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Microbiological and molecular characterization of environmental mycobacterium strains isolated from the Buruli ulcer endemic and non-endemic zones in Côte d'Ivoire

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***Mycobacterium ulcerans (MU)*, the causative agent of Buruli ulcer (BU), skin disease, is considered to be an environmental pathogen. The pathogenic virulence of *MU* is being linked to the expression of toxin called Mycolactone. Genetic analyses have shown the high diversity with variable number tandem repeats (VNTR) and mycobacterial interspersed repetitive units (MIRU) in *M. ulcerans* and in mycolactone producing Mycobacteria (MPMs). The purpose of this study is the molecular characterization of potentially pathogenic environmental mycobacteria strain, apart from the *M. ulcerans,* from aquatic environments in Côte d'Ivoire. A total of 473 samples were collected comprising of 251 water and 222 sediment based on sampling sites. The sediments were the most contaminated by mycobacteria with 60% as against 43.3% in water samples from the hyper endemic areas. In hypo-endemic areas, water was the most contaminated with 53.57% against 43.24% in sediment. Microscopy by Ziehl-Neelsen-staining, and PCR diagnostics using IS2404 and KR were performed on strains. 20% fast growing isolated mycobacteria species including *Mycobacterium mucogenicum*, *Mycobacterium peregrinum* and *Mycobacterium* sp. was found carrying the IS2404 gene previously found in *M. ulcerans.* 9.23% of strains carry the ketoreductase (KR) genes, one of the synthesis of mycolactone enzymes. In terms of genetic analysis using the MIRU/VNTR, the MIRU 1 was the most amplified sequence, and LOCUS 6 less amplified; no known profile have been identified in this study. This study is the first step taken in order to understand different skin infections encountered in Côte d'Ivoire.**

Key words: Mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR; ketoreductase, LOCUS 6, IS2404, *Mycobacteria*, Buruli ulcer.

INTRODUCTION

Mycobacteria are germs that cause lung infections, skin or lymph (Griffith et al., 2007). They are found in the environment like in the soil, water, aerosols, plants, aquatic animals (Winthrop et al., 2002; Sniezek et al.,

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2003; Marsollier et al., 2002, 2004). This Acid-Alcohol Bacillus Resistant (AFB) are not mandatory pathogens and for several years, the scientific community have not really shown interest in their study, but rather devoted to the study of TB epidemic. They were considered as saprophytic bacteria and their pathogenic potential are not recognized (Ziza and Desplaces, 2006). However, some mycobacteria such as *Mycobacterium liflandii* and *Mycobacterium fortuitum* produce toxins causing skin infections (Williamson et al., 2008; Kakou-Ngazoa et al., 2015). This is an extremely polymorphic bacterial genus that includes fast-growing species (less than 7 days), and slow growing species (7-60 days) and non-culturable outside animals species, *Mycobacterium leprae*, leprosy causing agent (Euzéby, 2010). Mycobacteria are divided into two major groups, they are complex tuberculosis and non-tuberculosis mycobacteria (NTM) also called environmental mycobacteria (mycobacteria of leprosy and atypical mycobacteria) (Inderlied et al., 1993; Caruso et al., 2009; del Rio Camacho et al., 2010). Although, molecular biology has helped to discover the existence of diversity in environmental mycobacteria (Domenech et al., 1994; Menendez et al., 2002; Williamson et al., 2008; Kakou-Ngazoa et al., 2015), culturing of species however, remains the most efficient means of knowing their physiology and their antibiotic sensitivity (Kubica et al., 1964; Trujillo et al., 2004). The most important ones are *Mycobacterium avium*, *Mycobacterium intracellulare, Mycobacterium kansasii, Mycobacterium xenopi* and *Mycobacterium abscessus* (Falkinham, 1996; Dailloux et al., 2010). The complex *M. avium, M. kansasii, M. chelonae* and *M. xenopi* are responsible for the majority of infections in the developed countries (Horsburgh, 1996), while *M. ulcerans* is responsible for ulcerative diseases in tropical and subtropical countries (Pedley et al., 2004). The distribution of commonly isolated species is in constant change in most countries studied and new species emerge (Martin-Casabona et al., 2004). They are part of the group of non-pigmented genes atypical mycobacteria such as *M. abscessus, M. chelonae, M. fortuitum* and *M. smegmatis* (Brown and Wallace, 1992).

