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Investigating drug resistance of *Mycobacterium leprae* in the Comoros: an observational deep-sequencing study



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Summary

Background Despite strong leprosy control measures, including effective treatment, leprosy persists in the Comoros. As of May, 2022, no resistance to anti-leprosy drugs had been reported, but there are no nationally representative data. Post-exposure prophylaxis (PEP) with rifampicin is offered to contacts of patients with leprosy. We aimed to conduct a countrywide drug resistance survey and investigate whether PEP led to the emergence of drug resistance in patients with leprosy.

Methods In this observational, deep-sequencing analysis we assessed *Mycobacterium leprae* genomes from skin biopsies of patients in Anjouan and Mohéli, Comoros, collected as part of the ComLep (NCT03526718) and PEOPLE (NCT03662022) studies. Skin biopsies that had sufficient *M leprae* DNA (>2000 bacilli in 2 µl of DNA extract) were assessed for the presence of seven drug resistance-associated genes (ie, *rpoB*, *ctpC*, *ctpI*, *folP1*, *gyrA*, *gyrB*, and *nth*) using Deeplex Myc-Lep (targeted next generation deep sequencing), with a limit of detection of 10% for minority *M leprae* bacterial populations bearing a polymorphism in these genes. All newly registered patients with leprosy for whom written informed consent was obtained were eligible for inclusion in the survey. Patients younger than 2 years or with a single lesion on the face did not have biopsies taken. The primary outcome of our study was the proportion of patients with leprosy (ie, new cases, patients with relapses or reinfections, patients who received single (double) dose rifampicin-PEP, or patients who lived in villages where PEP was distributed) who were infected with *M leprae* with a drug-resistant mutation for rifampicin, fluoroquinolone, or dapsone in the Comoros.

Findings Between July 1, 2017, and Dec 31, 2020, 1199 patients with leprosy were identified on the basis of clinical criteria, of whom 1030 provided a skin biopsy. Of these 1030 patients, 755 (73.3%) tested positive for the *M leprae*-specific repetitive element-quantitative PCR (qPCR) assay. Of these 755 patients, 260 (34.4%) were eligible to be analysed using Deeplex Myc-Lep. 251 (96.5%) were newly diagnosed with leprosy, whereas nine (3.4%) patients had previously received multidrug therapy. 45 (17.3%) patients resided in villages where PEP had been administered in 2015 or 2019, two (4.4%) of whom received PEP. All seven drug resistance-associated targets were successfully sequenced in 216 samples, 39 samples had incomplete results, and five had no results. No mutations were detected in any of the seven drug resistance-related genes for any patient with successfully sequenced results.

Interpretation This drug resistance survey provides evidence to show that *M leprae* is fully susceptible to rifampicin, fluoroquinolones, and dapsone in the Comoros. Our results also show, for the first time, the applicability of targeted sequencing directly on skin biopsies from patients with either paucibacillary or multibacillary leprosy. These data suggest that PEP had not selected rifampicin-resistant strains, although further support for this finding should be confirmed with a larger sample size.

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Introduction

Highly effective multidrug therapy, consisting of rifampicin, dapsone, and clofazimine, has been used for leprosy since the 1980s. In instances of rifampicin resistance, other drugs, such as ofloxacin, minocycline, and clarithromycin, can also be used. However, despite the worldwide availability and implementation of multidrug therapy, the global incidence of leprosy has not decreased since 2006, with around 200 000 new patients with leprosy diagnosed annually.¹ Emergence of drug resistance has

been reported in several countries, with the highest burden of drug-resistant *Mycobacterium leprae* reported in Brazil and India. Dapsone-resistant and rifampicin-resistant *M leprae* is transmitted in Guinea and the Philippines.^{2,3} Several studies report resistance to rifampicin, dapsone, or fluoroquinolones in patients with leprosy, as well as in patients with relapsed leprosy.⁴⁻⁶ Resistance to rifampicin is mediated by missense mutations in *rpoB*, and possibly the *ctpC* and *ctpI* genes. Resistance to ofloxacin is mediated by missense mutations in the *gyrA* gene, and resistance to

