Emergence of *Mycobacterium leprae* rifampicin resistance evaluated by whole-genome sequencing after 48 years of irregular treatment

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ABSTRACT

A case of *M. leprae* rifampicin resistance after irregular anti-leprosy treatments since 1971 is reported. Whole-genome sequencing from four longitudinal samples indicated relapse due to acquired rifampicin resistance and not to reinfection with another strain. A putative compensatory mutation in *rpoC* was also detected. Clinical improvement was achieved using an alternative therapy.

Short Form paper

A sixty-four year-old male, born and residing in Rio de Janeiro, Brazil, presented to the National Leprosy Reference Center FIOCRUZ clinic in November 2015 with several nodules and infiltrated plaques spread all over the body, and with swollen hands and feet consistent with lepromatous leprosy (1). The patient was initially diagnosed in 1971 with the lepromatous form of the disease (**Figure 1A**) showing diffuse infiltration, mainly affecting the ear lobes, a collapsed nose and a positive bacillary index (BI) in slit skin smears. After two months of dapsone monotherapy, he abandoned treatment. Later, in March 1975, he presented with a similar clinical condition. He restarted 100 mg dapsone until February 1977. In October 1978, signs and symptoms were unchanged. At that time, the Brazilian Ministry of Health (MoH) recommended dapsone treatment until the clearance of the bacilli. But the treatment was not improving his condition, so the patient received three months of 600mg of rifampicin and 100mg of dapsone, followed by 100mg of dapsone until bacterial clearance. However, he again abandoned dapsone treatment. In July 1985, he returned presenting with several lepromas and diffuse infiltration. He received 600mg of rifampicin for three months in combination with dapsone and then dapsone until

November 1990 when treatment was suspended following a negative BI. In October 1998, he presented with disseminated papular-nodular lesions, hypochromic macules and madarosis. Histopathological findings were compatible with lepromatous leprosy, revealing dermal histiocyte infiltration with Virchow cells and a BI of 3+. He took three doses of multidrug therapy (MDT), but abandoned treatment once again. In November 2002, his clinical conditions had worsened with diffuse infiltration, hypochromic macules, erythematous plaques, nodules and edema of the lower limbs. An archived skin biopsy from 2002 confirmed leprosy diagnosis exhibiting lymphocytic infiltrate, and macrophages containing abundant granular and foamy cytoplasm consistent with subpolar lepromatous leprosy (**Figure 1B**). The Wade staining (a modified Ziehl-Neelsen stain), showed a high 4.7+ logarithmic index of bacilli in biopsies (LIB).

MDT was restarted and again abandoned after eight supervised doses when the BI was 1.33+. He had an episode of *erythema nodosum leprosum* during this course of treatment in August 2003, confirmed by histopathology. He returned in November 2013 with leonine facies, ptosis, spread lepromas, swollen lower and upper limbs, plus neural pain affecting both legs. The BI was 3+ and no improvement was observed after 12 doses of MDT with a BI of 4.25+, suggesting treatment failure (2). Active disease was suspected as evidenced by abundant infiltrate of lymphocytes in a biopsy from a skin lesion at the end of the treatment in 2014. Wade staining revealed an increased density of granular materials and intact acid-fast bacilli (AFB), occasionally arranged in globi (**Figure 1C**) with increasing LIB of 6+.

He was then referred to our clinic, the National Leprosy Reference Center at FIOCRUZ in Rio de Janeiro, in November 2015 with no improvement in clinical conditions. Other parameters (temperature, hemodynamic, etc.) were normal and the Mitsuda test was negative. Serology for HIV and hepatitis B and C were negative. The patient received an additional 12 months of fixed MDT as recommended by the MoH regulations assuming treatment failure. After extended treatment (24-doses MDT, in November 2016), skin biopsies presented similar inflammatory infiltrates and Wade staining revealed the presence of AFB with LIB: 1+ (**Figure 1D**). Analysis of slit skin smears gave a BI of 2.5+. The clinical assessment showed diffuse infiltration, madarosis, ptosis, swollen hands and feet, with nodules and infiltrated plaques all over the body (**Figures 1E and 1F**), grade 1 disability and drug resistance was suspected (3). Molecular drug susceptibility testing was performed by PCR–sequencing on the DNA extracted from the skin biopsy sample, collected in 2016, as previously described (3). Drug resistance-associated Single Nucleotide Polymorphisms (SNPs) were not detected in *folP1* (dapsone) and *gyrA* (ofloxacin), but a mutation at codon 456 (Ser456Leu) of *rpoB,* known to cause resistance to rifampicin in *M. leprae,* was identified (4, 5).

