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Performance of a RT-PCR for the Diagnostic of Loiasis in an Endemic Area in Gabon

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Abstract

Loa loa infection is a growing public health issue in the endemic area where two-thirds of infected individuals have no detectable circulating microfilaræmia. Conventional microscopic-based diagnosis on the visualisation of the filarial worm is limited. Molecular tests are known to be sensitive, precise and fast. Here we evaluated the performance of a Real Time PCR assay for the diagnostic of *Loa loa* infection in blood sample from an endemic area of Gabon. Blood samples were analyzed by microscopy. Proven loiasis was defined by a positive conventional parasitological assay and subconjunctival migration of an adult worm. DNA from blood samples was extracted and tested by RT-PCR targeting the gene coding the *Loa loa*-15 kDa polyprotein antigen. Microscopic analyses identified 25/545 (4.59%) microfilaremic individuals. Thirty (30/545) samples were RT-PCR positive. According to the classification of infection cases, 26 individuals were positive for proven loiase and 4 individuals for non-loiasis; the test showed a sensitivity of 15.90% (95% CI [12.6 - 17.75]) and a specificity of 99.0% (95% CI [97.6 - 99.7]) for the diagnosis of proven loiase. The evaluated RT-PCR targeting the 15 kDa gene protein detected all microfilaremic cases but only a few amicrofilaremic ones. It is specific to *Loa loa* infection and might be used for the screening of at-risk populations in epidemiological and/or pretreatment surveys.

Keywords

Loiasis, Specificity, Microscopy, Antigen, Sensibility

1. Introduction

Loiasis is a neglected infectious disease that is caused by the filarial nematode parasite called *Loa loa* worm. The disease is also known as African eye worm. The filarial is transmitted to humans by tabanid flies belonging to the genus Chrysops commonly known as deer flies, mango or mangrove flies [1]. *Loa loa* is endemic in eleven African countries based on the prevalence of eye worm history through a Rapid Assessment Procedure for Loiasis (RAPLOA [2]. Symptoms of loiasis are mild (Calabar edema, pruritus, worm migration in the eye). However, severe adverse events (SAE) were recorded after treatment of individuals with high load *of Loa loa* microfilaremia (>30,000 microfilariae per millilitre, mf/ml) with ivermectin (IVM) or diethylcarbamazine (DEC) in regions where loiasis is co-endemic with onchocerciasis and/or lymphatic filariasis (LF) [3]-[6]. In endemic areas of loiasis, two-thirds of infected individuals do not have circulating microfilaria (amicrofilaremic individuals) [7]. Also, there are individuals with occult infection (individuals with ocular worm but without a microfilariae in their blood). These realities of *Loa loa* infection make the diagnostic difficult. Currently, in endemic areas the diagnostic of the disease is made by morphological identification of microfilariae in the blood using a light microscope [8]; this does not identify individuals without circulating microfilaria in their blood. Moreover, this method is tedious, requires expertise and takes a long time to identify the parasite. In order to overcome the problem, several alternative methods to improve the diagnosis of loiasis have been evaluated. Some serological tests were developed [9] [10], but they are not always effective, due to cross-reactions caused by co-infections between parasites. In fact, co-endemicity between filarial species is common in endemic regions [11]-[13]. As a result, accurate and efficient diagnostic methods for parasite detection are needed. Several polymerase chain reaction (PCR) techniques have been developed to detect *Loa loa* infections in humans from endemics and non-endemics regions, with a high degree of accuracy in detecting microfilaremics, amicrofilaremics and occult infections [14]-[16]. In fact, in 1997, a PCR targeting the specific 15 kDa *Loa loa* gene was developed for the diagnostic occult *L. loa* infections in blood sample from a *L. loa* endemic area [17]. Results showed that DNA was detected in all *L. loa* microfilaremic individuals and the quarter of the amicrofilaremic subjects. A year later, a team of researchers developed a nested PCR based on the same sequences of the gene (15 kDa). About 19 of 20 occult-infected and 23 of 30 amicrofilaremic samples that were positive [18]. More recently, three different PCR-based methods to detect *Loa loa* and *Mansonella perstans* have been compared. A quantitative PCR targeting the internal transcribed spacer one (ITS1) of the nuclear ribosomal gene of all filarial species, a Filaria-

Nested PCR designed from highly conserved regions of filarial 18S, 5.8S rDNA and ITS. The third PCR (COI PCR) is designed on the basis of regions of mitochondrial cytochrome oxidase I (COI) gene [14]. Results showed that q-PCR is more sensitive and specific compared to microscopy. It was able to detect a wide range of human filalariae compared to the other two methods. The aim of the study here is to evaluate the performance a Real Time-PCR targeted on the repeat 3 region of the gene coding for the *L. loa* 15-kD protein (15r3) for the diagnostic of *Loa loa* infection in an endemic area in Gabon.