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Institution of Tropical Medicine, Antwerp, Belgium (S Marijke Braet MSc, M Van Dyck-Lippens BSc, Prof L Rigouts PhD, Prof E Hasker PhD, B C de Jong MD); Department of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Antwerp, Belgium (S Marijke Braet, Prof L Rigouts); Research Foundation Flanders, Brussels, Belgium (S Marijke Braet); GenoScreen, Lille, France (A Jouet PhD, E Lenoir MSc); Sorbonne Université, INSERM, Centre d'Immunologie et des Maladies Infectieuses (Prof A Aubry PhD) and Centre National de Référence des Mycobactéries et de la Résistance des mycobactéries aux antituberculeux (Prof A Aubry), Paris, France; Damien Foundation, Brussels, Belgium (Y Assoumani MD, A Baco MSc); National Tuberculosis and Leprosy Control Program, Moroni, Comoros (A Mzembaba MD); Inserm UMR 1137 Iame, service de mycobactériologie spécialisée et de référence, APHP GHU Paris Nord, Hôpital Bichat, Paris, France (Prof E Cambau PhD); Laboratory of Molecular Biology applied to Mycobacteria, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil (S E G Vasconcellos PhD, P N Suffys PhD); U1019-UMR 9017-CIIL, Université de Lille, CNRS, INSERM, CHU Lille, Institut Pasteur de Lille, Center for Infection and Immunity of Lille, Lille, France (Prof P Supply PhD)

Correspondence to:
Sofie Marijke Braet, Institution of
Tropical Medicine,
Antwerp 2000, Belgium
sbraet@itg.be

Research in context

Evidence before this study

We searched PubMed for all studies published in English from database inception to Dec 1, 2021, in which drug resistance surveillance for leprosy results was reported, using combinations of the following keywords: ("resistance" OR "resistant" OR "drug resistance" OR "drug resistant") AND ("detection" OR "survey" OR "surveillance"). All studies identified in our search were included. Only a few region-wide resistance studies reported on drug resistance surveillance for leprosy because of the limitation that leprosy bacillus cannot be grown in laboratory conditions, thereby hampering studies of drug resistance in leprosy. In 2018, a study reporting on the global prevalence of drug resistance in patients with leprosy confirmed the presence of drug-resistant strains in several countries. There are no nationally representative data for leprosy drug resistance in the Comoros, which is among the six countries with the largest leprosy burden worldwide. Such data are even more urgent since WHO approved single-dose rifampicin in 2018 as prophylaxis for contacts of patients with leprosy.

Added value of this study

This study designed and implemented innovative field methods to collect, ship, and test skin biopsies for molecular

drug resistance surveillance for leprosy. Our results show that no traces of drug-resistance mutations were detected for rifampicin, fluoroquinolones, or dapsone in the Comoros. To our knowledge, this is the first nationwide survey of drug resistance among *Mycobacterium leprae* in the Comoros and the first to use Deeplex Myc-Lep to predict drug resistance profiles.

Implications of all the available evidence

The findings of this study provide evidence for the effectiveness of treatment (and prophylactic treatment) for leprosy and show the success of the leprosy control programme in the Comoros. Professionally supervised leprosy treatment in the Comoros ensures consistent exposure to drugs, which poses a lower risk of resistance selection than self-treatment by patients, even with the help of family members. The storage of biopsies at room temperature in alcohol allowed us to identify a full drug-resistance profile for *M leprae* months after collection. Our findings could inform the future nationwide approaches to drug resistance surveillance. The sensitivity of the Deeplex Myc-Lep tool to detect even traces of molecular resistance make it a potential tool for worldwide leprosy resistance surveillance.

dapsone is mediated by missense mutations in the *folP1* gene.⁷ Furthermore, nonsense mutations in the *nth* excision repair gene have been associated with greater sequence diversity and drug resistance.⁸ The attribution of resistance to particular mutations is complicated by the inability to grow *M leprae* in culture, requiring murine experiments for phenotypic resistance testing.⁹ This limitation could also introduce bias if mutations are not yet fixed, as wild-type populations without fitness loss might predominate.

Post-exposure prophylaxis (PEP) is one of the key interventions suggested to overcome plateaued leprosy incidence. A single dose of rifampicin (SDR; 10 mg/kg for adults and 10–15 mg/kg for children) is recommended by WHO, as several studies have shown that SDR-PEP is well tolerated and reduces the risk of leprosy by 50% over a 2-year follow-up period.¹⁰ Valid concerns about rifampicin resistance resulting from SDR, which could jeopardise the treatment of active leprosy and tuberculosis, were addressed at a consensus meeting in which (on theoretical grounds) this risk was considered negligible, yet repeated doses should be avoided.^{11,12} Molecular surveillance for drug resistance in patients with leprosy is therefore key to confirming that SDR-PEP does not jeopardise multidrug therapy. WHO have endorsed genotypic testing by analysing *rpoB*, *folP1*, and *gyrA* genes, either by Sanger sequencing or hybridising separately amplified PCR products with particular probes, such as GenoType LeptraeDR (Bruker, Germany). Resistance-associated (and strain typing) targets can be

simultaneously amplified and analysed by multiplexed targeted next generation deep sequencing (tNGS).¹³ Moreover, deep sequencing can greatly increase the sensitivity and the degree of confidence for detecting drug resistance-associated mutations, especially when borne by a minority bacillary population. This approach allows for the surveillance of existing or emerging resistance while providing phylogenetic information on circulating strains. Deeplex Myc-Lep (Genoscreen, France) is a next generation deep sequencing technique targeting drug resistance-associated genes, single nucleotide polymorphisms (SNPs), and variable number of tandem repeats to genotype *M leprae*.

