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Multicenter evaluation of the nitrate reductase assay for drug resistance detection of *Mycobacterium tuberculosis*

Anandi Martin[a,*], Ernesto Montoro[b], Dihadenys Lemus[b], Norberto Simboli[c], Nora Morcillo[d], Maritza Velasco[e], José Chauca[f], Lucía Barrera[c], Viviana Ritacco[c], Françoise Portaels[a], Juan Carlos Palomino[a]

[a]*Mycobacteriology Unit, Institute of Tropical Medicine, Nationalestraat, 155, Antwerp, B-2000, Belgium*
[b]*Instituto de Medicina Tropical "Pedro Kourí", La Habana, Cuba*
[c]*Instituto Nacional de Enfermedades Infecciosas ANLIS "Carlos G. Malbrán", Buenos Aires, Argentina*
[d]*Hospital Dr. A. Centrángolo, Vicente Lopez, Buenos Aires, Argentina*
[e]*Instituto de Salud Pública de Chile, Santiago, Chile*
[f]*Instituto de Medicina Tropical "Alexander von Humboldt", Universidad Peruana Cayetano Heredia, Lima, Peru*

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Abstract

The performance of the nitrate reductase assay was evaluated in a multicenter laboratory study to detect resistance of *Mycobacterium tuberculosis* to the first-line anti-tuberculosis drugs rifampicin, isoniazid, ethambutol and streptomycin using a set of coded isolates. Compared with the gold standard proportion method on Löwenstein-Jensen medium, the assay was highly accurate in detecting resistance to rifampicin, isoniazid and ethambutol with an accuracy of 98%, 96.6% and 97.9%, respectively. For streptomycin, discrepant results were obtained with an overall accuracy of 85.3%. The assay proved easy to be implemented in countries with limited laboratory facilities.

Keywords: Tuberculosis; Nitrate reductase assay; First line drugs

1. Introduction

Tuberculosis (TB) causes almost 3 million deaths a year with 98% occurring in developing countries. The global impact of TB is devastating; especially in developing countries where poverty and the prevalence of HIV infection facilitate further transmission (World Health Organization, 2004a,b). The Löwenstein-Jensen (LJ) or agar proportion method are the current standard methods recommended to perform susceptibility testing of *Mycobacterium tuberculosis* based on counting visible colonies (Canetti et al., 1963, 1969; Kent and Kubica, 1985). The proportion method (PM) is an inexpensive technique but time-

* Corresponding author. Tel.: +32 3 2476334; fax: +32 3 2476333.
E-mail address: amartin@itg.be (A. Martin).

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consuming and very slow. The BACTEC TB-460 system (Becton Dickinson) is also accepted as a gold standard method and widely used in reference laboratory facilities (Roberts et al., 1983; Siddiqi et al., 1981). This system provides results in only 5–10 days but involves a radioactive substrate and expensive equipment. The commercial BACTEC MGIT 960 (Becton Dickinson) is a non-radioactive system, rapid and reliable and could in the future replace the BACTEC TB-460 system (Scarparo et al., 2004) but is still expensive for some low-resource countries. Molecular techniques such as PCR and DNA hybridization assays provide results in 24 h, but require specialized equipment and highly skilled personnel. The search for rapid, sensitive, reliable and cost-effective drug susceptibility testing (DST) methods for *M. tuberculosis* has led to the development of new techniques that use viability markers not depending on heavy, sophisticated and costly equipment.

The nitrate reduction test is widely used to differentiate, among other tests, *M. tuberculosis* from *M. bovis*, to classify strains of *M. africanum* (some of them being nitrate reductase positive and some negative), and to classify some non-tuberculosis mycobacteria like *M. kansasii*, *M. terrae* complex or *M. szulgai* that are nitrate-reductase positive (Kent and Kubica, 1985). The chemistry of the detection used in the nitrate reduction was discovered in 1879 by JP Griess (Griess and Bemerkungen, 1879) and it was called the Griess method. Based on this concept, a nitrate reductase assay (NRA) for drug susceptibility testing of *M. tuberculosis* performed on LJ was recently described (Ängeby et al., 2002; Coban et al., 2004). In this multilaboratory study, we have evaluated for the first time the ease of use of the NRA performed in different settings to determine the susceptibility of *M. tuberculosis* to the first-line drugs rifampicin (RIF), isoniazid (INH), ethambutol (EMB), and streptomycin (SM) and the results were compared to those obtained with the most commonly used PM on LJ.

