Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Department of Biomedical Sciences

Using novel molecular approaches to understand transmission of *Mycobacterium leprae* in the Comoros.

Het in kaart brengen van de transmissie van *Mycobacterium leprae* in de Comoren met behulp van innovatieve moleculaire technieken.

Thesis for the degree of Doctor in Biomedical Sciences at the University of Antwerp to be defended by

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Contents

Abbreviations

95%CI	95% Confidence interval
AFB	acid-fast bacilli
AG	arabinogalactan
AIC	akaike information criterion
ATP	adenosine triphosphate
AUC	area under the curve
BB	borderline borderline leprosy
BCG	bacillus Calmette–Guérin
BE-PEOPLE	Bedaquiline Enhanced Post ExpOsure Prophylaxis for Leprosy (study)
BI	bacterial index
BL	borderline lepromatous leprosy
BL	bacterial load of sample
BLASTn	Basic Local Alignment Search Tool nucleotide
bp	basepair
BSA	bovine serum albumin
BT	borderline tuberculoid leprosy
CMI	cell-mediated immune
COMLEP	Improved Understanding of Ongoing Transmission of Leprosy in the Hyperendemic Comoros (study)
Cq	PCR cycle number at which your sample's reaction curve intersects the threshold line
CRP	C-reactive protein
ddPCR	digital droplet PCR
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
DPS	dapsone
DRDR	drug-resistance determining region
EDTA	ethylenediaminetetraacetic acid
ENL	erythema nodosum leprosum
ETS1	external transcribed spacer 1
FDA	U.S. Food and Drug Administration
FF	Fite-Faraco staining
FLA	fragment length analysis
FQ	fluoroquinolones
FWO	Fonds Wetenschappelijk Onderzoek Vlaanderen
HBL	high bacterial load
HHCs	household contacts
HLA	human leukocyte antigens
IgG	immunoglobulin G
IgM	immunoglobulin M

InDel	insertion or deletion
IPC	internal positive control
IQR	interquartile range
ITM	Institute of Tropical Medicine
LAM	lipoarabinomannan
LAMP	loop-mediated isothermal amplification
LCP	leprosy control programme
LEV	low elution volume
LFA	lateral flow assay
LID-1	leprosy IDRI diagnostic 1
LL	lepromatous leprosy
LM	lipomannan
LOD	limit of detection
LPA	line probe assay
LUMC	Leyden University Medical Center
M. leprae	*Mycobacterium leprae*
MB	multibacillary
MDT	multidrug therapy
MI	morphology index
MLVA	multi-locus VNTR analysis
MTBc	*Mycobacterium tuberculosis* complex
N.A.	not applicable
NA	not available
NC	non-coding
ND	not detected
NDO	disaccharide linked via an octyl carboxylic acid linker
NGS	next generation sequencing
NHDP	National Hansen's Disease Program
N_{neg}	number of negatives for the respective assay
N_{pos}	number of positives for the respective assay
NRC	nationel reference center
NSAIDs	nonsteroidal anti-inflammatory drugs
ODK	Open Data Kit
OR	odds ratio
PB	paucibacillary
PCR	polymerase chain reaction
PDIM	phthiocerol dimycocerosate
PEOPLE	Post ExpOsure Prophylaxis for LEprosy in the Comoros and Madagascar (study)
PEP	post-exposure prophylaxis
PGL	phenolic glycolipids
PGN	peptidoglycan
PROVEAN	Protein Variation Effect Analyzer
Q203	telacebec
qPCR	TaqMan quantitative PCR

R^2	the coefficient of determination
RFU	relative fluorescence units
RIF	rifampicin
RLEP	repetitive element *Mycobacterium leprae*
RLPM	repetitive element *Mycobacterium lepromatosis*
RNA	ribonucleic acid
ROC	receiver operating characteristic
ROM	rifampicin, ofloxacin and minocycline
RT-qPCR	reverse transcriptase qPCR
SB	skin biopsy
SD	standard deviation
SDDR	single double dose rifampicin
SDR	single dose rifampicin
SNP	single nucleotide polymorphism
SPC	sample processing control
SQ	starting quantity
SSS	slit-skin smear
TB	tuberculosis
TL	tuberculoid leprosy
TMM	trehalose mono-mycolate mycolic acids
tNGS	targeted NGS
TT	tuberculoid tuberculoid leprosy
UCP	up-converting phoshor
VNTR	variable number of tandem repeat
WHO	World Health Organization
ZN	Ziehl-Neelsen

Summary

This PhD thesis centers around the complex dynamics of leprosy, a debilitating disease caused by *Mycobacterium leprae (M. leprae)*. Despite being among the first human pathogens identified, the means by which it spreads among individuals remain enigmatic, complicating leprosy control even with available treatments. With an annual diagnosis of over 200,000 new cases worldwide and a significant proportion children, indicating the ongoing spread of the disease. Innovative approaches to leprosy control are essential to definitively halt transmission.

This study revisited transmission questions in an innovative manner. Initially, it examines the specificity of RLEP qPCR for *M. leprae*. Subsequently, it evaluated non-invasive, field-applicable tests to quantify bacterial levels in patients. Findings show that αPGL-I IgM levels in fingerstick blood correlate with bacterial load. Combining αPGL-I R-values ≥ 0.81 with a lesion count ≥25 predicts high bacterial load in a patient.

Pioneering the field, the study introduced targeted Next Generation Sequencing of *M. leprae* through the innovative Deeplex Myc-Lep assay with a detection limit of 80 M. leprae genomes. This approach enabled the first drug resistance survey of *M. leprae* in the Comoros, revealing no drug resistance. The Deeplex Myc-Lep also characterized *M. leprae* diversity in the Comoros, classifying distinct genotypes linked to patient residences, aiding in understanding transmission patterns. SNP subtypes detected are 1D-Malagasy and 1A. Among 265 patients with a full VNTR pattern, 79.7% cluster with at least one other patient based on identical VNTR profiles.

Additionally, the study explored if wild ticks from the Comoros carry *M. leprae* DNA, building on earlier evidence of their potential to transmit the bacterium. No *M. leprae* DNA is detected in these ticks, suggesting a limited role in leprosy transmission. These insights contribute to refining strategies for effective leprosy control, within the Comoros and beyond.

Samenvatting

Dit doctoraat draait om de complexe dynamiek van lepra, een slopende ziekte die wordt veroorzaakt door *Mycobacterium leprae*. Ondanks het feit dat lepra een van de eerste menselijke ziekteverwekkers was die werd geïdentificeerd, blijven de manieren waarop lepra zich onder individuen verspreidt raadselachtig, wat leprabestrijding bemoeilijkt, zelfs met de beschikbare behandelingen. Met een jaarlijkse diagnose van meer dan 200.000 nieuwe gevallen wereldwijd en een aanzienlijke proportie kinderen, duidt dit op de voortdurende verspreiding van de ziekte. Innovatieve benaderingen van leprabestrijding zijn essentieel om de overdracht definitief een te stoppen.

Deze studie herziet de transmissievraagstukken op een innovatieve manier. Eerst werd de specificiteit van RLEP qPCR voor *M. leprae* onderzocht. Vervolgens werden niet-invasieve, in het veld toepasbare testen geëvalueerd om bacteriële niveaus in patiënten te kwantificeren. De bevindingen tonen aan dat αPGL-I IgM-spiegels in bloed van vingerafdrukken correleren met bacteriële belasting. De combinatie van αPGL-I R-waarden ≥ 0,81 met een aantal laesies ≥ 25 voorspelt een hoge bacteriële lading bij een patiënt.

Dit onderzoek introduceerde als eerste in dit onderzoeksgebied gerichte Next Generation Sequencing van *M. leprae* door middel van de innovatieve Deeplex Myc-Lep assay met een detectielimiet van 80 M. leprae genomen. Deze aanpak maakte het eerste onderzoek naar geneesmiddelenresistentie van *M. leprae* in de Comoren mogelijk, waarbij geen geneesmiddelenresistentie werd aangetoond. De Deeplex Myc-Lep karakteriseert ook de diversiteit van *M. leprae* op de Comoren en classificeert verschillende genotypen die gekoppeld zijn aan de woonplaats van patiënten, wat helpt bij het begrijpen van transmissiepatronen. De gedetecteerde SNP-subtypen zijn 1D-Malagasy en 1A. Van de 265 patiënten met een volledig VNTR-patroon clustert 79,7% met ten minste één andere patiënt op basis van identieke VNTR-profielen.

Daarnaast onderzocht deze studie ook of wilde teken uit de Comoren drager zijn van *M. leprae* DNA, voortbouwend op eerder bewijs dat zij de bacterie kunnen overbrengen. Er werd geen *M. leprae* DNA gedetecteerd in deze teken, wat suggereert dat teken waarschijnlijk geen rol hebben in de overdracht van lepra op de Comoren. Deze inzichten dragen bij aan het verfijnen van strategieën voor effectieve leprabestrijding, op de Comoren en daarbuiten.

CHAPTER I
General introduction

1. Then and now: leprosy as a global public health burden.

No ailment is thought to have plagued mankind longer than leprosy. The disease was first described in an Egyptian papyrus document from between 1550 B.C. - 600 B.C. In India, evidence of leprosy was found in a 4,000-year-old skeleton [1].

Based on manuscripts from the ancient times, the disease spread by human migration out of Africa to India, to the Middle East and Europe, and subsequently from Europe to the Americas [2]. In medieval times, leprosy was endemic in Europe, where patients lived in confinement houses [3]. Leprosy is the first infectious disease for which the causative agent was discovered, in 1873 by Dr. Hansen, namely *Mycobacterium leprae* (*M. leprae*). The word leprosy was first used in a manual for nuns in the 13th century and since the nineteenth century, the disease is universally known as leprosy or Hansen's disease, of which the distribution in the late 19th century is shown in Fig. 1.1. Leprosy, a disease associated with poverty, has shown a gradual decline alongside increasing prosperity. Thus, poverty reduction has likely been (and is) a critical foundation for leprosy control.

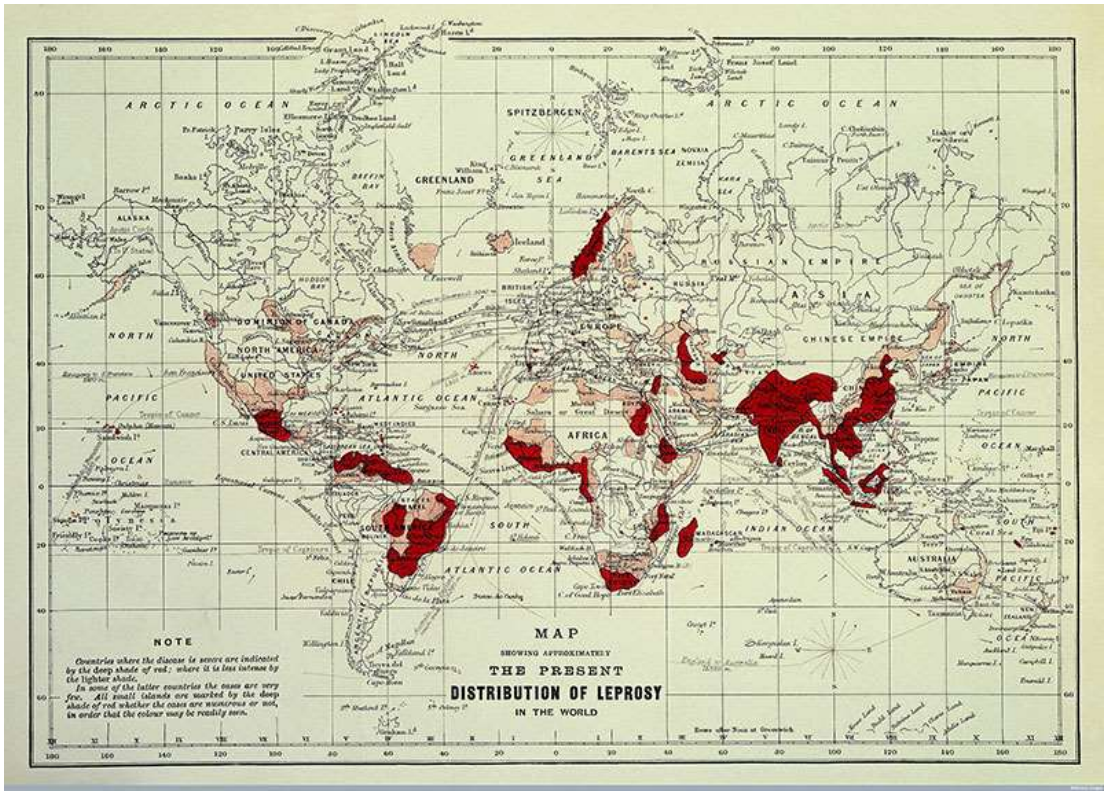


Figure 1.1: Global leprosy distribution in 1891. Map extracted from Leprosy by George Thin (1891) [4].

When the disease was no longer seen as a curse or sin, but an infectious disease, attempts at treatment changed. In the early 20th century, leprosy was treated with topical appliance or injections of oil from the chaulmoogra nut. In 1941, antibiotics, more in particular sulfone, were introduced as a treatment, followed by dapsone in the 1950s. As resistance developed against dapsone, the need for multidrug therapy (MDT) was recognized, which became available in the 1980s. The combination of rifampicin, clofazimine and dapsone is very effective, although

formal clinical trials were never conducted. Together with MDT, leprosy control programmes (LCP) were implemented in most endemic countries, providing free diagnosis, treatment and monitoring. This led to a dramatic decline (five million to 750,000) in leprosy prevalence from the eighties until 2000 (Fig. 1.2). Part of the decline was artificial, as the global leprosy burden was represented by the number of prevalent cases on treatment. A patient is no longer a prevalent leprosy case when cured after treatment, explaining the systematic drop in prevalence following the introduction of successful MDT that lasted 24 months and in 1998 was shortened to ≤12 months: patients get cured sooner and hence don't remain in the prevalence data >1 year. This declining trend does not coincide with a drop in new case detection, which has been quite stable until the year 2001 (Fig. 1.2), suggesting a relative steady rate of new cases developing the disease.

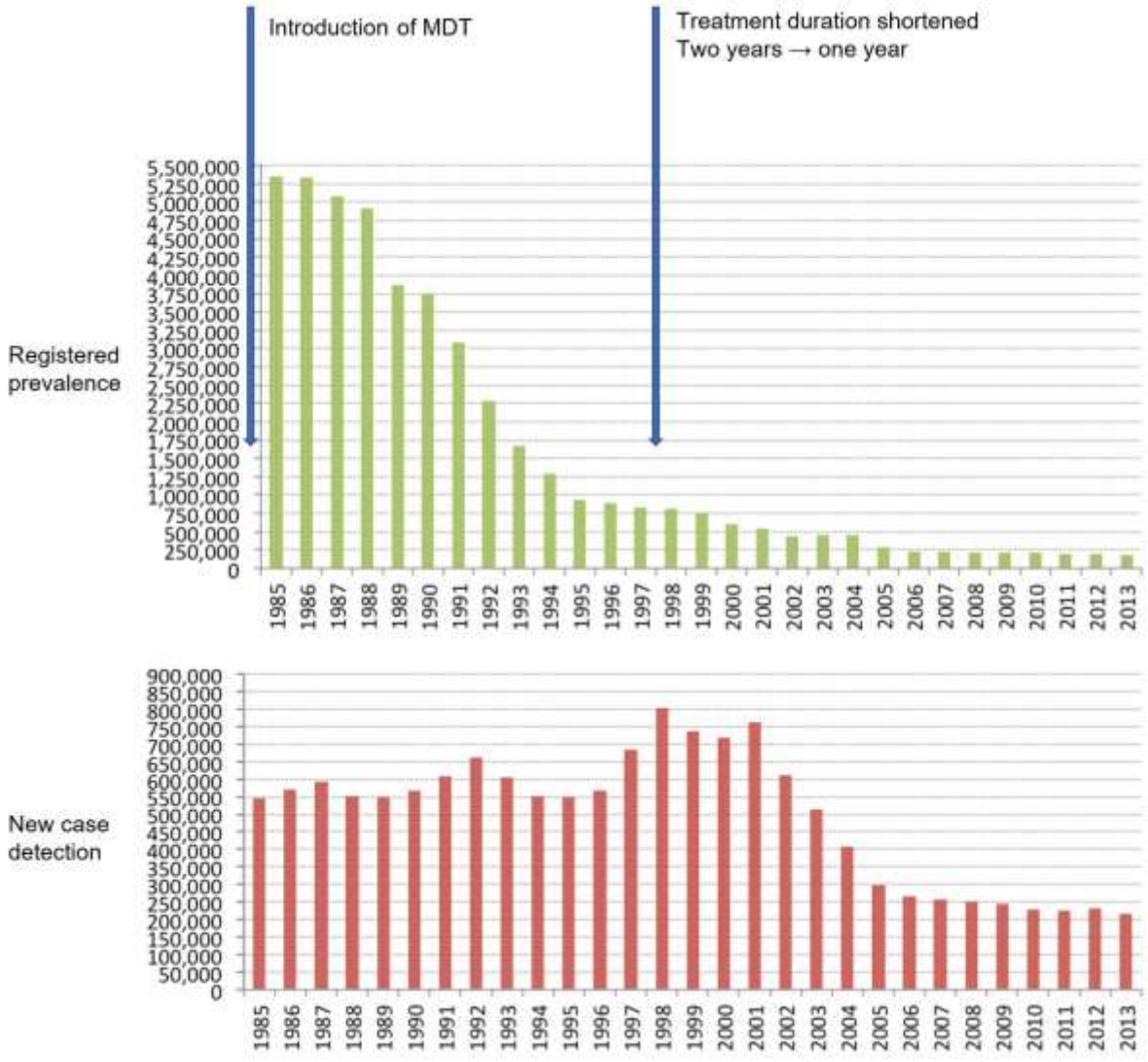


Figure 1.2: Global trends in registered prevalence (green) and new case detection (red) in leprosy from 1985 to 2013. Figure modified from The International Textbook of Leprosy (https://internationaltextbookofleprosy.org/)

The intensified efforts to eliminate leprosy in the last century, with almost 100% coverage of detected patients receiving MDT, led the World Health Organization (WHO) to call victory in 2000: as the global prevalence decreased to <1 patient per 10,000 population since the year 2000, leprosy was no longer considered to be a public health problem. From 2001 onwards, incident cases declined steeply, which may also be due to a decline in diagnostic efforts. Since 2006, the leprosy incidence has remained stable with approximately 200,000 new leprosy patients reported worldwide each year [5], often with heterogeneous distribution in high incidence 'pockets' (Fig. 1.3).

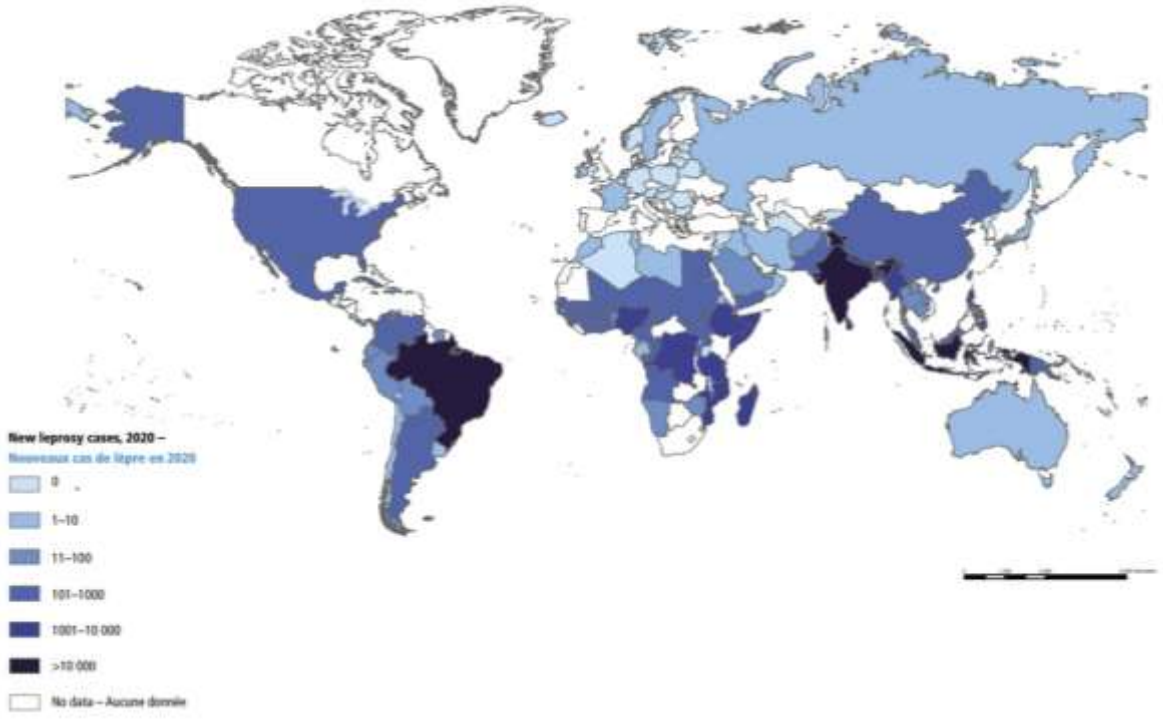


Figure 1.3: The current global situation of leprosy with regard to the new cases detected in 2020. (WHO REFERENCE NUMBER: WER No 36, 2021, 96, 421–444)

These persistently high incidence areas resemble the distribution of leprosy in the 19th century, except for high income countries where leprosy disappeared. A high incidence also persists in settings with a strong LCP (like the Comoros), where patients are diagnosed early and treated appropriately with highly effective treatment, before the characteristic disabilities have occurred. Moreover, in some regions 30% of the leprosy infections occur in children under 14 [6], which supports that transmission continues unabated.

2. Characteristics and reservoirs of the leprosy agents

Leprosy is a neglected tropical disease caused by two mycobacteria, *Mycobacterium leprae* and *Mycobacterium lepromatosis*. *M. leprae* triggers the classic spectrum of leprosy symptoms in humans (from the tuberculoid form with some hypopigmented patches to the lepromatous form with many patches and nodules), whereas patients infected with the much less common *M. lepromatosis* have to date only presented with lepromatous forms [7]. *M. leprae* infections

in humans are found worldwide while *M. lepromatosis* cases are mainly located in Mexico, the Caribbean region, and Central and South America, with sporadic cases in Asia [8]. In Mexico, pure *M. lepromatosis* infections as well as co-infections with *M. leprae* are reported, whereas elsewhere, *M. lepromatosis* is only reported as co-infection with *M. leprae* [7]. To date, no studies have investigated whether *M. lepromatosis* is partly responsible for leprosy endemics in the African continent. *M. leprae* naturally infects a wide-range of animal reservoirs such as armadillos [9], red squirrels [10] and chimpanzees [11]. *M. lepromatosis* was identified in red squirrels in the British Isles but no animal reservoir has been identified in the Americas to date [10]. In the US, leprosy is recognized as a zoonosis (nine-banded armadillo; 40% of the cases are related to zoonotic infection) with sporadic cases in the south-eastern part of the country while in other endemic countries, human-to-human transmission is regarded the main route of infection [8]. Ticks have recently been suggested as possible vector for *M. leprae* [12]. More specifically ticks belonging to the *Amblyomma* genus were identified as potential carriers for *M. leprae*, as in experimental environment the transovarial transmission and viability of *M. leprae* within female ticks and tick-derived cells was confirmed [13].

3. Pathogenicity

M. leprae and *M. lepromatosis* belong to the class Schizomycetes, order Actinomycetales, the family Mycobacteriaceae and the *Mycobacterium* genus.

M. leprae is a rod-shaped, non-motile and slightly bended bacterium, with a very thick cell wall with outer and inner layers (Fig. 1.4). The outermost layer, the capsule, is composed of specific phenolic glycolipids (PGLs) and a range of other lipids, like phthiocerol dimycocerosate (PDIM). The electron transparent zone is mainly composed of mycolic acids. The electron dense inner layer surrounding the plasma membrane comprises arabinogalactans and peptidoglycans. The cell wall also contains different lipid-linked polysaccharides going across different layers. *M. leprae* contains proportionally more mycolic acids than *M. tuberculosis*. These mycolic acids render the bacteria more resistant to chemical damage or dehydration, they provide a low permeability to hydrophobic antibiotic substances and give the bacteria the ability to survive inside of a phagosome.

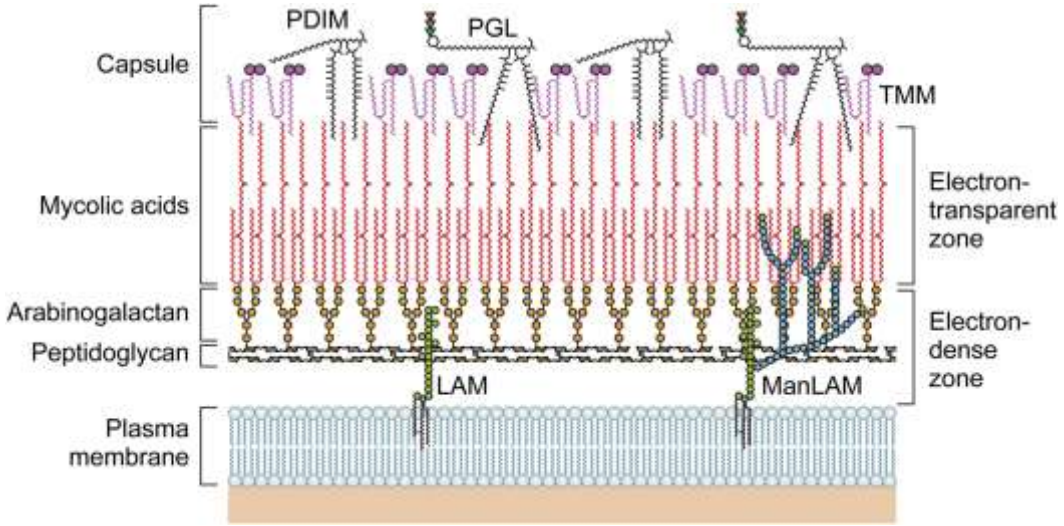


Figure 1.4: The structure of the M. leprae cell wall, consisting of an inner and outer layer that surround a plasma membrane. PGN: peptidoglycan; AG: arabinogalactan; LAM: lipoarabinomannan; LM: lipomannan; TMM: trehalose mono-mycolate mycolic acids; PDIM: phthiocerol dimycocerosate; PGL: phenolic glycolipids. [14]

The *M. leprae* bacillus is an obligate intracellular organism causing a chronic infection by invading macrophages in the skin and Schwann cells in peripheral nerves. The persistence of *M. leprae* infection depends on the type of the host immune response. Macrophages are crucial modulators of innate and adaptive immune responses and are the main cell types directly infected by the bacillus, which can lead to different immune responses, namely the humoral and cell-mediated. The initial interaction of the macrophage with *M. leprae* is essential for the polarization of the response toward a susceptible phenotype, favouring the survival of the bacilli. There are two types of macrophages (M1; pro-inflammatory, M2; anti-inflammatory), who differ in their surface markers, cytokine secretion and biological functions. *M. leprae* is being phagocytosed by the macrophage (Fig. 1.5A); the complex lipid PGL-1 & PDIM has shown to be implicated in the phagocytosis [14], and increasing infectivity by promoting host cell invasion [15]. Whether the macrophage is polarized towards a M1 or not, is potentially driven by the jagged 1 protein that has been observed in vascular endothelial cells of tuberculoid skin lesions [16]. M1 macrophages induce killing of the *Mycobacterium* by upregulation of the Th1-type response and subsequently initiating a predominant cell-mediated immune (CMI) response. Controversially, an abundance of M2 macrophages is observed in lepromatous lesions. M2 macrophages produce amongst others IL-10, which is known to suppress the Th1-response [17]. A recent study by Roy *et al.*(2020) investigated the transcriptome of *M. leprae* present in skin lesions of lepromatous patients, revealed that *M. leprae* transcribes an abundance of mRNA such as those implicated in the ESX-1 secretion system [18]. It has been observed that the ESX-1 secretion system is crucial for *M. leprae*'s cytosolic escape in the macrophages [19]. Schwann cells produce the myelin sheath around the axons of the peripheral nervous system. These axons with Schwann cells are surrounded by a basal lamina, which is been shown to be involved in the engulfment of *M. leprae*, and thereby the invasion of the Schwann cell (Fig. 1.5B). Laminin-2 isoform is one of the key components of the basal lamina,

specific to the Schwann cell, to which *M. leprae* preferentially binds [20]. Binding of *M. leprae* in the Schwann cells deregulates the communication of the axon-Schwann cell signalling, leading to breakdown of the myeline sheath. The bacterium is thought to reprogram the Schwann cells to the progenitor/stem cell stage to promote the spread of the infection. This can be done via two mechanisms. Firstly, through differentiation into mesenchymal tissues, skeletal muscles, and smooth muscles. Secondly, by inducing the formation of granuloma-like structures that later release macrophages containing bacteria [14].

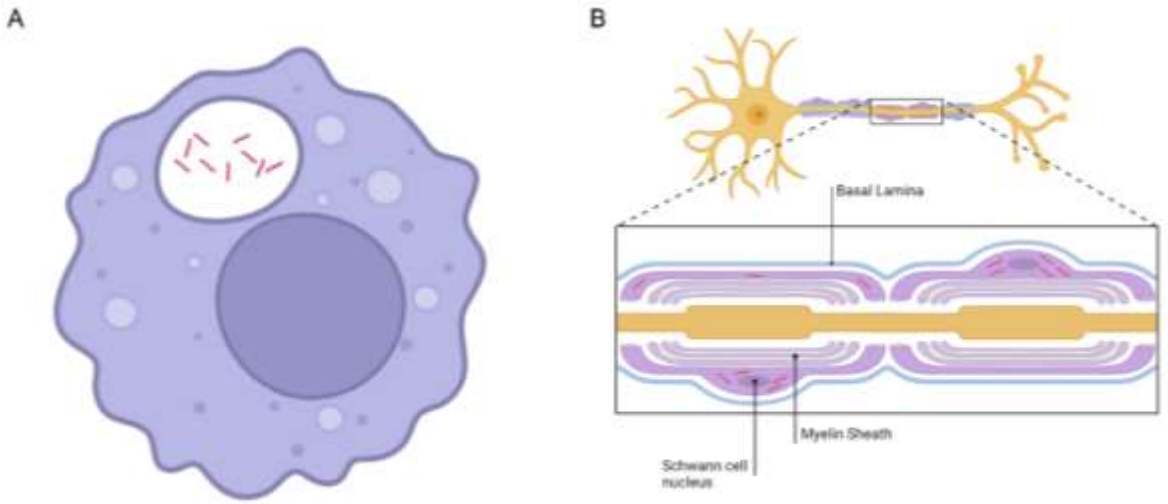


Figure 1.5: (a) M. leprae infected macrophages. (b) M. leprae infected Schwann cell. Created with BioRender.com.

M. leprae reproduces best at temperatures between 27°C and 30°C, explaining its predilection for skin, peripheral nerves, eyes and the mucosa of the upper respiratory tract. The bacillus regenerates very slowly (12-14 days) and therefore the clinical incubation time is 5 years on average but can go up to 20 years.

4. Transmission of Mycobacterium leprae and Mycobacterium lepromatosis

We still lack full understanding of transmission pathways of *M. leprae*, partly due the long incubation time for the disease and due to the inability to grow the bacterium *in vitro.* Based on the available evidence, possible transmission routes include skin-to-skin contact, aerosols/droplets to and from the nasal and oral cavities, and shedding of bacteria into the environment followed by inhalation [21-24]. Indeed, the upper respiratory tract is considered the main portal for entry and exit of *M. leprae* [22-24]. Contacts of index patients with a higher bacillary load (as determined from the earlobes with microscopy) have a higher leprosy incidence [25], although many new patients cannot be linked to an index case among their household contacts (HHCs). Untreated multibacillary (MB) leprosy patients (see 5.2) are often considered the main source of transmission, starting months to years before they are diagnosed, and probably before they develop symptoms. Once a leprosy patient starts MDT, it is assumed that transmission risk is drastically reduced, however no convincing evidence exists given the steady number of new cases detected yearly [26].

5. Clinical diagnosis

Current WHO guidelines base the diagnosis of leprosy entirely on clinical signs and symptoms, with limited microbiological confirmation. The diagnosis of leprosy relies on at least one of three cardinal signs, (i) definite loss of sensation in a pale (hypopigmented) or reddish skin patch; (ii) thickened or enlarged peripheral nerve with loss of sensation and/or weakness of the muscles supplied by that nerve; or (iii) presence of acid-fast bacilli in a slit-skin smear (SSS)(see 9.1) [27].

5.1 Clinical features of leprosy

5.1.1 Clinical features of the affected skin

Skin lesions most frequently identified in leprosy patients are de- or hypopigmented macules (<0.5cm diameter) and/or patches (>0.5cm diameter). Patients may also present with plaques – which are raised lesions (>0.5cm diameter) –, and nodules – which are raised palpable lesions (0.5cm-5cm diameter) (Fig. 1.6).

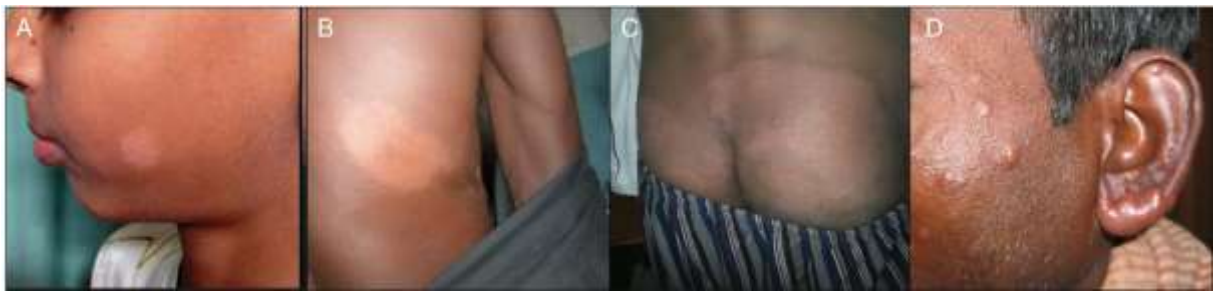


Figure 1.6: Some examples of leprosy skin lesions: (A) Macule. (B) Patch. (C) Plaque.(D) Nodules. Source : Leprosy Masquerading as Systemic Rheumatic Diseases. J Clin Rheumatol, 2016. 22(5)

5.1.2 Clinical features of the affected nerves

Enlarged (thickened) peripheral nerves are one of the cardinal signs for clinical leprosy diagnosis, caused by the bacterial invasion and subsequent inflammation of that nerve. The nerves most frequently affected are the ulnar and posterior tibial nerves. Also the greater auricular, radial, median, and lateral popliteal nerves can be affected (Fig. 1.7).

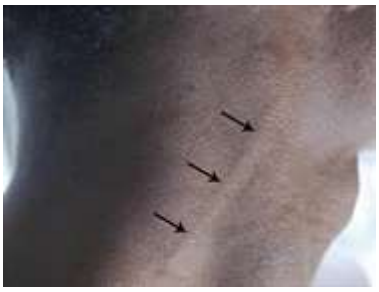


Figure 1.7: Thickened greater auricular nerve in a leprosy patient [28]. Source : Leprosy Masquerading as Systemic Rheumatic Diseases. J Clin Rheumatol, 2016. 22(5)

Nerve involvement can lead to sensory, motor and autonomic function impairment. For sensory dysfunction first heat sensation, followed by fine touch, and eventually pain sensations are affected. Motor weakness may occur gradually and slow, or sudden. Autonomic nerve

impairment will result in anhidrosis, which is the lack of sweating resulting in a very dry skin, which then can easily lead to cracks and ulceration in the skin. Scaling and itching do not occur in leprosy lesions, and rather point to other aetiologies, such as fungal dermatitis.

In tuberculoid- and borderline leprosy muscle weakness occurs more gradually and can manifest before the anaesthetic feeling in the skin lesions. On the lepromatous side of the Ridley Jopling-classification (see 5.2), nerves are commonly affected at a later stage of disease, and symptoms more generalized, with weakness in hands, feet and face. A skin lesion overlaying a nerve or nerve trunk, increases the chance of nerve impairment.

5.1.3 Clinical features of the nose of leprosy patients

The mucosa of the nose can be infiltrated with bacilli, leading to a stuffy nose. If not treated, involvement of the nasal cartilage/bone structure can result in a saddle nose, one of the widely recognized clinical characteristics of leprosy.

5.1.4 Ophthalmological features

Lagophthalmos, corneal ulceration, and uveitis are potential eye complications of leprosy. Lagophthalmos can be caused by facial nerve damage, while trigeminal nerve damage can cause anaesthesia and dryness at the level of the cornea and conjunctiva. Either of these complications increase the risk of corneal ulceration. The bacilli are also able to invade the iris and ciliary body.

5.2 Classification of leprosy patients

Classification of leprosy patients is important to initiate the optimal treatment duration for the patient, to predict the risk of complications and reactions, and to estimate the probability of transmission to contacts. The WHO- and the Ridley-Jopling classifications are the most widely used (Fig. 1.8).

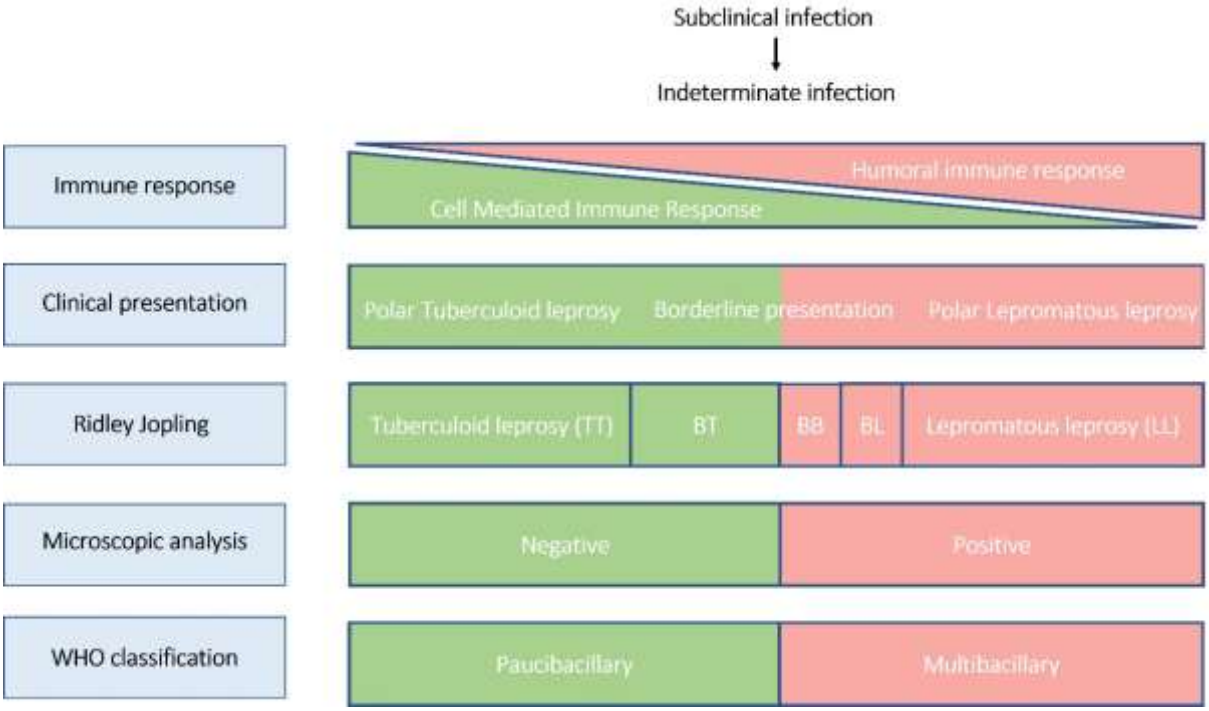


Figure 1.8: Classifications in relation to the immune response and microscopic analysis. BT: Borderline tuberculoid leprosy; BB: Borderline borderline leprosy; BL: Borderline Lepromatous leprosy

5.2.1 WHO classification: Multibacillary and paucibacillary

The WHO classification is more simple than the Ridley-Jopling, and based on counting lesions: 1-5 lesions for paucibacillary (PB) and more than five lesions for multibacillary (MB) leprosy [27]. This classification is quick, low cost and can be performed worldwide. In addition, a slit skin (tissue/dermal fluid) smear microscopic examination can detect the presence of *M. leprae* bacilli (limit of detection: 10^4 bacilli per gram tissue [29]), which automatically classifies a patient as having multibacillary leprosy regardless of the number of skin lesions. An untreated multibacillary patient can harbour more than 10^{11} bacilli.

5.2.2 Ridley-Jopling Classification

In addition to the clinical features, the Ridley-Jopling classification takes into account the histopathology (bacillary load and the involvement of CMI) and the bacterial load. This classification better captures the complexity of the pathogenesis, with five groups covering the spectrum from tuberculoid leprosy (TL), with a predominant Th1 CMI response profile and IFN-γ production, to the lepromatous leprosy (LL), with a Th2 type (B cell) response and high titres of anti-*M. leprae* antibodies [30]. The most abundant antibodies are made against phenolglycolipid I (PGL-I), ineffective at controlling this intracellular disease. In between, patients are classified as borderline leprosy forms from borderline tuberculoid (BT), borderline borderline (BB), to borderline lepromatous (BL).

Patients presenting with TT, have a very strong CMI, and by consequence only have one or two lesions that occur on one side of the body. Histopathology reveals no or few bacilli in the well-organized epithelioid granuloma. Polar TT leprosy can heal spontaneously [31]. The BT form is

characterized by a decreased CMI compared to TT, and more lesions than TT but still few and on one side of the body, and no or few bacilli are found in the epithelioid granuloma.

In BB patients the CMI is lower than in TT/BT. This unstable immunological state either progresses towards the tuberculoid- or the lepromatous pole of borderline leprosy. BB leprosy is characterized by a donut-like (punched-out) lesion, a plaque with hypopigmentation in the centre and raised borders. The lesions appear more symmetrically than in TT/BT. In histopathology, some bacilli can be identified and granulomas are formed with both epithelioid cells and macrophages.

At the lepromatous side of the spectrum, the CMI decreases progressively, while the humoral immune response predominates. BL is characterized by numerous lesions that may be symmetrical and widespread over the body, in the form of macules, plaques, papules and nodules. A lot of bacilli together with macrophage granulomas can be identified on histopathology. In this form nerve hypertrophy and/or neuritis is common. Lepromatous Leprosy (LL) has a very strong humoral immune response, which is ineffective to fight the leprosy bacilli growing intracellularly. LL therefore has lesions that are distributed over the entire body, in a symmetrical way. Due to the poorly organized immune response in LL, the histopathology reveals many foamy histiocytes and a very high bacterial load (bacillary index (BI): 5-6) that can be easily demonstrated.

Patients with indeterminate leprosy present with a solitary lesion. This form does not fit into this classification because this form presents early on in the disease process, before CMI involvement. Patients with intermediate leprosy might spontaneously heal or progress to any of the borderline forms on the spectrum.

5.2.3 Pure neural leprosy

Pure neural leprosy cannot be classified into the Ridley-Jopling spectrum or according to the WHO classification. This form is characterized by nerve involvement only, without dermatological signs. This form may be confirmed by the identification of bacilli in a nerve biopsy, and then the patient should be treated as MB. However, there is no consensus on PB or MB MDT for pure neural leprosy without a nerve biopsy and no WHO guidelines regarding treatment are available.

6. Treatment for leprosy

The first drug found to be effective against leprosy was promine, a sulfone used in the 1940's [32]. Its parent drug, dapsone, was demonstrated to be effective as well. Dapsone was used as monotherapy (lifelong) first as subcutaneous injection and later as daily oral treatment in the 50s and 60s, resulting in drug resistance (both primary (patient doesn't respond to treatment) and secondary (patient responds to treatment but develops resistance) in the 60s. From then on clofazimine was used as monotherapy throughout the mid- 70s. To date only two cases of clofazimine resistance have been reported [33, 34], since the mechanism of action remains poorly understood, the genetic foundation underlying clofazimine resistance remain unidentified. The gene rv0678, which is responsible for the efflux pump that can lead to

resistance to clofazimine in tuberculosis, is absent in *M. leprae* as it lacks an orthologous gene. In 1981, WHO proposed a multidrug treatment regimen, to prevent resistance form arising. No controlled clinical trials were performed to assess the efficacy of the regimen. Indeed, designing a trial for leprosy is complex as end-points are challenging; the BI reveals the presence of dead bacilli for years after successful treatment.

6.1 Multidrug therapy

Until 2018, for the tuberculoid (or paucibacillary) forms, the combination of dapsone and rifampicin was recommended while for the multibacillary forms clofazimine was added. The treatment duration was also shortened to 6 months for PB and 12 months for MB, which was beneficial for the treatment adherence and to prevent drug resistance emergence. As of 2018, MDT drug combination is the same for PB as for MB. MDT is highly effective and has few side effects. It is prepared in strips with pills for one month, for ease of self-administration.

Table 1.1: Treatment scheme for leprosy patients [27]

Monthly, supervised		Daily, self-administered		Treatment duration	
Adult (≥15yo)	Child (10-14yo)	Adult (≥15yo)	Child (10-14 yo)	Paucibacillary	Multibacillary
Rifampicin 600 mg, Clofazimine 300 mg	Rifampicin 450 mg, Clofazimine 150 mg	Clofazimine 50 mg, Dapsone 100 mg	Clofazimine 50 mg (alternate days), Dapsone 100 mg	6 months	12 months

Dapsone acts by hindering nucleic acid synthesis by inhibiting the dihydrofolic acid synthesis. It is a bacteriostatic drug, however when 100mg is administered daily it has a weakly bactericidal effect. Rare side effects of dapsone are anaemia and haemolysis, and even more rarely the dapsone hypersensitivity syndrome may occur, a severe and potentially deadly complication.

Clofazimine's mechanism of action against *M. leprae* is not entirely known. It is a bacteriostatic drug, and slowly bactericidal. The most common side effects of clofazimine are hyperpigmentation and dryness of the skin. Hyperpigmentation may be considered undesirable, which may lead to poor treatment adherence. Enteropathy may occur as well, caused by clofazimine crystal deposits in the intestines, which clinically presents as vomiting and abdominal pain. Clofazimine is also thought to be protective against the development of a type II hypersensitivity reaction (see point 7.2), however strong evidence is lacking.

Rifampicin, the core drug of MDT, inhibits the DNA-dependent RNA polymerase, and is the only bactericidal drug. This drug kills a lot of bacteria at once and therefore renders a person non-infectious in a few days. It should be well explained that transient harmless body fluid discoloration (orange) may occur. Hepatotoxicity is uncommon.

6.2 Alternative multidrug therapy

Rifampicin is the core drug in MDT, and lack of adherence to dapsone and/or clofazimine due to their side effects creates a risk for emergence of rifampicin resistance. Given the success of MDT, the adverse events mainly caused by dapsone and clofazimine rarely require use of other drugs. Other drugs such as ofloxacin, minocycline, rifapentine, moxifloxacin, bedaquiline, and telacebec have a more bactericidal effect. Kumar *et al* (2015) tested 6 months of rifampicin, ofloxacin and minocycline (ROM-6) against standard PB-MDT in almost 268 PB patients and found that less adverse events were recorded in ROM in comparison to MDT [35]. The cure rate at 2 years was 99% in the ROM-6 and 97% in the PB-MDT group, resulting in a comparable cure and relapse rate. Lockwood *et al.* (2022) recommended 12 monthly doses of ROM to be tested in a large clinical trial [36].

6.3 Post exposure prophylaxis

One of the interventions that is currently advocated to curb transmission of leprosy is post-exposure prophylaxis (PEP). The WHO recommends using single-dose rifampicin (SDR) as chemoprophylaxis, mainly based on two large field studies: a study in Indonesia (2000) comparing a blanket and contact approach on different islands, and a clustered randomized control trial (COLEP trial) in Bangladesh. The study in Indonesia revealed that a blanket approach was preferred. In the COLEP trial (2002-2007) in which SDR was given to close contacts of newly identified leprosy patients in the intervention arm, a 57% reduction in leprosy incidence (in comparison to the non-intervention arm) was detected two years after the intervention. SDR was found to be the most effective in non-blood related contacts [37]. For blood-related close contacts and close contacts of MB patients, a stronger PEP regimen should be sought. The MALTALEP trial (2012-2017) in Bangladesh evaluated the effectiveness of adding SDR after immunization with the bacillus Calmette–Guérin (BCG) vaccin (See 6.4). This study observed a 42% reduction in PB leprosy incidence among close contacts of MB patients who had received SDR 8-12 weeks after BCG vaccination, in comparison to BCG vaccination alone. The LPEP trial (2015-2019) in India, Indonesia, Myanmar, Nepal, Sri Lanka, Tanzania, Brazil and Cambodia was designed to evaluate the feasibility, effectiveness and impact of contact tracing and SDR-PEP combined. This trial did reveal that SDR-PEP is generally well-accepted by contacts of leprosy patients and health care workers, but this study did not address which contacts to offer SDR-PEP [38].

Ongoing trials are further investigating optimal dosing, target populations and treatment strategies. The PEOPLE trial (2018-2022) was designed to evaluate who benefits most from PEP in a wider circle of exposed contacts, as the trial in Indonesia indicated that a household contact approach alone would not suffice [39]. In the PEOPLE trial, a double dose of SDR is used, as it was shown previously for *M. tuberculosis* that the bactericidal effect of rifampicin is

proportional to the used dose, and safety is good and not dose dependent [40]. The PEP4LEP trial (2018-2023) compares the effectiveness and feasibility of a skin camp SDR-intervention to a health centre-based SDR-intervention in Mozambique, Ethiopia, and Tanzania [41]. The PEP++ trial (2020-2024), is testing an optimized regimen, three doses of rifampicin and clarithromycin to close contacts of new leprosy patients. The SIMCOLEP study models how many people should receive SDR-PEP in order to reach a 90% reduction of new cases detected. For the three countries with the highest absolute number of cases (India, Brazil, and Indonesia), SIMCOLEP modelled that in theory a total of 32.9 million people should receive PEP to achieve a 90% reduction over 22 years, which raises questions about feasibility given the fact that every person needs to be examined for signs of leprosy and tuberculosis before administering PEP [42]. However, it is promising that on a global level a 50% reduction in new cases detected could be achieved after 5-years [42]. SDR-PEP, is not a vaccine and will not induce lasting immunity like vaccines do, and therefore its effect is time dependent. The BE-PEOPLE trial (2022-2026), is testing safety and efficacy of a single dose of bedaquiline with rifampicin against SDR as PEP in the Union of the Comoros.

6.4 Immunoprophylaxis

The BCG vaccine, originally developed to protect against tuberculosis, also protects against leprosy [43], showing 41% protection in trials to 60% in observational studies. Additional doses of BCG do not improve protection against leprosy. The protective effect of BCG vaccination is higher in HHCs of leprosy patients than in the general population. BCG vaccination protects against both PB and MB.

Lepvax is a defined subunit leprosy vaccine candidate developed by Duthie *et al.* (2018) [44]. This vaccine is based on LEP-F1, which is a formed by a tandem linkage between four open reading frames encoding for the following proteins: ML2531, ML2380, ML2055, and ML2028. In mice, it has been proven to induce a LEP-F1 specific cellular immune response. A phase I trial in healthy individuals was successfully completed [45]. Currently the vaccine is undergoing phase II testing in endemic regions, intended for both pre- and post-exposure prophylaxis.

