Pan filarial-nested PCR with gelified tubes: a new approach towards point-of-care testing for human filariasis

Raquel Capote-Morales[1,2], Agustín Benito[1,3], Pedro Berzosa[1,3], Vicenta González[1], Luz García[1], Alba Sastre-González[4], Akeem Abiodun Akindele[5,6], Olusola Ojurongbe[6], Thuy-Huong Ta-Tang[1,3]/+

[1]Instituto de Salud Carlos III, National Centre of Tropical Medicine, Laboratory for Scientific-Technical Cooperation in Tropical Diseases, Madrid, Spain
[2]Complutense University of Madrid, Faculty of Biological Sciences, Madrid, Spain
[3]Biomedical Research Networking Centre of Infectious Diseases, Madrid, Spain
[4]Alcalá University of Madrid, Faculty of Sciences, Alcalá de Henares, Spain
[5]Ladoke Akintola University of Technology, Medical Laboratory Science Department, Ogbomoso, Nigeria
[6]Ladoke Akintola University of Technology, HRH-Centre for Emerging and Re-Emerging Infectious Diseases, Ogbomoso, Nigeria

BACKGROUND In recent years, traditional point-of-care tests (POCTs) for diagnosing human filariasis have demonstrated substantial limitations, raising concerns regarding their reliability in the current epidemiological context, in which numerous countries are approaching the elimination of human filariasis. Consequently, there is a need for new molecular tests that allow the simultaneous and reliable detection of multiple human filarial species, even in cases of very low microfilaremia.

OBJECTIVES The objective of this study was to convert the *Fn-PCR* from its original manual format to the new *gelified tube-filarial-nested polymerase chain reaction* (*Gel-Fn-PCR*), which overcomes some of the disadvantages of traditional molecular techniques thanks to its gelified tube format.

METHODS A total of 661 dried blood spots, along with their corresponding stained blood smears, were employed for the optimisation and validation processes. The results of the *Gel-Fn-PCR* were compared to the microscopic diagnosis.

FINDINGS The *Gel-Fn-PCR* demonstrated good sensitivity and specificity for single-species infections (91.6% and 83.0% for *Loa loa*, and 95.0% and 97.7% for *Mansonella perstans*, respectively). However, for mixed infections, the sensitivity was low (43.9%), while specificity remained high (99.0%).

MAIN CONCLUSIONS The assay proved to be effective for diagnosing single-species infections, even in cases with very low microfilaremia; however, its performance in identifying mixed infections was reduced, particularly when one species was present in much higher abundance than the other. Although not yet feasible as a fully deployable POCT, this study demonstrates the potential of integrating gelification with PCR for human filariasis diagnosis.

Key words: nested PCR - gelified tubes - point-of-care test - human filariasis - *Loa loa* - *Mansonella perstans*

Diagnostic tools are essential in the management and control of neglected tropical diseases (NTDs). Accurate and effective diagnostic testing facilitates the timely identification of cases and treatment, as well as critical activities such as disease mapping, population screening, and epidemiological surveillance.(1) These efforts are vital for accelerating progress toward the elimination of NTDs.

Currently, the commercially available point-of-care tests (POCTs) are immunoenzymatic tests that detect either circulating antibodies, such as Brugia Rapid Test (Reszon Diagnostics International Sdn. Bhd., Selangor, Malaysia) for detecting antibodies specific to *Brugia malayi* and/or *B. timori*, and Bioline™ Onchocerciasis IgG_4 rapid diagnostic test (Abbot, Lake Forest, IL, USA) for detecting IgG_4 antibodies to *Onchocerca volvulus* Ov16 antigen, or circulating antigens, like the BinaxNOW Filariasis test kit (Abbott, Chicago, IL, USA), Bioline™ Filariasis Test Strip (FTS) (Abbott, Chicago, IL, USA), and STANDARD Q Filariasis Antigen Test (QFAT) (SD Biosensor, Suwon, Republic of Korea) for the qualitative detection of circulating filarial antigen from *Wuchereria bancrofti*.(2,3,4,5,6) However, immunoenzymatic tests have notable limitations that reduce their effectiveness for monitoring ongoing transmission or assessing treatment efficacy in elimination programs. Antibody-based tests, like the Brugia Rapid Test or the Bioline™ Onchocerciasis, may generate false-positive results due to circulating antibodies that remain after treatment, therefore, not being able to distinguish between active and past infections. Antibody-based tests detect the host immune response rather than the presence of the parasite itself. Positive serology does not necessarily indicate active infection, be-

doi: 10.1590/0074-02760250337
Financial support: This study was funded by an Instituto de Salud Carlos III contract for the training of research personnel in health (ISCIII-PFIS), reference number TRPY-218/23-PFIS, and by the Biomedical Research Networking Center of Infectious Diseases (CIBERINFEC CB21/13/00120).
+ Corresponding author: tta@isciii.es | https://orcid.org/0000-0001-7058-4839
Received 15 November 2025
Accepted 05 May 2026

How to cite: Capote-Morales R, Benito A, Berzosa P, González V, García L, Sastre-González A, et al. Pan filarial-nested PCR with gelified tubes: a new approach towards point-of-care testing for human filariasis. Mem Inst Oswaldo Cruz. 2026; 121: e250337.
Handling editor: Adeilton Alves Brandão | https://orcid.org/0000-0001-5877-607X

CC BY

cause circulating antibodies can persist for long periods after successful treatment or parasite clearance. Another limitation of the Bioline™ Onchocerciasis is that, according to the study published by Gbakima et al. in 1996, the immunoglobulin G and E reactivity during *O. volvulus* infection in children from two to 15 years old, approximately 20% of individuals do not generate an antibody response against the Ov16 antigen, which causes false-negative results.[7,8] Antigen-based tests can also generate false-negative results due to either low or high antigen levels.[9] The BinaxNOW Filariasis test ki® has a three-month shelf-life, has to be cold-stored, and its results may vary over time.[4,7] Cross-reactivity with *L. loa* has also been reported for both the BinaxNOW Filariasis test kit® and Bioline™ Filariasis Test Strip tests.[10]

Nucleic acid-based tests, which target parasite DNA, offer greater sensitivity and specificity than immunoenzymatic tests. However, molecular approaches are often deemed unsuitable for POCT settings due to the high cost of reagents, the need for specialised equipment, multiple procedural steps, and the increased risk of contamination.[9,11] In 2010, Ta-Tang et al. developed a highly sensitive and specific nested polymerase chain reaction for the diagnosis of filariasis, capable of detecting all human-infecting filarial species simultaneously, known as *filaria-nested PCR* (*Fn-PCR*).[12] However, it faced the aforementioned usual problems, as well as others encountered when trying to implement the technique in laboratories located in endemic countries, such as the cost of transportation and the difficulty of keeping the reagents at an optimal temperature both during transportation and once stored at the destination.

To overcome these limitations, the process of gelification, developed by Biotools Biotechnological & Medical Laboratories S.A. (Madrid, Spain) in 2001, was taken into consideration.[13,14] This process enables the incorporation of all necessary PCR reagents, except water and isolated DNA, into a gel matrix within the PCR tube. The reagents remain stable, with no significant loss of enzymatic activity, for up to one month at room temperature and over six months at 4ºC.[14] Gelification is a more cost-effective alternative to traditional preservation techniques due to its complete automation and the capacity to process multiples of 96 tubes simultaneously.

Integrating a nucleic acid-based test with the gelification technique would align with the World Health Organization's roadmap for NTDs through 2030, which highlights the urgent need for the development of more sensitive, specific, non-invasive, cost-effective, and easy-to-implement diagnostic methods, particularly those integrated into multiplex platforms.[1] As onchocerciasis and lymphatic filariasis microfilaremia has significantly declined in several endemic regions due to the great effectiveness of the mass drug administration campaigns implemented through control and elimination programs, developing new feasible molecular methods has become essential for the diagnosis of human filariasis.[15-21]

Therefore, the objective of this study was to convert the original manual *Fn-PCR* into a gelified tube format, termed *gelified tube-filarial-nested PCR* (*Gel-Fn-PCR*) by applying the gelification process to the reaction components. This modification was intended to simplify the nested PCR workflow by reducing manual preparation steps and handling, thereby facilitating assay implementation in routine laboratory settings. In addition, the gelified format aims to improve standardisation, reduce the risk of contamination associated with multiple reaction preparations, and lower operational costs. Finally, the *Gel-Fn-PCR* was optimised and validated for the laboratory diagnosis of human filariasis.

MATERIALS AND METHODS

Microscopy - A total of 661 blood smears from Equatorial Guinea and Nigeria were analysed. The microscopic analysis of the thick blood smears from Equatorial Guinea was performed by researchers of the National Centre of Tropical Medicine (CNMT, Spain), while the microscopy of Nigerian samples was conducted by microscopists of Ladoke Akintola University of Technology (LAUTECH, Nigeria). Equatorial Guinea thick blood smears were obtained via finger-prick and stained with Giemsa (3%), whereas for Nigerian blood smears, venous blood was obtained from each patient, and initially, blood was directly examined for the presence of microfilariae using a wet blood film. Those filarial-positive samples, from 5 to 10 µL of blood were taken to prepare thin blood smears, fixed in methanol and stained with haematoxylin. Both in the CNMT and LAUTECH, all stained blood smears were examined by two different expert microscopists. Each thick blood smear contained approximately 20 µL. The entire smear was systematically screened and the number of microfilariae was recorded. Microfilarial density was subsequently calculated and expressed as microfilariae per millilitre of blood (mF/mL) according to the volume of blood examined. The detection of the microfilariae was performed under the 10x and 40x objectives, whereas identification of filarial species was achieved under the 100x oil-immersion objective to visualise the main morphological characteristics of the microfilariae.

Sample collection and preparation of dried blood spots (DBS) - To optimise and validate the *Gel-Fn-PCR* technique, a total of 661 DBS were used. The DBS samples belonged to the repository of CNMT (collection number C.0005278/ISCIII/Spain, Instituto de Salud Carlos III, in accordance with Spanish Law RD 1716/2011. Article 22.1). The samples from Nigeria were collected in 2015. The DBS samples were prepared impregnating the Whatman paper with the venous blood. Samples from Equatorial Guinea were collected in 2019, and the DBS samples were prepared by impregnating the Whatman paper with blood obtained via finger-prick.

DNA isolation - DNA isolation was performed using the Investigator STR GO! Lysis Buffer® (QIAGEN, Germany). A detailed description of the procedure was published in Capote-Morales et al.[22] The advantage of this DNA isolation method is the requirement of only basic and inexpensive laboratory equipment, such as a vortex mixer, a thermal block, and a centrifuge.

