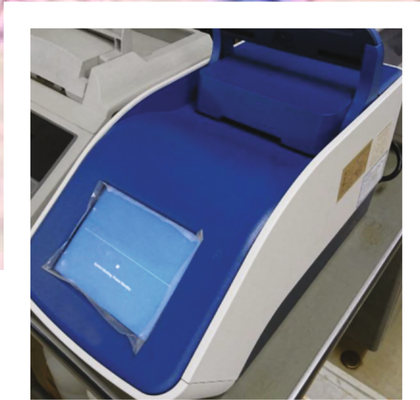

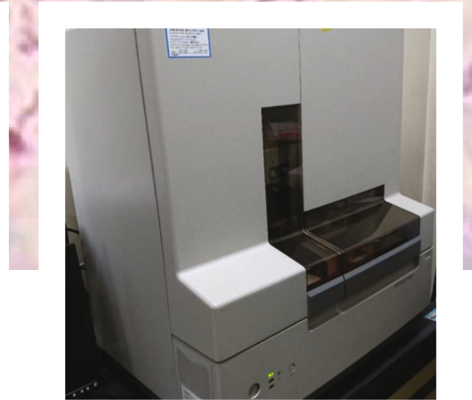

A guide for surveillance of antimicrobial resistance in leprosy

2017 update

World Health Organization

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Abbreviations and acronyms

AMR	antimicrobial resistance
BI	bacillary index
DRDR	dapsone resistance-determining region
GLASS	Global Antimicrobial Resistance Surveillance System
GLP	Global Leprosy Programme
IATA	International Air Transport Association
ILEP	International Federation of Anti-Leprosy Associations
MB	multibacillary
MDT	multidrug therapy
MoU	memorandum of understanding
NGO	nongovernmental organization
PB	paucibacillary
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QRDR	quinolone resistance-determining region
RLEP	*M. leprae*-specific repetitive element
RRDR	rifampicin resistance-determining region
TB	tuberculosis
WHO	World Health Organization

Executive summary

The emergence of drug resistance is a concern and a threat for many infectious disease intervention programmes, especially those that have secondary prevention (chemotherapy) as the main component of their control strategy. The fight against leprosy has been a great success, largely due to the development of multidrug therapy (MDT) in 1981. The roll-out of MDT in the late 1980s has been a major factor in bringing down dramatically the burden of leprosy until the year 2005, after which a plateau was observed in the number of cases on treatment, and a much slower reduction in notification. As rifampicin is the backbone of the MDT regimen, it is important to monitor the emergence of rifampicin-resistant strains, as recent reports and publications have indicated the existence of rifampicin resistance in several endemic areas. Prior to the introduction of MDT, patients were treated with dapsone monotherapy for several years. Resistance to dapsone has been reported since the early 1960s. In case of resistance to rifampicin, fluoroquinolones become the preferred category of second-line drugs. Unfortunately, quinolone-resistant strains of *Mycobacterium leprae* have also been reported in several countries, probably due to the extensive use of quinolones for treating several types of infections. Clofazimine resistance is still rare but this antimicrobial cannot be given alone. To meet the challenge of containing the disease and being able to respond to an increase in circulation of drug-resistant strains, it is essential to assess drug-sensitivity patterns globally, as well as to monitor resistance among both new and retreatment cases.

Objectives of the guide and its target population

1

This document aims to guide countries on how to test for drug resistance in leprosy. It highlights the clinical, field and laboratory support systems that need to be put in place to undertake this activity.

Antimicrobial resistance (AMR) is one of the key areas of intervention of the Global Leprosy Strategy 2016–2020, under its Pillar II “Stop leprosy and its complications”. Under this Strategy, all countries that detect leprosy are expected to set up a system to allow testing for AMR either in a national laboratory or in a laboratory located abroad.

This guide aims to promote the use of a twofold standardized approach:

(1) to detect primary and secondary drug resistance to antileprosy drugs, namely, rifampicin, dapsone and ofloxacin;

(2) to standardize reports related to the results of testing done and on patients' outcomes of treatment.

The availability of data on drug resistance in leprosy will allow global, national and subnational monitoring of drug resistance trends over time among new and retreatment cases of leprosy (*see* definitions in Section 5.2). It would also help to identify risk factors for drug resistance (age, sex, area of residence, patient's category/type).

The target audience for the updated guide consists of managers of national leprosy programmes, clinicians and personnel at referral facilities and sentinel sites, laboratory coordinators and technicians working in leprosy, as well as consultants working in the field of leprosy and of neglected tropical diseases (NTDs).

2 Background

The current network for drug-resistance surveillance was implemented jointly by the World Health Organization (WHO) and The Nippon Foundation, followed in later years by funding support from the International Federation of Anti-Leprosy Associations (ILEP) between 2008 and 2016. The countries that participated in the sentinel surveillance network were: Benin, Brazil, Burkina Faso, China, Colombia, Ethiopia, Guinea, India, Indonesia, Madagascar, Mali, Mozambique, Myanmar, Nepal, Niger, Pakistan, Philippines, Viet Nam and Yemen. In 2016 Sri Lanka started testing samples.

The countries that are part of the network either test samples in national laboratories, as is the case for Brazil (two testing laboratories), China, Colombia, India (four testing laboratories), Indonesia and Nepal, or send samples to the following four laboratories: National Hansen's Disease Programs Laboratory Research Branch, Baton Rouge, Louisiana, United States of America (USA); Global Health Institute, École Polytechnique Fédérale de Lausanne, Switzerland; National Reference Centre on Mycobacteria and Resistance to Anti-tuberculosis Drugs, Hôpital Lariboisière, Paris, France; and Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan. These laboratories are considered as international reference laboratories for AMR in leprosy. The reference laboratories that are part of the network in 2017 are listed in Annex 1. Some of the national laboratories are located within government hospitals and/or institutions, while others are located in hospitals managed by nongovernmental organizations (NGOs). WHO/ILEP support to laboratories consists of providing funding to some of the international laboratories (Lausanne and Paris), and funding a biannual meeting organized by WHO to discuss results and laboratory methods. The cost for testing at the national level is borne by national governments or international NGOs. The network became functional in 2008 following identification of mutations associated with drug resistance in specific genomic loci of *Mycobacterium leprae,* conferring resistance to dapsone, rifampicin and ofloxacin. Initially, testing was recommended only for relapse cases to assess the level of secondary resistance and the corresponding results were published in the *Weekly epidemiological record* (most recent publication was in June 2011: Wkly Epidemiol Rec. 2011;23 (86):2334). The first guidelines on drug resistance surveillance in leprosy were published in 2009 (*Guidelines for global surveillance of drug resistance in leprosy*. New Delhi: WHO Regional Office for South-East Asia; 2009).

Rationale for developing the guide

3

This guide has been prepared by WHO's Global Leprosy Programme (GLP) in coordination with the main leprosy stakeholders. Since 2011, some countries had already started testing new cases for drug resistance in addition to relapse cases to be able to assess the level of primary drug resistance. In addition, the difficulty of meeting the definition of relapse was a barrier to testing for secondary resistance. This may change in light of the new case definitions for leprosy included in the *Global Leprosy Strategy 2016–2020: Monitoring and Evaluation Guide* (*see also* Section 5.2).

Laboratories participating in the network were assessed for the quality of genomic amplification by the international reference laboratory in Japan in 2009 and 2011 with good concordance. Each of the involved laboratories uses its own techniques, such as polymerase chain reaction (PCR) products direct sequencing, DNA strip assay, high-resolution melting analysis or whole-genome sequencing. The detection of AMR in leprosy has not been standardized so far. Specimens collected consisted of slit-skin smears (as per the 2009 guidelines); in later years, skin biopsies have also been collected and analysed.

All data collected globally from 2010 to 2015 was synthesized during the global Consultation on Antimicrobial Resistance Surveillance in October 2016. Formal reports were received on a total of 1086 relapse cases and 776 new cases tested globally before the end of 2015, among which resistance to rifampicin was identified in 57 relapse cases (5.2% secondary resistance) and 16 new cases (2.1% primary resistance). These results pointed out the need for expanding the surveillance network not only in terms of laboratories and countries involved, but also in terms of scope, increasing the number of samples tested out of the total cases detected, and implementing a continuous surveillance model for secondary resistance. This is also in line with the space that AMR in leprosy has been given as one of the key areas of intervention under the Global Leprosy Strategy 2016–2020. The World Health Assembly issued a resolution on AMR in 2015 (WHA 68.20) together with a global action plan; therefore, this importance given under the Global Leprosy Strategy fits within a general global attention to drug resistance. For these reasons, GLP in collaboration with its main partners and stakeholders, has prepared this guide as an update to the surveillance guidance of 2009.

4 Global Leprosy Strategy 2016–2020 at a glance

2016-2020
GLOBAL LEPROSY STRATEGY

- Zero disease
- Zero transmission of leprosy infection
- Zero disability due to leprosy
- Zero stigma and discrimination

Further reduce the global and local leprosy burden

INDICATORS	2020 target
Number of children diagnosed with leprosy and visible deformities	0
Rate of newly diagnosed leprosy patients with visible deformities	<1 per million
Number of countries with legislation allowing discrimination on basis of leprosy	0

PILLARS AND COMPONENTS

1. Strengthen government ownership, coordination and partnership

- Ensuring political commitment and adequate resources for leprosy programmes.
- Contributing to universal health coverage with a special focus on children, women and underserved populations including migrants and displaced people.
- Promoting partnerships with state and non-state actors and promote intersectoral collaboration and partnerships at the international level and within countries.
- Facilitating and conducting basic and operational research in all aspects of leprosy and maximize the evidence base to inform policies, strategies and activities.
- Strengthening surveillance and health information systems for programme monitoring and evaluation (including geographical information systems)

2. Stop leprosy and its complications

- Strengthening patient and community awareness on leprosy.
- Promoting early case detection through active case-finding (e.g. campaigns) in areas of higher endemicity and contact management.
- Ensuring prompt start and adherence to treatment, including working towards improved treatment regimens.
- Improving prevention and management of disabilities.
- Strengthening surveillance for antimicrobial resistance including laboratory network.
- Promoting innovative approaches for training, referrals and sustaining expertise in leprosy such as eHealth.
- Promoting interventions for the prevention of infection and disease.