7. Leprosy reaction

Leprosy reactions can occur before, during and after MDT. Clinical vigilance is needed to recognize and treat them early, as they can equally lead to severe and irreversible disabilities. Reactions are caused by immunological reactions against antigens of (dead) *M. leprae.* They can occur acutely, and may wax and wane.

There are different symptoms/signs of reaction. At the level of the skin, the patches can be inflamed and erythematous nodules can be present. Nerves can be painful and/or tender, and new loss of sensation or new muscle weakness can be observed. The eyes can become red and painful, and even blindness is possible. Constitutional symptoms - like fatigue, malaise and fever – can be reported.

Three types of leprosy reactions can be distinguished: Type 1 leprosy reaction (delayed hypersensitivity reaction), Type 2 leprosy reaction (or Erythema Nodosum leprosum) and Type 3 leprosy reaction (or Lucio's Phenomenon)

7.1 Type 1 leprosy reaction (Reversal reaction)

A type 1 leprosy reaction, also known as the delayed hypersensitivity reaction, is a cell-mediated immune reaction against leprosy antigens. This is the most common type of reaction, and typically occurs more localized in the borderline forms on the Ridley-Jopling scale (BT, BB, BL). An episode is most likely to occur within the first six months of MDT or in the six months post-partum. With this reaction, inflammation of pre-existing patches can arise as well as ulceration of skin lesions. Nerves can be painful and/or tender, which can result in neuritis and thereby in new sudden loss of sensation/weakness, with subsequent deformities/paralysis. Oedema in hands, feet and face can occur. Systemic involvement is rare. Type 1 reversal reaction can occur in two forms, mild and severe (Table 1.2). A mild type 1 reaction is treated with nonsteroidal anti-inflammatory drugs(NSAIDs) for two to four weeks, if no improvement can be observed treatment with corticosteroids (e.g. prednisolone) should be started.

Table 1.2: Symptoms and signs of leprosy reaction according to severeness. Table is based on information from the book "Leprosy" by Bryceson et al. (1990) [46]

Type 1 Reversal Reaction	
Mild	Inflammation of pre-existing skin lesions (erythema and swelling without ulceration)
	No nerves affected
	No constitutional symptoms
Severe	Inflammation of pre-existing skin lesions with ulceration
	Nerves are affected: new or increased muscle weakness/loss of sensation
	Involvement of the face
	No response to NSAIDs
Type 2 Erythema Nodosum Leprosum	
Mild	Few crops of erythematous nodules
	No nerves affected
	Mild constitutional symptoms: fever ≤38°C
	No organ involvement
Severe	Multiple crops of erythematous nodules
	Nerves affected: new or increased muscle weakness/loss of sensation
	Constitutional symptoms: high fever >38°C
	Organ involvement
	More than 4 episode per year
	No response to NSAIDs

7.2 Type 2 leprosy reaction (Erythema Nodosum leprosum, ENL)

Type 2 reaction is caused by a humoral immunological reaction that produces an excess of antibodies against the antigens of *M. leprae*, and is therefore a more generalized systemic reaction. Thus, this reaction occurs more often in patients on the lepromatous side of the RJ-scale (BL,LL). This reaction is characterized by new painful erythematous subcutaneous nodules on the extremities and the face, which can be transient. Severe ulceration of the skin, inflammation of testes, bones, or lymph nodes (inguinal-femoral are common) are symptoms of type two reactions as well. Most of the time multiple organs are involved and it causes systemic illness. Recurrent inflammation of the eyes in type 2 reactions (uveitis, iritis), can result in blindness. ENL is treated with rest and NSAIDs in the mild form and with corticosteroids (e.g. prednisolone) in the severe form (Table 1.2). Wound care is important, to avoid superinfections.

7.3 Type 3 leprosy reaction (Lucio's Phenomenon)

Lucio's phenomenon is the least common leprosy reaction and has only been seen in patients with Mexican ancestry. It is only seen in patients with diffuse lepromatous leprosy and is characterized by vasculitis, which is sudden and can be necrotizing, presenting as punched-out ulcerations that may be extensive and potentially life-threatening. This reaction should be treated with antimicrobials and corticosteroids, and wound care is very important.

8. Occurrence of drug resistance

In the 1940s, sulphonamides, like promin and dapsone were found to be effective against *M. leprae*, which resulted in lifelong therapy for leprosy patients with these drugs (Fig. 1.9). After a decade of monotherapy with this drug, the first dapsone resistance was suspected, however no diagnostic test for drug resistance was available. In 1964, a first case of dapsone resistance was confirmed using the mouse footpad model. Between the 1960s and 1980s, for patients suffering from dapsone resistance, clofazimine or rifampicin was added to the treatment, as these drugs were found to be effective against leprosy as well. Such addition of a single drug to treatment for a dapsone resistant strain, resulted again in monotherapy. Nevertheless, these drugs were used for dapsone-resistant leprosy patients without any general approved treatment schedule for many years, which led to the first detection of rifampicin resistance in a leprosy patient in 1976 (Fig. 1.9). During this period, several other antibiotics were being assessed for their efficacy that are now used as second-line drugs, including minocycline, clarithromycin, and ofloxacin. In 1982, WHO recommended multidrug therapy, which regimen was globally implemented by 1997. In 1997, ofloxacin resistance was detected in a patient who already had resistance to dapsone and rifampicin, resulting in the first confirmed multidrug resistant *M. leprae* (Fig. 1.9).

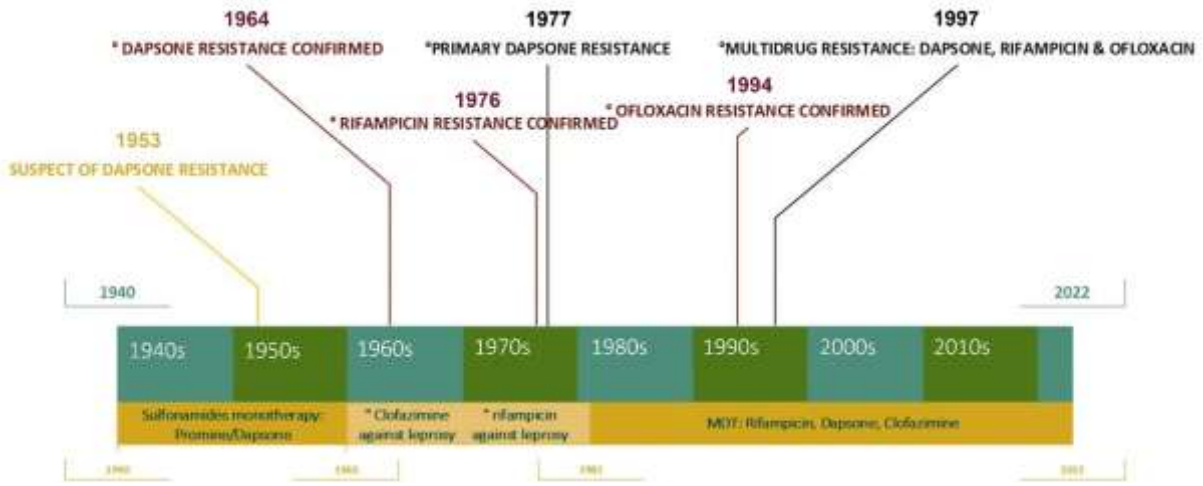


Figure 1.9: Timeline showing the introduction of anti-leprosy drugs and treatment, and the emergence of drug resistance.

Globally, dapsone resistance is found in 6.8% of relapsed patients and in 4% of new patients. Rifampicin resistance is found among 5.1% of relapse cases and in 2.0% of new cases worldwide. Brazil, India, and Colombia reported more than 5 rifampicin-resistant cases from 2009-2015 (Fig. 1.10). Ofloxacin resistance occur as much as rifampicin and dapsone resistance, as it is not part of the standard MDT. Ofloxacin resistance was observed in 1.3% of leprosy cases mainly in Benin, India and Brazil, 1.7% in relapses and 1% in new leprosy patients. Clofazimine resistance is rare. Drug-resistance emergence a pertinent knowledge gap in the leprosy control world. The surveillance of drug resistance among leprosy patients has become more pertinent since SDR was recommended by the WHO as prophylactic treatment in 2018 [27]. Several literature reviews raised concern of the use of SDR as a prophylactic treatment as it might induce drug-resistance to the most potent leprosy drug, although on theoretical grounds this is unlikely after a single dose. The main cause of treatment inefficiency is drug resistance. Systematic monitoring of drug susceptibility in leprosy is especially important since clinical signs of therapeutic inefficiency are seldom present before 12 months of standard MDT treatment. In addition, resistance to dapsone may be hidden by the bactericidal action of rifampicin, and most likely, dapsone resistant patients will relapse late after the end of treatment. In areas where drug resistance has been reported and drug susceptibility testing is available, such testing is recommended at the time of treatment initiation, and when treatment failure is suspected.

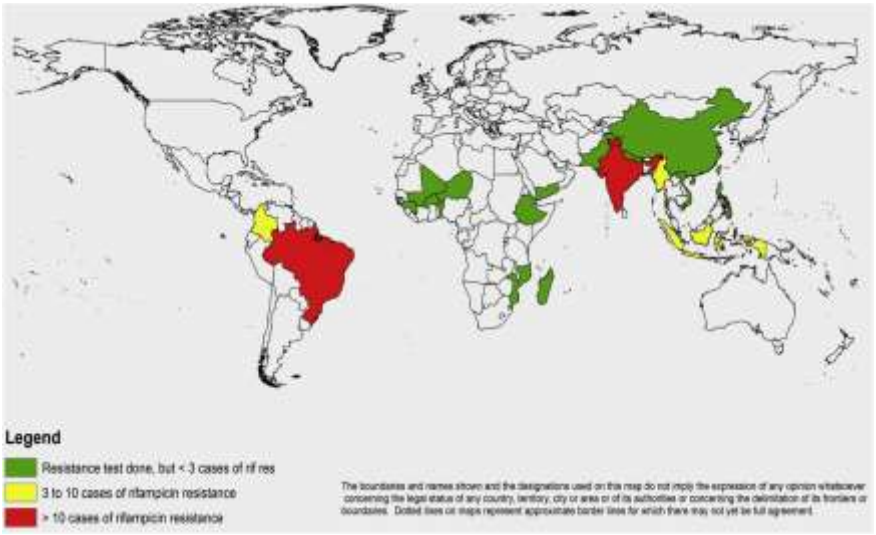


Figure 1.10: Map of countries reporting rifampicin resistance in leprosy between 2009 and 2015. Countries that reported more than ten rifampicin-resistant cases are coloured in red, those reporting between three and ten are coloured in yellow and those reporting fewer than three cases are shown in green. [47]

9. Laboratory methods for identification of the bacilli causing leprosy (extended/altered version of a chapter published in Springer review)

The genomes of *M. leprae* (3.26 Mbp [48] and *M. lepromatosis* (3.24 Mbp [49]) are one of the most reduced ones known for bacteria and contain a large proportion of pseudogenes. A consequence is the complete dependence of both bacilli on host energy metabolism and thus on intracellular growth. This observation also explains the lack of success in culturing these bacteria in vitro, which challenges their diagnosis. For primary isolation and subsequent passage, suspensions of biopsies from leprosy patients can be inoculated in the foot pads of mice and armadillos [50]. Growth of leprosy bacilli in an animal model requires a high inoculum and takes several months because of the pathogen's doubling time of 12 days [50]. These are technically challenging techniques for which only a handful of laboratories worldwide have the expertise. For research purposes, leprosy bacilli can successfully be maintained for short periods in cell culture such as macrophages. Presently, there is no reference standard for the microbiological confirmation of *M. leprae* that allows for early identification of patients, which would help in preventing transmission and severe forms of the disease.

The leprosy bacilli are mainly detected in skin and nerve tissue (Table 1.4) with various sensitivity depending on the disease's form [51]. MB patients (> 5 skin lesions) will harbour a high number of bacteria in skin lesions while few or no leprosy bacilli are expected in PB patients (≤5 skin lesions). Diagnosis of PB patients will often require several samplings (including sampling of a peripheral sensory nerve in case of pure neural leprosy), and methods to confirm the diagnosis microbiologically. SSSs and nasal swabs are less invasive than skin biopsies, but fewer bacilli are usually observed, decreasing the sensitivity of detection especially for PB patients. Moreover, a positive nasal swab in a healthy household contact of leprosy patients or

healthy individuals from endemic countries may not indicate leprosy disease, or even infection, representing a prognostic marker rather than a confirmatory diagnostic marker [52]. Furthermore, the presence of *M. leprae* DNA detected through polymerase chain reaction (PCR) analysis in nasal swabs from household contacts does not serve as a determinant for the progression of these contacts to active disease [53].

The edges of active skin lesions are the areas where the highest quantity of bacilli is found. Therefore, for diagnostic purposes, a skin biopsy (4mm) at the edge of an active lesion is preferred for downstream molecular applications (detection, genotyping and drug-susceptibility testing) as well as for inoculation in a mouse footpad. Nevertheless, SSS followed by microscopy to determine the BI is often used for diagnosis and allows for monitoring of the bacterial load during treatment. Given the tropism for skin and nerve cells, leprosy bacilli are rarely found in the systemic circulation, only in patients with a high burden of bacilli. Blood samples are thus less relevant for bacterial identification but can be used for serological or immunological diagnosis.

9.1 Identification and quantification of leprosy bacilli in skin lesions by microscopy

The most common method of identifying leprosy bacilli in tissue is microscopy on SSS, biopsy of a skin lesion and in rare cases on a nerve biopsy. Ziehl-Neelsen (ZN) staining is the standard coloration for acid-fast bacilli (AFB) in SSS. All mycobacteria (and several related genera) display AFB characteristics because of the presence of mycolic acid in their cell wall, which confers resistance to destaining when exposed to acid alcohol. However, since *M. leprae* and *M. lepromatosis* are only weakly acid-fast [54], the ZN procedure is performed with a shorter discoloration time. The adapted ZN method named Fite-Faraco staining is recommended for skin biopsies preserved in formalin and paraffin (Fig. 1.11) [55]. After staining, the rod-shaped bacilli turn uniformly pink and besides isolated bacilli they can be found grouped in globi, which is characteristic for an *M. leprae* infection.

After staining, the BI is counted based on a semi-logarithmic scale from 1+ to 6+ (Table 1.3). A patient's BI usually represents the mean of BIs calculated from SSS collected from the ear lobe, elbow and knee. The BI can be used to classify a patient according to both the Ridley-Jopling and WHO classification. This measure can performed before and at different time points during treatment. An increase of BI during treatment may indicate treatment inefficiency or failure. Conversely, patients presenting with new onset of symptoms after MDT may benefit from SSS to distinguish a reaction from a relapse. In case of relapse, which may occur many years after MDT, the patient typically develops a new skin lesion, and the BI has increased by 2+ from previous values.

Table 1.3: Bacillary index for leprosy patients, based on microscopic examination

Negative (0) – no bacilli per 100 high-power field
Positive (1+) – 1 to 10 bacilli per 100 high-power field
Positive (2+) – 1 to 10 bacilli per 10 high-power field
Positive (3+) – 1 to 10 bacilli per high-power field
Positive (4+) – 10 to 100 bacilli per high-power field
Positive (5+) – 100 to 1000 bacilli per high-power field
Positive (6+) – more than 1000 bacilli per high-power field

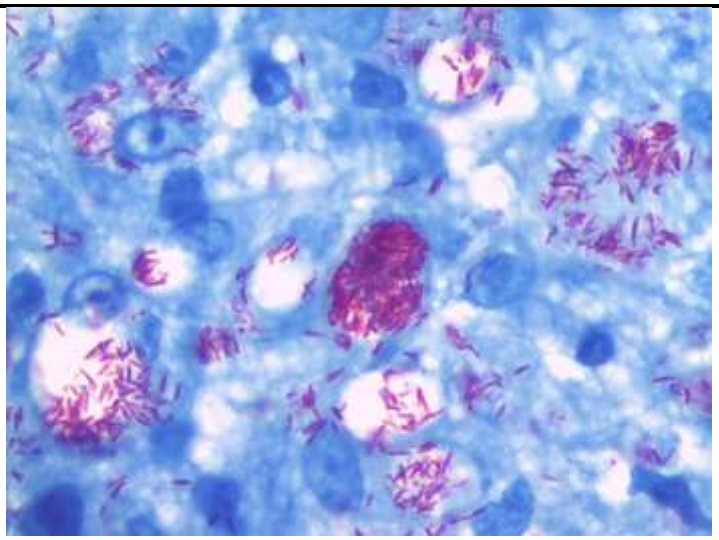


Figure 1.11: Bacterial index determination with Fite-Faraco staining (x100). Presence of bacilli in neural branches, macrophages, interstitial cells, in perivascular and periadnexal inflammatory infiltrates and occasionally in the walls of vessels and endothelium. In the center, macrophages have intracytoplasmic vacuoles filled with numerous bacilli (globi). Figure and caption adapted from [56].

9.2 Molecular diagnostic methods

In general the input material for molecular methods is extremely important for the reliability of the outcome of the assay. The sensitivity for detecting bacilli of different studies using the same molecular technique can differ considerably (especially for PB patients), given the choice of sample and the used DNA extraction technique. Given the thick cell wall of mycobacteria, there is a crucial role for mycobacterial DNA extraction to detect mycobacteria, thus also for *M. leprae* detection [57-59]. Different samples used in diagnosis of leprosy are mentioned in Table 1.4, however the perfect sample to diagnose all leprosy patients does not exist yet. With skin biopsies, the chances of collecting the most bacilli, are the highest, given the volume of the sample.

Table 1.4: Type of sample, location of sampling and whether anaesthetics are needed

Type of sample	Location of sampling	Anaesthesia needed	Reference for sampling
Slit skin smears	Earlobes, elbows, and knees	no	[60]
Skin biopsy	Edges of active patches	yes	[61]
Nerve biopsy	Thickened nerves in the ankle, forearm, or along a rib	yes	[62]
Nasal swab	Anterior nares	no	[63]
Nasal mid-turbinate swab	Turbinates	no	[64]
Nasopharyngeal swab	Nasopharynx	no	[65]
Finger stick blood	Finger tip	no	[66]
Venous blood draw	Vein in arm	no	N.A.

In the laboratories, where molecular methods are applied to detect the causative agent, different techniques are being used. In case of a skin biopsy, mechanical disruption of the tissue (bead beating, mortar and pestle, or GentleMacs) prior to DNA purification is important for efficient recovery of mycobacterial DNA [57]. After mechanical disruption, one can opt for a chemical pre-treatment before DNA purification which yielded more mycobacterial DNA in a study from Durnez *et al.* (2009) [59]. The DNA extraction and purification techniques used in a laboratory are often dependent on the available equipment and infrastructure. Freeze-boil DNA extraction is a cheap and rapid DNA extraction technique, however the yield and purity are not as high as with other DNA extraction techniques [67]. DNeasy Blood & Tissue (Qiagen, Germany) and QIAamp DNA Microbiome (Qiagen, Germany) and Maxwell RSC FFPE (Plus) Tissue LEV kits (Promega, USA) are often used to extract DNA from a sample with high yield and high purity DNA [68, 69].

Since 2001 the complete genome sequence of *M. leprae* is available, based on which several molecular techniques have been developed. The genome of *M. leprae* contains four families of dispersed repetitive elements, amongst which RLEP [70]. Thirty-seven copies of RLEP exist in the chromosome, each containing an invariant 545-bp core flanked in some cases by additional segments ranging from 44 to 100 bps. The molecular amplification of the conserved core region of this element is highly specific for *M. leprae* [71] and also sensitive, with a limit of detection down to three bacilli using TaqMan quantitative PCR (qPCR) (Table 1.5) [72-74]). In case of waning clinical acumen or presumptive early leprosy or PB patients, which are difficult to detect clinically or histologically, molecular detection using the RLEP target is the method of choice, provided that good practice and control of the qPCR conditions are applied to limit the risk of false positives. Two tests based on RLEP detection are commercially available. The GenoType LepraeDR from Hain LifeScience (Germany) is a reverse hybridization DNA strip that also allows detection of drug resistance, which requires a thermocycler and an automated washing and

shaking device called Twincubator [75]. The second test is a loop-mediated isothermal amplification, the RLEP LAMP assay soon to be commercialized by Amplex Diagnostics (Germany) [76]. The LAMP assay uses six primers and a Bst DNA polymerase, and can be executed at one single temperature, circumventing the need for a (q)PCR machine.

Only molecular assays can differentiate between *M. leprae* and *M. lepromatosis* infection. The analysis of both genomes has revealed genetic differences and singularities exploited to develop sensitive and specific molecular assay to differentiate both pathogens [48, 49]. There is no commercial test available yet for molecular detection of *M. lepromatosis* but infection by *M. leprae* or *M. lepromatosis* can be distinguished on the basis of their 16S rRNA sequence being only 98% identical [7]. This requires sequencing of the amplicon. Similarly, the pathogens can be differentiated based on a 45bp insertion in the *M. lepromatosis rpoT* sequence, seen as bands of different length on agarose gel [77]. However, in case of co-infection with *M. leprae*, 16S rRNA and *rpoT* amplification can be difficult to interpret. Singh and colleagues developed a specific 244bp PCR amplification assay targeting *hemN*, a gene absent in the genome of *M. leprae* [49, 78], but this gene is present in other mycobacteria making the assay non-specific. Recently, Sharma et al. developed a qPCR targeting the *M. lepromatosis* repetitive element RLPM [78], occurring 5-6 times in the *M. lepromatosis* genome and being highly specific. Other targets have been suggested and tested to be used to detect *M. leprae* and *M. lepromatosis* but with less sensitivity and specificity than the repeat regions [79].

Table 1.5: List of primers and probes to perform identification, quantification, and viability assay for the leprosy bacilli – Fw: forward; Rv: Reverse

	Target gene	Primer/probe sequences (5'-3')	References
Quantification of *M. leprae*	RLEP	Fw: GCAGCAGTATCGTGTTAGTGAA Rv: CGCTAGAAGGTTGCCGTAT P: CGCCGACGGCCGGATCATCGA	[80]
Quantification of *M. lepromatosis*	RLPM	Fw: TTGGTGATCGGGGTCGGCTGG Rv: CCCCACCGGACACCACCAACC P : AAGTGACGCGGGCGTGGATT	[77]
Drug resistance rifampicin*	*rpoB*	Fw : GTCGAGGCGATCACGCCGC Rv : CGACAATGAACCGATCAGAC	[81]
Drug resistance dapsone*	*folP1*	Fw : CCTGACGATGCTGTCCAGC Rv : CACCAGACACATCGTTGACG	[81]
Drug resistance ofloxacin*	*gyrA*	Fw : GATGGTCTCAAACCGGTACATC Rv : ACCCGGCGAACCGAAATTG	[81]
Viability *M. leprae*	*hsp18*	Fw: CGATCGGGAAATGCTTGC Rv: CGAGAACCAGCTGACGATTG P: ACACCGCGTGGCCGCTCG	[80]
	esxA	Fw: CCGAGGGAATAAACCATGCA Rv: CGTTTCAGCCGAGTGATTGA P: TGCTTGCACCAGGTCGCCCA	
Viability *M. leprae*	*16S rRNA*	Fw: GCATGTCTTGTGGTGGAAAGC Rv: CACCCCACCAACAAGCTGAT P: CATCCTGCACCGCA	[80]

*mutations conferring drug resistance and validated in mouse footpad are described by Aubry and colleagues [82].

9.3 Immunodiagnostics

Molecular methods are highly specific but lack sensitivity at early stages of disease progression when symptoms do not yet meet the cardinal signs, or to measure exposure and assess risk of disease progression in healthy contacts [83]. This is where host biomarkers can be complementary. However, due to the spectral variability in the immune response in leprosy patients, specific tests measuring antibody response (MB) and cell-mediated immunity (PB) are necessary to cover the full leprosy spectrum [83]. Several antigens are shown to mount a strong antibody response and include the native phenolic glycolipid I (PGL-I) that is part of the cell wall of *M. leprae* and *M. lepromatosis* (primarily IgM response), and the leprosy IDRI diagnostic 1 (LID-1), which is a fusion protein of two known *M. leprae* antigens, ML0405 and ML2331 (IgG response) [84]. However, their sensitivity remains low, especially for PB patients [84]. Additionally, when performed in an endemic country, healthy contacts can also have positive antibody response to these antigens, while the vast majority of these individuals will not

progress towards the disease [83]. The qualitative tests in a lateral flow test format are commercially available in Brazil for both Disaccharide linked via an Octyl carboxylic acid linker to (NDO)-LID and PGL-I antigens [85, 86]. A qualitative test assessing the antibody response to PGL-I is commercialized under the name "ML flow test" by Bioclin (Brazil) and a recently developed quantitative test is commercially available in The Netherlands, the Up-Converting Phoshor Lateral Flow Assay (UCP-LFA) for quantification of the anti-PGL-I response [87].

To cover the full spectrum of leprosy forms, a lateral flow test measuring multiple host proteins in fingerstick blood was recently developed and is currently being tested in several endemic countries [88]. This multi-biomarker test includes six previously identified host biomarkers able to diagnose all leprosy forms as well as differentiate between MB and PB cases [88].

10. Monitoring treatment efficacy

Drugs are administered by supervised dose (monthly), taken at the health center, associated with self-administered doses (daily) at home (Table 1.1). Rather than based on clinical parameters, treatment success is defined by full completion of the number of doses recommended [27]. Rarely, based on persisting clinical signs that are not ascribed to reactions, MDT can be pursued at the end of the recommended treatment period (treatment inefficiency) up to 24 months. After this period, persistence of symptoms is considered treatment failure [89] (47). A handful of laboratory methods are available to support clinical assessment.

10.1 Bacterial index and morphology index by microscopy

BI determination can be performed before and at different time points during treatment to monitor the evolution of the bacterial load in MB patients. BI determination is not a good measure of viability, as a slow decrease in AFB is commonly observed during treatment (one log/year). In contrast, the morphology index (MI) is a measure of bacterial viability based on cell integrity in AFB stained SSS. Solidly stained bacilli are considered viable while fragmented or granularly staining bacilli are deemed non-viable (Fig. 1.12) [90]. This method relies on previous observations that morphological changes of the leprosy bacillus correlate with effective treatment of lepromatous patients [91]. It is expressed as a percentage of viability measured on 200 bacilli [91]. The MI in untreated MB leprosy usually ranges between 25 and 75 and should decline to 0 after 6 months of effective chemotherapy. This method might reduce the variation seen with acid-fast staining and can be done subsequently to the BI measurement. However, the MI is considered an imperfect measure of viability since it is highly operator dependent and can only be performed on strongly microscopy positive samples.

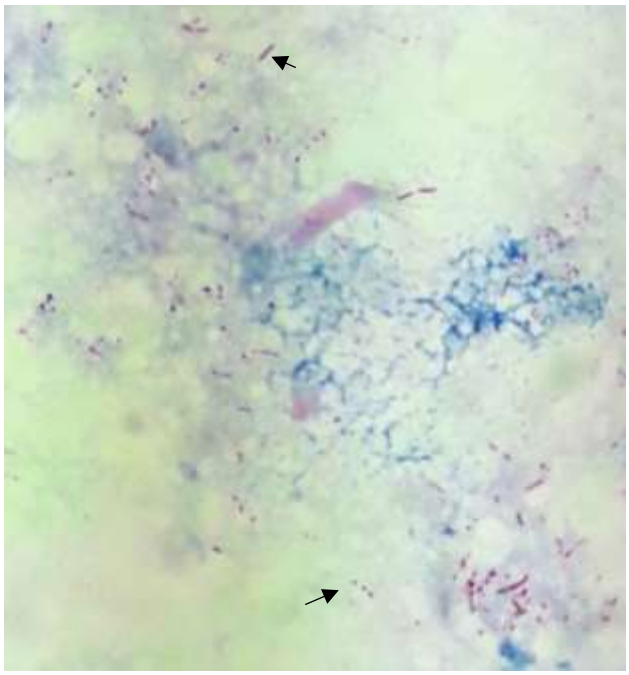


Figure 1.12: Morphological assessment of bacilli in microscopy – the arrow in the upper part represents a solidly stained bacillus considered viable while the arrow in the lower part of the figure shows a fragmented/granulated non-viable bacillus (x1000) [56].

10.2 In vivo inoculation

Alternatively, bacterial viability in clinical samples can be assessed by inoculation of bacteria from human skin biopsies in mouse foot pads of immunocompetent nude mice followed by the microscopic evaluation of bacterial replication 6 to 12 months later [92]. However, this method requires bacterial inoculation within 48 hours of skin biopsy and only few laboratories in the world (Bauru (Brazil), National Hansen Institute (USA) and Janvier Labs (Le Genest Saint-Isle, France) have the capacity to perform such experiments [92].

10.3 RNA-based approaches

Molecular viability assays (Table 1.5) are based on the quantification of transcripts, *hsp18* and *esxA*, or 16S rRNA measured on a defined number of *M. leprae* bacilli calculated by RLEP PCR [80, 93]. Both assays determine absolute viability based on the amplification of RNA and do not rely on a paired "pretreatment" sample [50]. Nevertheless, since patients' response to treatment is variable from one individual to another and because of the slow growth of the pathogen, longitudinal viability testing should be performed to properly measure the impact of the drugs on bacterial viability [93]. In addition, they were validated on samples from infected mice but only the 16S rRNA target was validated on clinical isolates [80, 93]. The dynamic range of these assays is yet to be determined, as the absence of mycobacterial RNA may not equate non-viability of a low bacterial burden.

11. Methods to monitor drug resistance

The WHO calls for strengthened universal drug-resistance surveillance. Empirically, the gold standard method for drug susceptibility testing is the Shepard method [94]. Briefly, the phenotypic method for drug-susceptibility testing relies on the isolation of at least 10^4 viable

bacilli from lesions of MB patients and inoculation within 48 hours following collection of the clinical specimen into the footpads of immunocompetent mice. The bacilli are recovered from the inoculated footpads after 6 to 10 months of treatment, compared to an untreated control group, and recovery of ≥ 1 x 10^5 bacilli per foot pad is considered positive growth. Because of the 12-days doubling time of *M. leprae* results may only be available after the end of the treatment of the patient [50]. This method is only applicable to patients with a high bacillary load. Mutations in specific parts of chromosomal genes encoding drug targets, known as drug-resistance determining regions (DRDR) [95] in *M. leprae* genes, are known to be responsible for drug resistance against dapsone, rifampicin and fluoroquinolones. This rather than horizontal gene transfer [96].

Leprosy patients with high level resistance against dapsone always seem to be infected with *M. leprae* harbouring a mutation in the drug resistant determining region of *folP1* (ML0224). *folP1* is a gene that encodes for dihydropteroate synthase (DHPS). Dapsone binds to the active site of DHPS, causing inhibition of folic acid synthesis in *M. leprae*. Thus, a mutation in *folP1* that causes an amino acid change in the active binding site of DHPS might result in drug resistance against dapsone. Patients with low or medium level resistance against dapsone, are not always found to be infected with a DRDR-mutated *folP1 M. leprae*. Thr53Arg and Pro55Arg are the most common mutations found in *folp1* that are associated with high level dapsone resistance (Table 1.6).

Rifampicin hampers mRNA production by preventing that DNA can bind to the ß-subunit of the DNA-dependent RNA polymerase. The ß-subunit is encoded by *rpoB*, and therefore mutation in the DRDR of this gene may result in rifampicin resistance. The most frequently observed codon changes are in codon 456 and 451, and to a lesser extent in 438, 440, 441 & 458 (Table 1.6). The genes responsible for ofloxacin resistance are *gyrA* and *gyrB.* A change in codon 91 of *gyrA* has been the most observed change to confer drug resistance against ofloxacin in leprosy patients, and to a lesser extent changes in codon 89. In *gyrB*, several mutations have been observed with a codon change as a result, however they are not proven to confer drug resistance. Mutations in *ctpC [97]*, *ctpI [97], fadD9 [96]*, *ribD [96]*, *ethA [96]*, *pks4 [96]*, and *nth* [96]have been found in strains that were drug resistant, however their roles in conferring drug resistance is yet to be confirmed. For clofazimine drug-resistance, which is still rare, the mechanism is unclear.

Molecular methods , using specific primers (Table 1.5),targeting the aforementioned DRDRs have been validated and implemented in some countries as part of the WHO drug resistance surveillance network [47]. Recommended methods include a polymerase chain reaction (PCR) step coupled with Sanger sequencing [81], the commercial DNA strip test GenoType LepraeDR [75] or, whole-genome sequencing [96]. There is currently no validated target for the leprosy drugs clofazimine, minocycline and clarithromycin [82].

Table 1.6: Mutations/insertions within anti-leprosy drug target genes that confer resistance in Mycobacterium leprae. In bold are the high frequency mutations. The numbering system is that of the M. leprae genome TN strain (GenBank AL583923) [81]

Gene	Nucleotide change (5'→3')	Amino acid substitution
rpoB	cag→gtg	Gln438Val
	Insertion	Met440Lys/Phe
	gat→tat	Asp441Tyr
	gat→aat	Asp441Asn
	cac→gac	His451Asp
	cac→tac	His451Tyr
	tcg→ttg	**Ser456Leu**
	tcg→atg	Ser456Met
	tcg→ttc	Ser456Phe
	tcg→tgg	Ser456Trp
	ctg→gtg	Leu458Val
	ctg→ccg	Leu458Pro
folp1	**acc→gcc**	**Thr53Ala**
	acc→agc	Thr53Arg
	acc→atc	Thr53Ile
	ccc→cgc	Pro55Arg
	ccc→ctc	**Pro55Leu**
	ccc→tcc	Pro55Ser
gyrA	ggc→ tgc	Gly89Cys
	gca→gta	**Ala91Val**

11.1 Drug resistance for *Mycobacterium lepromatosis*

Treatment of *M. lepromatosis* infection is empirically similar as for MB leprosy cases by *M. leprae [98]*. While it is likely that rifampicin is active against *M. lepromatosis*, it is yet not clear whether the bacterium is susceptible to dapsone or clofazimine [82]. There is currently no molecular test available to amplify the DRDR of *rpoB, folP1* and *gyrA* for *M. lepromatosis* and drug-susceptibility testing in mice has not yet been performed.

Table 1.7: Methods available for the detection, identification and viability measurement of leprosy bacilli in various clinical samples

Purpose of assay	Preferred sample type	Principle	Methods	Commercially available	References
Identification of leprosy bacilli					
Qualitative					
Immunodiagnostics	Venous blood or fingerstick	Antibody detection	ELISA Lateral flow test	Yes	[85, 88, 99]
		Cellular immunity	Lateral flow test		
Quantitative					
Microscopy	SSS Skin biopsies	Bacterial coloration (FF)	Bacillary index	No	[55, 100, 101]
Molecular methods	SSS Skin biopsies	Quantification of *M. leprae* and *M. lepromatosis* specific target from DNA samples	qPCR RLEP (*M. leprae*) RLPM (*M. lepromatosis*)	Yes (RLEP)	[72, 76, 78]
Viability of the leprosy bacilli					
Microscopy	SSS Skin biopsies	Assessment of bacterial integrity (solid *vs.* fragmented bacilli) after FF staining	Morphology index	No	[102]
In vivo	-	Inoculation from fresh skin biopsies in the footpad of immunocompetent mice	Shepard method	No	[94]
Molecular methods	Skin biopsies	Quantification of *M. leprae* transcripts in RNA extracts, measured on a defined number of *M. leprae* calculated by RLEP PCR	qPCR* *hsp18* and *esxA* 16S rRNA	No	[50, 80, 93]

*There is no viability assay developed for *M. lepromatosis; FF= Fite-Faraco staining ;SSS=slit skin smear*

12. Genotyping

12.1 SNP typing

Given the massive decay in the genome of *M. leprae*, there is an expectation of reduced variability among worldwide strains. Polymorphisms are only found, on average, once every 28kb, indicating a high level of genetic similarity. Nevertheless, all strains across the globe can be categorized into four types based on their single nucleotide polymorphisms (SNPs), with a genome that is 99.995% identical. Except for loci prone to mutation, such as Variable Number of Tandem repeats (VNTR) [103, 104], *M. leprae* is a highly clonal species. Three SNPs (Table 1.8) were discovered by WGS that allowed the separation of global isolates into four SNP types [104]. To further subdivide these SNP types into 16 subtypes, Monot *et al* (2009) determined additional informative SNPs (Table 1.9) [104].

Table 1.8: SNP typing between global isolates

<table>
<tr><th>SNP TYPE</th><th>SNP 1642875</th><th>SNP2935685</th><th>SNP14676</th></tr>
<tr><td>1</td><td>G</td><td rowspan="2">A</td><td rowspan="2">C</td></tr>
<tr><td>2</td><td rowspan="3">T</td></tr>
<tr><td>3</td><td rowspan="2">C</td></tr>
<tr><td>4</td><td>T</td></tr>
</table>

Table 1.9: Single Nucleotide Polymorphism (SNP) sub typing between global isolates, SNP positions are based on the M. leprae TN reference genome (GenBank: AL450380.1)

<table>
<tr><td></td><td></td><td colspan="13">SNP positions based on the M. leprae TN reference genome</td></tr>
<tr><td>SNP TYPE</td><td>SUB TYPE</td><td>8453 (gyrA)</td><td>313361</td><td>61425</td><td>3102778 (ML2597)</td><td>1104232 (ML0934)</td><td>2751783</td><td>1295192</td><td>2312059</td><td>413902</td><td>20910</td><td>INS 978586/ 978589</td><td>DEL 1476522/ 1476519</td></tr>
<tr><td rowspan="4">1</td><td>A</td><td>T</td><td rowspan="2">A</td><td rowspan="3">A</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>B</td><td rowspan="3">C</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>C</td><td rowspan="2">G</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>D</td><td>G</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="4">2</td><td>E</td><td></td><td></td><td></td><td>A</td><td rowspan="2">C</td><td rowspan="3">A</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>F</td><td></td><td></td><td></td><td rowspan="3">C</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>G</td><td></td><td></td><td></td><td rowspan="2">G</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>H</td><td></td><td></td><td></td><td>G</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="5">3</td><td>I</td><td></td><td></td><td></td><td></td><td></td><td></td><td>A</td><td rowspan="2">C</td><td rowspan="2">G</td><td rowspan="3">G</td><td></td><td></td></tr>
<tr><td>J</td><td></td><td></td><td></td><td></td><td></td><td></td><td rowspan="3">G</td><td></td><td></td><td></td><td></td></tr>
<tr><td>K</td><td></td><td></td><td></td><td></td><td></td><td></td><td rowspan="2">G</td><td></td><td></td><td></td><td></td></tr>
<tr><td>L</td><td></td><td></td><td></td><td></td><td></td><td></td><td rowspan="2">A</td><td></td><td></td><td></td></tr>
<tr><td>M</td><td></td><td></td><td></td><td></td><td></td><td></td><td>A</td><td></td><td></td></tr>
<tr><td rowspan="3">4</td><td>N</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>DEL</td><td rowspan="2">T</td></tr>
<tr><td>O</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td rowspan="2">T</td></tr>
<tr><td>P</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>DEL</td></tr>
</table>

12.2 VNTR typing

Genotyping of *M. leprae* for epidemiological use is currently done by multiple-locus variable number of tandem repeat (VNTR) analysis (MLVA). MLVA is based on a set of VNTRs (microsatellites and/or minisatellites) that proved to have sufficient resolution for *M. leprae* differentiation [105, 106]. VNTRs are useful as molecular markers because they are highly polymorphic within geographically confined populations, in contrast to typing based on a selection of lineage defining SNPs. MLVA-based genotypic clustering is considered a proxy for a direct chain of transmission and thus permits identification of risk factors as well as 'hotspots' for recent transmission. MLVA analysis is, however, time consuming and requires sufficient *M. leprae* DNA, resulting in limited sensitivity when applied to DNA extracts from SSS and nasal swabs NS. Without accurate, powerful and easily implementable genotyping tools for *M. leprae* it will continue to be difficult to track and eliminate this pathogen as a public health problem.

Ten microsatellites [(AC)8a, (AC)8b, (AC)9, (AT)15, (AT)17, (TA)10, (TA)18, (GGT)5, (GTA)9, (GAA)21] and seven minisatellites [6-7, 12-5, 18-8, 21-3, 23-3, 27-5, and *rpoT*], were described in Sakamuri *et al.* in 2009 [105] and Kumari *et al.* in 2009 [107]. Sakamuri *et al.* concluded that the VNTR profiles with these targets remain stable for at least as long as the incubation period in patients. VNTR profiles have also been found to be highly stable within one individual and are a robust method of establishing transmission links [105]. This suggests that if the appropriate specimens are collected for mycobacterial DNA extraction, evidence of direct transmission between individuals can be obtained from these VNTR patterns, especially when combined with additional SNP information. Other studies indicate that some of the AT and TA microsatellites are too variable to establish transmission links [108].

12.3 Phylogeographical

The origin and spread of *M. leprae* has been agreed on by several studies (Fig. 1.13) The progenitor was probably SNP type 2 that may have originated from east Africa, which gave rise to SNP type 1, which spread to Asia with human migration, and SNP type 3 that has gone along with migration to the Middle East and Europe, before giving rise to SNP type 4 that has a link with slave trade and is found in West Africa. The introduction of leprosy into Asia has happened via two routes: One southern route with SNP type 1 from East Africa and a northern route with SNP type 3 via Europe and the Middle East to Asia. In the Americas the most probable explanation for the introduction of leprosy is due to migration from Europe to the Americas.

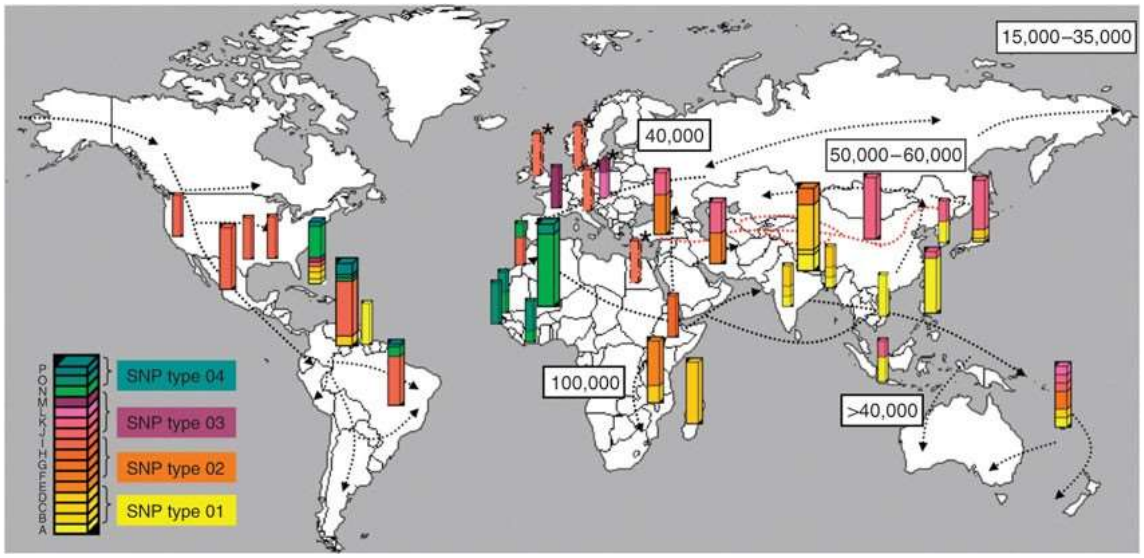


*Figure 1.13: Pillars are located on the country of origin of the M. leprae sample and colour coded according to the scheme for the 16 SNP subtypes shown. The thickness of the pillar corresponds to the number of samples (1–5, thin; 6–29, intermediate; ≥30, broad). The grey arrows indicate the migration routes of humans, with the estimated time of migration in years shown. The red dots indicate the location of the Silk Road in the first century, and * denotes results obtained from ancient DNA [104].*

13. Knowledge gaps and research goals: unravelling transmission of leprosy piece by piece

No disease has known to be around longer than leprosy, yet little is known in comparison to other diseases. This mutilating and highly stigmatized disease is still able to affect hundreds of thousands of people worldwide today. Since the control strategies have failed to impact global incidence over the last decade, the need for a better understanding of fundamental transmission dynamics is increasingly recognized, as a sine qua non for novel approaches for the prevention of incident leprosy. The Comoros is the only country in Africa to not have reached the elimination threshold of (<1/10.000 people) and even reports increased numbers of patients in the last decade. This archipelago provides the perfect cohort to unravel some of leprosy's mysteries. Improved understanding of the transmission of leprosy is key for developing novel approaches for its control, aiming for eradication. This improved understanding requires optimized molecular tools for the detection of *M. leprae* and to identify chains of *M. leprae* transmission.

There is a great need for a systematic standardization and optimization of DNA-extraction & -detection methods to maximize the clinical benefit. With the establishment of rapid and sensitive detection of *M. leprae* DNA we expect to better serve clinicians both in endemic and non-endemic countries. Therefore, a standardized confirmation tool that can reliably detect both PB and MB patients is crucial. Til date, in the largest part of the world, diagnosis of leprosy is solely based on clinical signs and symptoms. Therefore this thesis elaborately tested the

specificity of the highly sensitive RLEP-qPCR, which would be a good candidate for the reference test in leprosy diagnosis (CHAPTER II).

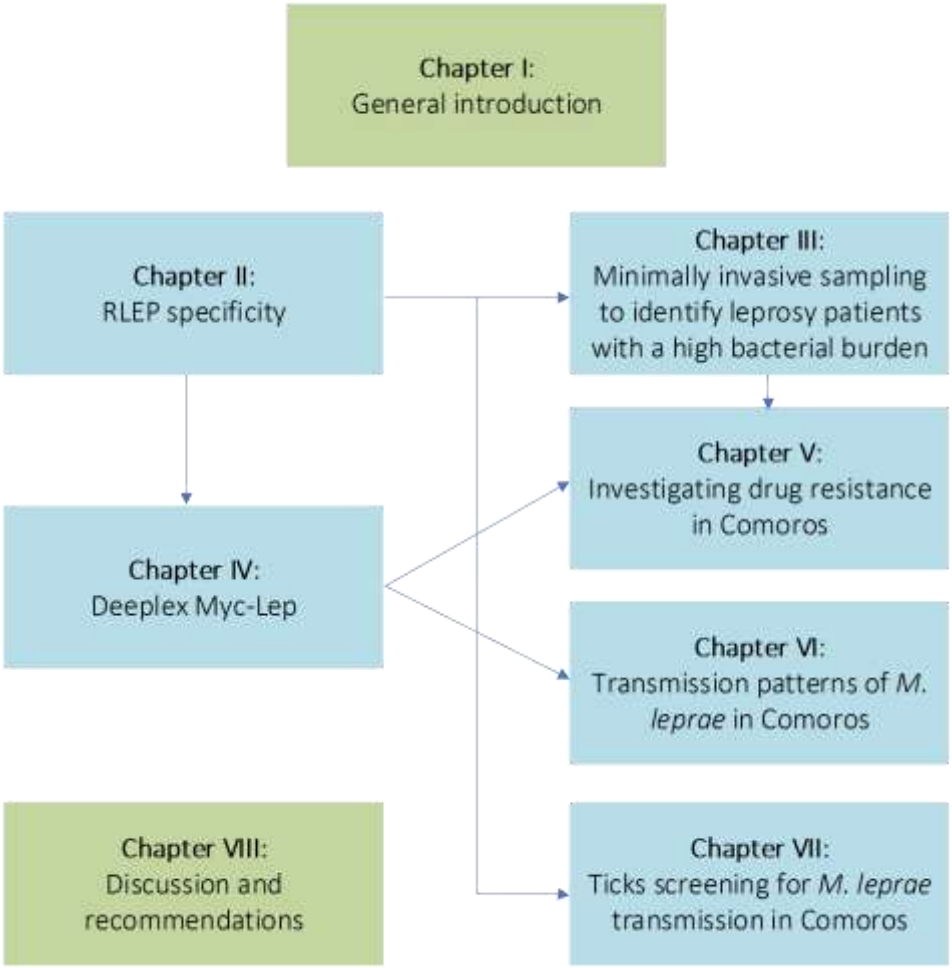
WHO recommended to use single dose rifampicin as a prophylaxis, however mass drug administration is not feasible. Therefore, it would be interesting to be able to identify patients with high bacillary loads in a less invasive way compared to a biopsy. This thesis is embedded in two parent cohort studies in the Comoros, the ComLep and PEOPLE study initiated by my promotors, which aim to identify who would most benefit from prophylactic treatment, for those not (yet) suffering from leprosy. A better tool to identify who would benefit most from prophylactic treatment to interrupt transmission is highly needed. To advance this part of research, this thesis evaluated the use of minimal invasive field-friendly tests as a tool to determine the bacterial load (CHAPTER III).

Thirdly, given the rise of next generation sequencing in the last decades we believe it is time to speed up the development and implementation of these new molecular techniques, to gain new insight in leprosy. Current MLVA genotyping techniques are laborious and require a lot of DNA. As *M. leprae* cannot grow *in vitro*, such large amounts are typically available in clinical samples from MB patients only. As clinical samples contain 99.9% host DNA, target amplification is necessary before next generation sequencing (NGS). We therefore collaborated with the Genoscreen company (Lille, France), to develop a novel *M. leprae* genotyping technique for epidemiological use, translating known hypervariable genomic and drug-resistance associated regions onto a targeted NGS (tNGS) platform (CHAPTER IV).

In the Comoros, a drug resistance surveillance has never been executed. It is of high importance to monitor whether drug resistance is present in the Comoros. Therefore in this thesis we investigated (with the tool developed in CHAPTER IV) whether drug resistance is present in the Comoros as a consequence of prophylaxis or that it might explain ongoing transmission in the Comoros (CHAPTER V). With that same tool we identified transmission chains, and measured genotypic clustering both inside and outside of households (CHAPTER VI).

In the Comoros, we are able to confirm 85% of the MB patients with RLEP qPCR. The fact that the other 15% is not being confirmed might be explained by missing the (few) bacilli in the lesion during sampling. Another explanation could be that the person has another differential diagnosis, such as leprosy caused by *M. lepromatosis,* that is not being captured by the RLEP qPCR. In the African continent no efforts were made so far to detect *M. lepromatosis*, so we cannot rule it out as causative agent for leprosy in the region. Thus, we investigated whether infection of these MB patients could be due to *M. lepromatosis* by performing Deeplex MycLep (CHAPTER VI). In light of their role as vectors for various infectious diseases, hard ticks have been suggested as potential contributors to leprosy transmission. In 2010, ticks residing on cattle were collected the Union of the Comoros. Therefore, in CHAPTER VII we screened ticks molecularly to assess their potential involvement as vectors in the transmission of leprosy.

We believe our findings will make an important contribution to bridging the numerous knowledge gaps that still exist for leprosy by offering new insights into the mycobacterial molecular diagnostics and epidemiology in the Comoros. It is our hope that this will contribute to paving the road towards improved leprosy prevention and control.



