

Full Length Research Paper

## Evaluation of real-time PCR for *Mycobacterium ulcerans* in endemic region in Côte d'Ivoire

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Buruli ulcer (BU) is caused by a mycobacterium called *Mycobacterium ulcerans*. The events of BU are the skin lesions. The lack of early diagnosis and treatment cause severe disability. Today the emergence to BU in Africa and particularly in Côte d'Ivoire needs faster diagnosis to control and to prevent the infection by *M. ulcerans*. The surveillance of BU is difficult, because the transmission of *M. ulcerans* occurs in rural regions where the transport of fresh collected sample is long, and the detection with culture technique needs several months. This study has allowed the application of polymerase chain reaction (PCR) technique in real time with two targets for molecular diagnosis of BU in Côte d'Ivoire. 63 samples (clinical, environmental, local strains and reference strains) were analyzed in real-time PCR by comparing the target of the Insertion Sequence (IS) 2404 and the sequence Ketoreductase-B (KR-B), located respectively on the chromosome and on the virulence plasmid. 49 samples (76%) were positive in real-time for both targets. The sensitivity of the PCR shows a detection limit of 0.25 genome copy for both targets. The capacity, speed and sensitivity of real-time PCR assays improve the diagnosis and contribute to strengthening the eradication of BU in Côte d'Ivoire.

**Key words:** Buruli ulcer, *Mycobacterium ulcerans*, real-time, insertion sequence, ketoreductase, Côte d'Ivoire.

### INTRODUCTION

*Mycobacterium ulcerans* is the third mycobacteria infection after tuberculosis and Leprosy and it causes Buruli ulcer (BU), a severe skin disease that has irreversible consequences in rural populations (MacCallum et al., 1993). The WHO was esteemed that 33 countries was endemic or semi-endemic for the Buruli ulcer (Asiedu et al., 2000; Janssens et al., 2005). The eradication of Buruli ulcer is difficult because, the lack of early diagnostic in rural endemic regions, and the unknown of the disease in medical national structures system. In the rural westlands and marshes of West Africa, children are most affected (Asiedu et al., 2000; WHO, 2008). The polymerase chain reaction (PCR) was become the gold method to confirm clinical and environmental samples (Ross et al., 1997a, b; Portaels et

al., 2001; Marsollier et al., 2004; Portaels et al., 2008). The most using targets were the insertion sequence IS2404 or IS26406 and the Ketoreductase B (KR-B), these are respectively located on the chromosome and on the virulence plasmid pMUM001 (van Werf et al., 2003). The cytotoxic mycolactone of *M. ulcerans* is produced from of three large multienzymes complexes called polypeptide synthases that are encoded by the genes *mlsA1*, *mlsA2*, and *mlsB* located on the plasmid (Stinear et al. 2004; 2005). The detection of *M. ulcerans* in clinical and in environmental samples was improved for the both most targets IS2404 and KR such aquatic insects, snails or in plants (Ross et al. 1994; Stinear et al. 2000; Eddyani et al. 2004; Philipps et al., 2005). In West Africa, the endemic region of BU is located in Côte d'Ivoire that has more 2000 confirmatory cases yearly (WHO, 2010). Molecular detection was the best method to confirm new human cases because of the low-growth of *M. ulcerans in vitro* (Portaels et al., 2001). Recently, de Vandelanoot

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et al. (2010) have detected *M. ulcerans* using real-time PCR in small animals and in water sample in a BU-endemic region. However, the sensitivity, the specificity and the capacity of real-time PCR are the major step forward to detect *M. ulcerans*. The application of real-time PCR in the Institute Pasteur contributes to increase the capacities of the national surveillance to eradicate the BU, by elucidating of the ecology and the transmission in the environment. The main aim of this study is to evaluate the performance of real time PCR as molecular diagnosis method, by using two targets IS2404 and KR-B to identify *M. ulcerans* in several types' samples in Buruli ulcer-endemic region in Côte d'Ivoire.

