min to allow cells to sediment before examination under a microscope at 10 × 10 magnification. Examination was conducted in a double-blind way by two technicians, each of them using one type of counting chamber. After 10 tests, counting chamber types were exchanged between technicians.

### Statistical analysis

The McNemar Chi-squared test was used to compare the dichotomised results obtained with MSC and DC. Bland–Altman difference plots were used to measure the repeatability of the Uriglass and of Fuchs–Rosenthal cell counting method and to assess any systematic difference between both methods (Bland & Altman 1986). The variance ratio test was used to assess the difference in repeatability between Uriglass and Fuchs–Rosenthal. The level of significance was set at 0.05.

## Results

### Modified Single Centrifugation *vs.* Double Centrifugation

We enrolled 94 patients. Most were in an advanced stage of the disease as evidenced by a mean of 323 WBC/$\mu$l (SD = 399) in their CSF. With MSC, trypanosomes were detected in the CSF of 85 patients, while with DC, the parasites were detected in the CSF of 46 patients (Table 1). MSC detected trypanosomes in significantly more patients than DC ($P < 0.0001$).

**Table 1** Number of patients with trypanosomes detected in their CSF with the modified single centrifugation (MSC) and the double centrifugation (DC)

| | DC | | |
|---|---|---|---|
| | Negative | Positive | Total |
| MSC | | | |
| Negative | 7 | 2 | 9 |
| Positive | 41 | 44 | 85 |
| Total | 48 | 46 | 94 |

### Fuchs–Rosenthal *vs.* Uriglass

We examined 189 patients. The mean age was 35 years (SD = 12). The cell counting results obtained in the four observations are given in Table 2. To assess the repeatability of the cell counts in each type of cell counting chamber, Bland–Altman difference plots were constructed (Figure 1). The variability in the differences between the two cell counts was significantly higher ($P = 0.002$) in Uriglass (SD of differences 2.03) than in Fuchs–Rosenthal (SD of differences 1.62) with limits of agreement between two measurements of −3.56 and 4.40 for Uriglass and −3.20 and 3.20 for Fuchs–Rosenthal.

To assess the agreement between cell counts in the two types of counting chambers, a Bland–Altman difference plot was constructed with the means of the two repetitions in each type of cell counting chamber (Figure 2). There was no bias between the two counting chambers (0.05, 95% CI −0.38 to 0.48), indicating the absence of a systematic difference in cell counts. The lower limit of agreement was – 5.85, the upper limit of agreement 5.96. Differences between the means ranged from −16 to 8.5 and tended to be higher in the higher count range.

## Discussion

In this study, we demonstrate that for the detection of trypanosomes in CSF of HAT patients, MSC is almost twice as sensitive as DC. For counting WBC in CSF, there is no systematic difference between the two methods, but repeatability remains worrisome.

Our study has some limitations. The total volume of CSF that was taken was limited to 5 ml, which had to be equally divided over two trypanosome detection techniques to compare them. In adults with normal CSF pressure, volumes up to 20 ml can be sampled and centrifuged (Kjeldsberg & Knight 1993). The real sensitivity of both techniques might therefore be an underestimation. For cell counting, we concentrated on cell counts

**Table 2** Cell count results obtained by two readers with two cell counting chambers

| | Uriglass | | Fuchs–Rosenthal | |
|---|---|---|---|---|
| | Count 1 | Count 2 | Count 1 | Count 2 |
| Min | 0 | 0 | 0 | 0 |
| Max | 35 | 32 | 32 | 29 |
| Median | 3 | 3 | 3 | 3 |
| 25% Quartile | 1 | 1 | 2 | 2 |
| 75% Quartile | 6 | 6 | 6 | 6 |



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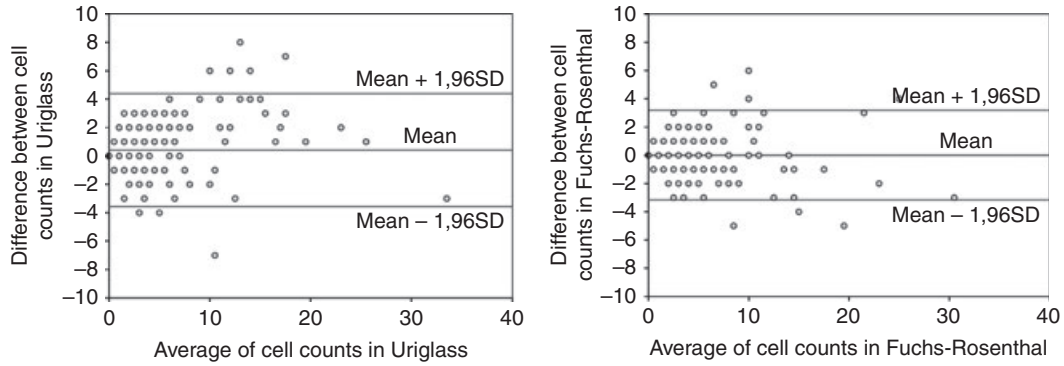


**Figure 1** Bland–Altman difference plots with the average cell counts in Uriglass and in Fuchs–Rosenthal cell counting chambers.

