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A Preliminary Study to Compare Recombinase Polymerase Amplification-Lateral Flow and Quantitative PCR in the Detection of Cutaneous Leishmania in Communities from the Volta Region of Ghana

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Abstract

Background: Leishmaniasis is a parasitic disease that mostly affects populations in tropical and subtropical countries. In Ghana, cutaneous leishmaniasis (CL) is the most common form of the disease affecting communities of the Volta Region. Conventional parasitological method (microscopy) is the commonly used test for CL diagnosis in many endemic countries, but has low sensitivity in chronic cases. Therefore, there is a clear need for a sensitive and easy-to-use point-of-care diagnostic method like an isothermal recombinase polymerase amplification-lateral flow (RPA-LF) test, suitable for use in austere and low-resource settings for the identification of CL cases. This study compared the efficacy of RPA-LF test with quantitative PCR (qPCR) in detecting *Leishmania* in suspected CL cases from the Volta Region.

Methods: Twenty-five participants between 5 and 14 years were enrolled in the study from whom a total of 26 samples were obtained. Lesion samples were collected using FTA® filter papers applied to ulcerated lesions for molecular diagnosis. DNA isolated from filter papers was used for both the RPA-LF test and qPCR.

Results: Twenty-two participants (88%) presented with one or two ulcerated active lesions per individual, while the rest of them had plaques or dried lesions. Among the 26 samples, 19/26 (73%) had concordant results when comparing the two diagnostic methods.

Conclusion: Data from this study suggest that the RPA-LF test can be used in addition to a conventional parasitological diagnostic test (microscopy) to detect CL cases in communities of the Volta Region.

Keywords: cutaneous leishmaniasis, RPA-LF test, qPCR, Endemic, Ghana

Introduction

Leishmaniasis is a neglected tropical disease caused by the protozoan of the genus *Leishmania* and transmitted through the bite of an infected blood-feeding female sandfly. It has a notable disease burden affecting 10–20 million people in 88 endemic countries (Alvar et al, 2012). The disease spectrum may present in three principal forms, namely, cutaneous leishmaniasis (CL), mucosal leishmaniasis with destructive nasal and oropharyngeal lesions, and visceral leishmaniasis (Pace, 2014). However, CL, a rural zoonotic disease, is the most widely distributed form. Clinical presentation of CL ranges from a painless, self-limiting lesion to a chronic nonhealing ulcer, characterized

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by disfiguring active lesions or scars that can impact an individual's functional status (Volpedo et al, 2021).

CL has been reported in Northern, Central, and Eastern Africa and has also been described in some West African countries, including Ghana (Kweku et al, 2011). Although leishmaniasis was first documented in West Africa in the early 1900s, it has only been sporadically recognized and reported (Boakye et al, 2005). Studies in Ghana have previously incriminated *Leishmania major* as the causative agent of CL (Kweku et al, 2011) and detected *Leishmania aethiopica* and *Leishmania tropica* in other settings, (Kwakye-Nuako et al, 2010; Nzelu et al, 2014), but more new members of the *Leishmania enriettii* complex were identified in patients from the Ho West district of the Volta Region (Kwakye-Nuako et al, 2015). Diagnosis of most CL cases is based on the presence of characteristic lesions, without further parasitological confirmation.

In addition, routine laboratory practice in many endemic regions is the microscopic examination for amastigotes from skin lesion scraping or aspirate material from culture (Adams et al, 2014). These methods require highly experienced personnel and may have decreased sensitivity caused by the low number of amastigotes present in the lesions during chronic infection (Weigle et al, 1987). PCR is the preferred method for CL diagnosis (Azmi et al, 2011; Bensoussan et al, 2006), but it is expensive and requires highly trained personnel, sophisticated equipment, and laboratory infrastructure. Consequently, it is difficult to implement in resource-limited settings, which are characteristic of CL endemic areas. On the other hand, recombinase polymerase amplification (RPA) is a novel diagnostic method that can be applied at a single temperature, without the expensive thermal cyclers or chemicals for heating, which are used in PCR.

