

Comparison of Three PCR-Based Methods to Detect *Loa loa* and *Mansonella perstans* in Long-Term Frozen Storage Dried Blood Spots

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Abstract

Background: *Loa loa* and *Mansonella perstans* are two filarial species very common in Africa, with overlapping geographic distribution in some areas. Microscopy is the traditional diagnostic method for human loiasis and mansonellosis, but is a time-consuming, labor intensive and tedious. Polymerase chain reaction (PCR) methods have emerged as an alternative approach for identification of filarial parasites. Dried blood spot (DBS) has been reported as a convenient way to keep DNA for epidemiological investigations and diagnosis of infectious diseases, and does not require venipuncture. The finding of a highly sensitive DNA extraction method for filarial nematodes is also required for a good molecular performance. The aim of this study was to compare three different molecular methods to diagnose human loiasis and mansonellosis using DBS as a medium of sample collection and storage. The saponin/Chelex method for extracting filarial DNA was also applied.

Methods: A total of 100 clinical samples were randomly selected for this study. Microscopy was used as the reference method for diagnosing and calculating the microfilaraemia. Filarial DNA was extracted using saponin/Chelex method from DBS. DNA isolated was assayed by three different molecular methods: qPCR, Filaria-Nested PCR, and cytochrome oxidase I PCR. All PCR-products were subsequently purified and sequenced. Statistical values for each molecular test were computed and compared.

Results: Overall, 64 samples were identified as negative by all the tests and 36 samples were positive by at least one of the methods tested. Microscopy detected 27 positive samples, meanwhile qPCR, Filaria-Nested PCR and COI PCR detected 30, 31 and 33 positive samples, respectively. The best overall results were obtained with COI PCR protocol (sensitivity 92.6%; specificity 89.0%; kappa index 76.3%).

Conclusions: Despite the good statistical values obtained for COI PCR, this method needs the sequencing of the fragment obtained to identify the filarial species; thus the optimal technique to diagnose filarial infection was qPCR, it was very similar in terms of sensitivity and specificity compared to microscopy and for its capacity to detect a wide range of human filariae. It is an appropriate method for filarial diagnosis in non-endemic settings.

Background

Human filariasis is a parasitic disease caused by nematodes with different tissue locations of adults depending on the species: subcutaneous (*Onchocerca volvulus*, *Loa loa*, *Mansonella streptocerca*, and *Mansonella ozzardi*), lymphatics (*Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*), and body cavity (*Mansonella perstans*) [1–4]. These filarial species are mainly anthroponotic; the causative agent reaches sexual maturity in the human host. Filariae have a remarkable specificity for their definitive mammalian host and obligate intermediate vector species [5].

This group of filarial nematodes has important social and economic impact, causing high morbidity and serious illnesses resulting in social stigmatization, marginalization, and loss of work for the afflicted [6]. Among all human filarial species, two of the most predominant species in Africa are *L. loa* and *M.*

perstans [7, 8], on which this study has focused *Loa Loa*, the parasite that causes loiasis (also known as African eye worm) [9], is endemic in eleven countries of Africa according to the Rapid Assessment Procedure for Loiasis (RAPLOA): Angola, Cameroon, Central African Republic, Chad, Congo, Democratic Republic of the Congo, Equatorial Guinea, Ethiopia, Gabon, Nigeria and Sudan [7, 10, 11]. Ten countries have areas where there are high rates of infection (more than 40% of the people who live in that area report that they have had eye worm in the past). An estimated 14.4 million people live in these areas of high rates of infection. Another 15.2 million people live in areas where 20–40% of people report that they have had eye worm in the past [7].

In several endemic areas, loiasis co-exists with onchocerciasis and lymphatic filariasis (LF), both classified as neglected tropical diseases (NTD) by the World Health Organization (WHO) [12]. The increasing importance of *L. loa* began when severe adverse events (SAE) presenting as encephalopathy following treatment with ivermectin occurred in areas where loiasis is co-endemic with onchocerciasis and/or LF, especially in very high microfilarial loads (≥ 30000 microfilariae per millilitre (mf/mL) [9, 10].

