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First Detection of Ketoreductase in the Genome of Rapid Growing Environmental Mycobacterial Strains in Cote D'ivoire

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Authors' contributions

This work was carried out in collaboration between all authors. Author CKJ designed the study, managed the sampling and wrote the first draft of the manuscript. Author VNS wrote the protocol, managed the sampling and the analyses of the study. Author CND managed the analyses. Author DAJ corrected the protocol. Author DM supervised the analysis. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

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. The ketoreductase (KR) gene, is one of the synthesis genes of mycolactone enzymes previously found in *M. ulcerans*. Genetic analyses using variable number tandem repeats (VNTR) and mycobacterial interspersed repetitive units (MIRU) have shown high diversity in *M. ulcerans* and in mycolactone producing Mycobacteria (MPMs).

Aim: The purpose of this study is to detect ketoreductase gene in the genome of environmental mycobacteria strain, apart the *M. ulcerans,* from aquatic environments in Côte d'Ivoire.

Place and Duration of the Study: The analysis of the samples took place in the laboratories of

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Institut Pasteur de Côte d'Ivoire in Abidjan City between June 2014 and December 2015. Sampling was done in some hypoendemic and hyperendemic sites of Buruli Ulcer of Côte d'Ivoire.
Methodology: A total of 473 samples were collected comprising of 251 waters and 222 sediments based on sampling sites. PCR diagnostics using IS2404 and KR were performed on strains.
Results: 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 carried the ketoreductase (KR) genes, one of the synthesis of mycolactone enzyme.
Conclusion: The results of this study proved the existence of ketoreductase (KR) genes in rapidly-growing mycobacteria. This study is one of the steps taken in order to understand different skin infections encountered in Côte d'Ivoire.

Keywords: Mycobacteria; buruli ulcer; environmental; ketoreductase (Kr); gene; rapid growing.

1. INTRODUCTION

Environmental mycobacteria, classified as "atypical", emphasizes the difference in terms of microbiological characteristics and pathogenicity of these mycobacteria when compared with other classical pathogenic mycobacteria such as *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium paratuberculosis, Mycobacterium leprae* and *Mycobacterium leprae murium*, which are strictly parasites with well-established specific pathogenicity [1]. The infections caused by these mycobacteria become difficult to diagnose and difficult to treat despite a suitable and prolonged antibiotic treatment. Buruli ulcer, a disabling disease, is a real public health problem. In Côte d'Ivoire almost all regions are affected. The mode of transmission and environmental sources are not well known. To understand the prevalence level of mycobacterial infections, it is important to identify the species involved in the ulcerations. As a result of phylogenetic comparisons, M. ulcerans is almost identical to *M. marinum*, mycobacterium responsible for infection in fish. This is due to the acquisition by *M. ulcerans* of a plasmid PMUM001 which would carry the genes of the biosynthesis of a toxin called mycolactone [2]. It has been identified in other slow-growing environmental mycobacteria called mycolactone-producing mycobacteria [3]. Using molecular technology, it has been shown that it also has other genes such as the IS2404 insert sequence common to other environmental mycobacteria such as Mycobacterium marinum, Mycobacterium liflandii and Mycobacterium pseudoshottsii [4,5]. This sequence, which is therefore a target for the diagnosis of the Buruli ulcer [6], could also be responsible for cutaneous Mycobacterial ulcerations. This study would be the first to demonstrate the existence of the IS2404 gene and ketoreductase in rapid-growing mycobacteria because, according to previous studies, they were identified with slow-growing mycobacteria [4,5].

2. MATERIALS AND METHODS

2.1 Sites and Scope of the Study

The study was carried out on sites considered to be hyperendemic to *Buruli ulcer* (Adiopodoumé, Tiassale, Adzopé) and hypoendemic sites (Agboville, Bouaké, Aghien) according to the National Program for the Control of *Buruli ulcer* in Côte d`Ivoire. However, the whole biological part of this work was carried out at the Institut Pasteur of Côte d'Ivoire.

2.2 Materials

The materials consisted of water and sediment samples from the different environments studied.

2.3 Methodology

Samples were collected monthly, from June 2014 to June 2015. A total of 22 collection stations were selected, 11 from the Lagoon of Aghien, 3 from Adzopé water retention. Two stations each were selected from Sokrogbo sites, Bodo and Adiopodoumé respectively. One station each selected, at the entrance of Agboville and Loka in Bouake water retention.

2.3.1 Collection of water and sediment samples

The sediment samples were collected using dump Eckmann at big water points and a sampler at the banks [7]. A 5 liter capacity bucket spouts allowed to draw water at the bank of water points and a hydrological bottle 1.5 L capacity for drawing water far away from the banks. The samples were then kept refrigerated at 4℃ during transportation, protected from light

Table 1. The sequences of the primers used in the study

Detectors	Primers	Sequences (5'-3')	References
IS6110	MYCGEN-F	AGAGTTTGATCCTGGCTCAG	Wilton and
	MYCGEN-R	TGCACACAGGCCACAAGGGA	Cousins [9]
IS2404	IS2404 F	ATTGGTGCCGATCGAGTTG	Ross et al.
	IS2404 R	TCGCTTTGGCGCGTAAA	[10]
	IS2404-probe	6 FAM-CACCACGCAGCATTCTTGCCGT-TAMRA	
KR	KR F	TCACGGCCTGCGATATCA	Fyfe et al.
	KR R	TTGTGTGGGCACTGAATTGAC	[11]
	KR-probe	6 FAM-ACCCCGAAGCACTG-TAMRA	
	Locus 6 R	GACATCGAAGAGGTGTGCCGTCT	

and taken to the laboratory within 24 hours of sampling.

