Available online on 15.03.2023 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

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Research Article

Genotypic characterization of *Mycobacterium leprae* strains resistant to rifampicin and ofloxacin in three health districts in Chad

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Article Info:

Article History:

Received 02 Jan 2023
Reviewed 11 Feb 2023
Accepted 20 Feb 2023
Published 15 March 2023

Cite this article as:

Kirga KA, Nadlaou B, Mahamat A, Cambau E, Penlap V, Godreuil S, Genotypic characterization of *Mycobacterium leprae* strains resistant to rifampicin and ofloxacin in three health districts in Chad, Journal of Drug Delivery and Therapeutics. 2023; 13(3):7-11

DOI: http://dx.doi.org/10.22270/jddt.v13i3.5748

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Abstract

Antimicrobial surveillance and identification of the genetic basis of antimicrobial resistance provides important information to optimize patient care. The present study was an analytical cross-sectional study aimed at determining the prevalence of rifampicin and ofloxacin resistance genes among *Mycobacterium leprae* strains in three health districts in Chad.

The determination of the folP1, rpoB and gyrA resistance genes was carried out by PCR-RLEP and confirmed by sequencing from 80 biopsy samples taken from patients with multibacillary leprosy, including 12 relapsed patients and 68 new cases. In the whole cohort, 1/80 (1.2%) showed resistance to rifampicin and 1/80 (1.2%) to ofloxacin. No mutations were detected for dapsone. The presence of *M. leprae* mutation associated with rifampicin resistance was observed in a relapsed patient and the mutation associated with ofloxacin resistance was observed in a patient with multibacillary leprosy who had not been sensitized by ofloxacin but should have used other quinolones. Both mutant strains revealed the emergence of secondary resistance.

This study, the first to highlight the emergence of resistance to rifampicin and ofloxacin in Chad. It raises the need to implement a robust surveillance system to detect resistance of *Mycobacterium leprae* in Chad and even in Central Africa.

Keywords: *Mycobacterium leprae*, resistance, Chad

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* or *Mycobacterium lepromatosis* [1]. The disease mainly affects peripheral nerves, skin and mucous membranes and can lead to nerve damage and disabilities [2]. Despite a remarkable decrease in the prevalence of leprosy following the global implementation of multidrug therapy (PCT) by the World Health Organization (WHO) in the 1980s [3]. The incidence rate has stagnated since 2005, indicating the continued active transmission of the disease. The first-line drugs used PCT against leprosy is composed of dapsone, rifampicin and clofazimine [14, 15] Second-line drugs include ofloxacin, minocycline, and clarithromycin. Due to the lack of effective alternative antileprosy drugs, resistance to first-line drugs could surely affect leprosy control programs [4, 9].

Resistance of *M. leprae* to anti-leprosy drugs has been observed in several areas' endemic for leprosy, [5].

Drug-resistant leprosy can arise either by transmission of a resistant strain (primary resistance) or by mutation of the drug-sensitive strain during treatment (secondary resistance). Only a few cases of primary resistance to ofloxacin have been reported to date [6]. However, cases of primary rifampicin resistance are more often described, and they are cause for concern due to the limited availability of second-line leprosy drugs [7].

Chad is a country in sub-Saharan Africa that achieved the elimination of leprosy in 1997, despite these 446 cases were reported in 2019, making it a country where the disease burden of leprosy is the lowest. highest in Central Africa [8]. No information on drug resistance in this region is available. The objective of this study was to determine the resistance genes to rifampicin and to ofloxacin among strains of *Mycobacterium leprae* in three health districts (Abéché, Bebedjia and N'Djamena) in patients with leprosy in Chad.

MATERIAL AND METHODS

Framework of the study and progress of the research work.

This is an observational, cross-sectional and analytical study, conducted in three health districts for the recruitment of people affected by leprosy in Chad.

Biopsy samples collected from multibacillary patients for *Mycobacterium leprae* research were tested:

- To the laboratories of the Hospitals of the three health districts;

- At the CHU Montpellier laboratory in France, as part of the South-North collaboration and evaluation of our results where all the stages of molecular diagnosis were carried out.

A total of 80 unduplicated *Mycobacterium leprae* isolates were collected from 80 people affected by leprosy to determine the rpoB, folP and gyrA genes.