References

1. Robbins, G., et al., *Ancient skeletal evidence for leprosy in India (2000 B.C.).* PLoS One, 2009. **4**(5): p. e5669.
2. Santacroce, L., et al., *Mycobacterium leprae: A historical study on the origins of leprosy and its social stigma.* Infez Med, 2021. **29**(4): p. 623-632.
3. Rawcliffe, C., *Leprosy in Medieval England*. 2006, Woodbrige: Boydell Press.
4. Thin, G., *Leprosy*. 1891, London, UK: Percival and Co.
5. Reibel, F., E. Cambau, and A. Aubry, *Update on the epidemiology, diagnosis, and treatment of leprosy.* Med Mal Infect, 2015. **45**(9): p. 383-93.
6. Hasker, E., Baco, A., Younoussa, A., Mzembaba, A., Grillone, S., Demeulenaere, T., Groenen, G., Suffys, P. & De Jong, B. C, *Leprosy on Anjouan (Comoros): persistent hyper-endemicity despite decades of solid control efforts.* Leprosy review, 2017. **88**(3): p. p 334-342.
7. Han, X.Y., et al., *A new Mycobacterium species causing diffuse lepromatous leprosy.* Am J Clin Pathol, 2008. **130**(6): p. 856-64.
8. Han, X.Y., et al., *Analysis of the leprosy agents Mycobacterium leprae and Mycobacterium lepromatosis in four countries.* Am J Clin Pathol, 2014. **142**(4): p. 524-32.
9. Sharma, R., et al., *Zoonotic Leprosy in the Southeastern United States.* Emerg Infect Dis, 2015. **21**(12): p. 2127-34.
10. Avanzi, C., et al., *Red squirrels in the British Isles are infected with leprosy bacilli.* Science, 2016. **354**(6313): p. 744-747.
11. Hockings, K.J., et al., *Leprosy in wild chimpanzees.* Nature, 2021. **598**(7882): p. 652-656.
12. Ferreira, J.D.S., et al., *Ticks as potential vectors of Mycobacterium leprae: Use of tick cell lines to culture the bacilli and generate transgenic strains.* PLoS Negl Trop Dis, 2018. **12**(12): p. e0007001.
13. Tongluan, N., et al., *Mycobacterium leprae Infection in Ticks and Tick-Derived Cells.* Front Microbiol, 2021. **12**: p. 761420.
14. Sugawara-Mikami, M., et al., *Pathogenicity and virulence of Mycobacterium leprae.* Virulence, 2022. **13**(1): p. 1985-2011.
15. Tabouret, G., et al., *Mycobacterium leprae phenolglycolipid-1 expressed by engineered M. bovis BCG modulates early interaction with human phagocytes.* PLoS Pathog, 2010. **6**(10): p. e1001159.
16. Kibbie, J., et al., *Jagged1 Instructs Macrophage Differentiation in Leprosy.* PLoS Pathog, 2016. **12**(8): p. e1005808.
17. de Souza Sales, J., et al., *The role of indoleamine 2, 3-dioxygenase in lepromatous leprosy immunosuppression.* Clin Exp Immunol, 2011. **165**(2): p. 251-63.
18. Roy, S., et al., *ESX secretion system: The gatekeepers of mycobacterial survivability and pathogenesis.* Eur J Microbiol Immunol (Bp), 2020. **10**(4): p. 202-209.
19. van der Wel, N., et al., *M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells.* Cell, 2007. **129**(7): p. 1287-98.
20. Rambukkana, A., et al., *Role of alpha-dystroglycan as a Schwann cell receptor for Mycobacterium leprae.* Science, 1998. **282**(5396): p. 2076-9.
21. Kotteeswaran, G., C.J. Chacko, and C.K. Job, *Skin adnexa in leprosy and their role in the dissemination of M. leprae.* Lepr India, 1980. **52**(4): p. 475-81.

22. Martinez, T.S., et al., *Oral mucosa as a source of Mycobacterium leprae infection and transmission, and implications of bacterial DNA detection and the immunological status.* Clin Microbiol Infect, 2011. **17**(11): p. 1653-8.
23. Hatta, M., et al., *Distribution and persistence of Mycobacterium leprae nasal carriage among a population in which leprosy is endemic in Indonesia.* Trans R Soc Trop Med Hyg, 1995. **89**(4): p. 381-5.
24. Araujo, S., et al., *Unveiling healthy carriers and subclinical infections among household contacts of leprosy patients who play potential roles in the disease chain of transmission.* Mem Inst Oswaldo Cruz, 2012. **107 Suppl 1**: p. 55-9.
25. Sales, A.M., et al., *Leprosy among patient contacts: a multilevel study of risk factors.* PLoS Negl Trop Dis, 2011. **5**(3): p. e1013.
26. Richardus, J.H. and J.D. Habbema, *The impact of leprosy control on the transmission of M. leprae: is elimination being attained?* Lepr Rev, 2007. **78**(4): p. 330-7.
27. Organization, W.H., *Guidelines for the diagnosis, treatment and prevention of leprosy.* 2018.
28. El-Gendy, H., et al., *Leprosy Masquerading as Systemic Rheumatic Diseases.* J Clin Rheumatol, 2016. **22**(5): p. 264-71.
29. Georgiev, G.D.a.M., A.C., *Skin smears and bacterial index (BI) in multiple drug therapy leprosy control programmes: an unsatisfactory and potentially hazardous state of affairs.* International Journal of Leprosy, 1988. **56**: p. 101-104.
30. Bhat, R.M. and C. Prakash, *Leprosy: an overview of pathophysiology.* Interdiscip Perspect Infect Dis, 2012. **2012**: p. 181089.
31. Smith, W.C. and P. Saunderson, *Leprosy.* BMJ Clin Evid, 2010. **2010**.
32. G. H. Faget, R.C.P., F. A. Johansen, J. F. Dinan and C. G. Eccles, *The Promin Treatment of Leprosy. A Progress Report.* Public Health Reports (1896-1970), 1943. **58**(48).
33. Kar, H.K., V.N. Bhatia, and S. Harikrishnan, *Combined clofazimine- and dapsone-resistant leprosy. A case report.* Int J Lepr Other Mycobact Dis, 1986. **54**(3): p. 389-91.
34. Warndorff-van Diepen, T., *Clofazimine-resistant leprosy, a case report.* Int J Lepr Other Mycobact Dis, 1982. **50**(2): p. 139-42.
35. Kumar, A., A. Girdhar, and B.K. Girdhar, *A randomized controlled trial to compare cure and relapse rate of paucibacillary multidrug therapy with monthly rifampicin, ofloxacin, and minocycline among paucibacillary leprosy patients in Agra District, India.* Indian J Dermatol Venereol Leprol, 2015. **81**(4): p. 356-62.
36. Diana N. J. Lockwood, G.O.P., Saba Lambert, Vivek Vasudev Pai, Stephen L. Walker, *Safer and newer antimicrobial drugs for leprosy – time to test monthly ROM in an adequately powered randomised trial?* Leprosy Review, 2022.
37. Moet, F.J., et al., *Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: cluster randomised controlled trial.* BMJ, 2008. **336**(7647): p. 761-4.
38. Peter Steinmann, A.C., Ann Aerts, Sunil Anand, Mohammad Arif, Sarady Sao Ay, Tin Maung Aye, Tanja Barth-Jaeggi, Nand Lal Banstola, Chuda Mani Bhandari, David Blaney, Marc Bonenberger, Wim Van Brakel, Hugh Cross, V.K. Das, Ansarul Fahrudda, Nilanthi Fernando, Zaahira Gani, Helena Greter, Eliane Ignotti, Deus Kamara, Christa Kasang, Burkard Kömm, Anil Kumar, Sambath Lay, Liesbeth Mieras, Fareed Mirza, Beatrice Mutayoba, Blasdus Njako, Tiara Pakasi, Paul Saunderson, Bakhuti Shengelia, Cairns S. Smith, René Stäheli, Nayani Suriyarachchi, Tin Shwe, Anuj Tiwari, Millawage Supun D Wijesinghe, Jan Van Berkel, Bart Vander Plaetse, Marcos Virmond, Jan Hendrik

Richardus, *The Leprosy Post-Exposure Prophylaxis (LPEP) programme: update and interim analysis.* Leprosy Review, 2018. **89**(2): p. 102-116.
39. Ortuno-Gutierrez, N., et al., *Protocol, rationale and design of PEOPLE (Post ExpOsure Prophylaxis for LEprosy in the Comoros and Madagascar): a cluster randomized trial on effectiveness of different modalities of implementation of post-exposure prophylaxis of leprosy contacts.* BMC Infect Dis, 2019. **19**(1): p. 1033.
40. Boeree, M.J., et al., *A dose-ranging trial to optimize the dose of rifampin in the treatment of tuberculosis.* Am J Respir Crit Care Med, 2015. **191**(9): p. 1058-65.
41. Schoenmakers, A., et al., *PEP4LEP study protocol: integrated skin screening and SDR-PEP administration for leprosy prevention: comparing the effectiveness and feasibility of a community-based intervention to a health centre-based intervention in Ethiopia, Mozambique and Tanzania.* BMJ Open, 2021. **11**(8): p. e046125.
42. Blok, D.J., et al., *The long-term impact of the Leprosy Post-Exposure Prophylaxis (LPEP) program on leprosy incidence: A modelling study.* PLoS Negl Trop Dis, 2021. **15**(3): p. e0009279.
43. Merle, C.S., S.S. Cunha, and L.C. Rodrigues, *BCG vaccination and leprosy protection: review of current evidence and status of BCG in leprosy control.* Expert Rev Vaccines, 2010. **9**(2): p. 209-22.
44. Duthie, M.S., et al., *LepVax, a defined subunit vaccine that provides effective pre-exposure and post-exposure prophylaxis of M. leprae infection.* NPJ Vaccines, 2018. **3**: p. 12.
45. Duthie, M.S., et al., *A phase 1 antigen dose escalation trial to evaluate safety, tolerability and immunogenicity of the leprosy vaccine candidate LepVax (LEP-F1 + GLA-SE) in healthy adults.* Vaccine, 2020. **38**(7): p. 1700-1707.
46. Bryceson, A. and R.E. Pfaltzgraff, *Leprosy*. 3rd ed. Medicine in the tropics series. 1990, Edinburgh ; New York: Churchill Livingstone. vi, 240 p., 4 p. of plates.
47. Cambau, E., et al., *Antimicrobial resistance in leprosy: results of the first prospective open survey conducted by a WHO surveillance network for the period 2009-15.* Clin Microbiol Infect, 2018. **24**(12): p. 1305-1310.
48. Cole, S.T., et al., *Massive gene decay in the leprosy bacillus.* Nature, 2001. **409**(6823): p. 1007-11.
49. Singh, P., et al., *Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium lepromatosis.* Proc Natl Acad Sci U S A, 2015. **112**(14): p. 4459-64.
50. Adams, L.B., *Susceptibility and resistance in leprosy: Studies in the mouse model.* Immunol Rev, 2021. **301**(1): p. 157-174.
51. Frade, M.A.C., et al., *Semmes-Weinstein monofilament: A tool to quantify skin sensation in macular lesions for leprosy diagnosis.* Indian J Dermatol Venereol Leprol, 2021. **87**(6): p. 807-815.
52. da Silva, M.B., et al., *Latent leprosy infection identified by dual RLEP and anti-PGL-I positivity: Implications for new control strategies.* PLoS One, 2021. **16**(5): p. e0251631.
53. Gama, R.S., et al., *Prospects for new leprosy diagnostic tools, a narrative review considering ELISA and PCR assays.* Rev Soc Bras Med Trop, 2020. **53**: p. e20200197.
54. Scollard, D.M., et al., *The continuing challenges of leprosy.* Clin Microbiol Rev, 2006. **19**(2): p. 338-81.
55. Fite, G.L., *The staining of acid-fast bacilli in paraffin sections.* Am J Pathol, 1938. **14**(4): p. 491-507.

56. Deps PD, R.P., *Agentes Etiologicos e principal metodos pae detecçao do M. leprae.*, in *Hanseníase na prática clínica*. 2022
57. Radomski, N., et al., *The critical role of DNA extraction for detection of mycobacteria in tissues.* PLoS One, 2013. **8**(10): p. e78749.
58. Affolabi, D., et al., *Effects of decontamination, DNA extraction, and amplification procedures on the molecular diagnosis of Mycobacterium ulcerans disease (Buruli ulcer).* J Clin Microbiol, 2012. **50**(4): p. 1195-8.
59. Durnez, L., et al., *A comparison of DNA extraction procedures for the detection of Mycobacterium ulcerans, the causative agent of Buruli ulcer, in clinical and environmental specimens.* J Microbiol Methods, 2009. **76**(2): p. 152-8.
60. Laboratory, N.H.s.D.P.-C. *https://www.hrsa.gov/hansens-disease/diagnosis/skin-smears.html*. 2018.
61. Laboratory, N.H.s.D.P.-C. *https://www.hrsa.gov/hansens-disease/diagnosis/biopsy.html*. 2022.
62. Chambers, A.R. and K. Song, *Sural Nerve Biopsy*, in *StatPearls*. 2022: Treasure Island (FL).
63. Prevention, C.f.D.a.C.a. *https://www.cdc.gov/coronavirus/2019-ncov/testing/How-To-Collect-Anterior-Nasal-Specimen-for-COVID-19.pdf*. 2020.
64. Prevention, C.f.D.C.a. *https://www.cdc.gov/coronavirus/2019-ncov/downloads/lab/NMT-Specimen-Collection-Infographic.pdf*. 2020.
65. Prevention, C.f.D.C.a. *https://www.cdc.gov/coronavirus/2019-ncov/downloads/lab/NP-Specimen-Collection-Infographic.pdf*. 2020.
66. Corstjens, P., et al., *Fingerstick test quantifying humoral and cellular biomarkers indicative for M. leprae infection.* Clin Biochem, 2019. **66**: p. 76-82.
67. Woods, S.A. and S.T. Cole, *A rapid method for the detection of potentially viable Mycobacterium leprae in human biopsies: a novel application of PCR.* FEMS Microbiol Lett, 1989. **53**(3): p. 305-9.
68. Avanzi, C., et al., *Population Genomics of Mycobacterium leprae Reveals a New Genotype in Madagascar and the Comoros.* Front Microbiol, 2020. **11**: p. 711.
69. Braet, S.M., et al., *Investigating drug resistance of Mycobacterium leprae in the Comoros: an observational deep-sequencing study.* Lancet Microbe, 2022. **3**(9): p. e693-e700.
70. Cole, S.T., P. Supply, and N. Honore, *Repetitive sequences in Mycobacterium leprae and their impact on genome plasticity.* Lepr Rev, 2001. **72**(4): p. 449-61.
71. Braet, S., et al., *The repetitive element RLEP is a highly specific target for the detection of Mycobacterium leprae.* J Clin Microbiol, 2018. **56**(3).
72. Truman, R.W., et al., *Enumeration of Mycobacterium leprae using real-time PCR.* PLoS Negl Trop Dis, 2008. **2**(11): p. e328.
73. Beissner, M., et al., *Development of a combined RLEP/16S rRNA (RT) qPCR assay for the detection of viable M. leprae from nasal swab samples.* BMC Infect Dis, 2019. **19**(1): p. 753.
74. Manta, F.S.N., et al., *Development and validation of a multiplex real-time qPCR assay using GMP-grade reagents for leprosy diagnosis.* PLoS Negl Trop Dis, 2022. **16**(2): p. e0009850.
75. Cambau, E., et al., *Detection of antibiotic resistance in leprosy using GenoType LepraeDR, a novel ready-to-use molecular test.* PLoS Negl Trop Dis, 2012. **6**(7): p. e1739.
76. Saar, M., et al., *RLEP LAMP for the laboratory confirmation of leprosy: towards a point-of-care test.* BMC Infect Dis, 2021. **21**(1): p. 1186.

77. Dwivedi, P., et al., *Simultaneous detection and differentiation between Mycobacterium leprae and Mycobacterium lepromatosis using novel polymerase chain reaction primers.* J Dermatol, 2021. **48**(12): p. 1936-1939.
78. Sharma, R., et al., *Isolation of Mycobacterium lepromatosis and Development of Molecular Diagnostic Assays to Distinguish Mycobacterium leprae and M. lepromatosis.* Clin Infect Dis, 2020. **71**(8): p. e262-e269.
79. Martinez, A.N., et al., *PCR-based techniques for leprosy diagnosis: from the laboratory to the clinic.* PLoS Negl Trop Dis, 2014. **8**(4): p. e2655.
80. Collins, J.H., et al., *A Sensitive and Quantitative Assay to Enumerate and Measure Mycobacterium leprae Viability in Clinical and Experimental Specimens.* Curr Protoc, 2022. **2**(2): p. e359.
81. New Delhi: World Health Organization, R.O.f.S.-E.A. *A guide for surveillance of antimicrobial resistance in leprosy: 2017 update [Internet].* . 2017 update.
82. Aubry, A., et al., *Drug resistance in leprosy: An update following 70years of chemotherapy.* Infect Dis Now, 2022. **52**(5): p. 243-251.
83. van Hooij, A., et al., *Quantitative lateral flow strip assays as User-Friendly Tools To Detect Biomarker Profiles For Leprosy.* Sci Rep, 2016. **6**: p. 34260.
84. Espinosa, O.A., et al., *Accuracy of Enzyme-Linked Immunosorbent Assays (ELISAs) in Detecting Antibodies against Mycobacterium leprae in Leprosy Patients: A Systematic Review and Meta-Analysis.* Can J Infect Dis Med Microbiol, 2018. **2018**: p. 9828023.
85. Teles, S.F., et al., *Association between NDO-LID and PGL-1 for leprosy and class I and II human leukocyte antigen alleles in an indigenous community in Southwest Amazon.* Braz J Infect Dis, 2020. **24**(4): p. 296-303.
86. Duthie, M.S., et al., *Use of protein antigens for early serological diagnosis of leprosy.* Clin Vaccine Immunol, 2007. **14**(11): p. 1400-8.
87. Zhou, Z., et al., *Detection and Monitoring of Mycobacterium leprae Infection in Nine Banded Armadillos (Dasypus novemcinctus) Using a Quantitative Rapid Test.* Front Microbiol, 2021. **12**: p. 763289.
88. van Hooij, A., et al., *Prototype multi-biomarker test for point-of-care leprosy diagnostics.* iScience, 2021. **24**(1): p. 102006.
89. F.B. Neto, R.B.B.F.M.S.d.S., *Daily Moxifloxacin, Clarithromycin, Minocycline, and Clofazimine in Nonresponsiveness Leprosy Cases to Recommended Treatment Regimen.* International Journal of Tropical Diseases, 2020. **3**(2).
90. DS., R., *The SFG (solid, fragmented, granular) index for bacterial morphology.* Leprosy review, 1971. **42**(2): p. 96-97.
91. Unit., W.H.O.L., *Laboratory techniques for leprosy [Internet].* . 1986.
92. Adams LB, L.R., *Cultivation and Viability Determination of Mycobacterium leprae.*, in *The International Textbook of Leprosy*, G.T. Scollard DM, Editor. 2016.
93. Martinez, A.N., et al., *Molecular determination of Mycobacterium leprae viability by use of real-time PCR.* J Clin Microbiol, 2009. **47**(7): p. 2124-30.
94. Shepard, C.C., *The Experimental Disease That Follows the Injection of Human Leprosy Bacilli into Foot-Pads of Mice.* J Exp Med, 1960. **112**(3): p. 445-54.
95. Williams, D.L. and T.P. Gillis, *Drug-resistant leprosy: monitoring and current status.* Lepr Rev, 2012. **83**(3): p. 269-81.
96. Benjak, A., et al., *Phylogenomics and antimicrobial resistance of the leprosy bacillus Mycobacterium leprae.* Nat Commun, 2018. **9**(1): p. 352.

97. Singh, P., et al., *Genome-wide re-sequencing of multidrug-resistant Mycobacterium leprae Airaku-3.* Clin Microbiol Infect, 2014. **20**(10): p. O619-22.
98. Fernandez, J.D.P., et al., *Mycobacterium leprae and Mycobacterium lepromatosis Infection: a Report of Six Multibacillary Cases of Leprosy in the Dominican Republic.* Jpn J Infect Dis, 2022. **75**(4): p. 427-430.
99. Buhrer-Sekula, S., et al., *Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy.* J Clin Microbiol, 2003. **41**(5): p. 1991-5.
100. Trombone, A.P., et al., *Optimized protocols for Mycobacterium leprae strain management: frozen stock preservation and maintenance in athymic nude mice.* J Vis Exp, 2014(85).
101. Scollard, D., *Histopathological Diagnosis of Leprosy. Cleverson*

Teixiera Soares. Leprosy review, 2021.
102. Ridley, D.S., *The SFG (solid, fragmented, granular) index for bacterial morphology.* Lepr Rev, 1971. **42**(2): p. 96-7.
103. Shinde, V., et al., *VNTR typing of Mycobacterium leprae in South Indian leprosy patients.* Lepr Rev, 2009. **80**(3): p. 290-301.
104. Monot, M., et al., *Comparative genomic and phylogeographic analysis of Mycobacterium leprae.* Nat Genet, 2009. **41**(12): p. 1282-9.
105. Sakamuri, R.M., et al., *Population-based molecular epidemiology of leprosy in Cebu, Philippines.* J Clin Microbiol, 2009. **47**(9): p. 2844-54.
106. Fontes, A.N., et al., *Genotyping of Mycobacterium leprae present on Ziehl-Neelsen-stained microscopic slides and in skin biopsy samples from leprosy patients in different geographic regions of Brazil.* Mem Inst Oswaldo Cruz, 2012. **107 Suppl 1**: p. 143-9.
107. Kimura, M., et al., *Rapid variable-number tandem-repeat genotyping for Mycobacterium leprae clinical specimens.* J Clin Microbiol, 2009. **47**(6): p. 1757-66.
108. Lima, L.N.C., et al., *Genotyping comparison of Mycobacterium leprae isolates by VNTR analysis from nasal samples in a Brazilian endemic region.* Pathog Glob Health, 2018. **112**(2): p. 79-85.

CHAPTER II

The repetitive element RLEP is a highly specific target for the detection of *Mycobacterium leprae*

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LETTER

Leprosy, caused by *Mycobacterium leprae* (*M. leprae*), is a mutilating and highly stigmatized disease that still affects hundreds of thousands of new patients annually. The diagnosis relies entirely on clinical findings, per WHO guidelines, although confirmation of clinically doubtful presentations requires reliable diagnostic tools. Early detection and treatment interrupts transmission and prevents severely debiliting disease. Since 2001 the complete genome of *M. leprae* is available, which was the basis for several molecular techniques to detect *M. leprae* (1). Martinez *et al*. (2011) compared four different quantitative real-time PCR (qPCR) assays for leprosy diagnosis using skin biopsies (SBs) from patients (2). They concluded that the qPCR targeting 36 RLEP copies per genome, described by Truman *et al.* (2008) (3), was the most sensitive assay, presenting high sensitivity (100%) for multibacillary (MB; >5 lesions) patients and 84.6% sensitivity for paucibacillary (PB; <5 lesions) patients. Housman *et al.* (2015) tested the RLEP qPCR in both experimentally infected and non-infected armadillos and reported a false positivity rate of 40% (4), raising concerns about test specificity. The specificity might be affected by the presence of homologous sequences in other environmental and understudied *Mycobacterium* species, which could yield false positives (2). Alternatively, the high sensitivity also makes the assay more prone to contamination as a source of false positives, or the samples tested included true positives in whom leprosy had clinically not been correctly diagnosed, i.e. misclassification of test samples. Thus, our study aimed to revisit the specificity of the RLEP qPCR.

Specificity was first determined *in silico*; the RLEP qPCR primer & probe sequences were compared against the NCBI's non-redundant database using BLASTn (07/12/2017) (5), including 148 sequenced mycobacterial genomes from recent studies (6, 7). This did not identify any potential cross reactivity. Subsequently, specificity was experimentally tested. Among SBs from 28 non-endemic and 31 endemic non-leprosy controls tested, no RLEP qPCR amplification was observed. In addition, none of 61 isolates from different mycobacterial species, including the closely related *M. szulgai* and *M. haemophilum,* showed amplification for the RLEP qPCR. Confirming sensitivity, all 110 samples from clinically confirmed patients (10 SBs from MB patients and 91 slit skin smears, including 27 Acid Fast Bacilli (AFB) negative and 64 AFB positive), were positive with the RLEP qPCR. We notified the Institute of Tropical Medicine's Institutional Review Board about testing de-identified surplus diagnostic samples from patients from Brazil, Belgium and the Comoros, who had provided informed consent.

These results suggest 100% specificity of RLEP qPCR for *M. leprae*. However, due to the possible presence of homologous RLEP sequences in unidentified, unculturable, or understudied mycobacteria closely related to *M. leprae,* the reported specificity will always be provisional. The absence of identical primer/probe binding sites in the current NCBI's database decreases the probability that new mycobacterial species with homologous RLEP sequences will emerge. Our results suggest that false positives would more likely represent contamination issues. This study supports RLEP qPCR as the gold standard for laboratory confirmation for leprosy, even when sensitivity in PB samples is still imperfect.

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REFERENCES

1. Cole ST, et al.,. *Massive gene decay in the leprosy bacillus*. Nature, 2001. 409:1007-11.

2. Martinez AN, Ribeiro-Alves M, Sarno EN, Moraes MO.. *Evaluation of qPCR-based assays for leprosy diagnosis directly in clinical specimens.* PLoS Negl Trop Dis, 2011. 5:e1354.

3. Truman RW, Andrews PK, Robbins NY, Adams LB, Krahenbuhl JL, Gillis TP. *Enumeration of Mycobacterium leprae using real-time PCR*. PLoS Negl Trop Dis;, 2008. 2:e328.

4. Housman G, Malukiewicz J, Boere V, Grativol AD, Pereira LC, Silva Ide O, Ruiz-Miranda CR, Truman R, Stone AC. *Validation of qPCR Methods for the Detection of Mycobacterium in New World Animal Reservoirs.* PLoS Negl Trop Dis, 2015. 9:e0004198.

5. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. *Basic local alignment search tool.* J Mol Biol, 1990. 215:403-10.

6. Fedrizzi T, Meehan CJ, Grottola A, Giacobazzi E, Fregni Serpini G, Tagliazucchi S, Fabio A, Bettua C, Bertorelli R, De Sanctis V, Rumpianesi F, Pecorari M, Jousson O, Tortoli E, Segata N. *Genomic characterization of Nontuberculous Mycobacteria*. Sci Rep, 2017. 7:45258.

7. Tortoli E, Fedrizzi T, Meehan CJ, Trovato A, Grottola A, Giacobazzi E, Serpini GF, Tagliazucchi S, Fabio A, Bettua C, Bertorelli R, Frascaro F, De Sanctis V, Pecorari M, Jousson O, Segata N, Cirillo DM. *The new phylogeny of the genus Mycobacterium: The old and the news.* Infect Genet Evol, 2017. 56:19-25.

CHAPTER III

Minimally invasive sampling to identify leprosy patients with a high bacterial burden in the Union of the Comoros.

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ABSTRACT

The World Health Organization (WHO) endorsed diagnosis of leprosy (also known as Hansen's disease) entirely based on clinical cardinal signs, without microbiological confirmation, which may lead to late or misdiagnosis. The use of slit skin smears is variable, but lacks sensitivity. In 2017–2018 during the ComLep study, on the island of Anjouan (Union of the Comoros; High priority country according to WHO, 310 patients were diagnosed with leprosy (paucibacillary=159; multibacillary=151), of whom 263 were sampled for a skin biopsy and fingerstick blood, and 260 for a minimally-invasive nasal swab. In 74.5% of all skin biopsies and in 15.4% of all nasal swabs, *M. leprae* DNA was detected. In 63.1% of fingerstick blood samples, *M. leprae* specific antibodies were detected with the quantitative αPGL-I test. Results show a strong correlation of αPGL-I IgM levels in fingerstick blood and RLEP-qPCR positivity of nasal swabs, with the *M. leprae* bacterial load measured by RLEP-qPCR of skin biopsies. Patients with a high bacterial load (≥50,000 bacilli in a skin biopsy) can be identified with combination of counting lesions and the αPGL-I test. To our knowledge, this is the first study that compared αPGL-I IgM levels in fingerstick blood with the bacterial load determined by RLEP-qPCR in skin biopsies of leprosy patients. The demonstrated potential of minimally invasive sampling such as fingerstick blood samples to identify high bacterial load persons likely to be accountable for the ongoing transmission, merits further evaluation in follow-up studies.

AUTHOR SUMMARY

Leprosy is the oldest infectious disease known to humankind. We still do not succeed in curbing its transmission, with more than 200,000 new patients detected worldwide each year. Identifying persons with a high burden of bacteria is key to curb transmission. To identify these persons, bacteria are counted in invasive and painful samples like slit skin smears and skin biopsies. We evaluated whether we can use less invasive samples, like fingerstick blood or nasal swabs, to determine the bacterial load. We found that the level of antibodies against *M. leprae* (αPGL-I IgM) in fingerstick blood correlates well with the bacterial load determined in skin biopsies from the same leprosy patient. Therefore, a high level of antibodies against *M. leprae* in fingerstick blood might identify persons who pose a potential risk for transmission of leprosy and could be prioritized for contact screening, which is essential for control of the disease.

INTRODUCTION

According to the World Health Organization (WHO) the global leprosy (also known as Hansen's disease) prevalence has decreased to <1 patient per 10,000 population since the year 2000, based on which leprosy is eliminated as a public health problem. However, the annual global incidence has stabilized since 2006, with approximately 200,000 new leprosy patients reported worldwide each year[1], often with heterogeneous distribution in high incidence 'pockets'.

These persistently high incidence areas also occur in settings with solid leprosy control programs, where patients are diagnosed early and treated appropriately with highly effective multidrug therapy. Moreover, in some regions 30% of the leprosy patients occur in children[2], which supports that transmission continues unabatedly. Although transmission pathways of *Mycobacterium leprae* (*M. leprae*) are still not fully understood, evidence shows that the main transmission route appears to be through aerosols/droplets to and from the nasal and oral cavities, also skin-to-skin contact and shedding of bacteria into the environment may play a role[3-5]. An infected contact is thought to be genetically predisposed to progress to either the paucibacillary or multibacillary form of the spectrum, although the majority of infected individuals never develop clinically overt leprosy[6]. Progressing to paucibacillary disease (WHO operational classification: ≤5 lesions), is associated with a predominant protective Th1 type response, while multibacillary leprosy (WHO operational classification: >5 lesions) infection links with Th2 type response and high levels of anti-*M. leprae* antibodies against phenolic glycolipid I (PGL-I), which are ineffective at controlling this intracellular disease[7]. Untreated multibacillary patients are considered a likely source of transmission, probably even before they develop symptoms[8]. Diagnosis is entirely clinical as stated by the WHO guidelines, relying on the cardinal signs of leprosy. Once a leprosy patient starts multidrug therapy, it is assumed that chances of transmission are drastically reduced since the numbers of viable bacteria are quickly reduced [9].

Since the decreased attention for leprosy in the last century, many clinicians/health care workers lost their acumen to diagnose leprosy, leading to late- and missed diagnoses[10]. Microbiological confirmation, including measurement of the bacterial load, is not standardized nor endorsed by WHO for leprosy diagnosis. The bacterial load can be determined microscopically and by an *M. leprae* specific quantitative real-time PCR (qPCR), typically on slit skin smears or skin biopsies which both represent invasive clinical samples. Unfortunately, access to laboratories facilitating molecular techniques tends to be limited in leprosy endemic countries. Therefore, a low complexity lateral flow assay (LFA) utilizing up-converting reporter particles (UCP) was recently developed to quantitatively detect IgM antibodies against the *M. leprae* specific PGL-I (αPGL-I) in human serum[11] and fingerstick blood [12], with documented applicability in *M. leprae*- and *M. lepromatosis*-infected squirrels[13,14]. This particular αPGL-I UCP-LFA on fingerstick blood (further referred to as αPGL-I test) was found to correlate with the bacterial index (BI) as determined by slit skin smear microscopy and qPCR on slit skin smear [12,15]. Molecular tests have greater sensitivity for detection of *M. leprae* than microscopy[16].

In 2011, Martinez *et al.* concluded that the qPCR assay targeting a specific repetitive element (RLEP)[17] was more sensitive than qPCR assays using 16rRNA*/sodA/Ag 85B*[18]. In 2018, we resolved that the RLEP-qPCR is also highly specific and that its sensitivity is superior to classical BI determination on slit skin smear[16]. In 2020, αPGL-I IgM levels were compared to RLEP-qPCR of slit skin smear[19].

The ComLep study is a cross-sectional study conducted in the Comoros on the island of Anjouan where the average annual incidence rate exceeds 7/10,000. Active case finding is in place since 2008 through Mini Leprosy Elimination Campaigns, which consist of outreach skin clinics at the village level, where anyone with skin problems (including leprosy) is invited for a free dermatological consultation and where free treatment is provided for common minor skin ailments. In the present study, we correlated the RLEP-qPCR based bacterial burden in skin biopsies of leprosy patients as reference, with nasal swab RLEP-qPCR and host-based αPGL-I test, aiming for less invasive proxy indicators for the total bacterial burden of leprosy disease.

MATERIAL & METHODS

Ethics Statement

The protocol from the ComLep study (clinicaltrials.gov: NCT03526718) was approved by the institutional Review Board of ITM, by the Ethical Committee of the University of Antwerp (B300201731571) and by the Ethical Committee on the island of Anjouan. Written informed consent was obtained from each participant or from the parent/guardian of each participant under 18 years of age. For minors aged 12-17 years, additional signed assent from the minor was obtained before participation in the study. Participant were allowed to (selectively) refuse sampling.

Study participants

From January 2017 until January 2018, on the island of Anjouan (Union of The Comoros) leprosy patients were diagnosed within the ComLep study. Diagnosis was based on the so-called cardinal signs, i.e. a typical hypopigmented patch with loss of sensation and/or enlarged peripheral nerves. Patients with ≤ 5 lesions were classified as paucibacillary and >5 lesions as multibacillary, according to the WHO operational classification. In addition, whether a patient had ≥ 25 lesion was added as a variable, since having ≥25 lesions, will automatically classify the patient as either borderline borderline, borderline lepromatous or lepromatous according the dermatological aspect of the Ridley-Jopling classification. Only patients who provided both a skin biopsy and a fingerstick blood sample were included in this study (S1 Fig). A contra-indication for a skin biopsy was a single lesion occurring in the face. Nasal swabs were collected in addition to these samples. For multibacillary patients who provided a slit skin smear the BI was determined by microscopy.

Modified Maxwell DNA extraction

The 4 mm skin biopsy and the nasal swab were stored directly after sampling in 1ml of Disolol (ethanol denatured with 1% isopropanol and 1% methyl ethyl ketone) in screw cap vials, which were shipped to ITM. Upon arrival in the laboratory the skin biopsies were manually grinded with mortar and pestle in 1ml PBS. The obtained skin biopsy suspensions and the nasal swab were treated with an inhouse lysis buffer (1.6 M GuHCl, 60 mM Tris pH 7.5, 1% Triton X-100, 60 mM EDTA, Tween-20 10%) followed by DNA extraction using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit, as described by the manufacturer.

qPCR assay for *M. leprae* detection

The *M. leprae* bacterial chromosome contains a family of dispersed repeats (RLEP) of variable structure and unknown function. The repetitive RLEP sequence is highly conserved. Thirty-seven copies of RLEP exist in the chromosome, each containing an invariant 545-bp core flanked in some cases by additional segments ranging from 44 to 100 bps. The qPCR detects the *M. leprae*- specific RLEP target. The qPCR assay targets 36 out of the 37 RLEP copies present in the *M. leprae* genome, yielding a highly sensitive test [17]. This RLEP-qPCR assay also has proven high specificity [16]. RLEP-qPCR was done for each sample in analytical triplicate following published protocols, using the StepOnePlus cycler [17]. To monitor for false negative results we included an internal positive control (Universal Exogenous qPCR Positive Control (Eurogentec, Belgium)) labelled with a different fluorescent probe than the probe detecting the RLEP target, which amplifies independently from the main RLEP-qPCR, to rule out qPCR inhibition in the sample.

Quantification was done by adding to each qPCR run a serial dilution of *M. leprae* reference strain NHDP ($3x10^6$-30 RLEP copies) (BEI: ref. number 19350). Based on the Cq-value, slope of the regression line and Y-intercept, the StepOnePlus Software v2.3 provides automatically the RLEP copy number per added template as starting quantity (SQ), to determine the amount of mycobacterial DNA present in a sample. Subsequently, the bacterial load (BL) of the samples was calculated by BL=(SQ x [volume of DNA extract/volume of template])/36 RLEP copy numbers.

αPGL-I UCP-LFA

The capillary fingerstick blood collected with a disposable 20 µl Minivette collection tubes (Heparin coated; Sarstedt), was diluted 1:50 by immediate mixing with 980µl assay buffer. The buffer was supplemented with 1% (*v*/v) Triton X-100 (100 mM Tris pH 8, 270 mM NaCl, 1% (w/v) BSA) to lyse blood cells. The diluted fingerstick blood sample (50µl) was flowed on lateral flow strips the same day after transportation to the central laboratory at ambient temperature. The lateral flow strips were transported to LUMC. By using an anti-IgM UCP reporter conjugate, the human αPGL-I IgM antibodies were detected as described in Corstjens et al. (2019) [12]. UCP materials ($NaYF_4$:YB3+,Er3+ polyacrylic-acid coated nano-sized particles, 980 nm excitation and 550 nm emission) were obtained from Intelligent Material Solutions, Inc. (Princeton, NJ,

USA). A Packard FluoroCount microtiterplate reader compatible with the UCP technology[20], was used to analyse the lateral flow strips. The results were displayed as the ratio value (R) between Test and Flow-Control signal based on relative fluorescence units (RFUs) measured at the respective lines. For this cohort, the αPGL-I UCP-LFA the R-value threshold was set at 0.29 according to and determined as described in previous studies [12].

Analytical controls

For molecular analyses, on each sampling day, (negative) environmental control swab samples were collected, to rule out contamination during sampling. A positive (suspension of mouse footpad infected with *M. leprae* Thai 53) and a negative extraction control (water) were extracted in parallel with the clinical samples in each run to check extraction performance and to rule out contamination during the extraction procedure. An internal qPCR control, a positive and negative (water) DNA qPCR control were run simultaneously with the samples to check performance of the qPCR and to rule out DNA contamination during the qPCR procedure.

For serological analyses, at the start of the study non-endemic - and endemic (non-diseased health care staff) control sera were included for the αPGL-I test.

Statistical analyses

Data from the assays were log transformed (zero values were replaced by the minimum value for that variable divided by two). Data obtained for the different laboratory assays were evaluated using parametric hypothesis tests. Comparing a quantitative result between the two groups was carried out using the Welch t-test. The difference between months on treatment and the binary outcome of assays, was evaluated with the non-parametric Mann-Whitney U test. The alternative hypothesis stating significant differences between two outcomes was accepted at a significance level of $\alpha = .05$.

The multiple regression models modelling bacterial load versus αPGL-I R-values used the 10-based logarithm of the bacterial load (determined by RLEP-qPCR) as dependent variable, with the 10-based logarithm of the R-values of αPGL-I test as the independent, continuous variable, and a grouping variable splitting the population into 3 groups: i) patients with a negative nasal swab and less than 25 lesions, ii) patients with either a positive nasal swab or ≥ 25 lesions, iii) patients with both a positive nasal swab and ≥ 25 lesions. To analyse whether the effect of R-value of the αPGL-I test on the bacterial load was uniform across the 3 groups, the interaction between αPGL-I R-values and nasal swab group was tested for significance. The inclusion of the different factors in the multiple regression model was based on a simple linear regression, modelling the effect of each factor on the log10(bacterial load in skin biopsy). Significant factors were entered into a multiple linear regression model (S1 Table). Subsequently, this regression model to predict the number of the bacilli was simplified using stepwise-forward selection based on the Akaike information criterion (AIC).

Patients having ≥50,000 bacilli in a 4mm biopsy were further referred to as high bacterial load (HBL) patients. To evaluate if it was possible to predict the odds of being a HBL-patient,

sensitivity and specificity were calculated for the three independent variables (number of lesions, αPGL-I R-value and nasal swab positivity) separately. For the presence of ≥25 lesions, for the nasal swab positivity by RLEP qPCR and for the continuous αPGL-I R-value, a ROC-curve was constructed, and the optimal cut-off value was determined by the Youden index[21], which was 0.81. Subsequently, 2x2 tables were constructed for the predicted versus observed outcome for each binary independent variable. Furthermore, to evaluate whether combining variables would increase the power of predicting the odds of being an HBL-patient, two multiple logistic regressions with presence of ≥25 lesions (yes or no), αPGL-I R-value (as a continuous variable) and nasal positivity (yes or no).

All analyses were conducted with R version 3.5.0 for Windows (The R foundation, Vienna, Austria).

RESULTS

From January 2017 until January 2018, 310 (paucibacillary=159;multibacillary=151) leprosy patients were diagnosed. For 263 (84.8%) patients, sampling was complete (fingerstick blood samples and skin biopsies), with 260 providing a nasal swab from both nostrils instead of a nasopharyngeal swab. Based on clinical examination, 117 patients were classified as paucibacillary and 146 as multibacillary leprosy. Of these, 137 multibacillary patients provided a slit skin smear, of which 62 had a BI >0. At the time of sampling, 53 of the patients (paucibacillary=18; multibacillary=35) had received no treatment, versus 210 (paucibacillary=99; multibacillary=111) who started their treatment (Table III.1). Female patients, patients under 17 years old and patients with no affected nerves are more likely to be paucibacillary (Table III.1). All environmental controls tested negative with qPCR, as did the different positive and negative controls included in the DNA extraction and molecular assays, suggesting accurate qPCR results. The endemic and non-endemic controls sera for the αPGL-I test all tested negative.

Table III.1: Representation of the patients included in this study according to different variables.

	Number of patients		% patients included in the study
Patients included in the study July 2017-January 2018	310		
Patients for whom sampling was complete (skin biopsy*, fingerstick blood)	263/310		84.8%
Patients for whom sampling was complete including nasal swab	260/310		83.9%
Classification			
Paucibacillary	117/263		44.5%
Multibacillary	146/263		55.5%
	Paucibacillary (%)	Multibacillary (%)	OR (95%CI)
Sex			
Female	60/117 (51.3)	54/146 (37.0)	1.79 (1.09 , 2.94)
Age (years)			
≤17	75/117 (64.1)	64/146 (43.8)	2.29 (1.39 , 3.77)
Number of lesions			
≥25	0/117 (0.0)	57/146 (39.0)	NA
Affected nerves			
0	49/117 (41.9)	21/146 (14.4)	4.29 (2.38 , 7.74)
Degree of disability			
0	117/117 (100)	125/146 (85.6)	NA
1		11/146 (7.5)	
2		10/146 (6.8)	
Treatment at sampling timepoint			
Treatment started	99/117 (84.6)	111/146 (76.0)	1.73 (0.92 , 3.26)

** Incomplete sampling was due to either a single lesion occurring in the face, which was a contra-indication for a skin biopsy, or due to (selective) refusal. OR: odds ratio, 95% CI: 95% confidence interval.*

Determination of bacterial load with RLEP-qPCR in skin biopsies

Of 263 skin biopsies, 196 (74.5%) tested positive for *M. leprae* DNA with the RLEP-qPCR (Table III.2). Of the paucibacillary patients 79/117 (67.5%) tested positive, versus 117/146 (80.1%) of multibacillary patients (Table III.2). As expected, bacterial load detected in skin biopsies was significantly higher in multibacillary patients (median: 2601.5 bacilli ; mean of log10(bacilli): 3.872) than in paucibacillary patients (median: 132.0 bacilli; mean of log10(bacilli): 2.12) (Fig III.1A; p=1.7e-10). Of the BI-negative multibacillary patients 51 out of 75 (68.0%) tested positive for the presence of *M. leprae* DNA in their skin biopsy (Table III.2). In all except one of the 62 BI-positive patients, *M. leprae* DNA was detected (98.4%). For 9 multibacillary patients no slit skin smear, and therefore no BI was available (Table III.2). There was no significant difference between the bacterial load detected in skin biopsies of paucibacillary and BI-negative multibacillary patients (p=0.7835). At the time of sampling, patients in whose skin biopsy no *M. leprae* DNA was detected, had on average been treated longer compared to patients with a detectable amount of *M. leprae* DNA in their skin biopsy (Fig. III.2A; p=0.005).

Nasal swab positivity determined with RLEP-qPCR

Of 260 nasal swabs, only 40 (15.4%) tested positive for *M. leprae* DNA with the RLEP-qPCR (Table III.1). The bacterial load detected in the nasal swabs was significantly higher in multibacillary patients than in paucibacillary patients (Fig III.1B; p=6e-07). Only 3 out of the 116 paucibacillary patients (2.6%) tested nasal swab positive, compared to 37/144 (25.7%) multibacillary patients (Table III.1). Of the BI-negative multibacillary patients, 1/74 (1.4%) tested positive for *M. leprae* DNA in their nasal swab (Table III.2), compared to 36/61 (59.0%) of the BI-positive (Table III.2). There was no significant difference in bacterial load in nasal swab of paucibacillary and BI-negative multibacillary patients (p=0.9796). However, patients in whose nasal swab no *M. leprae* DNA was detected, tended to have been treated longer prior to sampling compared to patients with a detectable amount of *M. leprae* DNA in their nasal swab (Fig. III.2B; p =0.0071).

Level of αPGL-I determined with the αPGL-I test

Fingerstick blood samples of 166/263 (63.1%) patients tested positive for αPGL-I, including 62/117 (53.0%) paucibacillary patients and 104/146 (71.2%) multibacillary patients (Table III.2). The level of αPGL-I was significantly higher in multibacillary (median Ratio (R)-value: 0.9; mean log10(R-value): -0.01) than in paucibacillary (median R-value: 0.3; mean log10(R-value): -0.56) patients (Fig III.1C; p=2.6e-08). For the BI-negative multibacillary patients 42/75 (56.0%) tested positive for αPGL-I whereas of the 62 BI-positive multibacillary patients, all but two tested positive for αPGL-I (96.8%). There was no significant difference between the levels of αPGL-I R-value detected in fingerstick blood samples of paucibacillary and BI-negative multibacillary patients (p=0.3298). Patients with a negative result for the presence of systemic αPGL-I had not been treated longer prior to sampling than patients with a positive result (Fig. III.2C; p=0.36).

Table III.2. Laboratory assay results.

	Paucibacillary			Multibacillary									
				BI=0*			BI>0*			BI=NA*			Total
	N_{Pos}	N_{Neg}	(%)	N_{Pos}	N_{Neg}	(%)	N_{Pos}	N_{Neg}	(%)	N_{Pos}	N_{Neg}	(%)	(%)
Skin biopsy RLEP-qPCR	79	38	**67.5%**	51	24	68.0%	61	1	98.4%	5	4	55.6%	**80.1%**
Nasal swab RLEP-qPCR	3	113	**2.6%**	1	73	1.4%	36	25	59.0%	0	9	0.0%	**25.7%**
Fingerstick blood αPGL-I UCP-LFA	62	55	**53.0%**	42	33	56.0%	60	2	96.8%	2	7	22.2%	**71.2%**

**BI = bacterial index as determined by microscopy on a skin slit smear; NA = not available, Multibacillary as per WHO clinical definition; Paucibacillary as per WHO clinical definition; N_{neg} = number of negatives for the respective assay; N_{pos} = number of positives for the respective assay*

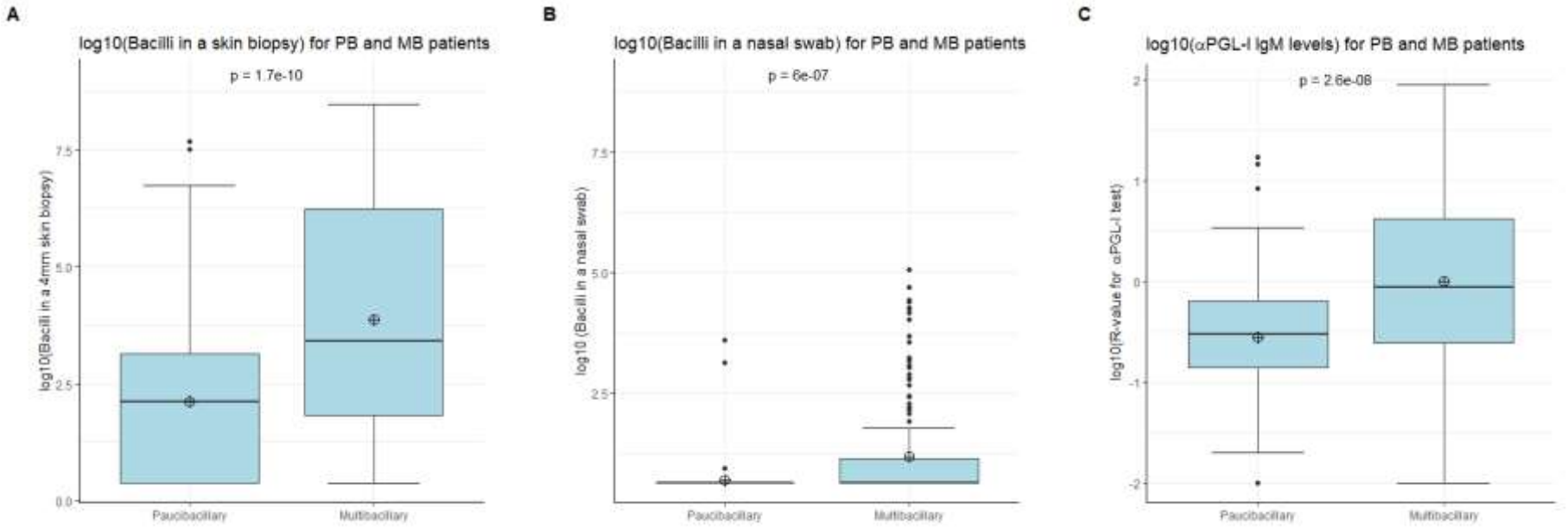

Figure III.1: Difference in outcome measure of the assays between paucibacillary and multibacillary patients as per WHO operational classification. (A) Outcome measure of RLEP-qPCR on skin biopsies as determined by the bacterial load in a 4mm skin biopsy of paucibacillary and multibacillary patients as per WHO operational classification. (B) Outcome measure of RLEP-qPCR as determined by the bacterial load in nasal swabs of paucibacillary and multibacillary patients. (C) Outcome measure of the αPGL-I test on fingerstick blood as measured by ratio (R) value, being relative fluorescence units measured at test line divided by the signal measured at the flow-control line of paucibacillary and multibacillary patients.

