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GUIDELINES FOR DRUG SUSCEPTIBILITY TESTING
FOR SECOND-LINE ANTI-TUBERCULOSIS DRUGS FOR DOTS-PLUS



Communicable Diseases
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Introduction

In 1997 the World Health Organization (WHO), the International Union Against Tuberculosis and Lung Disease (IUATLD) and partners world-wide released the first report of the global project on anti-tuberculosis drug resistance.¹ The data generated in this report were reinforced in a recently published second report.² Directly observed treatment short-course (DOTS), the WHO strategy for TB control cures virtually all patients with drug-susceptible TB and some drug-resistant TB through the administration of short-course chemotherapy with first-line drugs.³ However, patients with multidrug-resistant (MDR) tuberculosis (TB) to at least isoniazid and rifampicin are more likely to fail short-course chemotherapy. In recent years there has been encouraging evidence that patients with MDR TB can be cured with appropriate management based on second-line drugs.⁴⁻⁶ Unfortunately, second-line drugs are inherently more toxic and less effective than first-line drugs and reliable assessment of drug resistance is an essential prerequisite for appropriate use. Treatment is prolonged and significantly more expensive. Accurate laboratory drug susceptibility testing (DST) data to second-line drugs will support clinical decision making and help to prevent the emergence of further drug resistance in patients with MDR TB.

In order to meet the challenges posed by MDR TB, the WHO established the DOTS-Plus initiative to assess the feasibility and cost-effectiveness of using second-line drugs to manage patients with MDR TB primarily in middle and low-income countries.⁷ Reliable DST, including second-line drug testing, is a basic requirement of the DOTS-Plus strategy. Program officers and clinicians may not be aware that the intrinsic accuracy of susceptibility testing results (performed under the best circumstances) varies with the drug tested: it is most accurate for rifampicin and isoniazid and less for streptomycin and ethambutol.⁸ Data of comparable quality for classical second-line drugs are often fragmentary.

Second-line DST is unnecessary in first-line drug-susceptible cases. In the absence of drug resistance, first-line drugs are highly effective and second-line drugs should not be used except in the context of severe drug intolerance.

To ensure accuracy of in-vitro susceptibility results for second-line drugs only laboratories with experience and well-documented competency in performing first-line drug testing should consider offering testing for second-line drugs. Consideration should be given to centralising these analyses where appropriate. For example, a large body of clinical and bacteriological data exists for early trial drugs such as p-aminosalicylic acid (PAS) but little exists for the use of fluoroquinolones and other new agents in the management of TB.

In preparing this document the writing committee has tried to indicate the degree of evidence supporting our recommendations regarding appropriate methodologies for the analysis of second-line drug resistance.

Objectives for guidelines

- To guide TB programme managers and physicians in the appropriate use of second-line drug-susceptibility testing data
- To permit determination of the true scope of second-line drug resistance for surveillance purposes, in the long term

Objectives for the laboratory

- To recommend standardised methodologies and protocols for second-line DST
- To recommend appropriate classes of drugs for DST
- To recommend the scope of internal quality control measures that should be undertaken by laboratories performing second-line DST
- To provide a framework for the evaluation of new therapeutic compounds

Technical Issues

1. Drugs to be tested

Second-line drugs considered in these guidelines are those listed in the WHO Guidelines for the Management of Drug Resistant Tuberculosis.⁹ This list comprises the following drugs:

- Aminoglycosides for Kanamycin and Amikacin
- Polypeptides for Capreomycin, Viomycin, and Enviomycin
- Fluoroquinolones for Ofloxacin, and Ciprofloxacin
- D-cycloserine (Terizidone)
- Ethionamide, Prothionamide
- p-aminosalicylic acid (PAS)

When several drugs are mentioned for a class, only one of them needs to be tested because of complete cross-resistance between members of that class. Testing selection within a class is not to be considered as a recommendation for preferential clinical use.