The recombinase, coupled with its cofactor, forms a nucleoprotein complex with oligonucleotides (primers) that seek homologous sequences in the DNA template. At the sites of recognition, strand exchange occurs and the resulting strands are stabilized by single-stranded DNA binding proteins, after which strand extension occurs by isothermal strand displacement amplification. This occurs without the high temperatures that are usually applied to melt double-stranded DNA. The RPA reagents do not require cold storage and can thus be transported to and moved around in low-resource settings with ease. The RPA-lateral flow (LF) has been used successfully to detect *Leishmania infantum* in dogs (Castellanos-Gonzalez et al, 2015; Crannell et al, 2014; Piepenburg et al, 2006).

In Ghana, initial diagnosis of CL is based on the presence of active lesions. However, not all lesions are due to *Leishmania* infections, as skin ulcers/lesions caused by *Treponema pallidum* subsp. *pertenue* and *Haemophilus ducreyi* have been reported in CL endemic regions in Ghana and could be misdiagnosed as CL (Akuffo et al, 2021a). The CL endemic districts in the Oti Region, formerly part of the Volta Region, have an estimated prevalence of 12.5% (Akuffo et al, 2021a) and are located farther than 150 km from the Noguchi Memorial Institute for Medical Research (NMIMR) in Accra, which is one of the few national laboratories with molecular diagnostic capacity for CL.

Thus, a point-of-care (POC) test for leishmaniasis would significantly improve the diagnostic capabilities of the health system and increase disease incidence data that can guide future interventions. The University of Texas Medical Branch (UTMB) has designed and evaluated primers and probes for RPA, capable of detecting parasites from *Leishmania* and *Viannia* subgenera, which encompass the major species producing visceral or CL (Saldarriaga et al, 2016). This sensitive isothermal molecular test coupled with the LF for reading results with the naked eye is amenable to field implementation due to its minimal equipment and laboratory infrastructure requirements. In this study, we evaluated the diagnostic performance of the RPA-LF test in comparison to quantitative PCR (qPCR) in active suspected CL cases from the Volta Region of Ghana.

Methods

Study sites, enrollment of participants, and sample procurement

Using a cross-sectional design, this study recruited participants with suspected CL from endemic communities in the Volta Region of Ghana, from July to September, 2018. Patients 5–14 years of age presenting with cutaneous papules, nodules, or ulcers suggestive of CL were enrolled. The communities where participants were enrolled were Goviefe-Todzi (Afadzato South District), Gekrong, and Keri (Nkwanta South District) (Supplementary Fig. S1). Child assent, along with parental permission for those <18 years old, was obtained from eligible participants, after which demographic information and samples were acquired.

The data included age, gender, height, weight, size, number, and site(s) of the lesion(s) and type of lesion(s), including ulcers, plaques, or dried lesions. The lesions were cleaned with sterile saline solution and photographed, after which secretions were absorbed into Whatman FTA® classic card (GE Healthcare Companies, Arlington Heights, IL) by gently applying pressure with the card on the lesions. The cards were allowed to air dry before being placed in zip lock bags with silica gel desiccant. All samples were transported at room temperature (25°C) to the NMIMR laboratory for further processing. Subsequently, the FTA papers were shipped to UTMB for analysis by qPCR and RPA-LF.

DNA isolation. At UTMB, the FTA papers were subjected to two different DNA extraction methods based on the diagnostic test used. In the first extraction method, three 3 mm FTA paper discs collected from patients were immersed in 1.5 mL Eppendorf tubes containing 650 μL of FTA Purification Reagent (WB120204) for 5 min at room temperature with occasional vortexing. The buffer was then decanted and the discs were washed twice more with 500 μL FTA reagent.