## MATERIALS AND METHODS

### Mycobacterial strains

Local strains were gathered from patients with Buruli ulcerative form in Cote d'Ivoire endemic regions. Those strains were isolated and maintained within Abidjan Pasteur Institute Mycobacterial Laboratory. Fresh swab clinical specimens were spilt for DNA extraction and culture technique. Environmental samples were collected in 2009 from different endemic regions of BU and were inclusive with strains isolated from water insects Reference strains were provided from the Institute of Tropical Medicine, Antwerp, Belgium (ITM) and from Centre Pasteur of Yaoundé, Cameroon (Table 1).

### DNA extraction

#### Clinical specimen

DNA from clinical samples was extracted using Phenol extraction method as previously described (Ekaza et al., 2004; Coulibaly et al., 2010).

### Bacterial strains

DNA from bacterial strains was extracted by a phenol/chloroform method. Briefly five to ten colonies were harvested from Löwenstein-Jensen slants and suspended in 250 µl of lysis buffer. After overnight incubation at 37°C, phenol chloroform extraction was applied. The DNA was washed in ethanol and the pellet was dried and eluted in 50 µl of sterile water.

### Environmental samples

Aquatic insects were placed in sterile box and add to liquid nitrogen, the matrix was crushed and 2 ml of 50 mM NaOH were added. 1 ml of sample was added to 150 µl of 0.1 M Tris and heated 20 min, 95°C. The extraction protocol of Mo Bio kit was applied following the instructions of the manufacturer. The pellet was eluted in sterile water and stored at -20°C.

### Real-time TaqMan PCR assays

Real-time PCR was performed using PCR Reagents Kit (Promega, Madison, WI, USA) and the 7300 real-time PCR (Applied Biosystems, Forster City, USA). The reaction took place in a final

volume of 25 µl and contained 5 µl of DNA, 0.3 µM of each primer, namely IS2404R and IS2404F (Rondoni et al., 2003) or KR-B-F and KR-B-F (Fyfe et al., 2007), and 0.25 µM of labelled IS2404 probe or KR-B probe, 20 µl PCR-Mix containing 25 mM MgCl<sub>2</sub>, Rox Dye Pure (Invitrogen, Cergy Pontoise, France), 10 mM dNTPs, 5X Buffer, 0.2 µl Go-Taq Flexi DNA Polymerase (Promega, Madison, WI, USA).

PCR consisted of 35 cycles of melting at 95°C for 5 s, annealing and extension at 60°C for 1 min. A DNA segment of 58 and 65 bp for IS2404 and KR respectively in length was thus amplified. Fluorescence of FAM liberated from the probe by TaqMan was measured to determine the amplification threshold cycle (Ct), which was the first cycle at which fluorescent emission was 10-fold higher than the standard deviation of the mean baseline emission. Negative controls were performed with 5 µl of sterile water. A serial 10-fold dilution of 2x10<sup>7</sup> CFU/µl extracted DNA was used to establish the standard curve. Each sample was duplicated and tested in real time assays.

### Sensitivity test of real-time PCR assays

To determine the detection limit of both assays, serial dilutions of extracted DNA from 2.5 x10<sup>4</sup> CFU/µl from 2MU II strain (unpublished) and 5 µl were tested duplicate in real-time assays.

## RESULTS

### Sensitivity of the real-time PCR of *M. ulcerans*

The serial dilution of DNA solution containing 2.5x10<sup>4</sup> CFU/µl of *M. ulcerans* (2MUII) shows the detection by dilution (10<sup>-6</sup>) with the target IS2404 by Ct 39.27 and with the Ketoreductase-B by Ct 35.29 (Table 2) The detection limit of both assays was corresponding of 0.25 genome copies from *M. ulcerans*. The IS2404 is more sensitive with similar detection limit than the assay using Ketoreductase-B gen.

