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Virulence factors expressed by *Mycobacterium ulcerans* strains: Results of a descriptive study

AKA N'guetta[1]*, KAKOU-NGAZOA E. Solange[2], COULIBALY N. D.[2], VAKOU Sabine[1], KOUADIO Kouamé[3], AOUSSI Serge[2], KADIO M. Constance[4], KOUAKOU Hélène[2], AKE Julien[5], N'GUESSAN Raymond[1] and DOSSO Mireille[1]

[1]Unit of Mycobacteria, Institut Pasteur, Côte d'Ivoire.
[2]Platform of Molecular Biology, Institut Pasteur, Côte d'Ivoire.
[3]Department of Environment and Health, Institut Pasteur, Côte d'Ivoire.
[4]ONG AFRISOL-Côte d'Ivoire, Institut Pasteur, Côte d'Ivoire.
[5]MAP International, Institut Pasteur, Côte d'Ivoire.

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***Mycobacterium ulcerans* is a slow-growing mycobacterium responsible for Buruli ulcer. The pathogenic virulence of *MU* is being linked to the expression of toxin called Mycolactone, whose form varies according to the origin of the productive strains. Forms A and B are secreted by African and Malaysian strains, C by Australian strains and D by Asian strains. Forms E and F of mycolactone are secreted by animal mycolactone producing mycobacteria strains. The genes for the biosynthesis of Mycolactone are located on a plasmid called pMUM001. We investigated the circulation of Mycolactone in body fluids coupled to the detection of virulence factors in *MU* strains. Suspicious BU patients and healthy subjects (negative controls) were selected in three Ivorian endemic areas. Exudates, fine needle aspiration (FNA) and blood samples were collected. Microscopy by Ziehl-Neelsen-staining, culture and PCR diagnostics using IS2404 and KR were performed in patient samples. The Mycolactone detection by HLPC coupled to MS was performed in patient and control samples. PCR using IS2404, IS2606, KR and ER were also performed in *MU* strains. Ziehl-Neelsen-microscopy detected acid-fast bacilli in 19% of samples while PCR were positive in 76.2% for IS2404 and 52.4% for KR. Mycolactone A/B was detected in 31% of exudates and in 42.8% of sera. No Mycolactone was detected in control subjects. 17 strains isolated from exudates possessed both IS2404 and IS2606. 70.6% of those strains were positive for KR and ER gene. The study shows that Mycolactone A/B was actually present in most of BU patients selected in three Ivorian endemic areas. With the methods used we detected very low concentrations in patient fluids. Plasmid and ER gene were found in the majority of *MU* strains. But they were not found in about 30% of strains. Mycolactone was detected only in patients infected by strains in which plasmid was found.**

Key words: Buruli ulcer, *Mycobacterium ulcerans*, insertion sequence, virulence factor, Mycolactone.

INTRODUCTION

Mycobacterium ulcerans (MU) is a slow-growing mycobacterium responsible for Buruli ulcer (BU). The cutaneous mycobacteriosis is mostly seen in tropical and subtropical countries (Anne-Caroline et al., 2013; Emmanuelle et al., 2007; Hong et al., 2005; Laurence et al., 2009; Mac et al., 1948). The pathogenicity of *MU* is known to be linked to the secretion of a toxin named mycolactone (Caroline et al., 2009; Emmanuelle et al.,

2007; Kathleen et al., 1999; Sarojini et al., 2005; Yoshito, 2011). The forms of Mycolactone are classified according to the origin of the productive strains. Forms A and B are secreted by African and Malaysian strains; C by Australian strains and D by Asian strains (Kathleen et al., 1999; Sacha et al., 2005). Forms E and F have been found in mycobacteria strains isolated from animals (Armand et al., 2005; Brian et al., 2006; Hong et al., 2008; Sylvain et al., 2008). Mycolactone is responsible for the tissue necrosis and the diverse immune reactions of the host (Armand et al., 2003; Emmanuelle et al., 2007; Kathleen et al., 1999; Hurtado et al., 2009; Phillips et al., 2006; Sarojini et al., 2005; Travis et al., 2001). The biosynthesis of Mycolactone is linked to some genes located on a giant circular 174 Kb plasmid named pMUM001 (Mac et al., 1948; Timothy et al., 2004; Timothy et al., 2005; Timothy et al., 2005). More than half of the plasmid is devoted to six genes among which three encode the synthesis of four types of polyketide synthetases (PKS). The molecular diagnosis of BU is performed by PCR targeting insertion sequences of *MU* (IS2404, IS2606). This search can be coupled by detection of the plasmid which carries the genes encoding the synthesis of Mycolactone (Elise, 2011). Sarfo et al. (2011) showed in 2011 that Mycolactone circulates actually in BU patients at various stages of antibiotic therapy as recommended by WHO. However, in other infected people of that cohort, Mycolactone was found, neither in the exudates nor in the serum (Sarfo et al., 2011). In this study we sought to understand why Mycolactone was not detected in all BU patients. The first objective was to characterize Mycolactone in body fluids of patients who had got no antibiotics treatment by rifampicin and streptomycin. The second objective aimed at detecting the virulence factors in clinical samples and in *MU* isolated strains.