Subsequently, the discs were washed twice as above with template buffer (TE) (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and a final suspension in 300 μL of this buffer was incubated at 95°C for 30 min. Samples were allowed to cool and were processed for RPA-LF and qPCR, and stored at −80°C for future analysis. The second extraction method was done using a commercial kit following the manufacturer's recommendations (Qiagen DNeasy Blood and Tissue Kit, Hilden, Germany) and used for qPCR at UTMB.

RPA-LF test. RPA-LF was performed using methods we have previously described (Castellanos-Gonzalez et al, 2015; Saldarriaga et al, 2016). The FTA filter papers containing the

clinical samples were boiled at 96°C for 2 min in 0.5 mL DNase-free water. All samples were evaluated by RPA-LF using newly designed primers and probes to detect parasites of the *Leishmania* subgenus.

The amplification mixture comprised the following: (1) forward primer (13A2, 5′-GTGGGGGAGGGGCGTTCT-3′) (2.4 μL, 5 μM); (2) biotinylated reverse primer (RV2, 5′-CA ACCCCCAGTTTCCACCGCCGGAGCCGAA-3′) (2.4 μL, 5 μM); (3) FAM-labeled probe (5′FAM-AAATGGGTGCA GAAATCCCGTTCAAAAAT-[THF]GGCCAAAAATGC CAA-[C3-spacer] 3′) (0.3 μL, 5 μM); 4) magnesium acetate (1.25 μL, 288 mM); (5) Betaine (3.3 μL, 3 M stock in water); and (6) the TwistAmp™ nfo RPA kit rehydrated cocktail (TwistDx, UK). DNA (2.5 μL) was immediately added to the mixture and subjected to amplification at 40°C for 40 min using a dry bath incubator (VWR International, Radnor, PA). The RPA product was diluted at 1:50 in 100 μL of dipstick assay buffer in a 1.5 μL Eppendorf tube. The bottom tip of the lateral flow strip (UStar Biotechnologies, Hangzhou) was then immersed in the sample making the amplification product run upwards by capillarity. Seven discordant samples were re-processed with 4 μL of DNA to confirm the results of the assay.

Quantitative PCR. In the laboratory at UTMB, the RPA-LF sensitivity was compared with SYBR Green qPCR using the primers described by Pita-pereira et al (2012), which detect both subgenera (*Leishmania* and *Viannia*), and performed with iTaq Universal SYBR Green (Bio-Rad) qPCR. The reaction mixture contained 2×iTaq Universal SYBR Green Master Mix (Bio-Rad), 0.25 μmol of each primer (Forward: 5′-GGCCCACTATATTACACCAACCCC-3′ and Reverse: 5′-GGGGTAGGGGCGTTCTGC GAA-3′), and 2 μL of template DNA and distilled ultra-pure water for a final reaction volume of 10 μL. The reactions were set up, in triplicate, in a 384-well optical reaction plate in an ABI ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA).

The PCR conditions were as follows: an initial 2.5-min incubation step at 94°C, followed by 40 cycles of 30 s at 94°C, and 30 s at 60°C. The generation of amplification plots and analyses were detected at the end of each cycle. After amplification, the melting curve was performed per standard operating procedures to allow for confirmation of amplicon identity. Stringent measures to control sample contamination included six nontemplate negative controls (NTC—reaction mix without DNA and distilled water alone), six non-TE negative controls, and nonrelevant DNA controls (*Trypanosoma cruzi* and Vero cells) in each reaction plate. Positive controls (100 and 10,000 *Leishmania* parasites) were also included (Castellanos-Gonzalez et al, 2015). The cutoff Ct value for positive qPCR was established at 37.5.

Data analysis

The McNemar test was used to compare the percentage positivity of the qPCR and RPA-LF. Cohen's Kappa (κ) test was used to evaluate the agreement between the two diagnostic tests, (a k value of 1 would indicate complete agreement). The concordance and discordance of RPA-LF and qPCR were also determined. All the analyses were done using GraphPad Prism version 9.