2. Methods

2.1. Study Location and Population

The sampling was done in three different sites of the Haut-Ogooué region in the southeast of Gabon; Franceville, Moanda and Mvengue. Samples were collected from May to July and October to December 2018.

Participants from 10 to 70 years of age, with or without loiasis symptoms, have been enrolled for the study. All the participants or parents/guardians of children provided informed consent to participate in the study. Relevant information was collected: demographic data (age, gender); clinical presentation data, with past or actual eye worm migration, Calabar swellings, and pruritus. This study was approved by the National Ethics Committee of Gabon (PROT N00001/20/6/3/SG/CNE).

2.2. Blood Collection

Peripheral blood samples collected from each participant were used for microscopic and PCR analyses. Field laboratory facilities were set up in healthcare centers in each area of the three study sites. Blood was collected between 9 AM to 2 PM in 4 ml EDTA tubes. The wet blood (10 µl) was directly used for examination under light microscope. Blood sample was then centrifuged at 10,000 g for 3 minutes. Plasma was separated from blood pellet and each were stored at −20˚C for further analyses.

2.3. Conventional Parasitological Assays

Conventional parasitological diagnosis was based on the detection of *L. loa* microfilariae via direct examination of 10 µl of whole blood under light microscope (×100 magnification). May-Grunewald Giemsa stained thin blood films allowed filariae species identification, according to key morphological features [8], and the quantification of the microfilaremia (microfilaria count per ml of blood). For patients presenting with current subconjunctival migration of an adult worm, the adult worm was removed and kept in the physiological serum and kept at −20˚C for later use.

2.4. Clinical Case Definition of *Loa loa* Infection

We classified individuals in 3 groups: 1) Proven loiasis, defined by a positive con-

ventional parasitological assay (microfilaremia (microscopy) and/or subconjunctival migration of an adult worm); 2) Suspected Loa-infection group define by the presence of clinical symptoms as Calabar swelling. 3) Non-Loa infection group define by absence of microfilaremia, absence of ocular adult worm and absence of Calabar swelling symptoms.

2.5. DNA Extraction from Blood Samples

Briefly, DNA isolation was performed using EZ1 Advanced XL methods with EZ1® DNA blood Tissue Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's standard protocols with minor modifications. Briefly, 200 µl of blood pellet was pretreated by FastPrep Lysing Matrix B (MP Biomedicals) in a 2 mL tube with 1.4 mm ceramic spheres (MP biomedical, Germany) and 500 µl of lysis buffer (NucliSENS easyMAG, France).

2.6. Real-Time PCR Assays

The PCR targeting the gene coding for the 15 kDa *Loa loa* protein was performed on the DNA template extracted from blood samples by using the primers and probe provided by Eurogentec (Angers, France). The PCR reaction was performed on a CFX96™ Real-Time PCR detection system (BIO-RAD Life Science, Marnes-la-Coquette, France). Each real time (RT)-PCR reactions was carried out in 20 µL total volume in a 96-well plate (Roche Diagnostic) containing 10 µL Master mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 µM of each primer (Loa-F: CGAAAAATTATAGGGGGAAAC and Loa-R: TCGTAGACCAAACTGCGAAC), 0.125 µM (FAM-TCAAGAGCCGATATACTGAAAGCTATC-TAMRA) probe, and 5 µL DNA template. The thermal cycling parameters: were 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 s and 60˚C for 30s. Each sample was tested in duplicate. Negative control (nuclease free water) and positive control (highly loaded DNA of loa-microfilaremic blood sample) were used in each run. The RT-PCR efficiency was evaluated by plotting a standard curve with a serial 10-fold dilution of microfilaria stock (10,000 microfilariae/ml to 0.1 Microfilariae /ml. Amplification efficiencies range from 90% to 110%, with a 0.98 R^2 value. PCR results were considered negative when the cycle threshold (Ct) value exceeded 39 or no amplification curve was obtained. Each PCR was performed on both undiluted and 1/20 diluted DNA template to account for the presence of potential PCR inhibitors that might cause false negative PCR result.

2.7. Statistical Analysis

Data were entered in the Microsoft ExcelTM software. The data were then exported into Epi Info 7 software version 7.1.3.3 (Centers for Disease Control and Prevention, Atlanta, GA) for statistical analysis. Statistical values such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) were calculated and compared to microscopy as reference method. The confi-

dence intervals (CI) were established at 95%.

