Molecular detection of mutations in \textit{rpoB} gene involved in rifampin resistance in leprosy patients from Côte d’Ivoire

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Abstract

The control measures recommended for treatment of leprosy with multidrug therapy should limit spread of drug-resistant strains; however, rifampicin resistance continues to be reported in several countries. Resistance to anti-infectious used in multidrug therapy has not yet been molecularly established in Côte d’Ivoire. This preliminary study presents results of evaluation of level rifampicin resistance in Côte d’Ivoire. For each case of clinically confirmed multibacillary leprosy (new or relapsed cases) a dermal juice sample was examined by microscopy after Ziehl-Neelsen staining and a specific PCR targeting \textit{RLEP} repeat sequence. 48 isolates were used to perform amplification of the \textit{rpoB} gene involved in rifampicin resistance. The origin of the resistance was then investigated after sequencing and mutations analysis. Thus, 24 isolates (50 \%) had mutations, 11 (22. 91 \%) of which had mutations conferring rifampicin resistance. The mutations correlated with resistance were as follows: Gln438Val (9. 09 \%), Asp441Asn (18. 18 \%), Ser456Leu (45. 50 \%) and Ser456Phe (27. 27 \%) in 9 health districts in Côte d’Ivoire. These mutations were observed in both relapsed and new cases. In conclusion, cases of rifampicin resistance exist in Côte d’Ivoire and an active surveillance should be carried out to monitor the evolution of this resistance. In addition, this information can be used to control the spread of drug-resistant \textit{M. leprae} strains.

Keywords: Leprosy; \textit{Mycobacterium leprae}; Rifampicin resistance; Resistance gene; Mutation

1. Introduction

Leprosy is an infection of the skin and peripheral nerves that can lead to disability and social stigma. It remains endemic in many parts of world. Leprosy is of particular concern because it can progress to peripheral neuropathy and permanent progressive deformity. Thus, leprosy is classified as a neglected tropical disease like buruli ulcer and tuberculosis. The causative agent \textit{Mycobacterium leprae} (\textit{M. leprae}) was discovered by GA Hansen in 1873. It is a mycobacterium that cannot be grown on axenic medium [1], which limits the study of the disease. The increase in cases of drug-resistant leprosy is a real, worrying phenomenon and a concern in many countries. However, although prevalence of leprosy has decreased significantly since the introduction of World Health Organization (WHO) multidrug treatment regimen (MDT), the incidence remains high, with a ratio of approximately 215 656 cases worldwide in 2016 [2]. For most infectious diseases for which secondary prevention is provided only by chemotherapy, the emergence of drug resistance eventually becomes a concern and a threat to intervention programs. In addition, drug resistance has been reported since 1964 for dapsone [3], 1976 for rifampicin [4] and 1996 for ofloxacin, alternative drug [5]. Historically, antibiotic susceptibility testing has been performed using the mouse foot pad test, as \textit{M. leprae} does not develop in vitro, but has been successfully replaced by molecular detection of resistance [6]. This allowed first antimicrobial resistance (AMR) rates in endemic countries to be estimated through a World Health Organization (WHO)
surveillance network [7]. As rifampicin is the main drug used in MDT and the only bactericidal drug, it is very important to monitor the emergence of rifampicin-resistant mutants in leprosy patients. Indeed, PCR-based DNA sequence analysis of the rpoB gene of M. leprae has been shown to be consistent with rifampicin susceptibility testing [8]. To overcome the challenge of containing the disease and to maintain the current downward trend in leprosy in endemic countries, it is essential to monitor drug susceptibility patterns in current settings. With regard to infection with M. leprae, causative agent of leprosy, no studies on resistance to anti-infectives used in multidrug therapy have yet been carried out in Côte d’Ivoire. This first study presents results of an evaluation of the molecular resistance level to anti-leprosy drugs in Côte d’Ivoire.

In this study, we analysed DNA sequences from specific regions of M. leprae rpoB gene, which is responsible for rifampicin resistance. Several M. leprae isolates showed point mutations in this gene. These results suggest emergence of rifampicin-resistant strains of M. leprae in Côte d’Ivoire.