### Evaluation of real-time PCR for Insertion sequence IS2404 and Ketoreductase-B by different samples of *M. ulcerans*

From 63 samples tested in real-time assays, 47 samples (74%) were positive by IS2404 and 40 samples (77%) by KR. Both assays show identical performance by the detection of local strains and clinical samples (Table 3). References strains show similar results by both real-time assays. No environmental sample was detected in both assays. 31 clinical samples (96%) were detected in both assays and confirm the sensitivity of real-time assays. 14 samples (26%) were detected negative for both detection targets and 3 samples (5%) were negative by IS 2404 real-time or by KR-B real-time assay.

## DISCUSSION

Buruli ulcer was endemic in Côte d'Ivoire. The infection

**Table 1.** Types samples of *M. ulcerans* tested in this study.

<b>Samples</b>	<b>Source or strain</b>	<b>References</b>
ITM 9540	References strain	Stieger et al., 2006
ITM 980912	References strain	Stieger et al., 2006
ITM 842	References strain	Stieger et al., 2006
ITM 7922	References strain	Stieger et al., 2006
ITM 940662	References strain	Stieger et al., 2006
ITM 990006	References strain	Stieger et al. unpublished
ITM 94821	References strain	Stieger et al. unpublished
ITM 5150	References strain	Stieger et al. 2006
ITM 970483	References strain	Stieger et al. 2006
ITM 051459	References strain	Stieger et al. 2006
CIV-B043	Local laboratory strain	This study
CIV-B058	Local laboratory strain	This study
CIV-01-0104	Local laboratory strain	This study
CIV-01-098	Local laboratory strain	This study
CIV-01-0119	Local laboratory strain	This study
CIV-01-044	Local laboratory strain	This study
CIV-06-046	Local laboratory strain	This study
CIV-06-0120	Local laboratory strain	This study
CIV-01-054a	Local laboratory strain	This study
CIV-009	Local laboratory strain	This study
CIV-090	Local laboratory strain	This study
CIV-091	Local laboratory strain	This study
CIV-028	Local laboratory strain	This study
CIV-077	Local laboratory strain	This study
CIV-01-093	Local laboratory strain	This study
CIV-01-043	Local laboratory strain	This study
01-021b	Local laboratory strain	This study
U064	Local laboratory strain	This study
U044	Local laboratory strain	This study
02-43	Local laboratory strain	This study
B12	Local laboratory strain	This study
06-108	Local laboratory strain	This study
06-117	Local laboratory strain	This study
01-078	Local laboratory strain	This study
B043	Local laboratory strain	This study
02-003	Local laboratory strain	This study
06-120	Local laboratory strain	This study
01-104	Local laboratory strain	This study
01-109	Local laboratory strain	This study
08U-045	Local laboratory strain	This study
U059	Local laboratory strain	This study
01-098	Local laboratory strain	This study
UB09-960	Clinical sample	This study
UB09-973	Clinical sample	This study
UB09-988	Clinical sample	This study
UB09-989	Clinical sample	This study
UB09-995	Clinical sample	This study
UB09-997	Clinical sample	This study
UB09-1009	Clinical sample	This study
UB09-1025	Clinical sample	This study
UB09-1027	Clinical sample	This study

**Table 1** Contd.

UB09-1039	Clinical sample	This study
UB09-1042	Clinical sample	This study
UB09-1043	Clinical sample	This study
UBE1	Environmental sample	This study
UBE2	Environmental sample	This study
UBES1	Environmental sample	This study
UBES2	Environmental sample	This study
UBES3	Environmental sample	This study
UBSE4	Environmental sample	This study
UBSE5	Environmental sample	This study
UBSE6	Environmental sample	This study
UBSE7	Environmental sample	This study