The biopsy samples were taken from 2019 to 2020 in the health districts of Abèche, Bebedjia, N'Djamena in Chad. Patient volunteers (each of them having signed a consent form) were recruited by convenience sampling at different stages of treatment in order to search for mutant strains of *M. leprae.*

The study included 12 relapsed patients and 68 new cases under treatment or not without clinical improvement of the lesions during the year. Bacterial DNA was extracted from skin biopsies using the Dneasy Blood & Tissue Qiagen kit (Invitrogen) according to the manufacturer's protocol [10]. The extracted DNA was stored at -20°C until processing.

Detection of antimicrobial resistance genes by PCR-RLEP amplification

DNA amplification by PCR targeting RLEP repeat sequences was done as previously described [10]. Strict precautions have been taken to avoid cross-contamination. Sterile tubes and tips were used in all experiments. PCR reactions were performed in a 25 µL reaction mix consisting of 5 µL DNA template, 0.2 mM deoxynucleotide triphosphates, 0.5 M primers and 1 U Taq polymerase. The forward primer for RLEP was 5-TGC ATG TCA TGG CCT TGA GG3' and the reverse primer was 5' CAC CGA TAC CAG CGG CAG AA3'. RLEP PCR was performed at 95°C for 2 minutes (initial denaturation), followed by 45 cycles of 94°C for 30 seconds, 58°C for 2 minutes, 72°C for 2 minutes and 72°C for 8 minutes (final extension) and kept at 4°C. Each amplification reaction was analyzed by 2% agarose. The gels were stained with ethidium bromide and photographed using a transluminator. A 100 bp DNA marker was included on each gel in order to be able to observe the expected 129 bp fragment.

PCR was performed using primers to detect mutations in the rpoB, gyrA and folP1 genes in the *M. leprae* genome. The PCR mix containing 12.5 µL of hot start Taq polymerase PCR Master Mix (X) (Qiagen, Hilden, Germany), 1.25 µL of forward and reverse primers at a final concentration of 0.5 µM and 5 µL of DNA sample of the processed sample. The final volume of the reaction mixture was generated up to 25 µL with nuclease-free water. The primer sequences used in this study were as follows: folP:folPF primer CTTGATCCTGACGATGCTGT; folPR CCACCAGACACATCGTTGAC, rpoB primer: rpoBF GTCGAGGCG ATCACGCCGCA; rpoBR CGACAATGAACCGATCAGAC, and gyrA primer: gyrAF ATGGTCTCCAAACCGGTACATC; gyrAR TACCC GGCGAACCGAAATTG. The reaction was repeated 40 times at 94°C, 60°C and 70°C for 1 min each; starting with an initial denaturation at 95°C for 15 min; and finished with a final extension at 72°C for 10 min. Each response parameter contains a negative control and a positive control. After detection of the PCR product on a 2% agarose gel, the amplicons were processed according to the Sanger tube sequencing protocol [19].

RESULTS

Mapping of the cities surveyed in three Provinces of Chad

Figure 1 illustrates the cities surveyed: Abéché, Bebedjia and N'Djamena.

Chad is located between 7th and 24th degrees North latitude and 13th and 24th degrees East longitude. It covers an area of 1,284,000 km2; it is the fifth largest country in Africa after Sudan, Algeria, Zaire and Libya. From North to South, it extends over 1,700 km and, from East to West, over 1,000 km. It shares its borders with, to the North, Libya, to the East, Sudan, to the South, the Central African Republic and, to the West, Cameroon, Nigeria and Niger. The city of Abéché is located 900 km north of N'Djamena, shares its borders with, to the north, Libya, to the east, Sudan and that of Bebedjia 530 km to the south Chad in contiguity with the Republic Central African Republic and, to the west, Cameroon. The country belongs politically and economically to Central Africa, but due to the similarities in climatic conditions, it is also attached to the Sahelian countries. The geolocation of Chad would certainly have contributed to the transmission of leprosy in 446 cases of leprosy reported in 2019 after the eradication of the disease in 1997.

