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Subtyping of Clinical Strains of *Mycobacterium leprae* Using Single Nucleotide Polymorphism (SNPs) Markers in Leprosy Patients in Côte d'Ivoire

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Abstract Leprosy is a disease caused mainly by *Mycobacterium leprae*. The mode of transmission has yet to be elucidated. In order to understand its etiology, *Mycobacterium leprae* was genotyped into 4 types and 16 subtypes using molecular biology techniques. The overall aim of this study was to identify the subtypes of genotype 4 identified in clinical strains *of M. leprae* in leprosy patients in Côte d'Ivoire, using Single Nucleotide Polymorphisms (SNP) genetic markers. The biological material used consisted of dermal juices and nasal mucus collected from 40 patients clinically diagnosed with leprosy at the Institut Raoul Follereau of Côte d'Ivoire (IRFCI). Conventional PCR amplification revealed a high rate of positive *M. leprae* detection, i.e. 77.5% (62 /80). 5% (4 /80) of the strains identified had all three types of mutation (insertion, deletion and HPT). However, only one strain identified subtype O, characterized by the insertion of a T base at position Ins: 978589 and by the presence of a T base at position Del: 1476519. Subtype O of the identified *M. leprae* genotype 4 is thought to circulate in Côte d'Ivoire.

Keywords: *Mycobacterium leprae, PCR, SNP marker, Subtype O, Côte d'Ivoire*

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1. Introduction

Leprosy is a skin condition caused by *Mycobacterium leprae* (*M. leprae*) or *Mycobacterium lepromatosis*, two uncultivable pathogens with exceptionally long generation times ranging from 5 to 20 years [1,2]. It is classified as one of the world's 20 neglected tropical diseases (NTDs) and is the third most common mycobacteriosis after tuberculosis and Buruli ulcer [3,4]**.** Leprosy remains a disease of public health concern as it causes often irreversible disabilities, deformities, stigmatization and discrimination of those affected [5].

To combat leprosy, the World Health Organization (WHO) introduced treatment with multidrug therapy (MDT) back in 1981 [6]. The introduction of MDT significantly reduced the prevalence of leprosy by 80% in 10 years and spared a million patients from disability worldwide [7]. However, many endemic sites still exist. Indeed, 2021, WHO mentioned 140,594 new cases, with 66% of cases reported in Southeast Asia. In 2021, Africa was ranked by the WHO as the second continent with the highest number of leprosy cases in the world, after Asia. In Côte d'Ivoire, leprosy is endemic. The World Health Organization (WHO) has reported that 514 new cases will be detected in 2021, compared with 515 in 2020, showing a reduction in the number of cases. However, the rate of irreversible disability (degree 2) has risen from 9% in 2020 to 24.32% in 2021 [8].

In order to control leprosy, the WHO has adopted the global strategy for the years 2021-2030 entitled "Towards zero leprosy", whose objectives include: (a) no new indigenous cases in 120 countries, (b) a 70% reduction in new annual cases, (c) a 90% reduction in the incidence of new grade 2 disability (G2D) cases. and (d) a 90% reduction in the incidence of pediatric leprosy cases [9].

The exact mode of transmission of leprosy bacilli has yet to be elucidated. However, direct contact via aerosol droplets spread by the respiratory route remains the main route of transmission of *M. leprae* [10]. Frequent exposure is necessary for the development of the disease. Consequently, relatives of untreated leprosy patients are at greater risk of contracting the disease [11]. In addition to bacterial exposure, other risk factors associated with leprosy development have been demonstrated, such as genetic polymorphisms, clinical type of leprosy index case within a household, immunosuppression and nutritional factors [12,13]. Also, recent studies have shown that the environment of leprosy cases as well as animals could be sources of contamination, notably armadillos in South America or chimpanzees in Côte d'Ivoire [14,15].

In view of these probable means of transmission, increased diagnosis of *M. leprae* is essential. In Côte d'Ivoire, diagnosis is essentially based on clinical examination, but also on microscopy for Acid-Alcohol-Resistant Bacilli (AARB) [16]. However, clinical diagnosis of the disease remains delayed due to the late onset of symptoms, resulting in a 24.32% increase in the proportion of grade 2 disability in 2021.

