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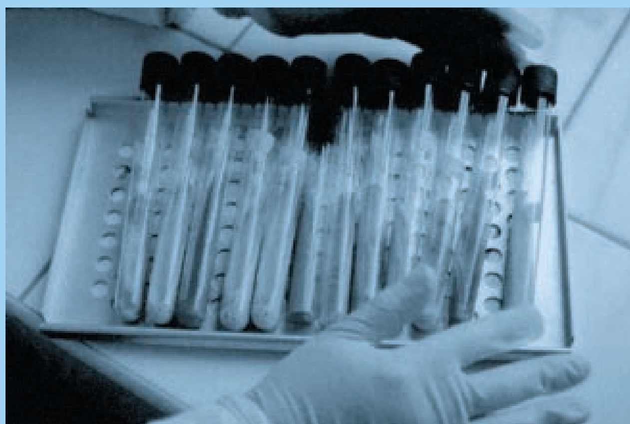
GUIDELINES *for* SURVEILLANCE *of* DRUG RESISTANCE *in* TUBERCULOSIS



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GUIDELINES FOR SURVEILLANCE OF DRUG RESISTANCE IN TUBERCULOSIS

SECOND EDITION

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CONTENTS

ACKNOWLEDGEMENTS 5

INTRODUCTION 7

Causes of anti-tuberculosis drug resistance 7
Establishing a drug resistance surveillance system 8
Choice of drugs 9

DEFINITIONS OF RESISTANCE 11

Acquired resistance to anti-tuberculosis drugs 11
Primary resistance to anti-tuberculosis drugs.......................................12
Importance of distinguishing patients who have been
previously treated from those not previously treated 13

LABORATORIES AND DIAGNOSTIC CENTRES 15

National Reference Laboratory 15
Diagnostic centres 15

SAMPLE SIZE AND SAMPLING STRATEGIES 17

Sample size 17
Sampling strategies 17

ORGANIZATION AND SURVEY OUTLINE 21

Suitable survey areas 21
National coordination team 21
Preparatory phase 21
Quality control of the survey 23

INTAKE OF PATIENTS 25

Inclusion criteria 25
Sputum collection........ 25
Registration........ 25
Collection and transport of sputum samples........ 26

NATIONAL REFERENCE LABORATORY 29

Decontamination 29
Cultures........ 29
Identification........ 30
Susceptibility testing 30
Quality assurance 32
Supranational Reference Laboratory........ 32

DATA MANAGEMENT AND ANALYSIS 35

Data collection 35
Data management 35
Data analysis 35
Interpretation of results 36

REFERENCES 39

ANNEXES

1 Drug resistance survey protocol checklist........ 41
2 Weighted cluster sampling 45
3 Safe shipment of infectious material 49
4 Preparation of media........ 53
5 Proportion method........ 55
6 Sputum shipment - form 1 57
7 Clinical information - form 2........ 59
8 Results of bacteriological examination - form 3........ 63
9 Anti-TB drug resistance results 65
10 Proficiency testing report form........ 67
11 Supranational laboratory list........ 69

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One of the aims of ensuring effective management of tuberculosis (TB) is to minimize the development of drug resistance. Surveillance of anti-TB drug resistance is therefore an essential tool for monitoring the effectiveness of TB control programmes and improving national and global TB control efforts. The use of drug sensitivity tests for monitoring and guiding TB treatment programmes was recommended many years ago *(1-3)*, and information available at the time suggested that there could be high and increasing levels of drug resistance in many parts of the world *(4-8)*.

In 1994, the World Health Organization (WHO) joined forces with the International Union Against Tuberculosis and Lung Disease (IUATLD) and launched the Global Project on Anti-tuberculosis Drug Resistance Surveillance. The aims of the Global Project are to measure the prevalence and monitor the trend of anti-TB drug resistance worldwide using a standardized methodology, and to study the correlation between the level of drug resistance and treatment policies in the different countries. The overall goal of the Project is to improve the performance of national TB control programmes (NTPs) through policy recommendations on drug management *(9-12)*.

These guidelines have been developed to assist NTPs in adopting country-specific anti-TB drug resistance surveillance systems measure susceptibility to the first-line drugs. The standardization of quality assurance and proficiency testing methods, through various centres worldwide that function as Supranational Reference Laboratories (SRLs) *(3)* will ensure that the results are comparable both within and between participating countries.

As the decisions on treatment regimens and programme management can be made only at the country level, prevalence of drug resistance should be monitored primarily at that level.

These guidelines update the 1997 *(13)* edition that was agreed upon in a meeting bringing together world experts in mycobacteriology and TB control. This revision, requested at the IUATLD meeting in Madrid 1999, is based on the lessons learned by the Global Project since 1994.

CAUSES OF ANTI-TUBERCULOSIS DRUG RESISTANCE

Resistance of *Mycobacterium tuberculosis* to anti-TB drugs is a man-made problem. Wild strains of *M. tuberculosis* that have never been exposed to anti-TB drugs are almost never clinically resistant *(5)*.

Exposure to a single drug - as a result of poor adherence to treatment, inappropriate prescription, irregular drug supply, and/or poor drug quality - suppresses the growth of bacilli susceptible to that drug but permits the multiplication of pre-existing drug-resistant organisms. This phenomenon is called ***acquired resistance***. Subsequent transmission of such resistant bacilli to other persons may lead to disease that is drug-resistant from the outset, a phenomenon known as ***primary resistance***.

The emergence of drug-resistant *M. tuberculosis* has been associated with a variety of management, health provider and patient-related factors. In some countries, management

factors may include the lack of availability of a standardized therapeutic regimen, or poor implementation compounded by frequent or prolonged shortages of drug supply in areas with inadequate resources or political instability. Use of anti-TB drugs of unproven quality is an additional concern, as is the sale of such medications over the counter and on the black market. Moreover, incorrect management of individual cases, difficulties in selecting the appropriate chemotherapeutic regimen with the right dosage, and patients' non-adherence to prescribed treatment contribute to the development of drug resistance.

In summary, anti-TB drug resistance results from the absence of a system properly organized to ensure prompt diagnosis and effective treatment within a well implemented TB control strategy.

Data presented in the first and second Global Reports suggest that HIV infection is not an independent risk factor for drug resistance *(9, 10)*. However, in settings with high HIV prevalence, the number of TB cases is increasing dramatically. It is therefore important to take TB/HIV co-infection into consideration when anti-TB drug resistance surveillance (DRS) protocols are being developed and implemented.

ESTABLISHING A DRUG RESISTANCE SURVEILLANCE SYSTEM

Since 1994, several countries have established national anti-TB DRS projects. Those projects adopted a standardized methodology for susceptibility testing with the assistance of the SRLs. Establishing surveillance of drug resistance at the country level requires strict adherence to the following three principles:

1. The sample of specimens should be representative of the TB patients in the country/geographical setting under study and the sample size should be determined to permit standard epidemiological analysis. It is recommended that anti-TB drug resistance surveillance covers the whole country/geographical area and that the sample size is derived from the total number of new sputum-positive cases in this country.
2. The patient's history should be carefully obtained and available medical records reviewed to clearly determine whether the patient has previously received anti-TB drugs. This is essential to distinguish between drug resistance among newly diagnosed cases and drug resistance among previously treated cases.
3. The laboratory methods for anti-TB drug susceptibility testing (DST) should be selected from among those that are internationally recommended. Four DST methods have been standardized and are widely used throughout the world *(15)*:
   - proportion method and its economic and standard variants
   - resistance ratio method
   - absolute concentration method
   - BACTEC 460® radiometric method.

Comparability of data resulting from any of the above four methods is assured by the quality assurance (QA) and proficiency testing performed by the SRL network.

CHOICE OF DRUGS

Four of the six anti-TB drugs used in first-line treatment - isoniazid (H), rifampicin (R), streptomycin (S), and ethambutol (E) - should be tested by all countries adopting these guidelines. These drugs were chosen because they have been, and continue to be, widely used throughout the world, and resistance can be reliably measured by standardized techniques and has been studied for many years. Given the difficulties in standardizing susceptibility testing for pyrazinamide, this drug should not be routinely included in the panel of anti-TB drugs to be tested for surveillance purposes. Guidelines for DST for second line anti-TB drugs are detailed elsewhere *(16)*.

Resistance to each of the four anti-TB drugs routinely tested is defined according to the results of bacteriological testing (see “Susceptibility testing”, page 30). Multidrug resistance (MDR) is defined as resistance to both isoniazid and rifampicin, with or without resistance to other agents.

