Detecting *Mycobacterium ulcerans* in Clinical Specimens from Ghana: Comparative Evaluation of BU-LAMP and IS2404 PCR

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Abstract

**Background:** Conventional and nested polymerase chain reaction (PCR), and loop-mediated isothermal amplification (LAMP) have been used to identify *Mycobacterium ulcerans* in separate studies and different specimens. However, the sensitivities of these three techniques have not been compared in a single study.

**Objective:** This study compared the performance of two variant PCR techniques and LAMP assays to detect *M. ulcerans* in same clinical specimens.

**Methods:** Samples were collected from patients suspected of Buruli ulcer disease (BUD) in Southern Ghana. Ulcerative and non-ulcerative forms of the disease were swabbed and aspirated respectively. Insertion sequence 2404 (*IS2404*) *M. ulcerans* targets were detected in each sample using conventional polymerase chain reaction (PCR), nested PCR and loop-mediated isothermal amplification (LAMP) assay.

**Results:** In all, 141 suspected BUD patients were sampled (Amasaman, n = 52; Obom, n = 17; Paakro, n = 31; Nkawie, n = 21 and Tepa, n = 20). The reference technique, nested PCR, detected *M. ulcerans* in 122 (86.5%) whereas conventional PCR and BU-LAMP detected *M. ulcerans* in 104 (73.7%) and 119 (84.4%) samples respectively. Compared to nested PCR, conventional PCR performed poorly (χ² = 19.7, p < 0.01; k = 0.58; % agreement = 86.62) while BU-LAMP was as good as nested PCR (χ² = 0.457, p = 0.459; k = 0.88; % agreement = 97.16). Sensitivity of conventional PCR and BU-LAMP were 84.6% (95% CI: 76.9-90.4) and 97.5% (95% CI: 92.9-99.5) respectively. BU-LAMP and nested PCR detected *M. ulcerans* in ulcerative forms of the disease and category II lesions better while the three techniques did not differ in sensitivities in other clinical forms and lesion categories.

**Conclusion:** BU-LAMP assay is very comparable to nested PCR in detecting *M. ulcerans* from clinical specimens. More importantly, LAMP assay is user-friendly, fast, requires less instrumentation and easy to use in resource-limited laboratory with minimal user training.

**Keywords**

*Mycobacterium ulcerans*, BU-LAMP, Nested-PCR, Conventional PCR, Buruli ulcer, Ghana

**Abbreviations**

BUD: Buruli Ulcer Disease; DNA: Deoxyribonucleic Acid; IS: insertion sequence; LAMP: Loop-Mediated Isothermal Amplification; PCR: Polymerase Chain Reaction

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Introduction

*Mycobacterium ulcerans* causes Buruli ulcer disease (BUD), a tropical skin disease which presents with severe necrosis and chronic cutaneous infection [1]. After *M. ulcerans* inoculation, lesions appear as painless, and mostly ignored by patients [2]. Early stages of the disease present as papules, nodules, plaques and oedema [3]. Which later progresses to ulceration of subcutaneous layers and bones [4], if treatment is delayed.

Due to the disfiguration and lifelong loss of mobility associated with the disease [5], early diagnosis with sensitive, rapid, user-friendly and cheap technique is important. In view of the foregoing, several diagnostic techniques have been developed to improve diagnosis of BUD. Notwithstanding, rapid diagnosis of the disease using sensitive techniques has not been achieved due to various demerits associated with current testing methods. The most common diagnostic methods are Ziehl-Neelson microscopy technique [6], cultivation of the bacilli [7] and molecular detection of insertion sequence 2404 *(IS2404)* using either polymerase chain reactions (PCR) [8] and/or loop-mediated isothermal amplification techniques (LAMP) [9]. Microscopic detection and culture of *M. ulcerans* are very specific, however these techniques are slow (positive culture obtainable in 6-12 weeks and in some cases up to 36 weeks) and of low sensitivity (approx. 40% for microscopy and 20-60% for culture) [6,10,11]. PCR on the other hand is very sensitive [8] but the processes involved are time consuming, delaying results for clinical decision. Again, it is not easily exploited in laboratories of developing countries where the disease is endemic [12]. A quick method for diagnosis of BUD has been developed, based on the loop mediated isothermal amplification (BU-LAMP) technique. As it stands now, three different LAMP assays for laboratory confirmation of BUD have been published [13]. The assay described by de Souza, et al. [9] targeted the enoyl reductase gene of *M. ulcerans*. Enoyl reductase gene is involved in mycolactone synthesis, the most important virulence factor in BUD pathogenesis. However, technical validation of the assay was conducted only on a limited number of samples. Deployment of this assay on a large scale, with samples collected from different locations and from different clinical forms of the disease is essential towards point-of-care application of the LAMP assay. Hence, this study was designed to compare the efficiency of BU-LAMP to the conventional and nested PCR. Even though these techniques have been piloted in separate studies, their sensitivities have not been evaluated using the same set of samples.