The Comoros is among the six countries with the largest burden of leprosy, as defined by WHO, with a yearly new case detection rate ranging from three to seven patients per 10000 population for the period of 2013–19.¹ In the Comoros, the completion rate of leprosy treatment is high (>85%), the relapse rate is low (1.8%), and the grade 2 disability rate among people with newly diagnosed leprosy is below 2.5%, all suggesting that leprosy control in the Comoros is effective.^{14,15} As a pilot intervention, in 2015, 269 close contacts of 70 patients with leprosy in four villages across the island of Anjouan were given SDR-PEP. In 2017, 2019, and 2020, we revisited these villages as part of the ComLep and PEOPLE studies, and sampled patients who had been newly diagnosed with leprosy. In 2019, the first round of single (double) dose rifampicin-(SDDR) PEP was distributed to contacts of patients with leprosy in the

PEOPLE study. In this Article, we aim to present the findings of the first anti-leprosy drug resistance survey conducted in the Comoros, based on tNGS done on skin biopsies to detect minor bacterial populations within patients with leprosy. The assay included all gene targets recommended by WHO,¹⁶ as well as potential resistance-associated targets. We also aimed to associate these findings with villages where SDR-PEP and SDDR-PEP had been administered to verify the hypothesis that these prophylactic treatments do not select for drug resistance emergence.

Methods

Study design and setting

During the ComLep study, a cross-sectional survey from 2017 to 2019 conducted on the island of Anjouan (Comoros), patients were identified via active case finding (via skin camps [ie, teams that go into health centres in villages, which have been contacted in advance, and provide treatment to people with skin ailments for free]) and passive case finding. The PEOPLE study identified patients during 2019–20 through active, door-to-door screening in selected villages on the islands of Anjouan and Mohéli, and via skin camps and passive case finding covering the other villages of the islands. Patients were diagnosed on the basis of clinical symptoms, and classified as either having paucibacillary leprosy or multibacillary leprosy as per the WHO operational classification.¹⁷

All newly registered patients with leprosy for whom written informed consent was obtained were eligible for inclusion in the survey. Patients younger than 2 years or with a single lesion in the face did not have biopsies taken. A questionnaire was completed in an Open Data Kit application that covered demographics, leprosy treatment history (new or previously treated), and PEP administration.

The protocols from the ComLep (ClinicalTrials.gov, NCT03526718) and PEOPLE studies (ClinicalTrials.gov, NCT03662022) were approved by the institutional review board of the Institute of Tropical Medicine (Antwerp, Belgium), the ethical committee of the University of Antwerp (Antwerp, Belgium), the ethical committee on the island of Anjouan (ComLep), and the Comoros national ethical committee (PEOPLE). Written informed consent was obtained from each participant, or their parent or guardian if they were younger than 18 years. Written consent was obtained for people aged 12–17 years, in addition to their parents' or guardians' consent. Participants could selectively refuse sampling if they chose to. Both the ComLep and PEOPLE study are registered at ClinicalTrials.gov.

Procedures

The 4 mm skin biopsies were inactivated directly after sampling in 1 ml of Disolol (ethanol denatured with 1% isopropanol and 1% methyl ethyl ketone) in screw cap

vials at ambient temperature, and transported in batches to the Institute of Tropical Medicine (London, UK). Negative sampling controls and Copan FloqSwabs (Murrieta, CA, USA) that were exposed for a minimum of 1 min to air in the room where the biopsies were taken were included each sampling day.