3. Stop discrimination and promote inclusion

- Promoting societal inclusion through addressing all forms of discrimination and stigma.
- Empowering persons affected by leprosy and strengthen their capacity to participate actively in leprosy services.
- Involving communities in actions for improvement of leprosy services.
- Promoting coalition-building among persons affected by leprosy and encourage the integration of these coalitions and or their members with other community-based organizations.
- Promoting access to social and financial support services, e.g. to facilitate income generation, for persons affected by leprosy and their families.
- Supporting community-based rehabilitation for people with leprosy-related disabilities.
- Working towards abolishing discriminatory laws and promote policies facilitating inclusion of persons affected by leprosy.

Overview of the surveillance system on antimicrobial resistance in leprosy

5

5.1 Objectives

The objective of the surveillance system is to determine primarily resistance to rifampicin among leprosy cases, either alone or combined with resistance to dapsone and/or ofloxacin among new cases (a proxy for primary resistance) and among retreatment cases (a proxy for secondary resistance), and to monitor resistance rates over time. It would also provide disaggregated information so that a possible association between resistance and specific sex and age groups, and/or geographical location of leprosy cases can be identified.

5.2 Definitions

New case (of leprosy): a patient diagnosed with leprosy who has never been treated for the disease.

Retreatment case (of leprosy): a patient diagnosed with leprosy who has already received treatment for the disease in the past. Retreatment cases are further classified into the following groups:

- **Retreatment after loss to follow up:** a patient diagnosed with leprosy who has abandoned treatment before its completion and returns to the health-care facility to complete treatment.
- **Relapse:** a patient who has completed a full course of treatment for leprosy in the past and who returns with signs and symptoms of the disease that are not deemed to be due to a reaction according to the clinician.
- **Transferred in:** a patient who has started treatment in one facility and reports to another facility to continue treatment.
- **Other retreatment:** any leprosy case that does not fit in any of the above categories and requires treatment.

Both new and retreatment cases can be further classified as follows:

PB case: a case of leprosy with 1–5 skin lesions and without demonstrated presence of bacilli in a skin smear.

MB case: a case of leprosy with >5 skin lesions; or with any nerve involvement (including pure neuritis or any number of skin lesions and neuritis); or with demonstrated presence of bacilli in a slit-skin smear, irrespective of the number of skin lesions.

5.3 Resistance among new cases

Samples to be tested. Slit-skin smears or skin biopsies

Antimicrobial agents to be investigated. Samples are tested for mutations known to be associated with resistance to rifampicin, dapsone and ofloxacin.

Inclusion criteria. To detect primary resistance, a certain number out of the total number of new leprosy cases detected per year have to be tested. In order to have a positive PCR and successful testing for AMR, only smear-positive MB cases with a bacillary index (BI) >2+ will be tested, as these have a higher chance of a positive PCR.

Define the design of the system. Surveillance for primary AMR in leprosy can be carried out based on either:

- a continuous surveillance system that tests all new MB cases (recommended for countries with high laboratory capacity, adequate resources and a small number of cases ONLY);
- a cross-sectional survey (recommended for countries with no representative baseline data and with sufficient financial resources);
- a sentinel surveillance system with the aim of monitoring the trends in rifampicin resistance among new leprosy cases over a period of time in various regions/states (recommended for countries with nationwide baseline data/after a national survey). A sentinel system after a nationwide survey could be a reasonable approach to generate constant data and be able to analyse trends
- setting up an arbitrary sample size, calculating a target proportion of all new MB cases to be tested according to capacity/resources. This should NOT be below 10% of the total new MB cases notified in the previous year (recommended for countries with no nationwide baseline data and without the resources to carry out a nationwide survey).

National or international laboratories will be identified for performing the testing. Skin samples would be collected from selected facilities (study sites during surveys or sentinel surveillance sites) after getting patient's consent and transported to the corresponding assigned national or international testing laboratory. When samples are sent abroad by air, they should be shipped according to the regulations of the International Air Transport Association (IATA).

Selection of sample collection health facilities. As slit-skin smears or skin biopsies are not routinely collected at the peripheral level, the patients to be tested will have to be identified among those reporting to intermediate-level facilities where slit-skin smears and/

or biopsies are routinely performed. In the selected facilities, all new patients with a BI >2+ would be tested for drug resistance.

Information system. Records of the patients would be kept at the facility collecting the samples, at the laboratory and at the national level using a national AMR register (example in Annex 2).

5.4 Resistance among retreatment cases

Samples to be tested. Skin-slit smears or skin biopsies

Antimicrobial agents to be investigated. Samples are tested for mutations known to be associated with resistance to rifampicin, dapsone and ofloxacin.

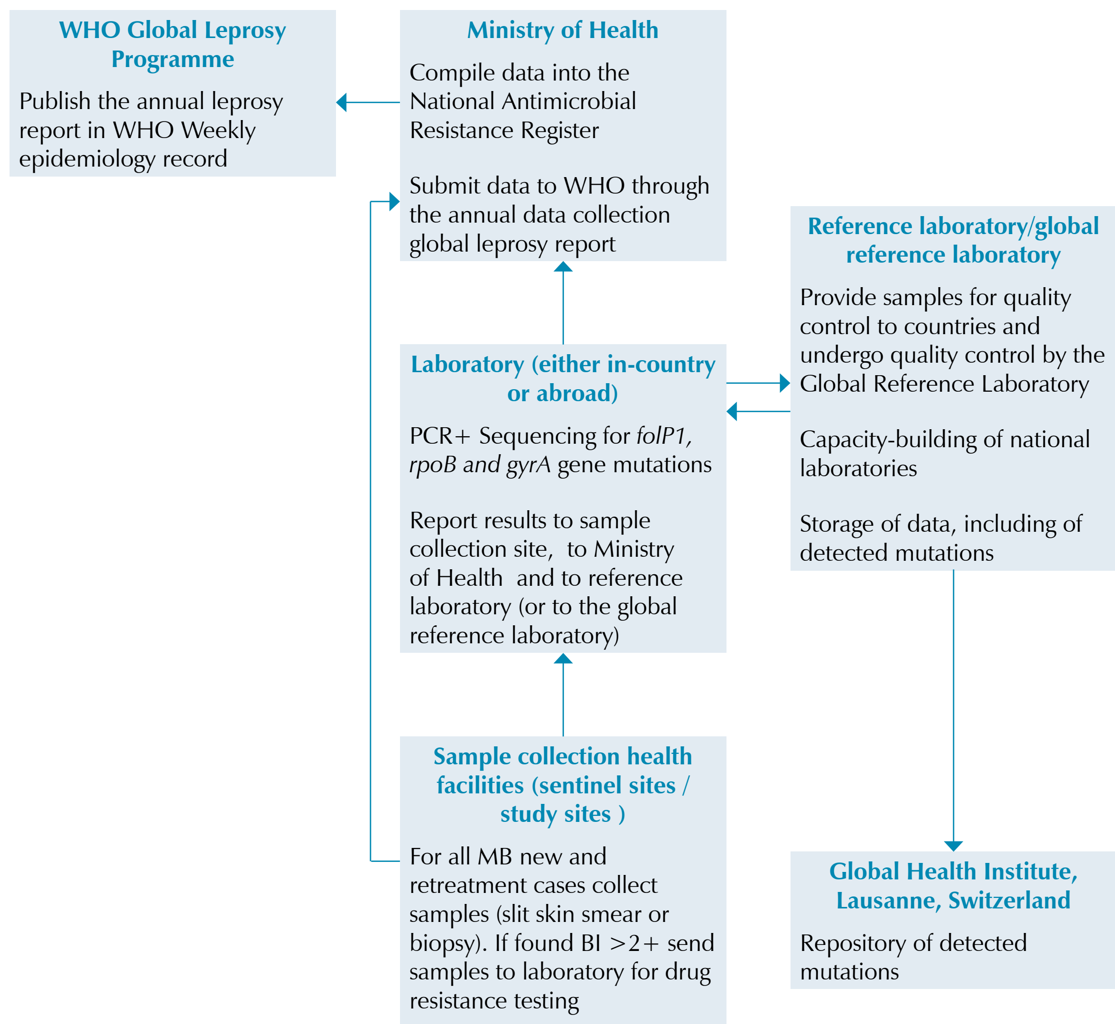
Inclusion criteria. To detect secondary resistance, **all** retreatment leprosy cases have to be tested with the exeption of transferred-in cases unless those cases are deemed to be at risk for AMR due to irregular treatment. In such cases, they shall be defined as "other retreatments" in the testing form. In order to have a positive PCR and successful testing for drug resistance, only MB cases confirmed to be smear positive with a BI >2+ would be tested, as these have a higher chance of a positive PCR.

Design of the system. Surveillance for drug resistance among retreatment cases in leprosy would be carried out by a **continuous surveillance method**, as the testing covers all retreatment cases (with BI >2+) throughout the years. National and international laboratories would be identified for performing the testing. Skin samples (biopsies or skin smears) would be collected from intermediate-level facilities identified for sample collection to test for AMR and transported to the corresponding designated national or international testing laboratory. When samples are shipped by air, IATA regulations should be followed (these can be found at http://apps.who.int/iris/bitstream/10665/254788/1/WHO-WHE-CPI-2017.8-eng.pdf?ua=1). To implement surveillance according to this methodology, all suspect retreatment cases would have to be referred to those specific intermediate-level facilities to confirm their diagnosis and collect samples for AMR surveillance purposes. This would have the indirect benefit of improving the quality of diagnosis for suspected retreatment leprosy cases and potentially also improving the management of reactions, since some of those patients might indeed be leprosy patients who have already completed treatment but still experience immune-based reactions that are extremely risky for developing disabilities and/or worsening an already existing impairment.