3. Results

3.1. Parasitological Analysis and Clinical Signs

Total blood samples of 545 individuals were examined under light microscope. *Loa loa* microfilaremia was detected in 25 (4.58%) samples; microfilaremia ranged between 100 mf/mL to 8300 mf/mL (mean: 1432 mf/mL). Regarding clinical manifestation, 151 individuals reported subconjunctival migration of an adult worm, of whom only 12 individuals were microfilaremic (range [400 - 8300] mf/mL, mean = 1333 mf/mL); 98 individuals reported Calabar swellings, of whom only 6 were microfilaremic (range [200 - 600] mf/m; mean = 340 mf/mL); and 43 individuals reported subconjunctival migration of an adult worm. In our population study, the prevalence of proven and suspected *Loa loa* infection was 30.1 % (95% CI [26.4 - 34.1]) and 9% (95% CI [6.60 - 11.40]) respectively (Table 1).

Table 1. *Loa loa* Real Time-PCR results in patients with proven, suspected, and no infection groups in a loiasis endemic area in Gabon.

Group	N	Positive *L. loa* RT-PCR
Loiasis		
Microfilaremia	13 (2.20%)	13 (100%)
Eye worm	139 (25.50%)	1 (0.72%)
Proven		
Both microfilaremia and eye worm	12 (2.20%)	12 (100%)
Total	164 (30.1%)	26 (15.85%)
Suspected		
Calabar swelling without microfilaremia or eye worm	49 (9%)	0 (0%)
Total		
Proven + suspected	213 (39.08%)	26 (12.21%)
Absent	332 (60.92%)	4 (1.2%)
Total	545	30

Group classification: 1) Proven loiasis: Samples with positive conventional parasitological assay (microscopy) and/or subconjunctival migration of an adult worm); 2) Suspected Loa-infection: Samples with presence of Calabar swelling. 3) Non-Loa infection group: Samples with absence of microfilaremia, absence of ocular adult worm and absence of Calabar swelling.

3.2. Performance of RT-PCR According to Defined Loiasis Status

Swelling This *L. loa* specific RT-PCR assay was evaluated on 545 blood samples. PCR was positive in all 25 (100%) of the microfilaremic samples and 16.85% of

the proven *L. loa* infection group (Samples with positive conventional parasitological assay (microscopy) and/or subconjunctival migration of an adult worm) (Table 1). There were only 4/381 positive PCR in the non-loiasis group (Table 1). Overall, the sensitivity for the diagnosis of proven loiasis of this RT-PCR targeting the gene coding the 15 kD protein antigen was 15.90% (95% CI [12.6 - 17.5]); its specificity was 99.0% (95% CI [97.60 - 99.7]); its positive predictive value was 86.7% (95% CI [68.9-95.6]); and its negative predictive value was 73.2% (95% CI [72.2 - 73.7]) (Table 2).

Table 2. Performance of Real Time-PCR according to defined loiasis status.

Assay		Proven loiasis*		Sensitivity 95% (CI)	Specificity 95% (CI)	PPV 95% (CI)	NPV 95% (CI)
		Positive	Negative				
RT-PCR	Positive	26	4	15.90 (12.6 - 17.5)	99.0 (97.6 - 99.7)	86.7 (68.9 - 95.6)	73.2 (72.2 - 73.7)
	Negative	138	377				

*Proven loiasis: Samples with positive conventional parasitological assay (microscopy) and/or subconjunctival migration of an adult worm).

4. Discussion

Define Diagnostic of *Loa loa* infection is difficult in endemic areas where most of the *L. loa* infected cases are amicrofilaremics and an accurate diagnostic test is highly needed. In this study, we report the performance of a RT-PCR assay targeting the *Loa loa* 15-kDa protein gene for the diagnosis of proven *Loa loa* infection. In fact, the 15-kDa protein antigen is a nematode polyprotein of allergen family [19]. It is present in all life cycle stages of *L. loa* [20]. It has already been used as a target for conventional PCR to detect *Loa loa* in microfilaremic and amicrofilaremic individual's samples [15] [17] [21]. A nested PCR for targeting the same *Loa loa* gene was also used for the detection of infection and occult infection [15] [18].

Here the RT-PCR was used for the diagnosis of samples from an endemic area to *Loa loa* infection. Samples were classified into groups of proven loiasis (microfilaremia and/or subconjunctival migration of an adult worm), suspected *Loa*-infection (presence of Calabar swelling and Non-*Loa* infection group (absence of microfilaremia, absence of any symptoms). We suggested categorizing cases of *Loa loa* infection in this way in order to arrive at a more accurate diagnosis of the infection. In the case of *Loa loa* worms, microfilaremia and/or subconjunctival migration of an adult worm are indisputable clinical manifestations of *Loa loa* infection because the microfilaria worm is observable through a microscope the adult worm is visible when it migrate under the eye. However, the presence of Calabar swelling in a patient is evidence of an allergic reaction which is just an indicator of *Loa loa* infection. For this reason, we have qualified them as a supicion of *Loa loa* infection. Loa non-infected individuals are those with no microfilare-

mia and no symptoms of infection; they appear to be uninfected by *Loa loa*. It is in this category of people that so-called amicrofilaremic cases are found.