Buruli ulcer, debilitating disease, is a serious public health problem. Almost all regions in Côte d'Ivoire are affected. The mode of transmission and environmental sources are unknown. To be able to understand the level of prevalence of skin infections in Côte d'Ivoire, it is important to identify species other than *M. ulcerans* that are involved. Given their large numbers, this study was undertaken with the aim to achieve the molecular characterization of potentially pathogenic environmental mycobacteria strains others than *M. ulcerans* from different aquatic environments in Côte d'Ivoire.

MATERIALS AND METHODS

Sites and scope of the study

The study was conducted at different sites considered to be Buruli ulcer endemic zones (Adiopodoumé, Tiassalé, Adzopé) and non-endemic zones (Agboville, Bouaké, Aghien) according to the national program against Buruli ulcer in Côte d'Ivoire. However, all biological part of this work was carried out at the Pastor Institute of Côte d'Ivoire.

Biological material

The biological material consisted of water and sediments sample from different studied environments.

Procedure

Samples were collected monthly, from June 2014 to June 2015. A total of 22 sampling stations were selected, 11 from the Lagoon of Aghien, 3 from Adzopé water retention. 2 stations were selected from Sokrogbo sites, Bodo and Adiopodoumé, respectively, 1 station each was selected, at the entrance of Agboville and Loka in Bouake water retention (Figure 1). The real prevalence of MU in the sampling site is unknown. But according the National reference Center of Buruli Ulcer (unpublished document), the rate of Buruli Ulcer confirmation in the region of the sampling site were: 62% Agboville; 56.36% Bouake, 30.77% Aghien and Adzopé.

Collection of water and sediment samples

The sediment samples were collected using dump Eckmann at big water points and a sampler at the banks (Schiavone and Coquery, 2011). A 5 L capacity bucket spout allowed drawing water at the bank of water points and a hydrological bottle 1.5 L capacity for drawing water far away from the banks. The sediments samples were put in sterile plastic bags and the water samples in sterile glass bottles of 1 L capacity. The samples were then kept refrigerated at 4°C during transportation, protected from light and taken to the laboratory within 24 h of sampling.

Culturing

In the laboratory, 500 g of each sediment and 100 mL of water sample was collected. In a Falcon tube 50 mL, 10 g of sediment were mixed with 40 mL of sterile distilled water following the method described by Kankya et al. (2011), slightly modified. After mixture of sediment and distilled sterile water, the supernatant was recovered in a new Falcon tube. Decontamination of water samples and recovered supernatant was carried out with cetylpiridium chloride (CPC) (Stinear et al., 2004), followed by neutralization with phosphate buffer.

The different culture medium: Loweinstein Jensen (LJ), Mac Conkey without purple crystal, ordinary agar and Middlebrook 7H10 agar was used for seeding. The samples in LJ and Middlebrook 7H10 agar were seeded in duplicate. One lot of each of them was packed in an aluminum foil for photoinduction test. The incubations were made at 23 and 37°C in the ovens. Daily observation were made until colonies were obtained.

An optical microscope (*Zeiss*®) was used for the observation of Acid-Alcohol Bacillus Resistant after Ziehl-Neelsen coloration (Barksdale, Kim. 1977). Classification of species was made according to the method described by Runyon and collaborators (1959). Biochemical identification of mycobacteria was done according to the method described by Metchock (1995). Briefly, for biochemical identification, the colonies obtained after culture was observed microscopically. The Acid-Alcohol Bacillus Resistant were cultured in the presence and absence of light on LJ medium for the test of photo-induction.

