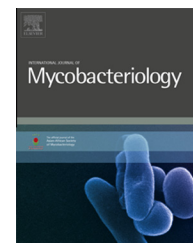


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Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy-positive sputum samples

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ABSTRACT

Aims and objectives: Current methods for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* (MTB) are either costly or slow. As the prevalence of multidrug-resistant (MDR) strains increases, the need for fast, reliable, and inexpensive methods is obvious. This study evaluated a rapid colorimetric nitrate reductase assay (NRA) for direct DST of MTB directly from clinical sputum samples.

Methods: A total of 111 sputa with positive microscopy results for acid-fast bacilli (AFB) with more than 10 AFB per high-power field were used in the study. The samples were decontaminated using the modified Petroff method. The NRA results were compared with the reference indirect proportion method.

Results: The sensitivity and the specificity of the direct NRA were 90% and 97.3%, 92.6% and 98.2%, 52.9% and 100%, and 28.6% and 100% for rifampin, isoniazid, streptomycin, and ethambutol, respectively. The results were in most cases available in 28 days (84.3%).

Conclusions: The direct NRA could be used as a rapid, inexpensive, and accurate method to determine rifampin and isoniazid susceptibility directly from sputum. The technique might become a valid alternative to traditional methods, especially in low-income countries.

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Introduction

Tuberculosis (TB) remains a major public health problem worldwide worsened by the emergence of multidrug-resistant tuberculosis (MDR-TB). In recent years, the incidence of TB has been rising, and the World Health Organization (WHO) has estimated the number of incident new cases at 9 million and 1.5 million people have died from

the disease in 2013. The proportion of new cases with MDR-TB was 3.5% [1].

In order to fight this situation, a rapid and inexpensive drug susceptibility test (DST) is needed to allow a rapid initiation of a correct antibiotic (ATB) therapy. Standard methods for DST, such as the proportion method, are used globally, but depend on culture on solid media and are regrettably time-consuming [2]. The time lag is a significant threat to

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the patient, the community and healthcare workers. The current techniques, genetic as well as phenotypic, have been developed [3–6]. But those methods are globally either costly or slow and are consequently not feasible in most low-income countries. In view of these considerations, alternative rapid methods have been suggested, among them, the nitrate reductase assay (NRA) on Löwenstein–Jensen (LJ) medium. It is simple to perform and has been successfully implemented in low-resources countries [7,8]. This test is based on the ability of *Mycobacterium tuberculosis* (MT) to reduce nitrate to nitrite, which is revealed as a color change in the culture medium, using the Griess method [9]. The indirect (using isolates) NRA yields results in less than 14 days, but requires an initial 3–4 weeks for the culture of the isolate. So far, only a few studies have evaluated the NRA applied directly on sputum samples.

The aim of the present study is to evaluate the performance of the NRA applied directly on microscopy-positive sputum samples from patients with pulmonary tuberculosis (PTB) for the detection of resistance to the first-line anti-tuberculosis drugs: rifampin (RIF), isoniazid (INH), streptomycin (STR) and ethambutol (EMB).

Material and methods

Setting

Currently, the laboratory receives samples from patients living in Sfax and suburbs and also from cities of the Tunisian South and Center. All strains are cultured on standard LJ medium, and the DST is performed with indirect proportion method (IPM). After processing, specimens are stored at -20°C .

Specimen processing

From January 2009 to April 2014, a total of 111 sputa with positive microscopy results with AFB (acid-fast bacilli) having a positivity score of 1+ or more were processed using the Petroff decontamination method [10]. One milliliter of sterile distilled water was added to the sediment.

IPM

An LJ tube was inoculated with 0.2 ml of undiluted decontaminated suspension and incubated for up to 60 days. Isolates from this tube were used for IPM performed using LJ medium according to standard protocol. The following critical concentrations were used: 0.2 $\mu\text{g/ml}$ for INH, 40 $\mu\text{g/ml}$ for RIF, 4.0 $\mu\text{g/ml}$ for STR, and 2.0 $\mu\text{g/ml}$ for EMB.