Recently, molecular diagnostics and the molecular epidemiology of infectious diseases have grown rapidly with advances in molecular biology techniques based on DNA analysis. The main diagnostic tool for leprosy is the polymerase reaction chain (PCR). It uses several targets, such as repeated elements in the *M. leprae* genome (RLEP), the enzyme Superoxide dismutase A (Sod A), Ribosomal RNA (16S rRNA) and the bacterial proline-rich gene (prA gene) [17]. However, RLEP would be the appropriate target for identification of *M. leprae* as it has greater sensitivity and specificity [17]. It is a 545 bp, 37-copy repeat sequence located throughout the bacterial genome. Following detection of *M. leprae*, molecular markers such as Variations in Genetic Nucleotide Repeats (VNTRs) and Single Nucleotide Polymorphisms (SNPs) are used for genotyping. Of these two markers, SNPs are said to provide a better understanding of the etiology, transmission and evolution of

M.leprae [18]. Recently, thanks to SNPs <<genotype 4>> has been identified as the *M. leprae* strain circulating in Côte d'Ivoire [19]. This genotype can be subtyped on the basis of Insertion-Deletion (InDel) SNPs and Homopolymer (HPT) sequences [18,20].

The subtypes associated with this strain of *M. leprae* circulating in Côte d'Ivoire have not yet been identified. Such identification could help in the choice of appropriate treatment against *M. leprae*

2. Materials and Methods

This study was carried out at the Institut Pasteur's molecular biology platform in Côte d'Ivoire, using samples from leprosy patients.

2.1. Ethical Approval

This study was approved by the National Ethics Committee for Life Sciences and Health (CNESVS) of Côte d'Ivoire as described in [19].

2.2. Study Population

The various samples used in this study came from the Institut Raoul Follereau of Côte d'Ivoire (IRFCI), a leprosy treatment center in Adzopé in the Mé region of Côte d'Ivoire. Forty (40) patients clinically diagnosed with leprosy were used in this study. Samples consisted of dermal juices obtained from the patients' right or left ear lobes and nasal mucus collected from nasopharyngeal swabbing.

2.3. Type of Study

This is an analytical study to be carried out in 2022.

2.4. DNA Extraction

DNA extraction was performed using the QIAamp® Mini Kit (QIAGEN) according to the manufacturer's protocol.

2.5. PCR Confirmation of Clinical Leprosy Cases

Confirmation of clinical cases was carried out by end-point PCR targeting *M. leprae* RLEP repeat elements, according to the protocol described by [17].

2.6. SNP Sequencing and Subtyping of *M. Leprae*

The PCR-SNP reaction was performed in a final volume of 25 µL, containing 5 µL genomic DNA, 10 µM of each primer, Gotaq G2 polymerase (PROMEGA, Madison, WI USA). SNP markers at positions HPT-741133, INS-978589 and DEL-1476519 were searched for.

Amplifications were performed using a GeneAmp® 9700 thermal cycler (Applied Biosystems, Singapore) according to the protocol of [20].; i.e. 94˚C for 1 min, 94˚C at 5s for 45 cycles, from 55˚C at 1min and 72˚C at 2 min, 10min at 72˚C. The primers shown in Table 1 were used for subtyping. PCR products were revealed from a 2% agarose gel (Sigma) incorporated with SybrSafe.

For sequencing, PCR products were purified using the promega swift... purification kit (PROMEGA) and sequenced on a 24-capillary Genetic Analyzer sequencer (Applied Biosystem). BioEdit software version 7.0.9.0 was used for sequence analysis and strain subtype characterization. Strain characterization was carried out by BLAST, comparing the reference sequence of Brazilian strain Br4923 with those obtained after strain sequencing and carrying mutations at positions HPT-741133, INS-978589 and DEL-1476519.

Table 1. Primers used for *M. leprae* subtyping

Markers	Primers	Sequences (5'-3')	Size (bp)
HPT	**HPT-741133 F**	ATTATGGTGTGCACCAATGC	296
	HPT-741133 R	ACGGTAGAGGCAAAGCTGAA	
INS	**INS 978589 F**	GCAGTTGCAGCACGATGG	276
	INS 978589 R	CGGTGGAATTACCCTAACGA	
DEL	**DEL 1476519 F**	TCGTGTTTCGGATCTCATCA	242
	DEL 1476519 R	AAATCCAGCACGACCAAGTC	

3. Results

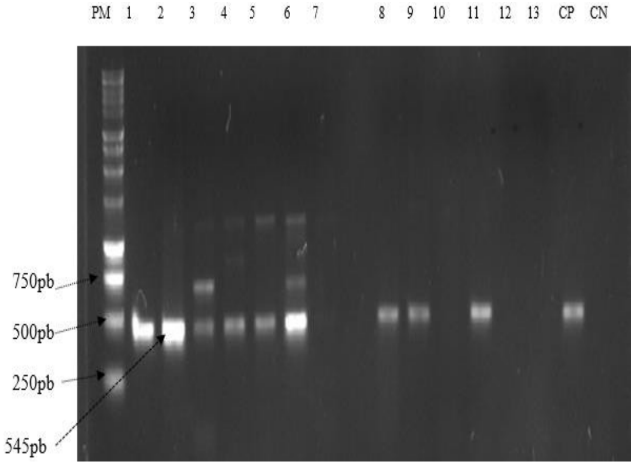
3.1. Confirmation of Leprosy Cases PCR

Conventional RLEP-PCR was performed on all extracted DNA, i.e. 80 DNA extracts.