At the Institute of Tropical Medicine, biopsies were manually grinded with mortar and pestle, or with an automated disrupter (GentleMacs [Bergisch Gladbach, Miltenyi Biotech, Germany]) in 0.5–1 ml phosphate buffered saline (pH 7.2, Oxoid, Hampshire, UK). The suspensions were treated with an inhouse lysis buffer,¹⁷ followed by DNA extraction using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit or the Maxwell 16 FFPE Plus Tissue LEV DNA Purification Kit, as described by the manufacturer (Promega, WI, USA). A positive (ie, a suspension of mouse footpad infected with *M leprae* Thai 53) and a negative (ie, molecular grade water) extraction control were included in each run. Samples were selected to be processed with the Deeplex Myc-Lep based on their estimated bacterial load (all samples [excluding one sample with <2000 bacilli in 2 µl of DNA included by mistake and one sample with >2000 bacilli in 2 µl of DNA not included by mistake] with more than 2000 bacilli in 2 µl of DNA extract were selected for sequencing, and for the group that had 100–2000 bacilli per 2 µl of DNA extract, some were selected) and treatment status (SDR or SDDR, or previously treated). The *M leprae* bacterial load in 2 µl skin biopsy DNA extract was calculated using *M leprae*-specific repetitive element(RLEP)-quantitative PCR (qPCR), as previously described.¹⁷ A positive and negative DNA qPCR control were included. DNA was amplified and sequenced using the Deeplex Myc-Lep prototype kit by the manufacturer. This prototype used ultra-deep sequencing of *M leprae* directly in clinical samples using a single, 42-multiplexed amplicon mix to identify the mycobacterial species (based on the *hsp65* gene) to type *M leprae* strains (based on SNPs in 18 gene regions and 11 variable-number tandem-repeat markers), and to detect potential resistance-associated SNPs in seven genes (rifampicin: *rpoB*, *ctpC*, and *ctpI*; dapson: *folP1*; and fluoroquinolones: *gyrA*, *gyrB*, and *nth*).⁸

Amplicons were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, CA, USA) and quantified by the Qubit dsDNA BR assay (Life Technologies, Paisley, UK). Paired-end libraries of 150-base pairs read length were prepared with the Nextera XT DNA sample preparation kit (Illumina, CA, USA) and sequenced on an Illumina MiSeq platform using standard procedures. Sequencing runs typically had >80% bases with a quality higher than or equal to Q30. Drug-susceptibility status was extrapolated from the sequences using the Genoscreen analytical pipeline. An SNP was considered fixed when it was observed in 90% of the reads. A 10% limit of detection for minority bacillary populations with a minimum of 40x depth at

For Open Data Kit see <https://getodk.org/>

	Total recruited patients (n=1199)	Patients selected for Deeplex Myc-Lep (n=260)	Univariate analysis sample rate ratio (95% CI)
Island			
Anjouan	1160 (96.7%)	254 (97.7%)	1 (ref)
Mohéli	39 (3.2%)	6 (2.3%)	0.6 (0.3–1.6)
New case, relapse, or reinfection*			
New case	1179 (98.3%)	251 (96.5%)	1 (ref)
Relapse or reinfection	20 (1.7%)	9 (3.5%)	0.7 (0.3–1.5)
Case finding			
Active	874 (72.9%)	124 (47.7%)	1 (ref)
Passive	325 (27.1%)	136 (52.3%)	3.0 (2.4–3.6)
Operational WHO classification			
Paucibacillary	675 (56.3%)	26 (10.0%)	1 (ref)
Multibacillary	524 (43.7%)	234 (90.0%)	11.6 (7.9–17.1)
History of treatment			
Not received treatment	715 (59.6%)	149 (57.3%)	1 (ref)
Started multidrug therapy	452 (37.7%)	100 (38.5%)	1.6 (0.9–1.3)
Previously completed paucibacillary multidrug therapy†	3 (0.3%)	0	NA
Previously completed multibacillary multidrug therapy†	17 (1.4%)	9 (3.5%)	2.5 (1.6–4.1)
Received SDR-PEP in 2015	4 (0.3%)‡	1 (0.4%)	1.2 (0.2–6.6)
Received SDDR-PEP during PEOPLE study in 2019	8 (0.7%)§	1 (0.4%)	0.6 (0.1–3.8)
Age (years)			
0–14	454 (37.9%)	46 (17.7%)	1 (ref)
15–24	358 (29.9%)	99 (38.1%)	2.7 (2.0–3.7)
25–34	144 (12.0%)	44 (16.9%)	3.0 (2.1–4.4)
35–44	92 (7.7%)	29 (11.2%)	3.1 (2.1–4.7)
45–54	54 (4.5%)	12 (4.6%)	2.2 (1.2–3.9)
55–64	38 (3.2%)	9 (3.5%)	2.3 (1.2–4.4)
≥65	57 (4.8%)	21 (8.1%)	3.6 (2.3–5.6)
Unknown	2 (0.2%)	NA	NA
Sex			
Male	725 (60.5%)	188 (72.3%)	1 (ref)
Female	474 (39.5%)	72 (27.7%)	0.6 (0.5–0.8)

NA=not applicable. SDDR-PEP=single (double) dose rifampicin as post-exposure prophylaxis (20 mg/kg). SDR-PEP=single-dose rifampicin as post exposure prophylaxis (10 mg/kg). *The terms reinfection and relapse are used interchangeably in this study. This was because it was impossible to distinguish between relapse and reinfection. †Multibacillary leprosy and paucibacillary leprosy were defined using the clinical definition given by WHO. ‡All four patients were sampled. However, the bacterial load exceeded 2000 bacilli per 2µl DNA extract for only one biopsy. §One out of eight patients did not have a biopsy because they had only a single facial lesion. In one biopsy, no *Mycobacterium leprae* DNA was detected, and in five biopsies the bacterial load was inferior to 2000 bacilli per 2 µl DNA.