They were then seeded on ordinaire Agar at different temperatures (37, 42, 45 and 52°C), on LJ medium with 5% NaCl with the manitol test, the test in presence of sodium citrate and test in the presence of ferric ammonium.

Table 1. List of primers and detectors used.

Detector	Primers	Sequences (5'-3')	References
IS6110	MYCGEN-F	AGAGTTTGATCCTGGCTCAG	Wilton and Cousin (1992)
	MYCGEN-R	TGCACACAGGCCACAAGGGA	
IS2404	IS2404 F	ATTGGTGCCGATCGAGTTG	Ross et al. (1997)
	IS2404 R	TCGCTTTGGCGCGTAAA	
	IS2404-probe	6 FAM-CACCACGCAGCATTCTTGCCGT-TAMRA	
KR	KR F	TCACGGCCTGCGATATCA	Fyfe et al. (2007)
	KR R	TTGTGTGGGCACTGAATTGAC	
	KR-probe	6 FAM-ACCCCGAAGCACTG-TAMRA	
MIRU1	MIRU1 F	GCTGGTTCATGCGTGGAAG	Stragier et al. (2005), Ablordey et al. (2005) and Hilty et al. (2006)
	MIRU1 R	GCCCTCGGGAATGTGGTT	
ST1	ST1 F	CTGAGGGGATTTCACGACCAG	
	ST1 R	CGCCACCCGCGGACACAGTCG	
VNTR-19	Locus 19F	CCGACGGATGAATCTGTAGGT	
	Locus 19R	TGGCGACGATCGAGTCTC	
VNTR-6	Locus 6 F	GACCGTCATGTCGTTCGATCCTAGT	
	Locus 6 R	GACATCGAAGAGGTGTGCCGTCT	

Molecular analysis of strains of mycobacteria

The DNA extraction was performed according to the method described by Ausubel et al. (1987). Molecular characterization was carried out using two types of PCR: Conventional PCR for the search of sequences IS6110 and MIRU-VNTR (Miru 1, Locus 6 VNTR19; ST1) and RT-PCR for the IS2404 sequences and ketoreductase (Kr). These analyses were done with all isolated colonies of Acid Alcohol Bacillus Resistant after culture. IS6110 and IS2404 were chosen to identify mycobacteria, MIRU-VNTR for typing the mycobacteria and the Kr sequence for the search of virulence factor.

Conventional PCR

The amplification of sequences IS6110 and Miru VNTR was made in the presence of specific primers for each sequence (Table 1). Each DNA extract (5 µL) was placed in the presence of 10X buffer, magnesium chloride (25 mM), DNTP 10 µM, copies of primers specific to each sequence (IS6110 and Miru VNTR), of 0.2 µl of DNA Taq polymerase (Hot Start Taq) in a final volume of 50 µL. The reaction mixture was incubated in a thermocycler of the type Gene Amp 9700 (Applied Biosystem®), according to the following schedule:IS6110 (94°C for 5 min; 94°C for 30 s; 62°C for 45 s; 72°C for 1 min 30; 72°C for 10 min and 4°C), Miru 1, Locus 6, VNTR 19 (95°C for 2 min; 94°C for 1 min; 58°C for 1 min; 72°C for 1 min; 72°C for 10 min and 4°C) ST1 (95°C for 2 min; 94°C for 1 min; 65°C for 1 min; 72°C for 1 min; 72°C for 10 min and 4°C).

The PCR products were separated by electrophoresis on agarose gel containing 2% ethidium bromide (ETB). The visualization was carried out under UV light from an automated system (Gel documention, Bio-Rad Laboratories USA).

Primers used

The sequences of the primers used are summarized in Table 1.

Real time PCR

Amplification of IS2404 and Kr sequences was performed in the presence of specific primers for each sequence (Table 1) in a final reaction volume of 50 µL. For markers used, a PCR –mixer of 20 µl containing water for injection (H_2O ppi), 5X buffer, magnesium chloride (25 mM), dNTP, a detector of 10µM, a Rox Dye, 0.2µl DNA Taq polymerase (Hot Start Taq) and 5 µl of our DNA extract. The reaction mixture was incubated in a STEP ONE PLUS device as follows : 50°C for 2 min; 95°C for 10 min; 95°C for 15 S; 60°C for 1 min.