Information system. Records of the patients would be kept at the sample collecting facility, at the laboratory and at the national level using a national AMR register (an example is shown in Annex 5.)

5.5 Scheme for surveillance of AMR in leprosy

Fig. 1. Scheme for surveillance of AMR in leprosy



Source: Table developed by Professor E. Cambau, Dr M. Matsuoka and Dr L. Gillini

Setting up or expanding a surveillance system at the national level

6

Several activities are required to set up and/or expand the surveillance system for AMR in leprosy:

(1) identifying the laboratory(ies) to carry out molecular tests for rifampicin, dapsone and ofloxacin resistance. Such laboratories could be located in the country or abroad (one of the four reference laboratories) and could be more than one per country, according to the needs;

(2) identifying a laboratory external to the country, among one of the four international laboratories (for countries testing in national laboratories only). A proportion of the samples would need to be sent periodically to the external laboratory;

(3) identifying the sample collection health facilities to test for drug resistance in new MB cases with a BI ≥2+;

(4) calculating the sample (number of new MB cases to be tested) at each sample collection facility that will have to be tested;

(5) setting up a transportation system for sending samples to the testing laboratory;

(6) systematically collect information related to the patients tested and corresponding results, including treatment outcomes (which could be done by the National Programme, by a national public health/research institute or by one of the national testing laboratories if adequate information technology [IT]/human resources are available);

(7) monitoring the trends in resistance over time and by geographical area, sex, age, category/type of patient and, if possible, by socioeconomic factors.

6.1 Selection of the testing laboratories involved

Current laboratory capacity should be strengthened to conduct surveillance activities for leprosy.

Surveillance for drug resistance among new cases would be an ongoing activity and participating centres – both national and international – would have to take into account the need to maintain the AMR work over a period of time, unless the model adopted is a point-in-time nationwide survey. However, even in the latter case, the work conducted to perform the survey would later on lead to the setting-up of a routine sentinel surveillance system to ensure the availability of data over time or be followed by a new survey after 3–5 years. One national or international laboratory is sufficient for testing in countries with a low burden of leprosy, while countries with a large number of cases annually should identify more

than one laboratory to be able to ensure testing of all retreatment cases and a proportion of new MB cases. The laboratories could be run by the government or an NGO. In case an NGO or international laboratory is identified, the National Leprosy Programme should develop a memorandum of understanding (MoU) to ensure access to resistance data by the Programme. National and international testing laboratories should test samples throughout the year until the indicated number of samples to be tested is reached.

While the practical performance of testing is the duty of the laboratory, ensuring the availability of a network for correct sample collection, transportation, storage of specimens and shipment, and collection and transmission of data to allow monitoring of resistance data are duties of the National Leprosy Programme. The possibility should be explored of establishing testing within either a tuberculosis (TB) laboratory or within the laboratory that acts as a reference for the national agency in charge of AMR when setting up the surveillance system.

6.2 Selection of laboratories for quality control purposes

As part of capacity-building and to ensure the reliability of national data, each country that tests samples at the national level should identify a reference laboratory to perform quality control. National leprosy programmes should develop an MoU to ensure access to services related to quality control. While the practical performance of quality control is a duty of the international reference laboratory, ensuring the regular shipment of samples for quality control purposes, recording and reporting of related information, and storage and revision of the outcomes of quality control activities are duties of the National Leprosy Programme.

Quality control of national laboratories would be conducted under the supervision by the international reference laboratory identified as a partner (Baton Rouge, USA; Tokyo, Japan; Paris, France and Lausanne, Switzerland; *see* Annex 1). To prepare standard material with the support of the international reference laboratory would send representative DNA and bacillary samples (no mutation, known mutation) once a year. In addition, country missions would be conducted by the reference laboratory once every 1–3 years to increase national laboratory capacity and as part of the quality control assessment.

All positive and negative skin specimens from each case are to be archived for quality assurance and further testing. Specimens should be kept frozen in the laboratory for at least 10 years. If necessary, a depository can be organized in a reference laboratory, especially for strains that show new mutations. These should be shared with the reference laboratories.

The international reference laboratories should also be subject to a quality control system once per year through monitoring coordinated by the laboratory of the Leprosy Research Center, National Institute of Infectious Disease, Tokyo, Japan.

While the practical task of complying with the quality control protocols by both the national and international laboratories is their task, ensuring that resources and mechanisms are in place for quality control is a duty of national leprosy programmes.

6.3 Identification of sample collection health facilities

Health facilities are identified for collecting samples of both new and retreatment MB cases to be tested for drug resistance. For convenience, it is suggested that the selected sites should be secondary- and tertiary-level centres where slit-skin smears/biopsies are routinely done to assess/classify patients. This would ensure that good-quality samples are collected and avoid referral of new patients seen at a peripheral centre to a higher level of care solely for antimicrobial surveillance purposes. All suspected retreatment cases should be referred in any case to confirm the diagnosis in a secondary-/tertiary-level facility. For big countries such as India and Brazil, at least one sample collection health facility per district should be identified among all the secondary-/tertiary-level facilities that provide leprosy services be it a government or NGO facility. If necessary, training should be provided for laboratory technicians at district hospitals. The facility at district level should be selected randomly if more than one secondary-/tertiary-level centre is available. For countries with fewer cases, sample collection health facilities should be selected only in big administrative jurisdiction (state/province/region). For such countries, the selection of sample collection health facilites per state/province/region should be done randomly among the secondary-/tertiary-level centres available in that state/province/region.

At each sample collection health facility, the appropriate forms should be filled in to accompany the shipment of samples; patient information should be recorded to allow communication of results and prescription of adequate treatment in case resistance is detected. The sample collection facilities should ask for written consent from each patient (or the parents in the case of a minor) after a brief information session on drug resistance in leprosy.

If one of the health facility selected is an NGO institution/hospital (whether as a sample collection health facility or as testing laboratory), the National Leprosy Programme should develop an MoU to ensure access to services and reporting of information related to AMR.

While the practical task of sample collection and shipment is a duty of each sample collection health facility, ensuring the regular shipment of samples for testing, recording and reporting of related information, and supervision to ensure that standard protocols are followed by all the facilities involved are duties of national leprosy programmes.

6.4 Calculation of sample size

For each sample collection health facilities (a study site in a survey or a sentinel site in a surveillance model), a sample size calculation should be performed as per the formula given in Annex 7.

For countries with no local baseline data on resistance, the sample size for each sample collection health facilities should be determined with the aim of testing at least 10% of the total new MB cases detected in the previous year. For all suspected retreatment cases, a system should be in place to ensure their referral to secondary-/tertiary-level facilities where their samples would be collected if confirmed to be retreatment cases, excluding transferred-in cases with no suspicion of resistance.

6.5 Systematic collection of samples

For all cases that meet the inclusion criteria, skin smear sampling would be done with the patient's consent according to the clinical practice and laboratory processing:

- either two slit skin smear samples of the lesion with a BI ≥2+ should be taken, with the earlobe being the preferred sampling site together with the most prominent skin lesion; or
- one skin biopsy (e.g. 4 mm punch biopsy) should be taken from a prominent lesion with a BI ≥2+.

For new cases, the systematic (routine) collection of samples should continue at each sample collection site until the number of samples to be collected is reached, as defined in the sample size calculation (section 6.4) or until reaching a number corresponding to at least 10% of the MB cases detected in that district-/state-level facility in the previous year.

Necessary patient consent must be obtained as per the ethical guidelines of the country. For patients below 18 years of age, parents' consent should be sought.

6.5.1 Slit skin smear samples

Slit skin smear samples are collected in the same manner as skin smears for BI examination, using a disposable stainless steel blade. Caution should be taken to prevent cross-contamination. The stainless steel blade containing the tissue scrapings should be rinsed into a 1.8 mL centrifuge tube (with screw cap) pre-filled with 1 mL of 70% ethanol (molecular biology grade absolute ethanol at 70% v/v + sterile deionized water from MilliQ or human

injection quality 30% v/v, the mix being prepared in the laboratory), making sure that the tissue scrapings are washed from the surface of the blade and are suspended in the solution. This will mean that from each case, two slit skin smear samples would be collected and labelled according to national practice (some countries use the leprosy registration number). Slit skin samples are also kept on a Whatman® FTA® card. Make as small a smear of the sample as possible on the card. Both materials can be stored at room temperature.