MATERIALS AND METHODS

Patient cohorts

Buruli ulcer suspected cases and healthy subjects (negative controls) were selected in three endemic areas of Côte d'Ivoire. Some patients were recruited in the last quarter of 2011 by local health workers in two endemic areas (Tiassalé and Djékanou), where there is a high prevalence of BU. The others were recruited in January 2012 by a mobile medical team actively screening the district of Abidjan, a hypo-endemic area. Patients were recruited if they met the WHO clinical case definition of Buruli ulcer disease (nodule, plaque, edema or ulcer). The control subjects were selected according to the following criteria: to have no suspicious lesion, to have never been infected, to have no apparent progressive pathology and to be from the same area as the patients. All subjects provided written informed consent (thumb print of parent or legal tutor in the case of children). The study protocol was approved by the National Ethic Committee.

Sampling procedure

Samples were collected on each lesion (nodule, plaque, edema and ulcer). Four swabs were performed on each ulcer after cleaning with normal saline. Two swabs were discharged into a tube containing 2 ml of Middlebrook 7H9 medium supplemented by Cetylpiridium chloride (0.5%) to achieve the isolation of *MU.* The other two were discharged into a tube containing 1 ml of pure ethanol for the extraction of lipids. A fine needle aspiration (FNA) was collected from pre-ulcerative lesions after application of a topical antiseptic. Each FNA was discharged into two tubes: one containing 2 ml of Middlebrook 7H9 medium and the other one 1 ml of pure ethanol. 10 ml of venous blood were also collected from each patient and control subject.

Diagnosis of Buruli ulcer disease in patients

Exudates and FNA samples were centrifuged at 3000 rpm for 20 min. The pellet was re-suspended in 2 ml of saline buffer to test three diagnostic methods. Four tubes of Löwenstein-Jensen medium were inoculated with 100 µl of each homogenate and incubated at 32°C. A daily supervision was instituted till the appearance of colonies. Microscopic examination to detect Acid-fast bacilli was performed by using dried smears made with 10 µl of homogenate and staining by the Ziehl-Neelsen technique (Adalbert et al., 2000). 1 ml of homogenate was washed twice by adding 1 ml of sterile DNA-free water. The mixture was centrifuged at 14000 rpm for 3 min, and the pellet was suspended in 400 µl of lysis buffer containing 20 mM Tris, 2 mM EDTA, 150 mM NaCl, 50 mM NaOH, 1% SDS and 20 µg/ml Proteinase K. The mixture was incubated at 37°C for 24 h. The phenol/chloroform method was used to extract and purify DNA. DNA was eluted in 50 µl sterile DNA-free water and stored at -20°C for molecular diagnosis. PCR was performed using the *GoTaq Flexi DNA polymerase* reagents Kit (Promega, Germany). The previous protocol was used to detect the IS2404 and KR in samples (Table 1). The reaction (25 µl) contains 5 µl of DNA, 0.3 µM of each primer, 0.25 µM of labeled probe (for real time PCR), and PCR-Mix. PCR consisted of 35 cycles of melting at 95°C for 5 s, aling and extension at 60°C for 1 min. The 7300 real-time PCR machine (Applied Biosystems, USA) was used and the fluorescence of FAM was measured to determine the amplification threshold cycle (Ct). Classical PCR were performed in 9700 PCR system Thermocycler (Applied Biosystems, USA) to confirm *MU* in isolated strains with the following targets: IS2404, IS2606 and ER. Negative controls were performed with 5 µl of nuclease free-water. Positive controls DNA were tested in duplicate.