2. Standard method: Löwenstein Jensen

Criteria for drug resistance testing on Löwenstein Jensen (L-J) using the proportion method have been elaborated for all drugs listed above except for fluoroquinolones.^{10,11} The following recommendations apply to indirect susceptibility testing of pure cultures of *Mycobacterium*

tuberculosis (*M. tuberculosis*). Other testing methods like the resistance ratio or the absolute concentration method might also be considered, if based on published evidence. Other variants of the proportion method such as those performed in Middlebrook 7H10, 7H11, 7H12 (Bactec 460TB) media can be similarly considered.

3. Concentrations

Drugs used for susceptibility testing should never be from the medicine used for treatment but pure compounds only, available from the manufacturer. The recommended critical concentrations ($\mu\text{g/ml}$) and critical proportions for the proportion method on L-J are the following:

Recommended critical concentrations ($\mu\text{g/ml}$) and critical proportions for susceptibility testing for the 2nd line drugs using the proportion method in L-J medium.

Drugs	Drug concentrations ($\mu\text{g/ml}$)		Solvents	Diluents	Stock Solutions (%)
	1% critical proportion	10% critical proportion			
Capreomycin	40	20	DW	DW	1
Cycloserine	40	30	DW	DW	1*
Kanamycin	30	20	DW	DW	1
Ofloxacin§	2	-	0.1N NaOH	DW	1
PAS	0.5	0.25	DW	DW	1*
Protionamide	40	20	DMSO	DW	1*
Thioacetazone	-	2	TG	TG	1*

DW=Sterile distilled water; DMSO=dimethyl-sulfoxide; TG=triethylene glycol or propylene glycol.

* Stock solutions of these drugs should be prepared freshly each time. Stock solutions of other drugs can be stored at 4° C for up to 1 month. The necessary amounts of drug should be calculated taking account of molecular formula and potency informed from the manufacturer. Drug containing media can be stored at 4° C for up to 1 month.

§ Only one concentration and its corresponding resistance criterium for ofloxacin are recommended because data are only available under these conditions.

4. Media preparation, dilutions, inoculation, incubation, and reading

For more details on this section please consult

Preparation of the media, bacterial suspensions and dilutions, inoculation of the media, incubation, reading schedule and reporting are those recommended for first line-drugs. In short, Lowenstein-Jensen (L-J) without potato starch with drugs incorporated before inspissation is used. The modification of the International Union Against Tuberculosis (IUAT) is recommended.¹²⁻¹⁴ Screw-capped tubes 17 mm in diameter, containing 7 ml of medium are inspissated at 85°C during 40-45 minutes. L-J medium with and without incorporated drugs can be stored at 4° C for one month.

The indirect drug susceptibility test is carried out from a primary isolation or a sub-culture on L-J medium. A representative portion of the culture is obtained by sampling as many colonies as possible within 1 or 2 weeks after appearance of growth. The sample is homogenised in a sterile screw-capped bottle (e.g. 14 ml McCartney bottle or 5 ml Bijoux bottle) containing 1 ml H₂O and 10 glass beads 3.0 mm in diameter. The mixture is homogenized on a Vortex mixer for up to a minute and if needed the opacity is adjusted by the addition of sterile, distilled H₂O, down to that of a standard suspension of 1.0 mg/ml of BCG. The suspension is left to settle for about 30 minutes.

There are two recommended methods of medium inoculation:

In the first one, serial dilutions of 10⁻¹ mg/ml to 10⁻⁵ mg/ml of the standard suspension are prepared by diluting sequentially 1.0 ml of the standard suspension (1 mg/ml) in tubes containing 9 ml of sterile distilled H₂O. Dilutions of 10⁻³ mg/ml and 10⁻⁵ mg/ml are inoculated on L-J and on drug containing L-J medium. For each isolate, two bacterial dilutions of the suspension are made; for each dilution of the suspension two control tubes are inoculated and one tube is inoculated for each drug concentration prepared. Therefore, for each drug 8 tubes have to be inoculated in total. The volume of the inoculum is 0.2 ml.

