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Preliminary comparison between an in-house real-time PCR vs microscopy for the diagnosis of *Loa loa* and *Mansonella perstans*

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ABSTRACT

Infections with the filarial nematodes *Loa loa* and *Mansonella perstans* are among the most neglected filarial infections. *L. loa* is endemic in 11 countries of Central and West Africa and loiasis is estimated to affect about 20 million people. *M. perstans* infection is widespread in more than 30 countries of sub-Saharan Africa. Due to the difficulty in diagnosing loiasis and *M. perstans* mansonellosis on a clinical basis, the diagnosis of infection with *L. loa* and *M. perstans* relies on laboratory techniques. Definitive diagnosis is based on the detection, identification, and quantification of circulating microfilariae (mf) by microscopy of concentrated blood. However, this is impractical for screening purposes as it requires expert laboratory personnel, considerable blood manipulation, and is time consuming, especially for the final issue of negative result reports, which are very common in the population visited outside endemic areas. The aim of the current work is the preliminary evaluation of the performance of the in-house real-time PCR described by Ta and colleagues compared to the routine microscopic approach for the screening of filarial infections in the clinical setting outside endemic areas, using samples from patients accessing the dedicated outpatient clinics for migrants and travelers of a reference centre for tropical diseases in Northern Italy.

1. Introduction

Infections with the filarial nematodes *Loa loa* and *Mansonella perstans* are among the most neglected filarial infections. *L. loa* is endemic in 11 countries of Central and West Africa and loiasis is estimated to affect about 20 million people (Metzger and Mordmüller, 2014; Molyneux, 2009). *M. perstans* infection is widespread in more than 30 countries of sub-Saharan Africa, while sporadic cases have been reported in Latin America, mostly in the Caribbean and along the Atlantic coast. Infection with *M. perstans* probably affects around 20% of inhabitants of endemic countries, with 114 million people estimated to be infected (Simonsen et al., 2011; Ta-Tang et al., 2018).

Similar to other filarial nematodes affecting humans, such as the most known and studied agents of lymphatic filariasis (*Wuchereria bancrofti* and *Brugia* spp), both nematodes are vector-borne, being transmitted through the bite of tabanid flies of the genus *Chrysops* in the case of *L. loa*, and of the genus *Culicoides* for *M. perstans*. Adult parasites reside in the subcutaneous tissue (*L. loa*) and serosal cavities

Abbreviations: CI, confidence interval; EDTA, Ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assays; IQR, Inter-Quartile Range; ITS1, internal transcribed spacer one; LAMP, loop-mediated isothermal amplification; mf, microfilariae; PCR-RFLP, PCR-restriction fragment length polymorphism; qPCR, real-time PCR or quantitative PCR; Tm, melting temperature.

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(*M. perstans*), therefore they are not accessible for the diagnosis of infection. The progeny, microfilariae (mf), circulate in the peripheral blood, where they are picked up by vectors during their blood meal and are accessible for the diagnosis by microscopy of the peripheral blood. However, infections with *L. loa* and *M. perstans* are extremely elusive. The main specific clinical manifestations of loiasis are transient subcutaneous edema named "Calabar swelling", and "eyeworm", i.e. the visualization of a worm migrating through the conjunctiva (Boussinesq, 2006), however these are not always present and patients are often asymptomatic or present unspecific complaints. Considered for a long time a benign disease, high *L. loa* microfilaraemia has been recently associated with an increased mortality risk (Eyebe et al., 2018). In the case of mansonellosis, the definition of clinical manifestations is even more difficult, symptoms being non-specific (including subcutaneous edema, rash, abdominal pain, and eosinophilia) and shared with other co-infections commonly occurring in affected people, and quantification of associated health consequences on human populations are still lacking (Simonsen et al., 2011).