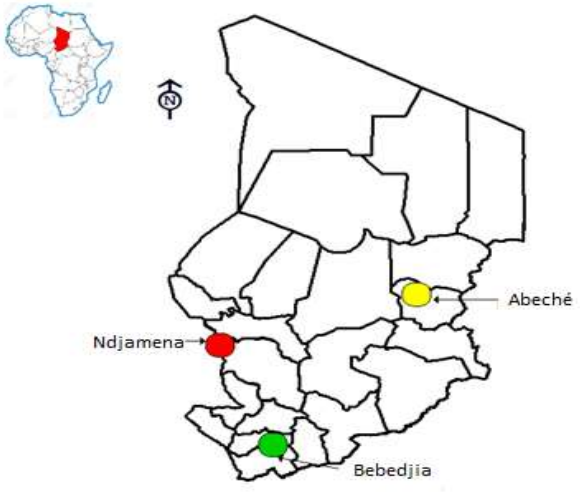


Figure 1: Map of surveyed cities

Study population, biological and demographic parameters of patients

The study involved 80 subjects examined, aged 12 to 77, including 45 (56.25%) men and 35 (43.75%) women (x^2 = 0.428<x^2_0<3.84, p = 0.50, dof = 1, difference not significant). This allowed us to affirm that with a margin of error of 0.05, the occurrence of a leprosy infection is not linked to sex. The average age of the patients was 38.2 years with the extremes of 12 to 77 years, and the sex ratio was 1.28.

Distribution of resistance genes according to the localities surveyed

Of the 80 biopsies, 48 (60%) were positive for PCR–RLEP. Sequencing revealed two mutant strains of *Mycobacterium leprae* associated with rifampicin and ofloxacin resistance. A missense mutation at codon 456rpoB (Ser (TCG>Leu (TTG)) was observed in a secondary case in favour of rifampicin resistance in a 46-year-old relapsed patient from N'Djamena. In addition, a missense mutation at codon gyrA 91 (Ala (GCA>Val (GTA)) associated with resistance to ofloxacin in a 37-year-old patient from Abéché who had not been sensitized by ofloxacin but has been in contact with other quinolones No mutation was observed on the flop1 gene.

Table 1: Results of PCR and DNA sequencing of mutations in the folp1, rpoB and gyrA genes of *Mycobacterium leprae*

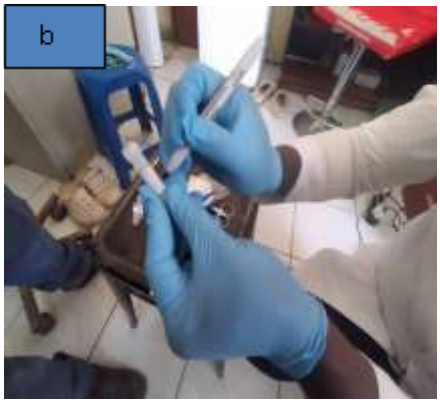
Sample Type	Effective	PCR-RLEP	flop1		rpoB		gyrA	
			PCR positive sample	Sample with mutation	Sample with mutation	Sample with mutation	PCR positive sample	Sample with mutation
New Case	68	45/68	40/68	0	32/68	0	29/68	0
Relapse	12	10/12	8/12	0	8/12	TCG>TTG (Ser>Leu) at codon 456	7/12	GCA>GTA (Ala>Val) at codon 91
Total	80	55	48	0	40	1	36	1

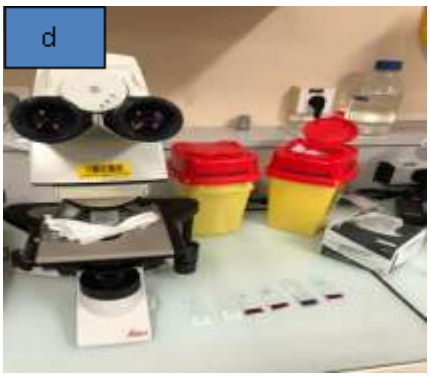
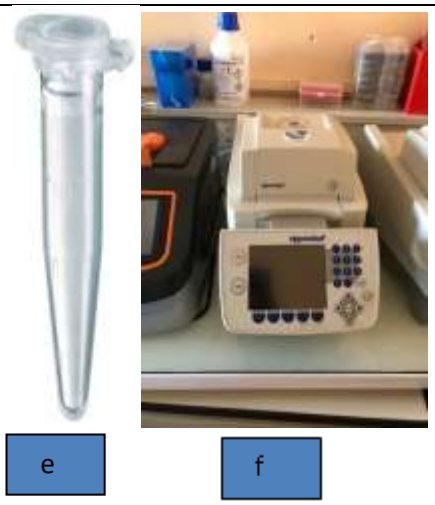
Table 2: Summary of multi-resistant cases by sequencing

Patient	Sex	Age	District	Type of leprosy	Sequencing Result		
					flop1	rpoB	gyrA
P1	M	46	N'Djamena	MB	No mutation	Mutation (TCG>TTG) **(Ser456>Leu)**	No mutation
P2	M	37	Abéché	MB	No mutation	No mutation	Mutation (GCA>GTA) **(Ala91>Val)**