Direct NRA DST

The NRA was performed as described previously by Ängeby et al. [11] on the difference in the use of sodium nitrate (NaNO_3) instead of potassium nitrate (KNO_3). Standard LJ medium was used with 1000 μg of NaNO_3/ml and with or without ATB. The same critical concentrations of ATB as those used in the IPM were applied.

Part of the decontaminated suspension was diluted 1:10 in sterile distilled water. For each specimen, 0.2 ml of the diluted preparation was inoculated into four drug-free LJ medium tubes containing only NaNO_3 (growth control tubes) and 0.2 ml of the undiluted suspension was inoculated into LJ medium containing NaNO_3 and each of the first-line ATB. The tubes were incubated at 37°C .

After 7 days of incubation, 0.5 ml of freshly prepared Griess reagent (1 part 50% concentrated hydrochloric acid, 2 parts 0.2% sulfanilamide, and 2 parts 0.1% *n*-1-naphthylethylenediamine dihydrochloride) was added to one drug-free tube. If any color appeared, the tube with ATB was developed with the Griess reagent. If not, the other tubes were re-incubated, and the procedure was repeated at day 10 (D10), day 14, and finally at day 28. The medium color changes to weak or strong pink. An isolate was considered to be resistant if there was a color change in the ATB tube equal or greater than that in the diluted growth control. An isolate was considered to be susceptible if there was no color change or a color change less than that in the diluted growth control (Figs. 1–3). NRA was considered to be invalid if the nitrate reaction was negative in the drug-free medium at day 28 despite the presence of colonies.

Quality control

For each batch of medium, internal quality control was done using two known susceptibilities of MT strains: one fully susceptible and one MDR isolate.

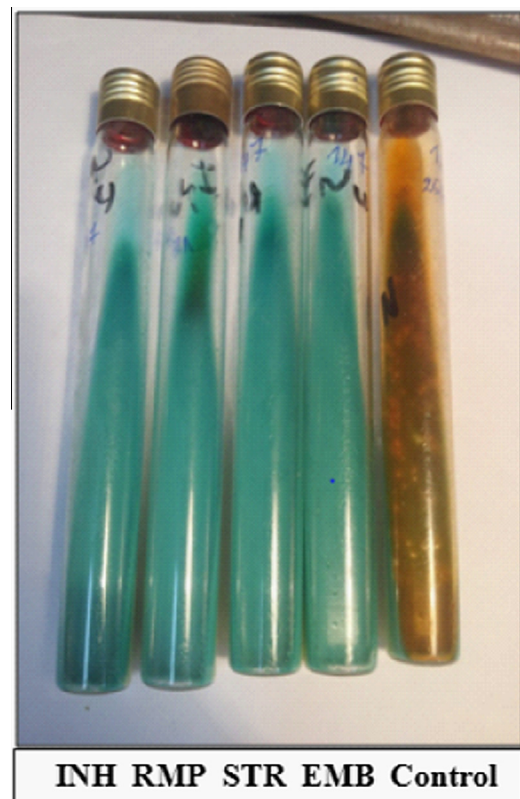


Fig. 1 – Sensitive strain to four antibiotics.