Outside endemic areas, where infection is imported and the goal is to treat all infected patients, the accurate diagnosis of all potentially infected subjects coming from endemic areas, such migrants, expatriated and tourist travelers, is pivotal. Due to the difficulty in diagnosing loiasis and *M. perstans* mansonellosis on a clinical basis, the diagnosis of infection with *L. loa* and *M. perstans* relies on laboratory techniques. Antigen-detection tests are not available for these filarial species, and serossays for the detection of antibodies are available, but cannot reliably distinguish between current and past infection and between filarial species, and cannot quantify mf burden, which are important information to guide clinical management. Therefore, definitive diagnosis is based on the detection, identification, and quantification of circulating mf by microscopy of concentrated blood (Antinori et al., 2012; Asio et al., 2009; Boussinesq, 2006; Fink et al., 2011; Touré et al., 1997). However, this is impractical for screening purposes as it requires expert laboratory personnel, considerable blood manipulation, and is time consuming, especially for the final issue of negative result reports, which are very common in the population visited outside endemic areas. In addition, mf of different species have different circadian periodicity, therefore at least two blood sampling time points, one by day and one by night, are required to diagnose filarial infections when strong clinical and/or geographic orientation is absent, which is extremely impractical and sometimes unfeasible in the clinical setting. Finally, the lack of identification of circulating mf cannot be used as a sure sign of absence of adult worms, as in loiasis microfilaraemia may be undetectable by microscopy-based techniques, especially in early infection in up to 80% of cases (Gobbi et al., 2019) and amicrofilaraemic *M. perstans* infections have been also reported (Cobo et al., 2015).

In this scenario, the implementation of high-throughput molecular techniques able to diagnose low-burden or mf-negative infections, and independently from the circadian mf periodicity, for the screening of subjects coming from endemic areas would be particularly helpful. Both conventional and real-time or quantitative PCR (qPCR) assays have been developed for the blood-based diagnosis of filarial infections, although none is commercially available (Fischer et al., 2000; Hassan et al., 2005; Klüber et al., 2001; McCarthy et al., 1996; Ta et al., 2018; Tang et al., 2010) . Nuchprayoon et al (Nuchprayoon et al., 2005) developed PCR-restriction fragment length polymorphism (PCR-RFLP)-based method to detect and differentiate a broad range of filarial species in a single reaction, which in theory is particularly appealing for screening purposes, but not easily applicable in a routine diagnostic lab setting. To overcome this shortcoming, Tang et al (Tang et al., 2010), designed a nested PCR able to amplify the internal transcribed spacer one (ITS1) of the nuclear ribosomal gene common to all filarial species, obtaining amplicon sizes different enough to allow differentiating the three human sympatric filariae known to inhabit the Amazon Region of Brazil, to be applied in filarial control programs in this area. Based on this protocol, Ta and colleagues (Ta et al., 2018) could detect infections with *Onchocerca volvulus, L. loa, M. streptocerca* and *M. perstans* in mf-microscopy negative samples from inhabitants Bioko Island (Equatorial Guinea) where an onchocerciasis control program was in place. In a similar way, but with immigrant patients from sub-Saharan Africa residing in a non-endemic country, Italy, it has been performed in this study also comparing with microscopy, unlike the study of Ta et al., 2018.

The aim of the current work is the preliminary evaluation of the performance of the in-house real-time PCR described by Ta and colleagues (Ta et al., 2018) compared to the routine microscopic approach for the screening of filarial infections in the clinical setting outside endemic areas, using samples from patients accessing the dedicated outpatient clinics for migrants and travelers of a reference centre for tropical diseases in Northern Italy.

2. Materials and Methods

2.1. Study setting and design

This is an exploratory, retrospective study conducted on 59 human blood samples collected between 2017 and 2018 obtained pre-treatment from patients coming from Sub-Saharan Africa (Guinea Bissau, Nigeria, Ghana, Cameroon, Senegal, Cote d'ivoire, Chad) with positive filarial serology and available in the biobank of the Department of Infectious-Tropical Diseases and Microbiology (DITM) of IRCCS Sacro Cuore Don Calabria Hospital, Negrar, Verona, Italy. The study was approved by the local competent Ethics Committee, Comitato Etico per la Sperimentazione Clinica delle Province di Verona e Rovigo, protocol number 39173.

