



Short technical report

Comparison of a new visual isothermal nucleic acid amplification test with PCR and skin snip analysis for diagnosis of onchocerciasis in humans



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ABSTRACT

Accurate, simple and affordable diagnostics are needed to detect *Onchocerca volvulus* infection in humans. A newly developed colorimetric loop-mediated isothermal amplification (LAMP) assay was compared to PCR and skin snip analysis for diagnosis of onchocerciasis. The robustness and simplicity of the assay indicates that it may be a useful field tool for surveillance in endemic countries.

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Onchocerciasis, or river blindness, is a neglected tropical disease afflicting more than 30 million people [1] and is caused by infection with the filarial nematode *Onchocerca volvulus*. Adult worms live in subcutaneous tissues and produce large numbers of microfilariae (mf) that migrate through the skin and eyes leading to skin lesions and blindness. The standard method to diagnose infection in humans relies on the detection and morphological classification of microfilariae in skin biopsies by microscopic examination [2]. Microscopy lacks the sensitivity required to detect light infections, for example a Community MicroFilarial Load (CMFL) <1mf/skin snip, that exist in areas where there is on-going Mass Drug Administration (MDA) with ivermectin [3]. MDA has progressed for several years in many countries [4] and accurate diagnosis is needed to guide the decision on when to stop treatment. Sero-surveillance has been used, however cross-reactivity with *Mansonella ozzardi* [5,6], as well as its failure to distinguish past from current infections [7] limit its usefulness.

Abbreviations: LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; O-150, *Onchocerca*-specific 150 bp repeat family.

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Nucleic acid-based diagnostic methods provide both specificity and sensitivity, and polymerase chain reaction (PCR) assays targeting an *Onchocerca*-specific repeat family (O-150), have been used for skin snip analysis [8,9]. However, current PCR methods are difficult to implement in resource-poor settings. Loop-mediated isothermal amplification (LAMP) is a simple method which rapidly synthesizes large amounts of DNA with minimal equipment [10]. We have developed a new visual LAMP assay and compared the performance with PCR and microscopy for detection of mf in skin snips. A consensus sequence alignment of 12 copies of O-150 (GenBank Accession Number: J04659) and Primer Explorer 4 (<https://primerexplorer.jp/e/>) were used to design LAMP primers (F3: 5'-TGGAAATTCACCAAAATATGGT-3', B3: 5'-GGGTACGTACCTTCAAACG-3', FIP: 5'-TGATGACCTATGACCCTAATCTCAACGAATATTTTCTTAGGACCAAT-3' and BIP: 5'-TGAAAATGCGTTTTTCGCCGGGTCCTAAGAAAAATATTCGACTA-3'). LAMP reactions were essentially performed as described [11,12] using 8 U Bst 2.0 DNA polymerase (New England Biolabs, Ipswich, MA, USA), 1.6 μM of each FIP and BIP, 0.2 μM of each F3 and B3 and various template DNAs. Reactions were carried out at 64 °C for 80 min then heated at 80 °C for 2 min to inactivate the DNA polymerase using a Loop Amp Real-time Turbidimeter (LA-320c, Eiken Chemical Co, Japan). Turbidity data were analyzed using