2. Material and methods

2.1. Study population and sampling

The different samples used in this study were taken from patients of 9 health districts at the Raoul Follereau Institute of Côte d’Ivoire (IRFCI). They were: Zuénoula, Daloa, Man, Adzopé, Agnibilekro, Daoukro, Gagnoa, Lakota and Bondoukou. Thus, the study involved multibacillary patients receiving or not an anti-leprosy chemotherapy at the time of sampling. The treatments and status of leprosy patients are listed in Table 1. Inclusion criteria were multibacillary leprosy positive for bacteriological examination according to the WHO protocol [9]. A total of 69 dermal juice samples from patients with relapsed and new cases of leprosy were obtained between 2016 and 2019. These samples were first examined by microscopy after a Ziehl-Neelsen staining according to protocol routinely used at the IRFCI microbiology laboratory.

| Isolats    | Polychemotherapy | Antibiotic       | Leprological Status |
|------------|------------------|------------------|---------------------|
| Lep 1 Adzope | Yes              | WHO regimen      | Relapse             |
| Lep 2 Adzope | No               | Dapsone          | New case            |
| Lep 3 Man   | No               | Rifampcin        | New case            |
| Lep 4 Daloa | Yes              | WHO regimen      | Relapse             |
| Lep 5 Daoukro | Yes              | WHO regimen      | Relapse             |
| Lep 6 Bondoukou | No               | Ofloxacin        | New case            |
| Lep 7 Agnibilekro | Yes          | WHO regimen      | Relapse             |
| Lep 8 Lakota | Yes              | WHO regimen      | Relapse             |
| Lep 9 Zuénoula | Yes             | WHO regimen      | Relapse             |
| Lep 10 Gagnoa | Yes              | WHO regimen      | Relapse             |
| Lep 11 Man  | Yes              | WHO regimen      | Relapse             |

2.2. Ethical declaration

This study was approved by National Research Ethics Committee of Côte d’Ivoire under approval number N/Ref: N°140/MSHP/CNER-km. All the participants approved the research protocol and signed the informed consent after reading information leaflet of study.

2.3. Cases definition

Relapse cases are defined as appearance of new skin lesions and/or an increase in bacteriological index of two or more units at same site compared with bacteriological index observed at same site on a previous examination, at any time after completion of a full course of treatment, after exclusion of reactions to leprosy [9]. They will therefore be used for
monitoring secondary resistance. New cases are defined as patients diagnosed with leprosy who have never received treatment for leprosy. These new cases have also been included to enable monitoring of primary resistance.

2.4. DNA extraction

The chemical extraction method used in this work is that described by Yoshikawa and al. [10] using guanidine thiocyanate as chemical compounds. Thus, this extraction will make it possible to isolate the DNA from the cells. The DNA thus extracted can be used to search for the presence of different gene targets specific to M. leprae. This phase will make it possible to release DNA and eliminate the PCR inhibitors. The concentration and purity of the samples were evaluated using the Qubit 3 (Invitrogen) equipment.

2.5. Case confirmation

Confirmation of cases was carried out by detection of RLEP gene by conventional PCR according to protocol previously described by [11]. Only samples positive for RLEP gene were amplified for the rpoB gene and sequenced.

2.6. Amplification and sequencing analysis of the rpoB gene

PCR was performed using Gotaq polymerase (PROMEGA, Madison, WI USA) in a 25 µl volume containing 5 µl of genomic DNA and concentrations of 10 µM primers, which were designed according to the sequence of the rpoB gene (Z14314) of M. leprae. The primers for rpoB gene were rpoB1 (5'-CAGACGCTGATCAATCCGT-3') and rpoB-2 (5' TACGGTGTTTCGATGAACCCG- 3'). The target region of rpoB gene was amplified by a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Singapore) with a program of 30 s at 95°C, 2 min at 50°C and 3 min at 72°C for 40 cycles. The DNA-free PCR reaction was used as a negative control (reagent control). The PCR products containing amplified fragments of target regions were subjected to electrophoresis in 3% agarose gel (Sigma) using Tris - Borate - EDTA (TBE) buffer at a constant voltage of 110 V for one hour. PCR products were recovered from agarose gels, purified by the GFX PCR DNA and Gel Band Purification Cleaning Kit, following the manufacturer’s protocol and sequenced using same primers as to generate PCR fragment of gene, using the Big Dye Terminator V3.1 reaction kit (Cycles sequencing Kit reference 4336917). Sequences were generated on ABI 3500 Genetic Analyser sequencer (24 capillary, Applied Biosystems) and compared with sequences from M. leprae NC002677 and z14314 available at GenBank (http://www.ncbi.nlm.nih.gov), and for analysis of mutation zones, sequences were entered into Mega 7 software.

3. Results

3.1. Bacilloscopic index (BI) determination

Ziehl Neelsen staining was performed on all previously treated samples. BI of all patients (new case and relapse) ranged from 1+ to 6+. All these samples had resistant acid-fast bacilli. The resistant acid-alcohol bacilli usually appeared microscopically as elongated sticks (refer to figure 1).

![Figure 1 Presence of Acido-Alcohol Resistant Bacilli (BAAR) Under the Microscope (Gx100) (IRFCI photo photo library)](image-url)
3.2. Case confirmation

For confirmation of leprosy cases, search for *M. leprae* by PCR detection of *RLEP* target gave 69 positives (100%) of samples. Figure 2 Agarose gel showing the result of PCR products migration on a 2% gel concentration.

