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Validation of a Real-Time PCR (qPCR) Technique for Detection of *Mycobacterium ulcerans* in Clinical and Environmental Samples

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Abstract

Introduction: Buruli ulcer (BU) is a serious skin disease caused by *Mycobacterium ulcerans*. According to WHO, 70% of BU cases should be confirmed by the PCR-*IS2404* gene amplification. The objective of this study is to validate a real-time PCR for the detection of *M. ulcerans* in clinical and environmental samples.

Methodology: A total of 70 clinical samples, 10 *M. ulcerans* strains and 15 environmental samples were tested by Ziehl-Neelsen staining technique, conventional PCR, real time qPCR and culture. The proportion of positive cases of *M. ulcerans* detection between the different tests was compared by the Chi-square test. The difference was statistically significant for a *P*-value ≤ 0.05.

Results: Out of 33/80 samples were cultured positive (41.25%) to *M. ulcerans*, 41/80 ZN staining were positive (51.25%) to AFB at the microscopy, 55/80 (68.75%) and 64/80 samples (80%) were positive to the IS2404 insertion sequence from conventional PCR and qPCR respectively. Both PCR techniques showed positivity rates significantly higher than microscopy and culture (*P*=0.002). However, no significant difference of positive rate was observed between the two types of PCR (*P*=0.38). In contrast, the detection limit for the real time qPCR was lower (0.1copies/μL) compared to conventional PCR. Only qPCR was able to detect *IS2404* in 2/15 environmental samples (13.3%).

Conclusion: This study showed the high sensitivity of the real time PCR for the detection of *Mycobacterium ulcerans* in clinical and environmental samples.

Keywords: Buruli ulcer; *Mycobacterium ulcerans*; conventional PCR; Real-time PCR; Ziehl-Neelsen; Culture

Introduction

Buruli ulcer (BU) is an emerging infectious disease caused by *Mycobacterium ulcerans*. This disease is characterized by extensive destruction of the skin and soft tissues with formation of significant ulcerations. The necrotizing and ulcerative character is induced by a diffuse cytotoxic macrolide enzyme, called mycolactone, which is produced by *M. ulcerans* virulence genes. Buruli ulcer cases are reported in more than 33 countries, mainly tropical or subtropical, but are also present in temperate climates such as Japan and southern Australia [1]. According to WHO, between 5,000 and 7,000 cases of Buruli ulcer are reported annually worldwide with more than 4,000 cases occurring in Sub-Saharan Africa. The highest number of reported cases are found in West African countries such as Côte d'Ivoire with about 2000 cases per year, Benin and Ghana 1000 cases per year [2,3].

The reporting of Buruli ulcer cases is based on laboratory confirmation using WHO recommended tests for the diagnosis of the disease, including direct examination of smears for acid-fast bacilli (AFB); in vitro culture and gene amplification (PCR) targeting the genome sequence *IS2404*. According to WHO, 70% of BU cases should be confirmed by the PCR-*IS2404* gene amplification technique [4]. The development of the conventional PCR technique targeting *IS2404* sequence, an insertion of more than 200 copies present in *M. ulcerans* genome has become a rapid technique for confirming cases of Buruli ulcer. This technique was also used to analyze environmental samples and to detect *IS2404*, thus showing the probable presence of *M. ulcerans* in water samples [5], aquatic insects [6]. Although this technique is sensitive and specific for *M. ulcerans* detection in clinical samples, its application on environmental samples remains difficult and non-specific due to the presence of PCR inhibitors. In order to increase specificity, confidence in the results of PCR and facilitate rapid analysis of clinical and environmental samples, Fyfe et al. have developed two real-time multiplex techniques targeting the *IS2404* insertion sequence [7].

In Togo, the availability of a national reference laboratory using the conventional PCR technique makes it possible to confirm each year about 30 to 65 new cases in the endemic districts of Zio and Yoto in the maritime region [8]. However, this technique required more time and overload work due to the gel preparation and DNA separation in electrophoresis. It is therefore important that this technique should be the most sensitive and specific to ensure a reliable diagnosis test. This study aims to validate a real-time PCR for the detection of *M. ulcerans* in clinical and environmental samples through *IS2404* sequence detection in order to improve the level of the sensitivity of the PCR in the diagnostic of Buruli ulcer.