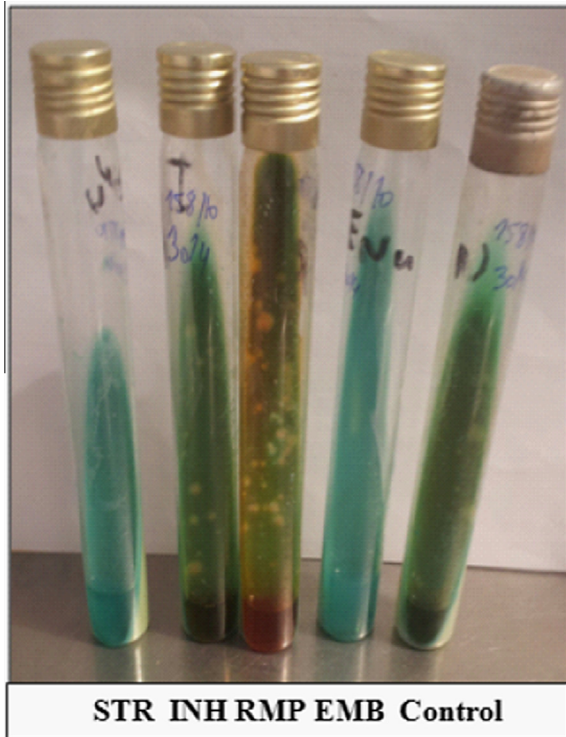


Fig. 2 – Sensitive strain to EMB and STR resistant to RMP and INH (MDR).

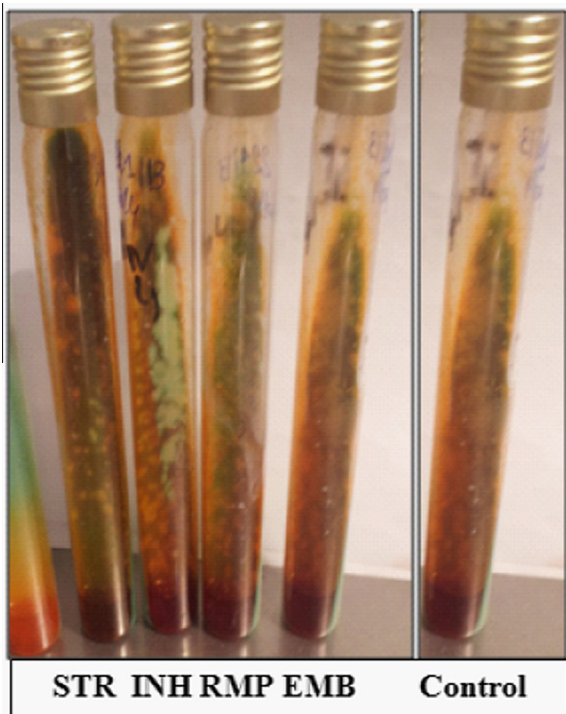


Fig. 3 – Resistant strain four ATB (MDR).

Data analysis

The performance of the NRA was evaluated in comparison with the IPM in terms of sensitivity, specificity, positive

predictive value (PPV), negative predictive value (NPV) and agreement.

Results

The NRA was completed on 83 of 111 sputa. Of the 28 specimens for which testing was not completed, 25 were culture-negative and three had invalid NRA results, with little or no color in the growth control tubes. In total, 55 (66%) strains were fully susceptible, 28 (34%) had different resistance patterns and 9 (11%) were MDR.

The comparison of NRA and IPM (Table 1) showed a sensitivity of 73.8%, a specificity of 98.9%, a PPV of 93.8%, an NPV of 94.4% and an agreement of 94.3%.

Good sensitivities and specificities were found for INH and RIF, while some problems were encountered when tested for sensitivities in detecting EMB and STR resistance (Table 1). Specificities and agreement were excellent for the four ATB. PPV and NPV gave satisfying results, except the PPV for RIF.

No positive results were obtained at D7 or D10. Time to result (TTR) was 14 days in 13 (15%) sputa and 28 days in 70 (85%) sputa, which leads to a time saving of 56 days in comparison with the IPM. TTR depending on AFB score (Table 2) shows that 95% (19/20) of 1+ specimens were positive at day 28 and 81% (51/63) of more than 1+ specimens were positive at day 28. There was no statistical correlation between TTR and AFB score ($p = 0.24$).

Discussion

This study demonstrated high sensitivity and specificity of the NRA, relative to IPM, in the identification of resistance of MT to INH (sensitivity: 92.6%; specificity: 98.2%) and RIF (sensitivity: 90%; specificity: 97.3%). Regarding the results, this study demonstrates the potential usefulness of NRA as a susceptible and specific screening tool, especially for the detection of INH and RIF resistance. This is essential because RIF and INH are the most valuable anti-TB agents. In addition, RIF resistance is mostly combined with INH resistance [12]. So, the NRA can be used as a marker of multidrug resistance in low-outcome countries, and clinicians can be highly confident of a diagnosis of INH or RIF resistance by this technique.