Mycolactone extraction and analysis by HPLC

Lipids were extracted from serum or from exudates by the method previously described by Sarfo et al. (2011). Briefly the samples were dipped in 1 ml of ethanol immediately after the sampling and stored in polypropylene tubes at -20°C, protected from light. The samples were concentrated by drying, using a Speed Vacuum and were suspended in 500 µl of ethanol. The lipids were extracted by sequential addition of 4/1 MeOH (v/v), 1/1 $CHCl_3$ (v/v), and 3/1 H_20 (v/v). The aqueous phase was eliminated and the organic phase was transferred into a new tube containing 3 ml of methanol. A double centrifugation was performed and the soluble organic phase was transferred each time into a new tube. It was dried and

*Corresponding author. E-mail: aka_nguetta@yahoo.fr.

Table 1. Primers used in this study.

Target	Sequence (5'-3')		References
IS2404	Mu5	GAT CAA GCG TTC ACG AGT GA	Stinear and coll., 1999
	Mu6	GGC AGT TAC TTC ACT GCA CA	
IS2606	Mu7	CCGTCACAGACCAGGAAGAAG	Stinear and coll., 1999
	Mu8	TGCTGACGGAGTTGAAAAACC	
Enoyl- reductase	ER-R	GAG ATC GGT CCC GAC GTC TAC	Mve-Obiang and coll., 2005
	ER-F	GGC TTG ACT CAT GTC ACG TAA G	
Keto-reductase	KR-F	TCACGGCCTGCGATATCA	Fyfe and coll., 2007
	KR-R	TTGTGTGGGCACTGAATTGAC	

Table 2. Results of methods applied to samples of patients and controls. Microscopy, culture and PCR methods were applied to exudates and FNA samples. HPLC was applied to samples of patients (exudates, FNA, sera) and controls (sera).

Clinical samples		ZN Microscopy	IS2404 Detection	KR Detection	Culture of *MU*	HPLC (Mycolactone)
Batch A (N=42)	FNA (10)	2	10	5	7	5
	Exudates (32)	6	22	17	10	8
Batch B (N=42)	Sera (42)	-	-	-	-	18
Batch C (N=8)	Sera (8)	-	-	-	-	0
Total	92	8	32	22	17	31
Percentage (%)	-	19	76.2	52.4	40.5	36.9

suspended in 500 µl of ethanol to achieve a Shimadzu HPLC. Fractions of interest were collected in glass tubes and analyzed on a QSTAR XL, AB-MDS-SCIEX mass spectrometer. The data were collected and processed through the Analyst QS 1.1 software from AB-MDS-Sciex.

RESULTS

Microbiological and molecular tests of clinical samples

42 patients and eight control subjects were selected in three Ivorian endemic areas. 25% of these patients had pre-ulcerative lesions (10/42) and 75% had ulcers of various size (32/42). From all subjects 32 exudates, 10 samples of FNA and 50 sera were collected for the study. The microbiology tests were cell culture and microscopy of the clinical samples.

Acid-fast bacilli were found in 19% of exudates and FNA samples. 76% of those samples were positive for genomic target IS2404 and 52% for plasmid located KR target. *MU* was isolated in 40% and Mycolactone was detected in 36% of patient samples. No Mycolactone was detected in control subjects (Table 2).

Mycolactone detection in clinical samples

Total lipids of 92 samples were extracted and analyzed using HPLC method. The analysis by HPLC detected some peaks corresponding to mycolactone in 31% of exudates (13/42) and in 42.8% (18/42) of the sera of patients. The detection of the mycolactone was more consistent when the sample was from a recent lesion (non-ulcerative form or early ulcer). At this stage, the toxin has been detected in 50% of exudates and in 60% of the sera of patients. Conversely, no mycolactone was found in the control serums (0/8). The concentrations of mycolactone A/B in samples were relatively weak compared to those of positive controls (Figure 1A).

The fractions of interest analyzed by Mass spectroscopic (MS) showed in each case the characterizing spectrum of the ion 765, in harmony with the presence of intact structure of the mycolactone A/B in the samples (Figure 2). In the serum of controls, this specific ion and the isotope mass were negative, while the spectrum obtained with a serum of patient from an active Buruli ulcer showed specific ion with a molar mass of 765.4 $g.mol^{-1}$, with generated ions at 359.2, 429.3, 565.4 and 659.3 $g.mol^{-1}$ respectively (Figure 2B).