Methods

Study sites

Clinical specimens used in this study were collected from major Buruli ulcer clinics in Southern Ghana. Namely, Ga West Municipal Hospital in Amasaman, Obom Health centre in Obom (Greater Accra Region), Paakro Health Centre in Paakro (Eastern Region), Nkwie-Toase Hospital in Nkwie and Tepa Government Hospital in Tepa (both in Ashanti Region). These health facilities serve as referral sites for Buruli ulcer disease from nine of 16 regions in Ghana.

Sampling techniques

Samples were collected from both ulcerative and non-ulcerative forms of the disease. Non-ulcerative and ulcerative lesions were aspirated using fine needle and swabbed using sterile swab sticks respectively. Both fine needle aspirates and swabs tips with necrotic tissues were put in Eppendorf tubes containing 1 ml of PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) transport medium. All samples were sent to Bacteriology Department of Noguchi Memorial Institute for Medical Research, University of Ghana for molecular analysis. Demographic parameters as well as clinical forms and categories of the lesions were also taken.

Inclusion and exclusion criteria

Suspected BUD patients included in the study were new patients (patients that have never been treated with anti-BUD drugs or have taken anti-BUD drugs for less than 1 month). Patients with category three lesions at critical sites such as eye, genitals, breast, joints and osteomyelitis were excluded in this study.

Place of laboratory work

DNA extraction, polymerase chain reactions and loop-mediated isothermal amplification assays for all samples were done in the Department of Bacteriology, Noguchi Institute for Medical Research, University of Ghana, Legon-Accra.

DNA extraction using the modified Boom’s method

DNA was extracted and purified based on the protocol described by Ablordey, et al. [14]. In brief, Portions 250 μl of the sample suspensions were lyed with 250 μl of lysis buffer (1.6 M GuHCl, 60 mM Tris pH 7.4, 1% Triton X-100, 60 mM EDTA, Tween-20 10%), 50 μl Proteinase-K and 250 μl glass beads. The mixtures were incubated horizontally at 200 rpm at 60 °C overnight. To capture the DNA, 40 μl of diatomaceous earth solution (10g diatomaceous earth obtained from Sigma-Aldrich Chemie GmbH in 50 ml of H2O containing 500 μl of 37% (wt/vol) HCl) was added to the suspensions and incubated at 37 °C with shaking (200 rpm) for 60 min. The mixtures were centrifuged at 14,000 rpm for 10 sec. and the resulting pellets washed twice with 900 μl of 70% ethanol (2-8 °C) followed by 900 μl of acetone. The pellets were dried at 50 °C for 20 min and resuspended in 100 μl milli Q purified water and centrifuged at 14,000 rpm for 10 sec. The purified DNA was used as templates for both *IS2404* nested-PCR and LAMP assays to detect *M. ulcerans*. 