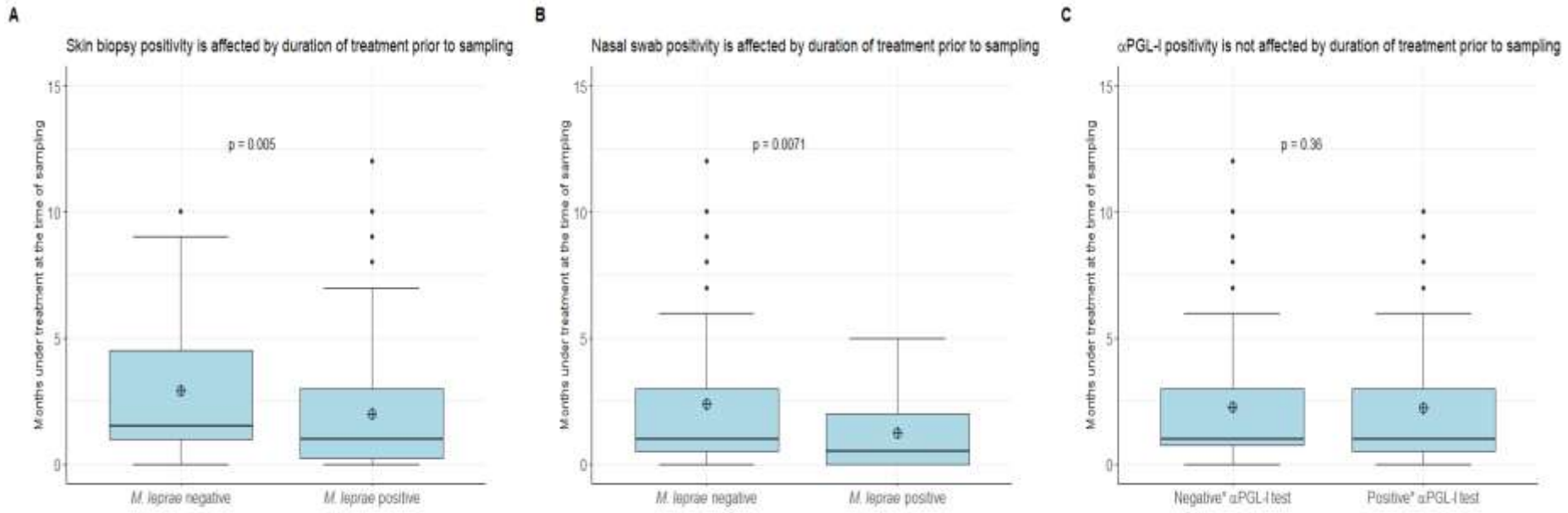

*Figure III.2: Patients with a negative assay result versus patients with a positive assay result with regard to how long they had been taking multidrug therapy prior to sampling. (A) Patients in whose skin biopsy no M. leprae DNA was detected, had on average been treated longer prior to sampling compared to patients with a detectable amount of M. leprae DNA in their skin biopsy (B) Patients in whose nasal swab no M. leprae DNA was detected, tended to have been treated longer prior to sampling compared to patients with a detectable amount of M. leprae DNA in their nasal swab (C) The presence of systemic αPGL-I is not affected by duration of treatment prior to sampling. R-values for the αPGL-I test on fingerstick blood.*Negative/positive means R-value < infection threshold and R-value ≥ infection threshold respectively.*

Regression model of the αPGL-I test with the bacterial load determined by RLEP-qPCR

To study the relationship between the αPGL-I IgM levels (measured as R-value of the αPGL-I test) and the bacterial load in skin biopsies, we fitted a multiple linear regression model, including 263 patients (Fig III.3). The patients were split in three groups: i) patients with a negative nasal swab and less than 25 lesions, ii) patients with either a positive nasal swab or ≥25 lesions, iii) patients with both a positive nasal swab and ≥ 25 lesions. We tested whether the αPGL-I R-value had an effect on the bacterial load, and if this effect was the same across the 3 groups. We observed an significant relation of the αPGL-I R-value and the bacterial load in a skin biopsy (Supporting information Table III.S2). This relation varied by group (p= 0.01346 for interaction between group and αPGL-I R-values), with the strongest association in the group with either a positive nasal swab or ≥ 25 lesions (Table III.3). Exact effect sizes are listed in Table III.3. None of the conclusions regarding significance and effect sizes were altered by adding months of treatment prior to sampling to the model.

Out of 263 patients, 64 had a bacterial load in a skin biopsy of ≥50,000 bacilli, further referred to as high bacterial load (HBL) patients. Of all the 64 HBL-patients that provided a fingerstick blood sample, 63 tested positive for αPGL-I test (R-value ≥0.29) , and 60 of those had a αPGL-I R-value ≥0.81. All HBL-patients except one provided a nasal swab, of which 34 (54.0%) contained *M. leprae* DNA. The 29 HBL-patients with a negative result for the nasal swab tended to have taken treatment longer at the time of sampling than the nasal swab positive HBL-patients (p=0.03006). Of the same 64 HBL-patients, 57 provided a slit skin smear, including 52 (91.2%) with a mean BI-value >0 (Table III.4). Out of the 64 HBL-patients, 7 (10.9%) were paucibacillary patients, including one with infiltrated lesions (Table III.4).

Table III.3. Linear relationship between log10(αPGL-I R-value) and log10 (bacterial load in a skin biopsy) for each of the three groups.

	Y-intercept	Slope	Effect Size F-statistic	P-value	Residual standard error	Adjusted R^2	Pearson's r
Group 1: < 25 lesions and Neg. nasal swab	2.65 (95%CI: 2.51- 2.79)	0.88 (95% CI: 0.71-1.05)	24.4	P= 1.696e-06	1.63 (192 df)	0.11	0.34
Group 2: ≥25 lesions or Pos. nasal swab	3.72 (95%CI: 3.46-3.99)	1.83 (95% CI: 1.55-2.10)	40.97	P= 2.609e-07	1.58 (34df)	0.53	0.74
Group 3: ≥25 lesions and Pos. nasal swab	6.50 (95%CI: 6.21-6.78)	0.82 (95% CI: 0.55-1.10)	8.543	P= 0.006792	0.85 (28 df)	0.21	0.46

** The y-intercept, slope, effect size, p-value, residuals standard error, adjusted R^2 and Pearson's r are given for the linear regression between log10(αPGL-I R-value) and log10 (bacillary load in skin biopsy) for each group*

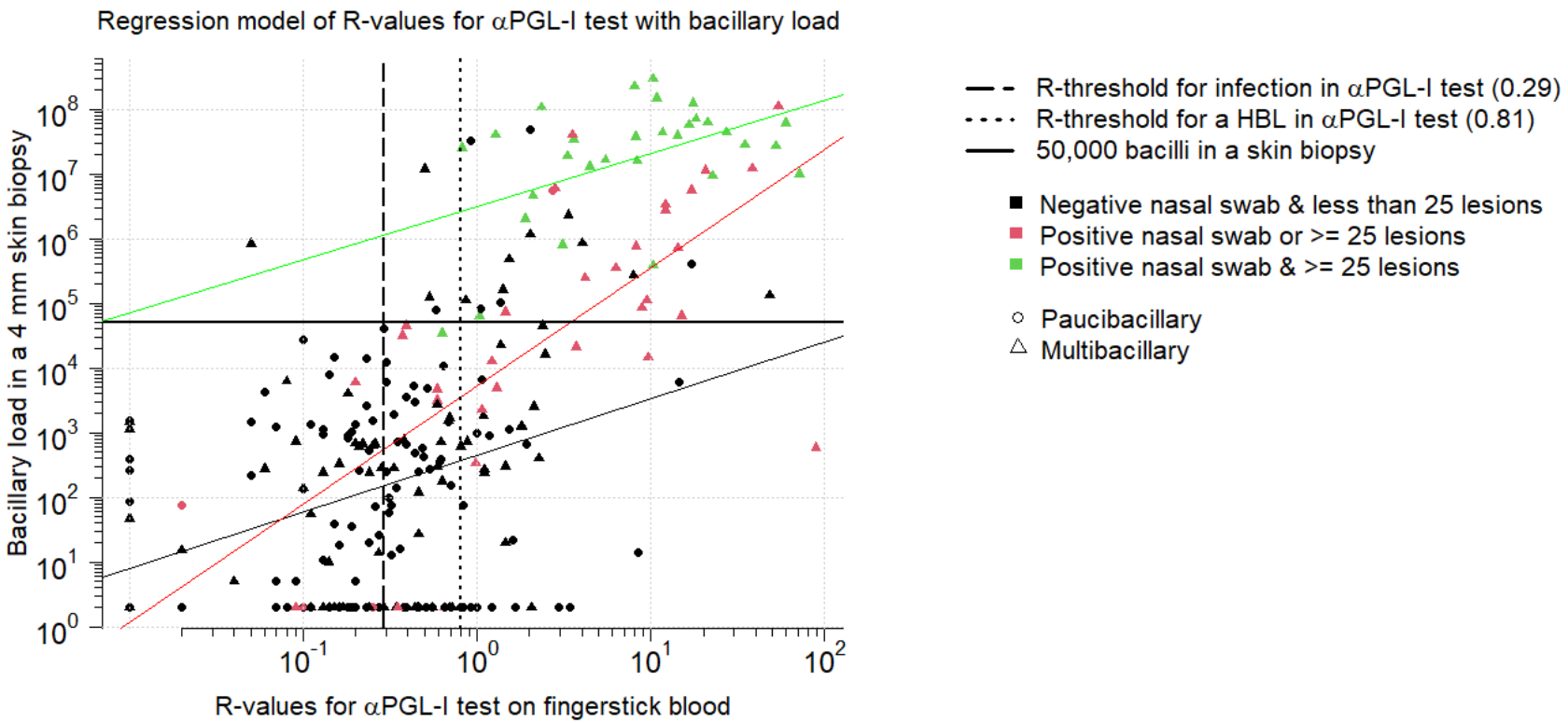

Figure III.3: Multiple regression model to estimate the bacterial load in skin biopsies based on the R-value of αPGL-I test, the number of lesions and the nasal swab qPCR result. R-values for the αPGL-I test on fingerstick blood; HBL = high bacterial load (≥50,000 bacilli in a skin biopsy); multibacillary as per WHO operational classification; paucibacillary as per WHO operational classification

Table III.4: Characteristics and treatment of the 64 high bacterial load (HBL) patients.

	Number of HBL-patients	
WHO operational classification		
Paucibacillary	7/64	
Multibacillary	57/64	
	Paucibacillary	**Multibacillary**
New case /Relapse		
New case	7/7	56/57
Relapse/reinfection*	0/7	1/57
αPGL-I test		
R-value <0.81	1/7	3/57
R-value ≥0.81	6/7	54/57
Slit skin smear		
BI N.A.	7/7	0/57
BI negative	0/7	5/57
BI positive	0/7	52/57
Number of lesions		
<25	7/7	15/57
≥25	0/7	42/57
Infiltrated lesions on clinical exam		
no	6/7	37/57
yes	1**/7	20/57
Treatment		
Paucibacillary treatment	6/7	0/57
Multibacillary treatment	1**/7	57/57
Treatment follow-up		
Completed	7/7	50/57
Lost to follow-up	0/7	7/57

*R-values for the αPGL-I test on fingerstick blood; BI = bacterial index as determined by microscopic examination of a skin-slit smear; Multibacillary as per WHO operational classification; Paucibacillary as per WHO operational classification *Relapse: the patient was sampled at a second episode of disease, either relapse or reinfection (indistinguishable in this study).** One PB patient was treated with MB treatment (12 months), due to the presence of infiltrated lesions. The nasal swab positive paucibacillary HBL-patient is not the paucibacillary HBL-patient with infiltrated lesions.*

Identifying HBL-patients.

The separate analysis of the three independent variables indicates that based solely on the clinical feature (≥25 lesions) a HBL-patient can be identified with 65.5% sensitivity and 92.4% specificity. The highest sensitivity (93.8%) for identifying a HBL-patient is obtained at αPGL-I R-value ≥0.81, however with a reduced specificity of 80.9%. The nasal swab result as predictor for HBL has the lowest sensitivity (54.0%) with the highest specificity (97.0%) (Table III.5). Combining predictors, two models resulted both in an AUC of 0.93; among these two models

we chose the simplest one, which includes having ≥25 lesions and the αPGL-I R-value as predictors, which increases sensitivity with 20% in comparison to solely counting lesions. Nasal swab positivity increased the specificity of HBL patient identification at the cost of sensitivity; overall the addition of nasal swab results did not improve the AUC (p-value= 0.99) (Table III.6).

Table III.5: Evaluation of the independent predictors for being an HBL-patients, with 2X2 tables and sensitivity/specificity.

	Number of lesions		αPGL-I R-value		Nasal swab RLEP-qPCR	
	<25	≥25	<0.81	≥0.81	Negative	Positive
Non HBL-patients	184	15	161	38	191	6
HBL-patients	22	42	4	60	29	34
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
	65.6%	92.4%	93.8%	80.9%	54.0%	97.0%

HBL= high bacterial load (≥50,000 bacilli in a skin biopsy); αPGL-I R-value= ratio (R) value being the relative fluorescence units measured at test line divided by the signal measured at the flow-control line of the αPGL-I test.

Table III.6: Multiple logistic regression models to predict being an HBL-patient

Logistic regression	Variables	AUC	95 % Confidence interval	Youden index	Sensitivity Youden index	Specificity Youden index
Multiple logistic regression	≥25 lesions and αPGL-I R-value	93.1%	89.4% - 96.8%	170.1	93.7%	77.4%
	≥25 lesions, αPGL-I R-value and nasal swab positivity	93.1%	89.2% - 96.9%	171.6	74.6%	98.0%

αPGL-I R-value= ratio (R) value being the relative fluorescence units measured at test line divided by the signal measured at the flow-control line of the αPGL-I test; AUC = area under the curve

DISCUSSION

Our findings confirm a strong correlation between αPGL-I IgM levels and the *M. leprae* bacterial load as measured by RLEP-qPCR in skin biopsies. To our knowledge this is the first assessment of the correlation of αPGL-I levels with a quantitative molecular measurement in a skin biopsy taken as a proxy for the bacterial load in a leprosy patient. Although a precise determination of the bacterial load by αPGL-I IgM levels is not possible, a range can be estimated. In 2019 Corstjens *et al* [12] demonstrated that the quantitative αPGL-I test results correlated well with the BI of multibacillary patients, and Tio Coma *et al*[19] extended this finding using nasal swab and slit skin smear of contacts and patients in Bangladesh.

The RLEP-qPCR on skin biopsies was able to confirm 74.5% of all clinically diagnosed leprosy patients, 80.1% of the multibacillary patients, and 98.4% of all BI positive patients. The RLEP-qPCR on nasal swab detected only 15.4% of all leprosy patients, 25.7% of the multibacillary patients, and 60.7% of all BI-positive patients, which is in line with previous data in an Asian cohort [19], yet is 41.2% and 49.6% lower for the paucibacillary and multibacillary patients respectively than in the study performed in non-treated patients conducted by Araujo *et al*[22]. The αPGL-I test on fingerstick blood of clinically diagnosed patients was positive in 63.1% of all patients included, 71.2% of the multibacillary patients, and increasing to 96.8% in those patients with a positive BI. These findings are in line with the genetic predisposition for T cell driven paucibacillary or B cell driven multibacillary leprosy. While the αPGL-I test specificity for leprosy could not be estimated in this cohort, given the absence of a large control group of individuals without leprosy, the αPGL-I IgM is acknowledged to be a marker of *M. leprae* infection rather than disease. The advantage of using the αPGL-I test on fingerstick blood to support clinical diagnosis, is that this test is minimally invasive, user-friendly and can be performed in remote laboratories such as in the Union of the Comoros, where no specialized facilities are present. Although the RLEP-qPCR on skin biopsies has overall better sensitivity than the αPGL-I test on fingerstick blood and RLEP-qPCR on nasal swab, the downside of using skin biopsies as a confirmation method, is the invasiveness of the sampling, for which local anaesthesia is necessary and a scar remains. Moreover, to avoid false positives, careful processing of the samples in advanced molecular laboratories needs to be ensured.

Avoiding misclassification using non-invasive sampling. Untreated leprosy, particularly lepromatous cases, can lead to serious irreversible nerve damage, often resulting in significant disfigurement and disabilities. Treatment is usually based on the spectral phenotype of the disease. Leprosy presents as a spectral disease, which is more complicated than paucibacillary/multibacillary determination, as demonstrated by the Ridley-Jopling classification for which histopathology is crucial[23]. The WHO operational classification, based on counting of lesions as a diagnostic method, has its shortcomings. In this study a negative BI distinguished a group of multibacillary patients whose assay results resembled paucibacillary patients. Also, 7 (10.2%) of the 64 HBL-patients (bacterial load in skin biopsy ≥50,000) would classify as paucibacillary patients following WHO operational classification. The leprosy control team decided at time of clinical diagnosis to categorize one of them as an multibacillary because of the presence of infiltrations. A second paucibacillary patient additionally had a positive nasal swab, which may be transient[24]. Hence, fingerstick blood αPGL-I testing in addition to counting lesions seem to improve differentiation within the spectrum of multibacillary leprosy patients. Ultimately, improve classification may guide more appropriate treatment.

Identification of high risk index cases is a critical knowledge gap. For leprosy it is extremely difficult to identify high risk index cases based on secondary cases as the incubation period of leprosy is exceptionally long (on average 5 years and can take up to 20 years or longer). Identification of high risk index cases is key to curb transmission, and therefore this remains an important knowledge gap, as identified during the COR-NTD conference (National Harbor, 2019). That multibacillary patients are primarily responsible for *M. leprae* transmission has been demonstrated several times[25-27] and Sales *et al.* found that patients with a positive BI were four times more likely to transmit the disease to their contact in comparison with multibacillary patients with a negative BI and eight times more likely with a BI>3[28]. To obtain a BI score, an invasive sample has to be taken. While αPGL-I IgM is acknowledged to be a biomarker of *M. leprae* infection rather than disease, our data confirm that in this cohort in the Union of the Comoros high αPGL-I IgM levels are indicative for a higher bacterial load, which is in line with findings of van Hooij *et al*. in 2017[15], where they demonstrate that αPGL-I IgM levels correlate with BI determined by microscopy. By applying arbitrary thresholds, an αPGL-I R-value of 0.29 could indicate tentative *M. leprae* infection, while a αPGL-I threshold of 0.81 (in addition to counting lesions) would allow to identify HBL-patients who may pose an increased risk for transmission. Adding the nasal swab, which may also be transiently positive, does not result in extra power for identifying HBL-patients. In the PEOPLE study (clinicaltrials.gov: NCT03662022) ongoing investigation for (close contact) secondary cases will allow to test the hypothesis that high risk index cases of *M. leprae* can be identified with non-invasive sampling. This will help resolve whether they should be prioritized for extensive contact screening beyond the household, to possibly provide prophylactic treatment to these contacts.

Limitations. Our patients were sampled at variable intervals since the start of treatment limiting our ability to determine diagnostic sensitivity at baseline for the different tests. To control for

potential bias, we added this variable to the regression models as a covariate. However, it was nowhere significant, neither in the full model with the interaction between antibodies response and different groups, nor in the linear regression models for the three separate groups (results not shown). None of the conclusions regarding significance and effect sizes were altered by adding months of treatment prior to sampling to the models. Therefore, the month of treatment was not included as a covariate in any of the models, and it is very unlikely that different treatment intervals would bias the results.

In this study we lack a non-exposed population control for the αPGL-I test, although we incorporated (non-)endemic controls sera. Inclusion of more non-diseased endemic controls may allow to identify titers indicative of (multibacillary) disease versus latent infection.

Way forward. The extension of the ComLep cohort in the ongoing PEOPLE study, including door-to-door screening for leprosy in highly endemic villages allows to identify the best predictors of transmission to close contacts. Additionally, the genotypic comparison of *M. leprae* can confirm transmission chains. Correlation with our findings on the bacterial burden may help identify high risk index cases and their characteristics, and allow us to test whether leprosy patients with higher outcome in the αPGL-I test, e.g. αPGL-I test R-value above 0.81, have significantly more secondary cases than patients with lower αPGL-I levels in a certain time frame. The ongoing adaptation of the αPGL-I, full integration of the test in an individually wrapped cassette and improved availability of readers will facilitate actual Point-of-Care testing.

In conclusion, improved approaches for microbiological confirmation of *M. leprae* utilizing less invasive sampling are desired and shown here to be feasible. The bacterial load is not routinely measured for leprosy patients, although it is a known correlate of infectiousness[28]. Counting the number of lesions together with the quantitative αPGL-I IgM levels can be used as a proxy for the bacterial load in leprosy patients and to better classify patients along the clinical leprosy spectrum. Ongoing studies (i.e. PEOPLE study) are expected to provide further evidence whether counting lesions and αPGL-I test on fingerstick blood can identify high risk index cases, who can then be prioritized for extensive contact screening beyond the household.

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REFERENCES

1. Reibel, F., Cambau, E., & Aubry, A., *Update on the epidemiology, diagnosis, and treatment of leprosy.* Med Mal Infect*,* 2015. *45*(9), 383-393.
2. Hasker, E., Baco, A., Younoussa, A., Mzembaba, A., Grillone, S., Demeulenaere, T., et al., *Leprosy on Anjouan (Comoros): persistent hyper-endemicity despite decades of solid control efforts.* Leprosy review*,* 2017*. 88*(3), p 334-342.
3. Martinez, T. S., Figueira, M. M., Costa, A. V., Goncalves, M. A., Goulart, L. R., & Goulart, I. M., *Oral mucosa as a source of Mycobacterium leprae infection and transmission, and implications of bacterial DNA detection and the immunological status.* Clin Microbiol Infect*,* 2011. *17*(11), 1653-1658.
4. Kotteeswaran, G., Chacko, C. J., & Job, C. K.. *Skin adnexa in leprosy and their role in the dissemination of M. leprae.* Lepr India*,* 1980*. 52*(4), 475-481.
5. Araujo, S., Lobato, J., Reis Ede, M., Souza, D. O., Goncalves, M. A., Costa, A. V., et al. M., *Unveiling healthy carriers and subclinical infections among household contacts of leprosy patients who play potential roles in the disease chain of transmission*. Mem Inst Oswaldo Cruz*,* 2012. *107 Suppl 1*, 55-59.
6. Cambri, G., & Mira, M. T., *Genetic Susceptibility to Leprosy-From Classic Immune-Related Candidate Genes to Hypothesis-Free, Whole Genome Approaches*. Front Immunol, 2018. *9*, 1674.
7. Bhat, R. M., & Prakash, C. , *Leprosy: an overview of pathophysiology*. Interdiscip Perspect Infect Dis*,* 2012. 181089.
8. Ranade, M. G., & Joshi, G. Y., *Long-term follow-up of families in an endemic area*. Indian J Lepr, 1995*. 67*(4), 411-425.
9. Wilder-Smith, E. P., & Van Brakel, W. H., *Nerve damage in leprosy and its management.* Nat Clin Pract Neurol*,* 2008. *4*(12), 656-663.
10. Geluk, A., *Challenges in immunodiagnostic tests for leprosy*. Expert Opin Med Diagn, 2013. *7*(3), 265-274.
11. van Hooij, A., Tjon Kon Fat, E. M., Richardus, R., van den Eeden, S. J., Wilson, L., de Dood, C. J., et al., *Quantitative lateral flow strip assays as User-Friendly Tools To Detect Biomarker Profiles For Leprosy*. Sci Rep*,* 2016. *6*, 34260.
12. Corstjens, P., van Hooij, A., Tjon Kon Fat, E. M., Alam, K., Vrolijk, L. B., Dlamini, S., et al., *Fingerstick test quantifying humoral and cellular biomarkers indicative for M. leprae infection.* Clin Biochem, 2019. 66, 76-82.
13. Schilling A, v. H. A., Corstjens PLAM, Lurz PWW, DelPozo J, Stevenson K, Meredith A, et al., *Detection of humoral immunity to mycobacteria causing leprosy in Eurasian red squirrels (Sciurus vulgaris) using a quantitative rapid test*. European Journal of Wildlife Research*,* 2019. *65*.
14. Avanzi, C., Del-Pozo, J., Benjak, A., Stevenson, K., Simpson, V. R., Busso, P., et al. (2016). *Red squirrels in the British Isles are infected with leprosy bacilli*. Science*,* 2016. *354*(6313), 744-747.

15. van Hooij, A., Tjon Kon Fat, E. M., van den Eeden, S. J. F., Wilson, L., Batista da Silva, M., Salgado, C. G., et al., *Field-friendly serological tests for determination of M. leprae-specific antibodies.* Sci Rep*,*2017. *7*(1), 8868.
16. Braet, S., Vandelannoote, K., Meehan, C. J., Brum Fontes, A. N., Hasker, E., Rosa, P. S., et al., *The repetitive element RLEP is a highly specific target for the detection of Mycobacterium leprae*. J Clin Microbiol*,* 2018. *56*(3).
17. Martinez, A. N., Lahiri, R., Pittman, T. L., Scollard, D., Truman, R., Moraes, M. O., et al., *Molecular determination of Mycobacterium leprae viability by use of real-time PCR.* J Clin Microbiol, 2009. *47*(7), 2124-2130.
18. Martinez, A. N., Ribeiro-Alves, M., Sarno, E. N., & Moraes, M. O., *Evaluation of qPCR-based assays for leprosy diagnosis directly in clinical specimens.* PLoS Negl Trop Dis, 2011. *5*(10), e1354.
19. Tio-Coma, M., Avanzi, C., Verhard, E. M., Pierneef, L., van Hooij, A., Benjak, A., et al., *Genomic Characterization of Mycobacterium leprae to Explore Transmission Patterns Identifies New Subtype in Bangladesh.* Front Microbiol, 2020. *11*, 1220.
20. Corstjens, P. L., Li, S., Zuiderwijk, M., Kardos, K., Abrams, W. R., Niedbala, R. S., & Tanke, H. J., *Infrared up-converting phosphors for bioassays.* IEE Proc Nanobiotechnol, 2005. 152(2), 64-72.
21. Youden, W.J., *Index for rating diagnostic tests.* Cancer, 1950. **3**(1): p. 32-5.
22. Araujo, S., et al., *Molecular Evidence for the Aerial Route of Infection of Mycobacterium leprae and the Role of Asymptomatic Carriers in the Persistence of Leprosy.* Clin Infect Dis, 2016. 63(11): p. 1412-1420.
23. Ridley, D. S., & Jopling, W. H., *Classification of leprosy according to immunity. A five-group system.* Int J Lepr Other Mycobact Dis*,* 1966. *34*(3), 255-273.
24. Klatser, P. R., van Beers, S., Madjid, B., Day, R., & de Wit, M. Y., *Detection of Mycobacterium leprae nasal carriers in populations for which leprosy is endemic*. J Clin Microbiol, 1993. *31*(11), 2947-2951.
25. Rao, P. S., Karat, A. B., Kaliaperumal, V. G., & Karat, S., *Transmission of leprosy within households.* Int J Lepr Other Mycobact Dis, 1975. *43*(1), 45-54.
26. Moet, F. J., Pahan, D., Schuring, R. P., Oskam, L., & Richardus, J. H., *Physical distance, genetic relationship, age, and leprosy classification are independent risk factors for leprosy in contacts of patients with leprosy.* J Infect Dis, 2006. *193*(3), 346-353.
27. Jesudasan, K., Bradley, D., Smith, P. G., & Christian, M., *Incidence rates of leprosy among household contacts of "primary cases".* Indian J Lepr, 1984. *56*(3), 600-614.
28. Sales, A. M., Ponce de Leon, A., Duppre, N. C., Hacker, M. A., Nery, J. A., Sarno, E. N., et al., *Leprosy among patient contacts: a multilevel study of risk factors.* PLoS Negl Trop Dis, 2011. *5*(3), e1013.

SUPPORTING INFORMATION

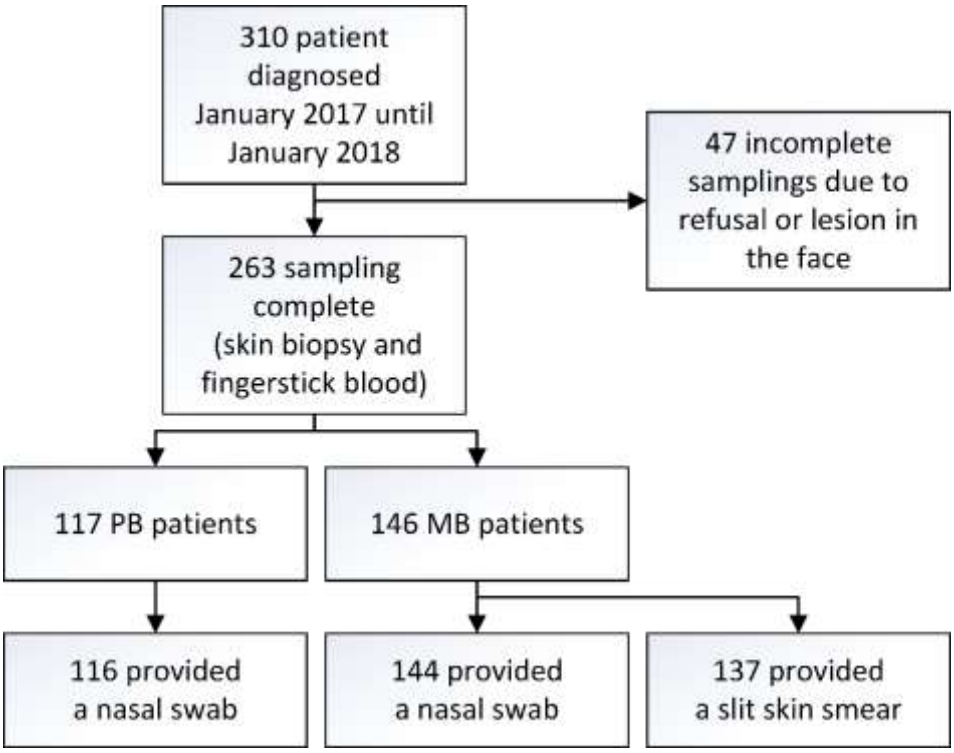

*Fig. III.S1 Patient flowchart. PB= paucibacillary according to the operational WHO classification; *MB= multibacillary according to the operational WHO classification*

Factor	Significance	Inclusion
αPGL-I UCP-LFA test value	P<0.001***	Inclusion
Number of lesion (≤or>25 lesions)	P<0.001***	Inclusion
Nasal swab positivity	P<0.001***	Inclusion
Nerves affected	P<0.05(.)	Not included
Painful nerves	P<0.01*	Not included
Hypertrophic nerves	P<0.01*	Not included
Plaques	P>0.05	Not included
Nodules	P<0.001***	Not included
Sensitivity loss	P>0.1	Not included

The inclusion of the different factors in the multiple regression model was based on a univariable analysis for each of the factors, estimating the influence of a factor to the bacillary load in the skin biopsy.

Table III.S1 Simple linear regression analysis. The inclusion of the different factors in the multiple regression model was based on a univariable analysis for each of the factors, estimating the influence of a factor to the bacillar load in the skin biopsy.

Coefficients:	Estimate	Standard Error	t-value	Pr(>|t|)
intercept	2.7711	0.1354	20.461	<2e-16***
Log10(αPGL-1 R-value)	1.1077	0.1415	7.828	1.31e-13***
Group 2: ≥25 lesions or Pos. nasal swab	1.1710	0.3090	3.790	0.000188***
Group 3: ≥25 lesions and Pos. nasal swab	3.4746	0.3677	9.449	<2e-16***
F-statistic 177.5 (256 df)	p-value: < 2.2e-16	Multiple R-squared: 0.5792,	Adjusted R-squared: 0.5743	Residual standard error: 1.574 (256 df)

Table III.S2 Coefficients for the multiple logistic regression, showing no signs of multicollinearity

Table III.S3 Table. Descriptive statistics for each assay per operational classification. PB= Paucibacillary operational WHO classification; MB=Multibacillary operational WHO classification

	Log10(Bacilli in skin biopsy)		Log10(bacilli in nasal swab)		Log10(αPGL-I R-value)	
	PB	MB	PB	MB	PB	MB
Mean	2.12	3.87	0.691	1.19	-0.557	-0.00598
Median	2.12	3.42	0.641	0.641	-0.523	-0.0605
Interquartile range	2.75	4.43	0	0.497	0.660	1.23
	Bacilli in skin biopsy		Bacilli in nasal swab		αPGL-I R-value	
	PB	MB	PB	MB	PB	MB
Mean	744633	12188796	46.6	2054	0.815	6.18
Median	132	2602	0	0	0.3	0.87
Interquartile range	1347	1808697	0	13.8	0.5	3.90
	Months of treatment					
	Skin biopsy RLEP-qPCR negative	Skin biopsy RLEP-qPCR positive	Nasal swab RLEP-qPCR negative	Nasal swab RLEP-qPCR positive	αPGL-I negative	αPGL-I negative
Mean	2.92	2.01	2.39	1.24	2.22	2.26
Median	1.5	1	1	0.5	1	1
Interquartile range	3.5	2.75	2.5	2	2.25	2.5

**PB= Paucibacillary operational WHO classification; MB=Multibacillary operational WHO classification*

Table III.S4 Table: Descriptive statistics for each assay per operational classification

	Log10(Bacilli in skin biopsy)		Log10(bacilli in nasal swab)		Log10(αPGL-I R-value)	
	PB	MB	PB	MB	PB	MB
Mean	2.12	3.87	0.691	1.19	-0.557	-0.00598
Median	2.12	3.42	0.641	0.641	-0.523	-0.0605
Interquartile range	2.75	4.43	0	0.497	0.660	1.23
	Bacilli in skin biopsy		Bacilli in nasal swab		αPGL-I R-value	
	PB	MB	PB	MB	PB	MB
Mean	744633	12188796	46.6	2054	0.815	6.18
Median	132	2602	0	0	0.3	0.87
Interquartile range	1347	1808697	0	13.8	0.5	3.90
	Months of treatment					
	Skin biopsy RLEP-qPCR negative	Skin biopsy RLEP-qPCR positive	Nasal swab RLEP-qPCR negative	Nasal swab RLEP-qPCR positive	αPGL-I negative	αPGL-I negative
Mean	2.92	2.01	2.39	1.24	2.22	2.26
Median	1.5	1	1	0.5	1	1
Interquartile range	3.5	2.75	2.5	2	2.25	2.5

**PB= Paucibacillary operational WHO classification; MB=Multibacillary operational WHO classification*

Table III.S5 Check for treatment bias in the full model

Coefficients:	Estimate	Standard Error	t-value	Pr(>|t|)
intercept	2.68624	0.14872	18.062	<2e-16***
Log10(αPGL-1 R-value)	0.86782	0.16967	5.115	6.21e-07 ***
Group 2: ≥25 lesions or Pos. nasal swab	1.05569	0.30864	3.420	0.000729 ***
Group 3: ≥25 lesions and Pos. nasal swab	3.83064	0.55656	6.883	4.61e-11 ***
Month of treatment prior to sampling	-0.01314	0.01447	-0.908	0.364766
F-statistic 61.83	p-value: < 2.2e-16	Multiple R-squared: 0.5945	Adjusted R-squared: 0.5849	Residual standard error: 1.554

Table III.S6 Table Check for treatment bias in the separate models

Coefficients: Group 1: <25 lesions or Neg. nasal swab	Estimate	Standard Error	t-value	Pr(>|t|)
intercept	2.68624	0.15574	17.300	<2e-16***
Log10(αPGL-1 R-value)	0.86606	0.17756	4.878	2.26e-06 ***
Month of treatment prior to sampling	-0.01596	0.01528	-1.045	0.297
F-statistic 12.75	p-value: 6.331e-06	Multiple R-squared: 0.1178	Adjusted R-squared: 0.1086	Residual standard error: 1.627

Coefficients: Group 2: ≥25 lesions or Pos. nasal swab	Estimate	Standard Error	t-value	Pr(>|t|)
intercept	3.3849	0.3226	10.491	4.81e-12 ***
Log10(αPGL-1 R-value)	1.7435	0.2795	6.237	4.81e-07 ***
Month of treatment prior to sampling	-0.01314	0.1243	1.875	0.0697
F-statistic 23.76	p-value: 4.057e-07	Multiple R-squared: 0.5902	Adjusted R-squared: 0.5653	Residual standard error: 1.525

Coefficients: Group 3: ≥25 lesions and Pos. nasal swab	Estimate	Standard Error	t-value	Pr(>|t|)
intercept	6.6778	0.3502	19.069	<2e-16***
Log10(αPGL-1 R-value)	0.7630	0.2897	2.634	0.0138 *
Month of treatment prior to sampling	-0.1144	0.1201	-0.953	0.3490
F-statistic 4.712	p-value: 0.01757	Multiple R-squared: 0.2587	Adjusted R-squared: 0.2038	Residual standard error: 0.8491

CHAPTER IV

Hi-plex deep amplicon sequencing for identification, high-resolution genotyping and multidrug resistance prediction of *Mycobacterium leprae* directly from patient biopsies by using Deeplex Myc-Lep

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S.M. Braet's Contribution: Conceptualizing and Formulating a Validation Plan, Editing, and Reviewing manuscript

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SUMMARY

Background: Expansion of antimicrobial resistance monitoring and epidemiological surveillance are key components of the WHO strategy towards zero leprosy. The inability to grow *Mycobacterium leprae in vitro* precludes routine phenotypic drug susceptibility testing, and only limited molecular tests are available. We evaluated a culture-free targeted deep sequencing assay, for mycobacterial identification, genotyping based on 18 canonical SNPs and 11 core variable-number tandem-repeat (VNTR) markers, and detection of rifampicin, dapsone and fluoroquinolone resistance-associated mutations in *rpoB and ctpC/ctpI, folP1, gyrA/gyrB*, respectively, and hypermutation-associated mutations in *nth.*

Methods: The limit of detection (LOD) was determined using DNA of *M. leprae* reference strains and from 267 skin biopsies and 75 slit skin smears of leprosy patients, with genome copies quantified by RLEP qPCR. Sequencing results were evaluated versus whole genome sequencing (WGS) data of 14 strains, and versus VNTR-fragment length analysis (FLA) results of 89 clinical specimens.

Findings: The LOD for sequencing success ranged between 80 and 3,000 genome copies, depending on the sample type. The LOD for minority variants was 10%. All SNPs detected in targets by WGS were identified except in a clinical sample where WGS revealed two dapsone resistance-conferring mutations instead of one by Deeplex Myc-Lep, due to partial duplication of the sulfamide-binding domain in *folP1*. SNPs detected uniquely by Deeplex Myc-Lep were missed by WGS due to insufficient coverage. Concordance with VNTR-FLA results was 99.4% (926/932 alleles).

Interpretation: Deeplex Myc-Lep may help improve the diagnosis and surveillance of leprosy. Gene domain duplication is a novel putative drug resistance-related genetic adaptation in *M. leprae*.

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KEYWORDS

Mycobacterium leprae, targeted next generation sequencing, antibiotic resistance, gene domain duplication, diagnostics, surveillance

RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed for *Mycobacterium leprae* genotyping and/or drug-resistance prediction methods published before January, 2023, using the following terms: ((test) OR (assay)) AND (Mycobacterium leprae) AND ((drug resistance) OR (antibiotic resistance) OR (genotyping) OR (diagnostics) OR (diagnosis)) AND (sequencing). Identified methods included phenotypic drug susceptibility and molecular testing approaches. Due to the inability to grow *M. leprae in vitro*, phenotypic testing requires the use of the mouse footpad model, which takes months for

obtaining results. Molecular tests comprise Sanger sequencing of amplicons, real-time PCR–high-resolution melt, microarray analysis, a line probe assay (LPA) based on post-PCR reverse hybridization and multi-locus VNTR analysis performed by fragment length analysis (MLVA-FLA). These methods detect only some, predefined variants in a limited number of *M. leprae* genomic regions, and/or require multiple PCR reactions. Whole-genome sequencing (WGS) and a targeted sequencing-based assay by Iwao *et al*. allow simultaneous genotyping and drug resistance prediction but they are costly and labor intensive, as they require *M. leprae* DNA enrichment procedures or three separate nested multiplex amplifications, respectively. The World Health Organisation (WHO) has called for improving surveillance, diagnosis and monitoring of (drug resistant) leprosy.

Added value of this study

Our study describes and evaluates a test called Deeplex Myc-Lep, which can both determine *M. leprae* strain type and detect drug resistance-associated mutations, directly from clinical specimens and by using a single hi-plex PCR mix followed by deep DNA sequencing. The assay analyzes the entire drug resistance-determining regions of all the known gene targets associated with resistance to the WHO-recommended anti leprosy drugs, along with 29 canonical markers (SNPs/indel and VNTR) for high-resolution genotyping of *M. leprae*, and a target for identification of both causal agents of leprosy, *M. leprae* and *M. lepromatosis*. Our experimental results obtained with DNA from *M. leprae* reference strains and from 213 biopsies from patients diagnosed with leprosy from the Comoros show that successful sequencing can be achieved with samples including a minimum number of genome copies in the range from 100 to 1,000. Our deep sequencing data demonstrate confident detection of strain genotypes as well as resistance-associated mutations, including those carried by bacterial subpopulations, potentially causing heteroresistance, down to a 10% proportion. All SNPs detected in targets by WGS were concordantly identified by targeted deep sequencing except in a clinical sample where WGS revealed two dapsone resistance-conferring mutations instead of one by Deeplex Myc-Lep, due to partial duplication of the sulfamide-binding domain in *folP1*. SNPs detected uniquely by Deeplex Myc-Lep were missed by WGS due to insufficient coverage. Concordance with VNTR-FLA results was 99.4% (926/932 alleles).

Implications of all the available evidence

The Deeplex Myc-Lep assay can substantially improve the diagnosis and surveillance of (multidrug resistant) leprosy, to help reach the goal set by the WHO of 120 countries with zero new autochthonous cases and a 70% reduction in the annual number of detected incident cases by 2030. Access to this test should be favoured by the global expansion of next generation sequencing capacity as a result of the COVID-19 pandemic response, including in many high-burden countries. Our results also show that Deeplex Myc-Lep worked well on Disolol-preserved samples (at ambient temperature), facilitating surveillance in regions where fast sample transport with adequate cold chains is challenging. Furthermore, a synergy could also be expected with the progressive deployment of Deeplex Myc-TB, used in more than 30

countries to date, given the same shared technical platforms and the large prevalence of tuberculosis in most settings affected by leprosy.

INTRODUCTION

Leprosy, also called Hansen's disease, is caused by infection with *Mycobacterium leprae* and more rarely, *Mycobacterium lepromatosis* (1). For several decades, the disease was treated using dapsone monotherapy, inevitably leading to emergence of resistance (2). The use of multidrug therapy recommended by the World Health Organization (WHO), with addition of rifampicin and clofazimine to dapsone (3), and of effective second-line drugs such as fluoroquinolones in case of rifampicin resistance, subsequently resulted in a decrease in the numbers of leprosy cases globally. Yet, the incidence of leprosy has plateaued since 2005 (4), and the disease is still present in 120 countries, with more than 200,000 new cases reported every year (5). Emergence of (multi-) drug resistant strains of *M. leprae* is reported in several world regions (6–9) and *M. leprae* transmission pathways are not fully understood, nor controlled (10,11). This situation calls for new tools for diagnosis and guidance of epidemiological tracing.

A number of biological and technical challenges must be overcome in order to determine both drug resistance profiles and high-resolution genotypes of *M. leprae* strains. Since *M. leprae* cannot be cultivated on artificial media, phenotypic drug susceptibility testing requires labour, time- and cost-intensive culture in the mouse footpad model (12,13). To circumvent this, molecular tests have been developed to detect genotypic resistance to rifampicin, dapsone and fluoroquinolones directly from clinical specimens, based on known resistance mutations located in the drug resistance determining regions (DRDRs) of *rpoB*, *folP1* and *gyrA*, respectively (14). In-house methods for mutation detection include Sanger sequencing (8), real-time PCR–high-resolution melt (15), and microarray analysis (16). A commercial line probe assay (LPA) is based on post-PCR reverse hybridization (17). However, these tests require multiple PCR reactions and/or identify only some, predefined high-confidence resistance mutations in these three genes.

M. leprae is moreover a clonal obligate pathogen with highly restricted genetic diversity (18). A typing system including 18 polymorphic sites (19,20), with single-nucleotide polymorphisms (SNPs) and DNA insertions/deletions (indels), canonically distinguishes *M. leprae* strains into four main types (1-4) and 16 subtypes (1A-4P), supported by whole genome sequencing (WGS)-based data and displaying a phylogeographical association (21,22). However, while this SNP/indel-based system is useful to identify relatively distant genetic relationships, analysis of short-range transmissions within a specific geographical setting requires markers with higher discriminatory power (23). VNTR loci present in the *M. leprae* genome (24) exhibit higher mutation rates compared to SNPs. Therefore, multi-locus VNTR analysis (MLVA), performed by fragment-length analysis (FLA), is often used to further type *M. leprae* strains (9,25,26). Like for SNP typing, MLVA-FLA similarly requires multiple PCR reactions, and accurate FLA-based determination of repeat numbers (alleles) can be challenging, especially for some loci with shortest, dinucleotide repeats.

WGS, done by short read sequencing (Illumina) for *M. leprae*, can simultaneously capture drug resistance-associated mutations and almost all genetic variation available for subsequent epidemiological inference (except in too complex/repetitive genome regions). However, this requires the use of costly and labor intensive *M. leprae* DNA enrichment procedures (27,28), and frequently results in relatively limited sequencing depth, restricting genome coverage and impeding confident variant detection, especially in case of minority variants potentially reflecting drug resistance emergence (heteroresistance) or mixed strain types.

As we showed for *M. tuberculosis* (29–31), targeted next-generation sequencing can offer an alternative solution for combined culture-free detection of drug resistance variants and determination of strain type, also allowing for high sequencing depth and higher multiplexing of samples per sequencing run. A method was recently described for detection of resistance mutations in *folP1*, *rpoB*, *gyrA* and *gyrB* and SNP-based typing of *M. leprae*. This method required six PCRs, consisting of three separate nested multiplex amplifications, before amplicon sequencing (32). Here, we describe and evaluate Deeplex® Myc-Lep, a culture-free targeted deep sequencing assay based on a single 44-plex PCR, commercially available as a ready-to-use amplification kit. The targets include (i) the *hsp65* gene for mycobacterial identification, (ii) 18 SNP/indel sites and 17 VNTR markers for high-resolution genotyping of *M. leprae* strains, and (iii) DRDRs of *folP1*, *rpoB, gyrA* and *gyrB* for drug resistance prediction, as well as gene regions of *ctpC*, *ctpI* and *nth*. Nonsense mutations in the excision repair gene *nth* are linked with hypermutated genomes and drug resistance profiles in *M. leprae* strains (21). *ctpC* and *ctpI* are included for exploratory purposes, as it has been suggested that missense mutations in these genes are associated with resistance to rifampicin, in a strain devoid of mutation in the *rpoB* DRDR (33). The evaluation was performed by comparison with reference data obtained from 342 clinical specimens of patients affected by leprosy, DNA of four *M. leprae* reference strains, and of *M. lepromatosis* NHDP-385 (see Table S1 for information on the datasets used in this study).

METHODS

Deeplex Myc-Lep assay

The Deeplex Myc-Lep assay starts with the amplification of 43 regions of the *M. leprae* genome as well as of one synthetic sequence used as internal control in a single multiplex PCR step (see Results, Assay design). Amplicon libraries are prepared using the Nextera XT kit and sequenced with 150bp or 250bp paired-end reads in a MiSeq (Illumina, CA, USA). Sequencing data analysis is performed using a pre-parameterized automated bioinformatic pipeline.

Clinical specimens, strains and *M. leprae* DNA quantification

The limit of detection (LOD) of Deeplex Myc-Lep was evaluated using DNA extracts from clinical specimens collected between 2017-2018 in the Comoros as part of the ComLep (Improved Understanding of Ongoing Transmission of Leprosy in the Hyperendemic Comoros, ITM IRB ref 1147/16) and PEOPLE (Post ExpOsure Prophylaxis for LEprosy in the Comoros and Madagascar, ITM IRB ref 1248/18 (34)) trial studies and purified DNA from the well-characterized strains

NHDP63, Thai-53, Br4923 (BEI resources) and Br14-3 (Fundação Oswaldo Cruz, Brazil) (see also Table S1). The 4 mm skin biopsies from the Comoros 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 (Antwerp, Belgium). The biopsies were preserved at ambient temperature up until months before analysis. Negative sampling controls, consisting of 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. DNA from these 213 biopsies from the Comoros were extracted as described in Braet *et al*. 2022 (35), by using the Maxwell 16 FFPE Tissue LEV DNA Purification Kit or the Maxwell 16 FFPE Plus Tissue LEV DNA Purification Kit (Promega, WI, USA). DNA from *M. leprae* NHDP63, Thai-53, Br4923 was obtained from the BEI Resources Repository (VA, USA) and genomic DNA of Br14-3 was obtained from cultures of corresponding strains on mouse footpads and purified with a modified protocol using the QIAamp® DNA Microbiome Kit (Qiaqen). Evaluation of the LOD using these reference strains and these 213 biopsies was done by utilizing kits from the same Deeplex Myc-Lep production lot.

Moreover, DNA from 107 additional samples including skin biopsies from the aforementioned studies as well as slit skin smears (SSS) from routine leprosy diagnostics at the Fundação Oswaldo Cruz (Brazil), extracted using the Maxwell 16 FFPE Plus Tissue LEV DNA Purification Kit (Promega, WI, USA), the modified Boom method (36) or the method described by Van Der Zanden *et al.* (37) were included as part of a supplementary analysis of the LOD of the assay (see Table S2 for details). *M. leprae* DNA was quantified from all samples using quantitative PCR (qPCR) based on the *M. leprae*-specific repetitive element (RLEP) region (38,39).

M. leprae strains studied by WGS are detailed in Table S3. DNA was extracted from human skin biopsies or mouse footpads as described by Woods and Cole (40) for crude extracts obtained by the freeze-boiling method, and by Avanzi *et al.* (41) , including human or mouse DNA elimination for obtaining WGS quality grade DNA.

DNA from *M. lepromatosis* NHDP-385 and *M. leprae* NHDP63, used in the *hsp65*-based species identification experiment, was obtained from the *National Hansen's Disease Program* (NHDP; *LA, USA) and BEI resources* (VA, USA), respectively.

Determination of the limit of detection

The LOD was assessed in terms of (i) the minimum number of RLEP copies enabling correct allele detection of all Deeplex Myc-Lep core markers, (ii) the minimum proportion of detectable minority variants in mixes of a resistant (Br14-3) and a susceptible (NHDP63) *M. leprae* strain as well as (iii) the minimum bacterial load, expressed as the RLEP qPCR Cq value, required for the sequencing of all Deeplex Myc-Lep core markers. Dilution series of four DNA extracts (with strains NHDP63, Thai-53, Br4923 and a mix of the former two) from 3.10^3 to 3.10^6 RLEP copies representing about 80 to 80,000 *M. leprae* genomes and a series of mixes of a resistant (Br14-3) with a susceptible (NHDP63) strain at total 6.10^6 RLEP copies were prepared for the first two

experiments, respectively. *M. leprae* was quantified by RLEP qPCR from clinical specimens. Cq values range from 13 to 30.

Species identification

Identification of mycobacterial species by Deeplex Myc-Lep was done based on amplification and sequencing of a *hsp65* gene segment, followed by best-match analysis of the obtained sequences against a database of *hsp65* sequences derived from Dai *et al*. (42), as for Deeplex Myc-TB (29).

DNA from *M. leprae* strain NHDP63 and *M. lepromatosis* NHDP-385 was quantified using the Qubit dsDNA High Sensitivity assay (ThermoFisher, MA, USA) and a series of mixes of DNA from the two strains was prepared using a total of 4.5ng of DNA in each mix.

Deeplex Myc-Lep results compared to VNTR-FLA and WGS

The ability of Deeplex Myc-Lep to correctly detect variants and VNTR marker alleles was assessed by comparing the assay's results to those of WGS and VNTR-fragment length analysis (FLA), respectively. In all cases, Deeplex Myc-Lep was performed on the same DNA extracts as those used for WGS or VNTR-FLA. Comparison to WGS was based on 11 skin biopsies with microscopy smear gradings from 2+ to 4+ collected from 2010-2018 as part of routine leprosy diagnostics by the National Reference Center for Mycobacteria (Paris, France) and 3 *M. leprae* strains cultivated in mouse footpads (Table S3). DNA extracted following the protocol published by Avanzi *et al.* (41) was sequenced using Nextera XT DNA Library Preparation Kit and a MiSeq with 150bp paired-end reads according to the manufacturer's instructions (Illumina, CA, USA). VNTR-FLA was performed as described by Jensen *et al.* (43) on 89 samples, comprising 35 slit skin smears collected in Brazil as part of routine leprosy diagnostics by the Fundação Oswaldo Cruz (Fiocruz Recife), 31 skin biopsies collected in the Comoros as part of the ComLep and PEOPLE trial studies, 20 skin biopsies and 3 *M. leprae* cultured strains provided by the Bichat-Claude Bernard Hospital (France).