Mansonella perstans is considered the most common of the mansonellosis parasites and is endemic in a large portion of sub-Saharan Africa, as well as a northern part of the Amazon rainforest stretching from equatorial Brazil to the Caribbean coast of South America. Overall, more than 100 million people may be infected by *M. perstans*, and it is estimated that 600 million people live at high risk of contracting an infection in Africa alone [8, 13]. *M. perstans* is co-endemic with *L. loa* in some African areas, like Cameroon, Equatorial Guinea or Democratic Republic of the Congo [9, 14].

Programmes to control or eradicate filarial infections need accurate diagnostic tools to be efficiently performed. Highly-sensitive methods are required to detect low microfilariae (mf) densities in blood and stringent specificity is required to differentiate species of mf that may coexist in one region or colonize the same tissues, such as *L. loa*, *M. perstans* and *W. bancrofti*, which have an overlapping geographic distribution and whose life cycles also include bloodstream mf, for an accurate assessment of prevalence and incidence in intervention programs [10].

Conventionally, laboratory diagnosis of human loiasis and mansonellosis depends on detection of mf on Giemsa-stained thick and thin blood films by microscopy requiring a properly collection and processing of blood specimens for a reliable diagnosis [14]. Basing on morphological descriptions (size and shape of the tail, presence or absence of a sheath, and the arrangement of terminal nuclei) mf of *L. loa* are unlikely to be confused with *M. perstans*, since *L. loa* mf are longer and thicker besides the presence of a sheath and *M. perstans* mf are smaller and thinner, sheathless, and their terminal nuclei are bigger than *L. loa* microfilariae [2, 3, 14, 15]. However, microscopy is a time-consuming, labor intensive and tedious method. The efficacy of mf detection is further decreased by the long pre-patency, the periodicity in the case of *L. loa* mf and mild or occult infection where the load of mf parasites is undetectable microscopically [1, 3].

DNA technology has provided an alternative approach for identification of the filarial parasites, like polymerase chain reaction (PCR) methods with high accuracy to detect single or mixed infections. In the

last years both conventional and real-time or quantitative PCR (qPCR) assays have been developed for detecting *L. loa* and *M. perstans* pathogens as a worthy alternative diagnostic method to microscopy [16–20]. These methods could easily diagnose infection by filariae even if the parasite could not be detected microscopically. These techniques have proved to be able to detect DNA down to the level of a single parasite.

Many of the optimized molecular methods used to diagnose human filariae *L. loa* and *M. perstans* employ venous blood samples [16–20], which must be adequately collected, immediately freeze-stored to guarantee high-quality PCR results and transported to special laboratories that provide such diagnostic services. This process requires regular health service structures and electricity, which are not always available in remote endemic areas.

For larger human filarial epidemiologic studies in endemic areas, improvements are needed in the sample collection and filarial DNA extraction techniques. The application of capillary blood collection on filter paper, known as dried blood spots (DBS), by the end of the 1990s has been reported as a convenient way to keep DNA for epidemiological investigations and diagnosis of infectious disease. This simplified method of collection and storage, cheaper, practical, safe, and does not require venipuncture has significantly contributed to disseminate the molecular analysis of various infectious pathogens in endemic countries [21–26]. Regarding DNA extraction, the finding of a highly sensitive DNA extraction method for filarial nematodes from DBS is critical for a good molecular performance.

The aim of this study was to compare three different molecular methods to diagnose human loiasis and mansonellosis using DBS as a medium of sample collection and storage. The saponin/Chelex method for extracting filarial DNA was also applied.

Materials And Methods

DBS samples were obtained from the Laboratory of the National Centre of Tropical Medicine's collection (Institute of Health Carlos III), registered according to the Spanish Law RD 1716/2011 (article 22. 1). DBS samples had been stored in double zip-lock plastic bags with silica gel absorbent at – 20 °C for seven years and came from West and Central Africa. DBS selected for the study derived from a finger prick which had been blotted onto Whatman 903™ paper (GE Healthcare Bio-Sciences Corp.).

The blood smears from the collection had been stained with 10% Giemsa solution. A total of 100 samples of this collection were randomly selected for our study.