ACQUIRED RESISTANCE TO ANTI-TUBERCULOSIS DRUGS

Patients diagnosed with TB who start anti-TB treatment and acquire resistance to one or more of the drugs used during the treatment are said to have developed “acquired drug resistance”. This can be ascertained only if the drug susceptibility pattern is determined before the start of treatment, as well as at a later point in treatment or at the end of treatment. Acquired drug resistance is thus a sensitive indicator of combined physician and patient adherence to internationally recommended treatment regimens. Such an approach is only possible in countries with the resources to perform serial susceptibility testing. In most of the world, systematic evaluation is not usually feasible and an alternative approach to estimating acquired resistance needs to be practical.

Proxy for acquired drug resistance: resistance among previously treated patients

Because acquired drug resistance is intimately linked to the use of anti-TB drugs, patients with a history of previous anti-TB treatment lasting at least 1 month should be grouped as “previously treated patients”. This group includes patients in any of the four following categories, which should be reported separately whenever feasible:

- Treatment failure – patients failing anti-TB treatment, i.e. patients who begin treatment for smear-positive pulmonary TB and who remain smear-positive, or become smear-positive again, at 5 months or later during the course of treatment.
- Relapse – patients who become smear-positive again after having been treated for TB and declared cured after the completion of their treatment.
- Return after default – patients who interrupt their treatment for more than 2 months after having received a total of at least 1 month of anti-TB treatment and who then return with bacteriologically confirmed tuberculosis (return after default)
- Chronic – patients who continue to be smear-positive after the completion of a re-treatment regimen.

Definition "Previously treated case"

For the purpose of surveillance, resistance in a previously treated patient is defined as the presence of resistant strains of *M. tuberculosis* in a patient who, in response to direct questioning, admits having been treated for tuberculosis for 1 month or more, or, in countries where adequate documentation is available, in a patient for whom there is evidence of such history.

PRIMARY RESISTANCE TO ANTI-TUBERCULOSIS DRUGS

Patients who are diagnosed with TB and who harbour organisms resistant to one or more anti-TB drugs, but have never been previously treated for TB or have been treated for less than one month, are said to have primary resistance. Primary drug resistance is a theoretical concept, as history of prior anti-TB treatment can never be entirely accurate. "Initial drug resistance" has thus been proposed in reference to patients presenting with an organism resistant to any anti-TB drug before the start of therapy. However, the systematic use of this term may discourage thorough investigation of prior anti-TB treatment history. Given the importance of distinguishing between primary and acquired resistance an approach to assessing primary drug resistance has to be as feasible and as accurate as possible.

Proxy for the prevalence of primary drug resistance: resistance among new cases

Patients should be interviewed in a standardized manner to exclude prior history of up to 1 month of anti-TB treatment. Patients who claim never to have received such treatment (which should be verified by checking TB registers) are said not to have been previously treated.

Definition "New case"

For the purpose of surveillance, resistance among new cases is defined as the presence of resistant strains of *M. tuberculosis* in a patient who, in response to direct questioning, denies having had any prior anti-TB treatment (for more than 1 month), and, in countries where adequate documentation is available, for whom there is no evidence of such history.

IMPORTANCE OF DISTINGUISHING PATIENTS WHO HAVE BEEN PREVIOUSLY TREATED FROM THOSE NOT PREVIOUSLY TREATED

Distinguishing between patients with and without history of at least 1 month of anti-TB treatment not only has implications for classification for the purpose of surveillance of drug resistance, but also has programmatic relevance for decisions on the type of treatment a patient is to receive. In general, it is expected to detect higher prevalence of resistance among previously treated cases than among new cases. Inaccurate classification of patients and/or combining drug resistance data from both categories will be misleading and will make the interpretation of data more difficult.

NATIONAL REFERENCE LABORATORY

The National Reference Laboratory (NRL), which should be the reference institution in the country, prepares cultures from the sputum samples and undertakes the identification of *M. tuberculosis* strains as well as drug susceptibility testing (DST). Basic laboratory equipment and materials must be available and functional in the NRL before the implementation of the surveillance. If there are peripheral culture laboratories, mycobacterial strains, rather than sputum samples, can be sent to the NRL for testing. One of the main tasks of the NRL is to ensure the quality of culture and of DST performed by regional or peripheral units by establishing a regular “on-site” supervision programme for those units, and by providing training in, and quality assurance systems for, the laboratory procedures. External quality assurance programmes, organized by the SRL, will validate the results of susceptibility tests done by the NRL. One NRL should be responsible for the surveillance of not more than 25-50 million people. In countries with larger populations, it is recommended that capacity for cultures and DST at the regional/provincial level be strengthened or that additional NRLs be established.

DIAGNOSTIC CENTRES

Diagnostic centres include all institutions where decisions on the diagnosis are taken and patients suspected of having TB are registered. Most diagnostic centres in control programmes with limited means are small, non-specialized health centres and clinics or outpatient departments of hospitals, operated by the government or by nongovernmental organizations. Private sector institutions and general practitioners are not included unless their activities are based on some agreement with the NTP and they are following national guidelines for diagnosis and treatment.

SAMPLE SIZE

The sampling universe for a survey on the prevalence of anti-TB drug resistance should include all newly registered sputum smear-positive TB patients in the country. The calculation of an appropriate sample size should be based on the following *(17)*:

- the total number of new sputum smear-positive cases detected in the previous year in the country or in the geographical setting to be studied;
- expected prevalence of resistance to rifampicin from available data (in the absence of available data, the best guess of investigators should be used);
- precision should be between 1-2%, while ensuring that obtaining the calculated sample is logistically feasible;
- a confidence interval of 95% should be used for estimated prevalence.

If the cluster sampling method is adopted, the cluster design effect needs to be taken into account, and the calculated sample size therefore needs to be multiplied by 2 *(13)*.

Finally, the calculated sample size needs to be increased by 5-20% to account for expected losses. These include patients diagnosed as smear-positive who do not return to the diagnostic centres and from whom it is not possible to obtain two sputum samples; patients whose culture is contaminated or does not grow; and patients whose susceptibility testing does not give interpretable results (unreadable or too few colonies).

SAMPLING STRATEGIES

Different sampling strategies can be adopted to select a representative sample of TB patients. In order to select a representative group of newly registered patients, a randomization step is essential *(14, 18)*. Simple random sampling of individual patients is not practical in TB diagnostic centres, mainly because routines – usually identical for most patients – would be disrupted and compliance of staff and patients would consequently be low and the quality of data poor. Involving all diagnostic centres can also give rise to logistic problems and high costs. Randomization can take place at the level either of diagnostic centres or possibly of health districts. In this way, routines would be slightly changed for some diagnostic centres, but remain identical for all newly registered smear-positive patients in a particular centre. In countries with significant logistic problems, sentinel site surveillance can be used, although this method, by definition, is not representative of the population under study. Some of the most useful sampling strategies are described below.

100% sampling of diagnostic centres

This sampling method is most suitable for small countries with relatively small numbers of TB diagnostic units, good infrastructure, and facilities for transportation of samples from all

diagnostic centres to the NRL. All eligible patients enrolling at each diagnostic centre within the same limited intake period are included. The functionality of this design is ensured by the inclusion of all diagnostic centres and by the use of the same enrolment period for each of them. Large and small diagnostic centres are equally represented without the need for a complicated sampling method. The intake period is calculated by dividing the sample size by the total number of sputum smear-positive patients per year in the country: for example, if around 7000 eligible patients are diagnosed per year and if a sample size of 600 patients is required, the enrolment period will be 600/7000 = 1/11.6 year, i.e. approximately 1 month. In this case, all consecutive eligible patients enrolled during 1 month in all centres should be included, which provides approximately a 10% sample of newly registered smear-positive patients.

The enrolment could be done either during the same month or on rotation – for example, centres in area 1 during the first month, centres in area 2 during the next month, and so on. In this way, the number of sputum samples sent to the central laboratory for culture and susceptibility testing would be approximately the same each month throughout the year. However, if the total time to complete the study exceeds 1 year, time trends will be more difficult to monitor. This technique of rotation avoids certain disadvantages, such as falsely assumed completeness of the sample, high costs, and overloading of the central laboratory, and affords the opportunity to instruct health centre staff and, where necessary, to correct procedures.

Cluster sampling

Cluster sampling methods are best used in situations where it is logistically difficult to cover the entire area of the country and where the number of TB diagnostic centres is high. With this design, centres are randomly selected, and all sputum smear-positive patients newly registered at these selected centres during a defined period of time are included in the survey. A defined intake period, which should not exceed 12 months, identical for all centres included in the survey, should result in a balanced sample, as centres are represented according to their burden of cases . This allows direct estimation of the prevalence of resistance from the proportion calculated in the sample.