It was used to diagnose 77.5% (62/80) (Figure 1).

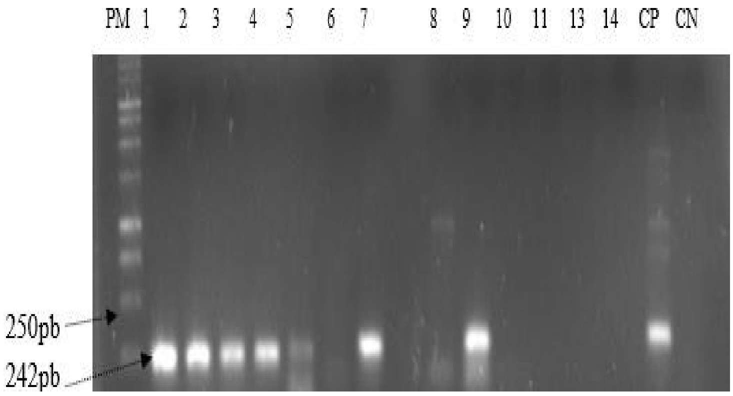
3.2. Confirmation of *InDel* Mutations and Homopolymer Sequences

Extracts used to confirm the presence of mutations were those diagnosed as positive for *M. leprae*, i.e. 46 extracts. Of these extracts, 36.96% (17/46) had insertion-type mutations, 47.83% (22/46) had deletion-type mutations and 15.21% (7/46) had HPT. Only 8.7% (4/46) of extracts had all three types of (Figure 2, Figure 3 and Figure 4).



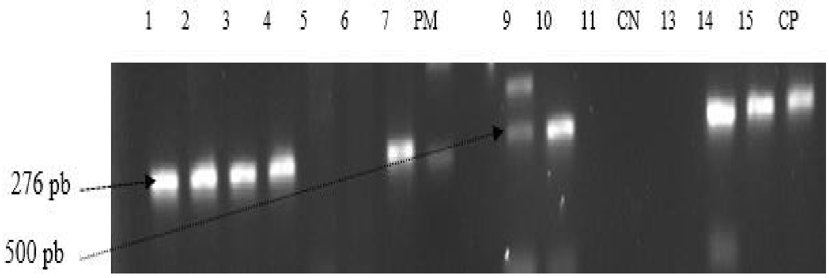
PM: Molecular weight marker (1kb)
CP: Positive Control
CN: Negative Control

Figure 1. RLEP electrophoretic profile



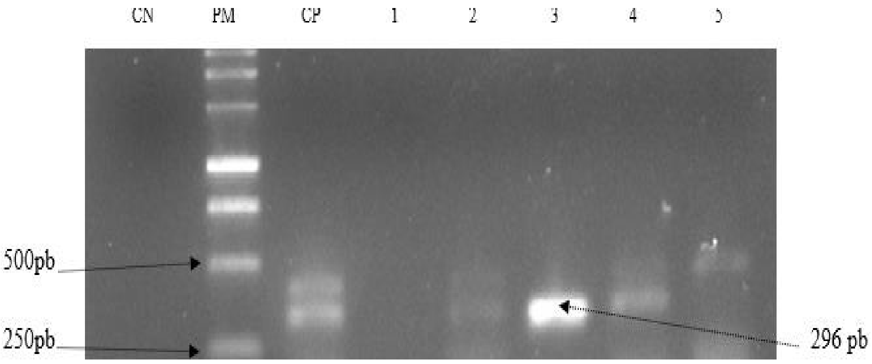
PM: Molecular weight marker (1kb)
CP: Positive Control
CN: Negative Control
Profile 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 represents samples

Figure 2. Electrophoretic profile of the Del marker mutation



PM: Molecular weight marker (1kb)
CP: Positive Control
CN: Negative Control
Profile 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 13, 14, 15 represents samples

Figure 3. Electrophoretic profile of the INS marker



PM: Molecular weight marker (1kb)
CP: Positive Control
CN: Negative Control
Profile 1, 2, 3, 4, 5 represents samples

Figure 4. Electrophoretic profile of the HPT marker

3.3. Sequencing

Subtype O, characterized by the insertion of a thymine (T) at position Ins: 978589 and the presence of another thymine at position Del: 1476519, has been identified as the *M. leprae* strain circulating in Côte d'Ivoire (Figure 5).