Table: Characteristics of the recruited patients with leprosy

that specific position was established. Controls for detection of minority bacillary populations consisted of a mixture of DNA from a susceptible (NHDP63) and a resistant isolate (Br14-3), resulting in different proportions of resistant strains in the mix, ranging from 10% to 100%. For this study, a gene target was considered successfully sequenced when the gene had an average read depth of at least 10x and ≥95% coverage of the target length.

Outcomes

The primary outcome of our study was the proportion of patients with leprosy (ie, new cases, patients with relapses or reinfections, patients who received SDDR-PEP, or patients who lived in villages where PEP was distributed) who were infected with *M leprae* with a drug resistant mutation for rifampicin, fluoroquinolone, or dapsone in the Comoros.

Statistical analysis

As recommended in WHO's guidelines for surveillance of antimicrobial resistance in patients with leprosy, for countries with no local baseline data for resistance, the sample size aims to cover at least 10% of the total multibacillary cases detected.¹⁸

The differences in characteristics between the patients who were selected for Deeplex Myc-Lep and other recruited patients with leprosy was evaluated using sample rate ratio calculations. Differences in time of treatment at the timepoint of sampling, differences in preservation time of biopsies in Disolol before extraction, and differences in bacterial load were calculated according to the sequencing success of the targets with the non-parametric Kruskal-Wallis test. The alternative hypothesis, stating significant differences between variables, was accepted at a significance level of $p=0.05$. All analyses were conducted with R version 4.1.2.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

On the islands of Anjouan and Mohéli, 1199 patients with leprosy were recruited between July 1, 2017, and Dec 31, 2020, (table) of whom 1030 (86%) provided a skin biopsy. Over a quarter (325 [27%]) of the patients were identified passively, and 439 (50% of the remaining 874 patients) were identified by door-to-door screening. All environmental and analytical controls included throughout the entire study assured the reliability of the obtained results. The median duration of Disolol preservation was 4 months (IQR 4), which did not affect the sequencing success of the targets ($p=0.69$). Three-quarters (755 [73.3%]) of 1030 patients) of the biopsies were confirmed to contain *M leprae* by RLEP qPCR. Of these, 260 (34.4%) were selected to be processed with the Deeplex Myc-Lep on the basis of their estimated bacterial load and treatment status (figures 1, 2). The median age of patients included in this drug resistance analysis was 22 years (range 4–95 years; IQR 21), 188 (72%) of the 260 patients were men and 72 (28%) were women (table).

Patients who were identified through passive case finding, men, and patients with multibacillary leprosy were more likely to have a higher bacterial load than

patients who were identified through active case finding, women, and patients with paucibacillary leprosy. Patients who were younger than 15 years had lower bacterial loads and were therefore under-represented in our study (table). Among 260 patients included, 104 (40%) had started multidrug therapy at the time of sampling (median time of 1 month ago; IQR 2·5), which did not influence the sequencing success of the targets ($p=0\cdot57$). Of these 104 patients, four (3·8%) had relapses or reinfections and 17 (16·3%) lived in villages where SDR-PEP was distributed to contacts of patients with leprosy in 2015. However, these patients did not receive SDR-PEP themselves at that time (figure 2). Among the patients who were not under treatment at the timepoint of sampling (156 [60%] of 260 patients), five (3·2%) patients had relapsed or had a reinfection, one (0·6%) had received SDDR-PEP in 2019 (1 year before the patient was diagnosed with leprosy), and 25 (16·0%) were identified in villages that had received PEP in 2015, of which one (4·0%) patient had received SDR-PEP that year (figure 2).

Of the 260 Deeplexed samples, all seven drug resistance targets were successfully sequenced in 216 samples (19 paucibacillary samples and 197 multibacillary samples), 39 samples (six paucibacillary samples and 33 multibacillary samples) had incomplete results, and five (one paucibacillary sample and four multibacillary samples) had no results. The bacterial load was significantly higher in the successfully sequenced samples ($p<0\cdot0001$) than in samples with an incomplete sequence result or no sequence result. The mean read depth for the failed group of samples was 1·9 reads (SD 1·1), compared with a mean read depth of 3190·8 reads (2018·3) for all 260 samples.