The second method uses calibrated inoculating loops. Two dilutions - 10⁻² mg/ml and 10⁻⁴ mg/ml - are made from the standard suspension by serially diluting a loopful (0.01 ml) into two screw-capped tubes (14 ml McCartney bottle or 5 ml Bijoux bottle) containing 1.0 ml of sterile, distilled H₂O. The inoculation of tubes is identical to the one described above, but the inoculum is a loopful (0.01 ml) of each dilution.

After inoculation, the tubes are incubated at 37° C in a slanted position, with the screw caps slightly loosened to allow for the evaporation of the inoculum. After 24-48 hours, screw caps are tightened and the tubes are further incubated.

The reading of results consists of three simple steps:

- the counting of colonies grown on the different slants
- the calculation of the proportion of resistant bacilli by comparing counts on drug free and drug containing L-J medium;
- the matching of the calculated proportion with the critical proportion of the drug in question to determine if the proportion is higher (resistant strain) or lower (susceptible strain).

The reading of results is carried out at the 28th and 40th day after inoculation. If at the 28th day reading, the proportion of resistant colonies is higher than the critical proportion, the strain can be reported as resistant. Also, if at the 28th day reading, there are no colonies on the lowest drug concentration inoculated with the richest dilution used, the strain can be reported as susceptible without further reading. Except for these two circumstances, all other results should be reported after the 40th day reading.

The results should be reported as “susceptible” or “resistant” only, to facilitate the interpretation by clinicians. The percentages of resistant mutants have to be kept in the labs for possible future reference.

5. Internal quality control procedures for drug susceptibility testing of *M. tuberculosis* by the proportion method on L-J.

Internal quality control of the media is an essential part of DST. Quality control tests should be carried out for each batch of medium produced. The recommended procedure includes the use of a reference strain i.e. H37Rv sensitive to all drugs tested. Two methods are recommended for internal quality control. One involves the plating of serial inoculation dilutions, which will detect the number of naturally occurring resistant mutants (test-undiluted suspensions and serial dilutions -1, -2, -3). The other one involves the use of serial drug concentrations to determine variations in minimal inhibitory concentrations.

Batches of media for DST should be kept at 4° C for no longer than 4 weeks.

5.1 Quality control of medium batches.

The test, carried out with the *M.tuberculosis* reference strain H₃₇ Rv, consists in determining for each drug and each drug concentration used, the proportion of drug resistant bacilli in this strain. If this proportion falls between the set limits indicated in Table I, the medium can be considered as satisfactory. If this is not the case, a new batch has to be produced and all tests repeated. As this proportion is very low, the quality control test differs slightly from the regular drug susceptibility test. The following dilutions of the standard bacterial suspension (1 mg/ml) are inoculated on a control tube and on a tube of each drug concentration tested:

1, 10⁻¹, 10⁻², 10⁻³ mg / ml

The following dilutions of the standard bacterial suspension (1 mg/ml) are inoculated on two control tubes:

10⁻⁵ and 10⁻⁶ mg / ml

The definitive reading of the test is done on the 28th and 40th day of incubation at 37° C. Colony

counting and the calculation of proportions are carried out as in a regular drug susceptibility test. This test should be carried out on each new batch of media.

5.2 Quality control of the validity of drug resistance criteria in the laboratory

This test is identical to the one described above except for the fact that it is carried out on five randomly selected “wild type” strains of *M.tuberculosis*. If the proportion of resistant bacilli in four out of the five selected “wild type” strains falls within the limit proportions shown on Table II, the recommended drug resistance criteria of the proportion method on L-J are validated. Accepting four of five “wild type” strains, allows for the low (but always possible) probability to pick a drug resistant strain, as one takes five “wild type” strains randomly nowadays. In practice only the four following drugs need to be tested: **isoniazid, streptomycin, PAS and ethionamide**. It is reasonable to test only four drugs, since the criteria will likely be met for the other drugs. This is a time saving recommendation; however, laboratories are also welcome to test all drugs.