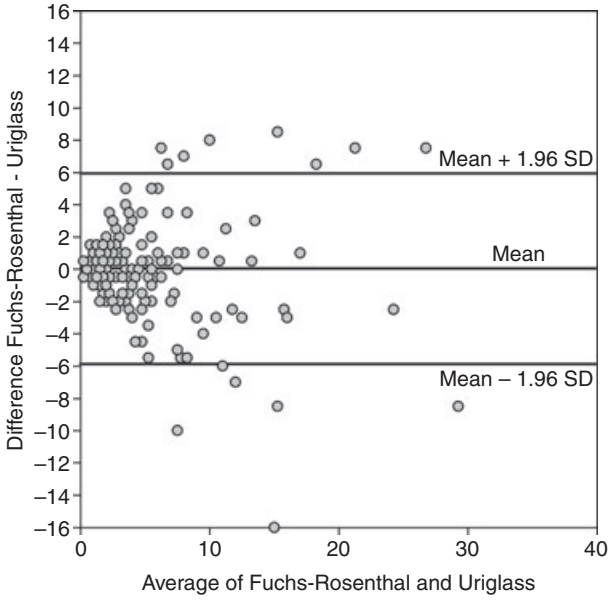


**Figure 2** Bland–Altman difference plots with the average of all cell counts in both cell counting chambers.

<30 WBC/$\mu$l, as cut-off values applied in HAT patient management are in this low range. Especially, for the Fuchs–Rosenthal technique, it might be expected that the technicians, knowing that they participated in a scientific study and that results would be compared afterwards, paid more attention to the correct mounting of the counting chamber than in routine practice. Its repeatability might therefore have been overestimated.

For trypanosome detection in CSF, our findings confirm earlier observations of Miézan *et al.* (2000) who detected trypanosomes in the CSF of 27 and 33 of 42 HAT patients with DC and MSC, respectively. The better sensitivity of MSC compared to DC might be explained by the limited number of manipulations and shorter operation time ($\leq$15 min) of MSC as CSF is centrifuged only once, and the sediment is examined directly in the centrifugation tube. We expect that the sensitivity of MSC may even be increased by using the plastic collector tubes designed for the mAECT test, without substantially increasing the cost (0.25 €/tube) (Büscher *et al.* 2009) to centrifuge 4 ml of CSF instead of the sealed 2-ml glass Pasteur pipettes used in this study. Still, it remains important that CSF is examined as soon as possible after collection because trypanosomes cannot survive for longer periods in this fluid (Cattand *et al.* 1988).

For counting the WBC in CSF, the agreement between the two methods under study is good but not perfect because differences can be as high as 16 in a single specimen. Most importantly, there is no systematic difference between the two methods (bias 0.05), so that both methods are interchangeable under the condition that the operating instructions are strictly adhered to, that the counting chambers are of high quality and not worn and that cell counting is repeated twice for low cell counts, as was the case in this study. Repeated cell counts when the first value is $\leq$20/$\mu$l have been recommended earlier to increase the accuracy of the measurement (Chappuis *et al.* 2005). The repeatability of the cell counts in the disposable Uriglass chambers was significantly lower than in the Fuchs–Rosenthal chamber. This could be explained by the three times lower volume of the Uriglass counting chambers (1 $\mu$l under the counting grid) than of the Fuchs–Rosenthal chamber (3.2 $\mu$l under the counting grid) leading to a higher precision of the latter chamber, particularly at cell counts that approach the detection limit of the counting chambers. In our opinion, the lower repeatability observed in the Uriglass chambers is balanced by the manipulating comfort. For correct application of non-disposable cell counting chambers, like the Fuchs–Rosenthal, proper training and original coverslips are essential, conditions that all too often are not met in

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the rural health centres or the mobile teams that deal with sleeping sickness control.

In sleeping sickness, decision on treatment regimen and assessment of cure are imperatively based on the examination of CSF. Even recently, cell counts and presence or absence of parasites in CSF have been proven to be the most reliable parameters that allowed shortening the after treatment follow-up from 24 to 6 or 12 months (Mumba Ngoyi *et al.* 2010; Priotto *et al.* 2012). Although promising alternative markers for stage determination and treatment outcome have been identified, translation of these findings into a simple ASSURED test for use in rural primary health care centres seems not for the near future (Peeling *et al.* 2006; Tiberti *et al.* 2012). Cell count and trypanosome detection will therefore continue to be applied.

In conclusion, for analysis of CSF in the context of sleeping sickness stage determination and follow-up after treatment, we strongly recommend the MSC for parasite detection, and we propose disposable counting chambers such as Uriglass or Kova to replace Fuchs–Rosenthal in conditions where proper application of the latter cannot be guaranteed, for example, due to lack of replacement of broken chambers and coverslips and lack of training. Furthermore, whatever technique is used, when the first cell count is $\leq 20$ cells/$\mu$l, we recommend to increase accuracy by repeating the counting procedure on the same CSF specimen and taking the average of both countings.

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