For rifampicin resistance surveillance, data for *rpoB* was available for 255 patients (247 new patients and eight who had been previously treated with multidrug therapy). Among these, 45 patients resided in villages where SDR-PEP and SDDR-PEP was distributed in 2015 or 2019, of whom one received SDR-PEP in 2015 and another received SDDR-PEP in 2019 (figure 2). None of the 255 patients had a mutation in *rpoB*, even not as minority bacillary populations (with an average *rpoB* read depth of $\geq 40\times$ for all samples). For 251 patients (243 patients who were newly diagnosed with leprosy and eight patients who had been previously treated) an interpretable result was available for both *ctpC* and *ctpI* (with an average read depth of $\geq 40\times$ read depth for both). Among these, 42 patients resided in villages where SDR-PEP and SDDR-PEP was distributed in 2015 or 2019, of which one received SDR-PEP in 2015 and another SDDR-PEP in 2019 (figure 2). None of these 251 patients had a mutation in *ctpC* or *ctpI*.

For dapson, *folP1* was successfully sequenced in 248 samples (240 who were newly diagnosed with leprosy and eight who had been previously treated), all of whom were wild type. For fluoroquinolones, *gyrA* was successfully sequenced in 253 samples (245 who were

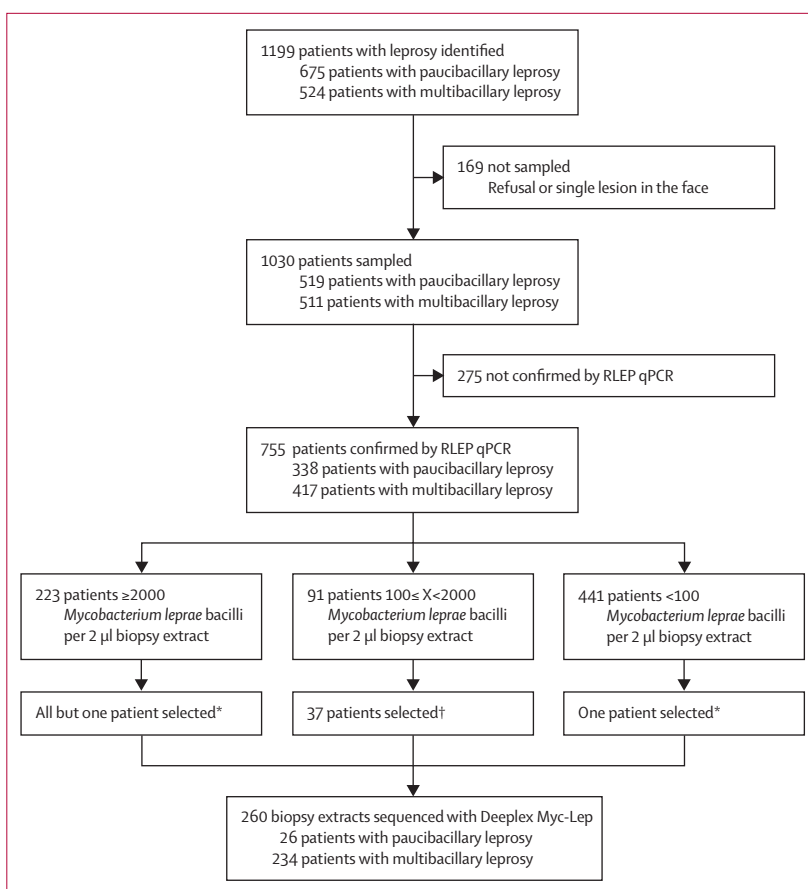


Figure 1: Flowchart for samples that were processed with Deeplex Myc-Lep, 2017–20
qPCR=quantitative PCR. RLEP=*M leprae*-specific repetitive element. *One high bacterial load sample was erroneously not processed with Deeplex Myc-Lep, and one sample with low bacterial load was processed with the Deeplex Myc-Lep. †12 patients were selected because they lived in villages where post-exposure prophylaxis was distributed to contacts of patients with leprosy in 2015 and 25 were selected to represent the scale of 100–2000 *M leprae* bacilli per 2 µl biopsy extracts.

newly diagnosed with leprosy and eight who had been previously treated), all of whom were wild type. *gyrB* was successfully sequenced in 230 samples (222 who were newly diagnosed with leprosy and eight who had been previously treated). Seven (3·0%) of 230 patients had a fixed non-synonymous uncharacterised SNP (Asp521Tyr) in *gyrB*. According to the results of the Protein Variation Effect Analyzer (PROVEAN) server, which provides a score that predicts the potential deleterious or non-deleterious effect of a mutation on protein biological function,¹⁹ this substitution is predicted to affect the gyrase's function. However, modelling done as previously described²⁰ indicated that the Asp521Tyr change is unlikely to affect susceptibility to fluoroquinolones as Asp521 is located in a loop of the Torpim domain, away from the drug binding pocket, and does not interact with other residues in the three-door closed conformation. *nth* was successfully sequenced in 216 samples (209 who were newly diagnosed with leprosy and seven who had been previously treated), none of which had an SNP in *nth*.