Microscopy: The thick and thin blood smears were utilized to detect mf and calculate the microfilaremia. Morphological identification for mf was performed in line with published guidelines [15] and examined by an expert microscopist from the Laboratory of the National Centre of Tropical Medicine. Mf densities were expressed as microfilariae per milliliter of blood (mf/mL) under at 10x magnification and to determine the filarial species at 100x magnification with immersion oil. All fields were examined before

declaring a slide negative. Mf densities were the average value found between the thick and thin films by microscopic examination.

DBS samples: The DBS were selected once we had the microscopic diagnosis. Positive and negative controls were prepared using DBS from infected and uninfected persons respectively, which was previously determined by microscopy on blood smear.

DNA extraction: DNA was extracted from DBS by one of the most frequently used protocol, the saponin/Chelex method, with slight modifications from the original protocol [27]. Two filter paper discs of 5 mm in diameter were punched from the center of a circle using handheld hole puncher. Filter paper discs were immediately placed into a 1.5 mL-tube, added 1 mL of 0.5% saponin (Fluka Biochemika. Sigma-Aldrich Chemie GmbH) in autoclaved phosphate-buffered saline (PBS) 1x, mixed thoroughly 2–3 times and incubated overnight at 4 °C or at 37 °C 1 hour. The brown solution was aspirated and replaced with 1 mL of PBS 1x autoclaved; the tube was incubated for 30 minutes at 4 °C. During this step, 100 mL of a solution of 5% Chelex-100 (Bio Rad, Richmond, CA) in water was heated at 100 °C in a magnetic stirrer. After aspiration of the PBS 1x, 200 µL of hot Chelex were added to the two paper discs, vortexed at high speed for 30 s, and placed in the heatblock at 98 °C for 10 min with a brief vortex again once during and once after the incubation. After centrifugation at full speed (13,000 rpm) for 2 min, the supernatant was carefully recovered so as not to take any Chelex. The isolated DNA was centrifuged again at full speed under the same conditions to remove any remaining Chelex and collected into a new tube. Supernatants were used for any DNA-based technique immediately or stored at -20 °C for future analysis.

Filter paper samples should be handled according to safety procedures to prevent contamination of samples with each other by disinfecting the handle punch in the NaOH 5 M solution.

Previously to molecular testing, the frozen DNA samples were thawed and centrifuged at 13,000 rpm for 2 min to place the remaining Chelex at the bottom of the tube. Chelex-100 is a chelating resin with a high affinity for multivalent metal ions. This can inhibit the PCR by binding to metal ions of Mg^{2+} [23].

Molecular methods: the presence of filarial parasites in DNA isolates was subsequently assayed targeting different genes by three different PCR-based methods previously described to diagnose filarial infections as briefly indicated

- Quantitative PCR (qPCR): targeting the internal transcribed spacer one (ITS1) of the nuclear ribosomal gene of all filarial species [16]. In this study the annealing temperature was changed by 48°C and the PCR mixture used was Luna® Universal qPCR Master Mix 2x (New England Biolabs). Cycling conditions consisted of initial denaturation step at 95 °C for 1 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 48°C for 20 s and extension at 60 °C for 30 s. A sample was considered positive for filarial parasite if the melting temperature (T_m) curve of the amplified fragments was $T_m = 77 \text{ °C} \pm 0.5 \text{ °C}$ and the species identification was according to amplified product size performed in QIAxcel Advanced (automatic electrophoresis).

- Filaria-Nested PCR: designed from highly conserved regions of filarial 18S, 5.8S rDNA and ITS1 [17]. This method was performed according to the original protocol without modifications.
- COI PCR: designed on the basis of regions of mitochondrial cytochrome oxidase I (COI) gene conserved among the nematodes species and yield single size amplicons of 649 bp [19]. The original protocol was followed except that the volume final was changed 20 μ L by 50 μ L and quantity of template used was 5 μ L.

Negative and positive clinical samples used as controls were always run in each reaction. Each sample was tested in duplicate.

For a more detailed overview of primer sequences, target gene, product size and other characteristics of the assay are described in Table 1.

