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Drugs Susceptibility Testing in Leprosy Patients from Côte d'Ivoire Reveals Multidrugs Resistance Combination Cases to Dapsone, Rifampicin and Ofloxacin

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Abstract Leprosy is a chronic tropical infectious skin disease caused by an obligate intracellular pathogen called Mycobacterium leprae. Until now no vaccine was available so early diagnosis and treatment were the basic strategy for leprosy control. The treatment is based on combined drug therapy including Dapsone, Rifampicin, and Ofloxacin according protocols recommended by the WHO. However, anti-leprosy drugs resistance has been reported in several leprosy endemic region. The drug susceptibility testing was done by detecting mutation after sequencing of the drug resistance determining region. Côte d'Ivoire like many African countries has reaching the threshold of elimination of the disease and the PCT is available nationwide. On the basis of recurrences of therapeutic failures that could be due to misobservance of patients drug therapy or eventually due to circulating resistant strains, we evaluated the drug susceptibility in 155 patients from a leprosy care center in Côte d'Ivoire. Patients were previously diagnosed by clinicians and confirmed by PCR then the genetic drug susceptibility was done by PCR-direct sequencing of the drug resistance determining region of rifampicin, dapsone and ofloxacin used in the treatment. Our results showed multiple cases of multiresistance to anti-leprosy drugs in Côte d'Ivoire. This should be an alert for antibiotic resistance observatories, and policies so that more active surveillance was carried out for the control and surveillance of *M. leprae* resistance to drugs.

Keywords: *leprosy, M. leprae, multidrug resistance, Côte d'Ivoire*

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1. Introduction

Leprosy is a chronic tropical infectious disease caused by an obligate intracellular pathogen called *Mycobacterium leprae* (*M. leprae*). The infection results in disfigurement, exclusion from society and, often, in or from poverty. Despite the efforts of the WHO to eradicate the disease, the transmission chain was not interrupted. 208 619 new leprosy cases were registered globally in 2018, according to official figures from 159 countries from the six (06) WHO Regions.

Until now no vaccine was available so early diagnosis and treatment was the basic strategy for leprosy control. The diagnostic is generally based on clinicals but the complexity of clinical presentation with other skin disease has hindered the development of molecular test to confirm cases. Concerning the treatment, it is based on combined drug therapy (MDT) including in first line Dapsone, Rifampicin, and clofazimine, and in second-line Ofloxacin, Minocyclin and Clarithromicin as recommended by the WHO [1,2]. However, the resistance of *M. leprae* to anti-leprosy drugs has been reported in several leprosy endemic region [3,4]. The drug susceptibility testing was done either by the mouse footpad method [5] which is cumbersome and time-consuming or by detecting mutation after direct sequencing of the drug resistance determining region (DRDR) [6]. Studies on anti-leprosy drug susceptibility testing had shown that drug resistance can occur either by transmission of a strain resistant (primary resistance) or most often by mutation of the wildtype drug-susceptible strain during therapy [7]. Côte d'Ivoire like many African countries has reaching the

threshold of elimination of the disease and the PCT is available nationwide. The diagnosis of leprosy in Côte d'Ivoire is essentially clinical and microbiological approaches were restricted to microscopy. On the basis of recurrences of therapeutic failures that could be due to misobservance of patient drug therapy or eventually due to circulated resistant strains, we evaluated the drug susceptibility in some patients from a leprosy care center. No study on leprosy drugs susceptibility was until now done in Côte d'Ivoire.

2. Materials and Methods

2.1. Study Population

155 patients were enrolled for this study. All patients were diagnosed suspected cases of leprosy by clinicians of the Raoul Follereau Institute of Côte d'Ivoire, a treatment center for leprosy in the south area of Côte d'Ivoire. Molecular analyses were done at the Pasteur institute of Côte d'Ivoire.

2.2. Ethics Statement

This study was approved by the National Ethic committee for research of Côte d'Ivoire: "Comité National d'Ethique de la Recherche de Côte d'Ivoire (CNER)" under the approval number N/Réf: N°140/MSHP/CNER-km. All participants approved the research protocol and signed the informed consent after read of the study information notice.