For more on PROVEAN see
<http://provean.jcvi.org/>

156 patients not receiving multidrug therapy at timepoint of sampling		104 patients receiving multidrug therapy at timepoint of sampling	
25	Patients living in villages where SDR-PEP was distributed in 2015	17	
1	Received SDR-PEP in 2015	0	
2	Patients living in villages where SDDR-PEP was distributed in 2019 before leprosy diagnosis		83
1	Received SDDR-PEP the calendar year before they developed symptoms within the PEOPLE study	0	
5	Patients who had relapsed or who had reinfection*	4	
122			

Figure 2: Selected samples presented according to treatment status

SDDR-PEP=single (double) dose rifampicin post-exposure prophylaxis (20 mg/kg). SDR-PEP=single-dose rifampicin post-exposure prophylaxis (10 mg/kg). *The terms relapse and reinfection are used interchangeably in this study. This was because it was impossible to distinguish between relapse and reinfection.

Discussion

Our findings show that there was an absence of any resistance to anti-leprosy drugs in patients positive for *M leprae* in the leprosy-endemic Comorian islands of Anjouan and Mohéli. Using Deeplex Myc-Lep, a novel and comprehensive tNGS approach, allowed us to exclude even the earliest signs of resistance to rifampicin and other leprosy drugs.

No mutations associated with rifampicin resistance were detected in *rpoB*, *ctpC*, or *ctpI*, not even as smaller subpopulations (heteroresistance), which are usually not detectable by classical Sanger sequencing. In other countries where leprosy is endemic, such as Brazil, China, Colombia, Guinea, India, Myanmar, and Philippines, rifampicin resistance was identified in 0.8–24.3% of patients.^{4,21,22} Differences in resistance rates across countries could be explained by use of supervised treatment versus self-treatment. In the Comoros, patients with leprosy were followed up every 1–2 weeks by a health worker, and at a minimum once monthly by the national leprosy control team, who supervised drug intake. Also, the low use of clofazimine in Brazil has been hypothesised to have contributed to higher rates of drug resistance in this country.

Similarly, no mutations were found in *folP1*, *gyrA*, and *gyrB*, including in patients who were already taking multidrug therapy and in patients who had relapsed or who had reinfection. These encouraging findings contrast with the globally reported resistance rate to dapsons of 5.3% in 2015–19, mainly in Brazil, China, India, Japan, and Viet nam.^{21,23,24} The global rate of resistance for fluoroquinolones in patients with leprosy was 1.3%.

Primary fluoroquinolone²³ resistance has been detected in Brazil, China, and India,²³ and is possibly associated with the use of fluoroquinolones to treat other bacterial infections. Finally, no mutations were found in the *nth* excision repair gene target, providing evidence that hypermutator strains, which are thought to be more prone to resistance acquisition, do not circulate in the Comoros.

The main SNP subtype from the Comoros was 1D, confirming findings by Avanzi and colleagues²² who, in 2020, published three genomes from the Comoros that belonged to 1D-Malagasy, in which no drug resistance associated mutations were found. Ofloxacin resistance was found in some strains from Madagascar. Although we did not subtype within the 1D SNP subtype, we expected a predominance of 1D-Malagasy.²²

In two patients who had previously received SDR-PEP or SDDR-PEP and developed leprosy afterwards, no rifampicin-resistance-related mutations were found. Although larger series are needed to confirm this finding, these preliminary data suggest that SDR-PEP or SDDR-PEP does not appear to select for rifampicin resistance in leprosy. Our results also provide evidence to show that experimental leprosy treatments that were used between 1981 and 1993 in the Comoros^{22,25–27} have not selected for drug resistance in the Comoros. Also, tuberculosis control might be jeopardised if SDR-PEP or SDDR-PEP could select for rifampicin resistance in *Mycobacterium tuberculosis*, although, during the study period, no mutations in *rpoB* were found by GeneXpert (Buckinghamshire, UK) in any of the 146 patients with tuberculosis (National Leprosy and Tuberculosis Program, personal communication).

The diagnosis of relapse in patients with multibacillary leprosy was complicated by the lengthy persistence of bacilli in slit skin smears and the slow resolution of clinical signs. Moreover, new onset symptoms could be due to leprosy reactions rather than relapse.²⁷ Although the absence of resistance in this treatment-exposed group was encouraging, biomarkers to confirm cure would greatly help the clinical management of such patients.