All qPCR reactions were performed using a Rotor Gene Q 5plex (QIAGEN GMBH, Germany). The rest of DNA amplifications were performed on an Applied Biosystems GeneAmp®PCR System 2700. Amplification products were detected by QIAxcel Advanced (QIAGEN GMBH, Germany), an analysis automated system of DNA fragments based on multicapillary electrophoresis.

DNA sequencing and sequence analysis: Amplified fragments were purified and sequenced to confirm the nucleotide sequence and the size of the fragment. All the products amplified by PCR techniques were purified using Speedtools PCR Clean Up Kit 250 rxns (Biotools, B&M Lab, SA) then sequenced using Big Dye Terminator v3.1 Cycle Sequencing in an ABI PRISM® 3700 DNA Analyzer, and were sequenced in both directions. Sequences obtained were compared to the genbank database using BLAST (Basic Local Alignment Search Tool) [28] and aligned using CLUSTAL W [29] to confirm the diagnosis and detect possible mutations.

Statistical analysis: Statistical values (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and kappa index) for each DNA amplification test compared to microscopy as reference method were calculated using the free software WinEpi: Working in Epidemiology [30]. The confidence intervals (CI) were established at 95%. Statistical values were calculated for the two filarial species together, although the three methods allowed differentiating both species.

Results

Overall, 64 samples were identified as negative by all the tests and 36 samples were positive by at least one of the methods tested (microscopy or molecular test). A list of only positive samples has been created to facilitate the following and understanding of the results (Table 2).

Microscopy: out of 100 thick and thin blood smears analyzed by microscopy, 73 (73%) were negative and 27 (27%) were positive: 13 were *L. loa*, 11 *M. perstans* and 3 were mixed infections by *L. loa* + *M. perstans*. The average microfilaremia for *L. loa* was 2766 mf/mL (parasite count range 200 – 12,200

mf/mL) and for *M. perstans* was 764 mf/mL (parasite count range 100-3,200 mf/mL) (Tables 2 and 3) (Fig. 1).

qPCR: this method was able to detect 30 (30%) positive samples: 11 *L. loa*, 17 *M. perstans* and 2 mixed infections. The remaining samples (70%) were negative. 7 *M. perstans*-qPCR positive samples were missed by microscopy, meanwhile 4 microscopy positive samples were missed by qPCR (2 *L. loa*, 1 *M. perstans* and 1 mixed infection) (Tables 2 and 3).

Filaria-Nested PCR: this nested PCR detected 31 (31%) positives: 8 *L. loa*, 15 *M. perstans* and 8 mixed infections. 69 samples (69%) were Filaria-Nested PCR negative. 8 samples were Filaria-Nested PCR positives (5 *M. perstans* and 3 mixed infections) but negatives by microscopy. On the other hand, 4 microscopy positive samples were missed by Filaria-Nested PCR (2 *L. loa*, 1 *M. perstans* and 1 mixed infection) (Tables 2 and 3).

COI PCR: this single PCR characterized 33 (33%) positive samples, which after sequencing turned out to be 14 *L. loa* and 19 *M. perstans*. 67 (67%) samples were negative. 8 *M. perstans*-COI PCR positives were not detected by microscopy, meanwhile two *L. loa*-microscopy positives were missed by COI PCR (Tables 2 and 3).

Of the 13 *L. loa*-microscopy positive samples, there were only two *L. loa*-microscopy positive samples (average microfilaremia was 400 mf/mL) which were not detected by any of the molecular assays. Regarding to *M. perstans*, 11 samples were *M. perstans*-microscopy positive, COI PCR was in total concordance to microscopy to detect *M. perstans*. Only one *M. perstans*-microscopy positive was missed by qPCR (the missed sample had 100 mf/mL) and another one by Filaria-Nested PCR (this one had 200 mf/mL).

Three mixed infections by *L. loa* + *M. perstans* were detected by microscopy, two of them were also detected by both qPCR and Filaria-Nested PCR. The only one mixed infection sample which was not detected by the pan-filarial methods was detected by COI PCR as *L. loa* mono-infection.