The control strain was *M. ulcerans* (ITM9540) provided by the molecular biology platform of Pasteur Institute of Côte d'Ivoire. The negative control was sterile distilled water.

RESULTS

A total of 473 samples were collected, 251 water and 222 sediment left based on sampling sites (Table 2). Based on morphological and biochemical characteristics, 65 strains (13.74%) were isolated and 17 species were identified (26.15%). From the water samples collected, 43.07% of species were identified, and 59.92% from sediments. These identified species were distributed according to the collection sites. 32.35% of the identified species are found in the hyper endemic sites, against 19.35% of species from hypo endemic sites. The *M. peregrinum* species, like *M. smegmatis*, *M. peregrinum*, *M. immunogenicum*, *M. chelonae*, *M. mucogenicum, M. abscessus, Mycobacterium* sp. were isolated in this study. *M. peregrinum* (13.84%) species was the most common in all sites, except in the sites of Bodo and Bouaké. The sediments are the most contaminated by *Mycobacteria* with 60% presence against 43.3% presence in water samples in hyper endemic areas. In hypo-endemic areas, water is the most contaminated with 53.57% presence against 43.24% in sediment samples. All species obtained were analyzed by PCR, focusing on IS2404 and Kr sequences. 9.23% of the isolated strains

Table 2. Samples collected according to sites and types.

Sample	Collection sites							
	Endemic sites				Non endemic sites			
	Adzopé	Adiopodoumé	Tiassalé		Agboville	Aghien	Bouake	Total
			Sokrogbo	Bodo				
Water	45	18	18	6	15	143	6	251
Sediments	16	18	18	6	15	143	6	222
Total	61	36	36	12	30	286	12	473

Table 3. Molecular characteristics of Identified mycobacteria species in each site.

Site	Samples collected	Mycobacteria species	IS6110	IS2404	Kr
Adzopé	Water	*M. smegmatis*	-	-	-
Adzopé	Water	*M. peregrinum*	+	-	-
Adzopé	Water	*M. mucogenicum*	+	-	-
Adzopé	Water	*M. mucogenicum*	-	+	+
Adzopé	Water	*M. mucogenicum*	-	+	+
Adzopé	Sediment	*M. peregrinum*	-	-	+
Abgoville	Sediment	*Mycobacterium sp.*	+	-	-
Abgoville	Sediment	*M. smegmatis*	-	-	+
Abgoville	Sediment	*M. immunogenicum*	-	-	-
Aghien	Sediment	*Mycobacterium peregrinum*	-	-	-
Aghien	Sediment	*Mycobacterium sp.*	+	-	-
Aghien	Water	*Mycobacterium sp.*	-	-	-
Aghien	Sediment	*Mycobacterium.peregrinum*	-	-	-
Sokrogbo	Sediment	*Mycobacterium sp.*	-	+	-
Sokrogbo	Water	*Mycobacterium sp.*	-	+	-
Sokrogbo	Sediment	*Mycobacterium sp.*	-	+	-
Sokrogbo	Sediment	*Mycobacterium sp.*	-	+	-
Bodo	Water	*M. mucogenicum*	-	+	-
Bodo	Water	*M. mucogenicum*	-	-	-
Adiopodoumé	Water	*M. peregrinum*	-	+	+
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	-
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	-	-
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	+
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	-
Adiopodoumé	Water	*Mycobacterium sp.*	-	+	-
Adiopodoumé	Sediment	*Mycobacterium sp.*	-	+	-

IS2404: Insertion sequence found in *Mycobacterium ulcerans* and other environmental mycobacteria; Kr: synthesizing enzyme mycolactone toxin found in *Mycobacterium* ulcerans and other MPM. ; (-): Negative result in the desired sequence; (+): Positive result in the desired sequence. Positive control DNA (ITM9540); negative control: (H_2O).

had ketoreductase genes (KR), these two sequences were found in the fast-growing isolated species. *M. peregrinum, M. mucogenicum* and *Mycobacterium* sp. (Table 3). All strains were identified by using Miru-VNTR. The isolated strains showed different profiles in the presence of the MIRU1, VNTR19, LOCUS 6 and ST1. Of The 4 markers used, the MIRU 1 is the most amplified sequence, and LOCUS 6 less amplified. None known profile was identified in this study (Table 4).