2.3.2 Molecular analyzes of mycobacterial strains

Molecular analysis was performed on strains of mycobacteria previously obtained in culture at the Pasteur Institute of Cote d'Ivoire. The extraction of the DNA was carried out according to the method described by Ausubel et al. [8].

The molecular characterization was carried out by two types of PCR: A conventional PCR for the search of the IS6110 sequences and an RT PCR for the IS2404 and Ketoreductase (Kr) sequences.

2.3.2.1 Conventional PCR

The amplification of sequences IS6110 and Miru VNTR was made in the presence of specific primers to each sequence (Table 1). Each DNA extract (5 µL) was placed in the presence of 10X buffer, magnesium chloride (25 mM), DNTP 10µM, copies of primers specific to each sequence (IS6110 and Miru VNTR), of 0,2µl of DNA Taq polymerase (Hot Start Taq) in a final volume of 50 µ L. The reaction mixture was incubated in a thermocycler of the type Gene Amp 9700 (Applied Biosystem®), according to the following schedule:

IS6110 (94℃ for 5 min, 94°C for 30 s, 62℃ for 45 s, 72℃ for 1 min, 72℃ for 10 min and 4℃). 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 documentation, Bio-Rad Laboratories USA)

2.3.2.2 Real time PCR

Amplification of IS2404 and Kr sequences was performed in the presence of specific primers to 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 10 µM, a RoxDye, 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℃ for 2 min; 95℃ for 10 min; 95℃ for 15 Seconds; 60℃ for 1 min.

3. RESULTS AND DISCUSSION

3.1 Results

A total of 473 samples were collected comprising of 251 waters and 222 sediments distributed according to the sampling sites (Table 2).

The *M. peregrinum* species, *like M. smegmatis, like M. peregrinum, M. immunogenicum, M. chelonae, M. mucogenicum, M. abscessus, Mycobacterium sp*. were isolated in this study.

All species obtained were analyzed by PCR, focusing on IS2404 and Kr sequences. The gene IS2404 were found in 20% of all the strains studies when the ketoreductase genes (Kr) was isolated in 9.23% of the strains. These two sequences were found in the rapid-growing isolated species: *M. peregrinum M. mucogenicum, Mycobacterium sp* (Table 3).

3.2 Discussions

Of the isolated strains, 20% were carriers of the IS2404 gene, in particular the species *mycobacterium mucogenicum, mycobacterium peregrinum and mycobacterium sp*. This could suggest the ability of these bacteria to cause skin ulcers or could be involved, in isolation areas in Côte d'Ivoire. The sites of Bodo and Adzopé are known to be endemic zone of cutaneous ulcers

attributed, rightly or wrongly to *Mycobacterium ulcerans* according to the National Program for the Control of *Buruli Ulcer*. *M. ulcerans* is a slow-growing mycobacterium whose culture is often lacking. The discovery in 1997 of the isolation sequence IS2404, specific to *M. ulcerans*, has been a catalyst for further research of the germ in the environment [10]. This sequence is also found in other environmental mycobacteria such as *Mycobacterium marinum, Mycobacterium liflandii and Mycobacterium pseudoshottsii* [4,5]. These slow-growing mycobacteria are also responsible for cutaneous ulceration [5]. This sequence, which is therefore a priority target for the diagnosis of the *Buruli ulcer* [6], could also be involved in Mycobacterial caused cutaneous ulcerations. Ulcerations of Buruli ulcer are caused by a toxin, mycolactone [12]. The presence of a 174 Kb plasmid called pMUM001 in the genome of the bacterium would carry the genes of Polyketide synthase (PKS), responsible for the synthesis of the latter [2,13]. It has been identified in other slow-growing environmental mycobacteria called mycolactone-producing mycobacteria [3]. The results of our study also prove its existence in rapidly- growing

Table 2. Number of samples according to type and sampling site

	Collection site							
	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. The distribution or presence of IS6110-, IS2404- and Kr-coding genes in mycobacterial strains isolated from environmental samples

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

IS2404: insertion sequence found in mycobacterium ulcerans and other environmental mycobacteria; Kr: mycolactone toxin synthesis enzyme found in mycobacterium ulcerans and other MPM. ; (-): Negative result in the desired sequence; (+): Positive result in the desired sequence

mycobacteria. Indeed, 9.23% of the isolated strains harbor the Ketoreductase (Kr) gene, one of the mycolactone synthesis enzymes according to [14]. Indeed, this enzyme would make it possible to confirm the presence of the plasmid in the genome of the bacterium [15]. The results of this study highlight the risk of contamination in humans, especially the populations in permanent contact with the environment. These discovered species could be responsible for ulcerations in the Ivory Coast, which could explain the very high level of endemicity. It is therefore important to identify the sequences of these species in order to develop a suitable and efficient method for diagnosis. This study could help to understand and identify the causes of cutaneous ulcerations with a negative search for *Mycobacterium ulcerans*. This would make it possible to better diagnose patients with skin infections other than *Buruli ulcer* and to consider strategies and means of protecting the population against all mycobacterial diseases by breaking the epidemiological chain.

A limitation of the study was to investigate more samples from non-endemic sites than from endemic sites, since more KR genes were obviously obtained in endemic areas.

4. CONCLUSION

Cutaneous ulceration is a public health problem in Côte d'Ivoire. This work showed a probable involvement of non ulcerans mycobacteria in the spread of this disease. Investigations must therefore continue in order to confirm this observation in clinical practice. All of which could help to determine the likely prevalence of skin ulcers due to *Mycobacterium* other than *M. ulcerans* to better adapt treatment in Côte d'Ivoire.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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