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New promising method to assess microfilarial *Loa loa* load on the peripheral blood

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ABSTRACT

Loiasis is a vector-borne parasitic disease caused by the filarial *Loa loa* (*L. loa*). Definitive diagnosis can be done by identifying and counting microfilariae in the peripheral blood by microscopy and with *L. loa*–specific PCR. An additional diagnostic method is the detection of *L. loa*–specific antibodies. Accurate methods are needed to automate quantification of microfilaria (mf) in peripheral blood. Indeed, the treatment procedure depends on the microfilarial *L. loa* load in blood. We report the first documented use of flow cytometry as a new method to count microfilaraemia in peripheral blood from a patient with *L. loa* infection. The diagnosis of loiasis was strongly suspected based on clinical presentation and rapidly confirmed by identifying typical features of *L. loa* in the peripheral blood. This diagnosis was achieved by flow cytometry using a specific fluorescence pattern for microfilaraemia count. The current report highlights the potential of flow cytometry to assess microfilarial *L. loa* load from a patient with loiasis infection.

1. Introduction

Loiasis is a vector-borne parasitic disease caused by the filarial nematode *Loa loa* (*L. loa*) and transmitted by the *Tabanid* vectors from the genus *Chrysops*. Epidemiological data collected from 11 African countries indicate that at least 10 million people are infected.(Emukah et al. 2018) Most of the *L. loa* carriers have symptomatic episodes in their medical history such as "Calabar swelling" or "eye worm". (Carme et al. 1989; Noireau et al. 1990) These symptoms are not permanent. Therefore, most infected people are asymptomatic at a given time. Disease manifestations are usually due to hypersensitivity reactions to the adult parasite migrating through subcutaneous tissue. Intense itching, urticaria, and elevated eosinophil counts may be seen during the course of illness.(Ali et al. 2008)

Occurrence of serious reactions to the treatment with diethylcarbamazine citrate (DEC) and ivermectin (IVM) drugs is related to the amount of *L. loa* microfilaraemia. The relative risk of developing marked or serious reactions is significantly higher when the *L. loa* load exceeds 8000 mf per mL and is particularly notable in patients with *L. loa* microfilarial density greater than 20,000 mf per mL.(Fain 1978; Gardon et al. 1997; Boussinesq et al. 2003; Ali et al. 2008; Chesnais et al. 2017; Herrick et al. 2017) Treatment of *L. loa* infection remains problematic. In hypermicrofilaraemic *L. loa*/baboon (*Papio anubis*) treated with IVM, it has been shown that *L. loa* encephalopathy may occur due to a vasculopathy associated with death and degeneration of microfilariae in the blood vessels of the brain.(Wanji et al. 2017)

Once a diagnosis made, it is imperative to effectively reduce the microfilarial *L. loa* load in blood. Accurate methods are needed to assess microfilarial *L. loa* load in peripheral blood as it is necessary for treatment decisions. The treatment strategy depends firstly on the risk of adverse events, which is related to the patient's *L. Loa* microfilarial density. The latter must mandatorily be quantified before any therapy decision. DEC and IVM can induce potentially fatal encephalopathy in persons harboring >30,000 mf per mL of blood (Ali et al. 2008). So consequently, a treatment strategy in four points was proposed by Boussinesq: 1) If the patient's *L. loa* microfilarial density is below 2000 mf per mL, DEC can be administered straight away (preferably in a hospital and together with oral antihistamines or corticosteroids). 2) In patients with *L. loa* microfilarial density between 2000 and 8000 mf per mL, it is advisable to begin treatment using IVM (a single dose of 150 μg/kg). Mild side effects similar to those observed with DEC can occur. 3) When the patient's *L. loa* microfilarial density is between 8000 and 30,000 mf per mL, IVM can also be given, but a close surveillance is needed,

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with hospitalization during the first 3–4 days. 4) If the patient's *L. loa* microfilarial density exceeds 30,000 mf per mL, albendazole (ALB) is probably the best option. Apheresis, which rapidly reduces microfilarial *L. loa* load by 75% after 3 sessions, have also been proposed.(Boussinesq 2012)

We present a rapid flow cytometry method allowing automated quantification of microfilariae in whole blood from patient with loiasis infection.