Ethics statement

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, ComLep ref 1147/16, PEOPLE ref 1248/18), the ethical committee of the University of Antwerp (Antwerp, Belgium, ComLep ref 17/05/052, PEOPLE ref 18/36/390, approved on 17/09/2018), the ethical committee on the island of Anjouan (ComLep, no ref, approved on 15/07/2017, PEOPLE ref 18-01/MSSPSPG/CNE, approved on 9/10/2018), 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. For the control strains used in the WGS analysis and provided by the NRC France, all subjects gave written informed consent in accordance with the Declaration of Helsinki. The genotyping of slit skin smears

samples was approved by the ethical committee from CPqAM/Fiocruz (ref CEP/CPqAM/FIOCRUZ 02/12). For human samples from which WGS was performed, all subjects gave written informed consent in accordance with the Declaration of Helsinki. Specimens were collected under the approval of the Centre de Ressources biologiques, Assistance publique-hôpitaux de Paris, France. DNA from mouse footpad specimens were obtained from previous work and were provided by Alexandra Aubry and Aurélie Chauffour (license number to carry out animal experiments C-75-13-01).

Role of funders

The funders had no role in study design, data collection, data analyses, interpretation, patient recruitment, or writing of this manuscript.

RESULTS

Assay design

The gene regions and sequence positions of *M. leprae* genome targeted by the assay are listed in Table IV.1. The reaction mix comprises an internal control sequence to detect potential PCR inhibition. After amplicon sequencing on an Illumina platform with 150 bp paired-end reads, the sequencing data are automatically analyzed using a proprietary, pre-parameterized bioinformatic pipeline, with integrated databases. A subsequently generated schematic representation of the results is shown in Figure IV.1, comprising identification of the mycobacterial species, detection of genotypic resistance, determination of the VNTR allelic profile and of strain type based on 18 canonical SNPs. Species identification is done by best-match analysis of reference *hsp*65 sequences from 168 mycobacterial taxa derived from Dai et al. 2011 (42). Via comparison with a proprietary reference database compiling amino acid changes reportedly associated with *M. leprae* antibiotic resistance, sequence variants in the relevant targets are reported as “Resistant” if known to be associated with resistance to either of the above antibiotics, or “Uncharacterised” if leading to a non-synonymous mutation not included in the current database. VNTR alleles are determined according to the numbers of repeats directly determined from the sequences amplified from the respective loci, also accounting for potential artefactual “stutter” peaks, as also seen for *M. tuberculosis* MIRU-VNTR markers (44). For the purpose of the analysis, markers were separated into two sets, defined as core and non-core, the latter category consisting of six VNTR markers that could be amplified only in a minority of the specimens of the test datasets using 150 bp read lengths. Of these, two VNTR markers, 18-8 and 27-5 that include longer repeat units and amplified alleles often exceeding analytic capacity with 150 bp sequencing, were recovered using longer 250 bp paired-end reads (see below).

*Table IV.1. M. leprae gene regions or positions targeted by Deeplex Myc-Lep, relative to the TN strain genome from MycoBrowser (45). NC, non-coding. * Expected lengths of VNTR marker alleles are reported in Table S4.*

Target	Genomic positions	Gene positions	Codons (Gene Name)	Information	
InDel_17915	17915-17936	433-454	pseudogene (ML0014)	Typing (SNPs/indels)	core
SNP-7614	7614	297	99 (*gyrA*)		
SNP-1642879	1642879	896	pseudogene (ML1378)		
SNP-2935693	2935693	753	pseudogene (*icl*)		
SNP-14676	14676	NC	NC		
SNP-8453	8453	1136	379 (*gyrA*)		
SNP-313361	313361	461	154 (*metS*)		
SNP-61425	61425	269	90 (*esxA*)		
SNP-3102787	3102787	452	151 (ML2597)		
SNP-1104235	1104235	239	pseudogene (ML0934)		
SNP-2751790	2751790	897	299 (*asd*)		
SNP-1295195	1295195	430	144 (ML1119)		
SNP-2312066	2312066	3	1 (ML1926c)		
SNP-413903	413902	275	92 (ML0324)		
SNP-20910	20910	1283	428 (*pknA*)		
Ins-978589	978589	89	30 (ML0825c)		
Del-1476522	1476522	NC	NC		
SNP-1527056	1527056	617	206 (*cydD*)		
6-3a	1190305-1190395	518-608	173-203 (*sigA*)	Typing (VNTRs*)	
AC8a	1531112-1531235	141-264	pseudogene (*cya*)		
AC8b	2210951-2211090	NC	NC		
AC9	1452501-1452646	NC	NC		

Target	Genomic positions	Gene positions	Codons (Gene Name)	Information	
GTA9	2583766-2583887	NC	NC		
GAA21	2785374-2785574	NC-77	NC-pseudogene (ML2344A)		
GGT5	2567170-2567330	NC	NC		
6-7	1816775-1816966	14-205	5-69 (ML1505)		
12-5	1381580-1381868	405-683	135-228 (PPE)		
21-3	73016-73195	492-671	164-224 (*espE*)		
23-3	2945411-2945600	NC	NC		
rpoB	2275546-2275343	1267-1470	423-490	Rifampicin	
folP1	296765-296914	70-219	24-73	Dapsone	
gyrA	7436-7638	119-321	40-107	Fluoroquinolones	
gyrB	6589-6842	1361-1614	454-538	Fluoroquinolones	
ctpC	889136-888916	1836-2056	612-686	Exploratory	
ctpI	3209132-3209379	4414-4661	1472-1554	Exploratory	
nth	2726174-2725850	318-642	106-214	Hypermutation	
18-8	1587513-1587860	188-535	63-179 (ML1334)	Typing (VNTRs*)	non-core
27-5	686961-687230	148-417	50-139 (ML0568)		
TA10	1743996-1744180	872-1056	pseudogene (ML1450A)		
TA18	984529-984670	231-372	pseudogene (ML0830c)		
AT15	948843-949041	NC	NC		
AT17	2597667-2597846	458-637	pseudogene (ML2183c)		
hsp65	405683-406083	165-565	55-188	Species identification	
Synthetic target	NA	NA	NA	Internal control	

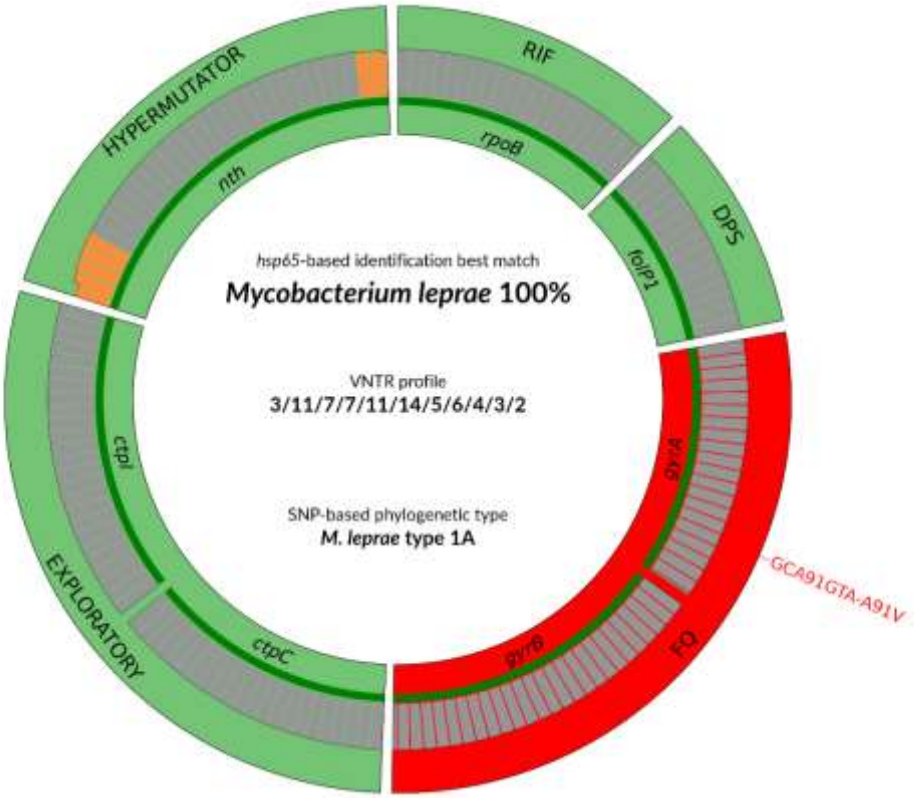

*Figure IV.1. Deeplex Myc-Lep results identifying a M. leprae strain of SNP type 1A genotypically resistant to fluoroquinolones. Results are shown for a Thai53 strain derivative, mutated in gyrA (see Table S3). Information on hsp65 best match-based identification, VNTR allelic profile and SNP-based phylogenetic type is shown in the center of the circle. Information on predictions of drug susceptibility and drug resistance for anti-leprosy drugs/drug classes and on hypermutator genotype is as follows. Target gene regions are grouped within sectors in a circular map according to the prediction feature (drug resistance, hypermutation) with which they are associated. Sectors in red and green indicate targets in which resistance- or hypermutation-associated mutations or no mutations are detected, resulting in predictions of resistant or susceptible phenotypes (for rpoB, folP1, gyrA, gyrB), or hypermutator strain (nth), respectively. The ctpC and ctpI sector (and their associated drug resistance or drug susceptibility predictions) are categorized as exploratory, based on previous work suggesting an association of missense mutations in these genes with resistance to rifampicin, observed in a single strain devoid of mutation in the rpoB DRDR (see text). Green lines above gene names represent the reference sequences with coverage breadth above 95%. Limit of detection (LOD) of minority variants (resulting from subpopulations of reads bearing a mutation) depends on the read depth at each sequence position and is shown either as grey (LOD 10%) or orange zones (LOD >10%) above reference sequences. Here, LOD is >10% at the extremities of the nth target only. In the VNTR profile, VNTR markers are ordered as follows: 6-3a, AC8a, AC8b, AC9, GTA9, GAA21, GGT5, 6-7, 12-5, 21-3, 23-3. *RIF: Rifampicin, DPS: Dapsone, FQ: Fluoroquinolones, VNTR, variable-number tandem-repeat, SNP, single nucleotide polymorphism.*

Identification of *M. leprae* and *M. lepromatosis*

The *hsp65* sequencing- and best match-based system versus the reference database derived from Dai *et al.* (42) used to identify mycobacterial species in Deeplex Myc-Lep is identical to that used in Deeplex Myc-TB (see Methods for detailed information). Its performance for species identification has previously been extensively described (29). Therefore, we evaluated here (co-)detection and distinction of *M. leprae* (NHDP63 strain) and *M. lepromatosis* (NHDP-385 strain), as the latter mycobacterium is the second causal agent of leprosy (1). For this, we applied the test on mixtures of genomic DNA from the two species at various ratios (4.5 ng total, Table IV.2). To note, these ratios were based on quantification of overall extracted DNA instead of specific quantification obtained by RLEP qPCR for *M. leprae*, since a specific qPCR was not performed for *M. lepromatosis*.

*Table IV.2. Theoretical and observed proportions of M. leprae NHDP63 and M. lepromatosis in mixes of genomic DNA from both species. ND: Not detected. *Species could not be specifically identified but was reported as "Other".*

M. leprae		*M. lepromatosis*	
Theoretical	*Detected*	*Theoretical*	*Detected*
100	99.4	0	ND
99	99.2	1	ND
95	81	5	18.9
90	71.3	10	28.5
80	64.8	20	35.1*
50	29.1	50	70.7
20	24.3	80	75.5
0	ND	100	99.8

In addition to correct identification in both controls including a single species, the presence of strains from both species was explicitly detected and reported in mixtures when the minority DNA exceeded a "theoretical 5%/detected 18.9%" proportion, except in the following case. At a detected 64.8% proportion of *M. leprae*, *M. lepromatosis* was reported as "Other" at 35.1%, reflecting a large part of species-specific variants in *hsp65* detected with frequencies close to 50%, making it impossible to unambiguously discriminate between *hsp65* sequences of both species. Excluding the controls with a single species, detected proportions of both species differed from theoretical proportions by an average of 14.6% (SD: ± 5.9), indicating semi-quantitative detection within the limits of DNA quantification accuracy indicated above. On top of this *hsp65*-based co-identification, the presence of *M. lepromatosis* in the mixes could also be inferred by detection of *M. lepromatosis*-specific variants in the resistance- and hypermutation-associated targets (up to 39 variants detected depending on the proportion of *M. lepromatosis*, Table S5).

Identification of SNPs, VNTRs and limit of detection using reference strains

Identification of SNPs and VNTR alleles, as well as the limit of detection (LOD) of the assay were first evaluated using DNA from reference *M. leprae* strains cultured from mouse footpads. The LOD was estimated both in terms of minimum number of genomes enabling at least 95% coverage breadth of resistance- and hypermutation-associated targets (fully comprising the DRDR region for *rpoB*, *folP1*, *gyrA,* and *gyrB*) at minimum depth of 5x (minimal threshold for base calling), correct marker allele detection and minimum proportion of detectable minority variants.

First, serial dilutions of four DNA extracts from genotypically drug susceptible strains NHDP63, Thai-53, Br4923, and an 85-15% mix of NHDP63 and Thai-53 were prepared. As estimated by RLEP qPCR, resulting amounts included per Deeplex Myc-Lep test ranged from 3.10^3 to 3.10^6 RLEP copies, representing about 80 to 80,000 genomes. While the read depth expectedly decreased with the number of genomes, all core markers (including typing SNP, VNTR and resistance- or hypermutation-associated markers) were completely covered, with a mean coverage depth of 1,718x even with 3.10^3 RLEP copies/80 genomes (Fig. IV.2), and all samples were identified as *M. leprae*. All expected alleles of the 18 typing SNPs and the 11 VNTR core markers were correctly called in all cases, and correct mixed SNP alleles were detected in the NHDP63/Thai-53 mix. For the 11 typing SNPs that were expected to be different between both strains, heterozygous calls were identified with the NHDP63 SNP alleles dominant as expected (see Table S6 for alleles detected in reference strains). Such detection of mixed typing SNPs was, and is, accordingly considered to report detection of mixed strain types in the sample. Further as expected, only a synonymous R99R SNP was detected in *gyrA* of NHDP63 and in the NHDP63/Thai-53 mixture (as a dominant allele), while no SNP was detected in any (other) resistance- or hypermutation-associated target in NHDP63, NHDP63/Thai-53, Thai-53 or Br4923. To note, because only the dominant VNTR marker alleles are called in this version of Deeplex Myc-Lep, these markers were not considered in the NHDP63/Thai-53 mixture. In contrast, less than half of the non-core VNTR alleles were called even with the highest tested numbers of genome copies, reflecting much lower read depth at these markers (average 12-108x vs 2,025-5,643x for core markers).

Second, a series of mixes of DNA were prepared from a multidrug-resistant strain (Br14-3, known to possess resistance-conferring mutations in *rpoB*, *gyrA,* and *folP1*, and a stop codon in *nth* (21)) and a drug susceptible (NHDP63) strain in various proportions. In these mixes, all minority variants from Br14-3, including typing and resistance/hypermutation-conferring SNPs, were detected if the strain represented at least 10% of the input DNA (Fig. IV.3). Below this level, part of the expected variants were missed, while other false positive variants were detected (one at a 5% ratio and 204 at a 1% ratio were observed). The LOD of Deeplex Myc-Lep for minority variant calls was therefore set at 10%.

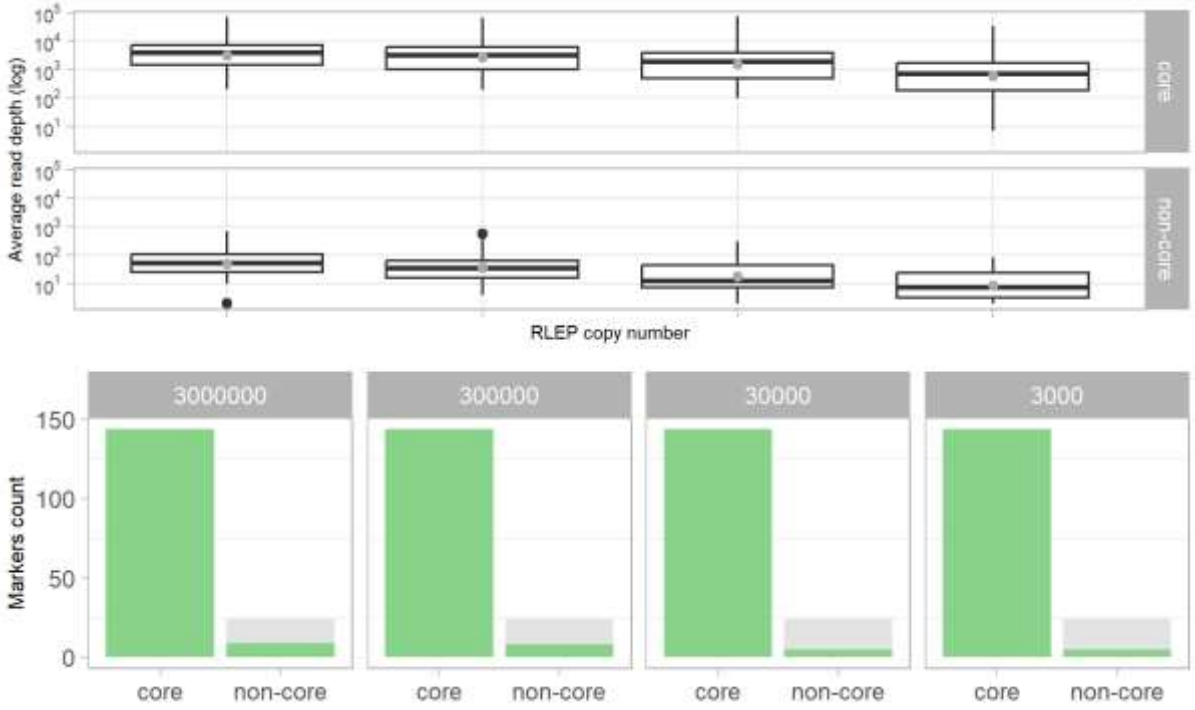

Figure IV.2. Limit of detection for correct allele calling of 42 Deeplex Myc-Lep markers. The LOD was evaluated with DNA extracts from three M. leprae strains and an 85-15% mixture of two strains. (Top) Read depth at core and non-core markers versus the number of RLEP copies. Median (line) and mean (grey dot) values as well as 25-75% quartiles are shown. (Bottom) For each serial dilution with 3.10^3, 3.10^4, 3.10^5 and 3.10^6 RLEP copies (80-80,000 M. leprae genomes), the fraction of correctly (green) and incorrectly detected or not sequenced (grey) alleles was determined for 144 core and 24 non-core alleles (36 core and six non-core markers times four DNA extracts, respectively).

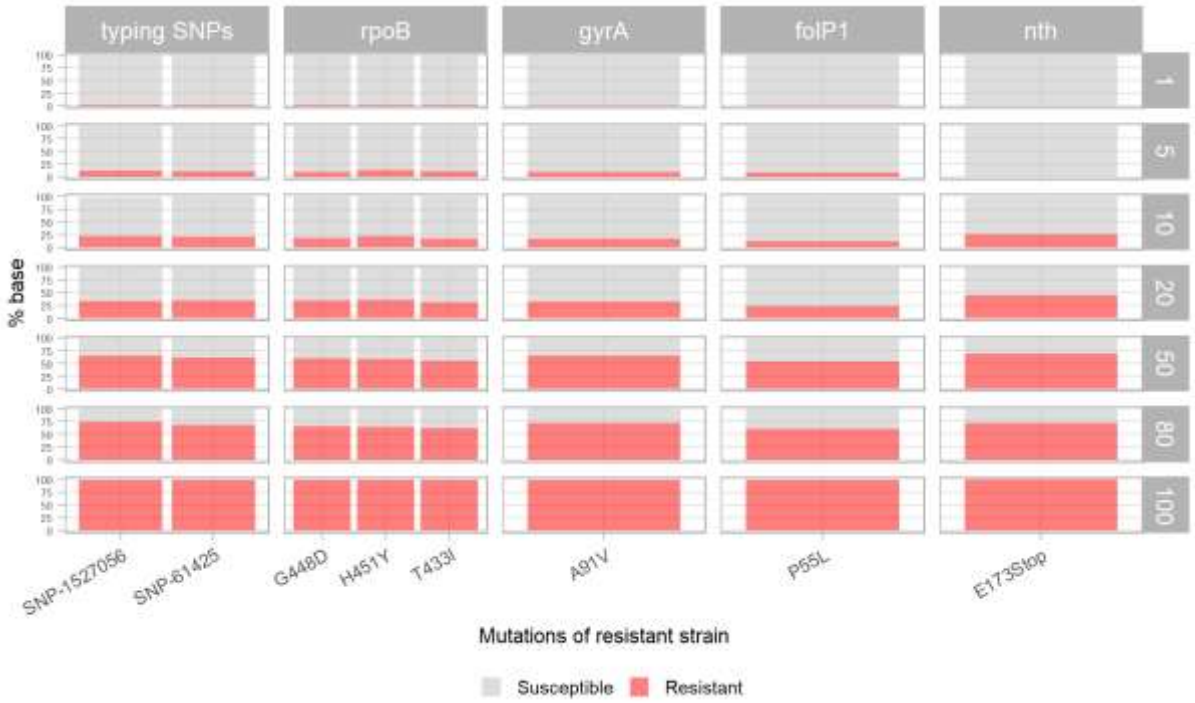

Figure IV.3. Limit of detection in terms of the minimum proportion of detectable minority variants, using M. leprae reference strains (VNTR markers not considered). Mixes of the

susceptible strain NHDP63 with 1, 5, 10, 20, 50 and 80% of the resistant strain Br14-3 were used to estimate the lowest fraction of detectable variant allele. The resistant strain was also analysed alone, as a control (100%, bottom). The resistant allele is depicted in red while the susceptible allele is in grey.

Limit of detection using clinical specimens

The Deeplex Myc-Lep LOD was evaluated on DNA extracted with Maxwell kits from 213 clinical specimens, consisting of skin biopsies collected from patients from Anjouan (Comoros), with leprosy diagnosed by conventional examination (Table S2). Out of these 213 samples, *hsp65* sequencing results of the Deeplex confirmed the presence of *M. leprae* in 186 (87.3%). We first determined the coverage depth and fraction of core markers with successful sequencing results as defined above depending on the *M. leprae* genome numbers, as estimated by RLEP qPCR (Fig. IV.4). Specimens with Cq values of 24 (corresponding to 3,243 genome copies, SD±1,591) or lower had almost systematically all core markers successfully sequenced (median of 36/36 markers). With Cq values between 25 and 29 (2,244-115 genome copies ±SD 3,946 for Cq values of 25; a single sample was available with a Cq of 29), medians of successfully sequenced markers still ranged between 34/36 (94%) and 28/36 (78%); a marked drop in the fraction of sequenced core markers was only observed at Cq values of at least 30 (median 12/37 sequenced core markers). Regarding non-core VNTR markers, alleles were successfully detected for more than half of the markers with Cq values of 21 or lower, but complete non-core allele profiles were never obtained, even with high bacterial load, due to low overall read depths (1-86x vs 36-2,206x at core markers, Fig. S1).

Of note, no substantial differences were seen when results were stratified by classification of samples from multi- (n=190) or paucibacillary (n=23) leprosy (according to WHO classification; Fig. S2), likely reflecting the limited quantitative information of this classification. Success rate for species identification and determination of core VNTR alleles were almost identical between both categories. The proportions of samples with determined SNP type/subtype and with coverage depth and breadth sufficient to detect potential variants at 10% or more (graded ++) or 80% or more frequency (+) in resistance/hypermutation targets were lower by only a few percent in the paucibacillary category.

Similar results and limits of detection were obtained in terms of RLEP Cq values when using other Deeplex Myc-Lep kit lots on sets of DNAs extracted from 33 skin biopsies with Maxwell kits or from 74 slit skin smears with the Boom method (36) and the method published in van der Zanden et al. (37). Rates of successfully sequenced markers versus Cq values appeared nevertheless slightly lower on DNA extracted from slit skin smears (Fig. S3, Table S2).

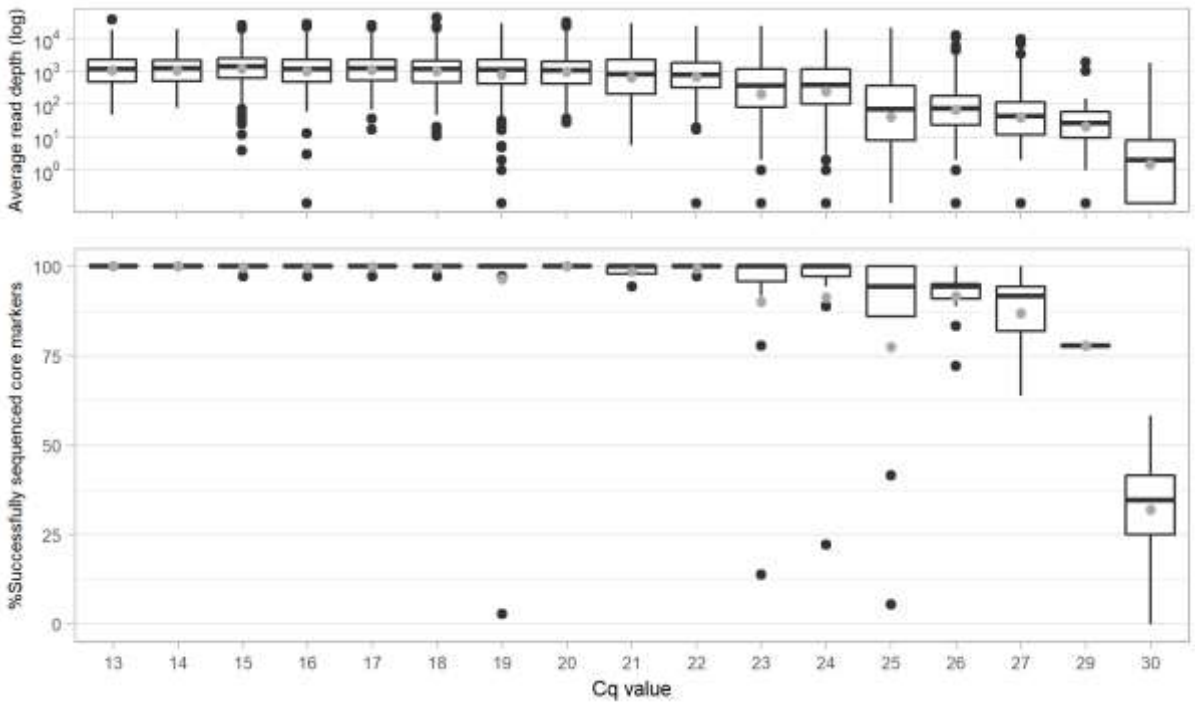

Figure IV.4. Limit of detection, in terms of RLEP qPCR Cq value, for the sequencing of 36 Deeplex Myc-Lep core markers (including the species identification target), determined by using 213 biopsies from patients affected by leprosy. (Top) Read depth at Deeplex Myc-Lep core markers versus RLEP PCR Cq values 13 to 30. (Bottom) Proportion of successfully sequenced Deeplex Myc-Lep core markers versus RLEP PCR Cq values 13 to 30 (Top & Bottom) Median (line) and mean (grey dot) values as well as 25-75% quartiles are shown.

Deeplex Myc-Lep versus WGS

Fourteen samples collected between 2010-2018 from various locations (Table S3) were sequenced using both Deeplex Myc-Lep and WGS, and variants in typing SNPs and Deeplex Myc-Lep resistance/hypermutation-associated targets were compared. Overall, 36 SNPs were concordantly detected by both methods including 3 typing SNPs and 3 resistance-associated variants (Table S7). However, 26 other SNPs, consisting exclusively of typing SNPs, were detected only by Deeplex Myc-Lep. The latter cases were straightforwardly explained by total absence of read coverage (n=19) or coverage by a single read only (n=7; below the threshold for confident variant calling) by WGS at the corresponding positions due to low bacillary load in the skin biopsies (BI of 1+ and 2+). In comparison, read depths were 8-4,615x (mean 833x) at these positions by Deeplex Myc-Lep (Table S7).

Unexpectedly, in one sample (WGS23), two variants were detected in *folP1* by WGS at ~50% (P55L and T53A) while Deeplex Myc-Lep only detected variant T53A as fixed (99.9%), even though read depths were high at both positions (>1,000x) with Deeplex Myc-Lep. Inspection of the WGS reads that mapped to *folP1* in the reference genome of the TN strain shows that, despite their proximal position in the *folP1* gene sequence, the two variants were systematically carried by different reads, indicating that they originate from two distinct regions in WGS23 (Fig. IV.5A). Moreover, the WGS coverage depth on the reference *folP1* sequence was up to two times higher compared to that on flanking regions (Fig. IV.5B). Detailed analysis of the

obtained mapping data excluded ambiguous mapping of reads from the *folP2* gene paralogue (not amplified by Deeplex Myc-Lep) present in *M. leprae* as a potential explanation for the WGS results. Taken altogether, these observations indicate a partial duplication of *folp1* in the WGS23 strain (spanning circa 350 bp, corresponding to the sulfamide-binding domain in the encoded enzyme), with T53A and P55L variants separately borne by the duplicated segments, and a possible rearrangement affecting *folP1* primer regions leading to amplification of T53A only by Deeplex Myc-Lep.

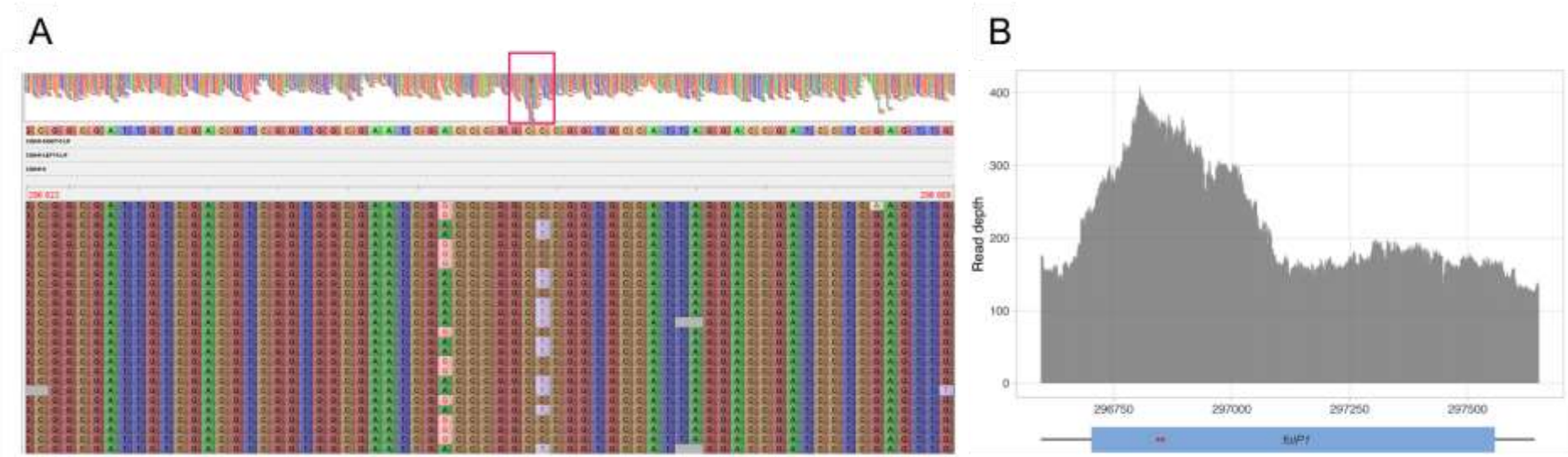

Figure IV.5. WGS reads of sample WGS23 mapped to the genome of the M. leprae TN strain, around folP1. (a) Variants detected in folP1 are carried by different reads, indicating that they originate from distinct regions. The top part of the figure shows read coverage depths at the folP1 and flanking regions. A red box indicates the folP1 region with a coverage depth up to twice as high as the depth of flanking regions. The bottom part shows a zoom-in of the folP1 region showing the aligned sequence reads. Genomic positions of the extremities of the region shown are indicated on the top right and top left. Different rows represent independent sequence reads. G and T variants are never found in combination in a read, resulting in mixed wild type/variant calls with a frequency of ~50% of the reads, at each of both variant positions. (b) Read depth at the folP1 region, with 100bp flanking sequences. Positions of the folP1 coding sequence and the two variants detected at a frequency of ~50% by WGS are represented by a blue rectangle and red stars, respectively. Variants are 7bp apart in the reference genome.

Deeplex Myc-Lep VNTR versus VNTR-FLA

The concordance of Deeplex Myc-Lep core VNTR results versus reference VNTR-FLA results was evaluated on 89 clinical specimens for which both result sets were generated. Results for two of the non-core VNTR markers (18-8 and 27-5) were also compared for a subset of 31 samples, with available 250bp read-based sequencing data. In total, results could be obtained from both methods in 932 out of 1041 (89.5%) tested markers across the specimen set. The same allele was concordantly called in 926 markers (99.4%) by both methods (Table IV.3, Table S8). Three tests showed a partial match, where a same allele was identified by both methods in addition to a second allele undetected by one method. In two cases, two alleles were detected only by Deeplex Myc-Lep, each with an identical number of reads. Absence of allelic concordance between MLVA and Deeplex Myc-Lep was seen only in three tests (0.6%; for AC9, with one-repeat unit discordance in one sample, and GAA21 with one-repeat unit discordance in one sample and four-repeat unit discordance in another sample).

Table IV.3. Comparison of Deeplex Myc-Lep and VNTR-FLA core and non-core VNTR analysis results from 89 M. leprae clinical samples. Non-core VNTR markers 18-8 and 27-5 were sequenced using 250bp paired-reads on a subset of 31 samples. Match, same VNTR marker allele detected by Deeplex Myc-Lep and VNTR-FLA; partial match, two alleles detected by one of the methods, including one matching with the other method; ND, not detected by Deeplex Myc-Lep and/or VNTR-FLA; mismatch, Deeplex Myc-Lep and VNTR-FLA detected different VNTR marker alleles.

VNTR	Total Tested	Match	Partial Match	ND	Mismatch	%Match
6-3a	89	86	0	3	0	100
AC8a	89	75	0	14	0	100
AC8b	89	86	0	3	0	100
AC9	89	82	0	6	1	98.8
GTA9	89	79	1	9	0	100
GAA21	89	82	1	4	2	97.6
GGT5	89	86	0	3	0	100
12-5	89	84	0	5	0	100
21-3	89	76	1	12	0	100
23-3	89	70	0	19	0	100
6-7	89	65	0	24	0	100
18-8	31	26	0	5	0	100
27-5	31	29	0	2	0	100
Total	**1041**	**926**	**3**	**109**	**3**	**99.5**

DISCUSSION

Expansion of antimicrobial resistance monitoring and effective epidemiological surveillance are key components of the first strategic pillar of the Global Leprosy Strategy of the WHO, aiming at 120 countries with zero new autochthonous cases and a 70% reduction in the annual number of detected incident cases by 2030 (46). The Deeplex Myc-Lep design is unique in that both components can be addressed in one single PCR assay, followed by NGS sequencing. This tool analyzes all the known (multi)drug resistance-associated gene targets (clofazimine resistance-associated gene(s) are as yet undetermined in *M. leprae*), along with 29 canonical SNPs/indel and core VNTR markers for high-resolution genotyping of *M. leprae*, and a mycobacterial speciation target for identification of both *M. leprae* and *M. lepromatosis*. Our results show the high degree of concordance, with an increment of superiority for some aspects as explained below, of this targeted NGS-based approach versus genome sequencing data (reference genomes and newly sequenced strains) and MLVA reference methods. We show that it can be applied directly on DNA extracts, and works best on clinical specimens with bacterial genome copies of ~100 per test or higher as pre-quantified by RLEP qPCR. This study further uncovered an unexpected evidence of gene duplication as a source of genome plasticity and as a possible alternative mechanism of (increased) drug resistance in *M. leprae*.

Compared to current clinically used methods, Deeplex Myc-Lep substantially extends the diagnostic spectrum and accuracy, for both diagnostic and biological discovery. The commercially available LPA test specifically identifies only two common mutations in *rpoB* (S456L, H451Y), one in *gyrA* (A91V), and one in *folP1* (P55L). Even if they may be less common, additional mutations in the DRDRs of these genes are known to confer resistance to rifampicin, fluoroquinolones, and dapsone (6,16,21,47). Mutations other than the four mentioned above can only be suspected in some codons within the respective DRDRs (432, 438-441, 451, and 456-458 for *rpoB*, 89-91 for *gyrA*, 53-55 for *foplP1*), in the absence of hybridization to "wild-type" probes, which requires additional PCR and sequencing for further assessment. Some suspected resistance mutations, such as *gyrA* S92A and *gyrB* D464N in the *gyrA* and *gyrB* DRDR segments, respectively, cannot be detected by such indirect analysis (47). Moreover, as shown for LPAs for *M. tuberculosis* resistance testing (48), potential synonymous or nonsynonymous mutations unrelated to resistance in these short segments could result in false inference of resistance if based on unbound wild-type probes. Microarray-based (16) and high-resolution melt-based (15) methods, similarly using a restricted set of pre-defined wild-type and mutant probes, or only distinguishing wild-type from mutant sequences in short DRDR segments, respectively, were exposed to the same limitations.

In contrast, Deeplex Myc-Lep analyses the entire (suspected) DRDRs of *rpoB*, *gyrA, gyrB,* and *folP1* by direct sequencing, allowing to unambiguously identify all mutations conferring resistance to the current multidrug therapy validated to date in the mouse footpad model or using surrogate mycobacteria (6). The obtained mean sequencing depths of 100x or more, with RLEP qPCR Cq values of 25 or lower on DNA extracts from skin biopsies, allow extensive and highly confident detection of variants on the targets of interest. In comparison, WGS can

frequently miss multiple variant positions, which were readily detected by Deeplex Myc-Lep as seen here, in case of bacillary load of 2+ or below. Our evaluation also showed that high read depths obtained by Deeplex Myc-Lep enable the detection of resistance alleles emerging within a sample, as low as a 10% heteroresistant subpopulation, which is also hard or impossible to reach with usual WGS depths or by Sanger sequencing. This deep sequencing capacity is expected to be especially useful for monitoring potential resistance emergence in the context of the anticipated scale up of preventive chemotherapy, currently done with a single dose of rifampicin, even if our preliminary data tend to be reassuring about this risk at least in the Comoros (35). In addition, targeted sequencing of relevant gene regions allows increased multiplexing of samples in a sequencing run (with, typically, 72 and 122 samples plus three controls in a single MiSeq run using 2x150bp or 2x250bp sequencing, respectively) compared to WGS, which thus reduces run cost.

Furthermore, new (candidate) resistance mutations, otherwise challenging to discover by phenotypic testing of *M. leprae*, could be identified as follows. Similar to WGS-based phylogenetic reconstruction (21), comparisons of the sequencing data of the *rpoB*, *gyrA/gyrB*, *folP1,* and the *ctpC*/*ctpI* targets with the SNP- and VNTR-based strain type information may allow detection of potential independent occurrence of the same variants in different genetic backgrounds (homoplasic mutations), indicative of positive selection likely associated with antibiotic pressure. In addition, as all hypermutated strains with nonsense *nth* mutations were previously found to be genotypically drug resistant, mutations detected in *nth* might serve as surrogate markers for inferring new candidate resistance mutations in the above targets, as well as for potential risk of treatment failure (21). Such systematic implicative relationship between *nth* mutations and drug resistance was not contradicted here. Indeed, *nth* variants were undetected in any of the 269 strains that had all resistance-associated targets successfully sequenced but showed no resistance mutation. Only one (Br14-3 (21) of the four strains with confirmed resistance mutations carried a nonsense *nth* mutation.

Automated, direct sequencing-based allele calling of 11 core VNTR markers - extensible to 13 markers when using 250 bp read -, on top of a canonical set of typing SNPs/indels, represents an additional valuable tool for epidemiological surveillance and investigation of leprosy transmission. The observed concordance of 99.4% with VNTR-FLA results shows the high accuracy of this approach. In comparison, accurate interpretation of our VNTR-FLA data required very meticulous and tedious comparative inspections of various stutter peaks and true allelic ladders across electrophoretic profiles of many samples, which necessitated extensive expertise in VNTR typing systems (including with short sequence repeats) also developed for other mycobacteria (49,50). Although they were used in previous studies, we found that the four remaining VNTR markers (all with dinucleotide repeats) from the 17 initially tested (covering the entire repertoire of VNTR genomic loci previously used for typing the bacterium) are essentially unexploitable in most cases. Besides more difficult amplification, these loci often include apparently large numbers (well above 10) of 2-nucleotide repeats, clearly affected by too strong stutter peak effects (see Fig. 9 in Jensen *et al.* (43)) and preventing any reliable allele

identification, whether by VNTR-FLA or by sequencing, as we reported for similar short sequence repeats in *M. paratuberculosis* (49). Irrespective of the exclusion of non-core markers, the relative allelic diversities among the retained VNTR markers were similar between the Comoros sample set and the set of samples from other, diverse origins (Fig. S4, Table S9), suggesting similar degrees of epidemiological resolution across various settings. Moreover, in the Comoros set, the degree of genotypic resolution obtained was close to that obtained by WGS (Braet et al., in preparation).

The discovered evidence for a partial duplication in *folp1* in one sample was unexpected, as the *M. leprae* genome is known to be otherwise prone to massive gene decay (51). The observation that each of the two partially duplicated copies carries a (different) dapsone resistance-conferring mutation known to alter the sulfamide binding site (52), with the duplication centered around the mutation positions, strongly suggests an original mechanism of domain duplication involved in (enhanced) drug resistance, reminiscent of kinase domain duplication involved in resistance of human tumoral cells to anticancer therapy (53). Of further interest, this strain neither showed resistance mutations in other resistance-associated gene targets, nor in *nth,* thus suggestive of a mechanism independent from hypermutation. Thus, this finding and the *nth*-mediated hypermutation in some other *M. leprae* strains (21) - without known counterpart in *M. tuberculosis* - suggest broader capacities for genetic adaptation than could have been anticipated for a bacterium with a greatly degraded genome. This gene domain duplication was seen only in a single case among the 14 clinical samples that were successfully analysed by WGS. Therefore, knowing whether this duplication mechanism occurs relatively frequently or not, in relation with dapsone resistance in particular, will require refined (re-)analysis of *M. leprae* WGS data, by inspecting potential unfixed mutations and local distribution of reads and coverage depth as we did here. To note, from a diagnostic perspective, detection of only one of the two *folP1* mutations was sufficient for dapsone resistance prediction by targeted sequencing.

In conclusion, based on one of the largest sample sets from a single study to date, our results show the potential of the Deeplex Myc-Lep assay to substantially improve the microbiological confirmation of the clinical diagnosis, and the surveillance of (multidrug resistant) leprosy, to help reach the ambitious goals set by the WHO for this disease. Access to and use of this test should be favoured both by its availability as a commercial kit, and global expansion of next generation sequencing capacity as a result of the COVID-19 pandemic response, including in many high-burden countries. Our results also show that Deeplex Myc-Lep worked well on Disolol-preserved samples (at ambient temperature), facilitating surveillance in regions where fast sample transport with adequate cold chains is challenging. Furthermore, a synergy could also be expected with the progressive deployment of Deeplex Myc-TB, used in more than 30 countries to date, given the same shared technical platforms and the large prevalence of tuberculosis in most settings affected by leprosy. Finally, our findings reveal also a probable, previously unsuspected mechanism involved in drug resistance of *M. leprae*, the prevalence of which is to be further investigated.

DECLARATION OF INTERESTS

A.J., C.G., G.B., N.B., E.L., A.F., M.C. and Y.L. are employees of GenoScreen (Lille, France). P.S. is a scientific consultant for the same company. Other authors declare that they have no other competing interests.

CONTRIBUTORS

Conceptualisation : A.J., S.M.B, C.G., G.B., E.C., B.C.J., P.N.S., P.S. ; Formal analysis : A.J., C.G., G.B., N.B, P.S.; Funding acquisition: B.C.J., Investigation: S.M.B, S.V., K.L., E.L., A.F., M.C., R.E.E.N.O.L., Y.Y.P.P. ; Resources : S.M.B, C.G., A.B.F., N.L., P.R., M.M, E.H., S.G., W.A., A.S., Y.A., N.A., E.C., P.S. ; Software : A.J., Y.L. ; Supervision : E.C. B.C.J., P.N.S., P.S. ; Visualisation : A.J., P.S., Writing – original draft : A.J., P.S., Writing – review and editing : A.J., S.M.B., E.C., B.C.J., P.N.S., P.S. All authors read and approved the final version of the manuscript. A.J. and P.S. verified the underlying data.

DATA SHARING STATEMENT

Sequence reads used in this paper were deposited in the Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI), under BioProject accession number PRJNA923280. A detailed description of the datasets is available in Supporting information Table IV.S1.

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REFERENCES

1. Han XY, et al. *A new Mycobacterium species causing diffuse lepromatous leprosy*. Am J Clin Pathol., 2008;130(6):856–64.
2. Pettit JHS, Rees RJW. *Sulphone Resistance in Leprosy: An Experimental and Clinical Study.* The Lancet. , 1964;2:673–4.
3. World Health Organization. *Chemotherapy of leprosy for control programmes*. 1982.
4. Chaptini C, et al, *Leprosy: a review on elimination, reducing the disease burden, and future research*. Lepr Rev. , 2015;86:307–15.
5. World Health Organization. *Weekly Epidemiological Record n°36*. 2020.
6. Aubry A, et al., *Drug resistance in leprosy: An update following 70 years of chemotherapy.* Infectious Diseases Now. Elsevier Masson s.r.l., 2022. p. 243–51.
7. Cambau E, et al., *Antimicrobial resistance in leprosy: results of the first prospective open survey conducted by a WHO surveillance network for the period 2009–15. Clinical Microbiology and Infection,* 2018 1;24(12):1305–10.
8. Kai M, et al., *Analysis of drug-resistant strains of Mycobacterium leprae in an endemic area of Vietnam*. Clinical Infectious Diseases, 2011 1;52(5).
9. Rosa PS, et al. *Emergence and transmission of drug-/multidrug-resistant mycobacterium leprae in a former leprosy colony in the brazilian amazon.* Clinical Infectious Diseases, 2020 6;70(10):2054–61.
10. Hambridge T, et al., *Mycobacterium leprae transmission characteristics during the declining stages of leprosy incidence: A systematic review*. PLoS Negl Trop Dis. 2021 1;15(5).
11. Ploemacher T, et al.,. *Reservoirs and transmission routes of leprosy; A systematic review.* PLoS Negl Trop Dis., 2020 1;14(4):1–27.
12. Levy L, Ji B. *The mouse foot-pad technique for cultivation of Mycobacterium leprae*. Lepr Rev., 2006;77(1):5–24.
13. Shetty VP, et al., *Viability and Drug Susceptibility Testing of M. LepraeUsing Mouse Footpad in 37 Relapse Cases of Leprosy*. Int J Lepr. ,2003;71(3):210–7.
14. World Health Organization. *Guidelines for Global Surveillance of Drug Resistance in Leprosy*., 2009.
15. Li W, et al., *Real-time PCR and high-resolution melt analysis for rapid detection of Mycobacterium leprae drug resistance mutations and strain types*., J Clin Microbiol., 2012;50(3):742–53.
16. Matsuoka M, et al., *A novel method for simple detection of mutations conferring drug resistance in Mycobacterium leprae, based on a DNA microarray, and its applicability in developing countries*. J Med Microbiol., 2008;57(10):1213–9.

17. Cambau E, et al., *Detection of antibiotic resistance in leprosy using GenoType LepraeDR, a novel ready-to-use molecular test.* PLoS Negl Trop Dis. 2012;6(7).

18. Schueneumann VJ, et al., *Genome-Wide Comparison of Medieval and Modern Mycobacterium leprae.* ,Science (1979)., 2013;341(179):undefined.

19. Truman RW, et al., *Probable zoonotic leprosy in the Southern United States., New England Journal of Medicine.,* 2011;364(17):1626–33.

20. Monot M, et al., *On the Origin of Leprosy.* Science (1979)., 2005;308:1040–2.

21. Benjak A, et al., *Phylogenomics and antimicrobial resistance of the leprosy bacillus Mycobacterium leprae.* ,Nat Commun., 2018 1;9(1).

22. Monot M, et al. *Comparative genomic and phylogeographic analysis of Mycobacterium leprae.,* Nat Genet., 2009;41(12):1282–9.

23. Singh P, Cole ST. *Mycobacterium leprae: Genes, pseudogenes and genetic diversity*, Future Microbiology., 2011. p. 57–71.

24. Cole ST, Supply P, Honoré N. *Repetitive sequences in Mycobacterium leprae and their impact on genome plasticity.* Lepr Rev. 2001;72(4):449–61.

25. Kimura M, et al., *Rapid variable-number tandem-repeat genotyping for Mycobacterium leprae clinical specimens.,* J Clin Microbiol., 2009;47(6):1757–66.

26. Lima LNC, et al., *Genotyping comparison of Mycobacterium leprae isolates by VNTR analysis from nasal samples in a Brazilian endemic region*. Pathog Glob Health. 2018 17;112(2):79–85.

27. Stefani MMA, et al., *Whole genome sequencing distinguishes between relapse and reinfection in recurrent leprosy cases*. PLoS Negl Trop Dis., 2017 15;11(6).

28. Tió-Coma M, et al., *Genomic Characterization of Mycobacterium leprae to Explore Transmission Patterns Identifies New Subtype in Bangladesh*., Front Microbiol., 2020 16;11.

29. Jouet A, et al. ,*Deep amplicon sequencing for culture-free prediction of susceptibility or resistance to 13 anti-tuberculous drugs*. European Respiratory Journal., 2021 1;57(3).

30. Makhado NA, et al., *Outbreak of multidrug-resistant tuberculosis in South Africa undetected by WHO-endorsed commercial tests: an observational study.* Lancet Infect Dis., 2018;18(12).

31. Tagliani E, et al. *Culture and Next-generation sequencing-based drug susceptibility testing unveil high levels of drug-resistant-TB in Djibouti: Results from the first national survey*. Sci Rep , 2017;7(1):1–9

32. Iwao Y, et al., *Simultaneous Determination of Mycobacterium leprae Drug Resistance and Single-Nucleotide Polymorphism Genotype by Use of Nested Multiplex PCR with Amplicon Sequencing*. J Clin Microbiol, 2021;59(10):814–21.

33. Singh P, et al., *Genome-wide re-sequencing of multidrug-resistant Mycobacterium leprae Airaku-3.* Clinical Microbiology and Infection. ,2014 1;20(10):O619–22.

34. Ortuno-Gutierrez N, et al., *Clustering of leprosy beyond the household level in a highly endemic setting on the Comoros, an observational study.* BMC Infect Dis., 2019 7;19(1).

35. Braet SM, et al., *Investigating drug resistance of Mycobacterium leprae in the Comoros: an observational deep-sequencing study.* Lancet Microbe., 2022 1;3(9):e693–700.

36. Boom R, et al., *Rapid and Simple Method for Purification of Nucleic Acids.* J Clin Microbiol., 1990;28(3):495–503.

37. van der Zanden AGM, et al., *Use of DNA extracts from Ziehl-Neelsen-stained slides for molecular detection of rifampin resistance and spoligotyping of Mycobacterium tuberculosis*. J Clin Microbiol., 2003 1;41(3):1101–8.