Statistical analysis results: The sensitivity and specificity were similar among the different molecular methods (range of sensitivity: 85.2%-92.6%; range of specificity: 89.0%-90.4%, Table 4). COI PCR showed the highest sensitivity (92.6%) and qPCR had the best specificity (90.4%). All the molecular methods demonstrated a good agreement respect to microscopy (kappa index range from 70.9% to 76.3%); the best concordance (kappa index: 76.3%) was for COI PCR method (Table 4).

Discussion

In this study, three molecular methods [16, 17, 19] capable of simultaneously detecting and identifying *L. loa* and *M. perstans* parasites was selected and compared to determine which of these three was the most convenient to use not only in resource-rich countries, where the relative infrequency of filarial infections limits the utility of conventional parasitology methods to confirm infection in immigrants and

travelers, but also in those low income countries having PCR technology available with minimal economic resources.

The molecular methods used were designed, optimized and validated using whole blood, which requires large quantities (100–500 mL), instead of DBS, where blood sample volumes are inevitably small (50–100 µL). Although our study has not made the comparison between whole blood and DBS, there are many previous works that have examined the use of DBS assays compared with recognized gold standards for the diagnosis and/or surveillance of infectious diseases, for both PCRs and serological assays, which support that similar sensitivities and specificities can be achieved with DBS instead of peripheral blood [31–34].

Some of the microscopy positive samples were missed by molecular methods: 4 samples were missed by qPCR and Nested-Filaria PCR, and 2 by COI PCR. These samples had a low load of mf. An important question to be considered is the distribution of mf on blood spot that is not homogenous and when the load of mf is very low, the possibility to take a punch with mf is lower. Parasite material seemed less concentrated towards the extreme edges of the blood spot, as it was shown by the experiment carried out by Baidjoe *et al.* in 2013 [35].

There was only one mixed infection sample that was not detected by the pan-filarial methods, but it was detected by COI PCR. However, it was not detected as mixed infection, but as *L. loa* mono-infection. This finding suggests that this sample could have been misdiagnosed as mixed infection by microscopy but we could not confirm it, as COI PCR amplifies a unique fragment and the sequencing is performed on the fragment at highest proportion [19].

It should be mentioned that qPCR, Filaria-Nested PCR and COI PCR detected seven, five and eight other *M. perstans*-positive samples respectively, which were not detected by microscopy. This demonstrated a greater sensitivity of the molecular methods compared to microscopy, as it was also shown in multiple previous studies when molecular methods were used [16–21].

In addition, the Filaria-Nested PCR was able to detect three more mixed infections. Mixed infections by *L. loa* + *M. perstans* are often underdiagnosed or are not recognized by microscopy because *M. perstans* mf are tiny and it is the smallest among the filarial species [15] and sometimes they go unnoticed [8].

In this work, DBS were used successfully to detect filarial infections yielding different performances for the three molecular assays, with sensitivities ranging from 85.2% (for qPCR and Filaria-Nested PCR) to 92.6% (for COI PCR) and specificities from 89.0% (for Filaria-Nested PCR and COI PCR) to 90.4% (for qPCR). However these values did not show high-quality results because of their sensitivity and specificity, which should be higher than 95% to be considered adequate for laboratory diagnostic tests, DBS PCR seemed to be a suitable alternative to whole-blood PCR. Smit *et al.* [33] reviewed the literature on the use of DBS for diagnosis of infectious diseases compared with recognized gold standards and they concluded that serological tests performed very well on DBS, with sensitivity and specificity close to

100%, but nucleic acid amplification tests performance were more variable because of the greater instability of nucleic acids, but mostly, it reached similar diagnostic accuracy [33].

It should be noted that DBS samples had been stored for 7 years. Although the samples were stored with desiccant and at -20°C, prior studies showed decreased PCR sensitivity for microscopically detectable infections after 4 and 7 years of storage and our findings were in agreement with these studies [22, 25, 36]. Despite this, DBS stored at -20°C with desiccant allows retrospective epidemiologic studies and the stability of filarial DNA onto paper filter has sufficient potential for practical DNA extraction in terms of cost, time, and sensitivity.