2. Case report

A 61-year-old Cameroonian male came to France and was hospitalized to explore a lytic lesion of the right humerus. CT scan showed a fracture of surgical neck of right humerus with numerous osteolytic lesions. Laboratory findings revealed bicytopenia moderate anemia (hemoglobin 9.8 g/dL) and leucopenia (white blood cell count 3.15×10^9/L), hypercalcemia 2.72 mmol/L, hyperprotidemia 129 g/L. Serum protein electrophoresis showed hypergammaglobulinemia, and serum protein immunofixation electrophoresis demonstrated a monoclonal lambda light chain IgG peak (>50 g/L). Bone marrow examination by light microscopy and immunophenotyping confirmed the diagnosis of multiple myeloma IgG light chain lambda. The patient reported a history of Calabar swelling, which was a strong clinical presumption for loiasis diseases. Therefore, venous blood testing by light microscopy was performed, which confirmed the presence of 1510 mf per mL. The microfilariae seen in blood smears stained with Giemsa stain showed typical features of *L. loa* (Fig. 1**, Panel A**). Microfilaraemia was then assessed by flow cytometry (AccuriC6, BD Biosciences, Le Pont de Claix, France) as described in the supplementary data. The analysis of blood sample by flow cytometry based on the forward and the side scatter delineated 2 populations which were identified to be: P1) leucocytes and P2) *L. Loa* microfilariae (Fig. 1**, Panel B1**). The same analysis based on the fluorescence pattern using anti-human immunoglobulin G coupled with Alexa-Fluor 568 [Life Technologies] and SYBR green for DNA labeling also delineated 2 distinct populations which were identified to be: 1) leucocytes and 2) *L. Loa* microfilariae (Fig. 1**, Panel B2**).