Materials and Methods

Biological material

Strains of *M. ulcerans*: Strains of *M. ulcerans* were obtained from the Reference Laboratory of Mycobacteria of Benin (LRM) (Table 1).

Samples	Origin	Country	Number
Clinical	CNRTUB	Togo	70
Control strains	LRM	Bénin	10
Environmental	National Laboratory, INH	Togo	15

Table 1: Sample Types used for real-time PCR validation of *M. ulcerans* detection.

CNRTUB: Centre National de Référence et de Traitement de l'ulcère de Buruli; LRM: Laboratoire de Référence des Mycobactéries

Clinical samples: Clinical specimens were collected from patients suspected of having Buruli ulcer according to WHO criteria (2014, 2001). Fine needle aspiration (FNA) was obtained from pre-ulcer lesions and swabs from ulcers. For each type of lesion, three samples were taken. The first one for culture was added to the transport medium consisted of 2 ml of Middlebrook 7H9 medium supplemented with PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin) and OADC (OADC: Oleic acid, Albumin, Dextrose and Catalase). The second sample put into the cell lysis solution (CLS) was used for PCR and the last sample was used to prepare a smear for Ziehl-Neelsen staining (Table 1) [4,9].

Environmental samples: Water, soil and plant samples were collected from the environment near the Zio River (Table 1).

Analytical methods

Ziehl-Neelsen staining: The smears were prepared by ejecting FNAs aspirate or by applying a swab directly to a glass slide and labeled. The slide was fixed to the Bunsen burner flame and then stained with carbolic fuschine and destained with a mixture of sulfuric acid (20%) and ethanol (70%). After adding methylene blue, the slides were rinsed with water and then air dried. They were examined under an optical microscope at the x100 objective and oil immersion.

Molecular analysis

DNA extraction: DNA was extracted from clinical samples and strains using the Gentra Puregene DNA extraction kit (Qiagen, Hilden, Germany) with minor modifications of the manufacturer's protocol. Briefly, the proteinase K (20 mg/ml) and lysozyme (10 mg/ml) were added to the sample previously put into cell lysis solution. The protein was removed using the Protein Precipitation Solution (PPS). After a centrifugation, the DNA was precipitated in supernatant with isopropanol [10,11].

Prior to DNA extraction of environmental samples of plant and soil were homogenized using the FastPrep-24 instrument (ver. 6004.2) at the laboratory of the Togolese agricultural research institute (ITRA, Lomé, Togo). DNA was extracted using the Fast DNA Spin Kit for Soil kit (MP Biomedicals, Cat No. 116560, Lot No. 93391) following the manufacturer's recommendations.

DNA amplification by PCR: *M. ulcerans* DNA detection was performed by using conventional and real-time PCR (qPCR). Firstly, all the DNA extracts from *M. ulcerans* strains and clinical samples were tested by conventional PCR according to the protocol described by Stinear [12]. Briefly, MU5 primers (AGCGACCCCAGTGGATTGGT) and MU6 (CGGTGATCAAGCGTTCACGA) were added in reaction tubes where primers were previously lyophilized. PCR beads (Ready-To-Go Illustra PuReTaq; GE Healthcare) containing Taq polymerase, dNTPs and Mg^{2+} were added and dissolved with water before adding the DNA samples.

The amplification reaction was carried out in the Eppendorf thermocycler according to the following program: pre-denaturation at 95°C-10 min following amplification of 40 cycles (95°C-30s, and 58°C-10s and 72°C-30s) and final extension of polymerization at 72°C-10 min. DNA fragments corresponding to *M. ulcerans* (492 bp) were identified on 1.5% agarose gel electrophoresis.

Real-time PCR (qPCR) was performed in a second step according to the method described by Fyfe [7] on all samples including those collected from the environment. The primers and probe sequences that target the *IS2404* insertion sequence are presented in Table 2.