DISCUSSION

Mycobacteria species are a large group distributed in

Table 4. Molecular typing of identified mycobacteria species according to sites.

Site	Mycobacteria specie	MIRU1	VNTR19	ST1	LOCUS 6
Adzopé	*M. smegmatis*	2	nd	nd	nd
Adzopé	*M. peregrinum*	nd	nd	2	nd
Adzopé	*M.mucogenicum*	4	nd	nd	nd
Adzopé	*M. mucogenicum*	nd	2	nd	nd
Adzopé	*M. mucogenicum*	nd	nd	1	nd
Adzopé	*M. peregrinum*	nd	nd	nd	nd
Abgoville	*Mycobacterium* sp.	nd	nd	nd	nd
Abgoville	*M. smegmatis*	4	nd	nd	nd
Abgoville	*M. immunogenicum*	3, 4	nd	nd	nd
Aguien	*Mycobacterium peregrinum*	nd	2	nd	nd
Aguien	*Mycobacterium* sp.	nd	nd	2	nd
Aguien	*Mycobacterium* sp.	nd	2	2	nd
Aguien	*Mycobacterium peregrinum*	nd	2	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Sokrogbo	*mycobacterium* sp.	nd	nd	nd	nd
Bodo	*M. mucogenicum*	nd	nd	nd	nd
Bodo	*M. mucogenicum*	nd	2	nd	1
Adiopodoumé	*M. peregrinum*	1	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	1, 2	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	1	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	nd	nd	2	nd
Adiopodoumé	*Mycobacterium* sp.	nd	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	4	nd	nd	nd
Adiopodoumé	*Mycobacterium* sp.	1	nd	nd	nd

nd: Not determined ; Miru1 (1 copy, 2 copies, 3 copies and 4 copies); VNTR 19 (2 copies); ST1 (1 copy and 2 copies) ; LOCUS 6 (1 copy); the negative control (H_20) has a negative outcome for all PCR.

various aquatic and terrestrial environments. Most are saprophytic, but some species are pathogens capable of infecting humans and animals (Von Reyn et al., 1993; Falkinham, 2009). Their no specific pathogenic characteristic differentiates them from mycobacteria causing tuberculosis. The objective of this study was to make the molecular characterization of environmental mycobacteria strains in Côte d`Ivoire. The high sediment contamination identified in the study highlight the fast growing of mycobacteria (Kirschner et al., 1992). The risk of transmission of cutaneous mycobacteriosis would therefore also be present in non-endemic sites like in the endemic locations as demonstrated by Williamson et al. (2008) in the case of Buruli ulcer. The prevalence rate of environmental mycobacteria was 13.74%. This rate was relatively lower than the 15.5% observed by Kankya et al. (2011) in Ouganda. Parashar et al. (2004) also observed variations in the effectiveness of decontamination methods depending on the origin of the samples. Indeed it is known that mycobacterial species do not have the same resistance to different decontamination procedures (Parashar et al., 2004). According to the classification of Runyon, all isolated mycobacteria belongs to the group of fast growing mycobacteria (Group IV). The following species have been identified in this study: species *M. peregrinum, M. chelonae, M. abscessus, M. mucogenicum, M. immunogenum, M. smegmatis, M. peregrinum* and *Mycobacterium* sp. The species *M. peregrinum* was the most common in all sites, except in the sites of Bodo and Bouaké. All these species identified in Cote d`Ivoire are responsible for skin ulcerations in some countries as reported by Zamarioli et al. (2008) and Buijtels et al. 2009. *M. chelonae*, is responsible for skin soft tissue infections as reported by Sniezek et al. (2003), *M. abscessus* responsible for skin and soft tissue infections with abscess formation or skin nodules, often appearing after trauma or surgical operation with infected materials (Griffith et al. 2007). Among the isolated strains, 20% were carriers of the gene IS2404, they include *M. mucogenicum, M. peregrinum* and *Mycobacterium* sp.