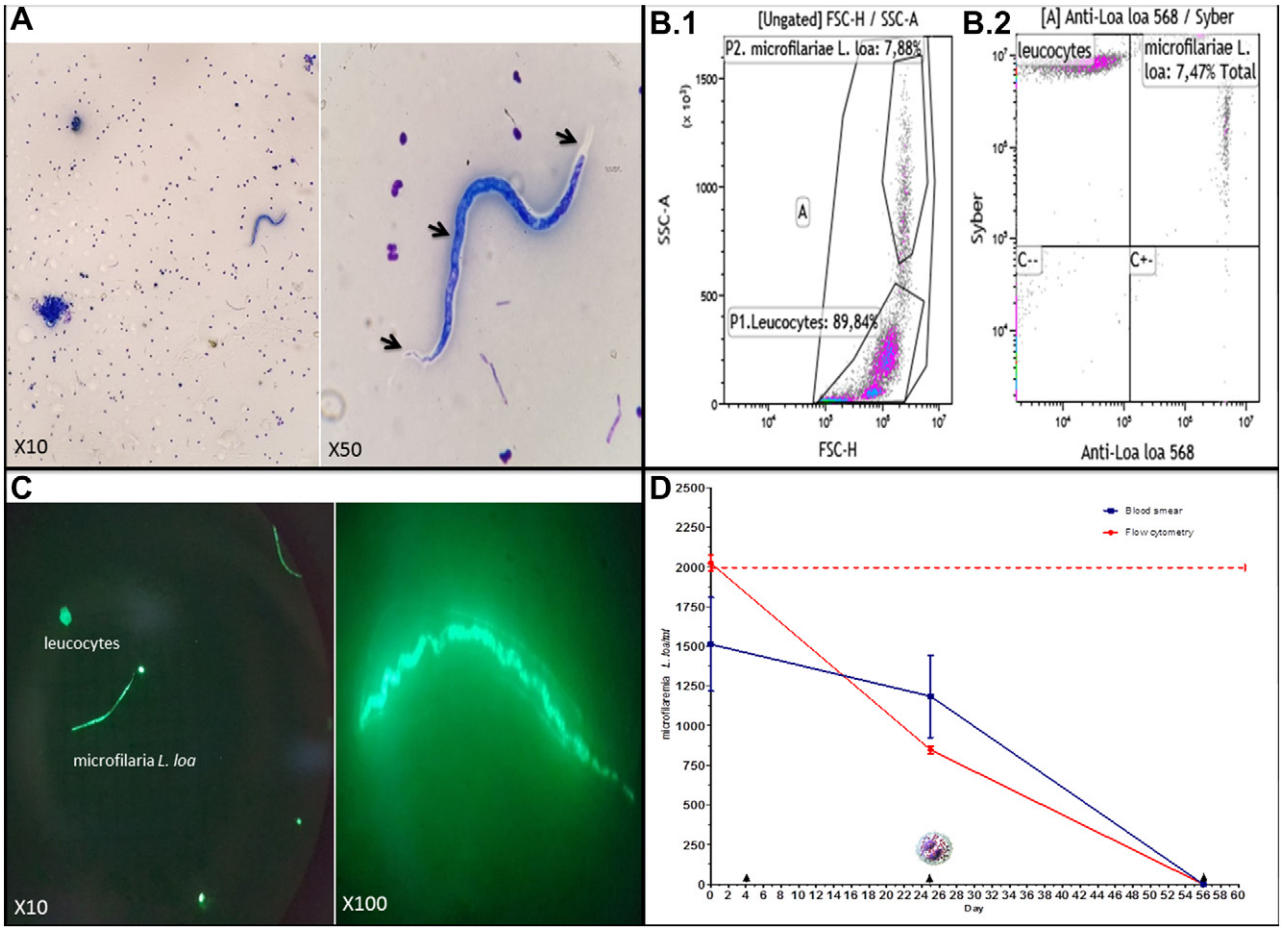

Fig. 1. Biological diagnosis of loiasis (Panel A, B, C, D). A. Typical features of *Loa loa on smear Giemsa staining*. *Loa loa (L. loa)* microfilariae in the blood (magnification: ×10, ×50). The resuspended pellet was smeared on a slide and stained with Giemsa reagent. Arrows indicate details of the cephalic space (1), the unstained sheath (2), and details of the nuclei extending into the tip of the tail (a typical feature of *L. loa*) (3) **B. Flow cytometry pattern. B1.** Analysis flow cytometry represented on Dot Plot based on the forward scatter (FSC) and the side scatter (SSC) delimitated 2 populations identified to be P1) leucocytes (granulocytes, monocytes, lymphocytes) and P2) *L. Loa* microfilariae (high forward scatter and high side scatter). **B2.** Analysis flow cytometry represented on Dot Plot based on the fluorescence using anti-human immunoglobulin G coupled with Alexa-Fluor 568 [Life Technologies] and SYBR green for DNA labeling also delimitated the 2 populations identified to be 1) leucocytes as a population (DNA positive) and 2) *L. Loa* microfilariae as a double positive population (DNA positive and anti-human immunoglobulin G anti *L. loa* positive) **C. Immunofluorescent assay (IFA).** *L. loa* microfilariae in blood (magnification: ×10, ×50) observed on IFA slides: Images were acquired by using a Leica DMI3000 microscope and Leica camera (Leica Microsystemes SAS, Nanterre, France) **D. Comparison of kinetics of the count of microfilaremia measured by flow cytometry versus smear microscopy.** *L. Loa* microfilariae counts are expressed in mean with the corresponding standard deviation as absolute mf per milliliter. Day 0 represents the positive diagnostic pretreatment microfilariae level in the blood [2025 (SD: +/−50) mf per mL flow cytometry; 1510 (SD: +/−300) mf per mL smear microscopy]. Day 4 was the first day after the patient received treatment with ivermectin. By day 25, a 62% decrease in microfilariae level still had occurred by flow cytometry [847 (SD: +/−23) mf per mL] versus 23% compared to the count obtained by smear microscopy [1185 (SD: +/− 260) mf per mL]. Day 27 was the first day after the patient received treatment with diethylcarbamazine citrate and corticosteroids. One month later (day 57), blood analysis confirmed a total clearance of microfilariae was observed.