An unbiased estimate of the prevalence of drug resistance necessitates the inclusion of a specified minimum number of centres (i.e. 30) if there is to be a good probability of including centres of different types (clinics, dispensaries and hospitals of different sizes) scattered throughout the country. In situations where the expected intake period will exceed the 12-month limit because of the small number of patients diagnosed in various centres, the number of clusters must be increased to more than 30.

While the main advantage of this technique is its simplicity, the principal disadvantage is the risk of missing the largest diagnostic centres, resulting in an unrepresentative assessment of resistance prevalence despite randomization.

The centres can be selected from a list of all diagnostic centres in the country, either by simple random sampling, assigning a sequential number to each from 1 to *X*, in any order, and selecting at random n numbers between 0 and *X*, or by systematic sampling. For

systematic sampling, a sampling interval should be calculated by dividing the total number of centres *X* by the number *n*. The first centre is selected from the list by finding a number between 0 and *X/n*, the second is found by adding the sampling interval *X/n* to this number, and so on consecutively. If the list of centres is ordered according to centre size (i.e. patient load), systematic sampling from the list would result in a sample stratified by centre size. However, it is always possible to miss some important centres, particularly where centre sizes vary widely and where the total number of centres is not large *(19)*.

Population-proportionate cluster sampling

To avoid the risk of drawing a sample that misses the largest diagnostic centres, a weighted cluster sampling technique can be used. Based on a list of all diagnostic centres with the numbers of newly registered patients per year, a cumulative population list is compiled. Assuming the minimum recommended number of 30 clusters is selected, the total number of patients registered per year in all the centres is divided by 30 to obtain the sampling interval. A random number between 1 and the sampling interval is picked, and this determines the first diagnostic centre on the cumulative list to be selected. The sampling interval is sequentially added to the random number to obtain the remaining clusters from the list. If centres are large, with 2-3 times more patients per year than the average, the sampling interval may well be more than one cluster per diagnostic centre.

To determine the number of patients per cluster, the required total sample size is divided by 30. If there is more than one cluster in a diagnostic centre, the number of clusters needed is multiplied by the size of the cluster to calculate the total number of patients needed from that centre. In all selected diagnostic centres, consecutive patients are included in the survey until the number required for one or more clusters is reached. See Annex 2 for a practical example of how to handle cluster selection and calculation of confidence interval.

Sampling of previously treated cases

Ideally, separate sample sizes should be calculated for new cases and for previously treated cases. However, the number of sputum-positive previously treated cases detected per year is usually small and, in order to achieve a statistically significant sample size, the intake period for previously treated cases may be too long. Instead, the best way to obtain an estimate of the drug resistance level among previously treated cases is to include all detected previously treated cases who arrive at centres during the intake period. This will not provide statistically significant data, but will give a rough estimate of drug resistance among previously treated cases.

Trend monitoring

In most national programmes the monitoring of trends, rather than the results of a single prevalence survey, may be considered the main objective of resistance surveillance. To

observe trends, proper sample size calculation is essential, and the survey on the prevalence of drug resistance as described in these guidelines should be repeated every 3 to 5 years. In some countries, continuous surveillance of anti-TB drug resistance may be considered. However, this is very costly and will depend upon the resources available to NTPs.

ORGANIZATION AND SURVEY OUTLINE *(SEE ANNEX 1)*

SUITABLE SURVEY AREAS

The country, state, or province considered as a survey area should have at least one functioning central culture laboratory linked by mail or messenger with the majority of TB diagnostic centres.

NATIONAL COORDINATION TEAM

A survey on the prevalence of anti-TB drug resistance involves three major operational issues:

- programme management (logistics, training, collection of clinical information, supervision of survey);
- laboratory techniques (drug susceptibility testing, proficiency testing, quality assurance);
- epidemiology (sampling, data entry and analysis).

A national coordination team, including one person from each of these fields, should be established. In general, the coordination team is composed of the head of the NTP and the head of the NRL, or persons designated by them, and an epidemiologist. This team is responsible for the preparation of the survey, for close coordination with the SRL, for supervision and quality assurance during the survey, and for the final collection and reporting of results. The coordinating team will require strong official backing from the authority responsible for health services.

PREPARATORY PHASE

Before the start of the survey, the following issues should be addressed by the principal investigator and the coordinating team: laboratory capacity and proficiency, sampling, training, logistics, supervision, funding, and data management. It is recommended that all technical, administrative, and logistic procedures involved be described in a simple survey manual or protocol, distributed to the health officers participating in the study. The quality of susceptibility testing performed by NRLs is critical. In order to ensure quality, it is recommended that staff from the SRL visit the NRL before the start of the survey and establish proficiency testing for drug susceptibility testing. It is also recommended that staff from the NRL make a supervisory visit to peripheral laboratories before the start of the survey.

Sampling

Once the diagnostic centres participating in the survey are identified by one of the sampling methods described in the previous chapter, a time schedule can be established, taking into account logistics, climatic conditions, and the NRL workload.

Training

Training should focus on the three essential parts of the survey:

- enrolment of patients into the survey;
- obtaining reliable and comparable data on previous treatment; and
- laboratory techniques.

The training activities must be planned carefully and include, if possible, each health worker who will be directly involved in the survey. As far as the intake of patients is concerned, it is strongly advised that the same minimal set of clinical information be collected in every country in order to allow international comparison. However, the coordinating team may adapt the intake form to the needs of their country by adding additional relevant questions. The medical officers/nurses in charge of the intake of patients and of the interviews should be identified in each diagnostic centre involved in the survey and properly instructed. In general, a meeting is an efficient way to inform, train, and motivate the officers involved, helping them to realize the need for reliable and comparable data in a national survey. As far as the peripheral laboratories are concerned, training or refresher courses should focus on preparation and reading of smears, decontamination of sputum samples, storage and transport of samples, and proper registration.

Logistics

Special attention must be given to the transport of the sputum samples in order to minimize the transport time and to prevent breakage and contamination. All the material necessary for the survey – such as sputum containers, forms, and laboratory equipment – should be available in sufficient quantities in each centre before the start of the survey. National and international guidelines on shipment of infectious material must be followed *(see Annex 3)*.

Depending on the local conditions, it could be useful to organize a time-limited pilot trial in a district to test logistics and quality of training, and to identify and solve unexpected problems.

Funding

The required budget must be carefully calculated. Accurate budgeting for all the activities will ensure the smooth running of the survey and avoid any interruption during the implementation. Funds must be available before the survey is started.

National tuberculosis control programmes should consider DRS as an important tool for monitoring programme efficiency and as a means of strengthening the capacity of the NRL to perform DST; NTPs should therefore allocate the necessary funds for such activities when planning the budget. Additionally, NTP managers who suspect a significant MDR-TB problem should use DRS as the prerequisite starting point for a clear description of the problem.

QUALITY CONTROL OF THE SURVEY

Quality control should be organized to detect system errors and to improve compliance with the survey procedures. It should be applied to all essential elements of the survey including:

- the sampling, i.e. the selection of patients included in the study;
- the clinical information, i.e. the distinction between never treated and previously treated patients; and
- the laboratory techniques used at the peripheral level and at the NRL.

Sampling

Patients included in the survey must be selected according to the sampling method chosen by the coordinating team to most accurately represent the population studied. When consecutive patients are to be included, they must be checked against the TB District Register and the TB Laboratory Register.

Clinical information

Classification of patients as never treated and previously treated is critical and has important implications for subsequent data analysis and interpretation. Special efforts are therefore needed during the survey to ensure the reliability of clinical data. First, the collected interview forms should be checked carefully for deficiencies. Second, the reliability of the information recorded should be assessed regularly. Several methods can be used depending on the characteristics of the country. For example, a representative sample of patients who deny any previous history of treatment can be re-interviewed by somebody assigned by the coordinating team; depending on the results of comparing the two sets of data, additional training of interviewers may be necessary. Alternatively, two different medical officers can independently interview each patient.

Laboratory techniques

At the peripheral level, the collection of sputum samples (including sputum quantity and quality), smear examination, and transport of sputum and forms must be carefully supervised. At the NRL, in cooperation with the SRL, a system of internal and external quality assurance of laboratory procedures should be established before the survey is started. Two components of an external quality assurance programme are yearly proficiency testing *(see annex 10)* and QA of a sample of DST survey results.

INCLUSION CRITERIA

A patient is eligible for inclusion in the survey if registered as a sputum smear-positive case (new or previously treated), according to the WHO/IUATLD definitions of smear-positive, during the intake period. Children under the age of 15 years who meet the admission criteria should also be included.