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Conventional PCR

Conventional PCR master mix per sample was prepared by adding the constituent reagents as follows; 2 µL Q-solution, 1.2 µL MgCl₂ (25 mMmol/L), 2 µl each deoxynucleoside triphosphate (200 µL), 1 µL forward primer (0.5 µmol/L), 1 µL reverse primer (0.5 µmol/L), 0.2 µL Taq DNA polymerase (HotstarTaq plus), 9 µL nuclease free water, and approximately 50 ng of DNA. The primer sets used were MU1 (5-’-GATCAAGC-GTTACAGGATGGA-3’) and MU2 (5-’-GGAGTTACCTCAGT-GCCA-3’) [15]. The PCR reaction mixture was spun shortly to remove water droplets from the inner wall of the PCR tube and inserted into a thermocycler for 40 cycles of DNA denaturation at 96 °C, primer annealing at 60 °C, and oligonucleotide extension at 72 °C. PCR products were electrophoresed in a 1.5% Tris-acetate- EDTA TAE (pH = 8.0) agarose gel with ethidium bromide. The amplicon sizes were estimated by comparison with DNA ladder (Invitrogen, USA) and visualized using Kodak Gel logic 100 imaging system.

IS2404 nested PCR reaction

Due to unavailability of IS2404 qPCR, nested PCR was used as reference technique to which all other techniques were compared. The IS2404 sequence was amplified in a 30 µl reaction mixture comprising 15.5 µL nuclease free water, 3 µL PCR buffer, 6.0 µL Q-solution, 2.0 µl dNTPs, 0.1 µL Hotstar Taq DNA pol, 0.3 µL each of pGp1 (pGp1: 5’-AGGGCAGCGCGTGATAGGG-3’) and pGp2 (pGp2: 5’- CAGTGGATTGGCGCATGAG-3’) primers and 3 µL DNA extract. The first run PCR reaction was done based on these amplification conditions; initial denaturation at 95 °C (15 minutes) and 40 cycles of denaturation, annealing and extension at 94 °C, 64 °C and 75 °C respectively. The second run of the IS2404 (nested) PCR was performed by using 1 µl of the first run PCR amplicons as the template DNA and 24 µL of master mix. The master mix for the nested reaction was the same as that for the first run PCR except that primer set pGp1 and pGp2 were replaced with the primer set pGp3 (5’-GGCCGAGATCAACTTGGCGGT-3’) and pGp4 (5’-CTGCGTGGTCTCTAAGGCG-3’) with the same volume. The same PCR amplification condition was used for the nested PCR except 35 cycles of denaturation, annealing and extension. The primers used have been previously used by Ablordey, et al. [14].

BU-LAMP reaction

The BU-LAMP reaction was made in 25.0 µL volume made up of 12.5µl of reaction mix, 1 µL of fluorescent detector, 2.6 µL of primer mix (FIP: 5’-GATCTTCCGC-GCCACCCCCACGCCAACAGGCCAAGGACCCTGA-3’; BIP: 5’-GTG-GTGCCCCCGCCCCGCCACTGTCG-3’; F3: 5’-AAGGATTGTCGGAGATTG-3’; B3: 5’-GGCCAGTGTC-CCTTGA-3’; FLP: 5’-GAGCTCTGGCCGGTC-3’; BLP: 5’-CATATACCCATTGTGGT-3’), 1 µL of Bst DNA polymerase (Eiken Chemical, Japan), 2.9 µL of nuclease free distilled water (Promega, USA) and 5 µL of the DNA extract in 0.2 ml microAmp™ tubes (Applied Biosystems, Singapore). The DNA amplification was done by incubating the reaction mixture on a heating block at a temperature of 65 °C for 1 hour and the Bst DNA polymerase was inactivated at 80°C for 5 minutes. The BU-LAMP primers used were designed by de-Souza, et al. [9]. The final reactions were determined by visual examination of the final colour production. A final greenish yellow colour development signified a positive reaction and a light brown final colour signified a negative reaction.

Quality control for molecular techniques

For every batch of 20 DNA isolation from clinical samples, two tubes of nucleic free water and two isolates of culture positive samples, found to be positive by IS2404 PCR, were used as negative and positive extraction controls respectively. Initial screening found the negative and positive controls negative and positive respectively before that batch of DNA from clinical samples were further processed. During the amplification phase of the techniques, another two tubes of nucleic free water and two IS2404 positive culture samples were used as respective negative and positive amplification controls. A respective corresponding negative and positive amplification outcomes were validated all PCR and LAMP assays.