The use of DBS samples also simplifies the sampling process by requiring a small sample volume and being minimally invasive. Given that DBS samples remain stable for long periods of time at room temperature, their

use reduces transportation costs and does not require special storage conditions.[23,24,25]

The combination of DBS samples with this DNA isolation method makes the process rapid and easy to perform, making it particularly suitable for implementation alongside the *Gel-Fn-PCR* in low-income settings.

Gel-Fn-PCR: optimisation - The *Gel-Fn-PCR*, adapted from the *Fn-PCR* developed by Ta-Tang et al. in 2010, targets partially the 18S (small subunit ribosomal RNA), ITS1 (first internal transcribed spacer), and partially the 5.8S regions for filarial detection and identification.[12]

The original *Fn-PCR* had a 50 µL final reaction volume, but the *Gel-Fn-PCR* was reformulated to a final reaction volume of 25 µL, according to the gelification process. The isolated DNA from positive and negative filarial samples was tested both pure and diluted with distilled water ten times. Likewise, the first PCR product to be incorporated into the second PCR was tested both non-diluted and diluted ten times in distilled water.

The optimisation process with DNA polymerase (Biotools, B & M Labs, S.A., Madrid, Spain) and primers was carried out by Biotools, preparing a series of formulas containing different concentrations, which were blindly checked in the laboratory to determine which one yielded the best results. Two different DNA polymerase enzymes from Biotools were evaluated to minimise non-specific amplification.

In addition, the FIL2F primer (GGTGAACCTGCGGAAGGATC) from the second PCR was replaced by the ITS1F-BIS primer (5′-GGTGAACCTGCRGMWGGATC-3′), a degenerate version of the original FIL-2F primer, was used in this study to improve amplification of genetically diverse *L. loa* isolates.

For each reaction performed, appropriate positive and negative controls were always included. Positive controls were DBS samples of *L. loa* and *M. perstans* diagnosed by microscopy, as well as by other validated molecular diagnostic methods available in our laboratory; negative controls were DBS samples of filarial microscopy-negative individuals from filarial endemic regions that were negative for filarial infection but could be positive or negative for other tropical parasites, such as *Plasmodium* spp. All samples were run in duplicate. If the duplicates varied (one positive, one negative), the samples were rerun in triplicate.

During the optimisation process, the gelified tubes were stored at 4ºC. Regarding the PCR amplification program, no modifications were introduced.

In the course of the initial optimisation phase, a subset of samples was analysed in parallel using both the original *Fn-PCR* and the *Gel-Fn-PCR* to confirm the equivalence of the two approaches. Once comparable performance was established, subsequent analyses were performed using only the *Gel-Fn-PCR* assay. For the evaluation of diagnostic performance in the full dataset, microscopy was used as the reference method, as it represents the routine diagnostic standard in the study settings.

Validation - To assess the diagnostic performance of the *Gel-Fn-PCR* and to determine its validity as a reliable alternative diagnostic method for filarial infections, the final optimised *Gel-Fn-PCR* was subjected to validation testing on 661 DBS clinical samples in the laboratory. The complete, final workflow of the *Gel-Fn-PCR* assay, encompassing all procedural steps from clinical sampling to result visualisation, is depicted in Fig. 1.

In selected cases with inconclusive or unexpected results, additional confirmatory molecular assays were performed using previously published methods.

Visualisation of amplified fragments - Since the *Gel-Fn-PCR* technique is a conventional PCR, amplified DNA fragments were visualised using the Qiaxcel Advanced automated electrophoresis system (QIAGEN, Germany). The identification of the filarial species was determined based on the size of the amplified fragment from the second PCR, and by comparing it with the size of the positive controls included in the PCR reaction.

The Qiaxcel automated electrophoresis system allows rapid, standardised, and high-resolution fragment analysis. However, conventional agarose gel electrophoresis can also be used as an alternative, particularly in resource-limited settings.

Statistical analysis - To evaluate the capacity of the *Gel-Fn-PCR* as a diagnostic method, the obtained results were compared with microscopy method, considered as the gold standard for diagnosing human filariasis. Statistical analyses were performed using WinEpi 2.0 software.[26] The calculated parameters included the sensitivity, specificity, positive predictive value, negative predictive value were calculated based on standard definitions for diagnostic test evaluation, and 95% confidence intervals (95% CI) were computed for each parameter. Agreement between methods was assessed using Cohen's kappa index with corresponding 95% CI. The recommendation was to have a high sensitivity and a high specificity for *Gel-Fn-PCR* method.[27]

Ethics committee statement - All clinical samples (DBS and thick blood smears) were authorised by the director of the National Centre of Tropical Medicine for its use in this study, with the unique purpose of optimising and validating the *Gel-Fn-PCR*. Samples were irreversibly anonymised and no additional ethical approval or informed consent was required for the present study.

RESULTS

Microscopic diagnosis - Filarial parasites were identified in 54.61% samples (361/661). Among the positive samples, 55.96% (202/361) were confirmed as mono-infections with *L. loa*, 5.54% (20/361) as mono-infections with *M. perstans*, and 38.50% (139/361) as mixed infections with *L. loa* and *M. perstans* (Fig. 2). A detailed depiction of the results is provided in Table I.

The median microfilaremia was 2700 mF/mL for *L. loa* in mono-infection, with microfilaremia ranging from 50 to 64850 mF/mL. For *M. perstans* mono-infection, the median microfilaremia was 850 mF/mL, with microfilaremia ranging from 500 to 2450 mF/mL.

Regarding mixed infections, the median microfilaremia was 2950 mF/mL (ranged 50-76700 mF/mL) and 150 mF/mL (ranged 50-5550 mF/mL) for *L. loa* and *M. perstans,* respectively.

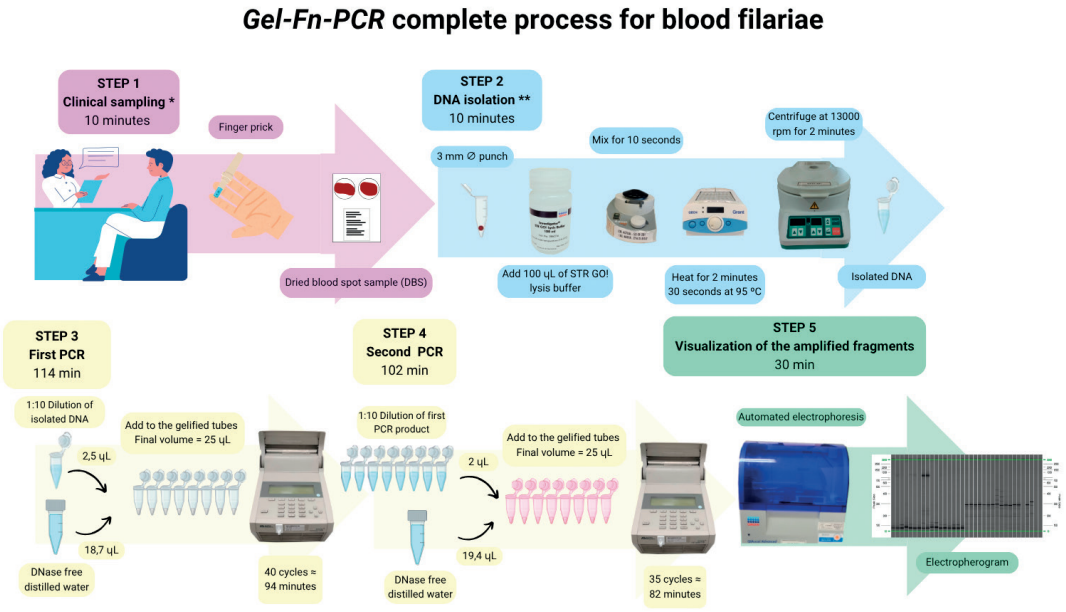


Fig. 1: depiction of the *Gelified tube-filarial-nested polymerase chain reaction (Gel-Fn-PCR)* complete process. Workflow of the *Gel-Fn-PCR* assay for the detection of filarial parasites. Asterisks (*) indicate steps that differ when the type of clinical sample are skin snips used for the detection of *Onchocerca volvulus*, a modified workflow is applied compared to the procedure used for blood samples targeting blood-dwelling filarial species (*e.g.*, *Wuchereria bancrofti*, *Loa loa* and *Mansonella perstans*). *In case of onchocerciasis suspicion, two skin snips from the iliac crest should be taken and put into an Eppendorf with 200 µL of physiologic solution. **Skin snips samples must undergo an additional 2 h of incubation with proteinase K at 56ºC before DNA isolation. Once skin snips are completely disrupted, 50 µL of the solution are taken and spotted onto Whatman paper.

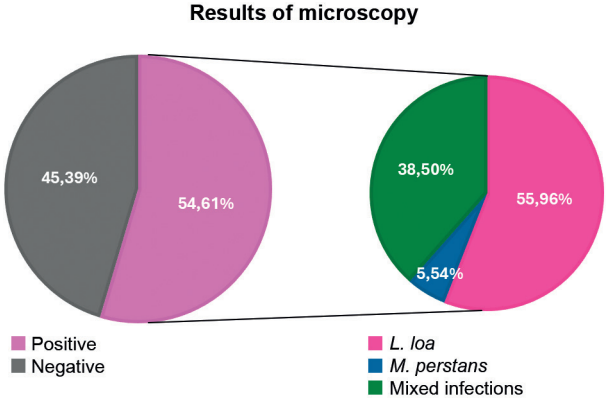


Fig. 2: summary of the results of the microscopic diagnosis. Sectoral chart illustrating the proportional distribution of the different infections and filarial species identified through microscopic examination.

Sample collection and preparation of DBS - Out of 661 DBS samples, 49.77% (329/661) were collected in Equatorial Guinea, and 50.23% (332/661) were collected in Nigeria. 76.60% (252/329) of Equatorial Guinea samples were positive: 55.16% (139/252) were mixed infections (*L. loa* and *M. perstans*), 36.90% (93/252) were *L. loa* mono-infection, and 7.94% (20/252) were *M. perstans* mono-infection. 32.83% (109/332) of Nigerian samples were positive, all of which were *L. loa* mono-infection.

Gel-Fn-PCR: final optimised conditions of the Gel-Fn-PCR assay - The following section describes the final optimised conditions of the *Gel-Fn-PCR* assay established after systematic evaluation of multiple reaction parameters.