2. Materials and methods

2.1. Participating laboratories

The results were generated by five laboratories: Institute of Tropical Medicine, Antwerp, Belgium; Instituto de Medicina Tropical "Pedro Kouri", La Habana, Cuba; Instituto de Salud Pública de Chile, Santiago, Chile; Instituto Carlos G. Malbrán, Buenos Aires, Argentina; Hospital Dr. A. Centrángolo, Vicente Lopez, Buenos Aires, Argentina. Laboratories were randomly numbered from 1 to 5 for the purpose of this publication.

2.2. Mycobacterial isolates

A panel of thirty clinical isolates of *M. tuberculosis* was coded by the Mycobacteriology Unit of the Institute of Tropical Medicine, Antwerp, Belgium and distributed to the participants with individual codes. As shown in Table 1 these clinical isolates have resistance to one or more drugs. Twelve were resistant to INH, 15 resistant to RMP, 9 resistant to EMB, 12 resistant to SM and 12 susceptible. The reference

Table 1
Profile DST of the 30 coded isolates

Coded isolates	RIF	INH	EMB	SM
001	R	R	S	R
002	R	R	R	R
003	S	S	S	S
004	S	S	S	S
005	S	R	R	R
006	S	S	S	S
007	S	S	R	S
008	R	R	R	R
009	R	S	S	S
010	S	S	S	S
011	R	S	S	S
012	R	R	R	S
013	S	S	S	S
014	S	S	S	S
015	S	R	R	R
016	S	S	S	S
017	R	R	R	R
018	R	S	S	S
019	R	R	R	R
020	S	S	S	S
021	S	S	R	S
022	R	R	R	S
023	S	S	S	S
024	S	S	S	S
025	S	R	R	R
026	S	S	S	S
027	R	R	R	R
028	S	S	R	S
029	R	R	R	R
030	S	S	S	S

strain H37Rv (ATCC 27294) from the American Type Culture Collection was used as the reference susceptible strain. All strains were freshly subcultured in each laboratory on LJ medium before being used.

2.3. Drugs

RIF, INH, EMB and SM were obtained in powder from Sigma-Aldrich (Bornem, Belgium). The stock solutions were prepared in advance at a concentration of 10 mg/ml in methanol for RIF, and 1 mg/ml in distilled water for the other drugs. Stock solutions were filter sterilized with a 0.2 μm membrane and kept at −20 °C until used. Stock solutions stored in this way remain active for 3–6 months.

2.4. Drug susceptibility testing (DST)

The PM was performed on LJ medium according to the standard procedures with the recommended critical concentrations of 40 μg/ml for RIF, 0.2 μg/ml for INH, 2 μg/ml for EMB and 4 μg/ml for SM. (Canetti et al., 1963, 1969).

2.5. Nitrate reductase assay (NRA)

The NRA uses the detection of nitrite as an indication of growth when used as a drug susceptibility test. The antibiotic was included in the LJ medium at a concentration of 40 μg/ml for RIF, 0.2 μg/ml for INH, 2 μg/ml for EMB and 4 μg/ml for SM; 1000 μg/ml of potassium nitrate (KNO_3) was also added.

The inoculum was prepared from a fresh Löwenstein-Jensen tube by adding a loop of colonies to 5 ml of phosphate buffer saline pH 7.3 (PBS) in a tube containing several glass beads. The bacterial suspension was vortexed for 2 min and allowed to sediment for 15 min. The supernatant was transferred to another tube and the turbidity was visually adjusted to a McFarland Standard tube no. 1. The suspension was further diluted 1:10 in PBS. The reagent mix consisted of 1 part 50% concentrated hydrochloric acid (HCl), 2 parts 0.2% sulfanilamide, and 2 parts 0.1% *n*-1-naphthylethylenediamine dihydrochloride. The method was performed as described by Ängeby et al. (2002). For each strain, 200 μl of the undiluted inoculum was added into the antibiotic tube, and 200 μl of the 1:10 dilution into 3 growth control tubes. The tubes were incubated at 37 °C. After 7 days, 500 μl of the reagent mixture was added to one growth control tube of each strain. If any color change appeared, all the tubes of that strain were developed with the reagent mixture; otherwise, the remaining tubes were re-incubated and the procedure repeated at day 10 and day 14. An isolate was considered resistant if color developed in the test tube (pink to red or purple) and if this color was greater than that appearing in the 1:10-diluted growth control.