**Table 2.** Sensitivity of the both real-time TaqMan assays.

Dilution of DNA CFU/ $\mu$ l	IS2404 Ct	KR-B Ct
2.5.10 <sup>4</sup>	20.88 $\pm$ 1.8	19.6 $\pm$ 1.2
2.5.10 <sup>3</sup>	24.85 $\pm$ 1.7	22.03 $\pm$ 0.7
2.5.10 <sup>2</sup>	28.35 $\pm$ 1.4	25.3 $\pm$ 1.1
2.5.10 <sup>1</sup>	31.82 $\pm$ 1.3	28.46 $\pm$ 1.4
2.5.10 <sup>0</sup>	35.45 $\pm$ 1.7	30.18 $\pm$ 1.3
0.25	39.27 $\pm$ 1.8	35.29 $\pm$ 1.2

**Table 3.** Detection of different samples for *M. ulcerans* in real-time assays.

Sample type	IS2404		KR-B	
	Positive	Negative	Positive	Negative
Clinical	8	4	8	4
Environmental	0	9	0	9
Local strains	30	2	31	1
References strains	9	1	10	0
Total	47	16	49	14
%	74	26	77	23

can be eradicated if the national surveillance becomes effective in all regions. Early diagnostic is the only step to prevent ulceration that has irreversible consequences for the patients. The number of cases of *M. ulcerans* in Côte d'Ivoire continues to rise, with more 2000 new human cases reported in 2009 compared with 1440 cases in 2008 and 1 case in 1978 (WHO, 2010). With increasing numbers of clinical specimens for diagnosis as well as the need to determine the mode of transmission and the natural reservoir of the mycobacteria, rapid, sensitive, and specific molecular tests are needed. Fyfe et al. (2007) have developed two multiplex, real-time TaqMan PCR assays for the detection of *M. ulcerans* DNA in clinical and environmental samples. Recently, de

Vandelannoote et al. (2010) have detected *M. ulcerans* in small animals in Ghana. To ameliorate the capacity of molecular diagnostic, we apply the real-time PCR with two targets in monoplex reaction to detect *M. ulcerans* in clinical and environmental samples. The IS2404 is the most target for *M. ulcerans* PCR used in several labours (Stinear et al., 1999). The Ketoreductase B domain is present in 15 copies within the mycolactone synthase gen and the real-time assay has shown gut sensitivity in previous assay (Rondoni et al., 2003). Real-time PCR is more 10-10.000 sensitive than classic PCR, however the PCR is recommended by WHO as the confirmatory test for clinical diagnosis of *M. ulcerans* infection.

The application in this study of real-time assays to

detect clinical samples of Buruli ulcer was chosen in rapidity and the capacity for the most endemic area of *M. ulcerans* in West Africa. Our results confirm the sensitivity of both assays and the detection limit was 0.25 genome copies. However, 96% of clinical samples have been confirmed the infection of *M. ulcerans* in this study. This detection limit was consistent of the results of Rondoni et al. (2003) with the detection of 0.2 genome copy in real-time of IS2404. Fyfe et al. (2007) have demonstrated that IS2404 or KR real-time PCR assays are able to detect all geographic strains of the world including African strains. The combination of both assays has facilitated to detect simultaneous mycolactone producing strains by the target KR-B and non-mycolactone producing strains by the target IS2404.

The application of several types of samples confirms the specificity of both real-time assays. Because of the long storage of references ITM strains DNA, several strains missed by real-time assays. No environmental sample shows the presence of DNA of *M. ulcerans* in both assays. The presence of inhibitors in environmental samples was investigated because of the correlation of the high incidence of BU in Côte d'Ivoire. Our results show gut performance of real-time assay for both targets to detect mycolactone producing strain and non producing strains. We will investigate in future studies to detect *M. ulcerans* in environmental samples.

In view of the high number of the samples received in the case of the confirmation of the cases of Buruli ulcer in Côte d'Ivoire and the search for the environmental reservoir of *M. ulcerans*. This technology is going to allow the avoidance of not only the long manipulations, but also the contaminations after PCR, and also to increase the sensibility and the specificity of the detection. However, the application of real-time PCR for *M. ulcerans* in Institute Pasteur will permit us to elucidate the ecology and the transmission of BU in Côte d'Ivoire.

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