The final *Gel-Fn-PCR* contained 1 U/µL of DNA polymerase in both the first and second PCR, 0.5 µM of each FIL1F and UNI1R primers (first PCR), as in the original *Fn-PCR*, 0.05 µM of each ITS1F-BIS and FIL2R primers (second PCR), concentrations higher than the *Fn-PCR,* at a final volume of 25 µL. The volume of isolated DNA diluted 1:10 in distilled water used for the first PCR was 2.5 µL, and 2 µL from the first PCR product diluted 1:10 in distilled water were used for the second PCR.

Validation - The *Gel-Fn-PCR* identified 54.92% (363/661) as filarial-positives and 45.08% (298/661) as negative for filarial parasites. Among the positive samples, 72.45% (263/363) samples were identified as *L. loa* infections, 9.37% (34/363) samples as *M. perstans* infections, and 18.18% (66/363) samples as mixed *L. loa* and *M. perstans* infections (Fig. 3). A comparison of the results of *Gel-Fn-PCR* against the reference method, microscopy, is presented in Table I. Overall agreement between microscopy and *Gel-Fn-PCR* was observed mainly along the diagonal (true concordant results), whereas discrepancies were observed in cases classified as mixed infections or negative by one of the methods.

Microscopic diagnosis was able to detect more mixed infections, 21.03% (139/661), than the *Gel-Fn-PCR*, 9.99% (66/661). Most mixed infections missed by *Gel-Fn-PCR* (76/139) were classified as mono-infection, predominantly as *L. loa* (62/139) and to a lesser extent as *M. perstans* (14/139). To overcome these discrepancies, the 76 sam-

TABLE I

Comparative results between microscopy and *Gelified tube-filarial-nested polymerase chain reaction (Gel-Fn-PCR)*

			Microscopy				
			Positives (54.61%, 361)				
			L. loa	*M. perstans*	Mixed infection	Negatives (45.39%, 300)	Total
Gel-Fn-PCR	Positives (54.92%, 363/661)	*Loa loa*	**185**	0	62	16	263
		Mansonella perstans	0	**19**	14	1	34
		Mixed infection	4	0	**61**	1	66
	Negatives (45.08%, 298/661)		13	1	2	**282**	298
	Total		202	20	139	300	661

Overall agreement between microscopy and *Gel-Fn-PCR* was observed along the diagonal of the matrix (true concordant results). Discordant results were primarily associated with differences in the detection of mixed infections and cases classified as negative by one of the methods.

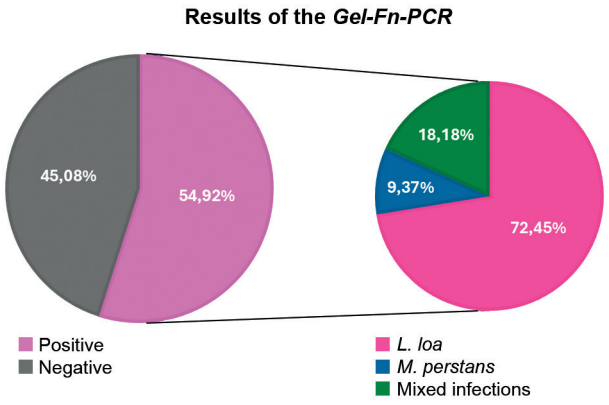


Fig. 3: depiction of the *Gelified tube-filarial-nested polymerase chain reaction (Gel-Fn-PCR)* results. Sectoral chart indicating the proportional distribution of different infections and filarial species detected by the *Gel-Fn-PCR* assay.

ples misclassified by *Gel-Fn-PCR* were re-analysed by the real-time PCR (*F-RT-PCR*) published by Ta-Tang et al. in 2022.[28] Among the 62 samples classified as *L. loa*, 52 were confirmed, while 10 were reclassified as mixed infections, in agreement with microscopy. Among the 14 samples classified as *M. perstans*, seven were confirmed, while the remaining were reclassified as mixed infections (n = 4) or *L. loa* (n = 3). Overall, 77.6% (59/76) of discrepant results were concordant with *Gel-Fn-PCR*.

The *Gel-Fn-PCR* showed reduced concordance with microscopy for mixed infections. Several samples identified as mixed infections by microscopy were classified as single-species infections by *Gel-Fn-PCR*, suggesting an underestimation of co-infections by the molecular assay.

Additionally, 13 samples positive for *L. loa* by microscopy were negative by *Gel-Fn-PCR*, whereas 16 microscopy-negative samples were positive for *L. loa* by *Gel-Fn-PCR*; all were confirmed by F-RT-PCR (Table I). Unexpectedly, the sample #171 from Nigeria with 34550 *L. loa* mF/mL was missed by *Gel-Fn-PCR*, and doubtfully detected by *F-RT-PCR*.[28]

Visualisation of amplified fragments - Identification of the species was performed according to the size of the amplified fragments from the second PCR, as described in the original *Fn-PCR* method, which were 286 bp for *L. loa*, 301 bp for *W. bancrofti*, 305 bp for *M. ozzardi*, 312 bp for *M. perstans*, and 344 bp for *O. volvulus*.

Statistical analysis - The *Gel-Fn-PCR* showed good sensitivity and specificity values for *L. loa*, 91.6% and 83.0% respectively, and high sensitivity and specificity values for *M. perstans*, 95.0% and 97.7%, respectively. However, for mixed infections, sensitivity dropped to 43.9%, and specificity remained high at 99.0%. The positive predictive value (PPV) was low for both mono-infections, 70.3% for *L. loa* and 55.9% for *M. perstans*, but remained high for mixed infections at 92.4%. Negative predictive value (NPV) was high for all infection categories, 95.7% for *L. loa* infections, 99.8% for *M. perstans* infections, and 86.9% for mixed infections. Cohen's Kappa coefficient was between adequate and moderate. The detailed results of the statistical analysis are presented in Table II.

DISCUSSION

In a landscape in which many endemic countries are close to eliminating human filariasis, the demand for reliable diagnostic methods is more critical than ever.[29,30,31] Nucleic acid-based assays, which directly target parasite DNA, offer higher sensitivity and specificity than traditionally used immunoenzymatic methods.[32] Therefore, this study developed the *Gel-Fn-PCR*, a reliable nucleic acid-based diagnostic method to detect all human filariasis while being more compatible with the POCTs criteria.

Considering microscopic diagnosis as the reference method, the *Gel-Fn-PCR* was able to identify most mono-infections with minimal discrepancies. Thirteen *L. loa* microscopy-positives were missed by *Gel-Fn-PCR* and, interestingly, all of them came from Nigeria. Twelve of those samples had a microfilariae load ranged 100-4150 mF/mL. The lower detection of microfilariae in some samples from Nigeria may be explained by the use of venous blood to prepare DBS. If blood samples were not fully homogenised prior to spotting, microfilariae may have been unevenly distributed, leading to potential loss of parasites in the analysed spots and

TABLE II

Statistical analysis of the *Gelified tube-filarial-nested polymerase chain reaction (Gel-Fn-PCR)*

	Loa loa	*Mansonella perstans*	Mixed infections
Sensitivity	91.6% (87.8%, 95.4%)	95.0% (85.4%, 104.6%)	43.9% (35.6%, 52.1%)
Specificity	83.0% (79.6%, 86.4%)	97.7% (96.5%, 98.8%)	99.0% (98.2%, 99.9%)
PPV	70.3% (64.8%, 75.9%)	55.9% (39.2%, 72.6%)	92.4% (86.0%, 98.8%)
NPV	95.7% (93.7%, 97.7%)	99.8% (99.5%, 100.2%)	86.9% (84.2%, 89.6%)
Cohen's Kappa Index	0.688 (0.613, 0.762) Adequate	0.692 (0.619, 0.765) Adequate	0.532 (0.462, 0.601) Moderate

CI: confidence interval (95%); PPV: positive predictive value; NPV: negative predictive value.

consequently affecting detection sensitivity. The failure to detect sample #171, despite its high microfilaremia, may be related to pre-analytical factors associated with sample preparation, incomplete homogenisation prior to spotting may lead to the absence of parasite DNA in the analysed punch. This highlights a potential limitation of DBS-based approaches, where parasite distribution within the sample may not be homogeneous. Because the result was uncertain, two *L. loa*-specific methods were also performed specifically for this sample: the nested PCR published by Touré et al.[33] and the LAMP published by Drame et al.[34] Surprisingly, both methods yielded negative results for sample #171. Suspecting that it could be a *L. loa*-like parasite, the nematode-specific PCR developed by Casiraghi et al. was carried out.[35] This nematode-specific PCR resulted in good amplification, and the sequencing of this fragment showed highest similarity with the species *L. loa*. To this day, the reason why discrepant results were obtained with different tests remains unclear. Since the available amount of blood was completely used during this study, and the microscopy slides were destroyed when diagnosis had been finished, further investigation was not possible.

Most discrepancies occurred with mixed infection samples. Microscopy was able to detect more mixed infections than the *Gel-Fn-PCR*, which detected most of these mixed infections as either single *L. loa* or *M. perstans* infections. *L. loa* microfilariae were usually found in greater proportion than *M. perstans* microfilariae in most mixed infections, and since *Gel-Fn-PCR* included a single forward and a single reverse primer targeting all filarial species equally, amplification was likely biased towards the more abundant species. This was the reason why the mixed infection detected by microscopy was identified mostly as *L. loa* by *Gel-Fn-PCR*.

On the other hand, eighteen samples that were negative by microscopy were positive by *Gel-Fn-PCR*. These results were confirmed by *F-RT-PCR*.[28] Most of these discordant results derived again from Nigerian samples, whose microscopic diagnosis was performed employing thin blood smears. Thin blood smears require only from 5 to 10 µL of blood, and it has less sensitivity than thick blood smears, which require up to 30 µL.[36] If Nigerian microscopists had used thick blood smears instead of thin blood smears, perhaps there wouldn't have been many discordant results between microscopy and *Gel-Fn-PCR*.

According to statistical analysis, the *Gel-Fn-PCR* demonstrated high sensitivity and specificity for the detection of mono-infections with 95% CI indicating consistent performance. These results support the reliability of the assay for diagnosing single-species filarial infections when compared with microscopy as the reference standard.

Regarding mixed infections, *Gel-Fn-PCR* was not a reliable method for diagnosing them. PPV was, as expected, comparatively lower for both mono-infections than for mixed infections, since they were influenced by the numerous mixed infections that were misdiagnosed as mono-infections, which likely confounded the apparent diagnostic performance of the *Gel-Fn-PCR* for mono-infections and prevented a clear interpretation of its true diagnostic capacity. In contrast, the NPV remained high for all types of infections, thereby highlighting the robust reliability of the assay in accurately identifying negatives.