6.5.2 Skin biopsy

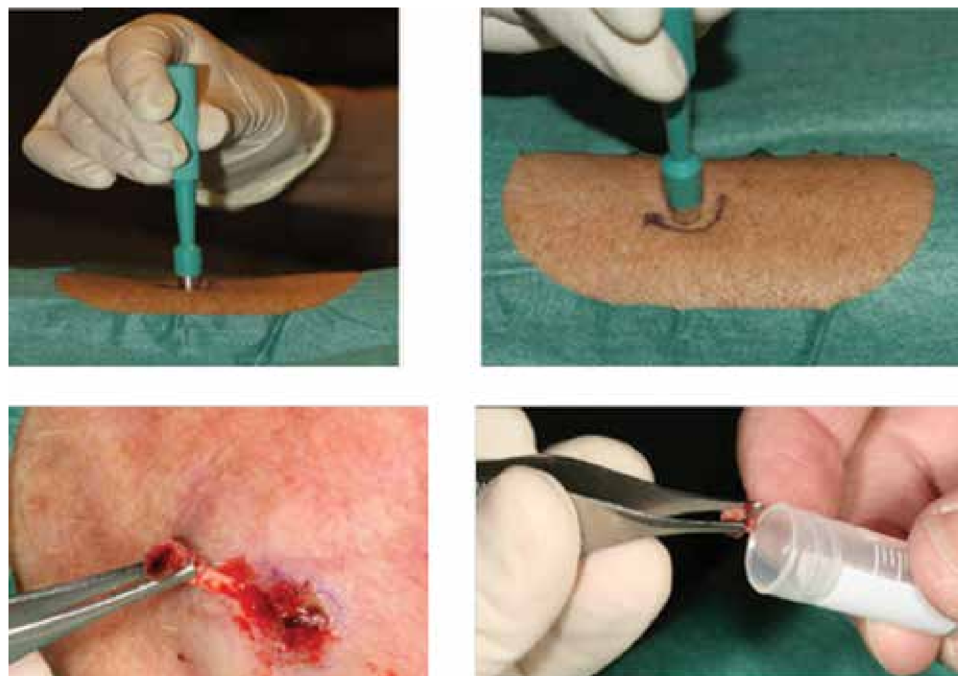
A skin biopsy is collected preferably using a punch of 4 mm for new cases. For retreatment cases, a surgical biopsy of 6 mm is preferred, especially if the BI is close to 2+. The biopsy is then placed in a 1.8 mL centrifuge sterile tube (with screw cap), pre-filled with 1 mL of 70% ethanol (molecular biology grade absolute ethanol at 70% v/v + sterile deionized water from MilliQ or human injection quality 30% v/v, the mix being prepared in the laboratory) as described above. If this cannot be prepared at the health facility, biopsies can stay in an empty 1.8 mL sterile centrifuge tube (with screw cap) without any preservatives.

Samples can be kept at room temperature until they are sent to the laboratory, possibly in batches, depending on the cost of transportation and on the number of samples per month. Bacilli are rapidly inactivated, which means that samples can be sent by routine transport without the need to control the temperature during transportation, or take additional precautions for biohazard control.

For cases in which molecular detection is negative (negative PCR results because of inhibition or a small amount of DNA), it might be necessary to collect additional samples from another lesion. This should be done after discussing with the testing laboratory and on a case-by-case basis.

Fig. 2. Skin smear examination and centrifuge tube with 1 mL of 70% ethanol for collection of tissue specimens

Fig. 3. Skin punch biopsy sampling and tube for transportation
(photo courtesy: Dr Beatrice Flageul)

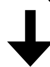


Personnel at the referral facility and sentinel sites should be trained in the proper collection of samples and transportation.

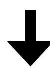
6.6 Laboratory tests

Once the testing laboratory, whether national or international, receives the samples, the following steps would have to be undertaken:

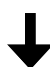
Amplification of drug resistance-determining regions of *rpoB*, *folP1* and *gyrA* genes



Each laboratory applies its own approved method. Sequencing of the amplified PCR products or hybridization with specific probes (the hybridization method can be applied in laboratories without sequencers. Reference laboratories are often using sequencers.)



Distinction between wild-type sequence and verify the presence of amino acid substitution (mutation)



Reporting of results

M. leprae DNA present in skin samples are amplified by PCR and mutations sought by sequencing or hybridization, according to the technique used by the laboratory. Several methods – all showing good performance – can be used. Three techniques are presented as examples in Annex 6: an in-house PCR + sequencing protocol, a commercially available PCR+ hybriditation kit and whole-genome sequencing. In case of negative PCRs for the *folP1*, *rpoB* or *gyrA* genes, it is recommended to perform a PCR targeting another DNA sequence, such as *M. leprae*-specific repetitive element (RLEP) for amplification. This permits assessment for the presence of *M. leprae* DNA in the sample. Whatever the techniques used by the laboratory, it is mandatory to be able to detect mutations known to confer resistance (*see* Fig. 4 and Table 1). Further research will be needed to identify additional mutations that confer resistance. For this reason, part of the scope of the surveillance network is to create a global "bank" of detcted mutations to be managed by the Global Health Institute, Lausanne.

From 1993 onwards, gene sequencing has been done for *M. leprae* strains known to be resistant in mouse footpad experiments and isolated from cases with treatment failure. This clearly showed that some missense mutations (mutations conferring an amino acid change) confer resistance to dapsone (mutation in the *folP1* gene), rifampicin (mutation in the *rpoB* gene) and the quinolones (mutation in the *gyrA* gene). As these mutations are clustered within each respective gene, regions determining drug resistance were described as dapsone resistance-determining region (DRDR), rifampicin resistance-determining region (RRDR) and quinolone resistance-determining region (QRDR) (*see* Bibliography).

The sequencing of the *M. leprae* genome has recently led to modification of the numbering system used previously.

Fig. 4. Nucleotide and amino acid sequences of the drug resistance-determining regions. The codons known to be most frequently involved in resistance are highlighted in rectangles.

```
A)                              folP1 DRDR

151 - gaa tcg |acc| cgg |ccc| ggt qcc att agg acc gat cct cga gtt gaa ctc tct cgt atc gtt
              | 53|     | 55|
 51 -  E   S  | T |  R  | P |  G   A   I   R   T   D   P   R   V   E   L   S   R   I   V

B)
                                rpoB DRDR
1261 - cgt ccg gtg gtc gcc gct atc aag gaa ttc ttc ggc acc agc cag ctg tcg |cag| ttc atg
                                                                         |438|
 421 -  R   P   V   V   A   A   I   K   E   F   F   G   T   S   Q   L   S  | Q |  F   M

1321 - |gat| cag aac aac cct ctg tcg ggc ctg acc |cac| aag cgc cgg ctg |tcg| gcg |ctg| ggc ccg
       |441|                                     |451|                 |456|     |458|
 441 - | D |  Q   N   N   P   L   S   G   L   T  | H |  K   R   R   L  | S |  A  | H |  G   P

C)                              gyrA DRDR

241 - acg atg ggc aat tac cat ccg cac |ggc| gac |gca| tcg att tat gac acg tta gtg cgc atg
                                     | 89|     | 91|
 81 -  T   M   G   N   Y   H   P   H  | G |  D  | A |  S   I   Y   D   T   L   V   R   M
```

Rifampicin binds the β-subunit (encoded by the *rpoB* gene) of the RNA polymerase and certain mutations in the *rpoB* gene lead to rifampicin resistance in *M. leprae* and *M. tuberculosis*. Missense mutations leading to the substitution of any one of the following amino acids (positions 438, 441, 451, 456 and 458) or insertion of nucleotides between position 439 and 440 confers rifampicin resistance on *M. leprae* (Table 1). Mutations observed at other positions (from 432 to 437) should be reported with caution and discussed with a microbiologist from one of the four international reference laboratories.

Table 1. Mutations within antileprosy drug target genes that confer resistance to *Mycobacterium leprae*

Gene	Amino acid substitution due to gene mutation	Nucleotide change (5′ –> 3′)
rpoB	Gln438Val	cag → gtg
	Asp441Tyr	gat → tat
	Asp441Asn	gat → aat
	His451Asp	cac → gac
	His451Tyr	cac → tac
	Ser456Leu	**tcg → ttg**
	Ser456Met	tcg → atg
	Ser456Phe	tcg → ttc
	Ser456Trp	tcg → tgg
	Leu458Val	ctg → gtg
	Leu458Pro	ctg → ccg
folP1	**Thr53Ala**	**acc → gcc**
	Thr53Arg	acc → agc
	Thr53Ile	acc → atc
	Pro55Arg	ccc → cgc
	Pro55Leu	**ccc → ctc**
	Pro55Ser	ccc → tcc
gyrA	Gly89Cys	ggc → tgc
	Ala91Val	**gca → gta**

Note. Mutant amino acids in **bold** *are high-frequency mutations for M. leprae associated with drug resistance. The numbering system is that of the M. leprae genome TN strain (GenBank AL583923; [1])*

Missense mutations in the sulphone resistance-determining region of the *folP1* gene (coding dihydropteroate synthase) and resulting in substitutions of amino acids at positions 53 and 55 confer dapsone resistance to *M. leprae* at an intermediate and high level in the mouse footpad assay.

Correlation between ofloxacin resistance and missense mutation in the *gyrA* gene has been confirmed at positions 91 (Ala91Val) and 89 (Gly89Cys). Mutations involving codons 92 and 95 may be regarded as possibly conferring ofloxacin resistance according to the results of *M. tuberculosis*, but should be reported with caution and discussed with a microbiologist from one of the four international reference laboratories.

After the laboratory tests have been performed, to report on the results, refer to the flowchart below:

Is the specific PCR for drug-resistance genes positive?

If PCR is negative; check if the sample contains enough *M. leprae* DNA (by RLEP PCR or another specific PCR); if the PCRs are all negative, ask for another BI+ sample

If PCR is positive, determine the sequence or hybridize with wild-type and mutated oligonucleotides

– if no mutation: report as susceptible

– if presence of mutation:

– Is it a silent mutation? (it does not change the amino acid encoded)

if yes, do not report

– Is it a missense mutation? (it confers a change/substitution of amino acid)

If yes, report the amino acid substitution with the numbering system as shown in Table 1.

– Is it one of the substitutions that confer drug resistance in leprosy as described in Table1?

If yes, report the strain as resistant.

If no, discuss how to report it with the international reference laboratory.

Send the laboratory report form to the referral facility and to the National Leprosy Programme Manager.