Data analysis

Data were analysed using SPSS Version 24 (Chicago, IL, USA). Differences in categorical data were determined using Chi square whilst Chi square goodness of fit was used to compare the performance of conventional PCR and BU-LAMP to nested PCR. Finally, sensitivities, specificities, positive and negative predictive values and their respective 95% confidence intervals as well as the inter-diagnostic technique test of agreement were determined based on Bayesian model. In all these analysis, significant levels were determined at p < 0.05.

Results

Study sites, age and gender distributions of the patients

A total of 141 suspected Buruli ulcer cases were sampled from the five study sites from August 2014 to May 2016. In absolute terms, majority of the cases were sampled from Ga West Municipal Hospital (46, 32.6%), Amasaman while minority of the cases sampled were seen in Obom Health Centre (17, 12.0%), Obom. Majority of the suspected cases were males (65.9%) and patients aged less than 19 years (36.9%). In all study areas, 80.0% or more of the suspected cases were confirmed to be infected with Buruli ulcer disease (BUD). The prevalence of BUD was also seen in both males and females with patients aged 0-19 years disproportionally affected (94.2%) (Table 1).
Sensitivity and specificity of the diagnostic techniques

The diagnostic performance of BU-LAMP and conventional PCR to detect *M. ulcerans* using nested PCR results as reference technique are presented in Table 3. The sensitivity of BU-LAMP was higher than conventional PCR even though both techniques were of equal specificity. According to the interpretation of Cohen’s kappa values by McHugh [16], there was a strong agreement between nested PCR and BU-LAMP (κ = 0.88) whilst nested PCR agreed with conventional PCR weakly (κ = 0.58).

Analysis of the efficiency of the protocols on clinical forms and lesions categorization

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Clinical forms of the disease, category of lesions and type of samples collected

Out of the 141 samples collected, 73% were ulcerative lesions, 16.3 were nodular lesions and 10.6% were oedematous form of the disease. Per the clinical forms of the disease encountered, swab samples were collected from ulcerative form of the disease while fine needle aspiration was done on the nodular and oedematous forms of the disease (27%). All the three categories of the disease were encountered; category 1 lesions (single lesions < 5 cm in diameter [5]) were 64 (45.3%), category II lesions (single lesions between 5-15 cm diameter [5]) were 24 (17.0%) and category III lesions (either a single lesion more than 15 cm diameter or multiple lesions or lesions at critical sites such as eye, genitals, breast, joints [5]) were 53 (37.6%).

Positivity rate of the techniques

Nested PCR detected *M. ulcerans* in 122 (86.5%) of the 141 samples tested while conventional PCR and BU-LAMP detected *M. ulcerans* in 104 (73.8%) and 119 (84.4%) samples tested respectively. Compared to nested PCR, the performance of conventional PCR was statistically different (χ² = 19.7, p < 0.01) while the performance of nested PCR did not differ from BU-LAMP assay (χ² = 0.457, p = 0.459) (Table 2).

| Diagnostic Technique | Positivity Rate n (%) | Negativity Rate n (%) | Chi square goodness of fit x² (p-value) |
|----------------------|-----------------------|-----------------------|----------------------------------------|
| Nested IS2404 PCR    | 122 (86.5%)           | 19 (13.5%)            | -                                      |
| Conventional IS2404 PCR | 104 (73.8%)       | 37 (26.25)            | 19.7 (p < 0.001)                       |
| BU-LAMP assay        | 119 (84.4%)           | 22 (15.6)             | 0.547 (p = 0.459)                      |

Chi square goodness of fit was determined at 95% confidence interval.
symptoms similar to that of BUD [17]. In furtherance to this, World Health Organization (WHO) has directed that all suspected cases of BUD are confirmed in the laboratory [18].

