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Nitrate Reductase Assay Using Sodium Nitrate for Rapid Drug Susceptibility Testing of *Mycobacterium tuberculosis* Directly on Sputum Samples

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Abstract

Multidrug-resistant tuberculosis is an increasing public health concern in many parts of the world, especially in low-income countries, where most cases occur. Traditional drug susceptibility testing is either time-consuming, such the proportion method on solid media, or expensive, such as the BACTEC 960 System. The aim of this study was to evaluate a nitrate reductase assay (NRA) using sodium nitrate ($NaNO_3$) on smear- positive sputum for the detection of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) and compared it with the nitrate reductase assay using potassium nitrate (KNO_3) and Proportion Method (PM) or Direct Proportion Method (DPM). The NRA-$NaNO_3$ results were compared with other methods for 91 sputum samples for which comparable results were available. The sensitivity (ability to detect true drug resistance) and specificity (ability to detect true drug susceptibility) of the NRA- $NaNO_3$, were 100% and 96%, 93% and 100%, 85% and 98%, and 76% and 97% for Rifampin, Isoniazid, Streptomycin and Ethambutol, respectively. The results were in most cases available in 10 days. NRA-$NaNO_3$ is simple to perform and provides a rapid, accurate, especially in low-income countries and might become alternative to traditional methods.

Keyword: Nitrate reductase assay, Sodium nitrate, *Mycobacterium tuberculosis.*

1. Introduction

Tuberculosis (TB) remains one of the major causes of morbidity and mortality from infection in humans. The World Health Organization (WHO) estimates that one third of the world population is infected with Mycobacterium tuberculosis, 9.4 million new cases of tuberculosis and 1.3 million deaths from tuberculosis occurring worldwide. The worldwide incidence is 140 cases per 100.000 population (WHO, 2010).

The emergence of multidrug-resistant (MDR) tuberculosis, defined as tuberculosis caused by strains resistant to the two first-line drugs (Isoniazid and Rifampin), and extensively drug-resistant (XDR) tuberculosis, defined as tuberculosis caused by strains resistant to the two above mentioned drugs, to at least one fluoroquinolone, and to at least one of three injectable second-line drugs (Amikacin, Kanamycin and Capreomycin) (Bwanga *et al*., 2009).

Data from more than 100 countries collected during the last decade show that 5% of all TB cases have MDR-TB. There were an estimated 500,000 new MDR-TB cases in 2007. Twenty- seven countries accounted for 85% of all MDR-TB cases. The top five countries with the largest number of MDR-TB cases are India, China, the Russian Federation, South Africa and Bangladesh, while XDR-TB has been found only in 58 countries to date (WHO, 2010).

Tuberculosis is one of the most important health problems worldwide. For this reason, the rapid diagnosis of TB drug resistance is a priority to avoid the spread of resistant strains (Palomino, 2005). There are different methods for detection of TB drug-resistance.

The BACTEC radiometric system has the advantage of being more rapid (5-10 days), but requires the use of radio-isotopes and can be costly to be performed routinely. Commercial tests (MGIT, E-Test) and molecular tools (INNO –LIPA) have been proposed, but are expensive and also impractical for routine use (Lemus *et al.*, 2004; Palomino, 2005).

For developing countries, it would be useful to have a simple and inexpensive test that could rapidly detect drug-resistant *M. tuberculosis* strains. Several methods have been reported, including colorimetric methods that use redox indicators (MTT and resazurin) and phage amplification technology (Martin *et al.*, 2003; Simboli *et al.*, 2005).

Conventional tests for the detection of drug resistance require several weeks to yield results. Recently, alternative rapid methods have been developed (Solis *et al.*, 2010). Among them, the colorimetric nitrate reductase assays (NRA), based on the ability of *M. tuberculosis* to reduce nitrate to nitrite,

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has been successfully applied on solid medium. This indirect method result in less than 14 days but requires an initial 3 to 4 weeks for cultivation of the isolate (Coban *et al.,* 2004). Another conventional method is proportion method (PM) or Direct Proportion Method (DST) for mycobacterial drug susceptibility testing requires several weeks of incubation to give results (Canetti *et al.,* 1989).

The aim of the present study was to comprise performance of a direct NRA, PM and using sodium nitrate ($NaNO_3$) with clinical sputum samples instead of bacterial isolates in determining the susceptibilities to rifampin (RIF), isoniazid (INH), streptomycin (STR), and ethambutol (EMB) of *M. tuberculosis* strains in microscopy-positive clinical samples from patients with pulmonary tuberculosis.