We recovered archived skin from four biopsies taken in 2002, 2003, 2014 and 2016 and submitted them to enzymatic host tissue and host DNA digestion, followed by enrichment of bacilli and whole genome sequencing on Illumina HiSeq and NextSeq instruments as described elsewhere (6). The read coverage ranged from 13.4 to 44.4X (**Table S1**) and was sufficient for comparative analysis at the single nucleotide level. All four isolates are SNP 4N genotypes and have nearly identical genomes, differing by only a few polymorphisms (**Table S2**). The isolate from 2002 differed from the three subsequent isolates by two SNPs, one in the intergenic region

between *ml2392* (unknown function) and *greA* (a transcription elongation factor, Supplementary Table 2), and another in *rpoC* (discussed below). Given the near identity between the strain from 2002 and the other isolates, and the presence of the same *rpoB* mutation in all cases, reinfection with a different strain seemed unlikely, especially considering that the second biopsy was from a type 1 reaction episode that occurred less than one year after the 2002 sample. Therefore, these two SNP differences likely reflect the intra-patient variation of the primary strain that occurred in the lesion from where the biopsy was taken. The non-synonymous SNP in *rpoC* (L527V) was detected from 2003 onward. Curiously, this mutation has been described in *M. tuberculosis* rifampicin-resistant strains but was felt to be unlikely to correspond to a compensatory mutation impacting fitness (7). Nevertheless, considering the chronology and the circumstances of the appearance of this mutation here, a compensatory effect appears probable in *M. leprae.* Three additional indel loci were polymorphic between all the isolates, occurring either in homopolymeric tracts or in Variable Number Tandem Repeats (**Table S2**), which tend to be polymorphic (8).

Confirmation of drug resistance led to the prescription of an alternative treatment. However, soon thereafter, the patient was diagnosed with larynx cancer and leprosy treatment was suspended until cancer chemotherapy was completed. At the time of treatment resumption in 2017, the smear test was BI: 2.25 + when compared to BI: 2.5 + performed at the end of the previous MDT. A final histological Wade staining indicated fragmented and intact AFB arranged in globi with higher LIB: 3.6+ (**Figure 1G**). From July 2017, the patient was treated with an alternative 2-year regimen replacing rifampicin (9) with daily clofazimine, 50mg; daily ofloxacin 400mg; and daily minocycline 100mg for 6 months; followed by daily clofazimine 50mg and

daily ofloxacin 400mg for 18 months. He has recovered well with reduction in infiltrates, BI and lesions (**Figures 1H and 1I**). On release from treatment (RFT) on June 2019, BI was 0.75 and LIB was 1+. The patient is still under observation returning every 6 months for re-evaluation to our clinic.

Due to its bactericidal action, rifampicin forms the backbone of MDT and treatment efficacy is seriously compromised when rifampicin must be replaced [9]. In our case, the combination of clofazimine, ofloxacin, and minocycline proved efficient after 24 months of treatment. After this alternative treatment, the patient has improved, although continuous observation of him and his household contacts is necessary as, in special cases, leprosy merits long term attention from health care providers.

Rifampicin resistance mostly occurs during irregular therapy [4-5,9-10]. Many factors are responsible for interruption of treatment in Brazil: socioeconomic difficulties, poor education, lack of knowledge about the disease, and inefficiency of health services, among others (10). Primary transmission of rifampicin-resistant strains also occurs in Brazil (11–13), although there is no evidence of it in the state of Rio de Janeiro (3). The long series of abandonment and retreatment of the case reported here delayed proper care and likely caused the emergence of drug-resistant *M. leprae* that led to the relapse in 1998. Emergence of the putative compensatory mutation in *rpoC* is also a cause of concern since this can increase the fitness of the drug-resistant bacteria and contribute to their spread in the population. The emergence of resistance warrants intensifying the efforts of the WHO sentinel network, which has already reported disturbing resistance trends that need following up (12). Therefore, educating and monitoring patients once a year after treatment

should be common practice because it can provide an early alarm signal for relapse that will need special care [9].