Results

A total of 25 participants, all minors, were enrolled in this study. Of these, 72% (18/25) were between 5 and 10 years of age and 60% (15/25) were females. Most participants (22/25; 84%) presented with one or two ulcerated lesions ranging from 1.3 to 26 mm in diameter, principally in the limbs (Table 1; Supplementary Fig. S2).

Table 1. Demographic Characteristics of Participants as well as Type, Number, and Diameter of Their Lesions

	Total, n *(%)*	*qPCR positive,* n *(%)*	*RPA-LF positive,* n *(%)*
Demographics			
Gender			
Male	10 (40.0)	7 (70.0)	7 (70.0)
Female	15 (60.0)	11 (73.3)	14 (93.3)
Age, years			
5–10	18 (72.0)	13 (72.2)	15 (83.3)
11–14	7 (28.0)	5 (71.4)	6 (85.7)
Height and weight			
Height (meters), median (range)	1.27 (1.1 – 1.5)	1.27 (1.0 – 1.5)	1.25 (1.1 – 1.5)
Weight (kg), median (range)	24.8 (15.1 – 44.5)	24.1 (15.9 – 44.5)	25.4 (15.1 – 44.5)
Type of lesion			
Ulcer*	22 (88.0)	17 (73.9)	19 (82.6)
Plaque	1 (4.0)	0 (0.0)	1 (100)
Dried lesion	2 (8.0)	1 (50.0)	1 (50.0)
No. of active lesions per individual			
1	11 (44.0)	10 (90.9)	9 (81.8)
2	11 (44.0)	6 (50.0)	9 (75.0)
3	2 (8.0)	2 (100)	2 (100)
4	1 (4.0)	0 (0.0)	1 (100)
Lesion diameter (range mm)	1.3–26	1.3–26	1.3–26
Total	25	18	21

*One participant had 2 lesions sampled that were both ulcers.
qPCR, quantitative PCR; RPA-LF, recombinase polymerase amplification-lateral flow.

Table 2. Correlation Analysis between Recombinase Polymerase Amplification-Lateral Flow and Quantitative PCR

Statistics	*Value*
Concordance	73.1%
Discordance	26.9%
Exact McNemar *p*-value	0.453
Kappa score	0.295

A total of 26 samples were obtained from 25 participants as one participant provided 2 samples from two different lesions. The CL positivity proportion for qPCR and RPA-LF was 69% and 81%, respectively. A total of 73% (19/26) of the samples had concordant results between testing modalities. The overall Kappa score ($\kappa = 0.295$) indicated a minimal agreement between the two tests (Table 2). Only three patients were negative by both tests, with two of them having ulcerative lesions and the third having a plaque. One study volunteer provided two samples from two separate lesions, which were discordant by the qPCR test. A number of samples (11/26) showed Ct values ranging between 25.2 and 35.0 with 7 samples from 35.1–37.5 and 8 samples greater than 37.6 (Table 3).

The five RPA-LF positive results that were negative by qPCR showed weak or very faint bands in the lateral flow strips, but for one that gave a clear band (Table 3). The positive predictive value and negative predictive value of the RPA-LF test were 76.20% and 60%, respectively.

Table 3. Performance of Recombinase Polymerase Amplification-Lateral Flow Test Compared to Quantitative PCR

Sample ID	*qPCR Ct value*	*RPA-LF*
LD-01	33.8	1
LD-02	36.8	1±
LD-03	35.1	1
LD-04	29.8	2
LD-05	33.4	1
LD-06	35.0	1±
LD-07	Negative	1±
LD-08	Negative	2
LD-09	25.2	3
LD-10	26.9	Negative
LD-11	36.6	1±
LD-12	Negative	Negative
LD-13	32.9	1±
LD-14	28.1	2
LD-15	32.9	3
LD-16	35.4	1
LD-17	36.1	2
LD-18	Negative	Negative
LD-20	35.6	1
LD-21	35.3	1±
LD-22	Negative	1
LD-23	Negative	1
LD-24	Negative	Negative
LD-26 RL	34.5	1
LD-26 LL	Negative	1±
LD-27	34.2	Negative
Positive control	11.7	3

