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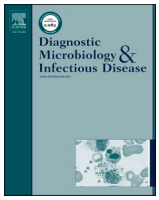

Original Article

The fully automated Sysmex XN-31 hematology analyzer can detect bloodstream form *Trypanosoma brucei*

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ABSTRACT

Background: For fully automated detection and quantification of *Plasmodium* parasites, Sysmex developed the XN-31 hemocytometer. This study investigated whether the XN-31 can also detect and quantify bloodstream form trypanosomes (trypomastigotes).
Methods: Axenic cultures of *Trypanosoma brucei brucei* were used to prepare two dilution series of trypomastigotes in the whole blood of a healthy donor, which were subsequently examined by the XN-31 as well as by microscopic examination of thin and thick blood films. Trypomastigote intactness during the procedures was evaluated by microscopy.
Results: The XN-31 hemocytometer detected trypomastigotes with a detection limit of 26 trypomastigotes/μL. Scattergram patterns of *Trypanosoma* and *Plasmodium* parasites were clearly distinct, but current interpretation settings do not allow the identification of trypomastigotes yet, and therefore, need future refinement.
Conclusion: Proof of concept was provided for an automated fluorescent flow cytometry method that can detect and quantify *Plasmodium* spp., as well as *Trypanosoma brucei* trypomastigotes.

1. Introduction

Microscopic examination of thick and thin blood films is still the most frequently used method to detect blood parasites that cause malaria (*Plasmodium* species) [1]. Although this gold-standard method is both sensitive and specific for the detection of *Plasmodium* parasites, it has the drawbacks that it is observer-dependent and labor-intensive. Furthermore, in non-endemic countries, proper microscopic examination of thick and thin blood films is a challenge as blood parasites are not frequently observed in clinical laboratories. This limits their experience and thereby the quality of the microscopic examination for blood parasites in travelers with fever who recently returned from malaria-endemic regions.

Recently Sysmex developed the CE ("conformité européenne") certified, fully automated Sysmex XN-31 hemocytometer for the detection and quantification of *Plasmodium* parasites. This hemocytometer can identify the *Plasmodium* species, discriminate asexual and sexual stages of *Plasmodium* spp., and determine the parasitemia [2–5] This analyzer has been evaluated in both endemic and non-endemic countries and proven to be reliable and accurate compared to other methods for the detection of *Plasmodium* species with a limit of quantification of 20 infected red blood cells/μL and a limit of detection of 6 infected red blood cells/μL [2–8].

Due to its excellent performance in the detection of *Plasmodium* parasites, the XN-31 hematology analyzer is increasingly used in clinical practice for patients presenting with fever in malaria-endemic areas or travelers from endemic areas. However, in addition to *Plasmodium* parasites other blood parasites can also cause a fever illness, such as the blood-dwelling flagellate *Trypanosoma brucei* spp. that causes Human African Trypanosomiasis (HAT), also known as African sleeping sickness. The concentration of bloodstream form trypanosomes (trypomastigotes) varies substantially during the disease course and in general tends to be high in *T. brucei rhodesiense* infections and low in *T. brucei gambiense* infections [9]. Diagnosis of human African trypanosomiasis is still cumbersome, especially in detecting infections with low parasitemia [9].

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Since the detection of *Plasmodium* parasites by the Sysmex XN-31 analyzer is based on the combination of cell morphology (classical forward-side scatter flow cytometry) with the detection of fluorescent staining of a DNA binding dye by violet laser technology, it is to be expected that this analyzer should also be able to detect other blood parasites. Therefore, in this study, we evaluated whether the Sysmex XN-31 analyzer can detect and quantify *Trypanosoma brucei* trypomastigotes in whole blood, which is of clinical interest since this parasite causes febrile symptoms similar to malaria.

2. Materials and methods

Monomorphic bloodstream-form *T. brucei brucei* strain 427 (cell line 449 [10]) was cultured in HMI-9 supplemented with 10 % fetal calf serum (Invitrogen) and 0.2 μg/ml phleomycin (Cayla) in a water-saturated incubator with 5 % CO2 at 37 °C as described previously[11]. On two distinct occasions, trypomastigotes from this axenic (free from other organisms) culture were used to prepare two independent serial dilution series in the whole blood of a healthy volunteer using EDTA as an anticoagulant. After a brief spin, the trypomastigote cell pellet was resuspended in whole blood, after which a two-fold dilution series was prepared by mixing 1 mL of trypomastigote-containing blood with 1 mL of fresh donor blood. This series covered the linear range of the XN-31 as per the Instructions for Use for *Plasmodium* detection and ranged from ca. 700 to 3 trypomastigotes/μL. Each dilution was processed in triplicate on the XN-31 and the average result was used for calculations. Blood films were prepared for microscopic examination. Thick and thin blood films were stained using Giemsa stain (Sigma-Aldrich, St Louis, Missouri, U.S.A). In addition, to determine whether the trypomastigotes stayed intact when exposed to the XN-31 reagents, the Lysercell M and Fluorocell M reagents of the XN-31 hematology analyzer were mixed with a small sample of the axenic *Trypanosoma brucei* culture and examined by fluorescence microscopy.