The fixed, non-synonymous, and uncharacterised SNP (Asp521Tyr) in *gyrB* that was detected in seven samples lies outside the known fluoroquinolone resistance determining region.²⁸ Modelling data indicated that the effect of Asp521Tyr on fluoroquinolone susceptibility is highly unlikely.²⁰ All but one of the seven patients harbouring this mutation were from two neighbouring villages in Anjouan. Moreover, these samples shared an identical variable-number tandem-repeat genotype, which was distinct from those of all other deep-sequenced samples. In addition, the same SNP was found in two of three Comorian strains that have been whole-genome sequenced in another study done in 2020.²² Taken together, these observations suggest that this *gyrB*, Asp521Tyr, is probably a phylogenetic marker of a particular *M leprae* clone circulating in the Comoros, and is unrelated to fluoroquinolone resistance.

Monitoring drug resistant leprosy remains a challenge in many countries where leprosy is abundant, as the tools or infrastructure are often inaccessible. A strength of our study is that it is, to the best of our knowledge, the first nationwide survey study to use tNGS deep sequencing on skin biopsies from patients with leprosy, which was applied on DNA extracted from Disolol-preserved biopsies transported and stored at ambient temperatures for months. As such, no cold chain was needed. Moreover, the Deeplex Myc-Lep limit of detection of 10% mutant population enabled an early warning system for the emergence of drug resistance.

However, this study also has some limitations. We restricted our analysis to patients with high bacterial burdens. Although there is no evidence that the prevalence of drug resistance differs between patients with a high-bacteria burden and a low-bacteria burden, the selection of resistant mutants could occur more readily in patients with a high burden. Our study involved only two patients who had themselves received SDR-PEP or SDDR-PEP, and the absence of any signs of resistance in their biopsies does not yet prove that SDR-PEP or SDDR-PEP cannot select for resistance. This issue requires an evaluation of a larger number of patients who have been previously exposed to PEP. Future studies could include tNGS analysis of *rpoB* (and other anti-tuberculosis drug resistance-associated targets) in patients with tuberculosis¹³ in settings where SDR-PEP is provided to contacts of patients with leprosy.

In conclusion, in this nationwide survey of leprosy drug resistance relying entirely on tNGS directly from skin biopsies, we found full susceptibility of *M leprae* to rifampicin, fluoroquinolones, and dapsone in patients with leprosy in the Comoros. These encouraging findings exclude drug resistance as a cause of the persistently high leprosy incidence and support the leprosy control efforts in place in the Comoros, including timely diagnosis, treatment, and follow-up of patients with leprosy. In addition, these preliminary data suggest that SDR-PEP and SDDR-PEP did not lead to the emergence of drug-resistant leprosy. In the PEOPLE study, annual door-to-door screening of included villages is still ongoing. Beyond the villages involved in the PEOPLE study, the control programme in the Comoros organises active skin camps, and conducts monthly follow-up of existing patients and their contacts. This programme and the PEOPLE study will allow continued surveillance for treatment outcome and for the detection of emerging drug resistance. Our approach, which used tNGS, was innovative and could detect the emergence of drug resistance at an early stage. Moreover, the drug resistance-testing and genotype-testing features of the assay are attractive for comprehensive surveillance in settings such as Brazil and India, where drug resistant *M leprae* has been shown to be transmitted.^{2,3,23} Use of this assay will also help to select effective treatment for

patients with multidrug-resistant leprosy, thereby curbing its transmission. Our results also show that Deeplex Myc-Lep worked well on Disolol-preserved samples, facilitating surveillance in regions where fast sample transport with adequate cold chains is challenging.

Contributors

SMB, AJ, YA, AM, PNS, EH, PS, and BCdJ designed the study. SMB, AJ, MVD-L, EL, and AB participated in the enrolment of patients and data collection. SMB, EH, LR, and BCdJ had access to all data. SMB, AJ, and AA analysed the data. EH, PS, LR, and BCdJ critically revised the manuscript. SMB, BCdJ, and EH verified the data. All authors contributed to the writing of the manuscript and approved the final version.

Declaration of interests

AJ and EL are employees of Genoscreen, who were involved in developing the Deeplex Myc-Lep. PS reports consultancy fees from Genoscreen. All other authors declare no competing interests.

Data sharing

All relevant data are within the manuscript. The data underlying the findings of this study are retained at the Institute of Tropical Medicine (Antwerp, Belgium) and will not be made openly accessible because of ethical and privacy concerns. Data can, however, be made available after approval of a reasonable request to the Institute of Tropical Medicine (ITMresearchdataaccess@itg.be).

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