This study also presented a classical strategy to extract parasites DNA from DBS samples, the saponin 0.5%/Chelex-100 5% method, which is extensively used for isolating DNA of malaria parasites from DBS [22, 25, 36, 37], and in this study was used to isolate filarial DNA. The saponin/Chelex DNA extraction method using double filter paper punches has resulted in an effective procedure to obtain template yielding positive amplification for DBS samples long-term frozen stored. These results were consistent with those obtained in other studies [22, 31, 32, 35].

According to statistical results, filarial DNA extraction from DBS employing the saponin/Chelex-100 method followed by COI PCR was identified as the most sensitive strategy (92.6%) with a good specificity (89.0%). However, this method could not be a suitable method for routine diagnosis for filariae in terms of cost and time because the fragment size obtained is common in all nematodes and only after sequencing, the filarial species could be determined [19].

After evaluating all the three molecular assays, it has been found that the optimal technique to diagnose filarial infection was qPCR because of the good sensitivity (85.2%), high specificity (90.4%) and the good agreement compared to microscopy (73.3%), and for its capacity to detect a wide range of human filariae in a single round. As stated by the results, it could be a very useful tool for the diagnosis of human filariasis. However, qPCR has the disadvantage that requires expensive equipment, laboratories with good infrastructure, reliable electrical supply and highly trained staffs besides a long amplifying process (2 hours). Because of these reasons, qPCR assay is not the most suitable method for application in filarial-endemic countries, although it could be for developed countries [16].

Filaria-Nested PCR was also a pan-filarial method like qPCR, with good statistical values (sensitivity 85.2%, specificity 89.0%) and good agreement (70.9%) comparing to microscopy. It was also a reliable technique to detect mixed infections (8 mixed infections by Filaria-Nested PCR vs 3 mixed infections by microscopy). Despite all this, Filaria-Nested PCR is not the best method to do filarial diagnosis because this test requires two rounds of PCR, which often leads to risks of contamination, and is more time consuming (4 hours).

Conclusions

In this study, qPCR results were similar to microscopy in terms of sensitivity and specificity to detect filarial parasites. Thus, qPCR is an appropriate method for filarial diagnosis in non-endemic settings but impractical if a rapid diagnosis of patients in endemic areas is required.

Blood samples spotted on filter paper along with saponin/Chelex 100 as a filarial DNA extraction method resulted in a proper combination way for filarial field epidemiologic studies from endemic countries. This method is a simple, practical and low-cost way to collect and store field samples and also a cost-effective and high performance approach for DNA extraction.

Abbreviations

BLAST: Basic Local Alignment Search Tool; bp: basepairs; °C: degrees Celsius; CI: confidence intervals; COI: cytochrome oxidase I; DBS: dried blood spot; DNA: Deoxyribonucleic acid; ITS1: internal transcribed spacer one; LF: lymphatic filariasis; mf: microfilariae; mL: milliliter; mm: millimeter; min: minutes; NPV: negative predictive value; NTD: neglected tropical diseases; PBS: phosphate-buffered saline; PCR: Polymerase Chain Reaction; PPV: positive predictive value; qPCR: real time PCR or quantitative PCR; rDNA: ribosomal deoxyribonucleic acid; WHO: World Health Organization; RAPLOA: Rapid Assessment Procedure for Loiasis; s: seconds; SAE: severe adverse events; T_m: melting temperature; µL: microliter.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data related to the present study are available in the manuscript.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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Authors' contributions

THTT, PB, JMR conceived and designed the research. THTT, BFS, DA performed the acid nucleic isolation and molecular techniques. THTT, PN, DA, AB carried out microfilariae microscopic diagnosis. THTT, BFS, PFS performed data processing and analyses. MRB analyzed and interpreted statistical data. THTT, BFS, PB, JMR drafted the manuscript with input from all other authors. AM, AB, ZH, PFS, MRB critically revised the manuscript for intellectual content. All authors reviewed and approved the final manuscript prior to submission.

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Tables

Due to technical limitations, table 1 to 4 xlsx are only available as a download in the Supplemental Files section.

