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Molecular Investigation of Rifampicin Resistance in Clinical Isolates of *Mycobacterium ulcerans* in Côte D'ivoire

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A B S T R A C T

M. ulcerans is the etiologic agent of Buruli ulcer. The clinical diagnosis of infection with this mycobacterium is based on microbiological analyzes, including PCR, which uses the nucleotide sequences of the primers to detect the targeted sequences. Cases of rifampicin resistance were recorded in mice experimentally infected with *M. ulcerans* strains and treated with rifampicin in previous studies. In this study, clinical isolates were confirmed as *M. ulcerans* strains. These isolates are from samples of patients with Buruli ulcer. The PCR gave a positivity rate of 92.10% for the detection of the *IS 2404* sequence and made it possible to highlight the virulence gene in most of the strains studied with a positivity rate of 94.74% for the detection of the sequence IS-KR. However, the *RpoB* gene could not be found in any of the strains. Thus it gives a positivity rate of 0% for the detection of this gene. The results show that most strains of *M. ulcerans* secrete mycolactone. The production of this toxin is the consequence of a mutation in the IS-KR gene. They also show that rifampicin has an effective bactericidal activity against *M. ulcerans* strains and that resistance to this antibiotic results from a mutation of the *RpoB* gene.

Introduction

The advent of antibiotic therapy in the 1940s completely revolutionized the medical field and resulted in a significant reduction in mortality associated with infectious diseases (Conly, 2002). But the bacterial world has adapted to antibiotics and this has resulted in the emergence of resistant strains in humans, animals and the environment. The existence of these resistant bacteria has consequences on therapeutics, public health and environmental hygiene (Guillot, 1989). Bacterial resistance to traditional antibiotics has rapidly become a global health problem (Conly, 2002). *M. ulcerans* is a Mycobacterium of the same family as that responsible for tuberculosis and leprosy. It causes extensive and disabling chronic skin ulcerations commonly referred to as Buruli Ulcer (Coulibaly *et al.,* 2011). It is the only mycobacterium known to date that produces a

toxin (mycolactone) responsible for the pathogenicity of bacilli (Asiedu *et al.,* 2000). The current drug treatment is based on the use of the combination of rifampicin and streptomycin (Etuaful *et al.,* 2005) almost always without prior antibiogram. However, one study revealed the risk of the emergence of resistance of some strains of *M. ulcerans* in an experimentally infected mouse treated only with rifampicin (Marsollier *et al.,* 2003).

A better understanding of the modes of action of different antibiotics will allow to consider the genotypic detection of most resistances. Thus, the detection of major mutations in the main genes could be an effective and rapid demonstration of antibiotic resistance, even if this means has until now been applied only for resistance to rifampicin (Cattoir, 2004).

This study makes it possible to evaluate the resistance to antimycobacterial agents and more particularly to Rifampicin by different approaches. It will identify ways to overcome the problem of antimycobacterial resistance through the elucidation of resistance mechanisms and the detection of resistance cases.

Materials and Methods

Study site

The present study was carried out at the Institut Pasteur of Ivory Coast, located in ADIOPODOUME (Km 17, road of Dabou) (Fig. 1).

Biological material

The biological material consists of 76 bacterial strains obtained from biological samples (exudates and biopsies) of patients with Buruli ulcer in Ivory Coast. The bacterial strains were obtained after culture on Löwenstein-Jensen medium and stored in glycerol at -20 ° C.

Methods

Detection of specific sequences (IS2404, IS KR, rpoB)

Extraction of genomic DNA

The extraction of the genomic DNA was carried out by thermal shock. The technique consisted of distributing a bacterial suspension in Eppendorf tubes due to 200 µl / tube. The tubes are centrifuged at 15,000 rpm at 4 ° C. for 15 minutes and the pellet of each tube is suspended in 100 µl of 50 mM NaOH. The tubes are then heated at 95 ° C. for 15 minutes and then a volume of 15 µl of 0.1M Tris-HCl is added to the bacterial suspension to neutralize the pH of the medium. The bacterial cells burst to release the DNA which is recovered by centrifugation. The liberated DNA is precipitated with 20.Mu.l (3M sodium acetate) and 500.Mu.l of absolute ethanol stored at -20.degree. The tubes are incubated at -20 ° C overnight and then centrifuged at 13000 rpm at 4 ° C for 20 minutes. The supernatant is removed and the bacterial pellets are washed in a volume of 1 ml of 70% ethanol previously stored at -20 ° C. The tubes are centrifuged again at 13000 rpm at 4 ° C for 5 minutes and the supernatant is removed. The pellets are dried at 50 ° C. for 20 min and then recovered in 100 µl of elution buffer TE (pH = 8) containing RNAse at 20 µg / ml.