2.3. Biological Samples

The biological samples to be analyzed consisted of nasal swabs and dermal pulp fluid in each patient clinically diagnosed for leprosy. Dermal pulp fluid was collected from each right and left earlobe when possible.

For microscopy, smears were performed from a nasal swab and dermal pulp fluid while for the molecular analyzes, the nasal swab and the dermal pulp fluid were recovered in a 2 ml microtubes containing 500 µl of phosphate buffered saline (PBS).

2.4. DNA Extraction and Molecular Cases Confirmation

DNA was extracted from samples using a modified guanidinium thiocyanate protocol described by Chomczinski P *et al.* [8]. Briefly 300 µl of each samples were pre-treated by boiling at 95°C for thirty minutes then DNA was extracted with a lysis buffer containing 5M guanidine thiocyanate, 50 mM Tris, pH = 8.0, 10mM EDTA, 5% 2-mercaptoethanol, 2% Triton X-100. After centrifugation the lysate was transferred into a sterile 1.5 mL Eppendorf tubes and DNA was precipitated with isopropanol and sodium acetate (3M). The DNA was then washed with absolute ethanol (96%) and finally resuspended with 150 µl of Tris -EDTA (TE).

Leprosy cases were confirmed by conventional PCR targeting the *Mycobacterium leprae* repetitive element RLEP as described by Woods and Cole [9]. PCR was performed in a final volume of 25 µl containing 5µl of DNA template, 3mM of MgCl2,0.4µM of each primers, 0.6 mM of dNTPs, 2X buffer and 0.04 U/µL of polymerase.

PCR conditions were done in a GeneAmp 9700 PCR System (Applied Biosystems) with the following program : an initial denaturation at 94°C for 5min following by 35 cycles consisted of denaturation: 94°C for 30s, annealing: 57°C for 30s, extension: 72°C during 60s and a final extension at 72°C for 10 min.

PCR products (545bp) were revealed using a GelDoc EZ imager (BioRad) after an electrophoresis in a 2% agarose gel containing SybrGreen.

2.5. Sequencing and Molecular Drug Susceptibility Test

Molecular drug susceptibility were performed only on positive RLEP-PCR patients who presented a bascilloscopic index superior or equal to +1.The level of resistance were not evaluate. The presence of mutations in the drug resistance determining regions (DRDR) of *RpoB, folP1*, and *gyrA* genes associated with Rifampicin, Dapsone, and Ofloxacin resistance, respectively were tested using *M. leprae* DRDR primers as described by Shinji Maeda et al. [10]. Each DRDR gene was amplified separately and attended PCR products were purified and sequences were obtained on a 24 capillary ABI 3500 XL Genetic analyzer (Applied Biosystem).

Bioinformatics analysis were done using MEGA software version 7.0 [11]; and for the mutations identification in the DRDR genes, sequences were aligned to the *M. leprae* Tamil Nadu (TN) reference strain sequence.

3. Results and Discussion

Of the 155 patients, 131/155 (85%) were tested positive for PCR RLEP. Among them 69 presented a bascilloscopic index greater than or equal to +1. They were distributed as follows: 52.90% of new cases, 28.39% of old cases and 18.71% of relapses cases. Thirty two out of the 69 (58,18%) analyzed patients were confirmed positive for the RLEP PCR using one of the nasal swab or dermal pulp fluid's type sample. Among the positive patients for the RLEP PCR, 26 of them had a null bascilloscopic index. These results show the interest of molecular diagnostic in confirming leprosy cases were traditional standard methods like microscopy or culture are limited. Effectively while the bascilloscopy has a poor detection limit (10^{e4} bacilli) [12], *M. leprae* culture is difficult on axenic media and time consuming using mouse footpad assay [13].