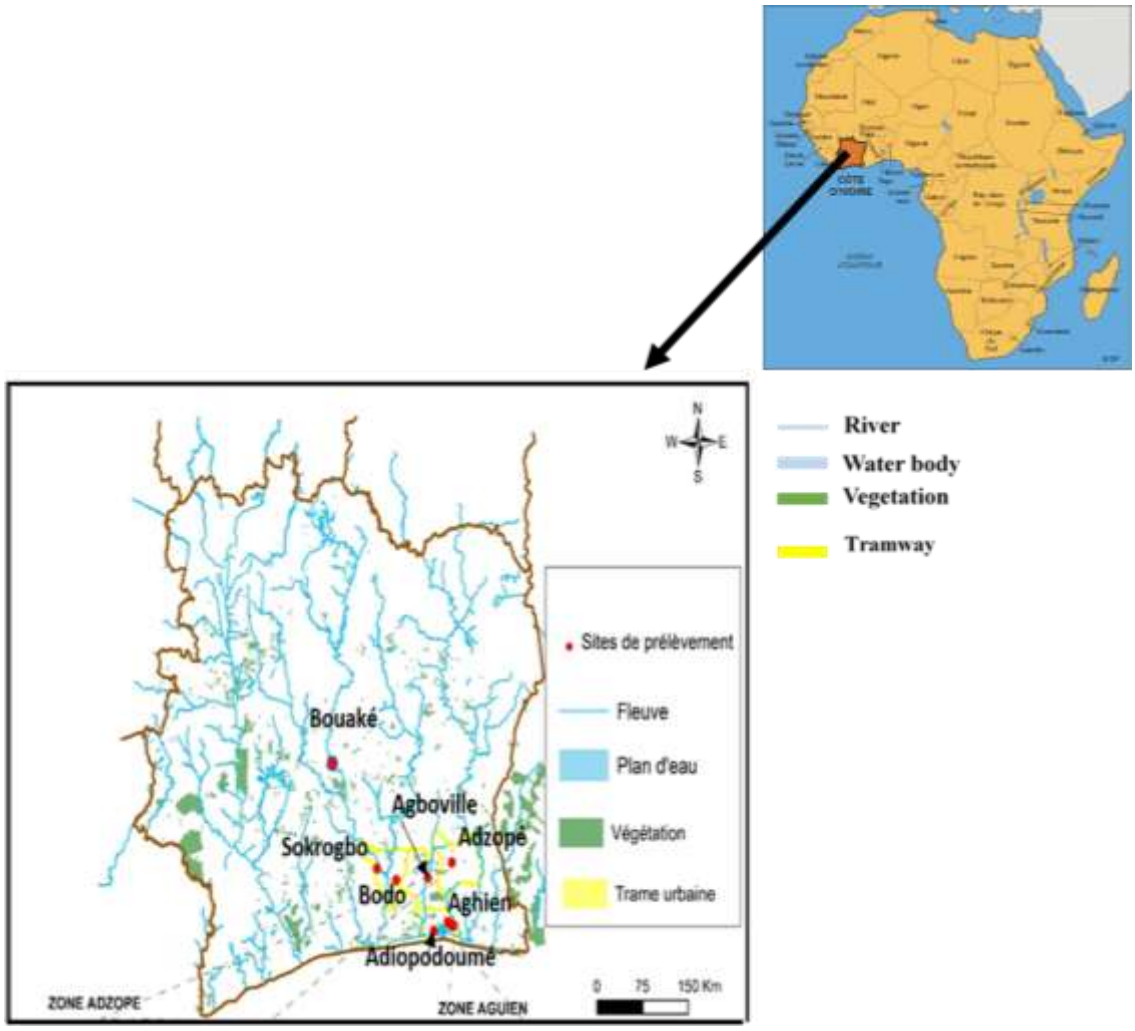

Figure 1. Sampling sites (Photo: Vakou Sabine, 2016).