However, the sensitivities in detecting resistance to STR and EMB were far too low to be acceptable (52.9% and 28.6%, respectively), whereas the specificities (abilities to find true drug susceptibility) were excellent (100%) for both drugs. This seemingly systematic discordance might be explained by the difficulty of achieving the phenotypic tests for STR and EMB admitted by several studies even by recommended standard methods [13]. The poor sensitivity of EMB can also be explained by the small number of resistant samples on which the sensitivity was calculated.

Musa et al. [13] found sensitivities for INH, RIF, STR and EMB, respectively, of 93%, 100%, 76% and 55%. Specificities were about 100% for INH, RIF and STR and 99% for EMB, whereas, Solis et al. [14], who worked only on RIF and INH, showed 100% of specificity and, respectively, 93.5% and 99.1% of specificity. These results were consistent with the

Table 1 – Sensitivity, specificity, PPV, NPV and agreement of the NRA compared with those of the IPM for *M. tuberculosis*.

		Nitrate Reductase Assay		Sensitivity	Specificity	PPV	NPV	Agreement
		S	R					
STR	S	66	0	52.9%	100%	100%	89.2%	90.4%
	R	8	9					
INH	S	55	1	92.6%	98.2%	96.2%	96.5%	96.4%
	R	2	25					
RIF	S	71	2	90%	97.3%	81.8%	98.6%	96.4%
	R	1	9					
EMB	S	76	0	28.6%	100%	100%	93.8%	94%
	R	5	2					
Total	S	268	3	73.8%	98.9%	93.8%	94.4%	94.3%
	R	16	45					

R: Resistant; S: Susceptible; VPP: positive predictive value; NPV: negative predictive value; STR: streptomycin; INH: isoniazid; RIF: rifampicin; EMB: ethambutol.

Table 2 – Time to result depending on AFB score.

AFB	D14	D28	TOTAL
1+	1	19	20
2+	7	24	31
3+	5	27	32
Total	13	70	83

D: Day.

majority of studies applied directly on sputa [13–16]. The results of studies on this type of sampling are worse than those made on MT strains because of the amount of bacilli in the sputum, which is significantly lower than that obtained after culture. Also, freezing sputum could induce an alteration of the bacterial wall which precludes its growth [17].

Agreement for INH and RIF was about 96.4%, 94% for EMB and 90.4% for STR. The recommendations of Laszlo et al. [18] permit researchers to judge the quality of their work. This study proposes as agreement rate targets of 99% RIF, 97% for INH and 92% for STR and EMB. Only EMB satisfies this criterion in the present work. Corrective measures should be conducted to improve these results, such as increasing the number of resistant samples and performing the IPM and the NRA in the same conditions.

In this study, results were obtained, at the latest, in 28 days. This represents a great advantage, since indirect methods require on average of 37 days (extremes: 27–51 days) for primary isolation prior to performing the DST, which needs 45 additional days for the final results. NRA leads to a time savings of 56 days in comparison with the IPM. Nevertheless, the majority (85%) of positive results were obtained at D28 and only 15% at D14. Further reductions in TTR are possible if another reading is taken at D21, as suggested in the original protocol [19]. This parameter will be considered in later studies.

As shown in Table 2, most sputa were positive at D28 independently of their AFB score, which is not coherent with the literature which found a proportional relationship between TTR and AFB scores [13,20].

Conclusion

In addition to its rapidity, the NRA has other obvious benefits that would facilitate its institution in resource-poor countries. Specifically, it requires very little training, because the method differs only slightly from the conventional method for DST on LJ medium. Furthermore, this method uses only inexpensive and easily obtained reagents, does not require maintenance of any specialized equipment, and requires minimal laboratory space and staffing. In light of the results of this study, it is believed that the NRA might then be used either as a rapid screening tool alone or in combination with other methods, especially in detecting INH and RIF resistance and so in detecting MDR strains.

Conflict of interest

None declared.

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