Cohen's kappa index indicated that the *Gel-Fn-PCR* had an adequate correlation with the microscopic diagnosis for mono-infections, while for mixed infections, the correlation was moderate, thereby corroborating our findings, and indicating that the *Gel-Fn-PCR* is a reliable diagnostic method for mono-infections, consistent with microscopy, although it is comparatively less consistent in the detection of mixed infections.

Various multiplex PCRs have also been developed and validated for the diagnosis of other parasites, including *Plasmodium* spp., *Schistosoma* spp., and soil-transmitted helminths. Still, the diagnosis of mixed infections remains a challenge for many researchers.[37-42] This limitation is intrinsic to the multiplex PCR technique. Preferential amplification of one target over the others can be attributable to the inherent properties of the target DNA or to the fluctuation in the interactions of PCR reagents, especially when there is a low concentration of the target DNA.[43]

Gel-Fn-PCR offers advantages over other filarial PCR assays previously described in the literature. The PCR developed by Casiraghi et al.[35] had high sensitivity and specificity, but species identification requires sequencing, thereby making it inapplicable as a POCT. In 2005, Nuchprayoon et al. developed a high sensitive and specific pan-filaria PCR, however, the method needs an additional restriction enzyme digestion step prior to the visualisation of the amplified fragments for species

identification.[44] This additional step increases the cost, duration, and technical complexity of the assay, further limiting its feasibility for implementation as a POCT.

Several multiplex PCR assays targeting filarial parasites have also been reported. Nicolas and Scoles developed in 1997 a multiplex PCR capable of detecting *Dirofilaria immitis* and *W. bancrofti*, although its application was limited to vector samples.[45] Dyab et al.[46] also published a multiplex PCR that detected *D. immitis*, *W. bancrofti*, and *D. repens*, yet this assay had only been evaluated in vectors.[46] Finally, Mishra et al. proposed another multiplex PCR restricted to the detection of *B. malayi* and *W. bancrofti*, which was tested in both blood and vector samples.[47] The *Gel-Fn-PCR* demonstrates a notably broader diagnostic spectrum compared with all the aforementioned methods, allowing the detection of all eight filarial species known to infect humans within a single assay.[45,46,47]

The *Gel-Fn-PCR* developed in this study has the same advantages of the *Fn-PCR* in terms of versatility, but with the addition of gelification technology benefits, such as convenient storage at 4ºC, shipment conditions, minimal set-up time, fewer errors, as well as being more user-friendly. Furthermore, *Gel-Fn-PCR* is cost-effective, 0.78€ per reaction. Although the *Gel-Fn-PCR* is still a relatively complex procedure and requires specialised equipment, and therefore cannot yet be considered a true POCT, the findings of this study demonstrate that PCR-based methods are approaching practical feasibility as POCTs in the diagnosis of human filariasis.

In conclusion - The *Gel-Fn-PCR* represents an optimised adaptation of the previously developed conventional *Fn-PCR,* maintaining comparable sensitivity and specificity for the detection of single filarial infections while offering important operational advantages. In particular, the gelified format simplifies the nested PCR workflow by reducing reagent preparation and handling steps, improving assay standardisation, and lowering the risk of contamination associated with multi-step procedures. These features make the *Gel-Fn-PCR* more practical and cost-effective for routine laboratory use. Sensitivity for mixed infections was low, though specificity remained high. While the assay was suboptimal for detecting mixed infections, particularly when one species was predominant, it performed effectively for single-species detection. Overall, the *Gel-Fn-PCR* is particularly advantageous in laboratory settings requiring simplified workflows and standardised procedures, while retaining the diagnostic performance of the original *Fn-PCR*. The *Gel-Fn-PCR* demonstrated limitations as a deployable POCT in resource-limited areas; nonetheless, this study highlighted the feasibility of integrating gelification technology with nucleic acid-based assays. It underscores the potential of using gelified tubes as a platform for further development of multiplexed and field-adaptable molecular tools.

Study limitations - A limitation of this study was the comparison between *Gel-Fn-PCR* and the original *Fn-PCR* only performed on a limited subset of samples during the optimisation phase, rather than across the entire dataset. Given that the *Gel-Fn-PCR* has only been tested on *L. loa* and *M. perstans* positive samples, further validation with additional blood-borne filarial species will be essential to confirm its broad diagnostic utility. Similarly, *Gel-Fn-PCR* should be tested on skin snip samples to determine whether this method would be valid for detecting *O. volvulus* microfilariae. Moreover, the Nigerian samples had been stored for a relatively long period of time, approximately ten years, and DBS samples have been prepared from venous blood, which may have affected the test results.

ACKNOWLEDGEMENTS

To all individuals and institutions who contributed samples to the DBS and blood smear repository used in this study. We also acknowledge Daniel F García Rabell for his assistance with the English language revision.

AUTHORS' CONTRIBUTION

RCM - Methodology, validation, formal analysis, investigation, writing-original draft preparation, writing-review and editing and visualization; AB - resources, supervision, project administration and funding acquisition; PB - investigation, resources, writing-review and editing, visualisation and supervision; VG and LG - investigation; ASG - methodology and validation; AAA and OO - methodology, writing-review and editing; THTT - conceptualisation, methodology, validation, formal analysis, investigation, writing-original draft preparation, writing-review and editing, visualisation, supervision and project administration. All authors have read and agreed to the published version of the manuscript. The authors declare no conflict of interest related to their research work.

DATA AVAILABILITY

After publication the data will be available on demand to corresponding author.

REFERENCES

1. WHO - World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021-2030. 2021. 196 pp. Available from: https: // www.who.int/publications/i/item/9789240010352.
2. Rahmah N, Taniawati S, Shenoy RK, Lim BH, Kumaraswami V, Anuar AK, et al. Specificity and sensitivity of a rapid dipstick test (Brugia Rapid) in the detection of Brugia malayi infection. Trans R Soc Trop Med Hyg. 2001; 95: 601-4. doi: 10.1016/S0035-9203(01)90091-4.
3. Nikièma AS, Koala L, Unnasch TR, Diendéré J, Compaoré J, Ouédraogo MW, et al. Field sensitivity and specificity of the SD BIOLINE onchocerciasis IgG4 Rapid Diagnostic Test in children < 10 years old from endemic areas in Burkina Faso. Parasit Epidemiol Control. 2024; 25: e00352. doi: 10.1016/j.parepi.2024.e00352.
4. Weil GJ, Curtis KC, Fakoli L, Fischer K, Gankpala L, Lammie PJ, et al. Laboratory and field evaluation of a new rapid test for detecting *Wuchereria bancrofti* antigen in human blood. Am Soc Trop Med Hyg. 2013; 89: 11-5. doi: 10.4269/ajtmh.13-0089.
5. Graves PM, Scott JL, Berg Soto A, Widi AYN, Whittaker M, Lau CL, et al. Laboratory comparison of rapid antigen diagnostic tests for lymphatic filariasis: STANDARD Q Filariasis Antigen Test (QFAT) versus Bioline Filariasis Test Strip (FTS). Trop Med Infect Dis. 2025; 10: 23. doi: 10.3390/tropicalmed10010023.
6. Scott JL, Mayfield HJ, Sinclair JE, Martin BM, Howlett M, Mut-

tucumaru R, et al. Field laboratory comparison of STANDARD Q Filariasis Antigen Test (QFAT) with Bioline Filariasis Test Strip (FTS) for the detection of Lymphatic Filariasis in Samoa, 2023. PLoS Negl Trop Dis. 2024; 18: e0012386. doi: 10.1371/journal.pntd.0012386.

7. Pietrzak D, Łuczak JW, Wiśniewski M. Beyond tradition: exploring cutting-edge approaches for accurate diagnosis of human filariasis. Pathogens. 2024; 13: 447. doi: 10.3390/pathogens13060447.

8. Gbakima AA, Nutman TB, Bradley JE, McReynolds LA, Winget MD, Hong Y, et al. Immunoglobulin G subclass responses of children during infection with *Onchocerca volvulus*. Clin Diagn Lab Immunol. 1996; 3: 98-104. doi: 10.1128/cdli.3.1.98-104.1996.

9. Bharadwaj M, Bengtson M, Golverdingen M, Waling L, Dekker C. Diagnosing point-of-care diagnostics for neglected tropical diseases. PLoS Negl Trop Dis. 2021; 15: e0009405. doi: 10.1371/journal.pntd.0009405.

10. Hertz MI, Nana-Djeunga H, Kamgno J, Njouendou AJ, Chunda VC, Wanji S, et al. Identification and characterization of *Loa loa* antigens responsible for cross-reactivity with rapid diagnostic tests for lymphatic filariasis. PLoS Negl Trop Dis. 2018; 12: e0006963. doi: 10.1371/journal.pntd.0006963.

11. Alhassan A, Li Z, Poole CB, Carlow CKS. Expanding the MDx toolbox for filarial diagnosis and surveillance. Trends Parasitol. 2015; 31: 391-400. doi: 10.1016/j.pt.2015.04.006.

12. Tang T-HT, López-Vélez R, Lanza M, Shelley AJ, Rubio JM, Luz SLB. Nested PCR to detect and distinguish the sympatric filarial species *Onchocerca volvulus*, *Mansonella ozzardi* and *Mansonella perstans* in the Amazon Region. Mem Inst Oswaldo Cruz. 2010; 105(6): 823-8. doi: 10.1590/s0074-02762010000600016.

13. Madejon Seiz A, Limones Lopez G, Haro Castuera A, De Grado Sanz M, Franco de Sarabia Rosado PM, inventors. Stabilised composition for fluorimetric, colorimetric or chemoluminescent assay, kits containing same and production method thereof. Espanha patent EP 1 598 418 A1 2003 Feb 26. Available from: https://patentimages.storage.googleapis.com/49/bd/c9/e114707a29fdd4/EP1598418A1.pdf.

14. Sun Y, Hogberg J, Christine T, Florian L, Monsalve LG, Rodriguez S, et al. Pre-storage of gelified reagents in a lab-on-a-foil system for rapid nucleic acid analysis. Lab Chip. 2013; 13: 1509. doi: 10.1039/c2lc41386h.

15. Lahane V, Sagar VVSS, Naik S, Shukla S, Acharya S, Kumar S. Microfilaria causing nephrotic syndrome: Highlighting the importance of diethylcarbamazine provocation test for diagnosis. J Fam Med Prim Care. 2022; 11: 4002-5. doi: 10.4103/jfmpc.jfmpc_2410_21.

16. Rajamanickam A, Babu S. Unraveling the dynamics of human filarial infections: immunological responses, host manifestations, and pathogen biology. Pathogens. 2025; 14: 223. doi: 10.3390/pathogens14030223.