6.7 Systematic recording of information

The results of the testing will be utilized by the sample collection/study/sentinel site facility for appropriate patient management and by the National Leprosy Programme for appropriate monitoring. Therefore, the results of testing from the laboratory need to reach both the facility that is managing the patient and the National Leprosy Programme, to be entered into the **National Leprosy Drug Resistance Register** (Annex 2). In order to achieve this, standard clinical information forms have been developed to record data that will be sent to the reference laboratories and back to the health centres (Annexes 3, 4 and 5). At the end of treatment of cases detected with resistance, a copy of the patient chart should be sent to the National Leprosy Programme to allow recording of the treatment outcomes (Annex 5).

The National Leprosy Programme will have to report to WHO annually on the following:

(1) number of new MB cases tested, and number and proportion of resistant cases (to one drug and to more than one drug);

(2) number of retreatment MB cases tested, and number and proportion of resistant cases (mono- and multi-resistant).

The global data submission results in a publication on leprosy in the WHO *Weekly epidemiological record*, which from 2017 onwards will include data on drug resistance, in line with the importance this aspect has been given in the Global Leprosy Strategy 2016–2020. Globally, some countries are implementing an online platform to report and monitor AMR, which is called the Global Antimicrobial Resistance Surveillance System (GLASS). At the moment, GLP is exploring the possibility of integrating the drug-resistance data for leprosy into the online GLASS platform.

For countries that count on a national public health/research institute and/or with collaborating centres for leprosy with adequate IT/public health tools, including human resources, the data collection and analysis could be done by such a centre on behalf of the National Leprosy Programme. However, even under such an arrangement, the ownership of and responsibility for monitoring trends in drug resistance remains with the National Leprosy Programme.

6.8 Monitoring antimicrobial resistance

National leprosy programmes will have to monitor the trends over time of resistance among new and retreatment cases, along with monitoring the proportion of patients tested. The results of retreatment should be analysed by type of retreatment, while trying to identify

risk factors for resistance such as loss to follow up. Of particular concern would be a rising proportion of cases resistant to rifampicin, especially if these are among new cases, and whether the total new leprosy cases remain stable in number over time. It is suggested to duly compile the drug resistance register to be able to analyse data among different categories and monitor treatment outcomes. This would help to identify risk groups for resistance by age, sex, place of residence and/or socioeconomic factors, and category of patient (for instance, retreatment after loss to follow up), as well as risk factors for treatment failure. For countries with a high burden of leprosy, it is suggested that a national computerized database be established and periodically shared with the reference laboratory to identify more frequent mutations and possible transmission patterns through studies, including whole-genome sequencing done by the laboratory at the École Polytechnique Fédérale de Lausanne.

Countries could also use the database as a tool to periodically conduct operational and scientific research to identify additional potential risk factors such as delay in diagnosis, treatment sought in the private sector, comorbidities, etc. Factors associated with treatment outcomes with second-line drugs could also be identified.

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Annexes

Annex 1

International reference laboratories

National Reference Centre on Mycobacteria and Resistance to Anti-tuberculosis Drugs, Assistance publique-Hôpitaux de Paris (APHP)
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Annex 2

National leprosy drug resistance register

Name												
Surname												
Leprosy register number												
Type of patient (new/ retreatment)												
Type of retreatment												
Date of start of MDT treatment												
Name & location Treating Facility												
Date of sample collection												
Name & Location Facility collecting the sample												
Skin site of sample collection												
BI index slit skin smear in national lab												
Results of national lab												
Results PCR of national lab												
Results PCR reference lab												
Results seq Rifampicin national lab												
Results Seq Rifampicin reference lab												
Results seq Dapsone national lab												
Results Seq Dapsone reference lab												
Results Seq Ofloxacine national lab												
Results Seq Ofloxacine reference lab												
Results Seq Ofloxacine reference lab												
Treatment regimen (for drug- resistant cases)												
Start of treatment (for drug-resistant cases)												
End of treatment (for drug- resistant cases)												
Treatment Outcomes												
Remarks												

Annex 3

Form 1. Clinical report form for MB leprosy cases to be tested for drug resistance

I. **Reporting details**

- Leprosy register number: ______________________________
- Name of country: ___________________________________
- Type of facility reporting:
  - ☐ PHC/other: name and address: ______________________________
  - ☐ Sentinel site: name and address: ______________________________
- Date of report (DD/MM/YYYY): ____/____/_______
- Date of specimen collection (DD/MM/YYYY): ____/____/_______

II. **Demographics and medical history of the case**

- Date of birth: ____________ Sex: M / F
- Patient citizenship or residence: __________________
- Country of origin: _____________________________
- Patient consent to participate: Yes / No (If no, skip following steps)
- Chemoprophylaxis: Yes / No

If yes, which drug was given: _________________________

- Previous TB treatment: Yes / No
- Previous treatment with ofloxacin or other quinolones for longer than one month: Yes / No

III. **Case classification**

- New
- Retreatment:

☐ Retreatment after loss to follow up	☐ Relapse
☐ Transferred in	☐ Other retreatment

IV. Treatment history (for retreatment cases only)

- Treated with MDT, year:
- Treated with rifampicin, dapsone, clofazimine (not in MDT package), year:
- Treated with other medications: specify

V. Clinical presentation at time of referral for testing for resistance

- Clinical features: ____

  ____

  Number of skin lesions: __________

- Skin smear results from specific sites (if any, along with the date of test):

  1. Site: ____________ BI: _______ Date of smear: ___/___/_____

  2. Site: ____________ BI: _______ Date of smear: ___/___/_____

  3. Site: ____________ BI: _______ Date of smear: ___/___/_____

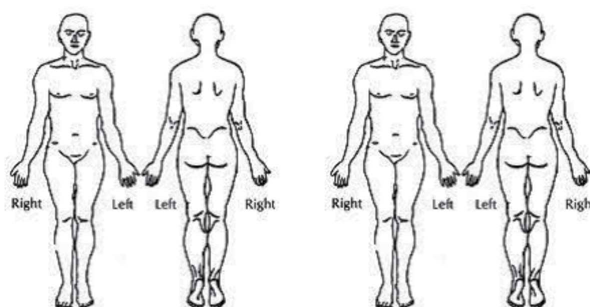
- Disease classification :

  A: (MB/PB)

  B: Ridley–Jopling – (BB/BT/TT/BL/LL)

VI. Type of sample

- Biopsy, number of samples: ___________ Site of collection: ___________
- Skin smear, number of samples: ________ Site of collection: ___________



Annex 4

Form 2. Report of the testing laboratory

Name of testing laboratory: ____

____

Address: ____

____

Telephone: Fax: Email: ________________

Case identification

Case leprosy register number:

Case initials:

Age:

Sex:

Name of institute sending specimen

____

Address: ____

____

Telephone: ________________ Fax: ____________

Email: ____

Type of case:

☐ New

☐ Retreatment

Date of acceptance: _____/_____/________ (dd/mm/yy)

Type of specimen received: slit-skin smear biopsy
(Circle both if more than one type of sample is received)

Sequencing results

***rpoB* gene**

Negative PCR (other PCR tested: __________________)

No mutation (do not report silent mutation)

Presence of a mutation known to confer drug resistance

nucleotide mutation (X to Y at nucleotide Z*) ____________________

amino acid substitution (A to B at position C*) : __________________

Presence of another mutation (A to B at position C*)

***folP1* gene**

Negative PCR (other PCR tested: __________________)

No mutation (do not report silent mutation)

Presence of a mutation known to confer drug resistance

nucleotide mutation (X to Y at nucleotide Z*) ____________________

amino acid substitution (A to B at position C*) : __________________

Presence of another mutation (X to Y at position Z*)

***gyrA* gene**

Negative PCR (other PCR tested: __________________)

No mutation (do not report silent mutation)

Presence of a mutation known to confer drug resistance

nucleotide mutation (X to Y at nucleotide Z*) ____________________

amino acid substitution (A to B at position C*) : __________________

Presence of another mutation (X to Y at position Z*)

*numbering system of *M. leprae* genome strain TN (NCBT accession number AL4550380.1)

Name of corresponding biologist (and signature)

Signature Date of reporting: ___/___/________ (dd/mm/yy)

Note:

The presence or absence of mutations known to confer drug resistance should be reported as:

- "no mutation known to confer resistance" if these mutations are not found and the sequence brings up wild-type codons;
- "presence of mutations known to confer resistance" if at least one of these mutations is observed. The substitution (e.g. Ser456Leu) should be stated on the basis of the numbering system of the *M. leprae* genome from the TN strain (*see* Table 1 and Fig. 4);
- "another missense mutation": this could be a new mutation or a previously described mutation but not known to confer resistance. The mutation should be written with regard to the substitution (e.g. Lys411Asn), with the nucleotide change in brackets (e.g. aaa to aac) on the basis of the numbering system of the *M. leprae* genome from the TN strain (*see* Fig. 4). Corresponding DNA sequences and data concerning the description of new mutations will be also sent to a common database located at Ecole Polytechnique Federale de Lausanne;
- do not report silent mutations, i.e. mutations that do not change the amino acid.