A calibration curve was constructed from a standard DNA dilution series made of recombinant plasmid containing 10^8 copies/μL of IS2404 (Table 3).

The amplification reaction was performed with a volume of 2 μL of the extracted DNA and 18 μL of the mastermix consisting of 0.4 μL of internal control DNA (IPC), 2 μL of the IPC control mix, 1 μL of the primer *IS2404* sense, 1 μL of the *IS2404* primer antisense, 1 μL of the Taqman probe, 8.6 μL of water and 4 μL of qPCR Mix Plus. The reaction was conducted on ABI 7300 thermal cycler (Applied Biosystems) under the following conditions: 95°C-15 min and 40 cycles of 95°C-15s and 60°C for 60s. The negative extraction control (check cross contamination during extraction process), negative PCR control (check contamination of water and reagent by DNA) and positive PCR control (check run), as well as the inhibition control (check false negative result) were included into the reaction.

Culture: The cultivation of *Mycobacterium ulcerans* was made from 10 strains obtained from Benin and 70 clinical samples. The strains from Benin stored at -80°C were previously thawed and then inoculated onto the Middlebrook 7H9 liquid medium (BD BBL MGIT) supplemented with OADC (oleic acid, albumin, dextrose and catalase) and PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin). After four weeks of incubation at 31°C., the cultures were subcultured onto Lowenstein-Jensen medium supplemented with glycerol.

Clinical specimens were initially decontaminated by the modified Petroff method and then were cultured as describe for the strains [4,9,13].

Data processing and analysis: Statistical analysis was performed by SPSS software (Statistical Package for Social Science, Version 24.0, SPSS Inc., Chicago, IL). The Chi-square test (or Fisher exact test) was applied to determine the difference between the observed proportions. This difference was considered significant if the p-value ≤ 0.05.

Results

Microscopy

AFB were detected using Ziehl-Neelsen staining in 31 out of 70 clinical specimens (44.28%) analyzed and 100% for strain samples (10/10) (Figure 1).

Primer and Probe	Sequence (5'-3')	Position of nucleotides	Size of amplicons
IS2404 TF	AAAGCACCACGCAGCATCT	27746-27762	
IS2404 TR	AGCGACCCCAGTGGATTG	27787-27804	
IS2404 TP	6FAM-CGTCCAACGCGATC-MGBNFQ	27768-27781	59

Table 2: List of primers and probe sequences for real-time PCR Targeting *IS2404* Insertion.
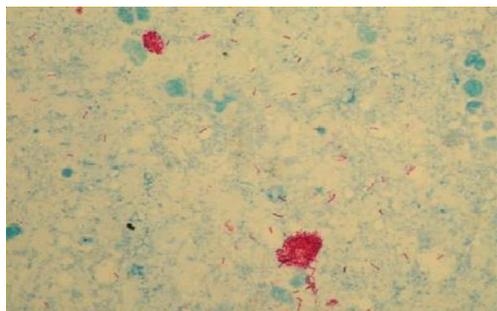

Figure 1: Illustration of a Ziehl - Neelsen stain of a smear from a lesion of a Buruli ulcer. Extracellular acid-fast bacilli appear in red on a blue background.
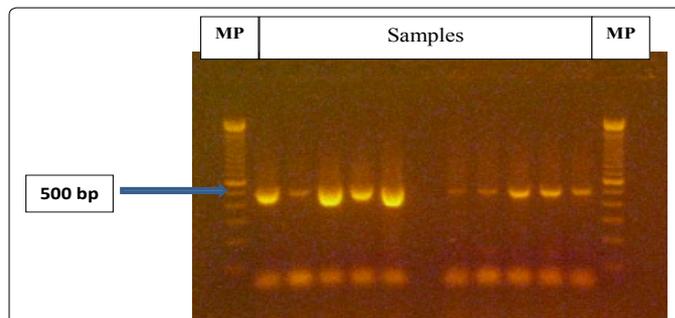