38. Martinez AN, et al., *Evaluation of qPCR-Based assays for leprosy diagnosis directly in clinical specimens.* PLoS Negl Trop Dis., 2011;5(10).

39. Truman RW, et al., *Enumeration of Mycobacterium leprae using real-time PCR.* PLoS Negl Trop Dis., 2008;2(11).

40. Woods SA, Cole ST. *A rapid method for the detection of potentially viable Mycobacterium leprae in human biopsies: a novel application of PCR*. FEMS Microbiol Lett., 1989;65(3):305–9.

41. Avanzi C, et al., *Population Genomics of Mycobacterium leprae Reveals a New Genotype in Madagascar and the Comoros*. Front Microbiol., 2020 11;11.

42. Dai J, et al., *Web-accessible database of hsp65 sequences from Mycobacterium reference strains.* J Clin Microbiol., 2011;49(6):2296–303.

43. Jensen RW, et al., *DNA fingerprinting of Mycobacterium leprae strains using Variable number tandem repeat (VNTR) - fragment length analysis (FLA).* Journal of Visualized Experiments. 2011;(53).

44. Supply P, et al., *Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units*. J Clin Microbiol., 2001;39(10):3563–71.

45. Kapopoulou A, et al., *The MycoBrowser portal: A comprehensive and manually annotated resource for mycobacterial genomes*. Tuberculosis., 2011 ;91(1):8–13.

46. World Health Organization. *Towards zero leprosy. Global leprosy (Hansen's Disease) strategy 2021–2030* [Internet]., 2021.

47. Chauffour A, et al., *A systematic review of Mycobacterium leprae DNA gyrase mutations and their impact on fluoroquinolone resistance*., Clinical Microbiology and Infection. Elsevier, 2021. p. 1601–12.

48. Ajileye A, et al., *Some synonymous and nonsynonymous gyrA mutations in Mycobacterium tuberculosis lead to systematic false-positive fluoroquinolone resistance results with the Hain GenoType MTBDRsl assays*. Antimicrob Agents Chemother., 2017 1;61(4).

49. Thibault VC, et al., *Combined multilocus short-sequence-repeat and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing of Mycobacterium avium subsp. paratuberculosis isolates.* J Clin Microbiol. 2008;46(12):4091–4.

50. Ablordey A, et al. *Multilocus variable-number tandem repeat typing of Mycobacterium ulcerans.* J Clin Microbiol., 2005;43(4):1546–51.

51. Cole ST, et al. *Massive gene decay in the leprosy bacillus.* Nature., 2001 22;409(6823):1007–11.

52. Williams DL, Gillis TP. *Drug-resistant leprosy: Monitoring and current status*. Lepr Rev. 2012;83:269–81.

53. Kemper K, et al. *BRAFV600E Kinase Domain Duplication Identified in Therapy-Refractory Melanoma Patient-Derived Xenografts.* Cell Rep., 2016 28;16(1):263–77.

CHAPTER V

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

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RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed for all studies published before December, 2021, in which drug resistance surveillance for leprosy results were reported, using combinations of keywords: ("resistance" OR "resistant" OR "drug resistance" OR "drug resistant") AND ("detection" OR "survey" OR "surveillance"). Only a few studies, report on drug resistance surveillance for leprosy, due to that detection of drug resistance in leprosy is challenged by the leprosy bacillus not growing in laboratory conditions. In 2018, a first study reporting on the global rate of drug resistance in leprosy patients confirmed the presence of drug-resistant strains in several countries. Nationally representative data on leprosy drug resistance in the Comoros, among the six highest burden countries worldwide, are lacking. Such data are even more urgent since the World Health Organization (WHO) approved single-dose rifampicin as prophylaxis for contacts of leprosy patients.

Added value of this study

This study has designed and set-up field friendly and innovative ways to collect, ship, and test skin biopsies for molecular drug resistance surveillance. The results show that not even traces of drug-resistance mutations are detected to any of the following drugs: rifampicin, fluoroquinolones, and dapsone in the Comoros.

Implications of all the available evidence

The findings of this study reassure the effectiveness of (prophylactic) treatment in the Comoros, and are a testimony to the strong leprosy control programme. Professionally supervised leprosy treatment in the Comoros favors consistent exposure to drugs, posing a lower risk of resistance selection than self-treatment by patients, even with the help of family members. As storage of the biopsies at room temperature in alcohol allowed us to identify a full drug-resistance profile for *Mycobacterium leprae* months later, our findings may inform national programs' approaches to drug resistance surveillance. Moreover, the sensitivity of the Deeplex Myc-Lep tool to detect even traces of molecular resistance make it an attractive tool for worldwide leprosy resistance surveillance.

ABSTRACT

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.

Funding

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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* (*M. 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, 5, 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 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 LepraeDR (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 2013–2019(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 2019–2020 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 (Antwerp, Belgium). 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 0.5–1ml 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*; dapsone: *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 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 V.1) 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 (Fig. V.1, Fig. V.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 V.1).

Table V.1: Characteristics of the recruited leprosy patients.

	Total recruited patients (1199)		Patients selected for Deeplex Myc-Lep (260)		Univariate analysis Sample rate Ratio (95% CI)
	Number	Proportion (%)	Number	Proportion (%)	
Island					
Anjouan	1160	96.7%	254	97.7%	1 (ref)
Mohéli	39	3.3%	6	2.3%	0.6 (0.3-1.6)
New/relapse/reinfected					
New	1179	98.3%	251	96.5%	1 (ref)
Relapsed/Reinfected*	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 MDT	452	37.7%	100	38.5%	1.6 (0.9-1.3)
Previously completed PB MDT	3	0.3%	0	0.0%	N.A
Previously completed MB MDT	17	1.4%	9	3.5%	2.5 (1.6-4.1)
Received SDR in 2015	4	0.3%	1	0.4%	1.2 (0.2-6.6)
Received SDDR during PEOPLE study in 2019	8	0.7%	1	0.4%	0.6 (0.1-3.8)
Age					
0–14 years	454	37.9%	46	17.7%	1(ref)
15–24 years	358	29.9%	99	38.1%	2.7 (2.0-3.7)
25–34 years	144	12.0%	44	16.9%	3.0 (2.1-4.4)
35–44 years	92	7.7%	29	11.2%	3.1 (2.1-4.7)
45–54 years	54	4.5%	12	4.6%	2.2(1.2-3.9)
55–64 years	38	3.2%	9	3.5%	2.3(1.2-4.4)
≥ 65 years	57	4.8%	21	8.1%	3.6(2.3-5.6)
Unknown	2	0.2%	N.A.	N.A.	N.A
Sex					
Male	725	60.5%	188	72.3%	1(ref)
Female	474	39.5%	72	27.7%	0.6 (0.5-0.8)

**reinfection/relapse is indistinguishable in this study. MDT= multidrug therapy; MB=Multibacillary as per WHO clinical definition; PB= Paucibacillary as per WHO clinical definition; SDR-PEP= single dose rifampicin as post exposure prophylaxis (10mg/kg); SDDR-PEP= single (double) dose rifampicin as post exposure prophylaxis (20mg/kg)* [a] *All four patients were sampled, however only for one biopsy the bacterial load exceeded 2000 bacilli per 2µl DNA extract.* [b] *One out of eight did not have a biopsy due to a single facial lesion, in one biopsy no M. leprae DNA was detected and in five biopsies the bacterial load was inferior to 2000 bacilli per 2µl DNA; N.A.= not applicable*

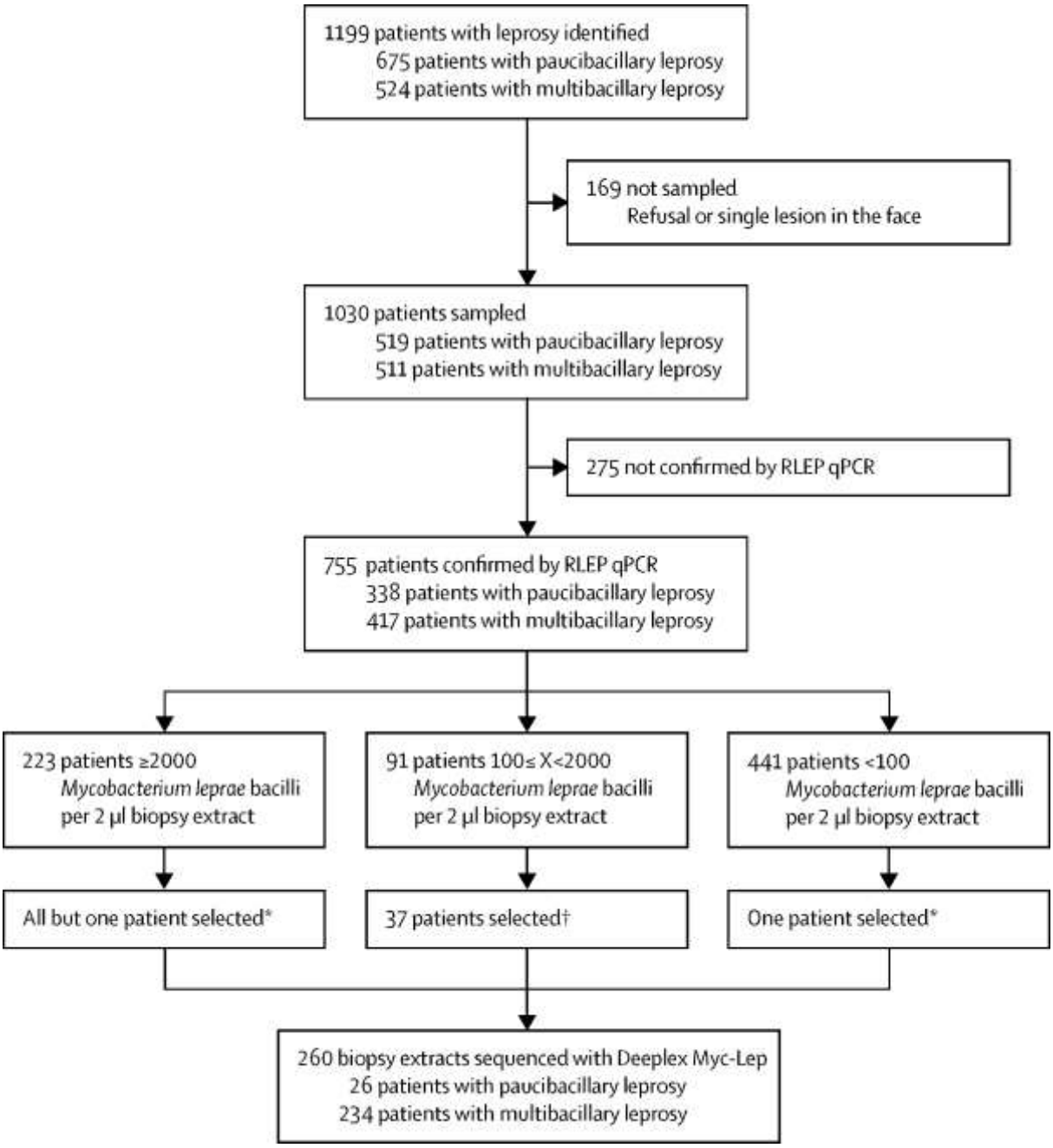

*Figure V.1: Flowchart for samples that were processed with Deeplex Myc-Lep, 2017–2020. qPCR=quantitative PCR. RLEP=M leprae-specific repetitive element. *One high bacterial load sample was erroneously not processed with Deeplex Myc-Lep, and one sample with low bacterial load was processed with the Deeplex 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.*

*Figure V.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.*

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 V.1). 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=0.57). 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 (Fig. V.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 (Fig. V.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<0.0001) 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 (Fig. V.2). None of the 255 patients had a mutation in rpoB, even not as minority bacillary populations (with an average *rpoB* read depth of ≥40x 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 ≥40x read depth for both). Among these, 42 patients resided in villages were 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 (Fig. V.2). None of these 251 patients had a mutation in *ctpC* or *ctpI*.

For dapsone, *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 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 described (20) 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*.

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 dapsone of 5.3% in 2015–2019, mainly in Brazil, China, India, Japan, and Vietnam (21, 23, 24). The global rate of resistance for fluoroquinolones in patients with leprosy was 1.3%. Primary fluoroquinolone (23) 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 colleagues (22) 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 have not selected for drug resistance in the Comoros (22, 25, 26, 27). 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 tuberculosis in settings where SDR-PEP is provided to contacts of patients with leprosy (13).

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 due to ethical and privacy concerns. Data can, however, be made available after approval of a motivated and written request to the Institute of Tropical Medicine at ITMresearchdataaccess@itg.be.

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REFERENCES

1. World Health Organization, team Control of Neglected tropical Diseases. Global leprosy (Hansen disease) update, 2020: impact of COVID-19 on the global leprosy control. World Health Organization; 2021.
2. Avanzi C, Busso P, Benjak A, Loiseau C, Fomba A, Doumbia G, et al. Transmission of Drug-Resistant Leprosy in Guinea-Conakry Detected Using Molecular Epidemiological Approaches. Clin Infect Dis. 2016;63(11):1482-4.
3. Li W, Sakamuri RM, Lyons DE, Orcullo FM, Shinde V, Dela Pena EL, et al. Transmission of dapsone-resistant leprosy detected by molecular epidemiological approaches. Antimicrob Agents Chemother. 2011;55(11):5384-7.
4. Chokkakula S, Chen Z, Wang L, Jiang H, Chen Y, Shi Y, et al. Molecular surveillance of antimicrobial resistance and transmission pattern of Mycobacterium leprae in Chinese leprosy patients. Emerg Microbes Infect. 2019;8(1):1479-89.
5. Cambau E, Perani E, Guillemin I, Jamet P, Ji B. Multidrug-resistance to dapsone, rifampicin, and ofloxacin in Mycobacterium leprae. Lancet. 1997;349(9045):103-4.
6. Maeda S, Matsuoka M, Nakata N, Kai M, Maeda Y, Hashimoto K, et al. Multidrug resistant Mycobacterium leprae from patients with leprosy. Antimicrob Agents Chemother. 2001;45(12):3635-9.
7. Singh P, Benjak A, Carat S, Kai M, Busso P, Avanzi C, et al. Genome-wide re-sequencing of multidrug-resistant Mycobacterium leprae Airaku-3. Clin Microbiol Infect. 2014;20(10):O619-22.
8. Benjak A, Avanzi C, Singh P, Loiseau C, Girma S, Busso P, et al. Phylogenomics and antimicrobial resistance of the leprosy bacillus Mycobacterium leprae. Nat Commun. 2018;9(1):352.
9. Shepard CC. Statistical analysis of results obtained by two methods for testing drug activity against Mycobacterium leprae. Int J Lepr Other Mycobact Dis. 1982;50(1):96-101.
10. Richardus JH, Tiwari A, Barth-Jaeggi T, Arif MA, Banstola NL, Baskota R, et al. Leprosy post-exposure prophylaxis with single-dose rifampicin (LPEP): an international feasibility programme. Lancet Glob Health. 2021;9(1):e81-e90.
11. Lockwood DNJ, Krishnamurthy P, Kumar B, Penna G. Single-dose rifampicin chemoprophylaxis protects those who need it least and is not a cost-effective intervention. PLoS Negl Trop Dis. 2018;12(6):e0006403.
12. Mieras L, Anthony R, van Brakel W, Bratschi MW, van den Broek J, Cambau E, et al. Negligible risk of inducing resistance in Mycobacterium tuberculosis with single-dose rifampicin as post-exposure prophylaxis for leprosy. Infect Dis Poverty. 2016;5(1):46.
13. Jouet A, Gaudin C, Badalato N, Allix-Beguec C, Duthoy S, Ferre A, et al. Deep amplicon sequencing for culture-free prediction of susceptibility or resistance to 13 anti-tuberculous drugs. Eur Respir J. 2021;57(3).
14. Hasker E BA, Younoussa A, Mzembaba A, Grillone S, Demeulenaere T, Groenen G, Suffys P, de Jong, B. C. Leprosy on Anjouan (Comoros): persistent hyperendemicity despite decades of solid control efforts. Leprosy reviews. 2017.

15. Ortuno-Gutierrez N, Baco A, Braet S, Younoussa A, Mzembaba A, Salim Z, et al. Clustering of leprosy beyond the household level in a highly endemic setting on the Comoros, an observational study. BMC Infect Dis. 2019;19(1):501.
16. World Health Organization, team Control of Neglected tropical Diseases. Guidelines for the diagnosis, treatment and prevention of leprosy. 2018.
17. Diseases WgCoNr. Guidelines for the diagnosis, treatment and prevention of leprosy. 2018.
18. Braet SM, van Hooij A, Hasker E, Fransen E, Wirdane A, Baco A, et al. Minimally invasive sampling to identify leprosy patients with a high bacterial burden in the Union of the Comoros. PLoS Negl Trop Dis. 2021;15(11):e0009924.
19. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics. 2015;31(16):2745-7.
20. Chauffour A, Morel F, Reibel F, Petrella S, Mayer C, Cambau E, et al. A systematic review of Mycobacterium leprae DNA gyrase mutations and their impact on fluoroquinolone resistance. Clin Microbiol Infect. 2021;27(11):1601-12.
21. Beltran-Alzate C, Lopez Diaz F, Romero-Montoya M, Sakamuri R, Li W, Kimura M, et al. Leprosy Drug Resistance Surveillance in Colombia: The Experience of a Sentinel Country. PLoS Negl Trop Dis. 2016;10(10):e0005041.
22. Cambau E, Saunderson P, Matsuoka M, Cole ST, Kai M, Suffys P, et al. Antimicrobial resistance in leprosy: results of the first prospective open survey conducted by a WHO surveillance network for the period 2009-15. Clin Microbiol Infect. 2018;24(12):1305-10.
23. Iwao Y, Mori S, Ato M, Nakata N. Simultaneous Determination of Mycobacterium leprae Drug Resistance and Single-Nucleotide Polymorphism Genotype by Use of Nested Multiplex PCR with Amplicon Sequencing. J Clin Microbiol. 2021;59(10):e0081421.
24. Avanzi C, Lecorche E, Rakotomalala FA, Benjak A, Rapelanoro Rabenja F, Ramarozatovo LS, et al. Population Genomics of Mycobacterium leprae Reveals a New Genotype in Madagascar and the Comoros. Front Microbiol. 2020;11:711.
25. Pattyn S, Grillone S. A 6 week quadruple drug regimen for the treatment of multibacillary leprosy. Lepr Rev. 2000;71(1):43-6.
26. Pattyn S, Grillone S. Relapse rates and a 10-year follow-up of a 6-week quadruple drug regimen for multibacillary leprosy. Lepr Rev. 2002;73(3):245-7.
27. Pattyn SR, Bourland J, Grillone S, Groenen G, Ghys P. Combined regimens of one year duration in the treatment of multibacillary leprosy--I. Combined regimens with rifampicin administered during one year. Lepr Rev. 1989;60(2):109-17.
28. Joshi R. Clues to histopathological diagnosis of treated leprosy. Indian J Dermatol. 2011;56(5):505-9.
29. Ahmed N, Saini V, Raghuvanshi S, Khurana JP, Tyagi AK, Tyagi AK, et al. Molecular analysis of a leprosy immunotherapeutic bacillus provides insights into Mycobacterium evolution. PLoS One. 2007;2(10):e968.

CHAPTER VI

Transmission patterns of *Mycobacterium leprae* in the Comoros

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INTRODUCTION

The Comoros islands, located between Madagascar and Mozambique, continue to be hyperendemic for leprosy. Enhanced case finding through skin camps is conducted across the island of Anjouan, resulting in early case detection and treatment initiation, which rapidly lowers infectiousness. Nevertheless, the high proportion of leprosy in children (31% of leprosy cases were <15y old in 2015) and a low relapse rate (<2%) suggest that transmission continues unabated in the Union of the Comoros. Globally, *Mycobacterium leprae* genotypes show geographical associations. Genotyping by multiple-locus variable number of tandem repeats (VNTR) analysis (MLVA) appears sufficiently polymorphic within geographically confined populations to differentiate *M. leprae,* hence allowing to detect transmission events. MLVA is however time-consuming and requires sufficient amounts of *M. leprae* DNA, resulting in limited sensitivity when applied to paucibacillary patients. Thus, the present investigation examines the potential application of targeted deep sequencing of VNTR markers, employing the Deeplex Myc-Lep assay, for transmission analysis in the Comoros.

MATERIAL AND METHODS

Setting

The Comoros, situated in the Indian Ocean between Madagascar and Mozambique, is comprised of three islands, collectively accommodating a total population of 821,625. Among these islands, Anjouan (340,000 population, 424km^2), and Mohéli (38,000 population, 290km^2), stand out as two territories where the prevalence of leprosy is exclusive. Notably, the island of Grand Comore, endowed with greater affluence, remains untouched by endemic leprosy. Additionally, a fourth island within the Comorian geographical archipelago falls under French governance, experiencing recurrent incidents of illicit migration.

Of the two islands with endemic leprosy, Anjouan is most endemic, with a high population density due to the island's rugged terrain, with the habitable zones predominantly concentrated along the coastal periphery. The Comoros is one of the six countries with the largest burden of leprosy, as defined by the World Health Organization (WHO). From 2013 to 2019, they found that every year, there were three to seven new cases for every 10,000 people. The National Leprosy Control Programme has been implementing enhance case identification measures since 2008, notably in the form of monthly skin camps. Because of these efforts, leprosy treatment is usually finished by over 85% of the people, very few people have a relapse (just 1.8%), and when new cases are found, less than 2.5% of them have grade II disabilities. Moreover, the absence of detected drug resistance in leprosy cases further underscores the efficacy of leprosy control endeavors within the Comoros [1].

Study Design and Sample Collection

The ComLep study (ClinicalTrials.gov Identifier: NCT03526718), a cross-sectional survey conducted on the island of Anjouan (Comoros) from 2017 to 2019, identified patients through enhanced case finding using skin camps in addition to routine passive case finding. Meanwhile,

the PEOPLE study (ClinicalTrials.gov Identifier: NCT03662022) identified patients from 2019-2022 by conducting annual door-to-door screening in selected villages on the islands of Anjouan and Mohéli, as well as skin camps and passive case finding in other villages. Diagnosis of patients was based on clinical symptoms and distinction between paucibacillary (PB) or multibacillary (MB) leprosy is done according to the WHO operational classification . The National Leprosy Control Programme uses a standardized form to record the location of a patient's lesions, as well as any patient's known leprosy contacts, and their relationship. For patients showing genetic clustering, the anthropological team carried out in-depth interviews with patients who could be relocated. The aim was to uncover connections among these patients without revealing the identities of the patients.

From consenting leprosy patients we collected 4mm skin biopsies, which were immediately inactivated by storage in 1 ml of molecular grade Disolol (ethanol denatured with 1% isopropanol and 1% methyl ethyl ketone) in screw cap vials at ambient temperature. These samples were transported to the Institute of Tropical Medicine (Antwerp, Belgium) in batches once or twice per year. Negative sampling controls (Copan FloqSwabs that were exposed for ≥ 1 minute to air in the room where the biopsies were taken) were included on each sampling day.

DNA Isolation From Skin Biopsies

At the Institute of Tropical Medicine, the skin biopsies were processed as described previously [1]. Briefly, suspensions were treated with an in-house lysis buffer, followed by DNA extraction in the semi-automated Maxwell system (Promega, USA). Each run included a positive (*M. leprae* Thai 53 suspension from mouse footpad) and a negative (molecular grade water) DNA-extraction control.

RLEP qPCR, Deeplex Myc-Lep and whole genome sequencing

The *M. leprae* bacterial load in 2 µl skin biopsy DNA extract was calculated using an *M. leprae*-specific repetitive element (RLEP)-quantitative PCR (qPCR), as previously described [1]. A positive (BEI resources: 19350) and negative DNA qPCR control were included. Samples were selected for Deeplex MycLep based on their estimated bacterial load (all samples with ≥2000 bacilli/2µl and some with ≥100bacilli/2µl) and treatment status (having received prophylaxis with single or double dose rifampicin, or full leprosy treatment). For a proof-of-concept a sub selection was made based on factors such as SNP subtype, VNTR cluster size, a mutation in *gyrB*, or geographical distribution of the VNTR cluster. From this selection samples with a RLEP Cq-value < 28 were selected to be processed with WGS.For the Deeplex Myc-Lep, DNA was amplified and sequenced using the prototype kit by the manufacturer [1, 2]. This assay allows ultra-deep sequencing of *M. leprae* directly in clinical samples, using a single, 37-multiplexed amplicon (+ internal control) mix to identify the mycobacterial species (based on the *hsp65* gene), type *M. leprae* strains (based on single nucleotide polymorphisms (SNPs) in 18 gene regions and 11 VNTRs), and detect potential resistance-associated SNPs in seven genes (rifampicin: *rpoB*, *ctpC*, and *ctpI*; dapsone: *folP1*; and fluoroquinolones: *gyrA*, *gyrB*, and *nth*). For the SNPs the minimal threshold for base calling was 5X to be considered successfully

sequenced, while for VNTRs the flanking regions of the VNTR marker must be detected in at least 5 independent reads.

For WGS, 1 µg of purified DNA was fragmented to 300-400 bp using Adaptive Focused Acoustics on a Covaris instrument. The DNA was cleaned using KAPA Pure beads (Roche, Switzerland), followed by library preparation using the KAPA HyperPrep kit and dual indexes. After quality and quantity were checked by Qubit dsDNA kit (Promega, USA) and Tapestation (Agilent, USA), libraries were multiplexed with the KAPA HyperCap Target Enrichment Probes and sequenced with paired-end 250bp reads on an Illumina MiSeq instrument (Illumina, USA).

M. leprae WGS was analyzed as previously described[3], using as cutoffs: overall coverage ≥ 5 non-duplicated reads, ≥ 3 non-duplicated reads supporting the SNP, mapping quality score >8, base quality score >15, and an allele frequency >80%. Repetitive regions are excluded from whole-genome sequencing analysis due to challenges in accurate read alignment, assembly, and variant calling caused by their propensity for inducing errors and ambiguities.

SNP (sub)-type determination

The classification of SNP subtypes was done using a typing system consisting of 18 polymorphic sites, which encompassed both SNPs and DNA insertions/deletions. This system effectively differentiates between the various global SNP (sub)-types of *M. leprae*, following the canonical guidelines initially outlined by Monot et al.[4].

Calculation of allelic diversity and discriminatory power per VNTR marker

In order to evaluate the VNTR discriminatory power, we used the Hunter-Gaston discrimination index (HGDI), with the formula: $HGDI\ (h) = 1 - \left(\frac{1}{N(N-1)}\right) * \sum_{j=1}^{s} n_j(n_j - 1)$, where n_j denotes the frequency of the nth allele at a given locus, and *N* is the total number of isolates in the sample [3]. Based on the resulting HGDI (h) values, the degree of polymorphism was classified as highly discriminatory (h > 0.6), moderately discriminatory (0.3 ≤ h ≤ 0.6), or weakly discriminatory (h ≤ 0.3) [4]

Cluster definition and genotype comparison

Clustering was defined by comparing the copy number of the VNTR loci, with genotypes considered identical if they had the same copy number for all 11 VNTRs. A cluster was defined as two or more patients having the exact same VNTR profile. A distance similarity matrix was made to differentiate variability within distinct VNTR clusters, subsequently the unweighted pair group method with arithmetic mean (UPGMA) from the Ape package was utilized to build the phylogenetic tree in R version 4.1.2 (The R foundation, Vienna, Austria) [5].

The clustering rate was computed using the formula (nc − c)/n, wherein nc stands for the total number of samples clustered by an identical VNTR profile, c represents the number of clusters formed, and n denotes the total number of samples in the dataset [6].

Sizes of population per village were retrieved from the official population figures from the Union of the Comoros 2017. Genotype diversity at the village level was determined by dividing

the number of distinct VNTR genotypes that were observed to be circulating within the same village by the number of patients with samples genotyped within a specific village.

Repetitive regions were omitted for phylogenetic inferences from WGS data; positions with missing data in <10% of samples were included. A concatenated SNP alignment was used for phylogenetic analysis with maximum likelihood approaches, including publicly available genomes, with *M. lepromatosis* as an outgroup.

Spatial analysis

For patients diagnosed during door-to-door screening, geographical coordinates (latitude and longitude) of the household were collected. For patients diagnosed during skin camps or at the hospital, geographical coordinates were collected during household visits for contact investigation. For patients for whom the household coordinates were not collected, we used the latitude and longitude of the central point of the patient's registered neighborhood of residence.

Statistical analyses

A univariate analysis of clustered versus non-clustered genotypes was done, to identify potential risk factors for recent transmission. All statistical analysis were done using logistic regression in R version 4.1.2 (The R foundation, Vienna, Austria), with clustered/non-clustered as a dependent variable. Additionally, the identified risk factors were mapped onto a phylogenetic tree based on the VNTR patterns to identify risk factors for genotypic clustering, as a proxy for recent transmission.

RESULTS

Between July 2017 and January 2022, a total of 1403 patients with leprosy were recruited on the islands of Anjouan (n=1364; 97.2%) and Mohéli (n= 39; 2.8%). Of these, 1213 (86.5%) provided a skin biopsy for analysis, 905 (74.6%) of which were confirmed to contain *M. leprae* by RLEP qPCR. Throughout the study, all environmental and analytical controls yielded the expected results (Fig. VI.1). From the RLEP positives, 290 (32.0%, 31 PB and 259 MB patients) were selected for further analysis with the Deeplex Myc-Lep based on their estimated bacterial load and treatment status: 35 patients with ≥2000 bacilli/2µl are not yet processed and one with <100 bacilli/2µl was erroneously processed. Two biopsies from this selection were derived from the same patient sampled in 2017 and 2020, while the remaining 288 were from different patients. For 256 (88.3%) of 290 biopsies, a full (11 markers) VNTR profile could be obtained.
In a proof-of-concept analysis, we successfully obtained WGS from 41 of 48 biopsies from patients with the highest bacterial load (>2000 bacilli/2µl), with a mean read depth ranging from 8.3X-149.3X and an average alignment rate of 96.39% (SD 5.53%). Seven failed due to too low read depth (0.0X-4.9X) and an alignment rate of on average 56.18% (SD 38.37%) after sequencing.

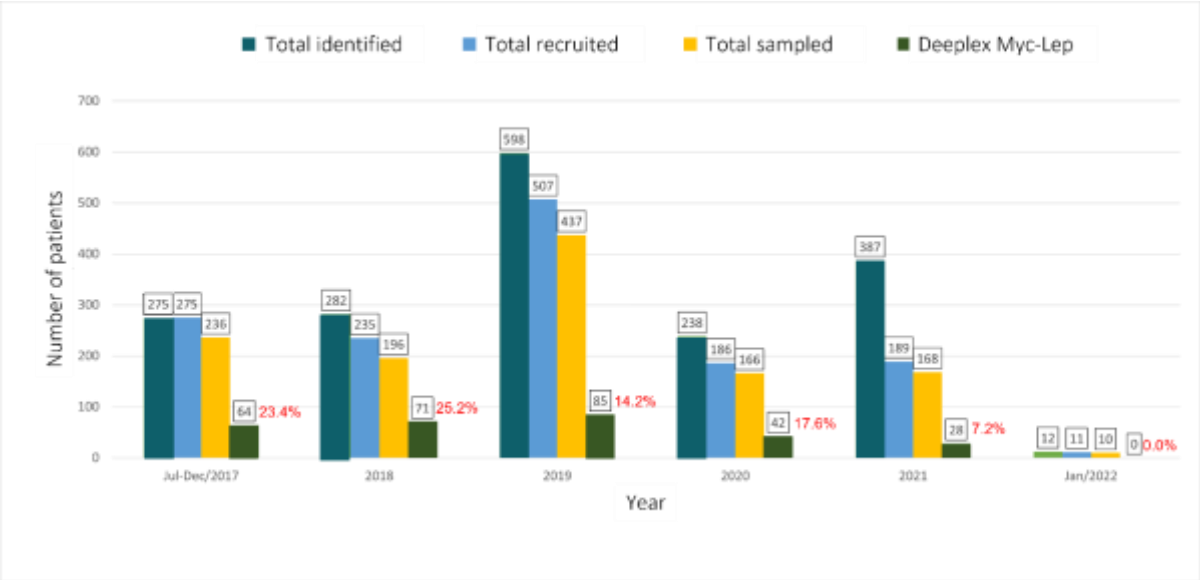

Figure VI.1 Sampling fraction. Red percentages are the fraction of patients genotyped with Deeplex Myc-Lep from total identified patients in that period.

M. leprae SNP (sub)types and their distribution

All samples showed pure *M. leprae* DNA based on the *hsp65* gene in the Deeplex Myc-Lep assay, i.e. no (co)infection with *M. lepromatosis* or other mycobacteria was found [2]. SNP subtype 1D was found in 220 (85.9%) and SNP subtype 1A in 34 (13.3%) patients with successful VNTR typing. While for two patients SNP type 1 could be inferred, the SNP subtype was not confirmed because SNP14676 (ID 297&2181), SNP61425 (ID 297&2181) and Ins-978589 (ID 297) were insufficiently covered.

When plotting the SNP-subtypes onto the VNTR-based phylogenetic tree, an almost perfect correlation of SNP-subtypes with VNTR-based branches can be observed (Fig. VI.2). Within our Comorian sample, the division based on SNP subtype (1A vs. 1D) can be attributed to VNTR locus 21-3, for which the 1A SNP subtype typically possesses three copies and 1D usually carries one or two copies. All the samples with 1D and 1x 21-3 cluster together in one single VNTR cluster except for one that differs by one VNTR marker (GAA21) with this cluster (Fig. VI.2) Interestingly, one sample exhibiting the 1D SNP subtype falls within the 1A SNP group due to

its VNTR profile, as it contains three copies of the VNTR 21-3 locus.

Figure VI.2: Left: Distribution of different SNP-(sub)types on the island of Anjouan (arrow indicates the location of the villages of Vassy, Vouani, and Dar Salama in the Pomoni district). Right: UPGMA phylogenetic tree based solely on the VNTR profiles, with SNP subtype plotted on the leaves (arrow indicates sample with SNP subtype 1D with 3x 21-3, accolade indicates SNP subtype 1D with 1x 21-3). Figure created with Microreact [7].

All 41 strains with successful WGS results were checked for their SNP (sub)type. All SNP-1D strains found by Deeplex Myc-Lep, belonged to the WGS-defined 1D-Malagasy group sequenced by Avanzi *et al* 2020 (Fig. VI.3), based on mutation A1015G at position 2921694 of the *M. leprae* reference genome. Comorian SNP- subtype 1D samples cluster with samples from Mayotte (samples starting with MY and Comore 1-3), which were not recruited within this study (Fig. VI.3). The SNP-subtype 1A strains from the Comorian cohort that were processed with WGS did not cluster with the 1A strains from Madagascar (Fig. VI.3).

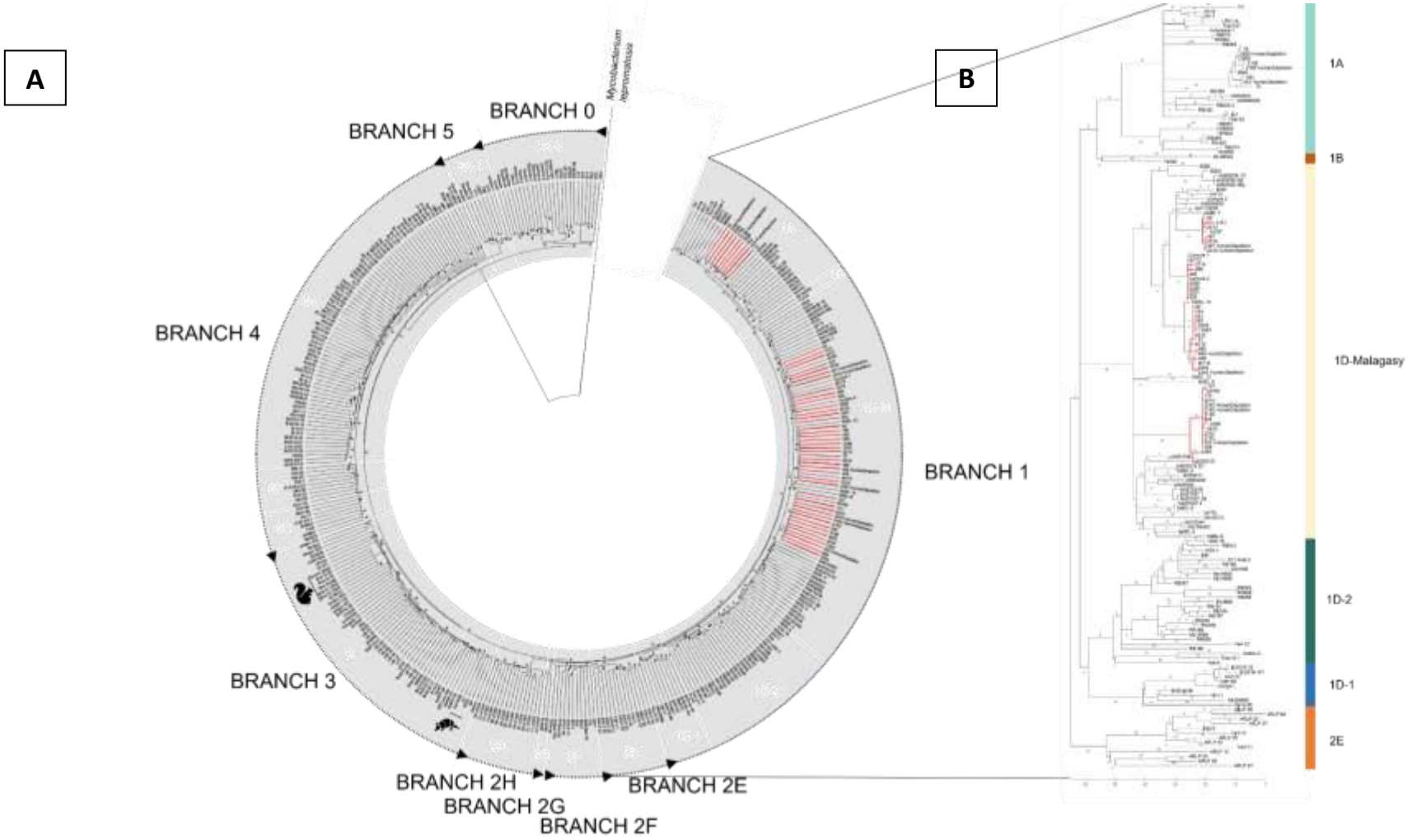

Figure VI.3: Global phylogeography of Mycobacterium leprae. Newly sequenced genomes from the Comoros are shown in red. Leaves are labeled with the sample ID. ID numbers starting with MY are samples from Mayotte. (A) Maximum parsimony tree of 372 genomes of M. leprae representing nine branches and 16 genotypes. Support values were obtained by bootstrapping 500 replicates. Branch lengths are proportional to nucleotide substitutions. The tree is rooted using Mycobacterium lepromatosis. (B) Zoom into branch 1 (genotypes 1A, 1B, 1D, and the 1D-Malagasy) and 2E of the maximum parsimony tree from (A).

While SNPsubtype 1D is widespread on both Anjouan and Mohéli, SNPsubtype 1A is more restricted with only one patient in Mohéli, and most cases found in the Anjouan district of Pomoni where 1A is the only circulating SNP subtype in the villages Vassy, Vouani and Dar Salama (Fig. VI.2:left).

We did not identify differences in leprosy spectrum among the different SNP sub-types, with MB proportions of 85.3% (95% CI 73.4%-97.1%) among the patients with 1A and 91.4% (95% CI 87.7%-96.7%) for 1D, albeit biased by inclusion of patients based on their bacterial load in biopsy.

Clustering based on VNTRs by Deeplex Myc-Lep

We examined the ability of 11 VNTR markers to differentiate among *M. leprae* obtained from skin biopsies from 255 different leprosy patients and one patient with two episodes of leprosy. The selected combination of VNTR loci demonstrated a high overall discriminatory index (h=0.98) (Table VI.1). The calculated HGDI values (h) ranged from 0.00 to 0.89 across the 11 separate VNTR markers, with markers 6-3a and 12-5 showing no discriminatory power (h=0.00), and GAA21 exhibiting the highest discriminatory power.

Table VI.1: H-values and general information on the copy number variants for each VNTR marker. A marker is considered highly discriminatory polymorphic if h > 0.6, moderately discriminatory if 0.3 < h ≤ 0.6 and weakly discriminatory if h ≤ 0.3.

VNTR marker	Repeat size (bp)	Variants in copy numbers	HGDI (h)	Discriminatory power	Min-max copy number in Comorian sample set	Most common copy number variant
GAA21	3	20	0.89	high	10-35	18 (23.04%)
6-7	6	6	0.64	high	5-10	9 (21.86%)
AC8a	2	6	0.62	high	7-12	9 (48.82%)
GTA9	3	6	0.57	moderate	8-14	9 (62.5%)
AC9	2	4	0.47	moderate	7-10	8 (69.92%)
AC8b	2	4	0.4	moderate	6-9	8 (75.78%)
21-3	21	3	0.31	moderate	1-3	2 (81.64%)
GGT5	3	3	0.2	weak	4-6	5 (89.06%)
23-3	23	2	0.02	weak	1-2	2 (99.22%)
6-3a	6	1	0	no	3	3 (100%)
12-5	12	1	0	no	4	4 (100%)
Set of 11 markers		97	0.98	high		

Our dataset contained a total of 52 unique VNTR profiles, and 45 VNTR profiles occurring more than once, resulting in 97 distinct VNTR profiles were identified in Anjouan and Mohéli (Table VI.1; Fig. VI.4A). The cluster sizes of these 45 profiles ranged from two to 14 patients, with the majority of clusters (n=20; 44.44%) comprising only two patients (Fig.4B) Of the 256 patients,

204 (79.7%) were clustered with at least one other patient based on an exact same VNTR-profile (Fig. VI.4A). The cluster rate was 60.2%.
When analysis the same data, but excluding GAA21 (the most variable VNTR, h>0.8)), the dataset contains only 26 unique VNTR profiles, and 31 VNTR profile occurring more than once, resulting in 57 distinct VNTR profiles across the islands. The cluster sizes of these 31 profiles range from two to 39 patients, with the majority of them (n=12; 38.7%) comprising only two patients. Of the 256 patients, 230 (89.8%) were clustered with at least one other patient based on an exact same VNTR-profile consisting of 10 markers. The cluster rate was 77.73%.

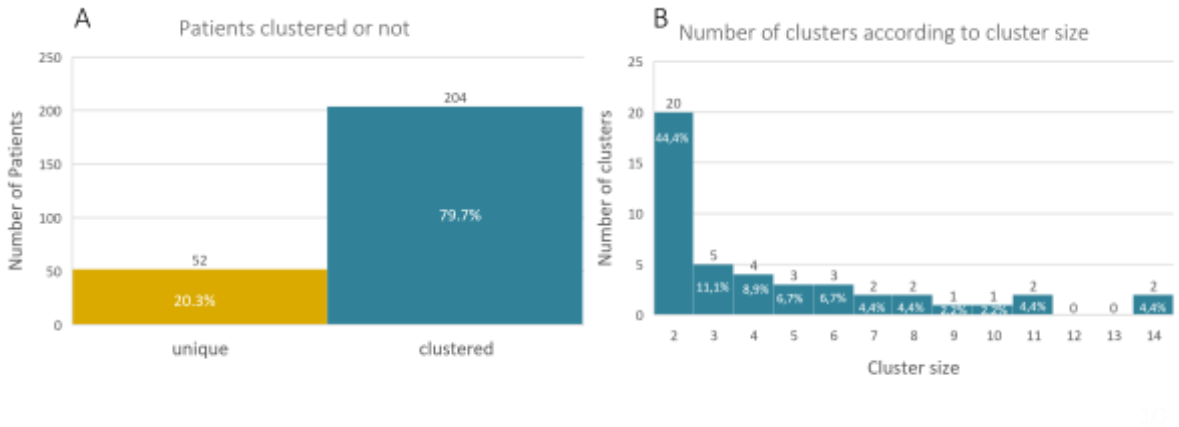

Figure VI.4: (A) Proportion clustered. (B) Distribution of cluster sizes. x-axis shows the number of patients included in such a cluster.

We examined these VNTR clusters in further detail, as evidence of ongoing transmission. The two biggest VNTR clusters identified (#2 & #19 with 14 patients each) were widely distributed across the island of Anjouan with no clear geographical pattern while some of the other larger clusters are more geographically bound, with the majority of the clustered samples found in one area (e.g. cluster #1, #6, #13 and #25) (Fig. VI.5B).

Fourteen (42.4%) of 33 patients with genotype 1A belong to the same VNTR cluster #2. Patients from cluster #2 are scattered over nine different villages with half of them from Vouani (Fig 5A). There are no known contacts between non-geographically clustered patients in this cluster. Cluster #2 samples were not selected for WGS.

In cluster #19, four pairs of patients resided in the same villages on Anjouan, namely Mirontsi, Mahale, Bougoueni and Dindri in the middle of the island, while the other eight patients were dispersed over the island of Anjouan (Fig. VI.5A) and one patient is from Mohéli. No social or family links could be identified between any of the patients from this cluster.
Eight patients from cluster #19 were successfully processed with WGS, which further split it down in two WGS clusters (#19a and #19b) that are 22 SNPs apart, one with maximum 5 SNPs difference among the samples and the other with maximum 2 SNPs difference (Fig. VI.6). Based on WGS only two cluster #19 samples are identical (427 & 2316), found in two patients from neighboring villages (Bougoueni and Mroumhouli) in the same district (Sima) (Fig. VI.5A).

Among the geographically more restricted clusters, #13 includes 10 patients of whom nine live in the same village (Jimilimé; Fig. VI.5B). Two patients belong to the same family and among the rest, no known contact could be identified. Eight cluster #13 patients had *M. leprae* WGS

done, of whom five patients showed the exact same WGS and one patient differed by one SNP (#13a)(Fig. VI.6). The remaining two patients (ID 2219/2226) fall in a separate WGS cluster (#13b), differing by 18 SNPs from the other eight patients. However, the coverage of these two samples for WGS ranges from 8-12X, so resolution might be missed by WGS because of coverage.

Cluster #6 consists of 11 patients, all belonging to SNP subtype 1D, except for one for which the SNP subtype couldn't be determined. Of these 11 patients, nine carry *M. leprae* with a non-synonymous SNP in *gyrB* (Asp521Tyr), not deemed to confer resistance to fluoroquinolones (Braet *et al* 2022). The majority of patients in this cluster (n=9) live in the same northwestern village Ouani (Fig. VI.5B) and one in the neigboring village Gnatranga (Fig. VI.5B). Two of them are known contacts, while the other cluster #6 patients living in southern Adda-Douéni are not known contacts. Five of 11 cluster #6 patients had successful WGS, with four of them showing identical genomes and one patient (ID 466) differing by only two SNPs (Fig. VI.6).

Even though cluster #6 and #13 are distinct VNTR-based clusters with different copy numbers in four VNTR loci (Table VI.2), a portion of cluster #13 shows an identical WGS profile with cluster #6 (Fig. VI.6). Patients from cluster #6 and #13 who share the same WGS profile are from neighboring villages, namely Ouani and Gnatranga, along with Jimilimé in the north (Fig. VI.5B).

Table VI.2: VNTR markers that have different copy numbers in cluster 13 and cluster 6

VNTR marker	h-value in Comorian bacterial *M. leprae* population	Discriminatory power	Copy number in Cluster #6	Copy number in Cluster #13
GTA9	0.57	moderate	8	9
GAA21	0.89	high	11	17
6-7	0.64	high	6	10
21-3	0.31	moderate	1	2
VNTR marker	h-value in Comorian bacterial *M. leprae* population	Discriminatory power	Copy number in Cluster #13	Copy number in Cluster #25
AC8a	0.62	high	9	12
AC8b	0.40	moderate	7	9
GAA21	0.89	high	17	19
6-7	0.64	high	10	8

The geographically more confined cluster #25 comprises 11 patients, with the majority (n=8) residing in the eastern village of Domoni (Fig. VI.5B). No direct social or family links were identified among them. Eleven patients' bacilli underwent successful WGS. One patient from northern Jimlimé (ID 2407) exhibited 42 SNPs difference compared to the other patients in the cluster (Fig. VI.6). Similar to the two cluster#6 samples, this patient falls within a WGS clade of cluster #13 even though the VNTR copies vary in multiple loci (Table VI.2). The remaining cluster #25 patients differ by maximum two SNPs. Notably, patients 489 (Ouani) and 2182 (Adda-Douéni) have identical WGS (Fig. VI.5B), despite living in other districts of the island. Although no self-declared direct link was found, the anthropological team found out that patient 2182

has never left Adda-Douéni, while her sister had lived in Ouani in the same neighborhood as patient 489, with frequent contact between the populations in Adda-Douéni and that neighborhood of Ouani. Furthermore, patient 2182 shares the same family name as the mother of patient 489, suggesting a potential (unconfirmed) family link.

Cluster #1 includes six patients of whom four live in two neighboring villages, namely Chamdra and Tsembehou (Fig. VI.5B). No direct social/family links could be identified. Samples from all patients of this cluster where sequenced with WGS, resulting in a separate clade in the phylogenetic tree grouping with other publicly available WGS belonging to SNP sub-type 1A (Fig. VI.6). Within this cluster #1 clade the maximum SNP difference is 12 (Fig. VI.6:ID 2365).