The performance of the three diagnostic techniques to detect \textit{M. ulcerans} in ulcerative forms of the disease significantly differed from the other. While nested-PCR and Bu-LAMP performed very well on ulcerative lesions ($\chi^2 = 9.68$, $p = 0.008$), the techniques were comparable on nodular lesions ($\chi^2 = 1.81$, $p = 0.403$) and oedematous forms of the disease ($\chi^2 = 2.50$, $p = 0.286$). Also, there were differences in the ability of the techniques to detect \textit{M. ulcerans} in category II lesions but nested-PCR and BU-LAMP detected \textit{M. ulcerans} in category II lesions with equal ability. In category I and III lesions, the techniques had equal ability to detect \textit{M. ulcerans} in their samples (Table 4).

### Discussion

The sensitivity of three molecular techniques for the diagnosis of Buruli ulcer disease (BUD) is reported here-in. Nested-PCR detected more infections in the clinical specimens than BU-LAMP and conventional PCR. However, BU-LAMP was superior to conventional PCR. The positivity rates for the techniques reflected in the sensitivities of the techniques. Comparing to nested-PCR, BU-LAMP was more sensitive than conventional PCR. However, the techniques were of equal specificities. Furthermore, nested-PCR performed better on all the categories and clinical forms of the disease. This study presents an alternative to laboratory diagnosis of BUD. Laboratory confirmation of BUD is a better diagnostic option compared to the widely used clinical diagnosis which represents a challenge due to the vast number of other skin infections or conditions that may present symptoms similar to that of BUD [17]. In furtherance to this, World Health Organization (WHO) has directed that all suspected cases of BUD are confirmed in the laboratory [18].

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### Table 3: Diagnostic indices and how nested PCR agreed with Bu-LAMP and conventional PCR.

| Indices          | BU-LAMP                  | Conventional PCR          |
|------------------|--------------------------|----------------------------|
| True positive    | 119                      | 104                        |
| True negative    | 19                       | 19                         |
| False positive   | 0                        | 0                          |
| False negative   | 3                        | 19                         |
| Sensitivity (95% CI) | 97.5% (92.9-99.5%)    | 84.6% (76.9-90.4%)         |
| Specificity (95% CI) | 100.0 (82.35-100.0)  | 100.0 (82.35-100.0)        |
| PPV (95% CI)     | 100.0                    | 100.0                      |
| NPV (95% CI)     | 86.4% (67.4-95.1%)       | 50.00% (39.81-60.19%)      |
| Kappa value      | 0.88% (0.564-0.741)      | 0.59% (0.51-0.69)          |
| % agreement     | 97.16                    | 86.62                      |

Values in parenthesis is 95% confidence interval.

### Table 4: Performance of the techniques on clinical forms and categories of the disease.

| Clinical forms of lesions | Nested IS2404 PCR | Conventional IS2404 PCR | BU-LAMP |
|---------------------------|-------------------|-------------------------|---------|
|                           | Positive n (%) | Negative n (%) | Positive n (%) | Negative n (%) | Positive n (%) | Negative n (%) | $\chi^2$ (p-value) |
| Ulcer (n = 103)           | 89 (86.4%)     | 14 (13.6%)     | 74 (71.8%)     | 29 (28.2%)     | 89 (86.4%)     | 14 (13.6%)     | 9.68 (0.008)     |
| Nodule (n = 23)           | 20 (87.0%)     | 3 (13.0%)      | 17 (74.0%)     | 6 (26.0%)      | 20 (87.0%)     | 3 (13.0%)      | 1.81 (0.403)     |
| Oedema (n = 15)           | 13 (86.7%)     | 2 (13.3%)      | 13 (86.7%)     | 2 (13.3%)      | 10 (66.7%)     | 5 (33.3%)      | 2.50 (0.286)     |
| Categorization of lesions |                   |                         |         |                   |         |         |
| Category I (n = 64)       | 58 (90.6%)     | 6 (9.4%)       | 55 (86.0%)     | 9 (14.0%)      | 58 (90.6%)     | 6 (9.7%)       | 0.96 (0.618)     |
| Category II (n = 24)      | 21 (87.5%)     | 3 (12.5%)      | 13 (54.2%)     | 11 (45.8%)     | 21 (87.5%)     | 3 (12.5%)      | 6.82 (0.033)     |
| Category III (n = 53)     | 43 (81.1%)     | 10 (18.9%)     | 36 (67.9%)     | 17 (23.1%)     | 40 (75.5%)     | 13 (24.5%)     | 2.47 (0.290)     |