In this centre, in the last 25 years, more than 100 cases of loiasis and of *M. perstans* infections have been diagnosed (Gobbi et al., 2017, 2014).

At DITM, screening for filarial infections on migrants and travelers from endemic areas is carried out by serology using a commercial enzyme linked immunosorbent assays (ELISA) test (Bordier®) based on an antigenic preparation from *Acanthocheilonema vitae* that detects IgG against various filarial nematodes affecting humans. In the presence of positive serology, hypereosinophilia (> 300 eosinophil/μl) and/or signs or symptoms suggestive of filarial infection (e.g. eyeworm or Calabar swelling in the case of loiasis), and based on the geographical origin of the patient, the modified Knott concentration technique is carried out on diurnal and/or nocturnal 9 ml blood samples, followed by Giemsa-stained thick blood smears and microscopy for species identification. Intensity of infection is expressed as microfilariae per milliliter (mf/ml) of blood and species identified based on morphology of mf observed at 100 x magnification under oil-immersion.

Demographic, clinical, and laboratory results of routine tests of the patients from whom samples were used for this study were retrieved from the electronic database of DITM.

2.2. DNA extraction, real-time PCR and species identification

For each patient a sample of 500 μl of blood in ethylenediaminetetraacetic acid (EDTA) was stored at - 20°C until analysis; DNA extraction was performed using the automatic DNA extractor MagnaPure LC2 (Roche), using the large volume protocol. The blood volume for analysis was chosen based on the available stored samples (500 μl). A multiplex-pan filarial real-time PCR targeting ITS1 was applied, as described by Ta el al (Ta et al., 2018), but with some minors modifications in the sequence of the primers as well as the annealing temperature, changed from 50°C to 62°C. The modifications performed in the primers (in bold) and expected size of the amplified fragment are detailed in Table 1. For each real-time PCR run appropriate positive *M. perstans, L. loa* and negative controls were included. Positive controls were "artificial controls" constructed with primers which amplified a large fragment used as product for the primers of the multiplex-pan filarial real-time PCR, as well as filariae-positive clinical samples,

Table 1
Name and sequence of the oligonucleotides used in the Multiplex-pan filarial qPCR to detect simultaneously human filariasis, and size of the amplified product.

Oligonucleotide name (original primers published in Ta et al., 2018)	Oligonucleotide sequence 5'-3'	Amplified fragment size (bp)
ITS-F Bis (FIL 2-Loa) JM-F-022R M-B (FIL2-R) JM-F-0022R L-O-D (FIL2-R)	GTGAACCTGCRGMWGGATC (**G**GTGAACCTGCRGMWGGATC) **TAGCATAAA**TGCTTATTAAGTCTACTTAA (TGCTTATTAAGTCTACTTAA) **TAGCTAAAA**TGCTTATTAAGTCTACTTAA (TGCTTATTAAGTCTACTTAA)	O. *volvulus*: 352 bp *M. perstans*: 320 bp *M. ozzardi*: 313 bp *W. bancrofti*: 310 bp, *L. loa*: 294 bp

isolated in the same and used the same volume in the qPCR way were also used; negative controls were filariae-negative samples from healthy persons came from filarial-endemic regions who are negatives for filarial infection but could be positive or negative for other tropical parasites, such as *Plasmodium* spp. In summary, in each real-time PCR run performed, five controls were always included: three clinical samples (one positive by *L. loa*, one positive by *M. perstans* and one negative by filarial parasites), and two amplicons (one *L. loa*-positive and one *M. perstans*-positive). In order to evaluate the correct DNA extraction, as internal control, we amplified the β-actin housekeeping gene.

The CFX96 Touch Real-Time PCR detection system (Biorad) was used for DNA amplification, starting with 3 min at 95°C, followed by 45 cycles of 5 s at 95°C and 30 s at 62°C.