2.6. Analysis of data

The MedCalc Software (Mariakerke, Belgium) was used to calculate the statistical parameters; sensitivity (ability to detect true resistance), specificity (ability to detect true susceptibility) and accuracy (proportion of correct results).

3. Results

DST for the first-line drugs RIF, INH, EMB, and SM was performed with a blind panel of 30 clinical isolates of *M. tuberculosis* sent to each participating laboratory. All sites performed the NRA based on a standard protocol sent by the Institute of Tropical Medicine, Antwerp, Belgium. The results were obtained in a median of 10 days and were compared to those obtained by the PM on LJ. Table 2 shows, for

Table 2
Susceptibility results of the 30 coded isolates performed by the nitrate reductase assay (NRA) compared to the proportion method (PM) for each site

NRA		Rifampicin		Isoniazid		Ethambutol		Streptomycin	
		PM		PM		PM		PM	
		R	S	R	S	R	S	R	S
Site 1	R	15	0	12	0	9	1	9	1
	S	0	15	0	18	0	20	3	17
Site 2	R	15	0	11	1	9	0	10	0
	S	0	15	1	17	0	21	2	18
Site 3	R	15	0	12	1	8	0	7	0
	S	0	15	0	17	1	21	5	18
Site 4	R	14	0	11	1	9	0	11	2
	S	1	15	1	17	0	21	1	16
Site 5	R	13	0	12	0	8	0	5	1
	S	2	15	0	18	1	21	7	17

Table 3
Sensitivity, specificity, and accuracy of the NRA to the first-line drugs for each study site

	Nitrate reductase assay											
	RIF (%)			INH (%)			EMB (%)			SM (%)		
	Sens*	Spec	A	Sens	Spec	A	Sens	Spec	A	Sens	Spec	A
Site 1	100	100	100	100	100	100	100	95.2	96.6	75	94.4	86.6
Site 2	100	100	100	91.7	94.4	93.3	100	100	100	83.3	100	93.3
Site 3	100	100	100	100	94.4	96.6	88.9	100	96.6	58.3	100	83.3
Site 4	93.3	100	96.6	91.7	94.4	93.3	100	100	100	91.6	88.9	90.0
Site 5	86.6	100	93.3	100	100	100	88.9	100	96.6	41.7	94.4	73.3

*Sens=sensitivity; Spec=specificity; A=accuracy.

each site, the number of resistant and susceptible strains obtained by the NRA compared to the number obtained by the proportion method on LJ. For RIF, no false positive was found. Site 4 had one false-negative and site 5 two false-negative results. For INH, two sites (2 and 4) had 1 false-negative result and sites 2, 3 and 4 had one false-positive result. For EMB, site 1 had a false positive result and sites 3 and 5 one false-negative result. For SM, sites 1 and 5 had one false-positive result and 3 and 7 false-negative results respectively; site 2 and 3 had 2 and 5 false-negative results respectively, and site 4 had one false-negative and 2 false-positive results. According to these results, sensitivity, specificity and accuracy values are shown in Table 3. For RIF, the sensitivity ranged from 93.3% to 100% and the specificity was 100% for all sites. The overall accuracy was 98%. For INH, the sensitivity ranged from 91.7% to 100% and the specificity from 94.4% to 100%. The overall accuracy was 96.6%. For EMB, the sensitivity ranged from 88.9% to 100% and the specificity from 95.2% to 100%. The overall accuracy was 97.9%. For SM, lower sensitivity and specificity were obtained with a sensitivity ranged from 41.7% to 100% and the specificity from 88.9% to 94.4%. The overall accuracy was 85.3%.