Annex 5

Reporting form for treatment outcomes of resistant cases

Reporting details

- Leprosy register number: ______________
- Initials/ sex: M / F /age (in numbers)
- Type of case: ☐ New ☐ Retreatment (type of retreatment)
- Date of report:__ / __ / ____ (dd/mm/yy)
- Date of treatment completion:__ / __ / ____ (dd/mm/yy)

Type of resistance

- Rifampicin alone
- Dapsone alone
- Ofloxacin alone
- Rifampicin + dapsone
- Rifampicin + dapsone + ofloxacin
- Rifampicin + ofloxacin
- Dapsone + ofloxacin

Treatment prescribed by:

Dr: ______________________________ Name of facility: ________________________

Treatment regimen prescribed

- MB-MDT

  Option A: 400 mg of ofloxacin + 100 mg of minocycline + 50 mg of clofazimine, daily for 6 months followed by 400 mg of ofloxacin + 50 mg of clofazimine for 18 months daily

  or

  Option B: 400 mg of ofloxacin + 100 mg of minocycline + 50 mg of clofazimine, daily for 6 months followed by 100 mg of minocycline + 50 mg of clofazimine daily for 18 months
- Other treatment (Specify): ____

Treatment outcomes

- Treatment completed
- Died
- Lost to follow up
- Transferred out
- Unsatisfactory response to treatment

Annex 6

Laboratory protocols

1. DNA extraction

Protocol A for skin slit smear samples: lysis buffer method (adapted from de Wit 1991; J Clin Microbiol. 1991; 29: 906–10)

- Centrifuge for 10–20 min at max speed (should be more than 15 000 x *g*).
- Discard the supernatant. Resuspend the precipitate in phosphate buffered saline (PBS). Allow to stand for more than 30 min before suspending in PBS.
- Centrifuge for 10–20 min at max speed.
- Resuspend the sediment in 50 μL of lysis buffer*. Overlay mineral oil to prevent evaporation of the buffer.
- Incubate overnight at 60 °C.
- Heat for 10 min at 97 °C to inactivate proteinase K.
- Transfer to a DNA low-binding tube such as Eppendorf product DNA LoBind.

*Lysis buffer

Stock solution I: proteinase K 1 mg/mL in 100 mM Tris-HCl, pH 8.5

Stock solution II: 0.5% Tween 20

Mix 1 volume of stock I and 1 volume of stock II

Dilute 5 times with 100 mM Tris-HCl, pH. 8.5 or distilled water

Usually 100 μL of stock solution I and II are mixed with 800 μL of 100 mM Tris-HCl, pH 8.5 or distilled water.

Protocol B for biopsy sample: freeze–boiling method (adapted from Woods 1989)

- If stored in 70% ethanol, place the tubes open under a safety hood for the ethanol to evaporate, until the biopsy is dry (24–48 h).
- Mince the skin biopsy and grind it with glass beads to obtain a biopsy suspension in Hanks solution or another buffer/sterile water.

- After inactivation at 95°C for 30 min, DNA is extracted as follows: 200 μL of the biopsy suspension is subjected to five series of heat–cold shocks, alternately boiling (1 min at 100°C) and freezing (1 min in dry ice) it. Then incubate at 95°C for 15 min followed by immersion in an ultrasonic bath (Branson) for 15 min if possible.

Protocol C for FTA card sample

- Remove one 3 mm sample disk from the centre of the smear using a biopsy punch.
- Transfer into a 1.5 mL tube.
- Add 500 μL of sterile H2O to the tube and immediately pulse vortex 3 times, for a total of 5 seconds.
- Using a sterile technique, immediately transfer the disc to a 0.5 mL microfuge tube containing 30 μL of sterile H2O. Ensure that the disc is completely immersed in the H2O by briefly centrifuging the tube for 10 seconds.
- Transfer the tube to a heating block at 95 °C for 15–30 min.
- At the end of the incubation period, remove the sample from the block and pulse vortex, or gently tap the sample approximately 60 times.

Protocol D for slit-skin sample by DNeasy blood and tissue (Qiagen)

- Slit-skin samples suspended in 70% ethanol by centrifugation for 10–20 min at max speed (should be more than 15 000 x *g*).
- Discard the supernatant. Resuspend the precipitate in 500 μL of PBS. Allow to stand for more than 30 min and replace ethanol with PBS.
- Centrifuge for 10–20 min at max speed.
- Suspend a sample pellet in 180 μL of enzymatic lysis buffer.
- Incubate for at least 30 min at 37 °C.
- Add 25 μL proteinase K and 200 μL of buffer AL (without ethanol). Mix by vortexing. Incubate at 56 °C for 30 min.
- Add 200 μL of ethanol (96–100%) to the sample, and mix thoroughly by vortexing.
- Pipette the mixture into the DNeasy minispin column placed in a 2 mL collection tube. Centrifuge at >6000 x g for 1 min. Discard the flow-through and collection tube.

- Place the DNeasy minispin column in a new 2 mL collection tube, add 500 μL buffer AW1, and centrifuge for 1 min at >6000 x g for 1 min. Discard the flow-through and collection tube.
- Place the DNeasy minispin column in a new 2 mL collection tube, add 500 μL buffer AW2, and centrifuge for 3 min at 20 000 x g to dry the DNeasy membrane. Discard the flow-through and collection tube.
- Place the DNeasy minispin column in a clean 1.5 mL or 2 mL microcentrifuge tube, and pipette 200 μL buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at >6000 x *g* to elute.

2. PCR sequencing

(a) In-house protocol for PCR sequencing

PCR mixture:
12.5 μL PCR master mix (2X)
0.5 μL Taq polymerase
1.25 μL forward primer at a final concentration 0.5 μM
1.25 μL reverse primer at a final concentration 0.5 μM
5.0–7.5 μL water to final volume 25 μL
2.5–5.0 μL template DNA
Thermal cycle:
Denature at 95 °C for 30 s
Denature at 95 °C for 15 s
Anneal at 57 °C for 15 s 40 cycles
Extend at 72 °C for 60 s
Final extension at 72 °C for 7 min

Primers for PCR

folP1

```
  1 gtgagtttgg cgccagtgca ggttattggg gttttgaacg tcactgacaa ttcgttctca
 61 gatggcggac gttaccttga tcctgacgat gctgtccagc acggcctggc aatggtcgcg
121 gaaggcgcgg cgattgtcga cgtcggtggc gaatcgaccc ggcccggtgc cattaggacc
181 gatcctcgag ttgaactctc tcgtatcgtt cctgtcgtaa aagaacttgc agcacagggg
241 attacagtaa gtatcgatac tacgcgcgct gatgttgcac gggcggcgct gcaaagcggc
301 gcacggatcg tcaacgatgt gtctggtggg cgagcagatc ccgcgatggc tcctctggtg
361 gctgaagccg gtgttgcgtg ggtgttgatg cactggcgac tgatgtcggc tgaacggccg
```

rpoB

```
1201 cgggagcgga tgaccaccca ggacgtcgag gcgatcacgc cgcagacgct gatcaatatc
1261 cgtccggtgg tcgccgctat caaggaattc ttcggcacca gccagctgtc gcagttcatg
1321 gatcagaaca accctctgtc gggcctgacc cacaagcgcc ggctgtcggc gctggggccg
1381 ggtggtttgt cgcgtgagcg tgccgggcta gaggtccgtg acgtgcaccc ttcgcactac
1441 ggccggatgt gcccgatcga gactccggag ggcccgaaca taggtctgat cggttcattg
1501 tcggtgtacg cgcgggtcaa ccccttcggg ttcatcgaaa caccgtaccg caaagtggtt
1561 gacggtgtgg tcagcgacga gatcgaatac ttgaccgctg acgaggaaga ccgccatgtc
```

gyrA

```
  1 atgactgata tcacgctgcc accaggtgac ggttctatac agcgggttga gccggtcgac
 61 attcagcagg aaatgcagcg cagctatatt gattacgcga tgagtgtgat tgtgggccgg
121 gcgttgcctg aagtccgcga tggtctcaaa ccggtacatc gtcgggtctt gtacgcgatg
181 ttagactccg gtttccgccc ggaccgtagc cacgctaagt cagcacggtc agtcgctgag
241 acgatgggca attaccatcc gcacggcgac gcatcgattt atgacacgtt agtgcgcatg
301 gcgcagccgt ggtcgctgcg gtatcccttg gttgatgggc aaggcaattt cggttcgccg
361 ggtaatgacc caccggcagc gatgcgttat tgtgtgtcag gaaattcctt ggtgaggttg
421 ctatttgggg aatcaatacg aatcggtgat atcgttactg gagctcagtt caattcggac
481 aatccgatcg acttgaaggt tcttgatcgg catggtaatc cggttgtagc cgattattta
```

Primers for Nested PCR

folP1

```
  1 gtgagtttgg cgccagtgca ggttattggg gttttgaacg tcactgacaa ttcgttctca
 61 gatggcggac gttaccttga tcctgacgat gctgtccagc acggcctggc aatggtcgcg
121 gaaggcgcgg cgattgtcga cgtcggtggc gaatcgaccc ggcccggtgc cattaggacc
181 gatcctcgag ttgaactctc tcgtatcgtt cctgtcgtaa aagaacttgc agcacagggg
241 attacagtaa gtatcgatac tacgcgcgct gatgttgcac gggcggcgct gcaaagcggc
301 gcacggatcg tcaacgatgt gtctggtggg cgagcagatc ccgcgatggc tcctctggtg
361 gctgaagccg gtgttgcgtg ggtgttgatg cactggcgac tgatgtcggc tgaacggccg
```

rpoB

```
1201 cgggagcgga tgaccaccca ggacgtcgag gcgatcacgc cgcagacgct gatcaatatc
1261 cgtccggtgg tcgccgctat caaggaattc ttcggcacca gccagctgtc gcagttcatg
1321 gatcagaaca accctctgtc gggcctgacc cacaagcgcc ggctgtcggc gctggggccg
1381 ggtggtttgt cgcgtgagcg tgccgggcta gaggtccgtg acgtgcaccc ttcgcactac
1441 ggccggatgt gcccgatcga gactccggag ggcccgaaca taggtctgat cggttcattg
1501 tcggtgtacg cgcgggtcaa ccccttcggg ttcatcgaaa caccgtaccg caaagtggtt
1561 gacggtgtgg tcagcgacga gatcgaatac ttgaccgctg acgaggaaga ccgccatgtc
```

gyrA

```
  1 atgactgata tcacgctgcc accaggtgac ggttctatac agcgggttga gccggtcgac
 61 attcagcagg aaatgcagcg cagctatatt gattacgcga tgagtgtgat tgtgggccgg
121 gcgttgcctg aagtccgcga tggtctcaaa ccggtacatc gtcgggtctt gtacgcgatg
181 ttagactccg gtttccgccc ggaccgtagc cacgctaagt cagcacggtc agtcgctgag
241 acgatgggca attaccatcc gcacggcgac gcatcgattt atgacacgtt agtgcgcatg
301 gcgcagccgt ggtcgctgcg gtatcccttg gttgatgggc aaggcaattt cggttcgccg
361 ggtaatgacc caccggcagc gatgcgttat tgtgtgtcag gaaattcctt ggtgaggttg
421 ctatttgggg aatcaatacg aatcggtgat atcgttactg gagctcagtt caattcggac
481 aatccgatcg acttgaaggt tcttgatcgg catggtaatc cggttgtagc cgattattta
```