Positive samples were determined by post reaction analysis of the melting temperature (Tm) curve of the amplified fragments, built by stepwise temperature increases of 0.5°C from 60°C to 95°C with fluorescence acquisition at each temperature transition. Species identification based on amplified product size was performed on the Tape Station, High Sensitivity D1000 (Agilent). The DNA of the discordant samples between microscopy and qPCR were re-analyzed and confirmed at the Malaria & Emerging Parasitic Diseases Laboratory, National Microbiology Center, Instituto de Salud Carlos III, Madrid, Spain.

2.3. Data analysis

Data analysis was performed using SAS software version 9.4. Collected data were summarized using descriptive statistics and estimated parameters reported with 95% confidence intervals. The diagnostic performance of the multiplex-pan filarial real-time PCR on blood samples was expressed as sensitivity and specificity calculated using microscopic examination as the reference test.

3. Results

A total of 59 blood samples from patients with a positive filarial serology, were assessed by microscopic examination of diurnally/nocturnally drawn blood and by multiplex-pan filarial real-time PCR Details on the serology used can be find in the paper published by Gobbi et al., (Gobbi et al., 2019).

Eleven (18.64%) patients were females and 48 (81.36%) males, with a median age of 27 years (Inter-Quartile Range – IQR- 23-38) and of 26 years (IQR 20.5-30.5), respectively. Sixteen patients (27.12%) had detectable mf on microscopy, 3/16 (18.75%) of *L. loa* and 13/16 (81.25%) of *M. perstans*, with median mf/ml loads of 10.02 (IQR 1.55-69.42).

A total of 13 (22.03%) samples were positive by qPCR, three [23.08%] identified as *L. loa* (all from the *L. loa* mf-positive patients) and 10 as *M. perstans* (9/13 [69.23%] from mf-microscopy positive patients and one [7.69%] from a mf-microscopy negative patient) (Table 2).

Table 2
Multiplex-pan filarial real-time PCR vs Microscopy considered as reference method.

		Microscopy Positive	Negative	Total
Multiplex-pan filarial qPCR	**Positive**	12	1	13
	Negative	4	42	46
	Total	16	43	59

The species identification was 100% concordant between molecular analysis and microscopy, there were not any discordancy at the level of filarial species identification. All the patients microscopically diagnosed as *L. loa*, were detected as *L. loa* by multiplex-pan filarial qPCR, likewise it occurred with *M. perstans* mf-positive patients.

Compared to microscopy, therefore, the multiplex-pan filarial real-time PCR had 75% (95% CI, 53.78%-96.22%) sensitivity and 97.67 % (95% CI, 87.71%-99.94%) specificity. For a more detailed overview of statistical values of the multiplex-pan filarial real-time PCR are described in Table 3.

These statistical values were calculated for the two filarial species together.

However, if statistical values were calculated separately for each species, sensitivities and specificities are the following as reflected in Table 4:

Summarizing, the multiplex-pan filarial real-time PCR has a high specificity and sensitivity for detecting *L. loa* species, but a poor sensitivity and a high specificity for amplifying *M. perstans* parasites.

Of the four samples that were microscopy-positive but qPCR-negative, the mean microfilarial load was 0.27 mf/ml (range: 0.14–0.61), while the mean mf load of microscopy and PCR-positive samples was 117.26 (range 2.5-511.56) (Table 5). Among the 4 samples with a median mf load of 0.16 per 500 ul, there was 1 sample with a higher mf load compared to the median (0.30 microfilariae per 500 ul).

Moreover, we evaluated if there was an association between the serological values and the qPCR results. To do so, we stratified the ELISA results in four categories based on the serological values obtained: 1) 1-1,5. 2) 1,6-2. 3) 2,1-3 4) >3 (Table 6). Among the 59 ELISA positive samples, we only had 13 positive samples at the qPCR and we could not find any association between these values (Fisher's exact test, p-value=0.2784). Moreover median values among the positive (2.3, Q1=1.7, Q3=2.4) and negative (1.8, Q1=1.2,Q3=2.5) groups did not differ (Wilcoxon test, p-value=0.2429).