- Positive control: *Leishmania (Leishmania) chagasi* for qPCR and RPA-LF. Cutoff point for qPCR: Ct 37.5 (based on three irrelevant DNA samples).
- Negative controls: Nontemplate Control—reaction mix without DNA and distilled water alone), 6 nontemplate buffer negative controls, and nonrelevant DNA controls (*Trypanosoma cruzi* and Vero cells) in each reaction plate.
- Numbers in the RPA-LF column: 3 = strong band; 2 = clear band; 1 = weak band; 1± = very faint band.
- LD-26 RL and LD-26 LL are specimens from the same individual, but from different lesions on the right and left legs, respectively.
- LD-19 and LD-25 are missing from the list of IDs because the samples were not adequate for laboratory analysis.

Discussion

This study indicated that the RPA-LF test has a sensitivity of 88.9% to detect CL in patients from Ghana using qPCR as a reference test. The applicability of the RPA-LF to CL diagnosis under austere conditions has been indicated by previous work in Colombia (Cossio et al, 2021). The RPA-LF primers used in that work were designed to amplify virtually all species within the *Viannia* subgenus (Saldarriaga et al, 2016). The test had a sensitivity of 87% and 75% under laboratory or field conditions, respectively, to detect *Leishmania panamensis* or *Leishmania braziliensis* (Cossio et al, 2021).

On the other hand, the RPA-LF primers and probes used in this study have the capacity to amplify *Leishmania donovani* and *L. major* (Supplementary Fig. S3) and were initially used to detect *L. infantum* infections in dogs (Castellanos-Gonzalez et al, 2015). Unpublished work in the lab at UTMB using Ghana strains showed that the RPA-LF test amplified strains of *Leishmania* (*Mundinia*) *enriettii*. We speculate that the low amplification of some *L. enriettii* strains may have been due to low DNA quality (unpublished data; Supplementary Fig. S4).

Therefore, it is possible that members of the *L. enriettii* complex, which have recently been described circulating in the Volta Region, were detected by this RPA-LF test (Kwakye-Nuako et al, 2015). However, one of the reasons why many discrepant results were observed may be that the primers used for qPCR only detected species within the subgenus *Leishmania* or *Viannia* and were eventually unable to amplify the *Mundinia* subgenus. Unfortunately, we were unable to retest the samples by qPCR and with *Mundinia*-specific primers due to the limited amount of DNA that was isolated from the samples.

Other species identified by prior studies in the Volta Region include *L. major* (Villinski et al, 2008), *L. aethiopica* (Kwakye-Nuako, 2010) and *L. tropica* (Nzelu et al, 2014). In this study, patients were enrolled from districts in the Volta Region that have not been studied in the past, specifically the northern part of the Volta Region, now the Oti Region. Recently, an epidemiological assessment conducted in the Oti Region (previously part of the north of Volta Region until the year 2019) detected a 41.8% prevalence of exposure to *Leishmania* spp. using the leishmanin skin test procedure (Akuffo et al, 2021b).

The study also detected a CL prevalence of 31.8% among persons with skin ulcers in these communities (Akuffo et al, 2021a). From the above, it is clear that the epidemiology of *Leishmania* infection in the Volta Region (including the newly studied Oti Region) is complex and, having a POC diagnostic procedure such as this RPA-LF could potentially improve the feasibility and cost-effectiveness of CL diagnosis.