![Figure 2](image)

**Figure 2** PCR amplification of representative targets of *RLEP* gene (545bp). M: 100 bp molecular weight marker, 1 to 3: samples, T-: negative control containing nuclease-free water, T+: positive control containing DNA from *M. leprae*.

3.3. Detection of *rpoB* gene by PCR

PCR identification yielded 60 positives, or (86.9%) of the samples. Only 48 DNA extracts out of the 60 PCR positive samples targeting the *rpoB* gene were used for sequencing. This choice was based on quality of fragments present in agarose gel. The extracts with highest DNA content were used. Figure 3 shows result of migration of amplicons on a 3% agarose gel.

![Figure 3](image)

**Figure 3** Electrophoreogram showing the result of *rpoB*-PCR (305bp) on a 3% agarose gel. M: 100 bp molecular weight marker, T+: Positive control containing *M. Leprae* DNA, 1-6: Samples, T1-: Negative control containing nuclease-free water and T2-: Negative control containing *M. ulcerans* DNA.

3.4. Frequency of mutation

A result of mutation study are as follows: out of 48 samples submitted to at sequencing, 24 had a mutation and 24 isolates with a wild-type profile. Thus, 11 isolates had mutations conferring resistance to rifampicin at a rate of 22.91% (Table II). The numbering system is that of *M. leprae* TN strain genome (GenBank AL583923). The mutations correlated
with resistance were as follows: Gln438Val (9.09%), Asp441Asn (18.18%), Ser456Leu (45.50%) and SerPhe (27.27%) (Table II).

**Table 2** Mutations in rpoB gene conferring resistance

| MUTE CODON | MUTATION | AMINO ACID CHANGE | FREQUENCY |
|------------|----------|-------------------|-----------|
| 438        | GAG→GTG  | Gln→Val           | 1 (9.09%) |
| 441        | GAT→AAT  | Asp→Asn           | 2 (18.18%)|
| 456        | TCG→TTG  | Ser→Leu           | 5 (45.50%)|
| 456        | TCG→TTC  | Ser→Phe           | 3 (27.27%)|
| TOTAL      |          |                   | 11 (100%) |

Of three new cases, two cases showed a previously reported mutation at codon 456 (Ser → Leu) and one case showed a mutation at codon 438 (Gln → Val). Of relapsed cases, three showed one mutation at codon 456 (Ser → Leu), two mutations at codon 441 (Asp → Asn) and three mutations at codon 456 (Ser → Phe) (Table III). All of these mutations were in the region of determination of rifampin resistance. The majority of isolates with a mutation were predominantly from male patients (9/11).

**Table 3** Summary of resistance (new case and relapse)

| ID PATIENT | Age | Status | BI | Mutations          | Gender |
|------------|-----|--------|----|--------------------|--------|
| lep 1 Adzope | 42  | Relapse | 4+ | Ser456Leu          | M      |
| lep 2 Adzope | 61  | New case | 2+ | Ser456Leu          | M      |
| lep 3 Man    | 45  | New case | 3+ | Ser456Leu          | M      |
| lep 4 Daloa  | 36  | Relapse | 6+ | Ser456Phe          | M      |
| Lep 5 Daoukro | 33  | Relapse | 5+ | Ser456Leu          | F      |
| Lep 6 Bondoukou | 60 | Relapse | 4+ | Ser456Phe          | M      |
| lep 7 Agnibilekro | 56 | New case | 6+ | Gln438Val          | M      |
| lep 8 Lakota | 41  | Relapse | 5+ | Asp441Asn          | M      |
| Lep 9 Zuenoula | 30 | Relapse | 2+ | Ser456Leu          | F      |
| Lep 10 Gagnoa | 60  | Relapse | 5+ | Ser456Phe          | M      |
| Lep 11 Man   | 54  | Relapse | 4+ | Ser456Leu          | M      |

4. Discussion

Although Côte d’Ivoire had reached leprosy elimination threshold since 2001 [12], a few leprosy cases were still registered. They are generally not reported to health authorities and are not investigated for rifampicin resistance. Systematic investigation of resistance in leprosy cases diagnosed in several endemic countries has revealed cases of secondary or primary resistance to rifampicin [13]. Although Côte d’Ivoire is not considered a high-risk country for leprosy, this may be a red herring.

Difficulties of this study are firstly: MDT resistance can only be studied in multibacillary cases; secondly, the sampling was not exhaustive because there is no mandatory reporting of leprosy in Côte d’Ivoire and we may have only received samples from selected cases; and thirdly, we chose to mix cases diagnosed in Adzopé with cases referred from other health districts (Daloa, Gagnoa, Man, Zuenoula, Lakota, Daoukro, Bondoukou and Agnibilekro).