Source: Guidelines for global surveillance of drug resistance in leprosy. New Delhi; WHO Regional Office for South-East Asia, 2009 (Drawn by Dr M. Matsuoka)

In-house sequencing reaction protocol

Mix for PCR sequencing reaction

1–3 μL (1–3 ng of 100–200 bp) purified PCR products

20 μL distilled water add up to the total volume

4 μL Bigdye terminator Ver 1.1

2μL 5XSEQ buffer

1 μL 3.3 M primer (same primer used for PCR is diluted 6X)

Thermal cycle reaction

Cycle 1: 96 °C for 30 s

Cycle 2: 96 °C for 10 s

Cycle 3: 50 °C for 10 s

Cycle 4: 60 °C for 3 min

Repeat cycle 2–4 25 times

Purification of sequencing reaction products (e.g. by Performa DTR gel filtration cartridge)

Preparation of samples for sequencing.

Add 25 μL of HI reagents (Applied Biosystems) to the sample tubes to resolve the dried sequencing products. Vortex thoroughly and spin down.

Transfer sample to sample tubes for sequencing.

Heat for 2 min at 95 °C then chill quickly in ice water; spin down.

Ready to apply to sequencer after resuspension in loading buffer.

b) Standardized commercial kit GenoType LepraeDR v1.0 for PCR sequencing (manufactured by Hain Lifesciences, http://www.hain-lifescience.de/en/)

After DNA extraction (*see* protocols above), the procedure is a multiplex amplification of drug resistance-determining regions (DRDRs) in *rpoB, folP1* and *gyrA* with biotinylated primers followed by a reverse hybridization (DNA strip test using PCR amplification (line probe assay).

The kit contains all reagents for multiplex PCR and hybridization, including internal quality controls, but the thermostable HotstartTaq DNA polymerase needs to be provided separately.

The primer nucleotide mix (PNM) contains biotinylated primers for the amplification of specific regions of the bacterial genome. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the

single-stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions that result from the combination of buffer composition and a certain temperature. Thus, the probes reliably discriminate several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye, which becomes visible on the membrane strips as a coloured precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

Each DNA strip includes five control zones:

- a conjugate control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction
- a universal control zone (UC) that detects, as far as is known, all mycobacteria and members of the group of Gram-positive bacteria with a high G+C content
- three locus control zones (rpoB, gyrA and folP1) that check the optimal sensitivity of the reaction for each of the tested gene loci.

Fig. 5. DNA strip of the kit GenoType LepraeDR. (A) Scheme of the strip. (B) Examples of DNA strips

(A) Scheme of the strip and details of probes

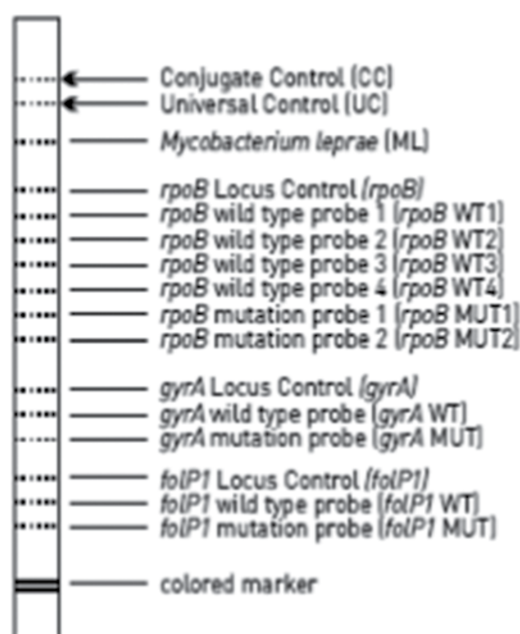


Table 1: Mutations in the gene *rpoB* and corresponding wild type and mutation bands (modified according to Cambau *et al.* 2002, Clin. Infect. Dis. 34: 39-45, Maeda *et al.* 2001, Antimicrob. Agents Chemother. 45: 3635-3639, Scollard *et al.* 2006, Clin. Microbiol. Rev., 19: 338-381)

Failing wild type band	Codons analyzed	Developing mutation band	Mutation
rpoB WT1	429-434		G432S
rpoB WT2	437-442		Q438V
			F439/M440 KF insertion*
			D441N*
			D441Y*
rpoB WT3	449-455	*rpoB* MUT1	H451Y
			H451D
			H451V*
rpoB WT4	455-459	*rpoB* MUT2	S456L
			S456M
			S456F
			S456W*
			L458V*
			L458P*

* These rare mutations have only been detected theoretically (in silico).

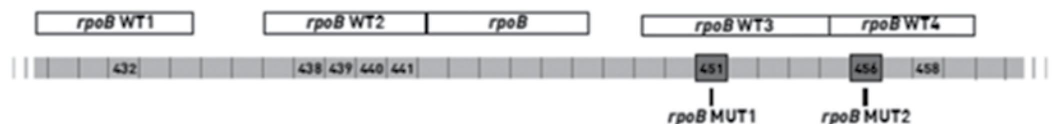


Table 2: Mutations in the gene *gyrA* and corresponding wild type and mutation bands (numeration according to *M. leprae* genome)

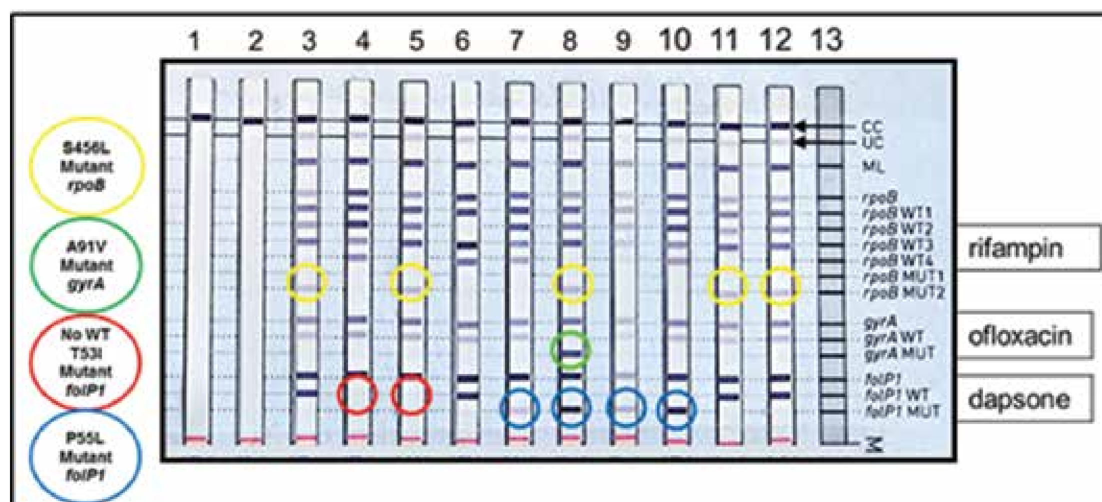
Failing wild type band	Codons analyzed	Developing mutation band	Mutation
gyrA WT	89		G89C*
	91	*gyrA* MUT	A91V

* This rare mutation has only been detected theoretically (in silico).

Table 3: Mutations in the gene *folP1* and corresponding wild type and mutation bands (numeration according to *M. leprae* genome)

Failing wild type band	Codons analyzed	Developing mutation band	Mutation
folP1 WT	53		T53A
			T53R
			T53I
	55	*folP1* MUT	P55L
			P55R

(B) Examples of DNA strips



c) *Whole-genome sequencing to find drug-resistance mutations*

For sequencing purposes, DNA can be extracted from human skin biopsies of known BI using the following customized protocol that removes most of the host DNA, followed by lysis of the bacteria and purification of *M. leprae* DNA on silica columns.

Punch biopsies (6 mm) in 70% ethanol are first rehydrated in 500 μL of Hank's balanced solution and then minced finely with scissors or disrupted by bead beating using 2.8 mm zirconium beads (Bertin Technology, France). Each extraction run could include a batch of 5–9 samples and one blank control. To digest human skin and tissue, the sample is incubated with a mixture of 0.5 U of collagenase and dispase (Roche, Switzerland) for 30 min at 37 °C; released cells are collected in a separate tube and the remaining tissue is digested by incubation with trypsin in PBS (10 mg/mL) at 56 °C until complete digestion. Released cells are pooled and centrifuged for 10 min at 10 000 *g* prior to resuspension in 1 mL of PBS. Cells are lysed with 500 μL of AHL buffer (QIAmp microbiome kit, Qiagen) for 30 min at room temperature with end-over-end rotation. The mixture is then centrifuged for 10 min at 10 000 *g* and resuspended in 190 μL of RDD buffer. Then 2.5 μL of benzonase is added to remove the host DNA (QIAmp microbiome kit, Qiagen) by incubating at 37 °C for 30 min. Next, the mixture is treated with proteinase K (20 mg/mL) at 56 °C for 30 min to inactivate the DNAase. Bacterial cells are then lysed mechanically by bead beating and enzymatically using proteinase K treatment (20 mg/mL) according to the manufacturer's recommendations prior to DNA purification on silica-based columns (QIAmp microbiome kit, Qiagen).