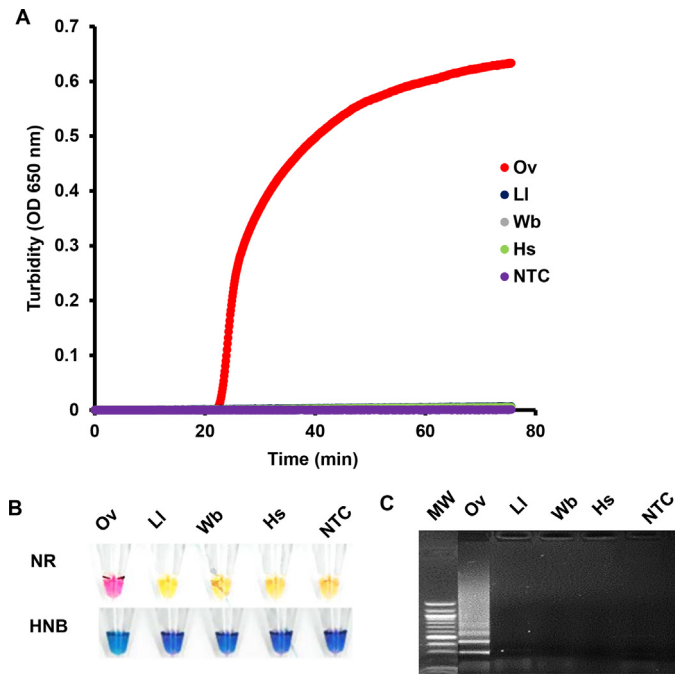


Fig. 1. Species-specificity of O-150 in LAMP and PCR. Assays were performed using genomic DNA (1 ng) from *O. volvulus* (Ov), *Loa loa* (LI), *Wuchereria bancrofti* (Wb), and *Homo sapiens* (Hs). Non-template controls (NTC) containing no DNA were also included. For LAMP, turbidity (A), and the pH sensitive dyes, neutral red (NR) and hydroxy naphthol blue (HNB) (B) were used to detect amplification. A 1.5% agarose gel showing DNA amplification (Ladder-like banding pattern) for *O. volvulus* using O-150 F3 and B3 LAMP primers in PCR (C). The molecular weight (MW) shown is the 100 bp DNA ladder (New England Biolabs).

the LA-320c software package and a positive reaction was defined as when the change in turbidity over time reaches a value of 0.1 [12]. For colorimetric LAMP, reactions were carried out at 64 °C for 60 min. Neutral red (NR) or hydroxy naphthol blue (HNB) (Sigma-Aldrich, St. Louis, MO, USA) dyes were employed as described to detect amplification [13,14]. Samples turn pink if positive or yellow if negative using NR. For HNB, samples changed from violet to sky blue when positive. For comparison, PCR reactions were carried out using the O-150 F3 and B3 LAMP primers listed above and Taq DNA polymerase in standard buffer (M0273S, New England Biolabs). Reactions were denatured once at 95 °C for 3 min, then cycled 35 times at 95 °C for 30 s, 52 °C for 1 min and 68 °C for 1 min followed by a 7 min extension at 68 °C using a Gene Amp PCR system 9700 (Applied Biosystems).

The specificity of O-150 LAMP and PCR was evaluated using genomic DNA (1 ng) from *O. volvulus*, *Loa loa*, *Wuchereria bancrofti*, and human, as well as non-template controls (NTC) containing no DNA (Fig. 1). Amplification was detected by turbidity (Fig. 1A), colorimetric dyes (Fig. 1B) or PCR (Fig. 1C) only when *O. volvulus* DNA was present, highlighting the specificity of O-150 in all diagnostic platforms. While both dyes changed color in the presence of *O. volvulus* DNA, it was much easier to visualize the yellow (negative) to pink (positive) transition when NR was used compared with changes in shades of blue for HNB. Due to the genus-specificity of O-150 [9,15], amplification was also observed when *Onchocerca ochengi* DNA was used as a template (data not shown).

The sensitivity of LAMP and PCR was compared using various concentrations of *O. volvulus* genomic DNA ranging from 0.00001 to 1.0 ng (Fig. 2). Average turbidity threshold times were 30–50 min using 0.0001–1.0 ng of DNA per reaction (Fig. 2A). For visual detection using NR a clearer distinction of positive and negative results was evident (Fig. 2B–C), even when 0.0001 ng DNA was present. Whereas for HNB, a positive was only observed using ≥ 0.001 ng