SPUTUM COLLECTION

In addition to the initial sputum sample used for diagnosis, the diagnostic centres selected will send another sputum sample so that the central laboratory (or NRL) will have a total of at least two samples, e.g. two spot samples or a spot and an overnight sample. **Treatment for any period of time will reduce the chance of culture positivity, therefore samples must be obtained before treatment is started.**

REGISTRATION

Each patient meeting the inclusion criteria should be assigned a serial number, which will be recorded on the intake forms. The serial number permits identification at the diagnostic centre in case of a resistant strain or when additional information is required. Three forms must be used – the sputum shipment form, the clinical form, and the laboratory results form.

Sputum shipment form (see Annex 6)

The sputum shipment form includes the following information:

- identification of the patient
- date of collection of the sputum
- result of the smear examination at the NRL.

This form will accompany the sputum sample to the NRL, and a copy will be kept at the diagnostic centre.

Clinical form (see Annex 7)

The main objective of the clinical form is to correctly identify the patient as a new case (never treated or treated in the past for less than 1 month) or as previously treated for TB.

The form consists of four sets of information:

- patient identification
- patient history
- documented data on previous treatment episodes
- final decision on history of previous treatment.

The form in Annex 7 contains a minimal set of information necessary for programme monitoring and for allowing international comparison of essential data. This information should be collected in every survey.

Countries may decide to collect additional information such as country of origin, HIV status, place of previous treatment, etc. In principle, only information that is obtainable, reliable, and useful from a programme perspective should be added, in a way that allows analysis. The denominator must be known for each variable collected. For example, if it is decided to stratify TB patients by country of origin, all patients must be asked to provide that information. If a decision is made to test all patients for HIV antibodies, it is recommended that a detailed protocol be prepared in order to ensure confidentiality and counselling for all patients *(2)*.

To help with the recall of any previous TB treatment and to standardize the questionnaire at the national and international level, a minimal set of questions should be asked of all patients who deny previous treatment (see Annex 7). However, these data do not need to be analysed centrally: their only purpose is to standardize and optimize the interview.

A copy of the clinical form should be sent to the coordinating team, while the original should be kept at the diagnostic centre. It should not be sent to the reference laboratory performing susceptibility testing, as knowledge of previous treatment could bias interpretation of the test.

Laboratory results form (see Annex 8)

The laboratory results form includes the following information:

- patient identification
- results of identification of *M. tuberculosis* in the two sputum samples sent to the NRL
- results of susceptibility testing done on only one sample.

A copy of the form should be sent to the coordinating team and the original should be kept at the NRL.

COLLECTION AND TRANSPORT OF SPUTUM SAMPLES

The correct collection and transportation of samples to the laboratory is essential in ensuring that results are accurate and reliable. Collecting a good sputum sample requires that the patient be given clear instructions. Aerosols containing *M. tuberculosis* may be formed when the patient coughs to produce a sputum specimen. Patients should therefore produce sputum, and not saliva, either outside in the open air or away from other people and not in confined spaces such as a room in the laboratory or toilets.

Sputum should always be treated with care. Suitable containers must be rigid to avoid crushing in transit and must have a watertight, wide-mouthed, screw top to prevent leakage and contamination. Containers should be packed in material that will absorb any leakage caused by accidents. All procedures involving the handling of specimens for culture and drug susceptibility testing should be carried out in a specially designed safety cabinet. Particular care needs to be taken when bottles are being opened, closed, or shaken and when materials are being centrifuged, all of which may lead to the production of infectious aerosols. The transportation of TB cultures presents special risks in the event of accidents or breakage of the container; it is therefore extremely important that the exchange of strains between the NRL and the SRL is carried out according to the regulations outlined in Annex 3.

Before transport, sputum samples are kept in a cool place, preferably a refrigerator at +4°C. For homogenization of the mucus and organic debris and for decontamination on transit, an amount of 0.6% cetylpyridinium bromide (CPB) or 1% cetylpyridinium chloride (CPC), equal to the volume of the sputum, is added if it is likely that the samples may be exposed to room temperature for more than 48 hours between collection and processing in the culture laboratory. The patient’s serial number in the centre’s register and a simple identification for the two successive samples from the same patient, such as A and B, are written *on the container* (not on the lid). The two samples are sent together with the sputum shipment form to the central laboratory. A copy of the form is kept in the patient’s file at the diagnostic centre.

DECONTAMINATION

Decontamination of sputum specimens has two objectives:

- destruction of bacteria other than mycobacteria
- homogenization.

The aim of decontamination is to kill as much of the contaminating flora as possible while harming as few mycobacteria as possible. Theoretically, many different techniques are available, but none of them is ideal.

The CPB/CPC method was proposed as a means of digesting and decontaminating sputum in transit (during transportation from peripheral diagnostic centres to the NRL). Worldwide, however, the preferred technique for achieving decontamination with a final maximum sodium hydroxide concentration of up to 2% (using an equal amount of 4% NaOH stock solution and sample) is that of Petroff *(20)*.

It should be noted that the use of both cetylpyridinium chloride and the Petroff method on the same sputum sample is harmful to the mycobacteria. The Petroff method should not be used for specimens containing CPB/CPC.

CULTURES

Before being processed at the reference laboratory, sputum samples should be kept in a refrigerator at +4 °C, and bacteriological examination should be carried out as soon as possible. However, when a transport medium containing CPC or CPB is used, samples should not be stored in a refrigerator because of the likelihood of crystallization at cool ambient temperature. Once crystallized, CPC/CPB cannot protect samples from contamination and will inhibit the growth of *M. tuberculosis* if transferred onto culture medium. Before inoculation of media, CPC/CPB in the samples should be discarded by centrifugation, which should be carried out without refrigeration to prevent crystallization. Samples other than those placed in transport media are decontaminated and homogenized with an equal volume of 4% NaOH. The samples can be inoculated onto medium after centrifugation, according to the Petroff method, or by a simple method without centrifugation. The centrifugation method is as follows.

The samples are decontaminated and further homogenized, according to the Petroff method. Equivalent volumes of the sample and 4% NaOH solution are mixed in a screw-capped tube, incubated at 37 °C for 15 minutes with occasional shaking, and then centrifuged at 2000–3000g for 15 minutes. Sediment is then neutralized and washed. (The total contact time between NaOH and the sample should not exceed 30 minutes unless the sample is strongly contaminated, in which case the incubation time may be extended by 15 minutes.) The sediment is inoculated on two tubes of Löwenstein–Jensen (LJ) medium and one of egg medium in which glycerol is replaced with sodium pyruvate. This latter medium is used to optimize growth of *M. bovis.* The cultures are incubated at 37 °C for 8 weeks or

until growth of colonies is observed; they are first inspected after 48 hours and then weekly. Each isolate strain is examined for morphology and pigmentation, and the week of appearance of the colonies is noted. If there is no growth by 8 weeks or in case of contamination, the cultures are discarded and the laboratory forms completed accordingly. All positive cultures are kept until retesting at the reference laboratory (NRL or SRL) has been completed or the strain has been excluded from further testing. Ideally they should be stored in a deep-freezer at –20 °C but they can also be kept for some time in the refrigerator at +4 °C.

In a simpler method, NaOH-decontaminated samples are inoculated directly onto acid-buffered medium (such as Ogawa medium) without centrifugation and neutralization. Sputum samples collected for drug resistance surveillance are smear-positive, may contain numerous bacilli, and do not require centrifugation to concentrate the TB bacilli. This method is more prone to contamination than the Petroff method.

IDENTIFICATION

Preliminary identification of the strains will be based on acid-fastness and cord formation. Definitive identification will be based on at least the niacin production test, the nitrate reduction test, and the thiophene carboxylic acid hydrazide (2 mg/l) (TCH) resistance test.[1] Identification using standard nucleic acid probe tests is also acceptable. If colonial morphology is consistent with *M. tuberculosis* complex, only one culture per patient needs to be identified. Mycobacterial strains other than *M. tuberculosis* will not be further considered for the purpose of the survey.

SUSCEPTIBILITY TESTING

It is recommended that participating laboratories use the DST method with which they are most familiar provided that it is one of the four that are internationally recommended – proportion method, absolute concentration method, resistance ratio method, and BACTEC®. This is to eliminate variability due to disruption of routine testing when changing to a new testing procedure.