4. Discussion and conclusion

Accurate diagnosis of infection with filarial nematodes can be challenging; infections may be asymptomatic, or present with non-specific signs and symptoms, and currently available diagnostic tests have shortcomings and suboptimal performances (Boussinesq, 2006; Hassan et al., 2005; Klüber et al., 2001; McCarthy et al., 1996). Outside endemic areas, the accurate diagnosis of all infected patients, independently from clinical manifestations, is particularly important as individuals cannot get re-infected, therefore the aim of treatment is parasitological cure

Table 3
Statistical values obtained for the qPCR method using microscopy as the reference method.

	Multiplex-pan filarial real-time PCR
Sensitivity % (95% CI)	75% (53.78%-96.22%)
Specificity % (95% CI)	97.67% (87.71%-99.94%)
PPV % (95% CI)	92.31% (66.69%-98.63%)
NPV % (95% CI)	91.3% (79.68%-96.57%)
Kappa index % (95% CI)	77.22% (58.1%-96.3) good agreement

PPV: positive predictive value. NPV: negative predictive value. CI: confidence interval

Table 4
Statistical values obtained for the qPCR method for each filarial species separately.

	Multiplex-pan filarial real-time PCR for *L. loa*	Multiplex-pan filarial real-time PCR for *M. perstans*
Sensitivity % (95% CI)	100% (100%-100%)	69.2% (44.1%-94.3%)
Specificity % (95% CI)	100% (100%-100%)	97.8% (93.6%, 102.0%)
PPV % (95% CI)	100% (100%-100%)	90.0% (71.4%, 108.6%)
NPV % (95% CI)	100% (100%-100%)	91.8% (84.2%, 99.5%)
Kappa index % (95% CI)	100% (100%-100%) very good agreement	73.1% (50.6%-95.7%) good agreement

PPV: positive predictive value. NPV: negative predictive value. CI: confidence interval

Table 5
Description of the four discordant samples (microscopy-positive and real-time PCR-negative).

Sample_ID	Microfilariae/ml	Microfilariae/500µl
8	0.14	0.07
2922	0.15	0.07
1800	0.18	0.09
5	0.61	0.30

Table 6
qPCR results VS Filarial ELISA results stratified per antibody titer.

Multiplex-pan filarial qPCR	Serology				
Serology values	1-1.5	1.6-2	2.1-3	>3	Total
Negative	19	13	9	6	47
Positive	2	3	5	2	12
Total	21	16	14	8	59

rather than morbidity control and reduction of the worm burden (Antinori et al., 2012; Gobbi et al., 2017, 2014). While positive serology may be a sensitive tool for the screening of patients from countries where filarial infections are endemic, limited data are available on the correlation between positive serology and current infection (Gobbi et al., 2019; Kwan-Lim et al., 1990). Besides the obvious risk of over-treatment if therapy is administered to all individuals with anti-filarial positive serology, no such "one-size-fits-all" approach exists for curative (i.e. macrofilaricidal) treatment of all filarial infections. Thus, both "general" diagnosis of filarial infection and identification of the involved species is required for the correct clinical management of patients.

Serology followed by microscopy in case of seropositivity is the diagnostic algorithm currently applied in our center for the diagnosis of filarial infections in patients from endemic areas (Gobbi et al., 2019). However, a general appraisal of results from the routine diagnostic activity of our centre of the past 25 years evidenced that around 70% samples positive for anti-filarial antibodies were microscopy-negative (Gobbi et al., 2017, 2014). Considering that a report of negativity can be issued only after performing thorough analysis of all concentrated material from all drawn blood, screening of blood samples from all serology-positive patients is particularly labor-intensive and time-consuming. Furthermore, microscopy must be carried out within a short time from blood collection, implying the physical presence or relative proximity of the patient and the diagnostic laboratory. Thus, a more automated, high-throughput method, such as PCR, for the triage of patients with active infection on which to perform microscopy is required.