The performance of this quality control test is indicated in the following circumstances:

- ❖ a few months after a laboratory begins testing for TB drug susceptibility, to make sure that a routine likely to be reproducible is attained in a laboratory which is just starting DST,
- ❖ every so often, in order to identify possible undetected changes in the growth characteristics of strains seeded on drug containing media,
- ❖ When strains isolated from untreated TB cases show an unusually high frequency of drug resistance. Testing on different batches of drug containing media is certainly a good way to check the validity of the original results

Good laboratory practice dictates that this test be performed at least semi-annually, preferably quarterly. It is important to state that when a laboratory starts DST, a linkage should be established with an external laboratory for quality control purposes.

Table I. Maximum, median and minimum number of drug resistant mutants in the H₃₇Rv strain per 10⁻⁶ bacilli. Reading at 40 days of incubation.

Isoniazid	CONC. (µg /ml)	0.1	0.2	1.0
	Minimum	2	0	0
	Median	2	4	2
	Maximum	150	32	14
Streptomycin	CONC. (µg /ml)	2.0	4.0	8.0
	Minimum	6	0	0
	Median	460	7	0
	Maximum	37,000	300	3
PAS*	CONC. (µg /ml)	0.25	0.5	1.0
	Minimum	2,000	17	1
	Median	38,000	900	22
	Maximum	700,000	50,000	450
Ethionamide*	CONC. (µg /ml)	10.0	20.0	30.0
	Minimum	12,500	120	38
	Median	95,000	3,500	400
	Maximum	700,000	70,000	8,200
Cycloserine	CONC. (µg /ml)	20.0	30.0	40.0
	Minimum	21	0	0
	Median	450	4	2
	Maximum	10,000	600	300
Viomycin	CONC. (µg /ml)	20.0	30.0	40.0
	Minimum	28	0	0
	Median	1,800	100	12
	Maximum	100,000	10,000	350
Kanamycin	CONC. (µg /ml)	10.0	20.0	30.0
	Minimum	900	1	0
	Median	6,200	80	7
	Maximum	400,000	4,000	150

* The proportion of PAS and ethionamide resistant mutants in the H₃₇Rv strain is much higher than in regular “wild type” strains.

Table II. Maximum, median and minimum number of drug resistant mutants in “wild type” strains of *M.tuberculosis* strain per 10⁻⁶ bacilli. Reading at 40 days of incubation.

Isoniazid	CONC. (µg /ml)	0.1	0.2	1.0
	Minimum	2	0.5	0
	Median	41	5	3
	Maximum	180	41	12
Streptomycin	CONC. (µg /ml)	2.0	4.0	8.0
	Minimum	100	0.7	0
	Median	5,000	40	2
	Maximum	100,000	400	12
PAS	CONC. (µg /ml)	0.25	0.5	1.0
	Minimum	1	0.1	0
	Median	800	25	5
	Maximum	11,000	100	32
Ethionamide	CONC. (µg /ml)	10.0	20.0	30.0
	Minimum	6,000	100	0
	Median	30,000	1,500	100
	Maximum	80,000	5,000	500

5.3 Quality control using serial drug concentrations to determine variations in minimal inhibitory concentrations.

For quality testing of each lot of culture medium, the minimal inhibitory concentration (MIC) of *M. tuberculosis* H37Rv shall be determined.

6. **Storage of mycobacteria in 10% skim milk solution.**

- i) Reconstitute skim milk powder in distilled water (100 grams per litre).
- ii) Dispense 1.6 ml into 1 oz. McCartney bottles containing 10 glass beads.
- iii) Autoclave at 15 lbs. and 121° C. for about 10 minutes. Tighten caps after cooling
(Medium can be frozen at this stage).

- iv) Add bacterial growth from 1 slope of L-J medium with 3+ growth.
- v) Mix on Vortex mixer for about 30 seconds.
- vi) Allow foam to subside for 2 hours.
- vii) Pipette bacterial suspension into 1 cryovial (Nalgene 2.0 ml).
- viii) Freeze at -20°C until needed.