Indirect susceptibility testing will be performed *on only one isolate for each patient.* Drug susceptibility tests will be performed using any of the four previously mentioned methods. However, most countries employ the simplified variant of the proportion method using LJ medium *(21)* as described below. Resistance is expressed as the percentage of colonies that grow on critical concentrations of the substances, i.e. 0.2 mg/l for isoniazid, 2 mg/l for ethambutol, 4 mg/l for dihydrostreptomycin sulfate, and 40 mg/l for rifampicin if LJ medium has been used. The interpretation will be based on the usual criteria for resistance, i.e. 1%

[1] In some areas such as south India or west Africa, the use of TCH is not advisable since many strains of *M. tuberculosis* and *M. africanum* are susceptible. In such cases TCH should be replaced by nitrate reduction.

for all drugs. The results of the tests are recorded on the laboratory forms, copies of which are collected by the national coordinator for analysis. It is recommended that a medium containing 500 mg/l of *p*-nitrobenzoic acid medium (PNB) is used, to rule out non-tuberculous mycobacteria. All members of *M. tuberculosis* complex are susceptible to PNB *(24).*

Proportion method for DST (simplified variant) (21, 22)

The LJ medium is used for all the resistance tests. Working drug dilutions should be prepared on the day of use; 1 ml of working solution added to 500 ml of LJ medium will yield final drug concentrations equivalent to the different critical concentrations. The medium is distributed in volumes of 6–8 ml in sterile 17 mm x 170 mm screw-capped test-tubes, coagulated at 85 °C for 45 minutes, and allowed to cool at room temperature for 24 hours; the screw caps are then tightened and the tubes stored at 4 °C. The control medium without drugs is prepared at the same time as the drug-containing media. The period of validity of the media stored at 4 °C is 2 months. Annex 5 gives details of the proportion method.

The resistance ratio method

The resistance ratio (RR) method utilizes the ratio of the minimum inhibitory concentration (MIC) for the patients' strain to the MIC of the drug-susceptible reference strain, $H_{37}Rv$, both tested in the same experiment *(23)*. Inclusion of the reference strain in each experiment is not only for quality control but also to standardize the results by taking into account the test variations within certain permissible limits. This feature makes the RR method the most expensive of the three conventional methods that use solid media *(23)*. Reading after 4 weeks of incubation defines "growth" on any slope as the presence of 20 or more colonies, and MIC is defined as the lowest drug concentration in the presence of which the number of colonies is less than 20. The range required for the test strain is determined by the variation in the MIC of $H_{37}Rv$, and by the need to determine a resistance ratio of 2 or less for sensitive strains and a resistance ratio of 8 or more for resistant strains *(21)*.

The absolute concentration method

The absolute concentration method was used originally to determine the MIC of isoniazid and streptomycin by adding a carefully controlled inoculum of *M. tuberculosis* to the control and drug-containing media. Media containing several sequential dilutions of each drug are used, and resistance is indicated by the lowest concentration of the drug that will inhibit growth, i.e. fewer than 20 colonies by the end of 4 weeks *(24)*.

BACTEC 460® radiometric method

The BACTEC® method is an indirect test in liquid medium. The liquid medium for this system – 7H12 broth, currently available in the so-called 12B vials (4.0 ml in each) – contains 7H9 broth base, casein hydrolysate, bovine serum albumin, catalase, and ^{14}C-fatty acid. Consumption of the ^{14}C-substrate by the growing bacteria results in the release of $^{14}CO_2$, the amount of which is expressed by the instrument as a growth index on a scale of 0 to 999 *(23, 25).*

This is a rapid test – DST results are available in about 10 days. The major disadvantages of the BACTEC® system are its high cost and the problems of disposal of a large volume of radioactive material.

QUALITY ASSURANCE

To ensure that results of susceptibility testing are reliable and comparable between different countries, a system of quality assurance is recommended. Susceptibility testing should be quality-controlled on three levels – internal, national, and international.

Internal quality control of susceptibility testing

Susceptibility testing should be performed on the standard $H_{37}Rv$ strain in each new batch of LJ medium and for each drug. It is also recommended that this internal quality control also include a known drug-resistant strain. Standardized procedures should be followed whether the proportion method, BACTEC®, resistance ratio, or other method is used for susceptibility testing and for formulation of media. As a part of internal quality control, the quality of the medium should be controlled for each batch. Drugs added to the medium must be pure drugs obtained from a reputable firm and the percentage of potency must be clearly indicated. Drug dilutions and their addition to the medium should be performed following accepted standards.

International quality control of susceptibility testing

Supranational Reference Laboratory

A SRL is a laboratory belonging to the WHO/IUATLD network *(see annex 11)*, which – at the time of this publication – is composed of 20 SRLs. It guides and advises the national coordinator during the preparation, implementation, and evaluation of the survey. The SRL also ascertains the accuracy of susceptibility test methods used in the NRL, thus allowing comparisons of surveillance data gathered in countries participating in the WHO/IUATLD.

Global Project and facilitating ongoing drug resistance surveillance projects in countries where surveys are carried out. The SRL should preferably be located in the region of the surveyed country – in a neighbouring country or in the same country if a state/province is selected as a survey area. However, this is not a rule, and if there is already an established relationship between an SRL and an NRL, this should be encouraged.

The SRL is familiar with all standard methods of culture and susceptibility testing required for the survey. Experienced laboratory staff must be available to visit the culture laboratories in the survey areas and to train their staff, if required. All SRLs agreed in 1994 and 1997 on the basic procedures of drug resistance surveillance as laid down in these guidelines. They ensure equal standards of susceptibility testing by a system of quality assurance, including proficiency testing, that should be established before any SRL assumes responsibility for supervising an NRL. That is particularly important when the SRL also functions as the central TB laboratory for the country or state/province in which it is located.

Proficiency of drug susceptibility testing within the network is assessed annually. The SRLs are asked to test the susceptibility pattern of the reference strains with their usual methodology and to classify the cultures as resistant or susceptible. The susceptibility results of *M. tuberculosis* strains are compared with a "gold standard" derived from the results obtained by the majority of the laboratories, i.e. a judicial criterion *(26)*.

As a result of the success of the SRL network, requests from laboratories around the world to participate in the network are increasing, and WHO and IUATLD have stimulated the creation of regional sub-networks of laboratories within the global network. For the purpose of proficiency testing, one or more SRLs located in specific geographical regions coordinate the distribution of the strains received from the global coordinating centre (Belgium, SRL) to other laboratories not directly linked with the global coordinating centre *(10).*

International quality control of susceptibility testing should be effected by exchanging samples of *M. tuberculosis* in two directions: from the SRL to the NRL, and from the NRL to the SRL.

- *From the SRL to the NRL (proficiency testing).* It is recommended that the SRL send a panel of coded strains to the NRL for retesting. The retest results from the NRL should be compared with the coded results at the SRL. The procedure should be double-blinded. The minimum required agreement should be defined for each drug and should be higher than 90% for isoniazid and rifampicin. Sensitivity, specificity, and reproducibility of susceptibility testing are calculated for each SRL and for each of the four drugs tested.

QUALITY ASSURANCE INDICATORS FOR DRUG SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* IN THE WHO/IUATLD SUPRANATIONAL REFERENCE LABORATORY NETWORK	
Sensitivity	Ability to detect true resistance
Specificity	Ability to detect true susceptibility
Efficiency or Accuracy	Ratio between the number of correct results and the total number of results
Predictive value for resistance	The rate of true resistance to total resistance
Predictive value for susceptibility	The rate of true susceptibility to total susceptibility
Reproducibility or reliability	Intra-laboratory agreement between duplicate cultures expressed as a percentage

Proficiency testing must be completed with good results before the survey is implemented. Similar methodology can be applied for external quality control from the NRL to regional laboratories in countries where regional laboratories are also performing susceptibility testing.

- *From the NRL to the SRL (quality assurance of survey results).* A sample of the strains isolated during the survey should be sent to the SRL to be retested. The results should be compared for agreement with respect to each drug. It is recommended that not less than 10% of the total sample of strains be included. In case of low numbers of resistant strains, it is recommended that 50–100% of resistant strains and 10–20% of susceptible strains be sent to the SRL. The DST method and percentage must be agreed upon in advance by the NRL and the SRL, and a schedule for the strain exchange should be arranged. This part of the international quality control can be performed either during the survey or when the survey is completed.

DATA COLLECTION

At regular intervals (not exceeding 2–3 months) during the intake period the coordinating team should tabulate all data produced by the diagnostic centres and the central laboratory. The national coordinator will make regular reports, based on these tables, to the managers of the NTP and the NRL. These reports should include information on field work, such as enrolment of patients, quality of clinical information collected, transport or logistic problems, and contamination of samples. If the data or comments suggest that a significant problem has occurred, the national coordinator and the managers of the NTP and NRL should analyse the situation and develop a plan of action.