Gene amplification

Reaction mixture for detecting IS2404 sequence and amplification

The amplification reactions targeting the insertion sequence IS2404 and generating 568 bp PCR products were carried out in a final reaction volume of 20 µl containing 12 µl of sterile distilled water; 2.5 µl of buffer (5X); 1.3 µl of MgCl 2 (25 µM); 0.5 µl of each deoxynucleotide triphosphate (10 µM

dNTPs); 0.75 µl of each primer (Mu-R / Mu-F) and 0.2 µl of the Taq polymerase. Positive and negative controls are included at each test to check for possible contamination of reagents or samples. The gene amplification is carried out in a thermocycler of GeneAmp 9700 type (Applied Biosystem) under the conditions mentioned below: It starts with an initial denaturation of 2 min at 50 ° C., followed by a cyclic step repeated 40 times, comprising a denaturation phase of 10 min at 95 ° C, a primer fixation phase of 15 sec at 95 ° C and an elongation phase of 1 min at 60 ° C. At the end of the cyclic phase, a final elongation of 5 minutes is carried out at 72 ° C.

Reaction mixture for detecting IS-KR sequence and amplification

Detection of the IS Kr insertion sequence and generating 330 pb PCR products, were carried out in a final reaction volume of 20 µl containing 12 µl of sterile distilled water; 2.5 µl of buffer (5X); 1.3 µl of $MgCl_2$ (25 µM); 0.5 µl of each deoxynucleotide triphosphate (10 µM dNTPs); 0.75 µl of each primer (Mu-R / Mu-F) and 0.2 µl of the Taq polymerase. Positive and negative controls are included in each trial. The gene amplification is carried out in a thermal cycler of GeneAmp 9700 type (AppliedBiosystem) under the following conditions: It begins with an initial denaturation of 2 min at 50 ° C, followed by a cyclic step repeated 40 times, comprising a phase denaturation time of 10 min at 95 ° C., a primer fixation phase of 15 sec at 95 ° C. and an elongation phase of 1 min at 60 ° C. At the end of the cyclic phase, a final elongation of 5 minutes is carried out at 72 ° C.

Rifampicin Resistance (RpoB) Gene Detection Mixture and Amplification

The amplification reactions targeting the rpoB gene sequence and generating 606bp PCR products were carried out in a final reaction volume of 20.mu.l containing 12.mu.l of sterile distilled water; 2.5 µl of buffer (5X); 1.2 µl of MgCl 2 (25 µM); 1.6 µl of each deoxynucleotide triphosphate (10 µM dNTPs); 0.3 µl of each primer (MuB-R / MuB-F) and 0.2 µl of Taq polymerase. Positive and negative controls are included at each test to check for possible contamination of reagents or samples.

The amplification is carried out in a thermocycler of GeneAmp 9700 type (AppliedBiosystem) under the conditions mentioned below: it starts with an initial denaturation of 2 min at 95 ° C., followed by a cyclic step repeated 40 times, comprising a phase denature of 20 sec at 95 ° C, a primer attachment phase of 10 sec at 63 ° C and an elongation phase of 15 sec at 70 ° C. At the end of the cyclic phase, a final elongation of 7 minutes is carried out at 72 ° C.