It is important to implement a CL-specific test to differentiate this infection from other pathogens present in Ghana, which produce skin lesions of a similar appearance. *Mycobacterium ulcerans* (Buruli ulcer) may produce a spectrum of lesions, some of which (papule, nodule, plaque, and ulcer) could be mistaken for CL. The disease is usually diagnosed on clinical grounds and although this method is highly specific (Mensah-Quainoo et al, 2008), it is possible that its specificity decreases in CL endemic areas leading to misdiagnosis and incorrect treatment. A similar situation could occur in patients infected with *H. ducreyi* (Pillay et al, 2016) and *T. pallidum* subsp. *pertenue* (Ghinai et al, 2015), two other pathogens that produce skin lesions and can be found in the Volta Region of Ghana.

A significant advantage of the RPA-LF is that samples are quickly processed (<1 h) compared to qPCR ($\approx$3 h), without the need for sophisticated equipment (Saldarriaga et al, 2016). DNA extraction was achieved, utilizing the boiling method, and samples could be stored on FTA Whatman filter paper for long periods, without the need for freezers, increasing the applicability of the RPA-LF for diagnosis in austere settings. RPA-LF is less complex than qPCR and does not require skilled or highly experienced technicians. These benefits have the potential to contribute to early diagnosis and subsequent treatment with less morbidity.

Study limitations

This initial field evaluation of the RPA-LF test involved a small number of participants. Some of the Ct values of qPCR from samples of dermal lesions were in general very high ($\geq$35), suggesting low parasite loads. We speculate that this could be, in part, responsible for the discrepant results found between RPA-LF and qPCR. However, we cannot rule out that these high Ct values could be due to suboptimal sample procurement or low parasite burden associated with more chronic lesions. Furthermore, the quantity of the samples was insufficient to run additional tests to identify the *Leishmania* spp. that was detected by both the RPA-LF and the qPCR tests. Further studies, including sequencing of several isolates, should determine whether one or more *Leishmania* spp. are circulating in districts of the Volta Region and which molecular diagnostic tools would be the more relevant for additional studies.

Conclusion

Economical techniques that can be applied in remote health settings like the communities in the Volta Region are of major advantage compared to other methods such as qPCR, which entails high costs, subject matter expertise, and more complex health infrastructure. The preliminary dataset of this small study suggests that the isothermal RPA-LF test could be used in the field to screen for CL, although confirmatory tests may be necessary. Nevertheless, a larger field validation will be necessary to confirm the applicability of this test as a POC in resource-limited settings.

Authors' Contributions

B.L.T.: study design and supervision, and article writing and review. T.R.S.: evaluation of clinical samples, and article writing and review. P.C.M.: support to test development, and article writing and review. N.A.: site-specific protocol writing, coordination of field and laboratory work, and article writing and review. C.Y.: laboratory and field work/coordination, and article writing and review. M.T.M. and S.O.A.: laboratory and field work, and article writing and review. E.B.: data management and analysis, and article review. B.A.: laboratory and field work, and article review. R.E.B.: laboratory work, and article writing and review. A.T.F.: study coordination, and article writing and review. M.W., D.B., and A.G.L.: study coordination and article review. R.A.A.: study data collection and article review.

Author Disclosure Statement

The authors declare no conflict of interest. Commander A.G.L. is a military Service member, while Dr. Anne T. Fox and Dr. Naiki Attram are employees of the U.S. Government. This work was prepared as part of their official duties. Title 17, U.S.C., §105 provides that copyright protection under this title is not available for any work of the U.S. Government. Title 17, U.S.C., §101 defines a U.S. Government work as a work prepared by a military Service member or employee of the U.S. Government as part of that person's official duties. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the article; or in the decision to publish the results.

Ethical Consideration

Ethical clearance for the study was obtained from the NMIMR (CPN 064/14–15), Ghana Health Service Ethics Review Committee [GHS-ERC] (GHS-ERC 07/05/15), and the Naval Medical Research Center Institutional Review Board [NMRC] (NAMRU3.2016.0014).

Data Availability Statement

All data supporting the findings have been included in this article.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4

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