Thus, rpoB sequence of positions 1243 to 1547 was amplified and analysed. Thus, mutations responsible for rifampicin resistance were sought in both new and relapsed leprosy cases. The following mutations in rpoB gene were found:
codons 438 (9.09%), 441 (18.18%) two types at codon 456 (45.5%) and (11.11%), with codon 456 having the highest frequency. This codon was associated with high resistance to rifampicin as well as cross-resistance to all rifampicins. The same types of mutations were observed in Asia by Maeda and al. [14] but in different proportions (numbering of E. coli), with codon 456 mutations still predominating. These same mutations were also reported in France by Chauffour and al. [13], according to these authors, the mutations correlated with resistance (numbering of the M. leprae TN genome) were as follows: Ser456Leu (2 cases) and Ser456Phe (1 case). The presence of mutations on the above-mentioned codons of rpoB gene could explain the treatment failure in some of our patients to first-line drugs including rifampicin. This is because rifampicin is the main active drug responsible for the efficacy of standard PCT treatment. Mutations in rpoB gene, coding for the β subunit of RNA polymerase, have been reported to cause rifampicin resistance (RMP) in several species of bacteria, including M. leprae [15]. This is because mutations (alterations) in DNA sequence alter amino acid sequence. Thus, a modification of amino acid sequence changes structure of the protein leading to a structural modification of the protein changes the function of latter [15]. However, RMP interferes with RNA synthesis by binding to β subunit of RNA polymerase according to Andre and al. [16]. RNA polymerase is an oligomer consisting of an enzyme nucleus formed by four α2β2 chains in association with a σ subunit that specifically stimulates transcription from promoters. The RMP binding site is located upstream of catalytic center and physically blocks RNA chain elongation. Therefore, structural modification due to mutations leads to a change in the RMP binding site, hence resistance. Rifampicin resistance of more than 15% has recently been reported in some endemic countries, which can be considered an alert to preserve the efficacy of treatment [17]. Molecular detection of mutations in rpoB gene is now best method for diagnosing anti-leprosy drug resistance in leprosy patients, as drug-resistant genotypes are highly consistent with mouse pad test phenotypes for rifampicin, dapsone and ofloxacin [18]. This is not possible for other anti-leprosy drugs, such as clofazimine or clarithromycin, as no resistance has been observed or mechanisms are unknown. Substitution mutations in the rpoB gene similar to those described in other studies have been observed: Ser456Leu (transition), Asp441Asn (transition) and a transversion-like substitution mutation Gln438Val [19]. The mutated codons varied between health districts. Good quality PCR products and sequences were not obtained from all samples, partly due to inclusion of samples with low bacterial load and the presence of PCR inhibitors. Indeed, presence of PCR inhibitors in dermal juice samples has already been described by Matsuoka and al. [19]. The observation that all resistant cases were predominantly male is consistent with results of other studies [20] and could be associated with a higher prevalence of males in MB leprosy and a higher number of irregular self-administered patients, mainly causing secondary resistance. This is confirmed by studies by Singh and al. [21] showing the absence of primary drug resistance, as evidenced by the low rate of drug-related mutations in strains of new leprosy patients.

These data show that the development of rifampicin-resistant M. leprae isolates would contribute to the relapse of leprosy in Côte d’Ivoire but that the possible causes are: bacterial persistence, host immunosuppression, presence of advanced leprosy, reinfection, and factors associated with failures in operational health care, such as late diagnosis, inadequate or irregular treatment of disease and misclassification of previous disease as suggested by some authors [3].

5. Conclusion

In this study, mutations in rpoB gene (codons 438, 441 and 456) associated with rifampicin resistance were observed. The protocol used is a specific and sensitive test for diagnosis of rifampicin resistance. It has advantage of having a very short lead time and gives the possibility to use a large number of samples at same time. This protocol can be used in diagnostic tests for multiresistance cases. However, well-trained technicians and an appropriate technical platform will be required to perform these types of analyses. Its implementation and popularization will reduce the waiting time before treatment. Moreover, these results show the emergence of rifampicin resistance in new leprosy cases. The emergence of new cases of rifampicin resistance indicates that resistant strains are actively circulating in endemic areas of Côte d’Ivoire from secondary resistance cases and infecting the naïve population at risk. This finding suggests that there is an urgent need to establish a policy for drug resistance surveillance and careful post-treatment follow-up of cured patients, in order to detect relapses earlier and rapidly identify secondary resistant strains for inclusion in a new drug regimen.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they do not have any conflict of interests.

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