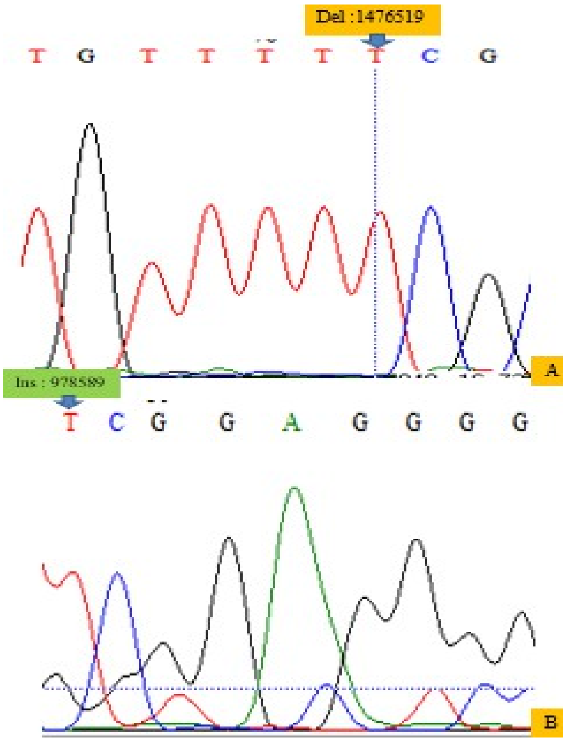


Figure 5. Chromatograms obtained from sequencing the deletion (A) and insertion (B) mutation regions of Ivorian strains *of M. leprae*

4. Discussions

Leprosy is present and still rampant in many countries around the world, particularly in Côte d'Ivoire, where diagnosis is mainly clinical. In order to control the spread of this disease, PCR-based molecular diagnosis has recently been developed [16]. In fact, many studies have focused on finding methods for better diagnosis of the disease, so that patients can be cared for rapidly in order to break the chain of transmission of leprosy**.**

To determine the molecular epidemiology of circulating strains in Côte d'Ivoire, single nucleotide polymorphism (SNP) marker analysis was carried out on samples from clinical specimens. In this study, samples samples were collected from patients diagnosed at the Institut Raoul Follereau of Côte d'Ivoire (Leprosy treatment Centre). Despite numerous studies on leprosy, in Côte d'Ivoire the genetic variability of *M. leprae* strains has not yet been properly elucidated. *M.leprae* branches are classified into specific SNP types or subtypes [1] including 4 SNP types (1-4) and 16 SNP subtypes (A-P), which provide information on the global distribution and circulation of leprosy [21]. Consequently, numerous analyses have been carried out as to the origin and global distribution of leprosy based on SNPs. Branch 0 is the most ancestral branch, found mainly in East Asia (China, New Caledonia and Japan). Branch 1, corresponding to type 1 SNPs, is mainly found in East and South Asia. Type 2 SNPs, corresponding to branch 2, are mainly found in South Asia and the Near East. Branch 3, corresponding to SNP type 3, is found in Latin America. Branch 4, corresponding to SNP type 4, has been detected in West Africa and South America. Recent studies by [19] have reported the presence of genotype 4 in patient samples from Côte d'Ivoire.

In this study, sequence analysis identified genotype O, characterized by the insertion of a T base at position 978589 and the presence of a T base at position 1476519 [20]. The SNP 4O genotype was thus found in Côte d'Ivoire. This result is in line with studies by [22], who detected a single case of the 4N/O genotype in a West African clinical sample**.** Several West African countries such as Mali, Guinea and Senegal have the SNP type 4O [23]. Analysis of *M. leprae* strains isolated in Côte d'Ivoire has shown that there is a good correlation between geographical origins *M. leprae* 4O genotype. The phylogeographic distribution of *M. leprae* strains worldwide is said to be associated host populations. SNP-type genetic markers have made it possible to learn about the distribution of leprosy in Côte d'Ivoire as it has about the distribution of the disease worldwide [19].

5. Conclusion

Of the genotype 4 subtypes, only genotype 4O has been determined in clinical samples from patients in Côte d’Ivoire. This genotype has been identified in West African countries. The *M.leprae* 4O genotype of leprosy cases diagnosed in Côte d’Ivoire is in line with their distribution in different regions of the world.

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Declarations Competing of Interest

The authors declare that they have no conflicts of interest

List of Abbreviations

INS: Insertion
DEL: Deletion
HPT: Homopolymer
SNP: Single Nucleotide Polymorphism
RLEP: Repetitive Element of *Mycobacterium leprae*
PCR: Polymerase Chain Reaction

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