bp: base pairs

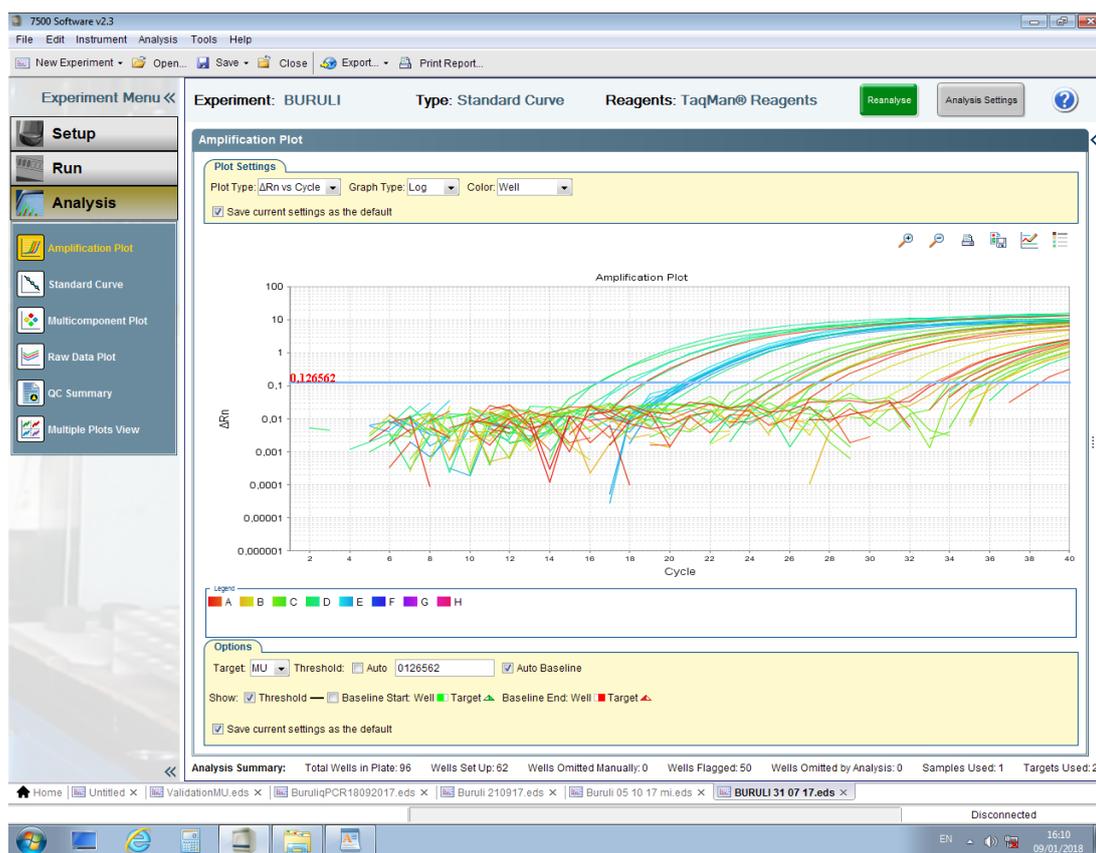**Figure 2**: Agarose gel showing *M. ulcerans* DNA fragments (492 bp).

Figure 3: Amplification curves of qPCR for detection of *M. ulcerans.*

Culture

Colonies of Mycobacteria were observed on 25 out of 70 of clinical samples (35.71%) cultured and 8/10 of strain (80%) after 4 weeks of incubation. The strain growth was observed after eight weeks after incubation at 31°C.

Conventional PCR

IS2404 insertion of *M. ulcerans* was detected in 49/70 (70%) of clinical samples tested, 21/35 (60%) for strain, including 6/10 for Benin strains and 15/25 for strains obtained from clinical samples (Figure 2).

Dilution ADN (Copies/µL)	Ct (IS2404)
10^8	13,0
10^7	13,8
10^6	14,9
10^5	16,5
10^4	21,1
10^3	25,9
10^2	28,2
10^1	31,7
10^0	34,3
10^{-1}	36,0
10^{-2}	38,0

Table 3: Limit of detection of qPCR according to the results of qPCR of the plasmid IS2404.