The sensibility of the Real Time PCR was low for the detection of proven loiasis was 15.90% (95% CI [12.6 - 17.75]) and the specificity was 99.0% (95% CI [97.6 - 99.7]). But the RT-PCR detected 25/25 of microfilaremic individuals. These results are similar to those obtained by Touré *et al.* in 1997 where the conventional and nested PCR targeting the 15 kDa assay identified correctly all microfilariae-positive samples as PCR positive [15] [17]. Also, only 1/139 of individuals with subconjunctival migration of worm were detected (**Table 1**).

In addition the RT-PCR was positive for 4/381 individuals belonging to the non-*Loa* infection group (samples with absence of microfilaremia, absence of ocular adult worm and absence of Calabar swelling). They are the amicrofilaremics individuals. The results are lower than those of Touré *et al.* which conventional PCR detected 15/20 amicrofilaremic individuals and 23/30 of samples that were positive for the nested PCR [18]. No sample of individuals with calabar swelling which belonged to the suspected group was detected by the RT-PCR. In fact, samples analysis by our RT-PCR shows an overall prevalence of 30.1% (95% CI [26.4 - 34.1]) for the proven loiasis. The result is similar to that obtained by Veletszky *et al.*, whose qPCR was positive for 37.33% of participants of their study [22]. These prevalences correspond to the affirmation made by Fain in 1981 which stipulated that the proportion of adults with microfilaremia is about 30% [7].

The performance of the essay was evaluated for the detection of proven loiasis (microfilaremia and/or subconjunctival migration of an adult worm). The sensibility was 15.90% (95% CI [12.6 - 17.75]) and the specificity was 99.0% (95% CI [97.6 - 99.7]). The sensitivity obtained here is low compared to the one obtained by Touré *et al.* in 1997 where the sensitivity of the conventional PCR was 95% in detecting occult loiasis [15]. The sensitivity is also low compared to the RT-PCR targeting LL-MF72 was 39% (95% CI [36.7 - 42.2]) [22]. But their specificity is quite similar to 99% CI [99.6 - 100]) [22]. The low sensitivity of our RT-PCR after analysis of the collected samples may be due to the fact that we sampled in an urban environment. It has been shown that in regions endemic to the infection, the prevalence of *Loa loa* infection is around 5% [13]. Also, it would appear that positivity of the PCR test targeted at the 15-kDa protein gene was associated with the appearance of microfilaires inperipheral blood [23]. Hence, low microfilemia in the population could have an important impact on the sensitivity of PCR targeting the 15 kDa gene. Hence, low microfilemia in the population could have an important impact on the sensitivity of PCR targeting the 15 kDa gene. PCR inhibitors have an effect on PCR sensitivity. But in the study, our samples underwent a pre-treatment phase extraction, in order to reduce the amount of PCR inhibitors in the DNA extracts, which allowed for a higher DNA yield. The low sensitivity observed could be due to the genetic variability of the target gene (15 kDa) within the studied population. This argument would be difficult to use due to the lack of information on the genetic diversity of this gene within the population. Neverthe-

less a preliminary study carried out in Cameroon suggested that the *L. loa* population in southern Cameroon is fairly heterogeneous genetically [24].

5. Limitations

We worked on a population from endemic region to the infection, but living in urban areas where the prevalence of infection is generally low [13]. This had maybe an influence on the sensitivity of the test. This is one of the limitations of our study. Also, the specificity of the test could have been better appreciated if the test had also been carried out on samples of blood from regions not endemic to *Loa loa*. Moreover, the performance of RT-PCR would have been more complete if it had been evaluated alongside other established molecular diagnostic methods, such as qPCR and nested PCR targeting different genes.

6. Conclusion

The RT-PCR targeting the gene coding for loa-15 kDa detected some amicrofilemic patients in an endemic area of loiasis and one patient with an occult infection, the patient had a history of an eyeworm. PCR sensitivity was low, but it could detect a few more samples than microscopy. The test can be used to improve diagnosis of *Loa loa* infection. It cannot be used routinely in endemic regions, but it could be used instead of microscopy for the diagnosis of microfilarial loiasis in clinical laboratories and population screening in epidemiological and pretreatment surveys.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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List of Abbreviations

DEC: Diethylcarbamazine

IVM: Ivermectin

MDA: Mass Drug Administration

RT-PCR: Real time polymerase chain reaction