3. Results

Since patients suffering from African sleeping sickness are scarce in non-endemic countries, blood samples of African sleeping sickness patients had to be mimicked by mixing bloodstream form trypanosomes (trypomastigotes) from an axenic culture with whole blood of a healthy donor. This experiment was performed twice at different times using two independent blood samples with circa 700 trypomastigotes/μL (see also Supplementary Fig. 2), which is in the relevant clinical range (10^7 to 10 cells/mL) [12,13]. Fig. 1A and B demonstrate the proper morphology of the *T. b. brucei* trypomastigotes after mixing with fresh whole blood, which validated the prepared samples as a proper mimic for the organism morphology present in blood specimens of African sleeping sickness patients. When trypomastigotes were exposed to the reagents of the XN-31, the trypomastigotes also remained intact as shown by fluorescence microscopy (Fig. 1C).

Analysis by the XN-31 of the two independent *T. b. brucei* dilution series showed that the analyzer reproducibly detects *Trypanosoma* trypomastigotes (Fig. 2B, C) with a lower limit of detection 26 trypomastigotes/μL. In the first experiment, the trypomastigotes were primarily misannotated as only *Plasmodium* gametocytes (Fig. 2B) and in the second experiment as *Plasmodium* gametocytes and trophozoites (Fig. 2C). Although the trypomastigotes were misannotated by the current software settings, they generated scattergram patterns that could easily be distinguished in both forward scatter (FSC) and side fluorescence scatter (SFL) from the scattergram of *Plasmodium falciparum* (Fig. 2A) and *Plasmodium vivax* (Fig. 2D). The side scatter (SSC) and forward scatter (FSC) scattergrams of blood samples with trypomastigotes also differed from those of *P. falciparum* and *P. vivax* (Supplementary Fig. 1). An important difference between the scattergrams of whole blood with trypomastigotes compared to whole blood with *P. falciparum* or *P. vivax* is the absence of events in the area where erythrocytes with *Plasmodium* ring forms (red particles in Supplementary Fig. 1).

The examination of the samples of two independent 2-fold dilution series showed a good correlation between the estimated concentration of trypomastigotes by microscopy and the XN-31 in the first experiment (798 ± 132 vs 688 ± 2 trypomastigotes/μL, respectively), but in the second experiment, there was a 2 to 3-fold difference between these examinations (690 ± 73 vs 251 ± 11 trypomastigotes/μL, respectively). This difference might indicate that the scattergram interpretation settings in the current XN-31 software might detect only a part of the trypomastigotes in some cases, which is not surprising as these detection and interpretation settings have been optimized for the detection of *Plasmodium*-infected erythrocytes and not yet for detection of trypomastigotes.

Finally, the examination of the two independent 2-fold dilution series showed an excellent linear quantification response, because a good correlation was observed between the expected number of trypomastigotes in the dilution series and the actual number of trypomastigotes detected by the XN-31 (Supplementary Fig. 2) with a linear agreement for both experiment #1 ($y = 0.08015 + 1.005x$) and experiment #2 ($y = -0.561 + 1.022x$). These results demonstrated that the XN-31 with the current scattergram interpretation settings can detect and quantify trypomastigotes in whole blood with an estimated limit of detection for trypomastigotes of circa 20 parasites/μL, which is equivalent to the theoretical limit of detection for blood parasites (such as *Plasmodium* spp.) by regular microscopic examination of thick blood films [1].

4. Discussion

Our study showed that the Sysmex XN-31 hemocytometer detected

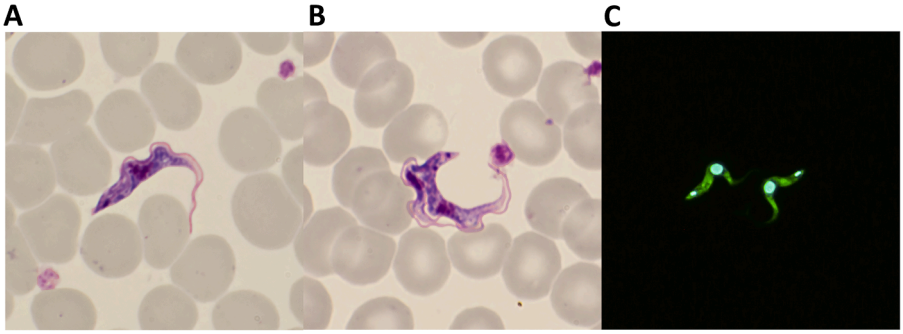

Fig. 1. Microscopic images of the prepared *Trypanosoma brucei brucei* samples. Panel A and B: Giemsa stained thin film with bloodstream form *T. b. brucei* from the *in vitro* culture after mixing with whole blood from a healthy donor. Panel B shows a dividing *T. b. brucei* through binary fission. C: fluorescence microscopy of *in vitro* cultured bloodstream form *T. b. brucei* combined with M-lysercell and M-fluorocell reagents of the XN-31showing bright staining of the nuclei and kinetoplasts. The trypomastigotes remained intact when exposed to reagents of the XN-31.