This result was confirmed by an immunofluorescent assay (IFA) using a Leica DMI3000 microscope (Leica Microsystems SAS, Nanterre, France) (Fig. 1, **Panel C**).

On admission, microfilaraemia of the patient was 2025 mf [standard deviation (SD): +/−50] per mL by flow cytometry and 1510 (SD: +/−300) mf per mL by smear microscopy. On day 4, the patient was treated with a single dose of 200 μg/kg IVM. On day 25, flow cytometric analysis of blood showed a microfilaraemia decrease of 58% from admission [from 2025 (SD: +/50) to 847 (SD: +/−23) mf per mL] and 22% only by the count obtained by smear microscopy [from 1510 (SD: +/−300) to 1185 (SD: +/−260) mf per mL]. As expected, besides this decreasing of microfilaraemia, a hypereosinophilia (0.7 giga/L) occurred. Then, on day 25, the patient was treated with DEC administered in incremental doses with 50 mg on day 1 followed by 50 mg 3 times per day for the next 3 days. The dose was gradually increased to 200 mg 3 times a day to complete 21 days of therapy associated with corticosteroids (Prednisone®) 40 mg/d for 3 days. One month later, flow cytometric analysis of blood confirmed a total clearance of *L. loa* microfilariae, with satisfactory clinical evolution and no adverse event (Fig. 1, **Panel D**).

3. Case discussion

Definitive diagnosis of loiasis can be done in most cases by morphological identification of the microfilariae in blood smears. Unique morphologic characteristics help differentiate this organism from other blood-borne microfilariae of *Wuchereria bancrofti* and *Mansonella perstans*, whose geographic distribution can overlap that of *L. loa* in Central Africa. Molecular methods such as loop-mediated isothermal amplification and quantitative PCR assays are alternatives to microscopy-based techniques. However, molecular methods remain impractical for rapid testing. Proteomic and immunological analyses of *L. loa*–infected human samples have identified *L. loa*–specific biomarkers, but it has not yet been applied for routine care testing. Finally, a portable smartphone-based microscope (LoaScope) has been developed to simplify and accelerate the counting of *L. loa* microfilariae. (Metzger and Mordmüller 2014; Pedram et al. 2017) Accurate methods are needed to assess microfilarial *L. loa* load in the peripheral blood as it is necessary for treatment decisions.

Flow cytometry has emerged as a powerful tool for counting and for classifying cells. The sensitivity of detection was 10^{-5} (1 positive cell/100,000 cells); average speed of acquisition and analysis on a cytometer was 1000 to 10,000 cells per second and, based on multiple parameters, it provides a result within a few seconds. The statistical significance of the count is much greater than that conventionally obtained by light microscopy. Moreover, the counting of parasite on smear microscopy is a nonautomated method, which may lack reproducibility and requires a trained technician. (Gaipa et al. 2018)

Flow cytometry offers the option to quantify *L. loa* microfilariae. Even if technologies are available to selectively label specific types of microorganisms, flow cytometry has not been extensively used as a tool for routine microbial analysis.(Álvarez-Barrientos et al. 2000; Shapiro 2001; Lloyd 2019) This has been mainly due to the high cost and complexity of equipment. The cost of blood examination by flow cytometry based on our laboratory experience is less than 10 euros. However, because flow cytometers have a wide spectrum of applications in medical biology, prices are dropping and cheaper versions are emerging. (Veal et al. 2000) On the other hand, the present approach is to assess the potential use of flow cytometry to quantify microfilaraemia in peripheral blood to facilitate rapid treatment decisions in loiasis.

The current report highlights the value of flow cytometry as a promising method for measuring microfilarial *L. loa* load consistent with the reference method (saponin hemolysis and counting by light microscopy) from a patient with loiasis infection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.114887.

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