Molecular identification of isolated strains

To identify isolated strains, a PCR was performed targeting Insertion Sequences (IS2404, IS2606), and plasmid (KR, ER). All *MU* strains were 100% positive for targets IS2404 and IS2606, whereas only 70% were

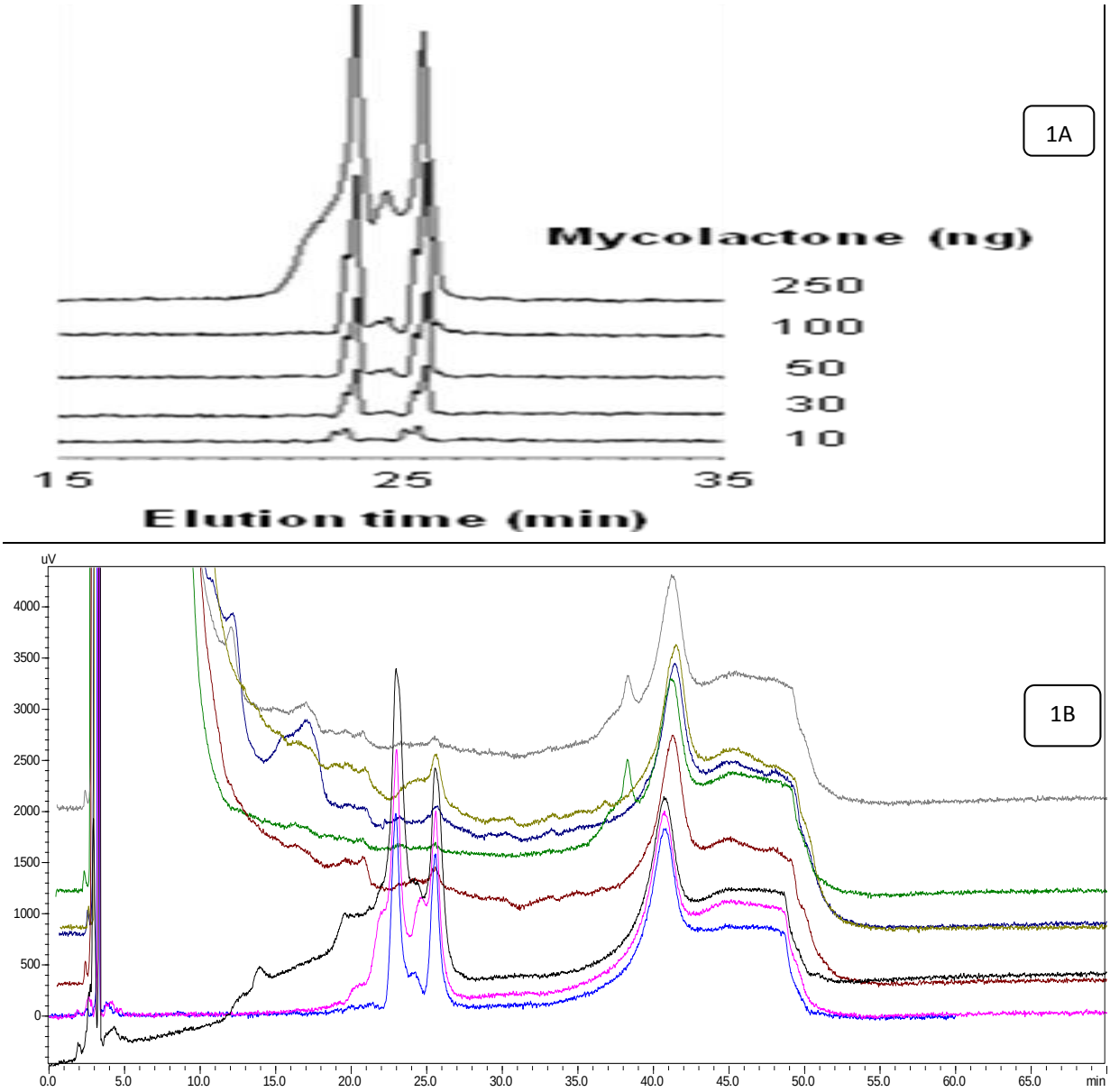

Figure 1. Chromatographic profiles of purified mycolactone (Figure 1A) and mycolactone in clinical samples (Figure 1B). **A**. Corresponds to the spectrum of 5 dilutions of the purified Mycolactone A/B. **B.** Shows signals obtained from clinical samples (exudates/sera). From bottom to top, the first three signals correspond to three concentrations of purified Mycolactone (50 ng; 100 ng; 250 ng). The curves 4 to 8 designate the signals given by the samples. Their mycolactone concentrations were respectively 20, 15.5, 26, 48 and 15 ng per ml. It is shown by weak amplitudes, compared to those of the dilutions of the purified Mycolactone.

positive for targets ER and KR (Table 3).