Figure Legend

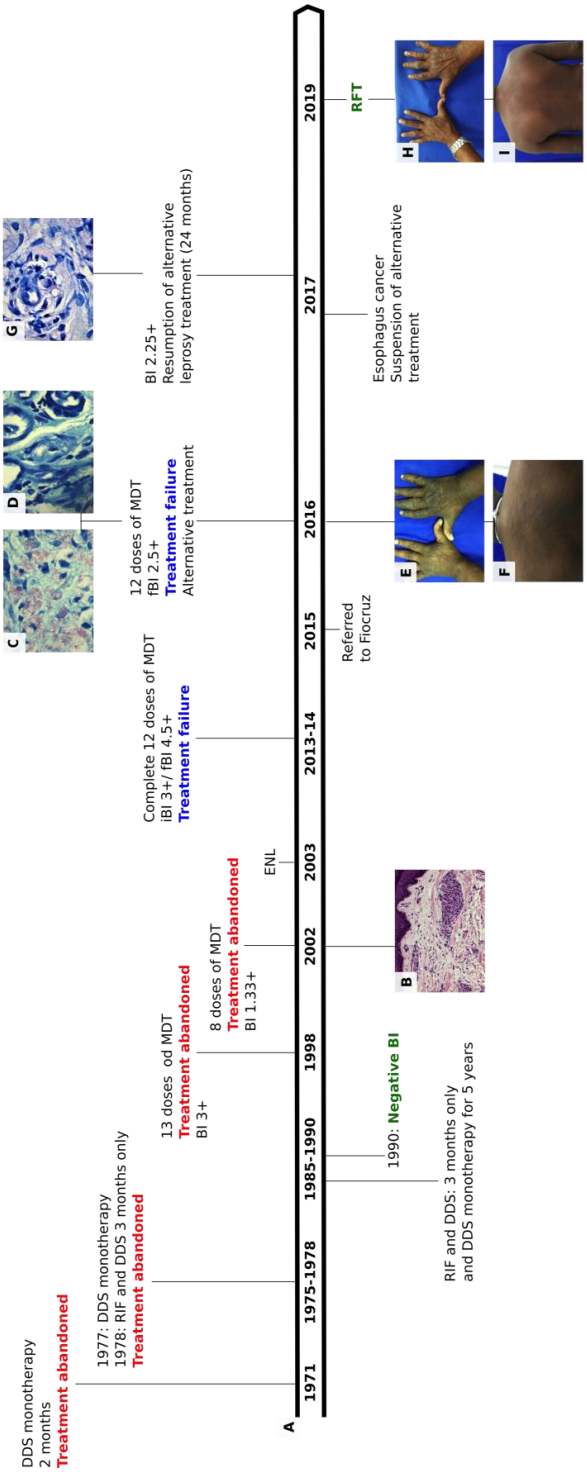

Figure 1 Information collected from medical records and observation of a relapsed leprosy patient with irregular treatment during 48 years **A-** Timeline of the patient's

abandonment and retreatment cycles during 48 years. Treatment abandonment, treatment failure and positive disease outcome are shown in red, blue and green, respectively. BI=bacillary index, DDS=dapsone, ENL=*erythema nodosum leprosum,* fBI=final BI after treatment, iBI=initial BI at treatment implementation, MDT=multidrug therapy, RFT=release from treatment, RIF=rifampicin, *date for which whole-genome sequence is available. **B-D, G** Histological analysis of retrieved skin biopsies consistent with leprosy diagnosis from 2002-2017. **B-** Superficial and deep perivascular, periadnexal and perineural lymphohistiocytic infiltrate. Macrophages containing abundant granular to foamy cytoplasm compatible with subpolar lepromatous leprosy, HE-20X (2002). **C-** High density of granular and intact AFB, occasionally arranged in globi, LIB: 6+, Wade-100X (2015). **D-** Presence of a few granular and intact AFB after 12 MDT doses, LIB:1+, Wade-100X (2016). **G-** Fragmented and intact AFB arranged in globi, LIB: 3.6+, Wade- 100X (2017). **E,F,G,I-** Clinical features of the patient at the resistance confirmation in 2016 and after 24 months of treatment (2019). Male, 64 y/o, born and residing in Rio de Janeiro, Brazil. Pictures showing the patient hands **(E)** and back **(F)** presented to the FIOCRUZ clinic in November 2016 with several nodules and infiltrated plaques spread all over the body, edematous hands and feet, besides increasing paresthesia of upper and lower limbs consistent with lepromatous form of the disease. Release from treatment in 2019 with complete remission of symptoms in the hands **(H)** and back **(I)**. [a]Nomenclature used as described by Groathouse et al.

AUTHOR CONTRIBUTION

Study design: MOM, STC, ENS

Patient diagnosis and patient follow-up: RCM, AMS, JAN

Sample collection: RCM, ROP

Histological analysis and microscopy: TPS, SJM, ENS, AM

PCR and Sanger sequencing: AFM FNM PS

Library preparation: CA, PB

Computational analysis: AB, CA

Formal analysis: MOM, CA

Manuscript draft: MOM, CA

All authors discussed the results and commented on the manuscript

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CONFLICT OF INTEREST

The authors declare that the submitted work was not subject to any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

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DATA AVAILABILITY

Sequence data are available from the NCBI Sequence Read Archive (SRA) under the bioproject PRJNA601236, biosamples SAMN13864306 to SAMN13864309