Revelation of amplification products by agarose gel electrophoresis

The amplified products are revealed after electrophoresis in 1.5% agarose gel containing SyberSafe DNA gel incorporated during the preparation of the gel, thus allowing visualization of the DNA under UV radiation. Electrophoresis was performed in TAE buffer (Tris Acetate EDTA, 90mM Tris, 90mM acetic acid, 2mM EDTA, pH 8.0). Thus, on a strip of parafilm paper, the different samples to be analyzed are labeled with a mixture of 4 µl of loading buffer (bromophenol blue) and 10 µl of genetic material. The labeled samples are deposited in wells migration gel, which gel bathes in a migration vessel containing a buffer solution TAE (1x). The migration vessel is energized at 135 volts for about 30 minutes. During migration, a molecular weight marker is used to verify that the resulting bands match the expected size. The gel is then placed in an automated gel reader: the GelDoc imaged where a software allows to photograph under

Ultra-Violet light (312nm) using a photo system.

The gel is then placed in an automated gel reader: the imaged GelDoc where a photo software can photograph under ultra-violet light (312nm) using a photo system. A result was considered positive, if the electrophoresis showed the presence of the specific sequence sought (band indicating the number of base pairs).

Results and Discussion

Detection of the *IS2404* insertion sequence

Detection of the *IS2404* insertion sequence from suspect *M. ulcerans* colonies confirmed the presence of this *M. ulcerans*-specific DNA sequence in 70 strains of the 76 suspect strains (Table 1).

Detection of the virulence gene KR in *M. ulcerans*

The confirmation of the presence of *M. ulcerans* in these microbial strains required complementary molecular analyzes such as the detection by PCR of a ketoreductase sequence present at the Kr genes encoding the polyketide synthetases. The IS-KR sequence was found in 72 out of 76 strains, a detection rate of 94.74% (Table 2).

Research of the rifampicin resistance gene (*rpoB*) at *M.ulcerans*

PCR also identified mutations in the *rpoB* gene conferring resistance to rifampicin. Thus, the detection of the *rpoB* gene was negative for all the strains studied. This study shows a positivity rate for the detection of resistant mutants of 0% (Table 3). The analysis of the results shows that of the seventy-six (76) suspicious strains of *M. ulcerans*, six (6) strains were not confirmed as *M. ulcerans* strains. In contrast, 70 strains were (Table 1). PCR has therefore confirmed the etiological diagnosis of the agent responsible for skin ulcers. Detection of the KR gene was negative in four (4) of the 76 strains and positive for 72 strains (Table 2). Demonstration of the IS-Kr sequence in isolated strains confirmed the production of mycolactone in these patients. Mycolactone is the only virulence factor identified in *M. ulcerans*. Due to its cytotoxic and immunosuppressive effects, this mycolactone is thought to be responsible for tissue necrosis (Stinear *et al.,,* 2004). These findings open new avenues for researching pharmaceutical agents targeting polyketide synthetase (Gomez *et al.,* 2004). This study also reveals not only that some mycobacteria other than *M. ulcerans* can cause ulcerations in patients but also other mycobacteria can grow in our culture conditions; hence the need for confirmation from bacterial colonies (Coulibaly *et al.,* 2010). No mutation in the resistance gene could be detected during this study (Table 3). According to this study, rifampicin remains active against *M. ulcerans* strains. The bactericidal action of rifampicin occurs both on intracellularly propagated bacteria and those with low metabolic activity (Campbell *et al.,* 2001). *M.ulcerans* is susceptible to many antibiotics in vitro. However, in vivo, given the nature of the lesions, antibiotics penetrate with difficulty into poorly vascularized, necrotic tissue. Because of the difficulties of diffusion of antibiotics within the lesions, the studies are moving towards combinations of antibiotics (Portaels *et al.,* 1998). But when used as monotherapy for the treatment of Buruli ulcer, some strains may become resistant to this antibiotic (Marsollier *et al.,* 2003).

The work of Marsollier *et al.,* showed that of 897 strains of *M. ulcerans* inoculated with mice and then treated with rifampicin only, three strains developed mechanisms of

resistance to this antibiotic. These studies also revealed mutations in the *rpoB* gene in *M. ulcerans*. These mutations have been identified in the codons corresponding to amino acids 416 and 420. They respectively cause the change of Serine (416) to Phenylalanine and Histidine (420) to Tyrosine. The results of their work indicate that rifampicin is effective against *M. ulcerans* in vitro, but should not be used as monotherapy in humans. The combination of rifampicin / streptomycin antibiotics has been shown to be effective in mice experimentally infected with *M. ulcerans* and allowed for a pilot study in an endemic area in Ghana. Both antibiotics have a bactericidal action. But only their combination protects against relapse and selection of resistant strains (Dega *et al.,* 2000). This antibiotherapy has major interests such as outpatient treatment for many patients and lower cases of relapse after antibiotic treatment. This represents less than 2% against 16 to 30% after surgical treatment.