This could imply the ability of these bacteria to cause skin ulcers in their isolated areas in Côte d'Ivoire. The sites of Bodo and Adzopé are known for their endemic zone to skin ulcers which has been attributed rightly or wrongly to *M. ulcerans* according to the National anti Buruli Ulcer Programe.

M. ulcerans is a slow-growing mycobacterium whose culture is often taken by default. The discovery in 1997 of the sequence of insertion IS2404 specific to *M. ulcerans* has been a catalyst for further research of the environmental germs (Ross et al. 1997). It shares this sequence with the other environmental mycobacteria such as *M. marinum, M. liflandii and M. pseudoshottsii* (Chemlal et al., 2002; Stragier et al., 2006). These slow growing mycobacteria would also be responsible for skin ulceration (Stragier et al., 2006). This sequence is an ideal for the diagnosis of Buruli ulcer (Portaels et al., 2009) could be good also for the diagnosis of Mycobacterial skin ulcerations.

Some differences were observed between the endemic sites and the non-endemic one. With the other level of contamination of water or sediments, the presence of *IS2404* gene was noticed only in the endemic sites. The Kr gene is most prevalent in endemic sites. This situation could be explain the sharing of genetic material between strains more frequently in endemic sites. But it could also be due to chance, in fact, Williamson et al. (2008), think that there would be no difference in the distribution of these genes between endemics and non-endemics sites. This study would be the first to reveal the existence of IS2404 gene in the fast-growing mycobacteria because according to previous studies, the IS2404 was the prerogative of slow growing mycobacteria (Chemlal et al., 2002; Stragier et al., 2006). In West Africa, Williamson et al. (2008), have also identified IS2404 gene in mycobacteria other than *M. ulcerans,* but they were the slow growing species. The ulcerations of Buruli ulcer are caused by one toxin, mycolactone (George et al., 1999).

A plasmid of 174 pb (pMUM001) present in the genome of the bacterium carries the polyketide synthetase genes (PKS), responsible for its' synthesis (Stinear et al., 2004, 2007). It was identified in other environmental mycobacteria that are slow growing, known to be mycolactone producing mycobacteria (Pidot et al., 2010). Results of this study also show its existence in the fast growing mycobacteria. Indeed, 9.23% of the isolated strains carry the ketoreductase gene (Kr), one of the enzymes involve in the synthesis of mycolactone according to Bali et al. (2006). Indeed, this enzyme would confirm the presence of the plasmid in the genome of the bacterium (Solange et al., 2015). Among the four specific markers, the MIRU 1 is the most amplified sequence, and LOCUS 6 the less amplified. No any known profile was identified in this study. These markers would be less appropriate to environmental isolated strains since they were tested only on samples from environmental and clinical strains (Stinear et al., 2007).

Conclusion

The results of this study highlight the potential risk of contamination in humans especially people in permanent contact with the environment. These species discovered, would be responsible for ulcerations in Côte d'Ivoire, which could explain the very high level of endemicity. It is therefore important to identify the sequences of these species in order to set up appropriate diagnostic methods. This study is just the first step, we want to understand the different skin infections encountered in Côte d'Ivoire. This will help to better diagnose patients suffering from skin infections other than Buruli ulcer and to consider strategies and means of protection of the population against all mycobacterioses by breaking the epidemiological chain.

In perspective, it would be good to continue studying the rapidly growing mycobacteria to determine their involvement in cutaneous ulcerations. The sequencing of the genes Kr and IS2404 isolated in the strains of this study for their eventual relationship with *M. ulcerans* could help for a better comprehension of the affection.

Conflict of interest

The authors declared that there is no conflict of interests.

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