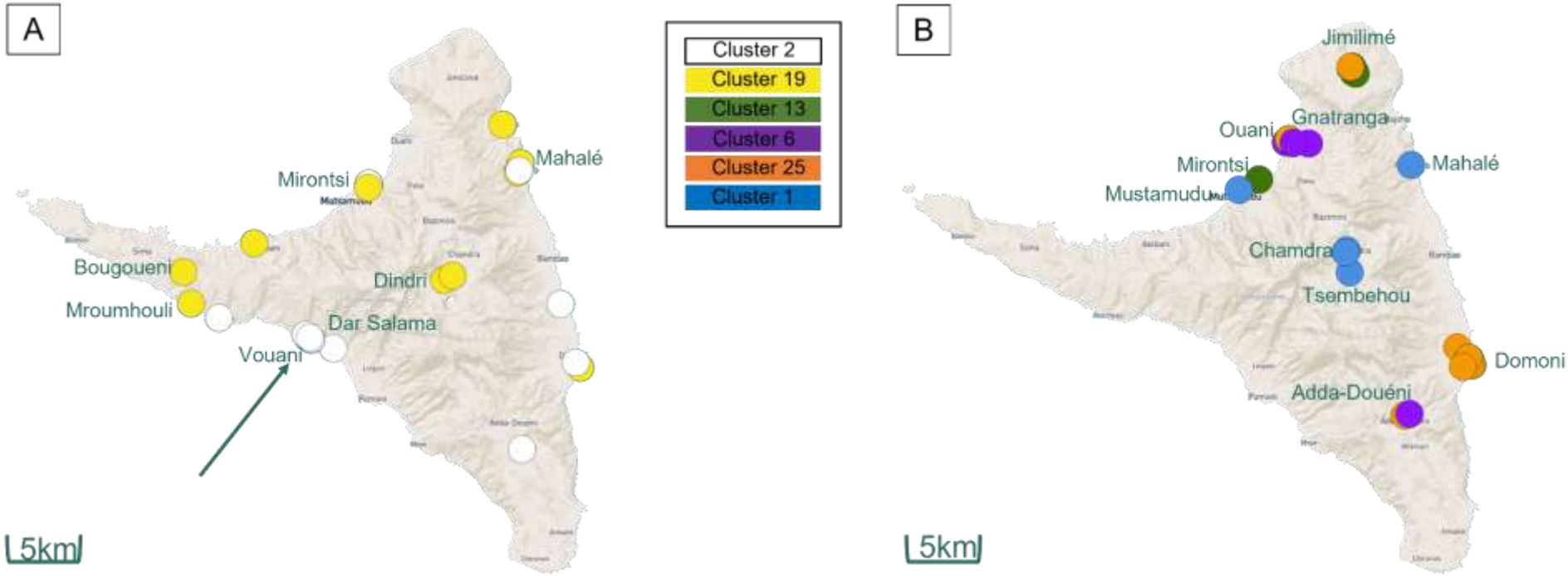

Figure VI.5: (A) VNTR clusters # 2 (SNP subtype 1A) and # 19 (SNP subtype 1D) are each comprising 14 patients in Anjouan dispersed over the island. Arrow indicates the villages of Vouani and its neighboring village Dar Salama. (B). VNTR clusters # 13, # 6, #25 & #1 ; each comprising 10,11, 11 and 6 patients respectively, are more geographically bound, with the majority of the cluster in one area. Figure created with Microreact[5]

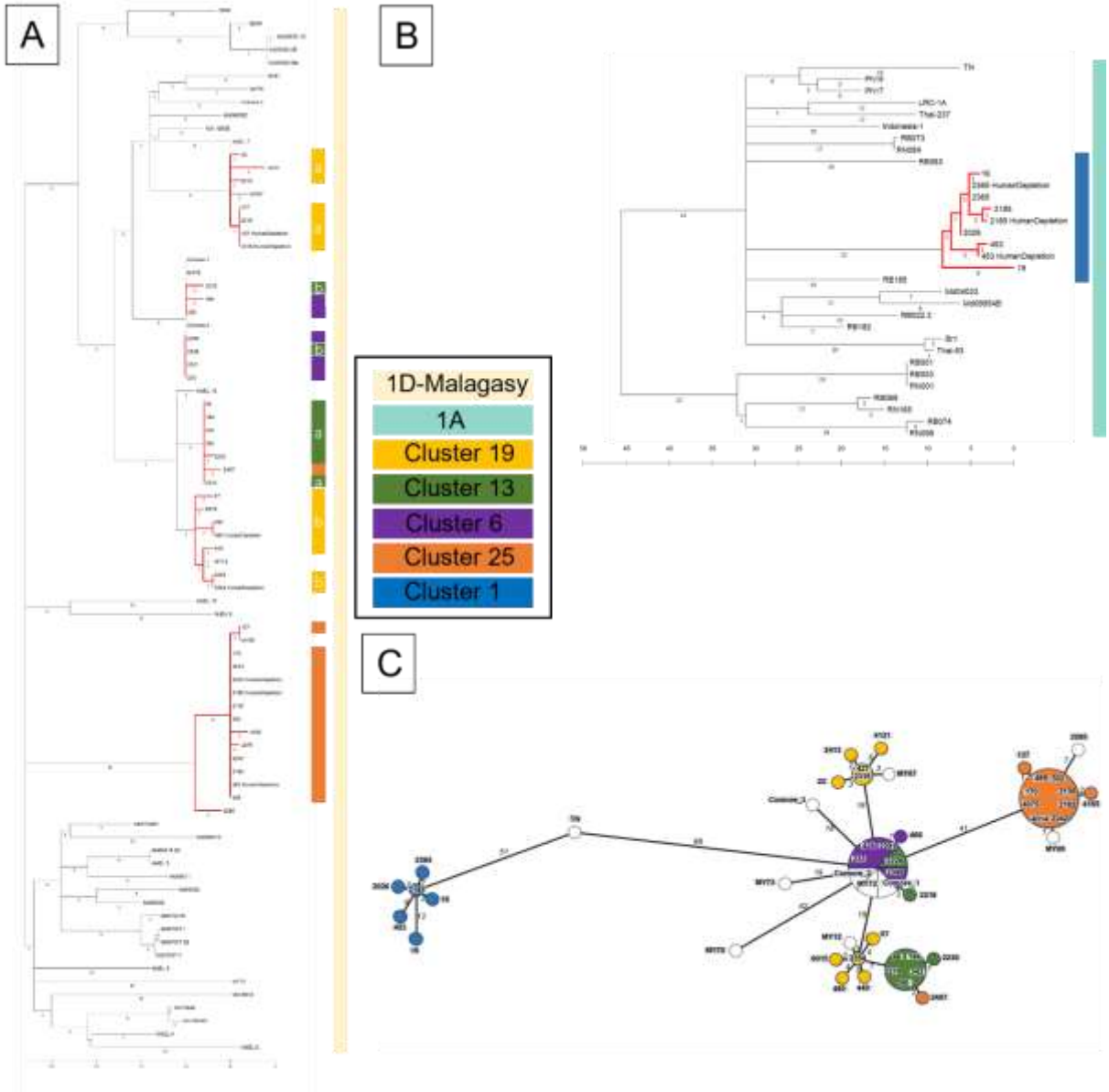

Figure VI.6: The WGS-based 1D-Malagasy (part A) and 1A (part B) M. leprae phylogenetic subtrees as shown in Figure 2, with indication of the VNTR-based clusters by color code. Branch lengths are proportional to nucleotide substitutions, which are represented under the branches. Samples sequenced in this study are shown in red. Part C represents the WGS-based minimum spanning tree created with Grapetree, with indication of the VNTR clusters by color code.

The majority (44.4%; n=20) of the identified clusters by VNTR comprised of two samples. One of these resulted from a patient being sampled twice (in 2017 and 2020), two clusters included patients who lived within 100m from each other and another one included two patients inhabiting the same neighborhood. In addition, patients from five other 2-sample clusters resided in the same village, including one cluster of patients with known contact. For the majority (11/20; 55%) of 2-sample clusters, however, no epidemiological link or geographical proximity could be identified: three clusters of two patients were from within the same district and eight clusters spanned across district borders.

Genotyping supporting (or not) transmission based on contact information

We investigated *M. leprae* VNTR profiles among known diseased contacts as declared by the patients at the time of diagnosis. Of ten contact relations identified, *M. leprae* transmission could be confirmed with identical VNTR profiles in five cases, while four contact links differed in only one VNTR locus and two in four VNTR loci (Table VI.3). The most variable VNTR locus was GAA21. Figure VI.7 illustrates a minimum spanning tree (MST) where patients having a social or family link but distinct VNTR profiles are highlighted; these patients, connected by one branch despite one or two VNTR differences, might still belong to the same transmission group. The other two pairs with four VNTR differences, are less likely to be linked, as the distance on the minimum spanning tree exceeds 1. By removing GAA21 from the analysis, some additional social links can be explained. Nevertheless, this leads to a greater number of unexplained genotypic links and the emergence of larger, more widely scattered clusters (data not shown).

Table VI.3: Summary of sociodemographic and genotypic information for patients with a known contact and an interpretable VNTR profile.

ID	Month Dx	Type of contact	Location Village (District)	VNTR Cluster #	VNTRs with observed difference	Copy numbers per VNTR variance	WGS available	Depicted in Figure 11
46	Aug/17	Common mutual leprosy contact diagnosed in 6/4/2013	Jimlime (Ouani): live in adjacent neighborhoods	13	NA	NA	yes	
161	Nov/17						no	
4096	Aug/20	Contacts of each other	Ouani (Ouani): live in same neighborhood	6	NA	NA	no	
4192	Jan/21						No, but*	
87	Oct/17	Same patient, relapse/reinfection	Paje (Mutsamudu)	2	NA	NA	no	
4090	Jul/20						no	
2370	Nov/19	Contacts of each other	Hajoho (Domoni)	35	NA	NA	no	
2530	Jun/21						no	
56	Jul/17	Daughter 1	Mirontsi (Mutsamudu)	Not tested	NA	NA	no	
57	Jul/17	Daughter 2		19	GAA21	16	yes	A
55	Jul/17	Son		Not tested	NA	NA	no	
2361	Oct/19	Father (recurrence, first episode 2012)		21	GAA21	15	no	A
4216	Nov/20	Daughter 1 (recurrence)		Not tested	NA	NA	no	
4217	Nov/20	Daughter 2 (recurrence)		Not tested	NA	NA	no	
4218	Nov/20	Son (recurrence)		Not tested	NA	NA	no	
18	Jul/17	Same household	Dindri (Tsembehou)	20	GTA9	3	no	B
309	Jan/18			unique		10	no	B

ID	Month Dx	Type of contact	Location Village (District)	VNTR Cluster #	VNTRs with observed difference	Copy numbers per VNTR variance	WGS available	Depicted in Figure 11
66	Sep/17	Contacts of each other	Sima (Sima)	3	AC9	8	no	C
364	Mar/18			unique		9	no	C
497	Dec/18	common mutual leprosy contact diagnosed in May 2014	Domoni(Domoni)	unique	GAA21	19	no	D
4075	Feb/20			25		21	yes	D
489	Nov/18	Contacts of each other	Ouani (Ouani): live in same neighborhood	19	AC8a|AC8b GTA9|GAA21	12|9|9|19	yes	E
2394	Nov/19			unique		9|7|10|13	no	E
2361	Oct/19	Contacts of each other	Mirontsi, Mutsamudu	21	AC8a|AC9 GTA9|GAA21	9|8|9|15	no	F
2389	Nov/19			30		8|7|10|25	no	F

NA = not applicable; * = 4192 has a SNP in gyrB

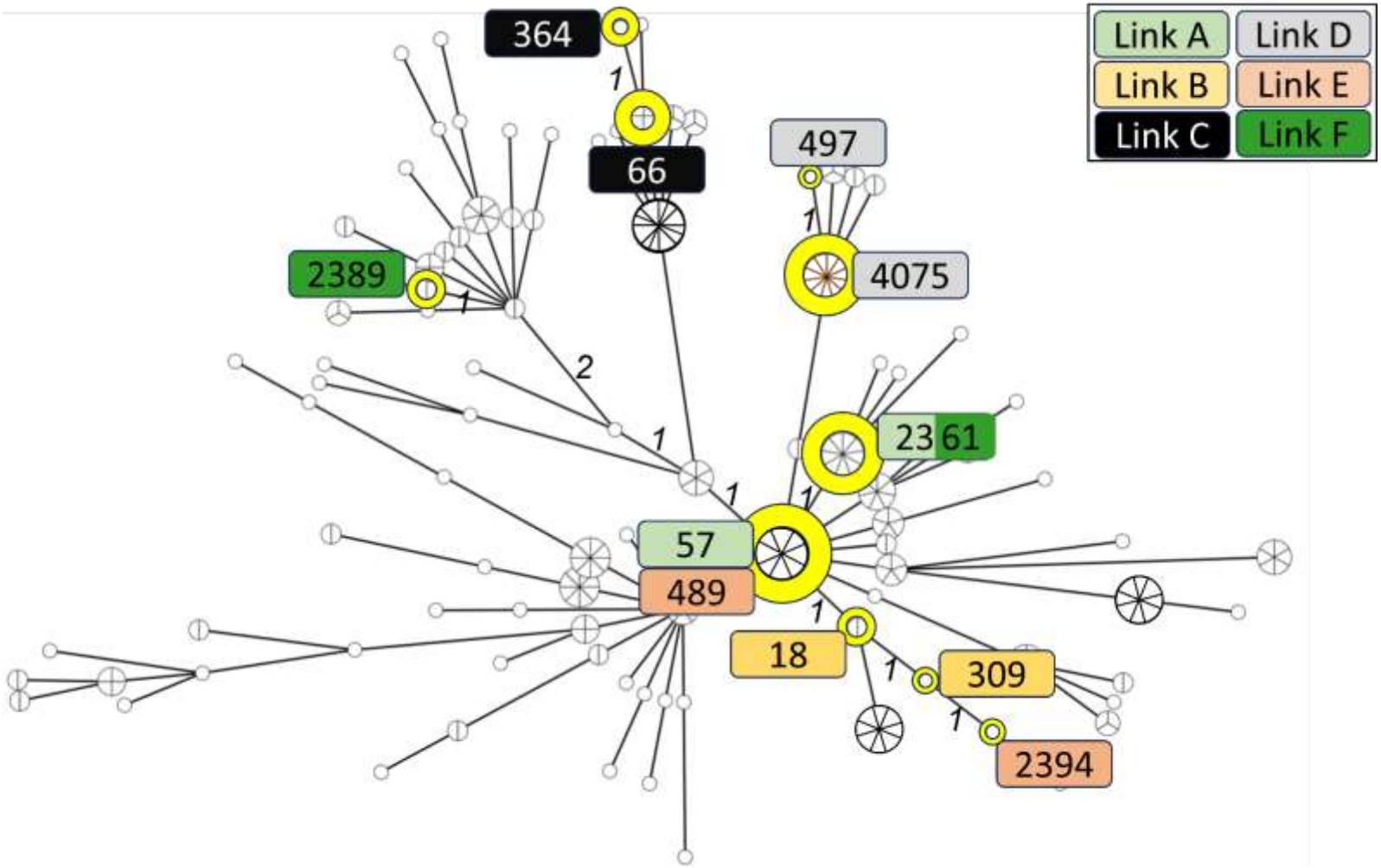

Figure VI.7. Minimum spanning tree created based on 11 VNTR markers with GrapeTree MSTV2 [8], highlighting pairs of patients with an identified epidemiological link: (Link A) Patient 57&2361 (Link B) Patients 18&309 (Link C) Patients 66&364 (Link D) Patients 497&4075 (Link E) Patients 489&2394 & (Link F) Patients 2361&2389.

VNTR-Clustered vs. non-VNTR-clustered

No identifiable risk factors (SNPtype, MB/PB, type of case finding, mutation in *gyrB*, bacillary load >50.000/2µl, sex) were found to be associated with clustered genotypes (data not shown). The calculated genotypic diversity can serve as an indicator of the prevalence of a specific strain and the corresponding level of circulation within the community. When the genotypic diversity ratio is 1, all genotypes are unique, while a ratio <1 indicates genotypic clustering within the village. Of the 40 villages for which we have genotypes available, 21 have a genotypic diversity <1. Jimilimé has the lowest genotypic diversity (0.29) (supporting information Table VI.S1), likely attributable to the geographically restricted cluster #13. The genotypic diversity ratio was not associated with a higher population density (data not shown, p>0.05).

DISCUSSION

This study was the first to use targeted deep sequencing of VNTRs to investigate transmission of *M. leprae* in a field setting. There is currently no consensus regarding the selection and number of VNTR markers to employ in leprosy transmission studies. The four dinucleotide VNTRs (TA10, TA15, AT18, AT17) exhibited very high variability in previous studies and are thus deemed unsuitable to cluster linked patients [9, 10]. The choice of VNTR markers seems to be highly dependent on the geographic region. For instance, marker 6-3a was found nonpolymorphic in the Comoros, India, Fortaleza in Brazil and the Brazilian amazon, while exhibiting minimal polymorphism in Thailand [9, 11-13]. Hence, the 6-3a marker might not be appropriate for investigating local transmissions. However, it could be better suited for studying transmissions within a continent, for example (to be investigated). While markers AC8b, GGT5, and 21-3 were polymorphic in the Comoros but non-variable in a Brazilian population in the amazon and Fortaleza, the opposite accounts for marker 12-5 [9, 12]. GGT5 was non polymorphic in Fortaleza (Brazil), but has some polymorphism in the Comoros.

In the Thai study, an association was found between marker GGT5 having five copies and marker 21-3 having three copies [5x GGT5/3x 23-3] or [5x GGT5/2x 23-3], which was also observed in global strains examined in 2009 [11]. In the Comoros population, these associations could not be systematically confirmed. The majority (n=288) displayed with 5x GGT5, of which 181 had two copies of marker 21-3, 35 had three copies and 12 had one copy. In addition, 25 strains displayed [6x GGT5/2x 21-3] and three strains had a [4x GGT5/2x 21-3] profile. The [4x 12-5/2x 23-3] pattern is a conserved feature previously observed in most of the Asian strains of *M. leprae* that have been genotyped, and this pattern was also observed in the Comoros population [11, 14, 15].

In this Comorian dataset, a substantial number of patients is clustered with at least on other patient 79.7%. This percentage is high compared to observations made in other regions: 60.4% in Fortaleza (Brazil), 38.0% in a former leprosy colony within the Brazilian Amazon, and 15.0% in China [9, 12, 15]. The higher clustering could potentially be attributed to the elevated endemicity within the Comoros, particularly concentrated on the island of Anjouan. In addition, although only one-fifth of the entire leprosy population is processed with Deeplex, which is high in comparison to these other studies. The presence of limited migration further contributes to

the insularity of this population, yielding a closed demographic structure. Therefore it could that certain genotypes have been deeply rooted on the island of Anjouan, causing high percentage clustering. No characteristic could be linked with clustering which is comparable to the study done in Fortaleza, where no clear patient characteristics could be linked albeit over a time period of two years. It could be that these kind of molecular epidemiological study are harder to perform in leprosy compared to TB given the long incubation period [12].

Half of the known contacts between leprosy patients from our study population, as identified through social networks, were confirmed by VNTR-typing, with another four potentially linked (1-2 VNTR difference). This finding supports the use of VNTR genotyping for confirming bacterial genetic relationships between leprosy patients. In leprosy, with a longer incubation period, our findings suggest that a declared or revealed relationship between patients may have lower specificity. However, not all known contacts were supported by the obtained genotypes, suggesting patients were infected through other routes, which risk is likely linked to the islands' hyperendemicity. Although population based, our genotypic sampling fraction covered only approximately one-fifth of all notified leprosy cases during the period, likely leading to an underestimation of clustering and a high probability that shared index cases may have been missed. In addition, the timespan over which patients were diagnosed was five years (2017-2022), while incubation time for leprosy is on average 2-4 years and can go up to 20 years, increasing the likelihood to miss genotypic links. This assumption is supported by the observation that certain genotype-based clusters correlated with geographical proximity, which could result from previous, unidentified social contact. An alternative explanation could be attributed to certain VNTR markers (such as GAA21 and GTA9), which might not consistently display a monomorphic profile within individual patients. This phenomenon was illustrated through an intra-patient comparison conducted by Lima *et al*. in 2016 [16].

The fact that other (larger) VNTR clusters could not be attributed to either proximity or known contact could reflect people's mobility not captured by the few in-depth interviews, and/or a lack of discriminatory power of the currently used panel of VNTR markers. Thus, for the larger dispersed clusters on the island they may overestimate transmission, as suggested by WGS, which further broke down cluster #19. Overall, WGS provided a higher resolution over Deeplex-MycLep, but it should be kept in mind that two VNTRs 27-5 and 18-8 are not included in this study (to be included in the next version of Deeplex Myc-Lep).

Nevertheless, some cases showed identical WGS profiles or only two SNPs but varied in copy number at 4 or 2 VNTRs respectively. The observation that WGS offers higher resolution than VNTR, while in other instances VNTR provides superior resolution than WGS, suggests to investigate the utilization of both methods in exploring transmission patterns. Deriving VNTR copy numbers from WGS is unlikely to provide a solution, given limited read depth.

Our study findings indicate that an lower genotypic diversity within villages could not be explained by a corresponding higher population density. This observation is exemplified in villages such as Jimilimé (ratio = 0.29) and Ouani (ratio = 0.56), which had nine patients

exhibiting the same genotype, Domoni (ratio = 0.57) with eight patients sharing an identical genotype, and Vouani (ratio = 0.54) with seven patients sharing the same genotype. For these villages, alternative explanations (other than population density) may account for the successful circulation of these strains. For instance, factors such as higher virulence or genetic susceptibility of the patients could be potential drivers of larger cluster sizes. Another risk factor could be poverty, given that Jimilimé is a village characterized by scarce resources when compared to the more developed and urbanized areas of Ouani and Domoni. Situated in the northern part of the island, Jimilimé stands as a highly isolated village accessible through only one road, potentially accounting for the higher circulation of one strain, and so less genotypic diversity.

Besides VNTR copy numbers, the Deeplex Myc-Lep also determines the SNP (sub)type. The majority (85.9%) of infections are caused by SNP subtype 1D, and 13.3% of the infections belong to 1A. In Madagascar in Eastern Africa, the same SNP subtypes were identified, albeit in different proportions, namely 97% SNP subtype 1D and 3% for 1A [17], with a larger SNP distance between 1A Madagascar and 1A Comoros than for 1D. The VNTR-based tree exhibits two large clades (Fig. VI.2), which can be divided into 1A and 1D SNP subtypes. The VNTR responsible for this division according to SNP subtype is 21-3, which has three copies for 1A. However, one strain with SNPtype 1D clusters by VNTRs within the 1A clade due to having three copies in 21-3. Previous research groups have shown a connection between specific VNTRs and SNP types as well, where in Brazil the allelic patterns of the minisatellite loci 27-5 and 12-5 exhibited a strong correlation with SNP type 3 [18] and in Colombia minisatellite [5x 27-5] and [4x 12-5] were frequently observed with SNP type 4 [19].

The remarkable distribution of 1A, primarily being found in Vouani, may be linked to the different migrations into Anjouan [20]. An original Bantu population may have been driven to central parts of the island when slave trading led to foreign occupation of coastal areas, which could suggest that 1D was introduced first, and therefore more widespread across the island of Anjouan. Or that the original Bantu population took 1A with them and then via slave trade 1D was introduced into the whole island. However, whether Bantu ancestry is linked to either 1A or 1D subtype, is still to be investigated, as this was not a parameter registered in this study.

The study's limitations include the fact that the sampling fraction is only one-fifth of the total, albeit being one of the highest fractions achieved. This is a result of systematically sampling every detected patient and conducting door-to-door screenings. Another constraint is that only a subset of samples underwent WGS processing as a proof-of-concept. Regarding the single outlier that clusters with different VNTR clusters based on WGS data, it's important to note that a limitation of the study is that we cannot completely eliminate the possibility of human error (e.g., ID2407 Fig. VI.5). However, we consistently used patient numbers and sample identifiers on the tubes to minimize the risk of sample mix-ups.

In summary, Deeplex Myc-Lep seems a suitable tool to identify *M. leprae* strain diversity. In our study, VNTR clustering is to a great extent explained by geographical proximity and/or social/family connections, while higher resolution from WGS may be required to detangle

strains with same VNTR profile but no known contact. Conversely, WGS may also benefit from information provided by VNTR clustering. Therefore, it is recommended to explore the combined use of both methods to establish a more comprehensive DNA-based transmission cluster definition or exploring other typing targets like e.g. the repetitive elements or other VNTRs. Whether these suggestions/conclusions are generalizable to other settings with lower endemicity is to be tested.

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SUPPORTING INFORMATION

Table VI.S1. Genotypic diversity that serves as an indicator of diversity of strains within the village.

Villages	Nr_genotypes	Nr_patients_genotyped	Genotypic Diversity ratio	Population	Max_nr_patients_per_genotype
JIMLIME	4	14	0.29	7539	9
MOYA	1	3	0.33	11699	3
DAJI	1	2	0.50	3100	2
VOUANI	7	13	0.54	3268	7
GNATRANGA_MOIOU	6	11	0.55	2902	4
OUANI	10	18	0.56	12389	9
ADDA_DAOUENI	4	7	0.57	9729	2
DOMONI	16	28	0.57	14115	8
CHAMDRA	3	5	0.60	6498	3
MAHALE	11	17	0.65	2337	3
BARAKANI	6	9	0.67	7717	2
BOUGOENI	2	3	0.67	3072	2
DINDRI	10	15	0.67	9055	3
KONI_DJODJO	2	3	0.67	10116	2
HAJOHO	3	4	0.75	3492	2
MARAHARE	3	4	0.75	1822	2
PAJE	3	4	0.75	2557	2
MIRONTSI	13	17	0.76	12316	2
HASSIMPAO	5	6	0.83	1609	2
VASSI	5	6	0.83	1347	2
MUTSAMUDU	10	11	0.91	29186	2
BAMBAO	5	5	1.00	5824	0
BANDAR_SALAMA	1	1	1.00	2025	0
BANDRANI_MTSANGANI	2	2	1.00	2742	0
BAZIMI	2	2	1.00	9087	0
BOINGOMA	2	2	1.00	1602	0
CHIRIRONI-SADAMPOINI	1	1	1.00	2965	NA

Villages	Nr_genotypes	Nr_patients_genotyped	Genotypic Diversity ratio	Population	Max_nr_patients_per_genotype
CHIRONKAMBA	1	1	1.00	2423	NA
CHITSANGACHELE	1	1	1.00	175	NA
DAR_SALAMA	1	1	1.00	1243	NA
GEGE	3	3	1.00	800	0
HAMAVOUNA	1	1	1.00	1148	0
HAMCHACO	2	2	1.00	2245	0
HANTSAHII	2	2	1.00	1498	0
HAREMBO	2	2	1.00	1679	0
JANDZA	1	1	1.00	405	NA
KANGANI	2	2	1.00	4407	0
KOKI	1	1	1.00	5991	NA
KONI_NGANI	2	2	1.00	4131	0
KOWE_COSINI	2	2	1.00	1597	0
LICOLI	1	1	1.00	NA	0
MAGNASSINI	1	1	1.00	5412	NA
MDJAMAOUE	3	3	1.00	1630	0
MJIMANDRA	1	1	1.00	2804	NA
MOIMOI_I	1	1	1.00	1686	NA
MOIMOI_II	2	2	1.00	887	0
MOUJIMVIA	1	1	1.00	1286	NA
MREMANI	5	5	1.00	7673	0
MROMOUHOULI	1	1	1.00	610	NA
NGADZALE	2	2	1.00	8207	0
ONGOJOU	1	1	1.00	7107	NA
POMONI	3	3	1.00	4204	0
SIMA	1	1	1.00	10793	NA
TSEMBEHOU	1	1	1.00	11353	NA

REFERENCES

1. Braet, S.M., et al., *Investigating drug resistance of Mycobacterium leprae in the Comoros: an observational deep-sequencing study.* Lancet Microbe, 2022. **3**(9): p. e693-e700.
2. Jouet, A., et al., *Hi-plex deep amplicon sequencing for identification, high-resolution genotyping and multidrug resistance prediction of Mycobacterium leprae directly from patient biopsies by using Deeplex Myc-Lep.* EBioMedicine, 2023. **93**: p. 104649.
3. Hunter, P.R. and M.A. Gaston, *Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity.* J Clin Microbiol, 1988. **26**(11): p. 2465-6.
4. Sola, C., et al., *Genotyping of the Mycobacterium tuberculosis complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics.* Infect Genet Evol, 2003. **3**(2): p. 125-33.
5. Paradis, E. and K. Schliep, *ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R.* Bioinformatics, 2019. **35**(3): p. 526-528.
6. Supply, P., et al., *Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis.* J Clin Microbiol, 2006. **44**(12): p. 4498-510.
7. Argimon, S., et al., *Microreact: visualizing and sharing data for genomic epidemiology and phylogeography.* Microb Genom, 2016. **2**(11): p. e000093.
8. Zhou, Z., et al., *GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens.* Genome Res, 2018. **28**(9): p. 1395-1404.
9. Rosa, P.S., et al., *Emergence and Transmission of Drug-/Multidrug-resistant Mycobacterium leprae in a Former Leprosy Colony in the Brazilian Amazon.* Clin Infect Dis, 2020. **70**(10): p. 2054-2061.
10. Monot, M., et al., *Are variable-number tandem repeats appropriate for genotyping Mycobacterium leprae?* J Clin Microbiol, 2008. **46**(7): p. 2291-7.
11. Srisungnam, S., et al., *Molecular epidemiology of leprosy based on VNTR typing in Thailand.* Lepr Rev, 2009. **80**(3): p. 280-9.
12. Fontes, A.N.B., et al., *Genotyping of Mycobacterium leprae for better understanding of leprosy transmission in Fortaleza, Northeastern Brazil.* PLoS Negl Trop Dis, 2017. **11**(12): p. e0006117.
13. Kuruwa, S., V. Vissa, and N. Mistry, *Distribution of Mycobacterium leprae strains among cases in a rural and urban population of Maharashtra, India.* J Clin Microbiol, 2012. **50**(4): p. 1406-11.
14. Sakamuri, R.M., et al., *Population-based molecular epidemiology of leprosy in Cebu, Philippines.* J Clin Microbiol, 2009. **47**(9): p. 2844-54.
15. Xing, Y., et al., *VNTR typing studies of Mycobacterium leprae in China: assessment of methods and stability of markers during treatment.* Lepr Rev, 2009. **80**(3): p. 261-71.
16. Lima, L., et al., *Intrapatient comparison of Mycobacterium leprae by VNTR analysis in nasal secretions and skin biopsy in a Brazilian leprosy endemic region.* Lepr Rev, 2016. **87**(4): p. 486-500.
17. Avanzi, C., et al., *Population Genomics of Mycobacterium leprae Reveals a New Genotype in Madagascar and the Comoros.* Front Microbiol, 2020. **11**: p. 711.
18. Fontes, A.N., et al., *Genetic diversity of mycobacterium leprae isolates from Brazilian leprosy patients.* Lepr Rev, 2009. **80**(3): p. 302-15.

19. Cardona-Castro, N., et al., *Mycobacterium leprae in Colombia described by SNP7614 in gyrA, two minisatellites and geography.* Infect Genet Evol, 2013. **14**: p. 375-82.
20. Sarasin, A., P. Munier, and F. Cartault, *How history and geography may explain the distribution in the Comorian archipelago of a novel mutation in DNA repair-deficient xeroderma pigmentosum patients.* Genet Mol Biol, 2019. **43**(1 suppl 1): p. e20190046.

CHAPTER VII

Ticks are unlikely to play a role in leprosy transmission in the Comoros as they do not harbour *Mycobacterium leprae* DNA

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ABSTRACT

Leprosy, one of the oldest known human diseases, continues to pose a global challenge for disease control due to an incomplete understanding of its transmission pathways. Ticks have been proposed as a potential contributor in leprosy transmission due to their importance as vectors for other infectious diseases. In 2010, a sampling of ticks residing on cattle was conducted on the islands Grande Comore, Anjouan and Mohéli which constitute the Union of the Comoros where leprosy remains endemic. To investigate the potential role of ticks as a vector in transmission of leprosy disease, molecular analyses were conducted. Out of the 526 ticks analysed, none were found to harbour *M. leprae* DNA, as determined by a quantitative polymerase chain reaction (qPCR) assay targeting a family of dispersed repeats (RLEP) specific to *M. leprae*. Therefore, our results suggest that in the Union of the Comoros, ticks are an unlikely vector for *M. leprae*.

INTRODUCTION

Leprosy is a mutilating disease caused by the intracellular bacilli *Mycobacteria leprae* (*M. leprae*) and/or *lepromatosis* (1). Despite the World Health Organization (WHO) removing leprosy from its list of public health concerns in 2001, the lack of significant reduction in new cases and the detection of leprosy in children indicate that transmission of the disease is still ongoing (2). This is evident in regions where active measures are taken to identify cases, such as door-to-door screenings, which consistently uncover new leprosy patients. Additionally, the prevalence of severe disabilities at the time of diagnosis in many countries suggests delayed detection and diagnosis (3). As a result, it is becoming increasingly apparent that we only see the tip of the iceberg of the global leprosy burden.

The most probable transmission route of leprosy is via the aerial route (4), caused by the prolonged close contact to leprosy patients. Especially multibacillary patients are considered to drive leprosy transmission, given the high bacterial load. However, the nine-banded armadillo (5,6), red squirrels (7) and chimps (8) have been confirmed as animal reservoirs and zoonotic transmission of *M. leprae* has been confirmed by genotyping (6) infected armadillos and leprosy patients in the US. Thus, the question as to whether the transmission pathway is direct or (partially) vector-driven remains unresolved (9).

For *M. leprae* the vector competence of *Amblyomma sculptum* from the family of hard ticks (Ixodidae) was demonstrated by Ferreira *et al.* (10) by artificially feeding adult females with *M. leprae* infected rabbit blood. Transovarial transmission of *M. leprae* was confirmed by the *M. leprae* specific RLEP qPCR and its viability by 16S rRNA RT-qPCR. These findings are supported by results of Tongluan *et al.* (11) who injected *Amblyomma maculatum* ticks at adult and nymph stage with an *M. leprae* Thai-53 suspension derived from infected nude mice footpads. They confirmed the presence of *M. leprae* DNA in F_1 larvae and F_1 nymphs via RLEP qPCR. Additionally, an examination of the normalized expression levels of the *esxA* gene in *M. leprae* revealed that the bacilli not only exhibited viability in cell lines derived from ticks but also retained infectivity for vertebrates (nude mouse footpad infection) (11).

The Union of the Comoros has the highest per capita incidence of leprosy in Africa (as high as seven cases per 10,000 individuals on Anjouan (12)), making it the only country of the African continent that did not reach the elimination target of less than 1 patient/10,000 population postulated by the WHO (3,13). Despite the persistent efforts of the National Tuberculosis and Leprosy Control Programme, including intensified screenings since 2008 and the administration of post-exposure prophylaxis within the framework of the PEOPLE and BE-PEOPLE study (Clinicaltrials.gov: NCT03662022 and NCT05597280), leprosy, a poverty-related disease, remains endemic on the islands Anjouan and Mohéli. In contrast, the wealthiest of the three islands, Grande Comore, is not considered a leprosy endemic region. Leprosy has a long incubation period of several months to decades, with an average of 2-4 years (14), which implies ongoing transmission of the disease by the high proportion of affected children on

Anjouan and Mohéli (2). The potential contribution of non-human animal and environmental reservoirs to the transmission of leprosy represents a knowledge gap towards interrupting leprosy transmission. Therefore, this study sought to investigate the presence of *M. leprae* DNA in a tick collection obtained from the Union of the Comoros as a means of further elucidating the potential involvement of ticks as a vector in leprosy transmission.

MATERIAL AND METHODS

Samples

A total of 526 ticks from a previously described collection (15) from the Union of the Comoros were screened for the presence of *M. leprae* DNA. Specimens were shipped and stored in molecular grade pure ethanol (Avantor, USA) at -20 °C. From the leprosy-endemic islands Anjouan (n = 134) and Mohéli (n = 129) 263 ticks were available. As a comparator, n = 263 ticks were selected from Grande Comore where leprosy is not endemic. All ticks were morphologically inspected and classified according to the guide by Walker *et al.* (16) before they were molecularly examined for the presence of *M. leprae* DNA.

DNA extraction

One half of each tick was used for DNA extraction. The ticks were ground with a mortar and pestle in 1 ml phosphate-buffered saline. To avoid DNA contamination mortars and pestles were autoclaved, treated with bleach, and rinsed prior to use and a new set was used for each sample. Subsequently, 200 µl of the resulting suspension were incubated with 200 µl in-house lysis buffer (Tris-HCl - pH 7.5, EDTA 0.5M pH 8, 6M GuHCl, Tween 20, Triton X-100, diatomaceous earth) and 20 µl proteinase K solution (Promega, USA) in a shaking incubator for 1h at 60 °C and 200 rpm. The lysed suspension was further extracted with the Maxwell® 16 FFPE PLUS Tissue LEV DNA purification Kit (Promega, USA), following the manufacturers' protocol. To control for contamination throughout the extraction procedure, each run included a negative (molecular grade water) and a positive extraction control (suspension of mouse footpad infected with *M. leprae* Thai-53, BEI reference number: 19352).

qPCR assay

To quantify *M. leprae* DNA in the tick extracts, a qPCR assay targeting a family of dispersed repeats (RLEP) (17) was used as described previously (18), using the StepOnePlus™ qPCR cycler and StepOne software v2.3 (Applied Biosystems, USA). With this assay 36 out of 37 RLEP copies in the *M. leprae* genome are detected. Samples were tested in triplicate and considered positive when two of the three replicates were under the positivity cut-off of 40 C_q. Non-template controls (molecular grade water) to control for contamination during the qPCR procedure and a gDNA (*M. leprae* NHDP, BEI reference number: 19350) standard curve for quantification with 1:10 dilutions from 3×10^5 to 3×10^1 RLEP copies were included in each run. An internal positive control (IPC, Eurogentec, Belgium) was spiked into each well to detect inhibition during the qPCR run.

Statistical analysis

To determine the significance of the difference between ticks selected from the leprosy endemic (Anjouan and Mohéli) and non-endemic (Grande Comore) islands, the one-proportion z-test was applied. The significance of the sample rate ratio of ticks investigated in this study compared to the complete tick collection by Yssouf *et al.* (15) was calculated with the Fisher's exact test. All statistical analyses were performed with R, version 4.3.0 for macOS (The R foundation, Vienna, Austria), the alternative hypothesis, stating significant differences between variables, was accepted at a significance level of alpha = 0.05.

RESULTS

Morphological classification of ticks

Of the 263 ticks from the endemic islands of Anjouan and Mohéli, 253 (96.2 %) were identified as Rhipicephalus microplus and 10 (3.8 %) as *Amblyomma variegatum* (Table VII.1). The sample rate ratio analysis of species classification showed that *A. variegatum* was slightly underrepresented in the subset examined in our study with a proportion of 3.8 % compared to 9.8 % in the complete original collection by Yssouf *et al.* (15) (Supporting information Table VII.S1).

Table VII.1: Species distribution of ticks over the three islands of the Union of the Comoros classified according to Walker et al(16)

Group	Island	*R. microplus*		*A. variegatum*		Total	
Islands endemic for *M. leprae* transmission	Anjouan	131/134	(97.8 %)	3/134	(2.2 %)	134	263
	Mohéli	122/129	(94.6 %)	7/129	(5.4 %)	129	
Island non-endemic for *M. leprae* transmission	Grande Comore	254/263	(96.6 %)	9/263	(3.4 %)	263	
Total		507/526	(96.4 %)	19/526	(3.6 %)	526	

In our study an additional classification of the ticks by developmental stage and sex was conducted. Most of the ticks from the endemic islands were adults (n = 184, 70.0 %), followed by ticks in the nymph stage (n = 77, 29.3 %). Only n = 2 larvae (0.8 %) were available for analysis (Table VII.2). The majority of collected ticks was identified as female (n = 109, 81.3 % from Anjouan; n = 102, 79.1 % from Mohéli; n = 167, 63.5 % from Grande Comore). For a small proportion of ticks (4.6 %) the sex could not be identified in our study because the determining features in some nymphs and larvae were inconclusive (Table VII.2).

Table VII.2: Distribution of developmental stages and sex of ticks classified and investigated in this study.

	Endemic (Anjouan + Mohéli)		Non-endemic (Grande Comore)	
Developmental stage				
Adult	184	(70.0 %)	243	(92.4 %)*
Nymph	77	(29.3 %)	20	(7.6 %)*
Larva	2	(0.8 %)	0	(0.0 %)
Total	263	(100 %)	263	(100 %)
Sex				
Female	211	(80.2 %)	167	(63.5 %)*
Male	43	(16.3 %)	81	(30.8 %)*
Undetermined	9	(3.4 %)	15	(5.7 %)
Total	263	(100 %)	263	(100 %)

** Proportions that are significantly different ($p < 0.05$) in the sample proportion from the non-endemic island compared to the endemic islands*

Detection of *M. leprae* DNA by RLEP qPCR

None of the 526 tested DNA extracts from ticks resulted in a positive result in the RLEP qPCR. The limit of detection of the RLEP qPCR assay is as low as 30 RLEP copies per 2 µl added to each qPCR reaction, which correlates with approximately one *M. leprae* bacillus. All positive extraction controls resulted in a positive qPCR result. Negative extraction controls and non-template controls were negative on qPCR, indicating the absence of DNA contamination during the extractions and qPCR assays. IPC was spiked into the DNA extracts before qPCR quantification. Results were consistent within each qPCR run which confirms the absence of qPCR inhibition. A summary of the qPCR results of RLEP and IPC can be found in Supplemental information File 1.

DISCUSSION

This study is the first to use molecular tools to screen wild, animal-derived ticks from a leprosy endemic country for the presence of *M. leprae*. The absence of *M. leprae* DNA was confirmed in all tested specimens from the Comoros. Next to *M. leprae*, *M. lepromatosis* can also cause leprosy disease in humans (1). We have tested the leprosy patient cohort in the Comoros for the presence of *M. lepromatosis* DNA by qPCR assay, with results suggesting that *M. leprae* is the only causative agent for leprosy on the Comoros (manuscript in preparation). Therefore, in this study ticks were only screened for the presence of *M. leprae* DNA.

In the search for drivers for leprosy transmission, two previous studies (10,11) identified ticks from the genus Amblyomma as potential competent vectors for *M. leprae*. More specifically, under experimental conditions the transovarial transmission and the survival of *M. leprae* in female ticks and tick-derived cells was confirmed. The majority of the wild tick collection

analysed in our study were adult females, which are able to harbour and transmit *M. leprae* under experimental conditions. The small proportion of nymphs, which is the developmental stage most likely to parasitize humans and transmit other tick-borne diseases such as lyme disease (19) and ehrlichiosis (20), could explain our inability to detect *M. leprae* DNA in the tick collection that was studied.

Further, the tick collection consisted of a small ratio of Amblyomma ticks, the species with proven capacity to harbour *M. leprae* (10,11), compared to *R. microplus*. Only 10 out of 263 (3.8 %) ticks from the endemic islands Anjouan and Mohéli were A. variegatum while Yssouf et al. (15) classified 73 out of 742 (9.8 %) ticks as *A. variegatum*. The reason for the different species distribution is that only a subset of the original collection was available for analyses at ITM, Antwerp. The selected number of ticks from Grande Comore, used as non-endemic controls, was matched to the species distribution found for the endemic islands in this study. Accordingly, the percentage of *A. variegatum* was smaller than the one found by Yssouf et al. on this island. However, both Rhipicephalus and Amblyomma ticks belong to the family of Ixodidae (or hard ticks). In their previous studies Tongluan *et al.* and Ferreira et al. were able to maintain *M. leprae* in Ixodes-derived cell lines which suggests a similar potential of all members of the Ixodidae family as a vector for *M. leprae*.

Even though the ticks analysed in our study were collected from cattle and goats and not from humans, feeding of cattle ticks on humans seems probable in situations where humans and livestock live closely together. For both *R. microplus* and *A. variegatum* which mainly feed on cattle and other large animals (21), such cross-over events have been reported (22–24). A recent publication by Faber *et al.* (25) is raising the hypothesis that a skin disease in water buffaloes described as lepra bubalorum could be caused by *M. leprae* and therefore act as animal reservoir. However, evidence for cases in Indonesia is only historical as there were no further reports for lepra bubalorum in cattle since 1961 (26) and there is no water buffalo population described in the Union of the Comoros (27).

Different other vectors have been suggested for the transmission of *M. leprae* e.g., arthropods such as mosquitos (Aedes, Culex, Rhodnius) (28–30), flies (Musea, Calliphora and Stomoxys) (31) and sand flies (Phlebotomus, Sergentomyia). The latter are unlikely vectors as they cannot maintain viable *M. leprae* bacilli (32). Early studies on mosquitos confirmed the presence of acid-fast bacilli in the proboscis of mosquitos (*A. aegypti* and *C. fatigans*) after experimentally feeding on untreated leprosy patients (28,29). However, viability determined by fluorescence microscopy reduced within seven days after feeding (29). Da Silva Neumann *et al.* have investigated *R. prolixus*, *A. aegypti* and *C. quinquefasciatus* as possible vector, with the result that only R. prolixus has the ability to defecate infective *M. leprae* up to 20 days after infection with *M. leprae* Thai-53 infected rabbit blood (30). Additionally, amoeba have been found to have vector potential as they can phagocytose *M. leprae*. In vitro experiments showed that *M. leprae* can survive up to 72 hours within the Acanthamoeba and up to 8 months in amoebal

cysts while retaining infectivity for a nude mouse model (33,34). However, for none of these vector candidates a clear correlation with leprosy infections in humans was identified.

Even though Ixodes ticks are potential competent vectors for *M. leprae* in vitro and pathogen transmission from livestock to humans via ticks is probable, all ticks from Anjouan, Mohéli and Grande Comore that were investigated tested negative for *M. leprae* DNA. This finding lessens the chance that leprosy is a tick-borne zoonosis in the Union of the Comoros, rather than spread by human-to-human transmission.

Our results support the hypothesis that most leprosy infections are caused by human-to-human interactions rather than by a non-human animal or environmental reservoir of *M. leprae* and that close contact to a leprosy patient is the driving force of transmission. For the definitive exclusion of the role of ticks in the transmission of leprosy disease, a larger number of ticks also from other leprosy endemic regions should be analysed. The exploration of human-derived ticks and particularly ticks parasitising leprosy patients should be the focus of such studies. Further, qualitative case control studies investigating daily activities of leprosy patients and healthy controls will be useful for the generation of new hypotheses on the driving factors of leprosy transmission.

CONFLICT OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

LK, EC, BJ and SB contributed to conception and design of the study. AY and PT were involved with the tick sampling and provided the tick collection. EC and MD were involved in the methodology, investigation and validation. LK, EC and SB performed the formal analysis and statistics. SB was the project administrator and together with BJ supervised the study. LK wrote the original manuscript draft and all authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Deps, P. and S.M. Collin, *Mycobacterium lepromatosis as a Second Agent of Hansen's Disease.* Front Microbiol, 2021. **12**: p. 698588.
2. Braet, S.M., et al., *Investigating drug resistance of Mycobacterium leprae in the Comoros: an observational deep-sequencing study.* Lancet Microbe, 2022. **3**(9): p. e693-e700.

3. WHO, *Global leprosy (Hansen disease) update, 2021: moving towards interruption of transmission.* 2021: Wkly Epidemiol Rec.
4. Araujo, S., et al., *Molecular Evidence for the Aerial Route of Infection of Mycobacterium leprae and the Role of Asymptomatic Carriers in the Persistence of Leprosy.* Clin Infect Dis, 2016. **63**(11): p. 1412-1420.
5. da Silva, M.B., et al., *Evidence of zoonotic leprosy in Para, Brazilian Amazon, and risks associated with human contact or consumption of armadillos.* PLoS Negl Trop Dis, 2018. **12**(6): p. e0006532.
6. Sharma, R., et al., *Zoonotic Leprosy in the Southeastern United States.* Emerg Infect Dis, 2015. **21**(12): p. 2127-34.
7. Avanzi, C., et al., *Red squirrels in the British Isles are infected with leprosy bacilli.* Science, 2016. **354**(6313): p. 744-747.
8. Hockings, K.J., et al., *Leprosy in wild chimpanzees.* Nature, 2021. **598**(7882): p. 652-656.
9. Bratschi, M.W., et al., *Current knowledge on Mycobacterium leprae transmission: a systematic literature review.* Lepr Rev, 2015. **86**(2): p. 142-55.
10. Ferreira, J.D.S., et al., *Ticks as potential vectors of Mycobacterium leprae: Use of tick cell lines to culture the bacilli and generate transgenic strains.* PLoS Negl Trop Dis, 2018. **12**(12): p. e0007001.
11. Tongluan, N., et al., *Mycobacterium leprae Infection in Ticks and Tick-Derived Cells.* Front Microbiol, 2021. **12**: p. 761420.
12. Ortuno-Gutierrez, N., et al., *Clustering of leprosy beyond the household level in a highly endemic setting on the Comoros, an observational study.* BMC Infect Dis, 2019. **19**(1): p. 501.
13. World Health Assembly 44. Forty-fourth World Health Assembly, Geneva, 6-16 May 1991: resolutions and decisions, annexes. 1991
14. Rogers L. Incubation Period of Leprosy. Ind Med Gaz . 1924 Feb;59(2):65–8.
15. Yssouf, A., et al., *Colonization of Grande Comore Island by a lineage of Rhipicephalus appendiculatus ticks.* Parasit Vectors, 2011. **4**: p. 38.
16 Walker AR, et al. *Ticks of domestic animals in Africa: a guide to identification of species*. Bioscience Reports; 2003.
17. Woods, S.A. and S.T. Cole, *A family of dispersed repeats in Mycobacterium leprae*. Mol Microbiol, 1990. 4(10): p. 1745-51.
18. Truman, R.W., et al., *Enumeration of Mycobacterium leprae using real-time PCR.* PLoS Negl Trop Dis, 2008. **2**(11): p. e328.
19. Matuschka, F.R., et al., *Stage-associated risk of transmission of the Lyme disease spirochete by European Ixodes ticks.* Parasitol Res, 1992. **78**(8): p. 695-8.
20. Des Vignes, F. and D. Fish, *Transmission of the agent of human granulocytic ehrlichiosis by host-seeking Ixodus scapularis (Acari:Ixodidae) in southern New York state.* J Med Entomol, 1997. **34**(4): p. 379-82.
21. Maina, A.N., et al., *High prevalence of Rickettsia africae variants in Amblyomma variegatum ticks from domestic mammals in rural western Kenya: implications for human health.* Vector Borne Zoonotic Dis, 2014. **14**(10): p. 693-702.
22. Kaur N, et al., *Rhipicephalus (Boophilus) microplus (arachnida: Ixodidae) larvae infestation of human eyelids.* A rare case. Acarologia. 2019;59(1):21–5.
23. Ali, A., et al., *Seasonal Dynamics, Record of Ticks Infesting Humans, Wild and Domestic Animals and Molecular Phylogeny of Rhipicephalus microplus in Khyber Pakhtunkhwa Pakistan.* Front Physiol, 2019. **10**: p. 793.

24. Jensenius, M., et al., *African tick bite fever in travelers to rural sub-Equatorial Africa.* Clin Infect Dis, 2003. **36**(11): p. 1411-7.
25. Faber, W.R., et al., *Lepra Bubalorum, a Potential Reservoir of Mycobacterium leprae.* Front Microbiol, 2021. **12**: p. 786921.
26. Ploemacher, T., et al., *Reservoirs and transmission routes of leprosy; A systematic review.* PLoS Negl Trop Dis, 2020. **14**(4): p. e0008276.
27. Minervino, A.H.H., et al., *Bubalus bubalis: A Short Story.* Front Vet Sci, 2020. **7**: p. 570413.
28. Narayanan, E., et al., *Persistence and distribution of Mycobacterium leprae in Aedes aegypti and Culex fatigans experimentally fed on leprosy patients.* Lepr India, 1978. **50**(1): p. 26-37.
29. Banerjee, R., et al., *Transmission of viable Mycobacterium leprae by Aedes aegypti from lepromatous leprosy patients to the skin of mice through interrupted feeding.* Lepr Rev, 1991. **62**(1): p. 21-6.
30. Neumann Ada, S., et al., *Experimental Infection of Rhodnius prolixus (Hemiptera, Triatominae) with Mycobacterium leprae Indicates Potential for Leprosy Transmission.* PLoS One, 2016. **11**(5): p. e0156037.
31. Geater JG. *The fly as potential vector in the transmission of leprosy*. Lepr Rev. 1975;46(4):279–86.
32. Sreevatsaet al., *Can sandflies be the vector for leprosy?* Int J Lepr Other Mycobact Dis , 1992. 60(1):94–6.
33. Lahiri, R. and J.L. Krahenbuhl, *The role of free-living pathogenic amoeba in the transmission of leprosy: a proof of principle.* Lepr Rev, 2008. **79**(4): p. 401-9.
34. Wheat, W.H., et al., *Long-term survival and virulence of Mycobacterium leprae in amoebal cysts.* PLoS Negl Trop Dis, 2014. **8**(12): p. e3405.

SUPPORTING INFORMATION

Table VII.S1: Primer and probe sequences and the respective qPCR conditions for each RT-qPCR assay.