Sample Type	Ziehl Neelsen		Culture		*IS2404* Conventional PCR		*IS2404* qPCR	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Clinical (Togo) n=70	31 (44.28%)	39 (55.71%)	25 (35.71%)	45 (64.28%)	49 (70%)	21 (30%)	54 (77.14%)	16 (22.85%)
Strains (Benin) n=10	10 (100%)	0 (0%)	8 (80%)	2 (20%)	6 (60%)	4(40%)	10 (100%)	0 (0%)
Environmental (Togo) n=15	NA	NA	NA	NA	NA	NA	2*	13*
Total	41 (51.25%)	39 (48.75%)	33 (41.25%)	47 (58.75%)	55 (68.75%)	25 (31.25%)	64 (80%)	16 (20%)

Table 4: comparison of results of microscopy, culture and PCRs of the clinical samples and strains of Benin.

Real-time PCR

By applying the real-time PCR, 54/70 (77%) of clinical samples were tested positive for *IS2404* sequence of *M. ulcerans* with threshold cycles ranging from 16.6 (10^5 copies/µl) to 34.3 (1 copy/µl).

All strains were tested positive for *IS2404* insertion (for strains from Benin and clinical samples) with cycles threshold ranging between 8.7 (>10^7 copies/µl) and 31.6 (10 copies/µL) (Figure 3).

The calibration curve constructed from *PCR-IS2404* results of the plasmid DNA showed a high sensitivity of detection up to 0.1 copies of the genome of the bacteria present in a sample (Table 3).

Comparison of different tests

All samples detected by conventional PCR were confirmed by qPCR. All negative samples from microscopy and culture specimens were tested by both method of PCR. The difference between two molecular tests was not significantly different (P=0.125). However, the two PCR techniques showed significantly higher positivity rates than microscopy and culture (P=0.002) (Table 4).

Discussion

WHO recommends that about 70% of Buruli ulcer cases should be confirmed by PCR [4] because it remains the gold technique for early diagnosis of the disease [14]. Currently, the conventional PCR has whether considered as a good technique to detect *M. ulcerans* through the *IS2404* insertion sequence. This technique is mostly applicable to clinical samples with a sensitivity limited for environmental sample due to the agarose gel revelation which required a great amount of DNA of the pathogen. The objective of this study was to validate the real time PCR for detection of *Mycobacterium ulcerans* in clinical and environmental samples.

This technique has been compared with other diagnostic techniques including microscopy, conventional PCR and culture.

Overall, 33 out of 80 samples (41.25%) cultured showed colonies of mycobacteria, AFB were observed in microscopy on 41 from 80 (51.25%)

slides performed, The *IS2404* sequence was detected in 55 of 80 samples analyzed (69%) in conventional PCR and 80% in qPCR (64/80 samples). The positivity rate obtained in conventional and real time PCR is in line with WHO standards and other studies [4,14-26]. Culture rate obtained is in conformity of WHO recommended rate which is ranged between 20 and 40%. This rate is similar to other studies [18,23]. Our microscopy rate is similar to those obtained in other studies [18,22,24].

The two PCR techniques showed significantly higher positivity rates than microscopy and culture. This study confirms the high sensitivity of PCR techniques. However, the sensitivity of conventional PCR is lower than the qPCR because 11% of negative sample from conventional PCR were detected positive to *IS2404* using the qPCR. The calibration curve has shown a high sensitivity of qPCR with a detection limit of 0.1 copy/µL in the genome of the bacterium [27]. Ngazoa-Kakou has demonstrated that the qPCR was very sensitive with a limit detection of 0.25 copies in the genome of *M. ulcerans* [28].

Conclusion

Molecular techniques (conventional PCR and real time PCR) are more sensitive for the detection of *M. ulcerans* in clinical samples and strains. However, the real-time PCR is more sensitive (lower detection limit) especially for environmental samples.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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