*No significant difference between techniques at significant level of 0.05. P-values were determined by Chi square at significant level 0.05. Category I lesions are single lesions < 5 cm in diameter; category II lesions are single lesions between 5-15 cm diameter; category III lesions are either a single lesion more than 15 cm diameter or multiple lesions [5].
which was later characterized in detail and designated IS2404 [22]. There are about 150-200 copies of IS2404 per M. ulcerans genome [23]. These large copies of IS2404 predestine their application as targets for sensitive molecular amplification tests for M. ulcerans.

On the other hand, loop-mediated isothermal amplification (LAMP) is a molecular method, which is relatively cheaper, simpler and faster compared to the PCR. The LAMP technology has been developed to detect several pathogens such as Trypanosoma species [24], Cryptosporidium species [25], Toxoplasma gondii [26], Babesia microti [27], Taenia species [28], Mycobacterium tuberculosis [29], Plasmodium spp [30] and rift valley fever virus [31]. In this study, LAMP assay was as good as nested PCR. This is because LAMP assays have been found to be very sensitive with the ability to detect as low as 20 - 30 copies of the ISs (about 0.1 to 0.15 genome of M. ulcerans) [32]. The analytical sensitivity of BU-LAMP has been found to be higher than conventional PCR [33] though lower than qPCR [34]. But equipment sophistication and the level of expertise needed to perform qPCR technique makes BU-LAMP an alternative choice in developing countries with resource limited settings. LAMP assays have several advantages over PCR assays. Comparing LAMP to PCR, it was realized that LAMP technology is less expensive in the sense that less instrumentation is required to achieve amplification at isothermal temperatures. Results obtained by LAMP were obtained within 1 hour but in the case of IS2404 PCR, the turnaround time was over 5 hours. Aside high sensitivity of BU-LAMP over PCR, the technique has been found to amplify DNA target from partially processed or unprocessed specimen [35]. Detection of LAMP products was found to be easy due to large amount of dsDNA formed and production of magnesium pyrophosphate allow visual detection of reactions. Considering the nature of LAMP technique, it is highly feasible in the field. These features associated with LAMP have been previously published [24,32,36,37].

In Plasmodium falciparum infections, analytical positive and negative rate of LAMP method was equivalent to that of nested-PCR methods [38]. These reports make LAMP assay a potential technique to replace PCR based techniques as a confirmation technique for infectious agents. Due to the specific nature of the action of LAMP primers, the amount of DNA produced is considerably higher than PCR based amplification. The corresponding release of pyrophosphate results in visible turbidity which allows easy visualization of amplification products [39]. LAMP has been observed to be less sensitive than PCR to inhibitors in complex samples such as blood, possibly due to the use of a different DNA polymerase (Bst DNA polymerase rather than Taq polymerase as in PCR). Several reports have described successful detection of pathogens from minimally processed samples [40,41]. This feature of LAMP may be useful in low-resource or field settings where a conventional DNA or RNA extraction prior to diagnostic testing may be impractical [42]. Also LAMP technique has been evaluated for point-of-care use to detected several pathogens [43-45]. Therefore, LAMP has the potential to be used as a point-of-care technique to detect M. ulcerans both in the laboratory and in the field.

Conclusion

This study has demonstrated that BU-LAMP is as good as nested PCR, hence it can be used as a reliable molecular technique to detect M. ulcerans infections from clinical samples. It is recommended that LAMP based assays be used at facilities in endemic communities to detect M. ulcerans in suspected lesions because it promised to perform well in resource-limited laboratories. Suffice it to say, LAMP assays have been found to be very cheap, user-friendly, rapid, highly sensitive and above all easy to read amplification reactions.

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