17. Dickson B, Graves P, McBride W. Lymphatic filariasis in mainland southeast Asia: a systematic review and meta-analysis of prevalence and disease burden. Trop Med Infect Dis. 2017; 2: 32. doi: 10.3390/tropicalmed2030032.

18. Chavarkar SP. Lymphatic filariasis: the importance of screening all peripheral blood smears in low power for detection of asymptomatic cases. Int J Res Med Sci. 2016; 5: 350. doi: 10.18203/2320-6012.ijrms20164577.

19. Medeiros ZM, Vieira AVB, Xavier AT, Bezerra GSN, Lopes MDFC, Bonfim CV, et al. Lymphatic filariasis: a systematic review on morbidity and its repercussions in countries in the Americas. Int J Environ Res Public Health. 2021; 19: 316. doi: 10.3390/ijerph19010316.

20. Ton TGN, Mackenzie C, Molyneux DH. The burden of mental health in lymphatic filariasis. Infect Dis Poverty. 2015; 4: 34. doi: 10.1186/s40249-015-0068-7.

21. WHO - World Health Organization / Region African. ESPEN Annual Report 2024. Expanded special project for elimination of neglected tropical diseases. [cited 2025 Sep 17]. Available from: https://espen.afro.who.int/tools-resources/documents/espen-annual-report-2024.

22. Capote-Morales R, Benito A, Berzosa P, de la Fuente IM, Akindele AA, Cruces R, et al. A novel filarial-multiplexed probe-quantitative PCR for the advance in the diagnosis of multiple infections with human filariasis. Trop Med Int Health. 2025; 30: 1097-106. doi: 10.1111/tmi.70016 PubMed PMID: 40775802.

23. Malsagova K, Kopylov A, Stepanov A, Butkova T, Izotov A, Kaysheva A. Dried blood spot in laboratory: directions and prospects. Diagnostics. 2020; 10: 248. doi: 10.3390/diagnostics10040248.

24. Demirev PA. Dried blood spots: analysis and applications. Anal Chem. 2013; 85: 779-89. doi: 10.1021/ac303205m.

25. Sharma A, Jaiswal S, Shukla M, Lal J. Dried blood spots: Concepts, present status, and future perspectives in bioanalysis. Drug Test Anal. 2014; 6: 399-414. doi: 10.1002/dta.1646.

26. WinEpi - Working in Epidemiology. Reflexiones sobre el COVID-19 de un epidemiólogo veterinario. [cited 2023 Sep 1]. Available from: http: //www.winepi.net/.

27. Akobeng AK. Understanding diagnostic tests 1: sensitivity, specificity and predictive values. Acta Paediatr. 2007; 96: 338-41. doi: 10.1111/j.1651-2227.2006.00180.x.

28. Ta-Tang TH, Febrer-Sendra B, Berzosa P, Rubio JM, Romay-Barja M, Ncogo P, et al. Comparison of three PCR-based methods to detect *Loa loa* and *Mansonella perstans* in long-term frozen storage dried blood spots. Trop Med Int Health. 2022; 27: 686-95. doi: 10.1111/tmi.13786.

29. WHO - World Health Organization. Global programme to eliminate lymphatic filariasis: progress report, 2023. [cited 2025 Sep 11]. 2024. Available from: https://www.who.int/publications/i/item/who-wer-9940-565-576.

30. WHO - World Health Organization. Elimination of human onchocerciasis: progress report, 2023-2024 = Élimination de l'onchocercose humaine : rapport de situation, 2023-2024. [cited 2026 May 20]. Available from: https: //iris.who.int/items/0627ee12-87ef-4eab-b9b4-0f2db4e2d71e.

31. Hietanen H, Pfavayi LT, Mutapi F. Unlocking the blueprint to eliminating neglected tropical diseases: a review of efforts in 50 countries that have eliminated at least 1 NTD. PLoS Negl Trop Dis. 2025; 19: e0013424. doi: 10.1371/journal.pntd.0013424.

32. Lubbers C, Amaral LJ, Colebunders R, Brattig N, Hadermann A. The last mile in onchocerciasis elimination: diagnostic challenges. Trends Parasitol. 2025; 41: 894-908. doi: 10.1016/j.pt.2025.08.006.

33. Touré FS, Bain O, Nerrienet E, Millet P, Wahl G, Toure Y, et al. Detection of *Loa loa*-specific DNA in blood from occult-infected individuals. Exp Parasitol. 1997; 86: 163-70. doi: 10.1006/expr.1997.4168.

34. Drame PM, Fink DL, Kamgno J, Herrick JA, Nutman TB. Loop-mediated isothermal amplification for rapid and semiquantitative detection of *Loa loa* infection. J Clin Microbiol. 2014; 52: 2071-7. doi: 10.1128/JCM.00525-14.

35. Casiraghi M, Anderson TJC, Bandi C, Bazzocchi C, Genchi C. A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts. Parasitology. 2001; 122: 93-103. doi: 10.1017/S0031182000007149.

36. Eberhard ML, Lammie PJ. Laboratory diagnosis of filariasis. Clin Lab Med. 1991; 11: 977-1010. doi: 10.1016/S0272-2712(18)30531-6.

37. Ten Hove RJ, Verweij JJ, Vereecken K, Polman K, Dieye L, Van Lieshout L. Multiplex real-time PCR for the detection and quantification of *Schistosoma mansoni* and *S. haematobium* infection in stool samples collected in northern Senegal. Trans R Soc Trop Med Hyg. 2008; 102: 179-85. doi: 10.1016/j.trstmh.2007.10.011.

38. Webster BL, Rollinson D, Stothard JR, Huyse T. Rapid diagnostic multiplex PCR (RD-PCR) to discriminate *Schistosoma haematobium* and *S. bovis*. J Helminthol. 2010; 84: 107-14. doi: 10.1017/S0022149X09990447.

39. Rubio JM, Benito A, Roche J, Berzosa PJ, García ML, Micó M, et al. Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of *Plasmodium vivax* infection in Equatorial Guinea. Am J Trop Med Hyg. 1999; 60: 183-7. doi: 10.4269/ajtmh.1999.60.183.

40. Kho WG, Chung JY, Sim EJ, Kim MY, Kim DW, Jongwutiwes S, et al. A multiplex polymerase chain reaction for a differential diagnosis of *Plasmodium falciparum* and *Plasmodium vivax*. Parasitol Int. 2003; 52: 229-36. doi: 10.1016/S1383-5769(03)00028-X.

41. Schols R, Carolus H, Hammoud C, Mulero S, Mudavanhu A, Huyse T. A rapid diagnostic multiplex PCR approach for xenomonitoring of human and animal schistosomiasis in a 'One Health' context. Trans R Soc Trop Med Hyg. 2019; 113: 722-9. doi: 10.1093/trstmh/trz067.

42. Phuphisut O, Yoonuan T, Sanguankiat S, Chaisiri K, Maipanich W, Pubampen S, et al. Triplex polymerase chain reaction assay for detection of major soil-transmitted helminths, *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, in fecal samples. Southeast Asian J Trop Med Public Health. 2014; 45: 267-75.

43. Markoulatos P, Siafakas N, Moncany M. Multiplex polymerase chain reaction: a practical approach. J Clin Lab Anal. 2002; 16: 47-51. doi: 10.1002/jcla.2058.

44. Nuchprayoon S, Junpee A, Poovorawan Y, Scott AL. Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction-restriction fragment length polymorphism analysis. Am J Trop Med Hyg. 2005; 73: 895-900. doi: 10.4269/ajtmh.2005.73.895.

45. Nicolas L, Scoles GA. Multiplex polymerase chain reaction for detection of *Dirofilaria immitis* (Filariidea: Onchocercidae) and *Wuchereria bancrofti* (Filarioidea: Dipetalonematidae) in their common vector *Aedes polynesiensis* (Diptera: Culicidae). J Med Entomol. 1997; 34: 741-4. doi: 10.1093/jmedent/34.6.741.

46. Dyab AK, Galal LA, Mahmoud AES, Mokhtar Y. Xenomonitoring of different filarial nematodes using single and multiplex PCR in mosquitoes from Assiut Governorate, Egypt. Korean J Parasitol. 2015; 53: 77-83. doi: 10.3347/kjp.2015.53.1.77.

47. Mishra K, Raj DK, Hazra RK, Dash AP, Supakar PC. The development and evaluation of a single step multiplex PCR method for simultaneous detection of *Brugia malayi* and *Wuchereria bancrofti*. Mol Cell Probes. 2007; 21: 355-62. doi: 10.1016/j.mcp.2007.05.001.

OPEN PEER REVIEW

Memórias do IOC thanks the anonymous reviewers for their contribution to the peer review of this work.

FIRST REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

While I can see that the presented article has been prepared by a team with a strong-track in developing molecular tools for filarial parasite detection, I am unconvinced the value of the novel Gel-Fn-PCR is adequately explained the current version of the authors 'manuscript. Thus, even though the article is written in good clear English, I feel there are a lot of presentational issues that prevent what has been done from being clearly understood. I do, however, trust that with some minor revisions the authors will be able to turn their submission into a valuable contribution to the field. Below, I have provided some suggestions as to how I think the article´s clarity could be improved by reconsidering how the results are presented and re-organizing the text. I have also highlighted specific passages of text that I think need to revised for accuracy and to improve clarity.

LINES 66-73: Here it is not made adequately clear that someone can test positive with one of the "antibody-based tests" they describe because they have been exposed to a given filarial parasite (e.g. o. volvulus), but may not have necessarily been infected with the parasite.

LINES 106-109: Please re-write this section to make clearer the benefits of the new Gel-Fn-PCR.

LINES 122-123: Please provide more information on how microfilariae counts were made. How much blood was examined to make these counts?

Line 127: this is not a good sub-title, please revise.

LINES 163: What is the sequence of the ITS1F-BIS primer? It is not provided in the cited reference. Also, why use a different primer from that described in Tang et al. (2010) if the claim in LINES 83-84 is true?

LINES 174-178: What were the positive controls used? What other filarial parasites might conceivably be detected in the study regions. What PCR fragment sizes would be generated from their DNA? Can you provide us with the gel results so that anyone wishing to employ the method can see how diagnostic calls get made?

LINES: 138-140 "is the requirement of basic and inexpensive" should surely be changed to something like "is the requirement of ONLY basic and inexpensive".

LINES 189: I recommend changing single infection (here and throughout) to single-species infections or mono-infections for clarity.

LINE 200: The subtitle needs changing; it is confusing. Are the blood samples placed on microscope slides not clinical samples?

LINES: 208-215: this belongs in the methods section.