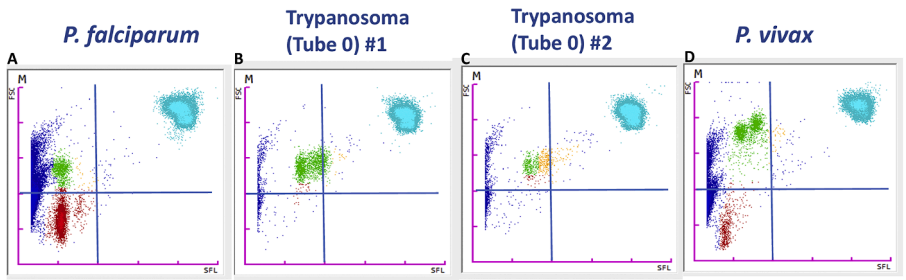

Fig. 2. Comparison of XN-31 scattergrams (FSC vs SSC) of *P. falciparum* (A), *Trypanosoma brucei brucei* from experiments 1 (B) and 2 (C), and *P. vivax* (D). *Trypanosoma* trypomastigotes were misannotated by XN-31 as *Plasmodium* gametocytes and trophozoites (green and yellow particles, respectively), but the scattergram pattern of trypomastigotes is very different from those of *P. falciparum* and *P. vivax*. Scattergrams with *T. b. brucei* show two distinct clusters that probably correspond to the trypomastigotes undergoing binary fission increasing SFL. FSC: forward scatter; SSC: side scatter.

and quantified *Trypanosoma* trypomastigotes. However, the fixed scattergram-interpretation algorithm of the Sysmex XN-31 is currently only developed for the detection of *Plasmodium*-infected erythrocytes, and therefore, by definition, it does not allow proper reporting of the presence of trypomastigotes. Based on the observed clearly distinct scattergram patterns of trypomastigotes compared to *Plasmodium*-infected red blood cells, it should be possible to properly discriminate these blood parasites. Examination of more trypomastigote-containing blood samples, including clinical specimens from patients suffering from human African trypanosomiasis, in combination with optimization of the interpretation algorithms of the XN-31 to ensure proper and specific gating and cluster ratio calculations, should then result in reliable identification and quantification of both *Trypanosoma* trypomastigotes from *Plasmodium*-infected red blood cells. In the meantime, in the event of trypomastigotes falsely identified as *Plasmodium* gametocytes and/or trophozoites, these scattergrams are deviant from those of regular malaria patients and these aberrant scattergrams should be noticed by the operator and trigger further investigation such as, for instance, manual microscopy.

The prepared blood samples containing trypomastigotes were also examined by a regular hemocytometer for routine diagnostics (Sysmex XN-1000). Surprisingly, the routine diagnostic setup did not detect any abnormalities. Analysis of the raw data obtained by the XN-1000 proved that the trypomastigotes were detected, but their forward-sideward scatter signal appeared in an area that is excluded for result interpretation (results not shown). Hence, the current settings of the Sysmex XN-1000 for routine diagnostics ignore the signal generated by trypomastigotes.

5. Conclusions

In conclusion, this study showed that the currently in use, fully automated fluorescence flow cytometry method for the detection, identification, and quantification of *Plasmodium* spp. infected red blood cells, can also detect bloodstream form trypanosomes (trypomastigotes) with a limit of detection of 26 trypomastigotes/μL. For clinical implementation, adaptation of scattergram interpretation algorithms is required after which validation by clinical specimens can be performed. Finally, the sensitivity of the current Sysmex XN-31 system for the detection of trypomastigotes is too low to detect infections with low parasitemia, and therefore, additional diagnostic methods (such as serological methods or microscopic methods in combination with concentration techniques) are still necessary to exclude human African trypanosomiasis [9]. However, the current settings of the XN-31 have been optimized for the detection of *Plasmodium* parasites, and therefore, its sensitivity is equivalent to parasite detection in a thick blood film. It can be expected that the sensitivity of the fluorescence flow cytometry method can substantially be improved if it is optimized for the detection of trypomastigotes by for instance enlarging the relatively small volume (60 μL) of examined blood.

Ethics approval

Ethical clearance for the use of residual blood samples for scientific purposes was granted by the Medical Ethics Review Board of the Erasmus MC, University Medical Center Rotterdam, the Netherlands (MEC 2012-047).

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Sysmex Europe GMBH provided free-of-charge reagents for the study. No monetary payments were made to any of the investigators.

CRediT authorship contribution statement

Tania Khartabil: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Ron HN van Schaik:** Writing – review & editing, Supervision. **Jurgen R. Haanstra:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Rob Koelewijn:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Henk Russcher:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Jaap J. van Hellemond:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. H Russcher reports equipment, drugs, or supplies was provided by Sysmex Europe GmbH. Sysmex Europe GMBH provided free of charge reagents for the study. No monetary payments were made to any of the investigators. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2024.116193.

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