Table.1 Positivity rate of detection of IS 2404 insertion sequence in suspicious strains of *M. ulcerans*

Effective some samples	PCR		SEQUENCE IS 2404	
	Negative	Positive	Negative	Positive
N= 76	00	76	06	70
Rate positivity (%)	00	100	7,89	**92,10**

N: Total number of microbial strains

Table.2 Positivity rate of the detection of the virulence gene (KR) in *M. ulcerans*

Effective some samples	PCR		***KR* GENE**	
	Negative	Positive	Negative	Positive
N= 76	00	76	04	72
Rate positivity (%)	00	100	5,26	**94,74**

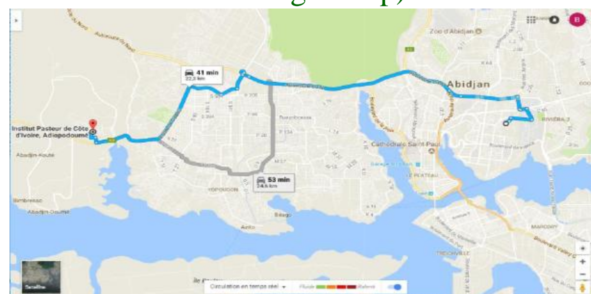
N: Total number of microbial strains

Table.3 Positivity rate of detection of the resistance gene (*rpoB*) in *M. ulcerans*

Effective some samples	PCR		***rpoB* GENE**	
	Negative	Positive	Negative	Positive
N= 76	00	76	76	00
Rate positivity (%)	00	100	100	**00**

N: Total number of microbial strains

Fig.1 Location of the Pasteur Institute of Côte d'Ivoire Adiopodoumé site (data obtained by Google Map)



WHO is currently recommending a combination of rifampicin and streptomycin for the treatment of Buruli ulcer and nodules and uncomplicated cases can now be treated as ambulatory (Etuaful *et al.,* 2005). The identification of rifampicin resistance is activated by mutations of the *rpoβ* gene. Resistance to rifampicin arises from mutations altering the residues of the rifampicin binding site to RNA polymerase, which results in a decreased affinity for rifampicin. Most of these resistant mutations are located on the *rpoβ* gene of the RNA polymerase encoding the β subunit. (Feklistov *et al.,* 2008). The acquired resistance of *M. ulcerans* to antibiotics is caused by a selection of resistant mutants during inadequate treatment: This is called secondary resistance (Campbell *et al.,* 2001). This selection takes place when a bacillary population is important and a single antibiotic is active. Also when the patient receives a monotherapy in principle (it takes only one of the prescribed antibiotics or the doctor prescribes only one antibiotic) or monotherapy de facto (the bacilli are resistant to other antibiotics prescribed simultaneously).

Buruli ulcer is an infectious disease of chronic evolution. Some aspects of its physiopathology remain unknown. Clinicians as well as researchers are striving to find an alternative to surgical treatment using effective antibiotics on *M. ulcerans*. The methods used to confirm the clinical diagnosis of Buruli ulcer are based on various microbiological analyzes, including the PCR technique. This technique uses nucleotide sequences of the primers to detect the insertion sequence IS2404 and the sequence of ketoreductase present at the genes encoding the polyketide synthetases of plasmid pMUM001. It is also used to detect mutations in the *RpoB* gene that confer resistance to rifampicin. This study found a large number of microbial strains positive for IS2404 and KR gene but negative for the *RpoB* gene. These results show that the mycolactone produced can be detected in human tissues infected with *M. ulcerans*. Also, the absence of a mutation in the *RpoB* gene makes it possible to show that rifampicin has an effective bactericidal activity on *M. ulcerans* strains. But the knowledge of the reasons (susceptibility factors) of the bacillus establishment in the cutaneous tissue must be identified.

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