LINES 219-221: While I think a table comparing the results of the two methodologies is useful, I feel the current matrix format is difficult to interpret. The current format makes it difficult to focus on the differences in diagnostic results observed between the microscopy and Gel-Fn-PCR approaches.

LINES 222-223: This needs to be we-written for clarity. As I understand it Gel-Fn-PCR routinely miss-reports co-infections as mono-infections. This should be made clearer, talking about detecting and not detecting makes the paper harder to follow. It would also be helpful to see gels from co-infection positive PCRs and PCR results where a co-infection is miss-reported by Gel-Fn-PCR as having a mon-infection.

LINES: 238-239: Please provide the PCR fragment sizes of all human filarial parasites known to cause blood parasitaemias in humans here.

LINES: 261-263: this belongs in the methods section

LINES: 264-265: this belongs in the results section.

LINES: 265-268: the belongs in the methods section

LINES: 268-272: Some of this sentence belongs in the methods section, some in the results section. None of it should appear in the discussion for the first time.

LINES: 287-288: This should be in the methods section.

LINES: 292-293: please clarify what statistical tests are being referred to and what definition of sufficiency is being used.

LINES: 328-331: this needs a reference or references.

LINES: 335-339: Is this really more cost-effective? I think a discussion of emergent microscope-based AI parasites identification tools is necessary here.

LINES: 341-349: The focus here needs to be on why Gel-Fn-PCR is better than conventional Fn-PCR assay this team previously developed. If it is only better in very specific situations, these situations need to be made clear. Most importantly, the authors need to discuss the automated QIAGEN electrophoresis system they use to diagnose species. How field friendly is it? Could it be replaced by an agarose gel electrophoresis system to make it more field-friendly?

LINES: 351-356: The Gel-Fn-PCR assay should have been compared to the standard Fn-PCR assay the team previously developed. This should be stated here. The differential handling of blood samples (which the authors discuss as having impacted their results) needs also to be mentioned here.

LINES: 560-565: This figure legend is less than helpful. Mentioning O. volvulus diagnosis and only O. volvulus here is very confusing. Please provide helpful information including information on the QIAGEN electrophoresis system they have used and show. More details on the QIAGEN electrophoresis system are also required in the methods section.

AUTHORS' RESPONSE TO THE REVIEWERS

RESPONSES TO THE REVIEWER'S COMMENTS AND SUGGESTIONS
06-Mar-2026

Dear Dr. TA-TANG:
Manuscript ID MIOC-2025-0337 entitled "Pan filarial-Nested PCR with gelified tubes: a new approach towards point-of-care testing for human filariasis

Running headline: "Gelified tubes for filaria molecular diagnosis" which you submitted to the Memórias do Instituto Oswaldo Cruz, has been fully evaluated by independent peer reviewers and substantial concerns were raised about the manuscript as it currently stands. These issues must be addressed before we would be willing to consider a revised version of your study.

Please be aware that the submission of a new revised version of your manuscript does not guarantee eventual acceptance, being subject to re-review before a decision is rendered.

If you are able to revise your manuscript along the lines recommended by the referees within 90 days, a list of specific responses to each of the reviewer's comments, noting where you have made such changes in the revision, is needed.

We will address all Reviewer comments and send the revised version, along with a detailed point-by-point response, within the requested timeframe and, preferably, before the 90-day deadline so that the article can be published as soon as possible.

Authors are not mandated to agree with every suggestion made by the reviewer. However, reasons for contesting any reviewer comments must be provided. If those unresolved comments appear to be fundamental to the work being described, the revision may be resubmitted to the Editors for final decision.

We will carefully analyze all Reviewer comments and provide clear and consistent responses. Where necessary, we will include detailed explanations to clarify our reasoning and the revisions made to the manuscript.

The specific response to the reviewer's comments, the new version of the manuscript, including tables and legends to figures must be submitted. If it is the case, all figures (in TIFF format 300dpi), must be uploaded again separately with legends. Please upload the new version of manuscript in the "manuscript files" section as main documents.

To revise your manuscript, log into https://mc04.manuscriptcentral.com/mioc-scielo and enter your Author Center, where you will find your manuscript under «Manuscripts with Decisions.» Under «Actions,» click on «Create a Revision.» Your manuscript number has been appended to denote a revision.

You may also click the link below to start the revision process for your manuscript. If you use the link below you will not be required to login to ScholarOne Manuscripts.

*** PLEASE NOTE: This is a two-step process. After clicking on the link, you will be directed to a webpage to confirm. ***

https://mc04.manuscriptcentral.com/mioc-scielo?URL_MASK=c9b885fd528c4a819949a0396678c5c6

You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript using a word processing program and save it on your computer. Please also highlight the changes to your manuscript within the document by using the track changes mode in MS Word or by using bold or colored text.

Once the revised manuscript is ready, you can upload and submit through your Author Center.

When submitting your revised manuscript, you will be able to respond to the comments made by the reviewer(s) in the space provided. You can use this space to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the reviewer(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Once again, thank you for submitting your manuscript to the Memórias do Instituto Oswaldo Cruz and I look forward to receiving your revision.

Sincerely,
Dr. Adeilton Brandão
Handling Editor
Memórias do Instituto Oswaldo Cruz

REVIEWER COMMENTS:

Reviewer: 1

While I can see that the presented article has been prepared by a team with a strong-track in developing molecular tools for filarial parasite detection, I am unconvinced the value of the novel Gel-Fn-PCR is adequately explained the current version of the authors 'manuscript. Thus, even though the article is written in good clear English, I feel there are a lot of presentational issues that prevent what has been done from being clearly understood. I do, however, trust that with some minor revisions the authors will be able to turn their submission into a valuable contribution to the field. Below, I have provided some suggestions as to how I think the article´s clarity could be improved by reconsidering how the results are presented and re-organizing the text. I have also highlighted specific passages of text that I think need to revised for accuracy and to improve clarity.

LINES 66-73: Here it is not made adequately clear that someone can test positive with one of the "antibody-based tests" they describe because they have been exposed to a given filarial parasite (e.g. o. volvulus), but may not have necessarily been infected with the parasite.

We thank the reviewer for this comment. We have clarified this point in the revised manuscript to better explain this limitation of serological assays. Antibody-based tests detect previous exposure and may not necessarily indicate active infection due to the persistence of circulating antibodies.

Revision in manuscript (Lines 69-72):

Antibody-based tests detect the host immune response rather than the presence of the parasite itself. Positive serology does not necessarily indicate active infection, because circulating antibodies can persist for long periods after successful treatment or parasite clearance.

LINES 106-109: Please re-write this section to make clearer the benefits of the new Gel-Fn-PCR.

We thank the reviewer for this suggestion. The objective of the study has been rewritten to better highlight the advantages of the *Gel-Fn-PCR* format. Specifically, we clarified that the gelified tube approach simplifies the nested PCR workflow by reducing reagent preparation and handling steps, facilitates standardization of the assay, lowers operational costs, and minimizes the risk of contamination, while maintaining the sensitivity and specificity of the original manual *Fn-PCR* method.

Revision in manuscript (Lines 109-118):

Therefore, the objective of this study was to convert the original manual Fn-PCR into a gelified tube format, termed gelified tube-filarial-nested PCR (Gel-Fn-PCR), by applying a gelification process to the reaction components. This modification was intended to simplify the nested PCR workflow by reducing manual preparation steps and handling, thereby facilitating assay implementation in routine laboratory settings. In addition, the gelified format aims to improve standardization, reduce the risk of contamination associated with multiple reaction preparations, and lower operational costs. Finally, the Gel-Fn-PCR was optimized and validated for the laboratory diagnosis of human filariasis.

LINES 122-123: Please provide more information on how microfilariae counts were made. How much blood was examined to make these counts?

We appreciate the reviewer's observation. Additional microscopy details have been included in the revised manuscript. The amount of blood used for microfilariae counting was approximately 20 µL and the entire thick blood smear was examined microscopically to count microfilariae. Microfilarial density was then expressed as microfilariae per milliliter of blood (mF/mL) based on the volume of blood examined.

Revision in manuscript (Lines 132-136):

Each thick blood smear contained approximately 20 µL. The entire smear was systematically screened and the number of microfilariae was recorded. Microfilarial density was subsequently calculated and expressed as microfilariae per milliliter of blood (mF/mL) according to the volume of blood examined.

Line 127: this is not a good sub-title, please revise.

We appreciate this helpful suggestion. The subtitle has been revised to better reflect the content of the section. It has been changed to "*2.2 Sample collection and preparation of dried blood spots (DBS)*" (Line 140).

LINES 163: What is the sequence of the ITS1F-BIS primer? It is not provided in the cited reference. Also, why use a different primer from that described in Tang et al. (2010) if the claim in LINES 83-84 is true?

We agree with the reviewer and have revised the manuscript accordingly. The sequence of the ITS1F-BIS primer has now been included in the revised manuscript. The primer sequence is 5′-GGTGAACCTGC**RGMW**GGATC-3′. This primer corresponds to a degenerate version of the original FIL-2F primer (GGTGAACCTGC**GGAA**GGATC). Degenerate bases were introduced to improve amplification of genetically diverse *Loa loa* isolates that were not efficiently amplified by the original primer sequence. Since the primer sequence was not explicitly reported in the previously cited reference, this citation has been removed and the primer information is now fully provided in the Materials and Methods section.

Revision in manuscript (Lines 176-180):

In addition, the FIL2F primer (GGTGAACCTGCGGAAGGATC) from the second PCR was replaced by the ITS1F-BIS primer (5'-GGTGAACCTGCRGMWGGATC-3'), a degenerate version of the original FIL-2F primer, was used in this study to improve amplification of genetically diverse Loa loa isolates.

LINES 174-178: What were the positive controls used? What other filarial parasites might conceivably be detected in the study regions. What PCR fragment sizes would be generated from their DNA? Can you provide us with the gel results so that anyone wishing to employ the method can see how diagnostic calls get made?

We thank the reviewer for these relevant questions.

Positive controls: Positive controls consisted of DBS samples previously diagnosed as positive for filarial infection by microscopy, as well as by other validated molecular diagnostic methods available in our laboratory.

What other filarial parasites might conceivably be detected in the study regions? In the samples analysed, only *Loa loa* and *Mansonella perstans* microfilariae were detected. These are among the filarial species known to circulate in the study regions. Other possible species that could potentially be detected in blood samples from Equatorial Guinea and Nigeria included *Wuchereria bancrofti*. Unfortunately (or fortunately for the people living in the region), no samples tested positive to *W. bancrofti*.