About halfway through the survey, the national coordinator and the managers of the NTP and the NRL should meet to discuss the quality of data collection, laboratory procedures, quality control results, and preliminary survey results.

DATA MANAGEMENT

A simple software programme – Surveillance of Drug Resistance in Tuberculosis (SDRTB) – has been produced by WHO for entering and analysing data from drug resistance surveys. The third version (SDRTB3) is based on Epi-Info and runs on DOS; and the newly available fourth version (SDRTB4) runs on Windows. SDRTB is simple and flexible: a programmed analysis can be run easily and summary tables produced with the prevalence of drug resistance for each drug and cumulative drugs. All SDRTB software is available free of charge from WHO.

To ensure accuracy, data should be entered twice, preferably by different people, and the two databases compared. This can be done easily using the "Validate" option in SDRTB3.

DATA ANALYSIS

To calculate the prevalence of drug resistance, the denominator is the number of cases for which drug susceptibility results are available. However, it is also important to report the number of results missing as a result, for example, of contamination, negative cultures, or insufficient growth for susceptibility testing. The following indicators should be included:

- *Analysis of patient intake.* It is useful to make a table comparing the number of patients included from each diagnostic centre with the expected number based on the sampling method.
- *Analysis of drug resistance patterns.* A table describing the proportion of patients with resistance to individual drugs, and to different combinations of drugs, among patients classified as newly diagnosed and those

classified as previously treated patients is essential. The presentation of data is based on mutually exclusive categories of resistance (mono resistance and combined resistance). The proper tabulation is shown in Annex 9. If appropriate, further comparisons based on age, sex, HIV status, country of origin, type of re-treatment case, etc. can also be made.

From a public health point of view, the extent of current transmission of drug-resistant strains is important. Young people are more likely than older people to have been recently infected. The prevalence of drug resistance in young age groups, therefore, provides more reliable information on recent patterns of transmission of drug-resistant TB. For the same reason, assessment of trends in drug resistance is more informative than data collected in a single survey. Surveys should be systematically carried out at regular intervals, e.g. 3–5 years, using similar methodologies, to monitor trends in drug resistance.

INTERPRETATION OF RESULTS

Interpretation of results of a survey on the prevalence of anti-TB drug resistance depends on local programmatic and epidemiological circumstances. The key indicators of programme performance are the levels of resistance among new and previously treated cases. High levels of resistance may be a serious threat to TB control and must be treated as such by the NTP.

Resistance levels among newly diagnosed cases provide an indicator of the quality and performance of an NTP over many years. An established NTP that adopts standardized chemotherapy and an effective control programme will see a subsequent reduction in primary drug resistance *(27)*. High levels of primary resistance may also indicate that some previously treated patients have been misclassified as new cases.

High levels of resistance in previously treated cases indicate poor programme performance. Even in the absence of results of cohort analysis, corrective action to improve cure rates may be required. Various factors promote acquired resistance, including unsupervised treatment, inadequate drug regimens, free availability of anti-TB drugs on the market, and poor quality of the drugs supplied.

In all situations in which high levels of drug resistance occur, reorganization of the programme, with an emphasis on strict adherence to recommended regimens and supervision of treatment, is urgently needed.

Use of data for routine management of cases

Data derived from the survey are intended primarily for surveillance activities and not for use in individual case management. Using these data for case management may be difficult for two primary reasons: drug susceptibility results may be delayed due to survey methodology, and communication of results is designed for a survey rather than for routine

purposes. Health care providers should therefore follow the policy established by the NTP for treatment of TB patients and for the use of susceptibility testing in routine case management.

Persons responsible for anti-TB drug resistance surveillance at the country level must report identified cases of MDR-TB to NTP managers. It is then the responsibility of the NTP to provide the best treatment available according to existing national treatment policies.

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DRUG RESISTANCE SURVEY PROTOCOL CHECKLIST

In developing a DRS protocol (and eventually a proposal for a grant), the following points should be included. (It should be noted that the information outlined below constitutes the minimum required information for a DRS protocol. The national programme is free to add any other relevant information judged necessary.)

1 Introduction and background

- Country profile, i.e. geography, population, etc.
- Epidemiological TB situation in the country.
- Information about the NTP, i.e. strategy, operational design, drug regimens used.
- Information about the NRL and the laboratory network in the country, with connections existing already with the SRL network.
- A summary of data from the previous cohort analysis (including case-finding and treatment outcome data).
- Data from previous DRS, if available.

2 Objectives

The objectives must be clearly specified in the form of bullet points, for example to:

- determine the levels and pattern of resistance to first-line anti-TB drugs among "newly diagnosed" sputum-positive cases in the country/geographical setting;
- estimate the levels and pattern of resistance to first-line anti-TB drug among "previously treated cases" in the country/geographical setting,
- establish the foundation for routine surveillance of drug resistance in order to observe trends over time.

3 Materials and methods

- Survey design and sampling strategy. The design chosen for the sample size of the survey has to be clearly described. The most commonly selected strategies are:
  - 100% sampling of diagnostic centres
  - cluster sampling
  - population proportionate cluster sampling (weighted cluster).

- Sample size. The statistical basis for the calculation of the sample size must be detailed, and the sample size and expected duration of the survey should be mentioned.

4 Intake of patients and logistics

Detail:

- intake period (especially if rotating)
- inclusion criteria
- sputum collection
- recording forms
  - sputum shipment form
  - clinical form
  - laboratory results form.

- Transportation of sputum/culture samples to regional or reference laboratory and other logistics.

5 Laboratory methods

Four DST methods have been standardized and are widely used throughout the world to measure anti-TB drug resistance:

- proportion method
- resistance ratio method
- absolute concentration method
- BACTEC 460® radiometric method.

It is recommended that participating laboratories use the DST method with which they are most familiar. This is to eliminate variability due to disruption of routine testing through changing to a new testing procedure. The chosen DST method must be detailed and explained step-by-step.

6 Training

The training plan for the staff should be detailed with regard to responsibilities, timing, location, etc.

7 Quality assurance

- *Internal QA of susceptibility testing:* should be performed on the standard strain $H_{37}Rv$ for each new batch of LJ medium and for each drug.
- *International QA of susceptibility testing and proficiency testing:*
  - from SRL to NRL (proficiency testing)
  - from NRL to SRL (QA of survey results).

It is recommended that this section be developed in collaboration with the chosen SRL.

8 Data management and analysis

- Data collection
- Data entry, possibly using SDRTB3/SDRTB4
- Data analysis

9 Resources needed (human and financial)

The coordinating team and the principal investigator should be identified. The budget needed to implement the above mentioned activities must be detailed.

CLUSTER SELECTION

Example. A sample size of 360 TB patients has been calculated after taking into account the effect of cluster sampling: 30 clusters of 360/30 = 12 patients will have to be selected. The following steps must be taken:

a. Establish the list of the diagnostic centres with their annual number of patients (see table below).

b. Calculate the cumulative number of patients and record them in an additional column. Cumulative number for second centre will be (number in first centre) + (number in second centre). Cumulative number for third centre will be (cumulative number for second centre) + (number in third centre) and so on. The total number of patients diagnosed in the country is 6322.

c. Determine the sampling interval: 6322/30 = **211**.

d. Select a number between 0 and 211 at random (using a table of random numbers or the last digits of a currency note, for example). In this case the number selected is **120**.

e. The first cluster is selected using this number **120**: it will be in the first centre because 120 falls between 0 and 246 (number of patients in the first centre).

f. Selection of the next clusters is done by adding the sampling interval 211 each time to this first number 120. The next number (120 + 211) = **331** falls between 246 and 1823 (cumulative number of patients for second centre); the second cluster is therefore selected in the second centre. The third number (331 + 211) = **542** also falls between 246 and 1823; the third cluster is therefore also selected in the second centre.

NAME OF DIAGNOSTIC CENTRE	NO. OF PATIENTS DIAGNOSED PER YEAR	CUMULATIVE NO. OF PATIENTS	CLUSTER NO.
A	246	246	1
B	1577	1823	2, 3, 4, 5, 6, 7, 8, 9
C	468	2291	10, 11
D	340	2631	12
E	220	2851	13
F	246	3097	14, 15
G	190	3287	16
H	1124	4411	17, 18, 19, 20, 21
I	61	4472	
J	154	4626	22
K	139	4765	23
K	60	4825	
M	14	4839	
N	38	4877	
O	19	4896	
P	41	4937	
Q	120	5057	24
R	455	5512	25, 26
S	51	5563	
T	26	5589	
U	199	5788	27
V	21	5809	
W	32	5841	28
X	69	5910	
Y	6	5916	
Z	145	6061	29
AA	129	6190	
BB	87	6277	30
CC	10	6287	
DD	35	6322	

Note: Reproduced from: ten Dam HG. Surveillance of tuberculosis by means of tuberculin surveys. Geneva, World Health Organization, 1985 (document WHO/TB/85.145).