DISCUSSION

This study follows a previous one carried out on samples from Ivorian and Ghanaian patients. We had pointed out that mycolactone was present in body fluids at various stages of *MU* infection. Those results were obtained by 2 chemical approaches using extracts of total lipids (TLC-Fluo and HPLC/MS). The weak performance of the TLC-Fluo had been accounted by a link between the mycolactone and some biomolecules that could prevent the access to organic solvent during the chemical extraction of Mycolactone. The HPLC coupled by mass spectroscopic permitted to bring out some Mycolactone A/B at all the stages of the infection with *MU* (before, during and after the treatment by antibiotics) (Sarfo et al., 2011;

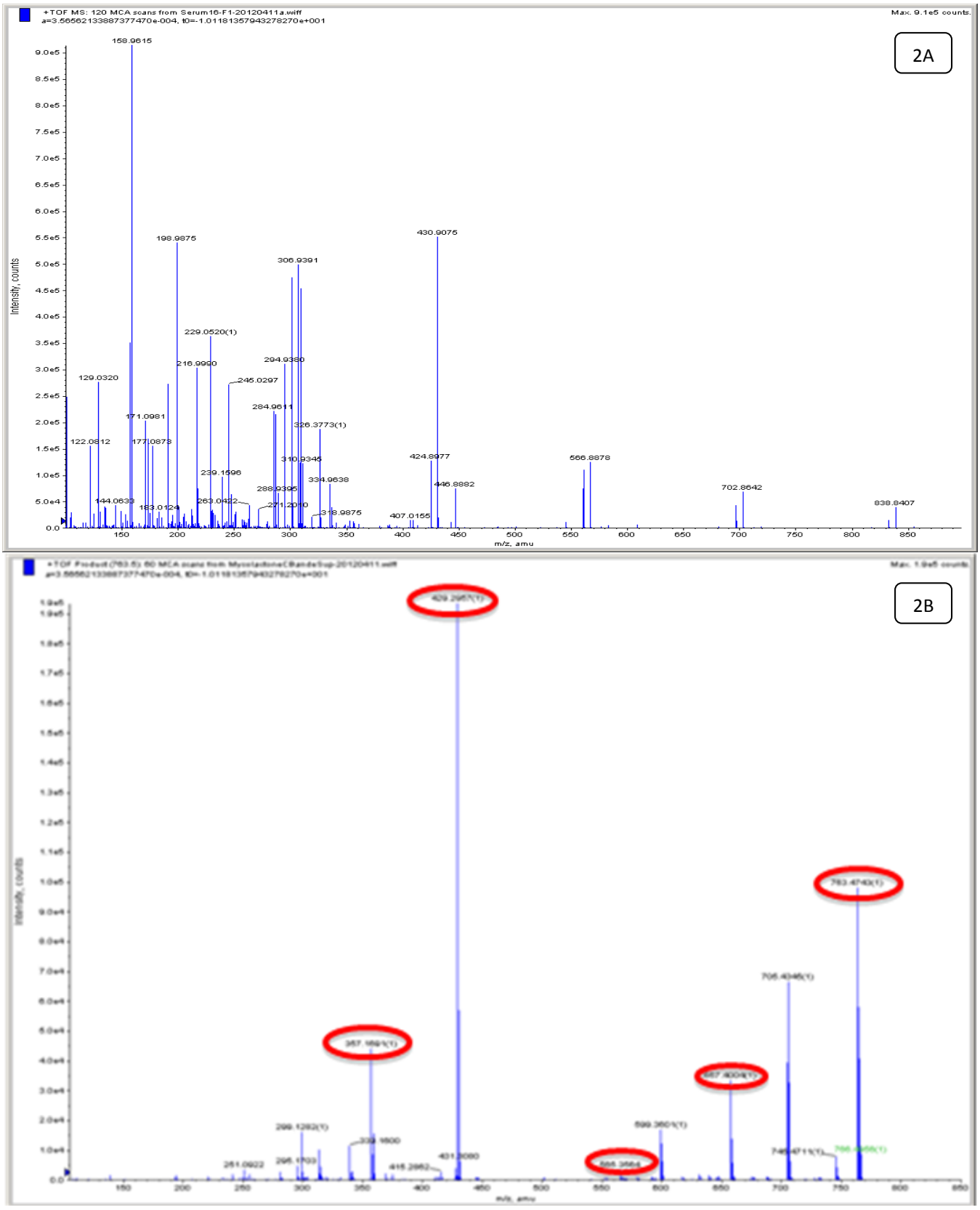

Figure 2. Mass spectrometry (MS) analysis of Acetone soluble lipids from fractions collected in the elution interval with HPLC in negative control (A) and in clinical sample for Buruli ulcer patient (B). The hydrolysis products of mycolactone and intact mycolactone can been identified at m/z 765.4.