PCR fragment sizes: The *Gel-Fn-PCR* developed in this study is based on the previously described nested filarial PCR method (2010), and the gelified format does not modify the amplification targets or the expected amplicon sizes. Therefore, the fragment sizes generated correspond to those described in the original method: *O. volvulus* 344 bp, *M. perstans* 312 bp, *M. ozzardi* 305 bp, *W. bancrofti* 301 bp, *Loa loa* 286 bp.

Gel visualization: Below, an example of Qiaxcel multicapilar electrophoresis results to illustrate the diagnostic band patterns obtained with the Gel-Fn-PCR. *Note: this image has been taken from the poster presented at the 34th ECCMID celebrated in Barcelona (Spain) 27-30 April 2024.

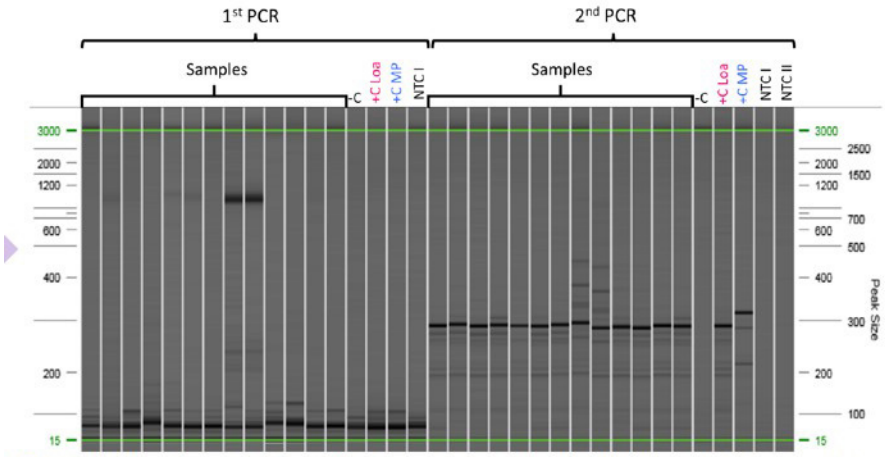


Figure 3. Visualization of amplified fragments; a band of 312 bp indicates infection with *M. perstans*, while 286 bp indicates *L. loa* infection.

In our study, PCR products were analyzed using the QIAxcel (QIAGEN) automated electrophoresis system, which provides a standardized and time-efficient alternative to conventional agarose gel electrophoresis. This platform offers higher resolution and reproducibility in fragment size determination, reducing variability associated with manual gel preparation and interpretation.

Although the cartridge-based system may involve higher initial costs, its advantages in terms of reduced hands-on time, automation, and improved consistency can offset these limitations in laboratory settings with moderate to high sample throughput.

We would like to clarify that the use of the QIAxcel system is not essential for the application of the *Gel-Fn-PCR*. The assay can also be performed using conventional agarose gel electrophoresis, which remains a suitable and more accessible option for laboratories with limited resources. This clarification has been included in the revised manuscript.

Revision in manuscript (Lines 181-188):

For each reaction performed, appropriate positive and negative controls were always included. Positive controls were DBS samples of L. loa and M. perstans diagnosed by microscopy, as well as by other validated molecular diagnostic methods available in our laboratory; negative controls were DBS samples of filarial microscopy-negative individuals from filarial endemic regions that were negative for filarial infection but could be positive or negative for other tropical parasites, such as Plasmodium spp. *All samples were run in duplicate. If the duplicates varied (one positive, one negative), the samples were rerun in triplicate.*

(Lines 211-213)

The Qiaxcel automated electrophoresis system allows rapid, standardized, and high-resolution fragment analysis. However, conventional agarose gel electrophoresis can also be used as an alternative, particularly in resource-limited settings.

LINES: 138-140 “is the requirement of basic and inexpensive” should surely be changed to something like “is the requirement of ONLY basic and inexpensive”.

Thanks for this correction. It has been changed in the revised manuscript (Line 151).

LINES 189: I recommend changing single infection (here and throughout) to single-species infections or mono-infections for clarity.

We think it is a good suggestion. In the revised manuscript “single infection” has been changed by “mono-infection” in the entire manuscript. Many thanks.

LINE 200: The subtitle needs changing; it is confusing. Are the blood samples placed on microscope slides not clinical samples?

Thanks for this objection. As in the M&M section, this subtitle has been changed to *3.2 Sample collection and preparation of dried blood spots (DBS)* in the revised manuscript (Line 238).

LINES: 208-215: this belongs in the methods section.

We respectfully disagree with the Reviewer’s suggestion. We would like to clarify that this study focuses on the development and optimization of a modified PCR format. Therefore, the experiments evaluating different concentrations of primers, enzymes, amount of DNA templates, etc were part of the optimization process and presented as M&M. However, the final conditions how the *Gel-Fn-PCR* were established must be in Results section.

The M&M section describes the general procedures, while the Results section presents the outcomes of the optimization process that led to the final standardized protocol.

To improve clarity, the section subtitle has been changed and a sentence has been introduced in the revised manuscript to present the established assay conditions rather than methodological procedures.

Revision in manuscript (Lines 246-248):

3.3.1 Final optimized conditions of the Gel-Fn-PCR assay

The following section describes the final optimized conditions of the Gel-Fn-PCR assay established after systematic evaluation of multiple reaction parameters.

LINES 219-221: While I think a table comparing the results of the two methodologies is useful, I feel the current matrix format is difficult to interpret. The current format makes it difficult to focus on the differences in diagnostic results observed between the microscopy and Gel-Fn-PCR approaches.

We thank the reviewer for this valuable comment because it has helped us to improve the manuscript. We agree that, while the full matrix provides comprehensive information, it may be difficult to quickly identify concordant and discordant results between methods.

To improve clarity, we have revised the presentation of the results by highlighting concordant results in the Table 1. This allows clearer visualization of the agreement and discrepancies between microscopy and *Gel-Fn-PCR*.

In the revised manuscript:

Table 1: Comparative results between microscopy and *Gel-Fn-PCR*

			Microscopy				
			Positives (54.61%, 361)			Negatives (45.39%, 300)	Total
			L. loa	*M. perstans*	Mixed infection		
Gel-Fn-PCR	Positives (54.92%, 363/661)	*L. loa*	**185**	0	62	16	263
		M. perstans	0	**19**	14	1	34
		Mixed infection	4	0	**61**	1	66
	Negatives (45.08%, 298/661)		13	1	2	**282**	298
	Total		202	20	139	300	661

Overall agreement between microscopy and *Gel-Fn-PCR* was observed along the diagonal of the matrix (true concordant results). Discordant results were primarily associated with differences in the detection of mixed infections and cases classified as negative by one of the methods.

In addition, a brief explanatory paragraph has been included in the Results section to guide the reader in interpreting the comparison between both methods.

Revision in manuscript (261-263)

Overall agreement between microscopy and Gel-Fn-PCR was observed mainly along the diagonal (true concordant results), whereas discrepancies were observed in cases classified as mixed infections or negative by one of the methods.

LINES 222-223: This needs to be we-written for clarity. As I understand it Gel-Fn-PCR routinely miss-reports co-infections as mono-infections. This should be made clearer, talking about detecting and not detecting makes the paper harder to follow. It would also be helpful to see gels from co-infection positive PCRs and PCR results where a co-infection is miss-reported by Gel-Fn-PCR as having a mon-infection.

We thank the Reviewer for this valuable comment. This validation section has been revised and almost entirely re-written to more clearly state that *Gel-Fn-PCR* tends to underestimate mixed infections, with some co-infections being classified as single-species infections.

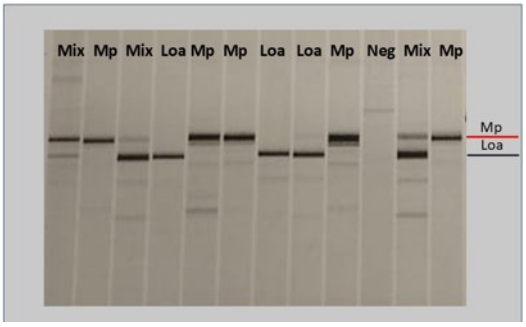
Revision in manuscript (Lines 265-291)

Microscopic diagnosis was able to detect more mixed infections, 21.03% (139/661), than the Gel-Fn-PCR, 9.99% (66/661). Most mixed infections missed by Gel-Fn-PCR (76/139) were classified as mono-infection, predominantly as L. loa (62/139) and to a lesser extent as M. perstans (14/139). To overcome these discrepancies, the 76 samples misclassified by Gel-Fn-PCR were re-analyzed by the real-time PCR (F-RT-PCR) published by Ta-Tang et al. in 2022. [28]several polymerase chain reaction (PCR Among the 62 samples classified as L. loa, 52 were confirmed, while 10 were reclassified as mixed infections, in agreement with microscopy. Among the 14 samples classified as M. perstans, 7 were confirmed, while the remaining were reclassified as mixed infections (n = 4) or L. loa (n = 3). Overall, 77.6% (59/76) of discrepant results were concordant with Gel-Fn-PCR.

The Gel-Fn-PCR showed reduced concordance with microscopy for mixed infections. Several samples identified as mixed infections by microscopy were classified as single-species infections by Gel-Fn-PCR, suggesting an underestimation of co-infections by the molecular assay.

Additionally, 13 samples positive for L. loa by microscopy were negative by Gel-Fn-PCR, whereas 16 microscopy-negative samples were positive for L. loa by Gel-Fn-PCR; all were confirmed by F-RT-PCR (Table 1).

Regarding the gel to visualize co-infection positive PCRs and mono-infection PCRs, a representative electrophoresis image obtained using the QIAxcel system has been include below. This figure illustrates both correctly identified mixed infections and cases where mixed infections may be underrepresented and interpreted as single-species infections by the *Gel-Fn-PCR*.



LINES: 238-239: Please provide the PCR fragment sizes of all human filarial parasites known to cause blood parasitaemias in humans here.

We thank the reviewer for this important comment. The expected PCR fragment sizes have now been included in the revised manuscript.

As the *Gel-Fn-PCR* is based on the previously described nested *Fn-PCR* method, the amplification targets and fragment sizes remain unchanged. Therefore, the fragment sizes correspond to those reported in the original method. Briefly, the expected fragment sizes for the main human filarial species are: *O. volvulus 344 bp, M. perstans 312 bp, M. ozzardi 305 bp, W. bancrofti 301 bp, Loa loa 286 bp.*

This information has now been explicitly added to the manuscript, with reference to the original publication.

Revision in manuscript (Lines 295-298)

Identification of the species was performed according to the size of the amplified fragments from the second PCR, as described in the original Fn-PCR method, which were 286 bp for L. loa, 301 bp for W. bancrofti, 305 bp for M. ozzardi, 312 bp for M. perstans, and 344 bp for O. volvulus.