Next, DNA samples are sonicated to obtain 400 bp fragments using an S220 Covaris instrument. Illumina libraries are prepared using the Kapa Hyper Prep kit (Kapa Biosystem, MA, USA) and PentAdpters (Pentabases, Denmark) or Illumina indexed adapters. Fragments are selected before amplification using AMPUre beads with a ratio of 0.5X for the first and 0.2X for the second selection. The concentration of indexed adapters and the number of amplification cycles are adapted with respect to the initial DNA concentration according to the manufacturer's instructions (Kapa Biosystem, MA, USA). After quality control (library concentration and size are determined using a fragment analyser), libraries may be multiplexed and sequenced as 100 base single-end reads on an Illumina HiSeq 2500 instrument (https://www.illumina.com). Raw reads are then adapter- and quality-trimmed using Trimmomatic v0.33 (Bolger et al. 2014) and mapped onto the *M. leprae* TN reference genome (NCBI accession number AL450380.1) with Bowtie2 (version 2.2.5; Langmead and Salzberg 2012) followed by variant calling using VarScan v2,3,9 (Koboldt et al. 2012) or other mapping programs. Single nucleotide polymorphisms (SNPs) can be called after applying quality checks, i.e. a minimum overall coverage of five non-duplicated reads, a minimum of three non-duplicated reads supporting the SNP, a mapping quality score >8, a base quality score >15 and an SNP frequency over 80%. These cut-offs are chosen to avoid false positive results.

Annex 7

Sentinel surveillance protocol

Sample size calculation. The sample size (*n*) will be calculated on the basis of the expected level of resistance as per the formula and related explanations below:

$$n = \frac{N*z^2*(1-g)}{(N-1)*d^2 g+z^2*(1-g)}$$

where:

N = annual average number of new MB leprosy cases registered at the sentinel site in the past five years

Z = z-value (from the standard normal distribution) that corresponds to the desired confidence level (narrowing the confidence interval from 95% to 90% will result in some reductions in sample size; if confidence interval is 95%, then z=1.96 and at 90%, z=1.65);

d = absolute precision (as a decimal 0.01 or 0.02, meaning an error within 1% or 2% of the true proportion);

g = previous estimate of the proportion of new cases with rifampicin resistance x (1 + anticipated change in previous estimate). The anticipated change can be considered as the change that the sentinel system should be able to detect. This change is expressed as a decimal, with a negative prefix (–) if a decrease is anticipated and a positive prefix (+) if an increase is anticipated. For example, a 4% increase from the previous estimate would be expressed as an anticipated change of +0.04; thus *g* = earlier estimate x (1 + 0.04) = earlier estimate x 1.04. Similarly, if a reduction of 10% is anticipated from the previous resistance level in the current year, then *g* = earlier estimate x (1–0.04) or *g* = earlier estimate x (0.96).

It is suggested to double the sample size calculated with the formula. This is done to neutralize the design effect and increase the precision of the sampling scheme (a sample size big enough to detect anticipated changes on a larger scale, considering the uncertainty of the baseline level of resistance and the lack of known trend in the majority of countries).

For countries in which the baseline level of resistance is not known at all, 10% of the total new MB leprosy cases detected per year will be tested either in a national or international laboratory.

Sample selection. After fixing the sample size, the next issue is how to select the sample from the new MB cases. The first step to be taken is to identify the number of endemic districts the sentinel sites serve, and then allocate the number of samples to be selected from each of those districts according to the average number of new MB cases registered in the past five years. After calculating the number of samples to be drawn from each endemic district, samples should be selected by the systematic scheme.

A step-by-step guide is given below:

- Suppose the above-mentioned formula returned a sample size of 20; then, after increasing the sample size say by 50%, i.e. 50% of 20 = 10, the total sample size will be 20 + 10 = 30.
- Further, suppose the sentinel site covers two districts only, A and B, with an average number of new MB cases of 150 and 250 (37.5% and 62.5% of total MB cases), respectively. Now, a total of 30 samples need to be distributed among these two districts proportional to their average number of new MB cases. This implies that from district A, 11 samples need to be tested whereas from district B, 19 samples need to be tested.
- To draw a circular systematic sample from district A, we have to find out the nearest integer k, which can be obtained as below:

$$\text{k} = \frac{average\ new\ MB\ cases\ in\ past\ 5\ years}{number\ of\ samples\ to\ be\ drawn}$$

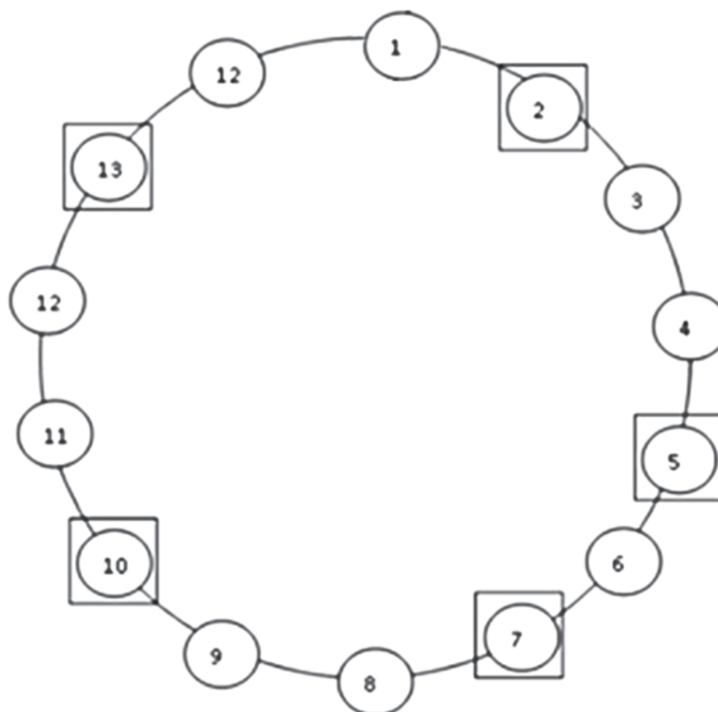
$$= \frac{150}{11}$$

$$= 13.66 = 14 \text{ (approximately)}$$

- Now, select a random number between 1 and 14. Say it is 12; then select every twelfth patient from the list of registered new MB patients in the current year and this will be your first sample from district A; now, select the second sample by adding k (14) to it, i.e. 12 +14 =26, patient serial number 26 will be the second and patient serial number 40 will be the third sample. Similarly, patient serial number 124 will be the ninth and serial number 138 will be the tenth sample. However, now 138 + 14= 152, and there are only 150 patients. Then the eleventh sample will be 152 – 150 =2, i.e. the patient with serial number 2 in the beginning will be the eleventh sample. In the same way, a sample for district B can be drawn.

Another pictorial example of a circular systematic sampling scheme is given with N = 14 and n = 5. Then k = nearest integer. Let the first number selected at random from 1 to

14 be 7. Then, the circular systematic sample consists of units with serial numbers 7, 10, 13, 16 – 14 = 2, 19 – 14 = 5.



Please note that the conclusion drawn about resistance relates only to the areas the sentinel site is covering.

Annex 8

Clinical management of resistant cases detected

All cases included in the surveillance should immediately be put on treatment with standard multidrug therapy (MDT) right after sample collection without waiting for the results from the reference laboratory on the status of drug resistance.

While GLP is in the process of preparing the guidelines on leprosy treatment, including for resistant cases, to date, the only guidance on treatment of resistant cases can be found in the report of the Eighth meeting of the Expert Committee for Leprosy held in Geneva in 2010. As per the report, the following is a guidance on the possible clinical management of resistant cases.

- If the results show a sensitive strain, MB-MDT treatment is to be continued.
- For patients who are reported to be resistant to dapsone only, standard MB-MDT can be continued, but the patient should be followed at the end of treatment and regularly examined for possible relapse.
- For patients with resistance only to quinolones, MDT should be continued.
- In case the reference laboratory reports rifampicin-resistant *M. leprae*, one of the following treatment regimens should be prescribed:

400 mg ofloxacin + 100 mg minocycline + 50 mg clofazimine, daily for 6 months; followed by 400 mg ofloxacin OR 100 mg minocycline + 50 mg clofazimine daily for an additional 18 months.

The above-mentioned treatment regimen is mandatory for patients harbouring both rifampicin- and dapsone-resistant *M. leprae*.

For cases resistant to rifampicin but susceptible to dapsone, the above treatment regimen is also suggested. Alternatively, a treatment regimen that includes dapsone might be discussed with the national referral centre.

It is compulsory to report treatment outcomes of leprosy patients detected with drug-resistant through the form in Annex 5 (either as in hard copy or electronically) and to send it to the National Leprosy Programme so that the information can be entered in the drug resistance register.

The current network for drug-resistance surveillance for leprosy has been led by the World Health Organization (WHO) since 2008 with the financial support of The Nippon Foundation initially and of the American Leprosy Missions and Fondation Raoul Follereau in more recent years. This guide has been prepared by WHO's Global Leprosy Programme (GLP). It expands the scope of surveillance to include also new leprosy cases. The new case definitions of the Monitoring and Evaluation Guide of the Global Leprosy Strategy 2016–2020 have also been incorporated. The antimicrobial surveillance guide promotes a much wider coverage of testing in accordance with the overall importance given to Anti-microbial resistance (AMR) as described in the Global Leprosy Strategy 2016–2020. In 2015 the World Health Assembly (WHA) passed resolution WHA68.20 to address AMR so the need was identified for heightened attention and improved surveillance systems including for leprosy. For these reasons, GLP, in collaboration with key partners and stakeholders, has prepared this guide as an update of the Guidelines for global surveillance of drug resistance in Leprosy (2009).

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