Previously, the assessment of chemotherapy efficacy in leprosy was difficult, since the only reliable method for determining whether *M. leprae* was viable depended on its growth on mouse footpads [14]; but today with new molecular technics based on PCR, those were not useful for follow-up of treatment, but for detection of drug resistance [15,16]. So *rpoB*, *folp1* and *gyrA* remains the main groups of *M. leprae* genes identified for their correspondence to drug resistance [17,18].

The amplification of the DRDR for Rifampicin, Dapsone and Ofloxacin as described by Shinji Maeda et al.

[10] showed the following results: 86.95% (60/69) of the patients amplified the *rpoB* gene associated with the rifampicin resistance (Figure 1) while 100% (69/69) of them amplified the *folp1* (Figure 2) gene associated with the dapsone resistance.

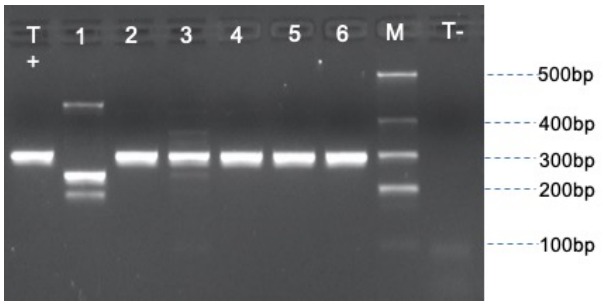

Figure 1. *rpoB* gene PCR amplification products (~305bp). Revelation was done on a 3% gel agarose electrophoresis. M: molecular weight marker; T+: Positive control; T-: Negative control(DNase/RNase free water); line 1 to 6: samples

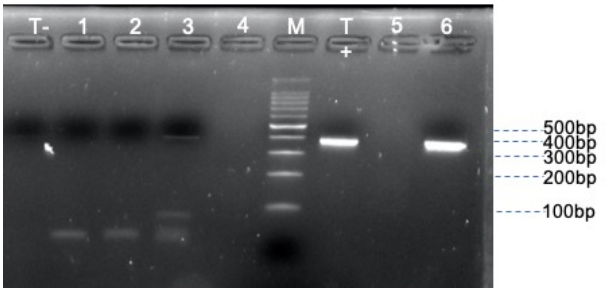

Figure 2. *folp1* gene PCR amplification products (~388bp). Revelation was done on a 3% gel agarose electrophoresis. M: molecular weight marker; T+: Positive control; T-: Negative control(DNase/RNase free water); line 1 to 6: samples

For the DRDR related to the fluoroquinolone resistance (ofloxacin), 52 out of 69 (75.66%) amplified it (Figure 3).

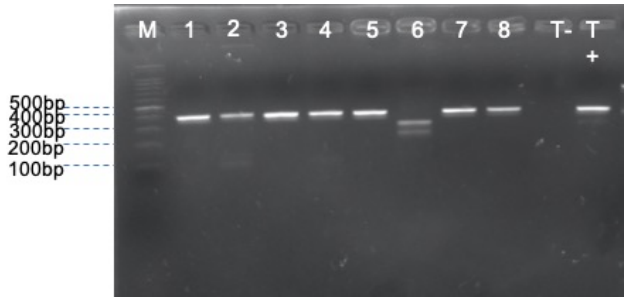

Figure 3. *gyrA* gene PCR amplification products (~342bp). Revelation was done on a 3% gel agarose electrophoresis. M: molecular weight marker; T+: Positive control; T-: Negative control (DNase/RNase free water); line 1 to 8: samples

48/69 samples amplified both of the *rpoB*, *folp1* and *gyrA* gene. The fact that all the samples had not amplified simultaneously both of the 3 genes conferring *M. leprae* drug resistance is probably due multiple alterations in *M. leprae* genome which composed of 70% pseudogenes [19]. Another reason could be due to a particularity of Ivoirian strains; unfortunally there is no study on Ivoirian *M. leprae* strains complete genome until now. Like Sekar B. *et al.* [20] suggested that the genetic detection of resistance has some inherited limitations such as resistance mechanisms not mediated by target gene mutations cannot be detected, or the gene may be present but not necessarily translated.