To use frozen vials

- i) Thaw cryovial at room temperature.
- ii) Pipette 2 or 3 drops onto a slant of medium and incubate at appropriate temperature.
- iii) Vials can be re-frozen 2 or 3 times.

7. Repository for well characterised strains

When a new method is validated, it is imperative that a large number of strains (several hundreds), including both drug-susceptible and -resistant, are tested at several sites and the results compared with a previously validated method. In addition, it is warranted that these strains should be well characterised, i.e., identified as susceptible or resistant, and if resistant, their mutation(s) identified. Further, to prevent any bias in the study, it is important that the strains are shown to be genetically dissimilar through DNA fingerprinting. Acquiring the background information on the strains will require close collaboration between laboratories, clinicians, and TB control officers. Only a few institutions have access to such well-characterised strains, and therefore, a repository of frozen strains should be formally organised and aliquots made available to evaluating laboratories. This approach would allow different procedures to be compared over time in different laboratories using the same group of well-characterised strains.

8. Further needs for research

Our knowledge today is very incomplete regarding how to best perform in vitro susceptibility testing of newer second-line drugs, and the clinical value of such tests. Studies to increase our understanding are needed both regarding methodology - which is the optimal test assay and breakpoint concentrations for each of the newer drugs? - and regarding the clinical relevance of such in vitro testing - can the outcome of the addition of a certain drug to a drug regimen in treating MDR-TB be predicted by its in vitro susceptibility/resistance?

Some examples of needed research follows:

Methodology:

- Pyrazinamide - what is the optimal way to test resistance to this drug? Broth or solid

medium? pH? Usefulness of enzyme testing such as pyrazinamidase? Other possible markers of PZA-resistance?

- ❑ quinolones - which is the best representative to test in this group, ofloxacin or ciprofloxacin or another one?
- ❑ quinolones - how should the test be carried out, solid culture, L-J or agar medium? a broth based system, Bactec?
- ❑ quinolones - which concentrations should be recommended for testing? Are these the same for all quinolones?

Similar methodological questions are relevant also for other new and coming drugs.

Clinical relevance:

Standardized outcome studies where in vitro data are analyzed in relation to the improved clinical outcome for the MDR-TB patients when an individual drug is added to an existing (standardized?) regimen are needed to document the relevance of in vitro DST.

9. Issues to consider

- ◆ It is important to report separately results regarding first-line drugs from those of second-line drugs. This is to emphasise that second-line drugs constitute a second option for the treatment of TB
- ◆ For clinical purposes, the utilisation of second-line drugs or new drugs is not justified without bacteriological confirmation of resistance to first-line drugs.
- ◆ In patients with a high load of bacilli in sputum and pulmonary cavities, smear microscopy may persist positive up to 3 months even if *M. tuberculosis* strain is fully susceptible to first-line drugs. Repetition of DST is unnecessary within a 3 month period even in patients with persistent positive sputum.
- ◆ In the event of patients with *M. tuberculosis* susceptible to first-line drugs that show poor response to directly observed treatment, before switching to another therapeutic option, several other factors should be investigated: interaction with other drugs (antiretrovirals), absorption, problems related to intestinal mobility or hepatic metabolism.
- ◆ Treatment for tuberculosis should follow the current recommendations from the WHO.

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Sample form to report drug susceptibility testing of *Mycobacterium tuberculosis***Name**

Age

Sex

Address

Specimen No.:

Date:

Smear microscopy results

Culture

Identification:

Susceptibility test (date):

Second-line drugs: **Yes**☐**No**☐

Drug	Result
Isoniazid	
Rifampicin	
Ethambutol	
Pyrazinamide	
Streptomycin	

Drug	Result
Thioacetazone	
Ethionamide	
Kanamycin	
Cycloserine	
Capreomycin	
PAS	
Ofloxacin	