LINES: 261-263: this belongs in the methods section

We appreciate the Reviewer's perspective; however, we consider that this part should be in discussion section to explain the discrepancies in Nigerian samples. We have reformulated the phrase to explain the potential impact of incomplete homogenization in the distribution of microfilariae that can affect the detection of the method.

Revision in Manuscript (Lines 323-327)

The lower detection of microfilariae in some samples from Nigeria may be explained by the use of venous blood to prepare DBS. If blood samples were not fully homogenized prior to spotting, microfilariae may have been unevenly distributed, leading to potential loss of parasites in the analyzed spots and consequently affecting detection sensitivity.

LINES: 264-265: this belongs in the results section.

We agree with the Reviewer and have revised the manuscript accordingly. The description of sample #171 has been revised to separate the objective observation from its interpretation. The result (failure of detection despite high microfilaremia) has been included in the Results section (Lines 292-293), while the potential explanation has been changed in the Discussion section.

Revision in Manuscript (Lines 292-293)

Unexpectedly, the sample #171 from Nigeria with 34550 L. loa mF/mL was missed by Gel-Fn-PCR, and doubtfully detected by F-RT-PCR.[28]several polymerase chain reaction (PCR

Revision in Manuscript (Lines 328-333)

The failure to detect sample #171, despite its high microfilaremia, may be related to pre-analytical factors associated with sample preparation, incomplete homogenization prior to spotting may lead to the absence of parasite DNA in the analyzed punch. This highlights a potential limitation of DBS-based approaches, where parasite distribution within the sample may not be homogeneous.

LINES: 265-268: the belongs in the methods section

We agree in part with the Reviewer and have modified the text to improve clarity. These additional PCR assays were not part of the standard methodology applied to all samples but were performed only for a single sample (#171) as confirmatory analyses due to an unexpected result. Therefore, we have retained their description in the Discusion section to reflect the case-specific investigation.

However, to improve clarity, we have added a brief statement in Materials and Methods section indicating that additional confirmatory molecular assays may be performed in selected cases when required.

Revision in manuscript (Lines 203-204)

In selected cases with inconclusive or unexpected results, additional confirmatory molecular assays were performed using previously published methods.

LINES: 268-272: Some of this sentence belongs in the methods section, some in the results section. None of it should appear in the discussion for the first time.

While we understand the Reviewer's point, we have retained this information in the Discussion section, as these additional analyses were performed exclusively for a single sample (#171) in order to investigate and interpret an unexpected result. Our intention in this section is to discuss the observed discrepancy and the steps taken to resolve it, rather than to describe standard methodological procedures applied across the study.

A general statement indicating that additional molecular methods may be used in selected cases has already been included in the Materials and Methods section. Therefore, we consider that the current structure appropriately distinguishes between general methodology and case-specific interpretation.

LINES: 287-288: This should be in the methods section.

We thank the reviewer for this comment, but we have maintained this information in the Discussion section, as it was intended to interpret the observed discrepancies between microscopy and *Gel-Fn-PCR* results rather than to describe the methodology itself.

Specifically, this statement provides a possible explanation for the discordant findings by highlighting how differences in microscopy techniques (thin versus thick blood smears) may influence diagnostic sensitivity. The general description of the microscopy methods has already been included in the Materials and Methods section. In addition, the volumes of blood used for thick and thin blood smears have now been explicitly specified in the Materials and Methods section to improve clarity (Line 129 for thin smears; Line 133 for thick smears). Therefore, we consider that its discussion in this context is appropriate.

LINES: 292-293: please clarify what statistical tests are being referred to and what definition of sufficiency is being used.

We agree with the Reviewer that this sentence is confusing. This sentence has been revised to explicitly refer to the statistical parameters calculated (sensitivity and specificity) and to avoid the use of the term "sufficient," which may be ambiguous. We have also incorporated reference to the 95% confidence intervals to better support the robustness of the results.

Revision in manuscript (Lines 359-363)

According to statistical analysis, the Gel-Fn-PCR demonstrated high sensitivity and specificity for the detection of mono-infections with 95% CI indicating consistent performance. These results support the reliability of the assay for diagnosing single-species filarial infections when compared with microscopy as the reference standard.

LINES: 328-331: this needs a reference or references.

Thank you for this note. We have now included appropriate references to support this statement (references 45–47 in the revised manuscript).

LINES: 335-339: Is this really more cost-effective? I think a discussion of emergent microscope-based AI parasites identification tools is necessary here.

We respectfully disagree with the Reviewer's suggestion. On one hand, the cost-effectiveness of the *Gel-Fn-PCR* primarily reduce reagent handling, simplified workflow, and operational efficiency compared to the conventional Fn-PCR.

On the other hand, emerging AI-based microscopy tools, while we acknowledge their relevance in the field of parasitological diagnosis, these approaches fall outside the scope of the present study, which is focused on the development and optimization of a molecular diagnostic method. Therefore, we have not included a detailed discussion of these technologies in discussion section.

LINES: 341-349: The focus here needs to be on why Gel-Fn-PCR is better than conventional Fn-PCR assay this team previously developed. If it is only better in very specific situations, these situations need to be made clear. Most importantly, the authors need to discuss the automated QIAGEN electrophoresis system they use to diagnose species. How field friendly is it? Could it be replaced by an agarose gel electrophoresis system to make it more field-friendly?

We thank the reviewer for this important comment. The Conclusions section has been revised to more clearly highlight the advantages of the *Gel-Fn-PCR* compared to the original *Fn-PCR* assay. Specifically, we now emphasize that the gelified format simplifies the nested PCR workflow by reducing reagent preparation and handling steps, improves standardization, and decreases the risk of contamination, while maintaining comparable diagnostic performance for single infections.

We have also clarified that the advantages of the *Gel-Fn-PCR* are particularly relevant for routine laboratory settings, whereas its lower sensitivity for mixed infections may limit its use in specific epidemiological contexts.

Regarding electrophoresis, we have clarified that although an automated QIAxcel (QIAGEN) system was used in this study to standardize fragment analysis, it is not required for the application of the method. The *Gel-Fn-PCR* can be performed using conventional agarose gel electrophoresis, making it adaptable to laboratories with more limited resources. This point has been explicitly included in both the Methods and Discussion sections.

Revision in manuscript (413-429)

The Gel-Fn-PCR represents an optimized adaptation of the previously developed conventional Fn-PCR, maintaining comparable sensitivity and specificity for the detection of single filarial infections while offering important operational advantages. In particular, the gelified format simplifies the nested PCR workflow by reducing reagent preparation and handling steps, improving assay standardization, and lowering the risk of contamination associated with multi-step procedures. These features make the Gel-Fn-PCR more practical and cost-effective for routine laboratory use. Sensitivity for mixed infections was low, though specificity remained high. While the assay was suboptimal for detecting mixed infections, particularly when one species was predominant, it performed effectively for single-species detection. Overall, the Gel-Fn-PCR is particularly advantageous in laboratory settings requiring simplified workflows and standardized procedures, while retaining the diagnostic performance of the original Fn-PCR. The Gel-Fn-PCR demonstrated limitations as a deployable POCT in resource-limited areas; nonetheless, this study highlighted the feasibility of integrating gelification technology with nucleic acid-based assays. It underscores the potential of using gelified tubes as a platform for further development of multiplexed and field-adaptable molecular tools.

LINES: 351-356: The Gel-Fn-PCR assay should have been compared to the standard Fn-PCR assay the team previously developed. This should be stated here. The differential handling of blood samples (which the authors discuss as having impacted their results) needs also to be mentioned here.

We thank the reviewer for this important comment. A direct comparison between the *Gel-Fn-PCR* and the original *Fn-PCR* assay was performed during the initial phase of the study as part of the optimization process. A subset of samples was analyzed in parallel using both methods to confirm that the gelified format yielded comparable results to the standard *Fn-PCR*.

Once equivalence between both approaches was established, subsequent analyses were carried out using the *Gel-Fn-PCR* assay. For the evaluation of diagnostic performance in a larger sample set, microscopy was used as the reference method, as it represents the routine diagnostic standard in the study settings.

We agree that this point was not sufficiently explained in the original manuscript. The text has been revised to clarify that an initial parallel comparison was performed and to explain the rationale for using microscopy as the reference method in the full dataset.

Revision in manuscript (Lines 191-196)

In the course of the initial optimization phase, a subset of samples was analyzed in parallel using both the original Fn-PCR and the Gel-Fn-PCR to confirm the equivalence of the two approaches. Once comparable

performance was established, subsequent analyses were performed using only the Gel-Fn-PCR assay. For the evaluation of diagnostic performance in the full dataset, microscopy was used as the reference method, as it represents the routine diagnostic standard in the study settings.

Furthermore, a new limitation has now been included in the revised manuscript in *6. Study limitations* (Lines 434-436)

A limitation of this study was the comparison between Gel-Fn-PCR and the original Fn-PCR only performed on a limited subset of samples during the optimization phase, rather than across the entire dataset.

LINES: 560-565: This figure legend is less than helpful. Mentioning O. volvulus diagnosis and only O. volvulus here is very confusing. Please provide helpful information including information on the QIAGEN electrophoresis system they have used and show. More details on the QIAGEN electrophoresis system are also required in the methods section.

We thank the reviewer for this comment and agree that the figure legend was not sufficiently clear.

The legend has been revised to clarify that the asterisks refer to differences in the workflow depending on the type of sample analyzed. Specifically, samples obtained from skin snips (for the detection of *Onchocerca volvulus*) follow a different processing workflow compared to blood samples used for the detection of blood-dwelling filarial parasites. This distinction has been explicitly clarified by changing the legend of Figure 1 to avoid confusion.

Revision in manuscript (Lines 643-647)

Workflow of the Gel-Fn-PCR assay for the detection of filarial parasites. Asterisks (*) indicate steps that differ when the type of clinical sample are skin snip samples used for the detection of Onchocerca volvulus, a modified workflow is applied compared to the procedure used for blood samples targeting blood-dwelling filarial species (e.g., Wuchereria bancrofti, Loa loa and Mansonella perstans).

In addition, further details on the QIAGEN electrophoresis system have been included in the Methods section to improve reproducibility and clarity.

SECOND REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

I am now satisfied that the authors have addressed all the concerns I raised in my first review of their work. I look forward to seeing their work published in the near future and wish them well!

DECISION: ACCEPT SUBMISSION | RECEIVED 15 NOVEMBER 2025 | ACCEPTED 05 MAY 2026