All 48 samples were sequenced and the sequences were aligned with the sequence of the reference strain TN in order to identify the mutations.

For the *rpoB* gene: 24 out of the 48 patient sequences (50%) showed a wild-type profile identical to the sequence of the reference strain TN. In 24 patients sequences, mutations were detected, 11 of which (22.91%) defined missense mutations conferring resistance to rifampicin. The various missens mutations observed were at the position 438 (Gln→Val) (1/11); 441(Asp→Asn) (2/11); 456(Ser→Leu) (5/11); 456(Ser→Phe) (3/11) (Table 1).

Table 1. Drug resistance gene and observed mutations in Ivoirian patients

Resistance gene	Wild type profile	Mutant profile	Observed mutations		
			Position	Type	Number
Folp1	20/48	28/48	53	Thr→Iso	10/28
			55	Pro→Arg	11/28
			55	Pro→Leu	7/28
rpoB	24/48	11/48	438	Gln→Val	1/11
			441	Asp→Asn	2/11
			456	Ser→Leu	5/11
			456	Ser→Phe	3/11
gyrA	39/48	9/48	91	Ala→Val	9/9

For the *folp1* gene: 20 of the 48 patients had their sequence identical to that of the reference sequence TN while 28 presented a mutation conferring resistance to dapsone. The mutations observed in the folp1 gene were varied: Thr53Iso (10/28); Pro55Arg (11/28); Pro55leu (07/28) (Table 2).

Table 2. PCT drugs resistance combination observed in Ivoirian patients

Drug associations	Number of resistance cases observed
Rifampin + Dapsone	8/48(16,66%)
Ofloxacin + Dapsone	4/48(8,33%)
Ofloxacin + rifampin	4/48(8,33%)
Rifampin + Dapsone + ofloxacin	3/48(6,25%)

As regards the *gyrA* gene involved in resistance to fluoroquinolone (Ofloxacin), in 9 out of 48 patients, Ala91Val-type mutations were observed.

In some patients, simultaneous search for mutations in the *RpoB*, *folp1* and *gyrA* genes has made it possible to observe cases of multi-resistance. Thus cases of dual resistance to: rifampicin / dapsone; ofloxacin / dapsone; ofloxacin / rifampicin were found with respective frequencies of 16.66% (8/48) and 8.33% (4/48) for the last two combinations.3 cases of triple resistance to the molecules used in multidrug therapy for the treatment of leprosy were also detected (Table 2).

In view of these results, it is not surprising to observe cases of genetic resistance to antibiotics used in the treatment of leprosy in Côte d'Ivoire. Indeed, many countries around the world have reported cases of resistance to multidrug therapy [13,18,21] and Africa does not was no less spared [7]. Dehe *et al.* [22] were already presented cases of resistance to rifampicin in Côte d'Ivoire

but not to the various antibiotics used in MDT. The MDT for 12 or 24 months against *M. leprae* infection was usually started without information of drug resistance or in some cases without molecular confirmation of clinical cases; although some clinical aspects of leprosy remain obvious to experienced practitioners, today many skin diseases may be related to it.

On the other hand, no effort to monitor *M. leprae*'s resistance to MDT [23] was done by governments in low, middle-, and endemic countries. Effectively, even if molecular methods are becoming more feasible and affordable in many low-cost countries laboratories, there is no active drug resistance surveillance for leprosy control in Côte d'Ivoire. Practicians are limited to the efficacity of the treatment without take care that due to the lack of several crucial metabolic pathways in *M. leprae*, it's accurate diagnosis and also monitoring of patients during and after treatment is often complicated.

4. Conclusion

Although the finding of our study is not new for many leprosy endemic countries, it is a first report of its kind in west Africa region of multiple dual and triple resistance cases to anti-leprosy drugs. This should constitute an alert for antibiotic resistance observatories and Ivoirian's health politics. This work reinforce Who strategy in its global surveillance system to monitor the development of drug resistance in *M. leprae*.

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Statement of Competing Interests

The authors have no competing interests.

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