4. Discussion

There is an urgent need for fast, reliable and inexpensive methods for DST of *M. tuberculosis*. The purpose of this multi-laboratory study was to evaluate the performance and the ease of use of the NRA set up in different countries to determine the susceptibility of *M. tuberculosis* to RIF, INH, EMB and SM. It is well known that EMB and SM are difficult drugs to test even by the conventional standard methods. According to the criteria established by the WHO-IUATLD supra-national laboratory network, DST accuracy levels of 99% and 97%, respectively, for RIF and INH and 92% for EMB and SM are proposed as reasonable performance goals for reference laboratory (Laszlo et al., 2002). In this multicenter study, agreement between the reference method and the NRA was very good for RIF and INH, almost at optimal levels. EMB gave also very good results with an overall accuracy of 97.9 %. For SM, significant discrepancies were observed. For this drug, the performance was not acceptable. In the only two other studies performed with the NRA (Ängeby et al., 2002; Coban et al., 2004) the sensitivity and the specificity was 100% for RIF and higher than 96% for INH. SM and EMB gave sensitivity and specificity values ranging between 75% and 100%. In this study, it was not the case for SM. The majority of the sites could not determine correctly the susceptibility pattern for SM. The possible interaction between the drugs and potassium nitrate is unknown and could be taken into consideration for future evaluations. One possible limitation of the NRA is that is can give positive results with non-tuberculous mycobacteria. Species that reduce nitrate are for example *M. kansasii, M. szulgai, M. flavescens, M. terrae* complex and some rapid growers (Kent and Kubica, 1985). *M. bovis* is nitrate negative. On the other hand, nitrate reductase-negative strains of *M. tuberculosis* are very unusual. We have prospectively tested the ability to reduce nitrate in 206 clinical isolates originating from several countries, mostly in Africa and found that only 7 were NRA negative. After identification of these negative isolates, 4 were identified as strain other than *M.*

tuberculosis and only 3 *M. tuberculosis* gave a NRA negative. It could be possible that these three strains did not growth very well or that the nitrite was reduced to nitric oxide which cannot be detected by the reagents used. This could explain in part the false negative results obtained in some laboratories in this study. This could be an inconvenience of this method and should be taken into account. In our experience, it is important to perform the NRA on a fresh culture to correctly determine its enzymatic activity, using old cultures could lead to negative results. On the other hand, the NRA has several advantages. One of them is that it is performed in a solid medium reducing the risk of production of aerosols during the manipulation. The medium used is the classical LJ medium with nitrate incorporated and with exactly the same concentrations of antibiotics used for the conventional PM on LJ. The NRA was also easy to perform without changing the organisation of the routine laboratory performing DST. This method does not need any expensive reagents or equipment and no microscopy observation is needed for the interpretation of results. As discussed by Panaiotov and Kantardjiev (2002) the NRA can be further simplified by using a cristalline nitrate reductase reagent that has a longer shelf life and is less toxic (Lampe, 1981; Warren et al., 1983) to replace the mix reagent. More recently, Syre et al. (2003) applied this technique in liquid medium and obtained results in five days. A number of new commercial and in-house methods for DST testing of *M. tuberculosis* have been described over the last decade, as the Mycobacterial Growth Indicator Tube (MGIT), E-test, Microscopy Observation Drug Susceptibility (MODS), phage amplification test, and colorimetric methods (Freixo et al., 2004; Lemus et al., 2004; Martin et al., 2003; McNerney et al., 2000; Moore et al., 2004; Morcillo et al., 2004; Palomino et al., 1999, 2002; Rusch-Gerdes et al., 1999; Símboli et al., 2005). Each method has some advantages and disadvantages. Our study shows that the NRA can be easily performed in middle-resource countries with limited laboratory facilities, working in different conditions to detect early the resistance to RIF and INH the two most important drugs in the treatment of TB and also to detect the resistance to EMB but for SM the accuracy was not good enough in this multicenter study. The method could be developed to perform DST to second-line antituberculosis drugs and applied directly to sputum smear-positive samples in order to reduce the turnaround time for drug resistance detection.

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