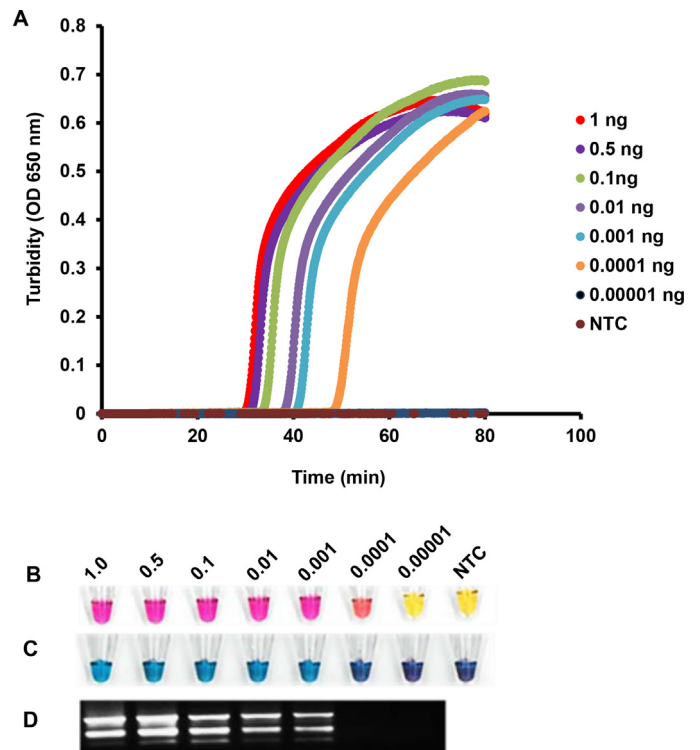


Fig. 2. Sensitivity of O-150 LAMP compared to PCR. Various dilutions of *O. volvulus* genomic DNA ranging from 0.00001 to 1.0 ng were amplified. LAMP reactions were visualized using turbidity (A); the colorimetric dyes, neutral red (B) and hydroxy naphthol blue (C). PCR was performed using O-150 F3 and B3 LAMP primers and products were analyzed by electrophoresis on a 1.5% agarose gel (D).

DNA. Consistent with studies in other systems [16], LAMP was found to be more sensitive (~10-fold) than PCR (Fig. 2D). We also performed PCR using previously published primers [17], and compared the results to those obtained using O-150 F3 and B3 LAMP primers. Identical sensitivities were obtained (data not shown).

To compare the performance of the various methods, skin snips were collected as previously described [18] from 70 individuals with different levels of *O. volvulus* infection living in 6 communities in the Northern and Brong-Ahafo Regions of Ghana (Table 1). For mf detection, skin snips were incubated in saline for 24 h. Following this treatment, between 10 and 20% of mf remain in the skin [2]. DNA was subsequently isolated from the skin using the Qiagen Tissue and Blood Kit (Qiagen, Valencia, CA, USA). Ethical approval for this study was obtained from the Council for Scientific and Industrial Research (CSIR) Institutional Review Board, Accra, Ghana.

Out of 70 samples, 22 (31.4%) scored positive based on microscopic detection of mf. In Agborlekame 1 and Tainso communities, more individuals harbored mf and their counts were generally higher (5–122 mf) (Table 1, Table S1). The nucleic acid detection methods were considerably more sensitive as a greater number of positives were detected despite the fact that DNA was extracted from skin snips after the emergence of mf where an estimated 10–20% of mf remain in the skin [2]. Using the O-150 F3 and B3 LAMP primers in PCR, successful detection of *O. volvulus* DNA was achieved in 26 samples (37.1%); 26 of these were also positive in LAMP using turbidity as a readout. An additional 20 samples scored positive in LAMP using turbidity as the readout (65.7%, Table 1, Table S1), highlighting the greater sensitivity of LAMP. When colorimetric LAMP was used on a smaller sample set from Agborlekame and Tainso, a similar trend was observed, with 24 samples scored positive compared with 22 by PCR. High levels of sensitivity are particularly important in areas where prolonged administration

Table 1
Comparison of microscopy, PCR and LAMP for the detection of *O. volvulus* in human skin snips.

Region ^a /Community	No. of samples	Mf Median (Range ^b)	No. scored positive			
			Mf microscopy	PCR ^c	LAMP (Turbidity)	LAMP (Color)
NR/Agborlekema 1	19	8 (28)	11	11	13	12
BA/Agborlekema 2	11	0 (6)	1	5	7	6
BA/Tainso	6	65.5 (94)	6	6	6	6
BA/Faoamang	2	4 (0)	2	2	2	ND ^d
BA/New Longoro	16	0 (0)	0	0	7	ND
BA/Nyire	16	0 (5)	2	2	11	ND
Total	70		22	26	46	24
Percentage			31.4%	37.1%	65.7%	66.7%

^a Region: NR = Northern region; BA = Brong-Ahafo.
^b Range = Max(mf load)-Min(mf load).
^c O-150 LAMP Primers F3 and B3 were used for PCR amplification.
^d ND = Not done.

of ivermectin has led to the reduction or elimination of *O. volvulus* [19]. The greater number of positives observed using LAMP compared with microscopy or PCR, is consistent with the greater sensitivity of LAMP. This was evident in samples collected from areas where the disease is less prevalent and/or infections are lighter (New Longoro and Nyire, Table S1). This may be due in part to the greater tolerance of LAMP over PCR to inhibitors which are known to be present in clinical samples [11,20].

In conclusion, we describe a simple LAMP assay with a clear visual readout using NR that is more sensitive than PCR and conventional microscopy for the detection of *O. volvulus* microfilariae in human skin snips. The method is highly specific and sensitive and has the potential to be developed further as a field tool for assessment of MDA programs and surveillance for onchocerciasis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molbiopara.2016.07.006>.

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