CONFIDENCE INTERVAL CALCULATION

If cluster selection is done with probability proportional to size (method described above) and if clusters have the same size, a simplified formula for the confidence interval (CI) around the drug resistance prevalence is:

$$CI = \pm 1.96 \sqrt{\frac{\sum_i (P_i - P)^2}{n(n-1)}}$$

where P is the prevalence calculated for the total sample,
P_i is the prevalence calculated in each cluster i
n is the number of clusters (30).

To calculate the sum of the $(P_i - P)^2$ over all 30 clusters the following table can be used:

CLUSTER NO.	P_i	$P_i - P$	$(P_i - P)^2$
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			

Note: Reproduced from: ten Dam HG. Surveillance of tuberculosis by means of tuberculin surveys. Geneva, World Health Organization, 1985 (document WHO/TB/85.145).

The total of the last column can then be used in the formula.

For international quality control of susceptibility testing, cultures have to be exchanged between the NRLs and the SRLs. Cultures of *M. tuberculosis* are enriched infectious material containing great numbers of viable organisms that can cause disease in humans. The hazard is compounded when cultures of resistant strains are transported.

Some international organizations, such as the Universal Postal Union, the International Civil Aviation Organization, and the International Air Transport Association, have developed guidelines and procedures designed to facilitate the safe and expeditious shipment of infectious substances while at the same time ensuring the safety of transport personnel and the general public *(1)*. These organizations have also developed agreed common definitions, and packaging and labelling requirements *(2, 3)*. Information on the documentation requirements should be obtained from the appropriate national authorities of the country where the cultures are sent.

Infectious substances and diagnostic specimens likely to contain infectious substances require triple packaging in accordance with the recommendations of the United Nations *(4)*. Cultures of mycobacteria should be shipped on solid medium in screw-cap tubes or freeze-dried in vials as primary watertight containers. Petri-dish cultures and cultures in liquid medium must not be shipped. The primary container should be entirely surrounded by at least 2 cm of absorptive material and enclosed in a second, durable watertight container. The tissue paper or cellulose wadding in the secondary container must be sufficient to absorb all of the fluid in the specimen in case of leakage of the primary container. Several primary containers may be enclosed in a single secondary container, if the total volume of all the primary containers does not exceed 50 ml and there is no contact between them *(3)*. Each set of primary and secondary containers should be enclosed in an outer shipping container made of corrugated fibre board, cardboard, wood or other material of equivalent strength.

One copy of the request forms, letters and other information that identifies or describes the specimen should be taped to the outside of the secondary container. Another copy should be sent by airmail to the receiving laboratory and a third retained by the sender. The outer container must bear the infectious substance (biohazard) label. The label should be about 10 cm large and printed in red on a white background. In addition to the sender's and recipient's addresses, the telephone numbers and fax numbers if available should also be put on the outside of the package.

Compliance with the shipment requirements is the responsibility of the shipper, who must be familiar with the regulations. Failure to comply may result in fines and other penalties. Hand carriage of infectious substances is strictly prohibited by international air carriers, as is the use of diplomatic pouches *(2)*.

Note 1: This diagram shows just one example of an acceptable packaging system. Several primary containers (cryovials) may be enclosed in a single secondary container, if the total volume of all the primary containers does not exceed 50 ml and there is no contact between them.
Note 2: This diagram is reproduced from pg. 108 of reference 2 with permission from the International Union Against TB and Lung Disease.

REFERENCES

1. *Infectious substances shipping guidelines,* 3rd ed. Montreal, International Air Transport Association, 2002:9052–9102.

2. *Guidelines for the safe transport of infectious substances and diagnostic specimens,* Geneva, World Health Organization, 1997 (document WHO/EMC/97.3).

3. Kent PT, Kubica GP. *Public health mycobacteriology: a guide for the level III laboratory*. Atlanta, GA, U.S. Department of Health and Human Services, Centers for Diseases Prevention and Control, 1985.

4. *Recommendations on the transport of dangerous goods. Model regulations (twelfth revised edition).* New York, United Nations, 2002 (ST/SG/AC.10/1/Rev.12).

LÖWENSTEIN–JENSEN MEDIUM[1]

Löwenstein–Jensen is the most widely used solid medium for TB culture. The modification introduced by the IUATLD is recommended and is described in detail. LJ medium containing glycerol favours the growth of *M. tuberculosis* while replacement of glycerol by sodium pyruvate enhances the growth of *M. bovis* and *M. africanum.*

Mineral salt base solution

- Potassium dihydrogen phosphate anhydrous (KH_2PO_4) 2.4 g
- Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) 0.24 g
- Magnesium citrate 0.6 g
- Aspargine 3.6 g
- Glycerol (reagent grade) 12 ml
- Distilled water 600 ml

Dissolve the ingredients, in order, in the distilled water by heating. Autoclave at 121 °C for 30 minutes to sterilize. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

Malachite green solution

- Malachite green dye 2.0 g
- Sterile distilled water 100 ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing in the incubator for 1–2 hours. This solution will not store indefinitely. If precipitation occurs or the solution becomes less deeply coloured, discard and prepare a fresh solution.

Homogenized whole eggs

Fresh hens' eggs (not more than 7 days old), from hens that have *not* been fed antibiotic-containing feed, are cleaned by scrubbing thoroughly with a brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution, then rinse them thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs, scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

Preparation of medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well :

- Mineral salt solution 600 ml
- Malachite green solution 20 ml
- Homogenized eggs (20–25 eggs, depending on size) 1000 ml

The complete egg medium is distributed in 6–8-ml volumes in sterile 14-ml or 28-ml McCartney bottles or in 10-ml volumes in 20 x 150 mm screw-capped test-tubes, and the

[1] This section is based on *Laboratory services in tuberculosis control. Part III: Culture.* Geneva, World Health Organization, 1998 (document WHO/TB/98.258).

tops are securely fastened. Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Coagulation of medium

Before loading, preheat the inspissator to 85 °C. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 85 °C. (Since the medium has been prepared with sterile precautions, this heating is to solidify the medium, not to sterilize it. Inspissation time begins when the inspissation chamber reaches 85 °C).

The quality of egg media deteriorates when coagulation is carried out at too high a temperature or for too long. Discoloration of the coagulated medium may be due to excessive temperature. The appearance of small holes or bubbles on the surface of the medium also indicates faulty coagulation procedures. Poor quality media should be discarded.

Sterility check

After inspissation, the whole media batch or a representative sample of culture bottles should be incubated at 35–37 °C for 24 hours as a sterility check .

Storage

The LJ medium should be dated and stored in the refrigerator and can be kept for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ should not be older than 4 weeks.

MODIFIED OGAWA MEDIUM

Modified Ogawa medium is used for the culturing of mycobacteria but not for drug susceptibility testing.

Formula

Base solution

- Potassium dihydrogen phosphate anhydrous (KH2PO4) 10.0 g
- Magnesium citrate 0.5 g
- Sodium glutamate 2.5 g
- Glycerol (reagent grade) 20 ml
- Distilled water 500 ml
- 2% Malachite green solution 20 ml
- Egg homogenate 1000ml

Preparation of medium

The procedure for preparing the medium is the same as for L-J medium.

PROPORTION METHOD[1]

Only one concentration per drug is used. The concentrations are as follows:

isoniazid	0.2	μg/ml
streptomycin	4	μg/ml

(dihydrostreptomycin sulfate, at a concentration corresponding to 4 mg/ml base)

rifampicin	40	μg/ml
ethambutol	2	μg/ml

INOCULUM

With a spatula, a representative sample of 5–10 mg is taken from the primary culture and placed in a spherical, flat-bottomed flask containing 30 glass beads of diameter 3 mm. The flask is shaken for 20–30 seconds; 5 ml of distilled water are added slowly under continuous shaking. The opacity of the bacterial suspension is then adjusted by the addition of distilled water to a standard suspension containing 1 mg/ml of tubercle bacilli (or BCG). Either a calibrated loop or pipettes may be used for further processing and for inoculation.