MB = multibacillary, P1 = patient one (1), P2 = patient two (2)

Table 3: Macroscopic characteristics of skin biopsy and molecular steps for the detection of folp1, rpoB and gyrA genes of *Mycobacterium leprae*

1	a : skin biopsy sample material b : Registration, information on direct debit codes		

2	C : skin biopsy sample d : microscopic research after ZIEHL NEELSEN staining		
3	e : DNA extraction f : RLEP-PCR: master mix and amplification g : sequencer: ABI 3130XL: gene sequencing: rpoB, flop1, GyrA		

DISCUSSION

The WHO has stated that MDT can effectively control the incidence of leprosy and contribute to the elimination of the leprosy burden in several countries, including Chad [12]. The emergence of leprosy drug resistance is a major concern for the implementation of disease intervention programs [11, 18]. No previous study has identified the presence of *Mycobacterium leprae* mutation in Chad. This study analyzed cases of drug-resistant leprosy by sequencing the sites to determine three potentially compensatory genes (flop1, rpoB, gyrA) associated with antimicrobial resistance, using samples from 80 patients with leprosy from 2019 to 2020 [16, 17, 20].

All the cases were patients with multibacillary leprosy, 68 were new cases and 12 were relapse cases and they completed treatment based on WHO guidelines [12, 15]. A mutation at codon 456 (Ser-Leu) of rpoB (Table 2), known to cause rifampicin resistance among 12 samples from relapsed cases, was identified. The frequency of drug resistance to rifampicin (1.2%) was lower than that of Niger (8.3%) [11] but higher than that of Senegal and Madagascar (0%) [13], The gyrA sequence also showed a single mutation in codon 91 with an amino acid change from alanine to valine (Table 2), demonstrating resistance to ofloxacin. Anyway, the frequency (1.2%) was high than in African countries [13] and 2.9% was lower than that found in China for ofloxacin [21]. Resistance was observed in a patient not sensitized to ofloxacin but to other quinolones. Fluoroquinolones are promising and widely used antibiotics that are being introduced into routine clinical practice for the treatment of infectious diseases in Chad. Given the easy availability and inappropriate use of these drugs, resistance to fluoroquinolone antimicrobials has emerged in Chad. In the present study, no dapsone-associated mutation (flop1 gene) was confirmed.

However, identifying drug resistance in leprosy patients involves clinical and financial challenges. Furthermore, switching therapy is not a common clinical practice because resistance testing is not accessible in Chad. Moreover, alternative treatments are not freely available for the fight against leprosy. The limitation of this study was the limited number of samples which did not allow drawing conclusions on primary resistance and differentiating between relapse and reinfection.

This study gives an alert on resistance to *Mycobacterium leprae* in Chad and even in Central Africa. The association between confirmed resistance mutations and relapse in leprosy patients was 100% because the patients in whom the mutations were detected were on PCT treatment. The resistance of *M. leprae* strains in 446 cases of leprosy reported in 2019 after the eradication of the disease in 1997 could be explained by non-compliance with MDT treatment by patients or the migration of populations from the sub-region of Central Africa in relation to the geolocation of Chad [22]. Given the resistance of bactericidal drugs used in the first- and second-line treatment of leprosy, antimicrobial resistance would constitute a real problem for the elimination of leprosy in Chad and even in the Central African sub-region. The leprosy control program should operationalize the surveillance of drug-resistant *Mycobacterium leprae*.

CONCLUSION

The results of this study show, for the first time, cases of secondary resistance to ofloxacin and rifampicin in Chad. Although the two strains of *Mycobacterium leprae* resistant to ofloxacin and rifampicin are considered a major threat to leprosy control, these are epidemiological indicators of active transmission of the disease. Moreover, our results underline

the interest of studies on the observance of MDT in endemic countries. The data from this study should encourage national programs to activate the surveillance system for resistant *Mycobacterium leprae*.

Acknowledgments

The authors thank the patients who participated voluntarily and by informed consent in this study. Our thanks also go to the Organization for the Coordination of Endemic Disease Control in Central Africa (OCEAC), and the German Federal Ministry for Economic Cooperation and Development (BMZ) through the German KfW Bank, the National Leprosy Control Program in Chad, the Raoul Follereau Foundation for the provision of data.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical consideration

This study was approved by the Bioethics Committee of Chad (N°171/PR/MESRI/SG/CNBT/2019), informed consent was obtained from the participants. The data was anonymous and confidentiality was strictly respected in the data analysis.

Author contributions

All authors have read and approved the final version of the manuscript.

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