In our retrospective study, the investigated multiplex-pan filarial real-time PCR was 100% concordant with microscopy in the identification of filarial species and reached an overall sensitivity of 75%, proving very promising. However, it missed four samples that were positive by microscopy, clearly showing that the technique requires further optimization. Since these samples had an extremely low mf load (0.14-0.61 mf/ml), meaning a theoretical presence of 0.13 mf in 500 ul of blood from which the DNA was extracted, further evaluation of the performance of the real-time PCR starting from a larger blood volume, either whole or after concentration of the cellular elements by centrifugation, is worth, to try increasing the sensitivity of the assay.

Of note, one sample was PCR-positive for *M. perstans* but mf could not be detected by microscopy. This patient came from Nigeria and presented hypereosinophilia and was considered as a probable case of occult mansonellosis. Although sequencing was not carried on the amplicon, the high specificity of the technique (Ta et al., 2018; Tang et al., 2010), and the results of control analyses (appropriate *L. loa* – *M. perstans* positive controls and β-actin amplification as DNA extraction control), could exclude a false-positive result. Therefore, merging the ELISA results and the evidence published by Gobbi et al (Gobbi et al., 2019), it can be assumed that infection with *M. perstans* was actually present in this patient. We did not find any association between the serological values and the qPCR results; indeed, we got a Fisher's exact test, with a p-value=0.2784. Furthermore, median values among the positive (2.3, Q1=1.7,Q3=2.4) and negative (1.8, Q1=1.2,Q3=2.5) groups did not differ (Wilcoxon test, p-value=0.2429).

While the results of Ta and colleagues (Ta et al., 2018), who detected filarial infections by *O. volvulus, M. streptocerca, L. loa* and *M. perstans* in 2% mf-negative skin snips in Bioko Island (Equatorial Guinea) individuals, may suggest that detection of circulating DNA from adult worms could be detected, it remains to be clarified whether in that study and in the present cohort, microcopy-negative qPCR-positive samples reflected truly non-patent infections, (i.e. with presence only of adult worms but not mf), or were patent infections with an extremely low mf load, which could be captured in the blood aliquot used for qPCR but not in the volume examined by microscopy. As the primers used here are designed to amplify also sequences from filarial parasites that hardly ever develop patent infection in humans, such as *Dirofilaria* spp, evaluation of the performance of this assay on samples from confirmed patients with *Dirofilaria* spp nodules could shed light on this point.

The relatively recent molecular tool loop-mediated isothermal amplification (LAMP) emerged in the year 2000 (Notomi et al., 2000) as an alternative to qPCR. This novel technique has not been considered in our study because at the time of the study, the LAMP methods available to diagnose human filarial infections identified separately each one of the human filarial species, leading to perform a test for each species of filariae, thus making the clinical diagnosis more expensive. Consequently the multiplex-pan filarial real-time PCR was preferred.

To conclude, our preliminary study in a clinical setting outside endemic areas indicate that the multiplex-pan filarial real-time PCR could be a very useful tool to integrate in the screening flowchart of patients coming from countries endemic for different filarial infections, and encourage further efforts to optimize the technique. Furthermore, further work is warrant to clarify whether this method can detect real non-patent infections.

Limitations of the study: The present study was conducted on a limited samples, thus, the findings may not be generalized to say the multiplex-pan filarial real-time PCR method has a high sensitivity and specificity to diagnose filarial infections, since some *M. perstans*-microscopy positive samples were undiagnosed. Additionally, the lack of higher blood volume availability did not allow testing the method with largest initial blood volume.

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Availability of data and materials

All data generated or analyzed during this study are included in the article.

Authors' contributions

F.F., T.H.T-T, C.P., F.P., F.G. and J.M.R designed the research, F.F., T. H.T-T., G.L.M., E.M.E. and B.P. carried out the research and performed data processing and analyses, R.S. performed the statistical analysis. F. F., T.H.T-T and F.T. drafted the manuscript with input from all other authors. All authors reviewed the manuscript prior to submission.

Consent for publication

Not applicable

Declaration of Competing Interest

The authors declare no competing interests.

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