Loop

The loop should be of platinum wire (diameter 0.7 mm) and should have an internal diameter of 3 mm, which delivers 0.01 ml. (Delivery volume must be verified by weighing 10 loopfuls of distilled water deposited on a filter paper.) The two bacterial dilutions required for inoculation with the loop are 10^{-2} mg/ml and 10^{-4} mg/ml; the two inocula are respectively
10^{-4} mg and 10^{-6} mg of bacilli for each slope. The dilution 10^{-2} mg/ml is produced by discharging two loopfuls of the bacterial suspension, standardized at 1 mg/ml, into a small tube containing 2 ml of distilled water, and shaking. Similarly, the dilution 10^{-4} mg/ml is produced by discharging two loopfuls of the dilution 10^{-2} mg/ml into a small tube containing 2 ml of distilled water, and shaking. Two slopes of medium without drug and two slopes of medium with drug are inoculated with a loopful of each dilution.

Pipettes

If pipettes are used, the inoculum for each slope is 0.1 ml. Accordingly, the two bacterial dilutions required for inoculation with the pipette are 10^{-3} mg/ml and 10^{-5} mg/ml; again, the two inocula are 10^{-4} mg and 10^{-6} mg of bacilli, respectively, for each slope. The dilutions are prepared by 10-fold dilution steps (0.5 ml of the bacterial suspension 1 mg/ml, discharged into 4.5 ml of distilled water, produces the dilution 10–1 mg/ml, discharged into 4.5 ml of distilled water, produces the dilution 10^{-2} mg/ml, etc., down to 10^{-5} mg/ml). The pipette is changed for each dilution. Two slopes of medium without drug and two with drug are inoculated with 0.1 ml of the two chosen dilutions.

[1] This section is reproduced from Canetti G et al. Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bulletin of the World Health Organization,* 1969; 41:21–43.

The inoculated slopes are plugged with cotton-wool; no rubber or screw caps are used. The slopes are put in a stand at a very slight angle from the horizontal and placed in the incubator at 37 °C. The liquid should cover as much surface of the medium as possible, without touching the cotton-wool plug. When the liquid part of the inoculum has evaporated (24–48 hours) the slopes are covered with rubber caps and left in the incubator at 37 °C.

READING OF TESTS

The results are read for the first time on the 28th day. The colonies are counted only on the slopes seeded with the inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). This inoculum may be the same for the control slopes and the drug-containing slopes, or it may be the low inoculum (10^{-6} mg of bacilli) for the control slopes and the high inoculum (10^{-4} mg of bacilli) for the drug-containing slopes.

The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum. Dividing the second figure by the first gives the proportion of resistant bacilli existing in the strain. Below a certain value – the critical proportion – the strain is classified as sensitive; above that value, it is classified as resistant. The proportions are reported as percentages.

If, according to the criteria indicated below, the result of the reading made on the 28th day is “resistant”, no further reading of the test for that drug is required: the strain is classified as resistant. If the result at the 28th day is “sensitive”, a second reading is made on the 42nd day: this provides the definitive result.

CRITERIA OF RESISTANCE

Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs – rifampicin, isoniazid, ethambultol, and streptomycin – is classified as resistant to that drug.

For calculating the proportion of resistant bacilli, the highest count obtained on the drug-free and on the drug-containing medium should be taken, regardless of whether both counts are obtained on the 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd day.

SPUTUM SHIPMENT – FORM 1

Country: .... Diagnostic Centre: ....

Code: .... Code: ....

IDENTIFICATION OF THE PATIENT

Name: ....

TB district number: .... Date registered: |____|____|____|
Day Mo Yr

Sex: |____| Male |____| Female

Age: .... years

Date of sputum collection: A .... B ....

Result of smear: ....

CPB/CPC added: |____| Yes |____| No

CLINICAL INFORMATION – FORM 2

Country: .... Diagnostic Centre: ....

Code: .... Code: ....

A IDENTIFICATION OF THE PATIENT

Name: ....

TB district number: .... Date registered: |____|____|____|
Day Mo Yr

Sex: |___| Male |___| Female

Age: .... years

Date of sputum collection: A .... B ....

Country-specific data (to be decided by the coordinating team), for example:

Country of origin |_______________________________|

HIV-status |_______|

History of drug-abuse |_______|

B HISTORY GIVEN BY THE PATIENT

B1 Previously treated for TB? No |___| Yes |___|

If the answer is no, go to B2, if yes, go to B3.

B2 Standardized history

- For how long have you been sick? ....
- Did you have the same symptoms prior to this episode? ....
- Did you have other symptoms of lung disease prior to this episode (haemoptysis, chest pain, cough)? ....

- Did you have X-ray examinations prior to this episode? ....
- Did you have sputum examinations prior to this episode? ....
- Did you ever take tuberculosis drugs for more than one month? ....
  If yes, what was the name? ....
- Did you ever have injections for more than one month? ....

Did the patient remember previous treatment for TB after these questions?

No |___| Yes |___|

If the answer is yes, continue with B3

B3 Information about previous treatment

- Where was the patient treated? ....
- When was the patient treated? ....
- How many times was the patient treated? ....
- Which drugs were used for treatment? ....
- By whom was the patient treated? ....
- Outcome of the last treatment according to the patient:

  Cured |___| Not cured |___| Unknown |___|

C MEDICAL RECORDS

After extensive checking through the medical files and other documents available in the health centre, have you discovered that the patient has been registered for tuberculosis treatment before?

No |___| Yes |___|

If the answer is yes, what was the outcome of the last course of chemotherapy:

Cured	|___|	Treatment completed	|___|
Defaulted	|___|	Failed	|___|
Transferred out	|___|		

D FINAL DECISION

D1 Patient has been previously treated for TB for more than a month:

Yes	|____|	(answer to question B1 or B2 and/or C was 'yes')
No	|____|	(answer to B1 and B2 and/or C was ‘no’)
Doubtful	|____|	

D2 If yes, what was the outcome of previous treatment ?

Cured/treatment completed	|___|
Failed	|___|
Defaulted	|___|
Chronic	|___|
Relapse/defaulter not distinguishable	|___|
Unknown	|___|

Responsible Officer: ....

ANNEX 8

RESULTS OF BACTERIOLOGICAL EXAMINATION FORM 3

Country: .... Diagnostic Centre: ....

Code: .... Code: ....

A PATIENT

Number: .... Date of receipt: |____|____|____|
Day Mo Yr

B IDENTIFICATION

Sample A:		Sample B:	
|___|	*M. tuberculosis*	|___|	*M. tuberculosis*
|___|	*M. bovis*	|___|	*M. bovis*
|___|	*M. africanum*	|___|	*M. africanum*
|___|	Negative	|___|	Negative
|___|	Contaminated	|___|	Contaminated
|___|	Other	|___|	Other

C SUSCEPTIBILITY OF *M. TUBERCULOSIS*

Susceptible to:		Resistant to:	
|___|	Isoniazid	|___|	Isoniazid
|___|	Rifampicin	|___|	Rifampicin
|___|	Ethambutol	|___|	Ethambutol
|___|	Streptomycin	|___|	Streptomycin

Date of recording: |____|____|____|
Day Mo Yr

Responsible Officer: ....

ANTI-TUBERCULOSIS DRUG RESISTANCE RESULTS

	NEW			PREVIOUSLY TREATED		
	No.	%	95% CI	No.	%	95% CI
Total no. of cases tested						
Sensitive to all four drugs						
ANY RESISTANCE						
Isoniazid (H)						
Rifampicin (R)						
Ethambutol (E)						
Streptomycin (S)						
MONORESISTANCE						
Isoniazid (H)						
Rifampicin (R)						
Ethambutol (E)						
Streptomycin (S)						
MULTIDRUG RESISTANCE						
H + R						
H + R + E						
H + R + S						
H + R + E + S						
OTHER PATTERNS						
H + E						
H + S						
H + E + S						
R + E						
R + S						
R + E + S						
E + S						

PROFICIENCY TESTING REPORT FORM

R = Resistant
S = Susceptible
NG = No Growth

COUNTRY ____________________

YEAR ____________________

SRL ____________________

NRL ____________________

#	Strain Code	National Reference Laboratory Drugs Tested				Supranational Reference Laboratory Drugs Tested			
		INH	RMP	EMB	SM	INH	RMP	EMB	SM
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									

SRL Concentrations		NRL Concentrations	
SRL Resistance Criteria		**NRL Resistance Criteria**	

Summary assessment				
INH	RMP	EMB	SM	
				Total Correct Results
				True Resistant
				False Resistant
				True Susceptible
				False Susceptible
				Sensitivity
				Specificity
				Efficiency
				Reproducibility

Method used SRL	
Method used NRL	

Turnover time	
Method used SRL	
Method used NRL	

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