Figures

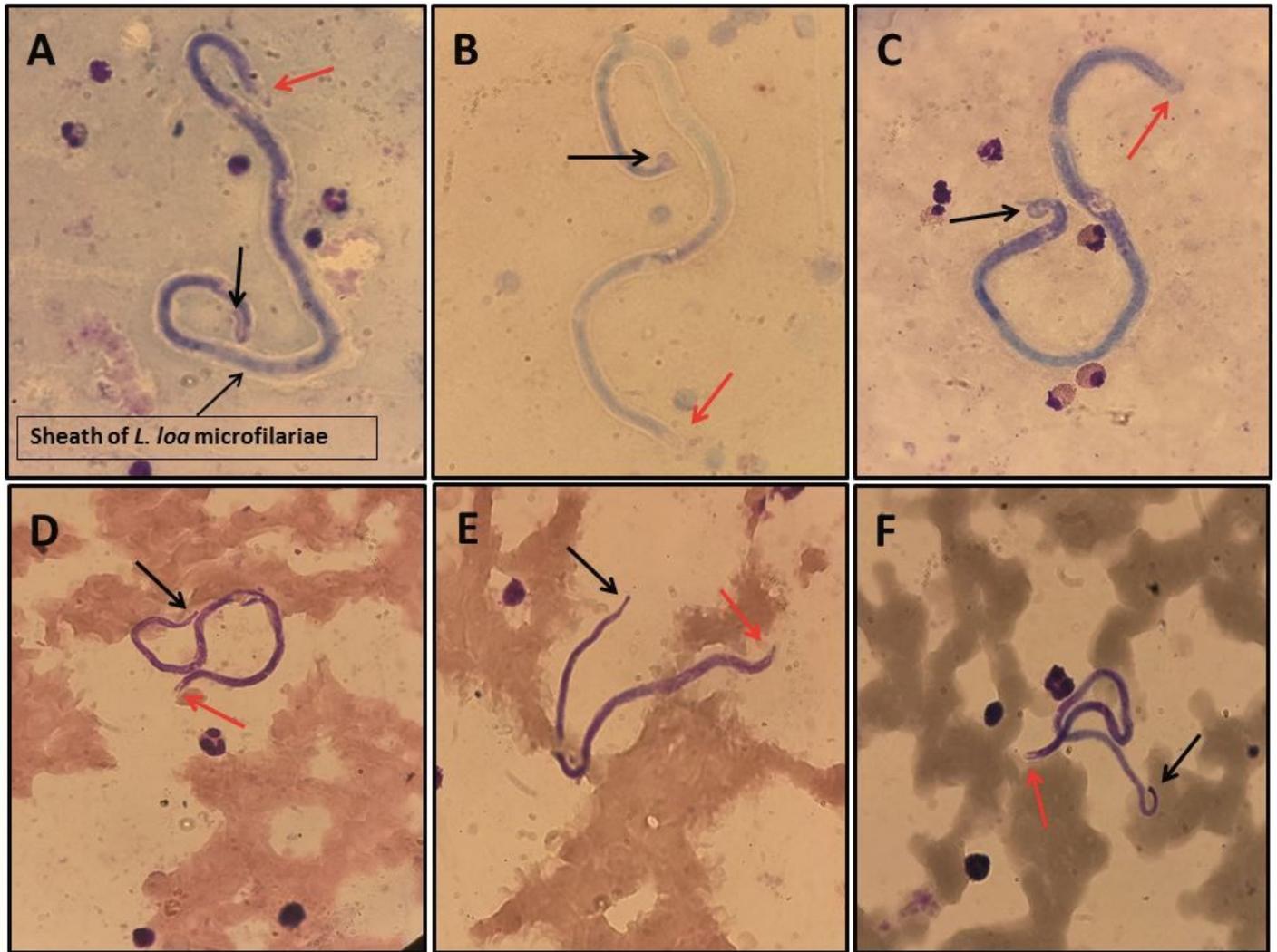


Figure 1

Giemsa-stained blood films. Red arrow points microfilariae head and black arrow marks the tail. A, B, C thick blood films containing *L. loa* microfilariae: sheath unstained by Giemsa, long cephalic space, nuclei reach the tip of the tail, long and attenuated tail. D, E, F thin blood smears with *M. perstans* microfilariae: no sheath, short cephalic space, nuclei reach the tip of the tail, short and blunt tail.

Supplementary Files

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