Name	Sequence 5' – 3'	qPCR conditions		Reference
MLRLEPTaq-F	gcagtatcgtgttagtgaa	1 x	95 °C, 10 min	Truman *et al.,* 2008
MLRLEPTaq-R	catacggcaaccttctagcg	45 x	95 °C, 10 sec	(18)
MLRLEPTaq-P	tcgatgatccggccgtcggcg		60 °C, 1 min	

Table VII.S 2: Differences in species distribution of the whole tick collection classified by Yssouf et al. (15) and the subset that was re-classified for this study. Only numbers from the endemic islands Anjouan and Mohéli are presented. The sample rate ratio was calculated with a Fisher's exact test.

Species	Yssouf *et al.* (1)		This study		Sample rate ratio (95% CI)	
Rhipicephalus microplus	669/742	(90.2%)	253/263	(96.2 %)	1	(ref)
Amblyomma variegatum	73/742	(9.8 %)	10/263	(3.8 %)	0.4	(0.2 – 0.7)

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CHAPTER VIII

Discussion, recommendations and policy

SUMMARY OF FINDINGS

Transmission of leprosy in the community may decrease through prompt and accurate diagnosis and rapid treatment initiation of the index cases. As there is no consensus on which assay to use as a reference standard for diagnosing infections with *M. leprae,* we investigated and confirmed in Chapter II the specificity of the sensitive RLEP qPCR. In Chapter III, we explored the possible use of skin biopsies followed by molecular quantification to determine a patient's bacterial load as a proxy for infectivity. We confirmed in the cohort in the Union of the Comoros that αPGL-I IgM levels correlate with the bacterial load using minimally invasive sampling, more specifically fingerstick blood. The combination of αPGL-I R-values ≥ 0.81 in fingerstick blood with a lesion count ≥25 predicts a high bacterial load (HBL; ≥50,000 bacilli in a 4mm biopsy) with 93.7% sensitivity and 77.4% specificity. Nasal swabs, on the other hand, did not increase sensitivity to microbiologically confirm leprosy patients, nor to identify HBL-patients. In Chapter IV, we describe the development of the Deeplex MycLep, a tool designed to detect drug resistance and type *M. leprae* for the purpose of tracking its spread. The timely identification of drug resistance improves patient care, while the tracking of *M. leprae* transmission informs how to interrupt its spread. The method employs SNP detection for drug-resistance calling and strain typing, complemented with analysis of 11 VNTR markers. MycLep's limit of detection (LOD) ranged from 80 to 3,000 genome copies depending on the sample type. Its capability to detect minority variants of 10% is a key feature, allowing for the early and prompt detection of drug resistance. Chapter V presents the first-ever survey of drug resistance in the Comorian cohort of leprosy patients, which included 34.4% (n=260) of the RLEP qPCR confirmed (n=755) leprosy patients, by using the newly developed Deeplex MycLep. Our study's results suggest that all *M. leprae* circulating in the Comoros is fully susceptible to rifampicin, fluoroquinolones and dapsone. Moreover, our findings demonstrate for the first time the successful application of targeted sequencing directly on (ethanol-preserved) skin biopsies from patients with either paucibacillary or multibacillary leprosy. In Chapter VI, our investigation focused on the *M. leprae* strains circulating within the Comoros region. Of the total number of leprosy patients confirmed with RLEP qPCR, 290 were selected for Deeplex Myc-Lep analysis, and a complete VNTR pattern was obtained for 256 patients. The findings of this chapter revealed that 85.9% of the patients were diseased with *M. leprae* SNP type 1D, and 13.3% with SNP type 1A, while the SNP subtype could not be determined for two patients. Notably, two VNTR markers, namely 6-3a and 12-5, exhibited no discriminatory power within the Comorian cohort tested (i.e. VNTR alleles of all tested isolates had the same copy number). The finding of having 79.7% of patients within a genotypic cluster is likely an underestimation considering the incomplete sampling fraction, although the resolution of VNTR typing is generally lower than that of WGS, with some cases of convergence with WGS. These results combined with a high leprosy prevalence in children confirm that *M. leprae* transmission persists in the Comoros. Furthermore, in Chapter VI we recommend the establishment of a transmission chain cut-off, which could be based on VNTR markers, SNPs in WGS, or a combination of both (see below). This section outlines the key findings and proposes potential measures to advance the control of leprosy through laboratory confirmation tests. Lastly, in Chapter VII we found that in a collection of mostly cattle

ticks obtained in the Comoros, no *M. leprae* DNA could be found, suggesting that ticks probably do not play a role as vector for *M. leprae*.

DISCUSSION & RECOMMENDATIONS

Even though our studies and findings contribute important new findings, numerous knowledge gaps in leprosy pathogenesis and transmission remain, which obstacles impede control measures.

More sensitive microbiological confirmation of paucibacillary patients

Early diagnosis and treatment of leprosy are crucial for preventing transmission of the disease and preventing or reducing the severity of disabilities. The clinical diagnosis by well-trained healthcare workers is the reference standard for the diagnosis of leprosy. Where diagnostic uncertainty exists, especially for less seasoned clinicians, laboratory diagnostics assume an important role, although their sensitivity is imperfect, especially in paucibacillary disease. As leprosy develops slowly, the team in the Comoros monitors the clinical evolution of a patient with an unclear presentation rather than start them on leprosy treatment. Rapid molecular testing could aid in clinical decision making for such cases. For optimal clinical benefit, DNA extraction & -detection methods need systematic standardisation and optimisation. With the establishment of rapid and sensitive detection of *M. leprae* DNA we expect to better serve clinicians and patients both in endemic and non-endemic countries.

Prior attempts were made to better capture paucibacillary patients with microbiological tools. One such tool is the RLEP qPCR, which amplifies repetitive DNA fragments specific to *M. leprae* (Truman et al 2008). In Chapters II and III we have shown that, by confirming 65% of clinically confirmed paucibacillary patients, RLEP qPCR is more sensitive than traditional techniques, such as slit skin smear microscopy, which is negative for most paucibacillary patients. Recent studies on MTB Droplet Digital PCR (ddPCR) have demonstrated its superior sensitivity in comparison to quantitative PCR (qPCR). As a result, it holds the potential to detect a greater number of paucibacillary leprosy patients. Nonetheless, the current limitation is that this technique is not widely accessible and necessitates sophisticated laboratory facilities, rendering it less practical for countries with a high prevalence of leprosy.

Samples that are most widely used in national program settings, albeit in a minority, are the painful slit skin smear, in which paucibacillary patients generally test negative. Alternative sampling techniques may be more efficacious for identifying paucibacillary leprosy patients. Other samples in current use, more so in a research context, are skin biopsies, nasal swabs and fingerstick blood, as described in Chapter III. Given that transmission likely occurs via the upper respiratory tract, other approaches to improve the detection may include the collection of oral biomass by tongue swabs or scrapers and the collection of exhaled bacilli through face mask sampling. These techniques have demonstrated promising outcomes in detecting tuberculosis (TB) among affected individuals [1, 2].

In Chapter III, we confirmed that increased αPGL-I antibody levels correlate to patients with a high bacterial load, while antibodies *are* generally not detectable in paucibacillary patients and BI-negative multibacillary patients. Hence, examining anti-αPGL-I antibody response is not sufficient to identify patients on the entire spectrum of the disease. Van hooij *et al.* identified an extended host biomarker signature encompassing αPGL-I IgM, IP-10, CRP, ApoA1, and S100A12, which can be detected in unstimulated fingerstick blood and serum [3]. This signature covers the humoral and cellular immunopathological spectrum of leprosy. Elevated levels of αPGL-I IgM, IP-10, and CRP were associated with MB leprosy, whereas ApoA1 and S100A12 were deemed critical in identifying both patient groups. In PB leprosy patients, ApoA1 emerged as the most significant biomarker. Additionally, the levels of ApoA1 and S100A12 facilitated differentiation between highly exposed contacts and endemic controls, potentially detecting *M. leprae*-infected individuals, although unable to distinguish between infection from disease [4].The goal is to integrate these markers into a single minimally invasive test that can detect both PB and MB patients.

Better tools to microbiologically detect and confirm leprosy patients, including the entire spectrum of the disease, is essential for effective disease control and management. Increasing the sensitivity of microbiological detection of *M. leprae* infection might also allow detection of asymptomatic and/or preclinical cases as they might also contribute to ongoing transmission, which is not known yet. Further research should be conducted to develop better diagnostic tools for leprosy, particularly applicable in regions where the disease is endemic, which are often resource-limited. Biomeme's portable DNA extraction and qPCR system have the potential to facilitate leprosy diagnosis in resource limited settings. Their M1 Sample Prep Cartridge Kits and qPCR system with lyophilized reagents are being tested by the American Leprosy Mission. The M1 sample prep cartridge eliminates the need for extra lab equipment, refrigeration, or a cold chain. The combination of Biomeme's portable qPCR system with lyophilized reagents in the 8-well strip allows for ambient temperature preservation, and necessitates only the addition of molecular grade water and DNA extract for activation. Future studies can test whether the extracted DNA from Biomeme's sample preparation allow for subsequent genotyping with Deeplex MycLep and/or WGS.

Better classification of different disease stages of leprosy and identification of potentially high risk index cases.

Another crucial aspect in the management of leprosy is the differentiation of active disease, residual lesions without viable bacilli, and reactions. The differentiation between an active lesion and a residual lesion that has not yet fully regressed is commonly performed through visual inspection, comparing with clinical records that contain drawings of the lesions at the onset of treatment and/or from previous visits. These drawings are compared with the current size and location of the lesion to ascertain whether it represents a new active lesion or a residual one. In contrast to active leprosy, reactions constitute a frequently encountered

complication that may arise during or after the course of leprosy treatment or may develop as a result of natural bacterial decay [5]. Such reactions can cause nerve damage and lead to disability, necessitating an immunomodulatory treatment. Notably, reactions may manifest during the active phase of the disease, during treatment, or most commonly, in the post-treatment phase, when the patient no longer exhibits signs of active disease. Remnant lesions do not require treatment as they just take time to disappear. Active disease and relapse, on the other hand, require antibiotic *M. leprae* treatment to prevent further transmission and prevent complications. In case of relapse without molecular confirmation of drug resistance, another round of MDT is given. However, distinguishing between relapse and reactions can be challenging, as the clinical presentations may resemble each other, especially for an untrained eye. The existing diagnostic tools are mainly focusing on detection of *M. leprae* DNA, which can remain present for a long time even after bacterial cell death. So DNA based tools are not ideal to differentiate between these conditions. In contrast, the development of a tool to determine the viability of *M. leprae* bacilli could distinguish between relapse (viable) and reactions (non-viable, unless on treatment for a short duration), and additionally inform about the infectiousness of an individual. Given that attempts to grow *M. leprae* in culture medium were not successful, at present, viability tests are conducted in *in vivo* models such as armadillos and mice, which are laborious and time-consuming. An alternative molecular viability test developed by Davis *et al.* in 2013 detects RNA (a proxy for viable bacteria) in relation to DNA (a proxy for both dead and live bacilli). Another potentially more sensitive assay is the RS ratio test, which was developed to test the viability of *M. tuberculosis* [6] and is now applied to *M. leprae* (ongoing research by Dr. Avanzi). This assay measures ongoing rRNA synthesis in *M. tuberculosis* by quantifying the abundance of precursor rRNA (short-lived spacer sequences of external transcribed spacer 1 (ETS1)) relative to stable mature rRNA (23S rRNA). Unlike existing viability markers, such as colony-forming units and 16S rRNA burden that enumerate the abundance of *M. tuberculosis*, the RS ratio measures the degree to which drugs and regimens interrupt rRNA synthesis. The decline in RS ratio is drug specific, and shows a dose response for rifampicin and bedaquiline [7]. The development of such a tool for leprosy could vastly improve accessibility and the turn-around time compared to *in vivo* viability testing, which is conducted only in a handful of laboratories worldwide and requires inoculation in mouse footpads to be done <48hours of sampling.

Differentiating between reactivation of a previous strain (or treatment failure) and reinfection with a different strain during clinical relapse is key to inform leprosy control programs, particularly in endemic areas like the Comoros, where 30% of cases are children, indicating ongoing transmission and high risk of (re-) exposure to *M. leprae*. Accurate identification of individual strains is thus crucial to unravel the leprosy dynamics. The Deeplex MycLep allows simultaneous strain typing and drug resistance determination for a specific strain, including those from different disease episodes.

Drug resistance surveillance in the context of optimized patient care and chemoprophylaxis

Besides late diagnosis and the lack of confirmation tools, another hypothesis for continued transmission is drug resistance. WHO calls for drug-resistance surveillance in at least 10% of the multibacillary patients diagnosed. In Chapter V, we used Deeplex MycLep to exclude resistance to rifampicin, fluoroquinolone or dapsone as reason of high leprosy endemicity in the Comoros. Drug resistance appeared to be nonexistent. Our efforts and data added the Comoros to five other countries within the African region where routine drug-resistance reporting is conducted: Benin, Guinea, Madagascar, Mali, Niger, and Senegal. Dapsone resistance in these countries ranged from 0%-16.9% and rifampicin resistance from 0%-4.5%, with primary dapsone- and rifampicin-resistant strains reported from Guinea [1, 2]. None of the countries in the Central African region has reported on *M. leprae* drug resistance.

The impact of drug resistance has likely been underestimated. The adjustment of treatment when drug resistance (mainly to rifampicin as it is the core drug) is present is crucial, to effectively treat leprosy. Currently, only three genes (*folp1, rpob*, and *gyrA*) have been identified where mutations can confer resistance to dapsone, rifampicin and fluoroquinolones respectively. Comprehensive screening of additional genomes may identify additional targets and associated mutations that confer drug resistance. The current understanding of *M. tuberculosis* suggests that for *M. leprae* numerous other genes and mutations likely confer drug resistance. Moreover, as strains continue to mutate and evolve, new and previously unknown mutations that confer resistance may emerge, requiring continuous genome monitoring. This is particularly important, as hyper-mutable strains were identified by Benjak *et al.* in 2018 [8]. Valuable insight can be gained by detecting the independent emergence of possible drug-resistance related mutations (convergence), especially within samples collected from patients who have undergone treatment but are still experiencing treatment failure or relapse. However, this necessitates conducting extensive studies to gather an adequate number of such patients for genome-wide analysis. In Chapter VI we have processed 43 strains with WGS and no potential drug resistance-related mutation were found. In a project recently submitted to the leprosy research initiative, we propose to do a cross-sectional observational study, where we will test whether drug resistance contributes to transmission of leprosy in four African countries. We propose to recruit leprosy patients in the national leprosy control programs of Ghana, Burundi, Cameroon and the Democratic Republic of Congo. We will screen biopsies from leprosy patients for the presence of *M. leprae* DNA. Positive DNA extracts will be tested for the three known resistance mutations beyond the three known targets (*folp1*, *gyrA*, *rpoB*) by Hain LPA testing. Furthermore, through more extended genotyping using directly-on-specimen targeted- or whole genome sequencing of leprosy patients, we will test if any other gene target is possibly related to drug resistance and whether genotypic clustering of *M. leprae* is suggestive of recent transmission within these countries.

In Chapter V of this thesis we already screened for a couple of additional drug-resistance-associated targets, like *ctpC/ctpI* for rifampicin resistance and *gyrB* for fluoroquinolone resistance and nth for the detection of hypermutable strains. However these targets are not

validated yet. After identifying potential drug targets/mutations, the next step is to validate them in the laboratory. This can be accomplished through various models such as mouse or armadillo inoculation (laborious and time consuming) combined with for example the above mentioned RS ratio assay as an outcome measure for viability. Alternatively, other models can be used, such as recombinant fast growing mycobacteria a surrogate for phenotypic culture-based drug susceptibility testing [9-11]. Nakata *et al.* (2011) successfully tested this approach for *folp1* and in 2012 for *rpoB*. It is important to note that the model's effectiveness relies on the gene of interest being essential for the rapid growth of the specific mycobacterium, such as *Mycobacterium smegmatis*. This requirement arises from the necessity to knock out the essential gene in the fast-growing strain of the bacterium for the model to function optimally, as the gene will be substituted with the homologous genes from *M. leprae*, where mutations of interest are introduced. [10, 11]. These models evaluate the effect of the mutation in a certain gene on drug efficacy. Laboratory validation of drug-resistance-associated targets is crucial to confirm that the identified targets are indeed conferring drug resistance. Identifying and validating these targets/mutations can lead to more precise screening of drug resistance, and it can provide valuable information about the potential widespread undetected resistance to the drugs (dapsone, rifampicine and clofazimine) used to treat leprosy since the 1940s. Ultimately, a better understanding of drug-resistance mechanisms can improve leprosy treatment outcomes, and continued research in this area is necessary. This information can enhance our understanding of the epidemiology of the disease as well, as the number of new cases detected is not decreasing since 2006. Undetected drug resistance could explain ongoing spread, as drug resistance it not tested routinely and treatment is therefore not adjusted.

Bedaquiline, a potent drug developed against *M. tuberculosis* after a 40-year gap in drug development, received conditional FDA approval in 2012 for treating multidrug-resistant tuberculosis. It specifically targets the subunit c of the ATP synthase in the respiratory chain, making it a game-changing treatment for TB patients with multidrug-resistant strains. Bedaquiline has a long half-life and is active against replicating and non-replicating bacteria, like rifampicin but unlike moxifloxacin and isoniazid [12]. In TB patients, (spontaneous) drug-resistance mutations have been found in *Rv0678*, a repressor of an efflux pump inhibitor, potentially leading to resistance against bedaquiline [13]. However, *M. leprae* lacks an orthologue of Rv0678 (transcriptional regulator), making bedaquiline a highly promising drug for treating *M. leprae* infections [14] This hypothesis is supported by the experiments done in murine models where the minimal oral dose was determined to effectively clear an *M. leprae* infection [15]. To interrupt transmission in the Comoros, we are currently conducting a clinical trial called BE-PEOPLE, which involves testing PEP enriched with bedaquiline, i.e. a single dose of rifampicin and bedaquiline [16]. In this study we monitor also for drug resistance, by examining the *atpE* gene in addition to the genes included in the Deeplex MycLep. The *atpE* gene, responsible for encoding the c subunit of ATP synthase, the direct target of bedaquline, is present in *M. leprae*. However, so far, drug resistance mutations in this essential gene are rarely observed in clinical samples from TB patients.

Telacebec (Q203), a QcrB inhibitor, is an exciting new drug with picogram level anti *M. leprae* activity. It exhibits great potential due to its highly effective bactericidal properties against *M. leprae* by specifically targeting the cytochrome bcc:aa3 terminal oxidase. In the case of Buruli ulcer, caused by *M. ulcerans*, a single dose of telacebec has demonstrated complete clearance of the infection in mice [17]. Since *M. ulcerans* lacks a functional cytochrome bd oxidase, inhibiting the cytochrome bcc:aa3 terminal oxidase leads to cell death, as the bacterium cannot compensate for this loss. Similarly, *M. leprae* lacks the gene responsible for encoding cytochrome Bd oxidase and solely relies on the cytochrome bcc:aa3 terminal oxidase. Indeed telacebec was shown to be highly effective against *M. leprae* in a murine model [18]. In a Phase 2 trial of TB patients who received two weeks of monotherapy with telacebec at different doses, telacebec proved safe and showed a dose response. Clinical trials are required to test safety and efficacy for the treatment of leprosy, with careful monitoring of potential side effects (including leprosy reactions), drug levels and potential acquired drug-resistance related mutations in the patient. Additionally, it is recommended to conduct viability testing on punch biopsies at regular intervals, such as weekly for the initial month (to record the initial “steep” decline) and subsequently monthly until the completion of treatment. Furthermore, a post-treatment assessment should be performed to evaluate the outcomes. Clinical trials of this nature have not been conducted thus far for the current multidrug therapy used against *M. leprae*. However, it is crucial to yet consider conducting such trials not only for the evaluation of alternative and potentially superior drug combinations in the future but also to enhance our understanding of effective treatment options.

Need for a better direct-on-sample WGS technique

As *M. leprae* cannot be grown in vitro, research into *M. leprae* diversity by necessity leads the way on innovations in culture-free approaches to apply mycobacterial whole genome sequencing on clinical samples. Avanzi *et al.* developed a procedure utilizing *M. leprae*-specific double-stranded DNA baits (KAPA HyperCap Target Enrichment Probes, Roche Diagnostics, USA) to capture *M. leprae* DNA from whole DNA extracts, leading to a breakthrough in sensitivity of WGS of *M. leprae* DNA extracted directly from patients' biopsies, in the sense that samples with lower bacterial load yield better read coverage in WGS. Additionally, more samples could be pooled making the whole approach cheaper than the previous approach with Mybaits Whole Genome Enrichment kit (Arbor Biosciences, USA). Using this approach In Chapter V we demonstrated successful WGS of DNA extracts derived from biopsies from Comorian patients with RLEP qPCR Cq-values of ≤27 that were preserved for months in ethanol. We also tested another approach by extracting DNA from biopsies of ten patients and simultaneously depleting human DNA, followed by the same capture technique with the dsDNA baits. We successfully obtained WGS data, however the quality of the WGS was not superior to the WGS generated from the DNA captured from the whole DNA extracts. Despite optimizations made to enable WGS of *M. leprae*, the technique requires further improvement to increase its sensitivity, as only the most heavily infected leprosy patients can currently be

examined with WGS. Globally, only around 300 *M. leprae* genomes are publicly available to which we contributed an additional 43 (Chapter V), increasing this database with almost 15%. The low number of publicly available genomes limits our ability to conduct drug-resistance surveillance and understand transmission dynamics, pathogen spread and metabolism.

To increase the sensitivity of the procedure, alternative methods for human DNA depletion could be explored, such as heat treatment to rupture human cells, followed by DNAse treatment to destroy only free DNA while leaving bacterial DNA intact inside its waxy cell wall, as has been done by Dippenaar *et al* 2022 [19]. Another possibility is to investigate if library amplification for WGS can be done within a drop, as a preliminary experiment with *M. tuberculosis* suggests that using the Xdrop droplet generator (Samplix, Denmark), a genome can be generated from as little as 1pg, as opposed to the current threshold of 1ng (poster presentation from Anzaan Dippenaar ESM 2022 titled "Optimising whole genome amplification for genome sequencing of minute amounts of *Mycobacterium tuberculosis* DNA").

Need for a recent transmission cluster definition, this depends on the molecular clock

WGS analysis and typing based on VNTR markers are potentially powerful tools for studying the genetic variation of *M. leprae* and its transmission dynamic. Single nucleotide polymorphisms (SNPs) in WGS and VNTRs can be important markers for cluster detection, which can provide valuable insights into the epidemiology of infectious diseases. In Chapter VI, we were not able to establish a cut-off for transmission neither for VNTR or SNPs, given the limited amount of samples that could be processed and the limited time span covered; 5 years, versus the long incubation time of leprosy (on average 2-4 years with wide variance). Nevertheless, the results of this thesis suggest that WGS might give more resolution than VNTR typing, yet that the combination of both methods could yield an even higher resolution. Another exploration might be that we try to reliably infer the VNTR regions from the WGS. To define a SNP cut-off in WGS for cluster detection, it is necessary to know the molecular clock of the marker used, which allows for the estimation of the time lapse since the most recent common ancestor of a cluster. Without a molecular clock, it is difficult to determine whether the observed SNPs reflect recent or distant transmission events, which can lead to false positives (in case of a mutation rate that is estimated too fast) or false negatives (in case of an mutation rate that is estimates too slow) in cluster identification. Typically, when two or more strains of a pathogen differ by a limited number of SNPs, it is assumed that recent transmission has occurred. However, this approach tends to underestimate transmission rates for groups of strains that evolve more rapidly (faster molecular clocks). Based on the investigations done for *M. tuberculosis* complex (MTBc) carried out by Menardo *et al*. in 2019, it is advisable to ascertain a distinct clock rate for different lineages[20], so it might be useful for the different SNP-types as well in *M. leprae* . However given, the timespan over which genomes and the number of genomes available for *M. leprae*, this will be difficult. Therefore, a relaxed clock model could be used, with variation per branch/SNP-type. The research sought to reassess the utility of the molecular clock by

evaluating various methodological approaches using identical datasets. Through these diverse approaches, it was discerned that distinct lineages for MTBc exhibit different molecular clocks. Therefore, the establishment of a reliable molecular clock is essential for accurate interpretation of clustered WGS data. Two estimations have been made for the *M. leprae* SNP rate, at 7.8x10-9 [8] and at 7.5x10-9 [21] based on ancient DNA from Europe. Given the unavailability of contemporary *M. leprae* DNA samples from Europe, it is improbable that the aforementioned clock estimates accurately depict the mutation rate prevalent in the currently circulating strains of *M. leprae*, and accurately depict the mutation rate for strains (e.g. Comorian 1A and 1D) not belonging to 2F, which is the SNP-subtype from the medieval strains, found in Europe, used for the analysis

To obtain more precise clock estimates, optimized WGS is required to gather more information from modern and older samples, like paraffin-embedded biopsies stored from the Comoros since the early 2000s, or even older samples from the African continent available at ITM. To do this, we will need to leverage the specialized expertise of researchers analyzing ancient DNA. By comparing recent and older *M. leprae* strains from the same island location, we hope to reduce the uncertainty around the substitution rate for this pathogen and enable more accurate cluster for recent transmission definition. In a future study we propose to improve the molecular clock estimates with these samples from the same island.

After obtaining a more precise molecular clock estimation, a more accurate recent transmission cluster definition can be achieved by integrating the WGS data with social network and spatial analysis as has been done by Walker et al in 2013 for *M. tuberculosis*. The WGS diversity within and between patients (and other potential hosts) can be evaluated. Initially, paired samples of patients over the course of the disease or from various body sites can be compared. Secondly, the diversity between patients within a household can be assessed, as they are presumed to have been infected by the same source or each other, or by patients who identify each other as known sources. Thirdly, the diversity of WGS can be examined among patients spatially belonging to the same cluster. Fourthly, the diversity between spatially or socially non-clustered patients can be investigated. Finally, VNTR markers can be incorporated into this analysis to determine if the combination of WGS and VNTR can improve the specificity for detection of transmission. Based on this comprehensive information, cluster definitions for recent transmission for VNTR, WGS, or for the combination of both can be obtained.

Subsequently, after establishing a recent transmission cluster, we could apply the method with Transphylo developed by Xu *et al* in 2019 [22]. By employing this approach, we will be able to examine small clusters within a 5-year cohort on the Comoros, providing insights into transmission links and the timing of transmission within these clusters. Additionally, this technique allows us to make inferences about any likely missing index cases and estimate the unsampled population, known as the hidden burden. The potential contribution of subclinical or pre-clinical cases to transmission remains unknown due to the lack of available detection methods for these cases. In the future study, we aim to include samples from the early 2000s,

which already could potentially contain missing index cases, considering the long incubation period of leprosy.

No consensus on where and when *M . leprae* originated.

There is no consensus about the origin of leprosy. With currently available data, it is believed that the progenitor of *M. leprae* may have originated from East Africa around 100 000 years ago and subsequently spread to Asia through human migration. Along with human migration to the Middle East and Europe, leprosy spread further. Slave trade eventually introduced leprosy in West Africa. Two introduction routes of leprosy into Asia are hypothesized: the southern route originating from East Africa, and the northern route by traveling through Europe and the Middle East to reach Asia. Migration from Europe to the Americas most likely introduced leprosy into the Americas.

However, these hypotheses lack comprehensive support from molecular epidemiological data, leaving open the possibility that the origin of leprosy could be traced back to central Africa. This region remains undersampled in terms of *M. leprae* genomes. By acquiring information about past and present strains in different geographic areas, we may gain unprecedented insights into the origin and spread of leprosy. Consequently, the genomes obtained through another future study will undoubtedly make significant contributions to addressing these research questions. For the countries included in this project (Burundi, Ghana, Cameroon, and the Democratic Republic of Congo) currently no *M. leprae* genomes are available.

Is *M. leprae* an environmental and/or zoonotic mycobacterium in Africa?

The presence of bacteria in the environment does not necessarily prove that infection is acquired from the environment, as for an airborne disease such as leprosy aerosols must be created and a sufficient amount of viable bacteria must be taken up by a susceptible individual. Although thousands of *M. leprae* bacteria are shed into the environment by MB patients [23], it is unlikely that these bacteria would survive and infect someone as they are obligatory intracellular bacteria. A study in 2014 demonstrates that common free-living amoebae (*Acanthamoeba*) are capable of phagocytosing *M. leprae*, allowing the bacterium to maintain viability for a period of up to 8 months within amoebic cysts, preserving infectivity in the nude mice model [24]. However, there is no empirical evidence in support of re-aerosolization of *M. leprae* once it has settled on a surface. A review conducted by Bratschi *et al.* in 2015 provided strong evidence for the transmission of leprosy among contacts, presumably by aerosols/droplets, and sporadic zoonotic leprosy in the southern states of the USA. There is no solid evidence supporting transmission through skin-to-skin contact, nor bacterial shedding into the environment followed by infection through dust or small wounds. [25].

In the context of the Comoros, a region devoid of recognized animal reservoirs such as armadillos, red squirrels, and chimpanzees for leprosy, the transmission of the disease is believed to be exclusively transmitted between human hosts. Ticks are known to be important vectors for infectious diseases due to their low host specificity and global distribution, and a

recent experimental study has shown their vector competence for *M. leprae,* though this has not yet been confirmed in wild ticks [26]. To investigate the presence of *M. leprae* DNA in Chapter VII wild tick collections from the Comoros islands, we extracted a collection consisting of 526 tick samples from 2011 (Yssouf *et al*) at the ITM using Maxwell DNA extraction and an RLEP qPCR assay, but found no ticks carrying *M. leprae*, nor *M. lepromatosis*.

In a recent investigation conducted by Chris Ruis and colleagues in 2023, a distinction between lung bacteria and environmental bacteria was established based on their overall mutation signatures [27]. The study revealed that lung bacteria consistently display a greater amount of C>A and C>T mutations, whereas environmental bacteria have a relatively higher frequency of T>C mutations. This observation could be attributed to the occurrence of known mutagens such as alkylating agents and nitro polycyclic aromatic hydrocarbons in the environment. The same team demonstrated that *M. leprae* has a high percentage of C>A and a low proportion of T>C, leading to a mutation signature that aligns with exclusively human lung bacterial clades. These findings support the hypothesis that *M. leprae* is a disease acquired solely through aerosol between hosts rather than supporting an environmental source of infection.

One prospective method for investigating the potential involvement of animals in the transmission of leprosy within the Comoros would be the implementation of a case-control study. By systematically monitoring (former) leprosy patients, as well as control subjects, throughout their routine daily activities, it becomes possible to generate hypotheses regarding differences in exposures that may offer insights into potential animal reservoirs or vectors associated with the disease.

Sloths, primarily found in the tropical rainforests of Central and South America, inhabit countries like Costa Rica, Panama, Brazil, Ecuador, and Venezuela. Similar to armadillos, known *M. leprae* reservoirs, sloths have a lower body temperature (30 to 34 °C) compared to other animals. Due to *M. leprae's* preference for lower temperatures to replicate, sloths could potentially also serve as a reservoir for *M. leprae*, especially considering their presence in leprosy-endemic areas such as Brazil. However, unlike armadillos in the southern United States, sloths are generally not hunted, eaten, or handled by humans. Given their limited interaction with humans, the likelihood of zoonotic transmission is unlikely.

Human genetics

Host genetics plays a significant role in the susceptibility and progression of leprosy. The HLA system, immune-related genes such as IL-10 and TNF-alpha, and genes coding for TLRs are all known to be associated with leprosy susceptibility. Understanding the genetic basis of leprosy will help in the development of new treatments and vaccines for the disease. However, leprosy is a complex disease that is influenced by a variety of other factors, including environmental and socioeconomic. The leprosy control programme of the Comoros, as well as members of an affected family, have wondered whether inherited risk factors play a role. We initiated a pilot study to address this question by performing genomic studies on human DNA. The whole

genome sequencing approach we propose to undertake seeks to identify rare non-synonymous variants or large insertions/deletions with strong impact on the disease.

Why do people develop leprosy after PEP?

Potential reasons that contacts still develop leprosy after PEP include drug resistance, inadequate treatment for incipient disease (although people who were diagnosed within 30days post PEP were not counted as incident leprosy), and reinfection, particularly in hyperendemic settings. The precise incubation period remains elusive, albeit estimated to be approximately three months at a minimum, with an average range spanning from 2 to 4 years. Considering the relatively short half-life of rifampicin, which is approximately six hours, the impact of PEP is restricted to individuals already infected, rendering it unlikely to prevent future infections. Beyond the pilot study on host genetics we aim to investigate the genetic factors contributing to post-PEP breakthrough leprosy. We will assess the contribution of host and pathogen factors to ongoing transmission of *M. leprae*. Specifically, we will determine if the population of Anjouan is hypersusceptible to *M. leprae*, leading to leprosy development despite receiving PEP. Our approach involves performing host WGS on children in Anjouan with early onset leprosy to identify genetic factors. Additionally, we will conduct a case-control study to impute HLA types and identify HLA alleles associated with post-PEP leprosy, as well as common variants associated with the previously identified genes from human WGS analysis.

POLICY

Based on the preliminary findings of the PEOPLE study, it appears that PEP provides an individual protective effect of approximately 40% in the short term. Door-to-door screening results in early identification and treatment of leprosy patients, which can effectively prevent further transmission. Yet door-to-door screening poses logistical challenges, such as accessibility of the included regions, which can limit its feasibility and cost-effectiveness for control programs. As also the control arm in our PEOPLE trial received door-to-door screening for leprosy, we can moreover not distinguish the relative contribution of screening versus PEP in the modest reduction (+/-20%) in incidence seen in the villages that received most PEP. Therefore, I would recommend implementing genotypic clustering analysis to identify hotspots of recent transmission, coupled with comprehensive systematic drug-resistance screening beyond the known genes. Subsequent door-to-door screening could be conducted in these hotspots every few years, while annual skin camps can be organized in the years that no door-to-door screening is planned and in regions with no hotspots.

CONCLUSION

In conclusion, our study contributes important findings on leprosy transmission and control. Rapid diagnosis and treatment initiation for index cases can reduce community transmission. The RLEP qPCR assay proved its accuracy for diagnosing *M. leprae* infections, while αPGL-I IgM levels correlated with bacterial load, aiding in predicting high bacterial load in leprosy patients. We developed the Deeplex Myc-Lep tool, enabling early detection of drug resistance and

genotyping *M. leprae*. All *M. leprae* strains in the Comoros remained susceptible to specific drugs. Targeted sequencing on skin biopsies revealed genotypic diversity in *M. leprae* with clustering within and outside villages, indicating the potential of using VNTRs for tracking transmission. Majority of patients in the Comoros were infected with SNP type 1D. *M. leprae* transmission persists in the Comoros, emphasizing the need for (targeted) interventions. Through the use of novel genetic tools and analytical methods, we anticipate a significant advancement in our ability to comprehend transmission networks and date transmission events related to leprosy—an area that has thus far remained unexplored. No evidence was found of ticks serving as vectors for *M. leprae* in the Comoros. Accurate diagnostics, drug resistance monitoring, and surveillance are crucial for leprosy control. Like within the PEOPLE study, the ongoing implementation of rounds of door-to-door screening, conducted at biennial intervals, will persist. As this process unfolds, we will progressively reduce the coverage area, allowing the circles of screening to gradually contract. Concurrently, we will intensify our efforts in developing more robust proactive measures (combination of bedaquiline and rifampicin as PEP), evaluating the comparative effectiveness of these measures in relation to door-to-door screening with new phylodynamics tools.

REFERENCES

1. Williams, C.M., et al., *Exhaled Mycobacterium tuberculosis Predicts Incident Infection in Household Contacts.* Clin Infect Dis, 2023. **76**(3): p. e957-e964.
2. Wood, R.C., et al., *Characterization of oral swab samples for diagnosis of pulmonary tuberculosis.* PLoS One, 2021. **16**(5): p. e0251422.
3. van Hooij, A., et al., *Application of new host biomarker profiles in quantitative point-of-care tests facilitates leprosy diagnosis in the field.* EBioMedicine, 2019. **47**: p. 301-308.
4. van Hooij, A., et al., *Household Contacts of Leprosy Patients in Endemic Areas Display a Specific Innate Immunity Profile.* Front Immunol, 2020. **11**: p. 1811.
5. Antunes, D.E., et al., *Number of leprosy reactions during treatment: clinical correlations and laboratory diagnosis.* Rev Soc Bras Med Trop, 2016. **49**(6): p. 741-745.
6. Walter, N.D., et al., *Mycobacterium tuberculosis precursor rRNA as a measure of treatment-shortening activity of drugs and regimens.* Nat Commun, 2021. **12**(1): p. 2899.
7. Reichlen, M.J., et al., *Standardized RS Ratio Metrics To Assess Tuberculosis Antimicrobial Efficacy and Potency.* Antimicrob Agents Chemother, 2023. **67**(1): p. e0148322.
8. Benjak, A., et al., *Phylogenomics and antimicrobial resistance of the leprosy bacillus Mycobacterium leprae.* Nat Commun, 2018. **9**(1): p. 352.
9. Lelovic, N., et al., *Application of Mycobacterium smegmatis as a surrogate to evaluate drug leads against Mycobacterium tuberculosis.* J Antibiot (Tokyo), 2020. **73**(11): p. 780-789.
10. Nakata, N., M. Kai, and M. Makino, *Mutation analysis of the Mycobacterium leprae folP1 gene and dapsone resistance.* Antimicrob Agents Chemother, 2011. **55**(2): p. 762-6.
11. Nakata, N., M. Kai, and M. Makino, *Mutation analysis of mycobacterial rpoB genes and rifampin resistance using recombinant Mycobacterium smegmatis.* Antimicrob Agents Chemother, 2012. **56**(4): p. 2008-13.
12. Pieterman, E.D., et al., *Advanced Quantification Methods To Improve the 18b Dormancy Model for Assessing the Activity of Tuberculosis Drugs In Vitro.* Antimicrob Agents Chemother, 2020. **64**(7).
13. Andries, K., et al., *Acquired resistance of Mycobacterium tuberculosis to bedaquiline.* PLoS One, 2014. **9**(7): p. e102135.
14. Machado, D., et al., *Insights on Mycobacterium leprae Efflux Pumps and Their Implications in Drug Resistance and Virulence.* Front Microbiol, 2018. **9**: p. 3072.
15. Aurélie Chauffour, N.L., Koen Andries, Vincent Jarlier, Nicolas Veziris, Alexandra Aubry, *Minimal effective dose of oral bedaquiline and activity of a long acting formulation of bedaquiline in the murine model of leprosy Bedaquiline for the treatment of leprosy.* BioRXiv, 2023.
16. Younoussa, A., et al., *Protocol, rationale and design of BE-PEOPLE (Bedaquiline enhanced exposure prophylaxis for LEprosy in the Comoros): a cluster randomized trial on effectiveness of rifampicin and bedaquiline as post-exposure prophylaxis of leprosy contacts.* BMC Infect Dis, 2023. **23**(1): p. 310.

17. Thomas, S.S., et al., *Toward a Single-Dose Cure for Buruli Ulcer.* Antimicrob Agents Chemother, 2020. **64**(9).
18. Lahiri, R., et al., *Sensitivity of Mycobacterium leprae to Telacebec.* Emerg Infect Dis, 2022. **28**(3): p. 749-751.
19. Dippenaar, A., et al., *Optimizing liquefaction and decontamination of sputum for DNA extraction from Mycobacterium tuberculosis.* Tuberculosis (Edinb), 2022. **132**: p. 102159.
20. Menardo, F., et al., *The molecular clock of Mycobacterium tuberculosis.* PLoS Pathog, 2019. **15**(9): p. e1008067.
21. Schuenemann, V.J., et al., *Ancient genomes reveal a high diversity of Mycobacterium leprae in medieval Europe.* PLoS Pathog, 2018. **14**(5): p. e1006997.
22. Xu, Y., et al., *High-resolution mapping of tuberculosis transmission: Whole genome sequencing and phylogenetic modelling of a cohort from Valencia Region, Spain.* PLoS Med, 2019. **16**(10): p. e1002961.
23. Ploemacher, T., et al., *Reservoirs and transmission routes of leprosy; A systematic review.* PLoS Negl Trop Dis, 2020. **14**(4): p. e0008276.
24. Wheat, W.H., et al., *Long-term survival and virulence of Mycobacterium leprae in amoebal cysts.* PLoS Negl Trop Dis, 2014. **8**(12): p. e3405.
25. Bratschi, M.W., et al., *Current knowledge on Mycobacterium leprae transmission: a systematic literature review.* Lepr Rev, 2015. **86**(2): p. 142-55.
26. Ferreira, J.D.S., et al., *Ticks as potential vectors of Mycobacterium leprae: Use of tick cell lines to culture the bacilli and generate transgenic strains.* PLoS Negl Trop Dis, 2018. **12**(12): p. e0007001.
27. Ruis, C., et al., *A lung-specific mutational signature enables inference of viral and bacterial respiratory niche.* Microb Genom, 2023. **9**(5).

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Curriculum Vitae

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Education & achievements

May - June 2015:	Short internship at the unit of Mycobacteriology unit in ITM.
August 2015:	Expand background and expertise by working at the unit of Mycobacteriology as a student.
December 2015 - June 2016:	Stay abroad for master thesis at MRCG in The Gambia, Africa.
	Master thesis: "Seasonal variation in malaria parasite diversity and multiplicity of infection in asymptomatic malaria patients in the Gambia Upper River Region"
28th of June 2016:	Graduated magna cum laude as Master of Science in Biomedical Sciences: Tropical and infectious diseases Biomedical Sciences at University of Antwerp
27th of June 2018:	Grant FWO aspirant fellowship (oct 2018- sept2020) (Research Foundation- Flanders)
24th of June 2020:	Grant Second mandate FWO aspirant fellowship(oct 2020-sept 2022) (Research Foundation- Flanders)
13th of December 2021:	FWO travel grant for a long stay abroad- V408322N (Research Foundation- Flanders)
January 2022 - April 2022:	Stay abroad at Colorado State University, Fort Collins, United States of America.
	Training: "Whole Genome Sequencing of *M. leprae* strains direct on samples"
16th of September 2022:	FWO travel grant for a conference abroad- K1C6122N ((Research Foundation- Flanders)
3rd of November 2022:	Invited speaker for the WHO Workshop on strengthening laboratory services for antimicrobial resistance (AMR) surveillance in leprosy, 14-15 November 2022, Karigiri (Vellore). India

Scientific curriculum

October 2016 – September 2017: Expand background on leprosy and laboratory expertise at the unit of mycobacteriology as a laboratory technician. Assisting in the ComLep study.

October 2017-September 2018: Research assistant in the unit of Mycobacteriology, supporting management of ComLep study and PEOPLE study

October 2018- 2023: Conducting PhD in molecular epidemiology of *M. leprae*.

- Research and management support of Comlep and PEOPLE study
- Trainings Comlep and PEOPLE study
- Promotors: Bouke de Jong & Leen Rigouts

October 2022-present: Junior researcher assistant in the unit of Mycobacteriology, supporting management of PEOPLE study and BE-PEOPLE study

Bachelor dissertation supervisions

1. Zakaria Benaamar (2021): 'Het effect van RNAlater, GTC-TCEP en DNA/RNA Shield op de leefbaarheid en het transcriptoom van *Mycobacterium tuberculosis*'

Master dissertation supervisions

1. Elien Chauvaux (2018-2019,UA): "Leprosy transmission on the Comoros: possible role of ticks and improvement of molecular epidemiological tools on historical samples"

2. Lena Krausser (2019-2020,UA): "Molecular Viability Assays for Ethanol-preserved Samples containing Mycobacterium tuberculosis and Mycobacterium leprae"

3. Nissad Attoumane (2021-2022,ITM): "Molecular confirmation of clinical diagnosis of leprosy by identifying *Mycobacterium leprae* and *Mycobacterium lepromatosis* in the Union of the Comoros"

Scientific publications

1. Braet, S., Vandelannoote, K., Meehan, C. J., Brum Fontes, A. N., Hasker, E., Rosa, P. S., Lucena-Silva, N., Rigouts, L., Suffys, P. N., & De Jong, B. C. (2018). The Repetitive Element RLEP Is a Highly Specific Target for Detection of Mycobacterium leprae. *J Clin Microbiol*, *56*(3). https://doi.org/10.1128/JCM.01924-17

2. Braet, S. M., van Hooij, A., Hasker, E., Fransen, E., Wirdane, A., Baco, A., Grillone, S., Ortuno-Gutierrez, N., Assoumani, Y., Mzembaba, A., Corstjens, P., Rigouts, L., Geluk, A., & de Jong, B. C. (2021). Minimally invasive sampling to identify leprosy patients with a high bacterial burden in the Union of the Comoros. *PLoS Negl Trop Dis*, *15*(11), e0009924. https://doi.org/10.1371/journal.pntd.0009924

3. Braet, S. M., Jouet, A., Aubry, A., Van Dyck-Lippens, M., Lenoir, E., Assoumani, Y., Baco, A., Mzembaba, A., Cambau, E., Vasconcellos, S. E. G., Rigouts, L., Suffys, P. N., Hasker, E., Supply, P., & de Jong, B. C. (2022). Investigating drug resistance of Mycobacterium leprae in the Comoros: an observational deep-sequencing study. *Lancet Microbe*, *3*(9), e693-e700. https://doi.org/10.1016/S2666-5247(22)00117-3

4. Maug, A. K. J., Hossain, M. A., Gumusboga, M., Decroo, T., Mulders, W., Braet, S., Buyze, J., Arango, D., Schurmans, C., Herssens, N., Demeulenaere, T., Lynen, L., de Jong, B. C., & Van Deun, A. (2020). First-line tuberculosis treatment with double-dose rifampicin is well tolerated. *Int J Tuberc Lung Dis*, *24*(5), 499-505. https://doi.org/10.5588/ijtld.19.0063

5. Maug, A. K. J., Hossain, M. A., Gumusboga, M., Decroo, T., Mulders, W., Braet, S., Buyze, J., Jimenez, D. A., Schurmans, C., Herssens, N., Demeulenaere, T., Lynen, L., de Jong, B. C., & Van Deun, A. (2021). Finding the right balance between efficacy and tolerability for TB treatment: the search continues. *Int J Tuberc Lung Dis*, *25*(1), 84-86. https://doi.org/10.5588/ijtld.20.0575

6. Ortuno-Gutierrez, N., Baco, A., Braet, S., Younoussa, A., Mzembaba, A., Salim, Z., Amidy, M., Grillone, S., de Jong, B. C., Richardus, J. H., & Hasker, E. (2019). Clustering of leprosy beyond the household level in a highly endemic setting on the Comoros, an observational study. *BMC Infect Dis*, *19*(1), 501. https://doi.org/10.1186/s12879-019-4116-y

7. Ortuno-Gutierrez, N., Mzembaba, A., Ramboarina, S., Andriamira, R., Baco, A., Braet, S., Younoussa, A., Cauchoix, B., Salim, Z., Amidy, M., Grillone, S., Rasamoelina, T., Cambau, E., Geluk, A., de Jong, B. C., Richardus, J. H., & Hasker, E. (2021). Exploring clustering of leprosy in the Comoros and Madagascar: A geospatial analysis. *Int J Infect Dis*, *108*, 96-101. https://doi.org/10.1016/j.ijid.2021.05.014

8. Ortuno-Gutierrez, N., Younoussa, A., Randrianantoandro, A., Braet, S., Cauchoix, B., Ramboarina, S., Baco, A., Mzembaba, A., Salim, Z., Amidy, M., Grillone, S., Richardus, J. H., de Jong, B. C., & Hasker, E. (2019). Protocol, rationale and design of PEOPLE (Post ExpOsure Prophylaxis for LEprosy in the Comoros and Madagascar): a cluster

randomized trial on effectiveness of different modalities of implementation of post-exposure prophylaxis of leprosy contacts. *BMC Infect Dis*, *19*(1), 1033. https://doi.org/10.1186/s12879-019-4649-0

9. Ortuno-Gutierrez, N., Mzembaba, A., Baco, A., **Braet, S. M.,** Younoussa, A., Salim, Z., Amidy, M., Grillone, S., Said, A., de Jong, B. C., Richardus, J. H., & Hasker, E. (2022). High yield of retrospective active case finding for leprosy in Comoros. PLoS Negl Trop Dis, 16(3), e0010158. https://doi.org/10.1371/journal.pntd.0010158

10. Krausser, L.[#], **Braet, S. M.**[#], Benaamar, Z., Van Dyck-Lippens, M., de Jong, B. C., & Rigouts, L. (2023). Mycobacterium tuberculosis retains viability in RNAlater buffer but not in GTC-TCEP and DNA/RNA Shield. *Diagn Microbiol Infect Dis*, *106*(1), 115905. https://doi.org/10.1016/j.diagmicrobio.2023.115905 *(# shared first author*)

11. Younoussa, A., Samidine, S. N., Bergeman, A. T., Piubello, A., Attoumani, N., Grillone, S. H., **Braet, S. M.,** Tsoumanis, A., Baco, A., Mzembaba, A., Salim, Z., Amidy, M., Grillone, S., Snijders, R., Corstjens, P., Ortuno-Gutierrez, N., Hoof, C., Geluk, A., de Jong, B. C., & Hasker, E. (2023). Protocol, rationale and design of BE-PEOPLE (Bedaquiline enhanced exposure prophylaxis for LEprosy in the Comoros): a cluster randomized trial on effectiveness of rifampicin and bedaquiline as post-exposure prophylaxis of leprosy contacts. *BMC Infect Dis*, *23*(1), 310. https://doi.org/10.1186/s12879-023-08290-0

12. Jouet A. #, **Braet S.M.#,** Gaudin C., Bisch G., Vasconcellos S., Nicacio de Oliveira do Livramento R.E.E., Palacios Y.Y.E., Fontes A.B., Lucena N.6, Rosa P., Moraes M., La K., Badalato N., Lenoir E., Ferré A., Clément M., Hasker E., Grillone S.H., Abdou W., Said A., Assoumani Y., Attoumani N., Laurent Y., Cambau E. , de Jong B.C.*, Suffys P.N.*, Supply P. * (2023) Hi-plex deep amplicon sequencing for identification, high-resolution genotyping and multidrug resistance prediction of Mycobacterium leprae directly from patient biopsies by using Deeplex Myc-Lep. *E Biomedicine Part of Lancet Dicsovery Science (# shared first author*) https://doi.org/10.1016/j.ebiom.2023.104649

13. **Braet S.M.,** P S Rosa, J S Spencer & C Avanzi (2023). Leprosy agents and principal methods of detection, identification and characterization of the leprosy agents. Springer, Book “Hansen’s Disease” chapter 5 (published in September 2023)

14. Krausser L. , Chauvaux E., Van Dyck-Lippens M., Yssouf A., Assoumani Y., Tortosa P., de Jong B.C., **Braet S. M**. Ticks are unlikely to play a role in leprosy transmission in the Comoros as they do not harbour *Mycobacterium leprae* DNA. *Front Med (Lausanne)* https://doi.org/10.3389/fmed.2023.1238914

Laboratory skills

Working in a biosafety level 2/3 laboratory
Mycobacterium tuberculosis culture
Mycobacterium tuberculosis/leprae genomic DNA extraction
Mycobacterium tuberculosis/leprae Ziehl-Neelsen microscopy
DNA extraction of mycobacteria from clinical samples
RNA extractions from mycobacteria
(q)PCR asay (development)-
Whole genome sequencing *Mycobacterium leprae*

Computer and programming skills

Programming Languages

Unix Shell scripting	Intermediate
R	Proficient
Python	Intermediate

Office Applications

Word	Proficient
Excel	Proficient
Powerpoint	Proficient
Outlook	Proficient
Access	Proficient

Language skills

Dutch	Native speaker
English	Fluent
French	Proficient