Table 3. Molecular tests of isolated M.ulcerans strains.

Origin of strains	Quantity	Target detection		
		IS2404	IS2606	ER/KR
FNA	7	7	7	5
Exudates	10	10	10	7
Total	17	17	17	12
Percentage (%)	-	100%	100%	70.6%

Fred et al., 2014).

Our results show the high positive rate of molecular tests for Buruli ulcer in all samples collected in endemic areas. We used IS2404 and KR targets to detect *MU* in clinical samples. The combination of those targets permitted to confirm BU disease in patients and to determine if plasmid was present. The results show that 76.2% of samples contained IS2404 and plasmid was present in 52.4%. IS2404 and IS2606 have demonstrated the higher positivity by 100% for isolated strains. The high copy number of IS2404 (205-209 copies) and IS2606 (91 copies) in genome explain the sensitivity of PCR using IS2404 or IS2606 (Stinear et al., 2007). Fyfe et al. (2007) have demonstrated the identical sensitivity of PCR-IS404 and PCR-IS2606 (Fyfe et al., 2007).

Concerning PCR using targets KR/ER located on the virulence plasmid, the positivity rate was at 70.6% for isolated strains of *MU* and at 52.4% for clinical samples. The evidence of the plasmid lost or poor DNA extraction efficiency and PCR inhibition can explain our results. Molecular detection of *MU* in clinical and in environmental samples in multicenter external quality control program has demonstrated the limitations and the progress of the diagnosis (Eddyani et al., 2009). The lower positivity of culture by 40.5% is similar with the results of (Eddyani et al., 2008; Miriam et al., 2014). We demonstrated that the isolation in solid medium was more sensitive (82.3%) when the samples were taken from non-ulcerative lesions or from early ulcers (unpublished data). In this study, Mycolactone was detected in 31% exudates/FNA and 42.8% in sera but not in control subjects. The rate of Mycolactone detection varied according to the stage of the lesion. Mycolactone was more frequently detected in the liquid from non-ulcerated lesions (50%) and in the exudates from early ulcers (45.5%).

In contrast, the performance of HPLC was very weak in larger lesions (less than 18%). Similar result was observed in the sera, but the frequency was high for larger ulcers (40%). Our results confirm the evidence that serum was the appropriate biological fluid to follow the kinetics of secretion of the mycolactone during the *MU* infection (Fred et al., 2011; Fred et al., 2014).

The characterization by MS pointed out a predominant secretion of entire molecule of mycolactone A/B (93.6%) and 6.4% of Mycolactone A/B with loss of an ion of “hydrogen”. Our results are similar with the results of Portaels et al. (2008) for mycolactone A/B from *MU* isolated from environmental (Portaels et al., 2008). The results confirmed that isolated strains of *MU* belong to African strains with typically expression of virulence toxin, Mycolactone A/B.

Among the strains, 70.6% had the plasmid and the Enoyl-reductase gene. We compared the results of gene detection to those of the mycolactone detection in body fluids. The analysis showed that in patients infected by *MU* strains having a plasmid, the mycolactone was detected in body fluids. In contrast, when the plasmid was not found in *MU* strains, no mycolactone was detected in patients. These results could be explained by three factors: the first factor could be related to limits of extraction methods used in this study (poor DNA extraction efficiency and PCR inhibition). The second factor could be related to the loss of plasmid by *MU* during the culture process. The third factor could be related to the circulating in endemic areas of *MU* strains with plasmid and those without plasmid.

Conclusion

This study shows that Mycolactone A/B was actually found in most of BU patients selected in three Ivorian endemic areas. With the methods used we detected very low concentrations of mycolactone in patient fluids. Plasmid and ER gene were found in the majority of *MU* strains. But they were not found in about 30% of strains. Mycolactone was detected only in patients infected by strains in which plasmid was found. Further investigations, including the study of the virulence in laboratory animals and analysis of the entire genome of *MU* strains will certainly clarify these issues.

Conflict of interests

The authors did not declare any conflict of interest.

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