2. Materials and Methods

2.1. Specimen Processing

From February to August 2012, a total of 100 smear-positive sputum samples from new and treated patients, with positive score of 1 + or more (>1 acid-fast bacillus-AFB) per field (WHO, 1998), were collected at the tuberculosis chest disease clinic in Basrah city. The samples (one per patient) were processed using the Modified Petroff Digestion Decontamination (WHO, 1999). The sediment was re-suspended in 1ml of sterile distilled water, and portions were plated onto NRA drug susceptibility testing medium and into a Lowenstein Jensen (LJ) tube without nitrate, which was later used for the Indirect Proportion Method (IPM)

2.2. Direct NRA Drug Susceptibility test (by using KNO_3)

The NRA was performed as described previously by Musa *et al.*(2005). We used standard LJ medium with 1.000 µg of KNO_3/ml and with or without Rifampin (RIF). For LJ medium with RIF, the critical concentration of 40µg/ml was used. Before NRA, part of the decontaminated suspension was diluted 1:10 in sterile distilled water. For each specimen, 0.2 ml of the undiluted suspension was inoculated into LJ medium containing KNO_3 and RIF, and 0.2 ml of the 1:10 dilution was inoculated into four drug-free LJ medium tubes containing KNO_3. The tubes were incubated at 37ºC.

The assay was performed as described previously by Angeby *et al.* (2002). After 10 days of incubation, 0.5 ml of freshly prepared reagent mixture (1 part 50% concentrated hydrochloric acid, 2 parts 0.2% sulfanilamide, and 2 parts 0.1% n-1-naphtyl-ethylenediamine dihydrochloride) was added to one drug-free tube. If any color appeared, the tube with RMP was developed with the reagent mixture. Otherwise, the other tubes were re-incubated, and the procedure was repeated at day 14 , day 18, and finally at day 28. An isolate was considered to be resistant if there was a color change in the RMP tube equal or greater than that in the 1:10-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 1:10-diluted growth control. 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.

2.3. Direct Proportion Method (DST) or Proportion Method (PM)

The technique was carried out on normal LJ medium according to the laboratory standard procedure (Canetti, 1993). The medium was prepared in 7-ml portions in 150-by-155 mm glass tubes with rubber plugs, with or without antimicrobial agents incorporated. Critical concentrations of antituberculosis drugs were the same as were used for NRA. The critical proportion values were 10% for RIF and STR and 1% for INH and EMB. For each strain, part of the suspension was diluted 1:100, and 0.2ml of the dilution was inoculated into two tubes of LJ medium without antibiotics. Then, 0.2ml of the undiluted suspension was inoculated into the tubes containing LJ medium with antibiotics. The tubes were incubated at 37ºC. Final susceptibility results were reported after 40 days following the laboratory standard procedure, but preliminary results could be reported earlier for resistant strains, sometimes as early as after 20 days.

2.4. Direct NRA by using Sodium Nitrate (NaNO3)

The method is similar of direct NRA drug susceptibity test but using sodium nitrate ($NaNO_3$) in replacement of potassium nitrate (KNO_3) (Maira *et al.*, 2012).

2.5. Quality

Internal quality control was done using the fully susceptible *M. tuberculosis* H37Rv and Known MDR *M. tuberculosis* isolate.

2.6. Statistical Analysis

In the present study, the term sensitivity reflects the ability to detect a true drug resistance in a strain, whereas specificity reflects the ability to detect a true drug susceptibility. Statistical analysis of data was carried out by using SPSS analysis (Moore, 2000).

3. Results

One hundred sputum samples of *M. tuberculosis* were analyzed by the Direct NRA-KNO_3, Direct NRA-$NaNO_3$ and DST methods. Table 1 shows the results obtained with Direct NRA-KNO_3 compared to DST method using sputum samples. The smear results for AFB were positive with more than 10 AFB per field (+++). Of the 100 smear microscopy-positive results, 9 had negative growth control as determined by the NRA method and could thus not be used in the comparison. Then, 91 sputum samples could be used for the comparison between three methods.

In table 1, for RIF, 60 isolates were found resistant and 24 susceptible by both methods. For INH, 64 isolates were resistant and 22 susceptible by both methods; four strain gave a discordant result being susceptible by DST method. For STR , 67 isolates were resistant and 20 susceptible by both methods; three isolates were susceptible by DST but resistant by NRA-KNO_3. In other hand, for EMB, 67 isolates were

resistant and 18 susceptible by both methods; 4 isolates were susceptible by DST but 2 isolates susceptible by NRA-KNO_3. The results were available in 10 days for 11 samples, in 14 days for 45 samples, and in 18 days for 35 samples.

Table 2 shows the sensitivity and specificity obtained with NRA using $NaNO_3$ and KNO_3 compared to the DST method. Drug susceptibility testing for RIF showed a sensitivity of 93% with KNO_3 and 100% with $NaNO_3$, but specificity was 96% for both nitrate sources. For INH the sensitivity was 90% with KNO_3 and 93% with $NaNO_3$ while the specificity was 97% and 100%. For STR the sensitivity was 80% with KNO_3 and 85% with $NaNO_3$ while the specificity was 94% and 98%. In addition, for EMB the sensitivity was 71% with KNO_3 and 76% with $NaNO_3$ while the specificity was 90% and 97%.

Figure 1 shows the comparison of three methods are NRA- $NaNO_3$, NRA-KNO_3 and DST.

Table 1. Comparison of the susceptibility results, sensitivityand specificity to the Direct NRA method by using KNO_3,$NaNO_3$ and DST for *M. tuberculosis* in sputum samples.

Drug	Direct proportion method (DST) determination	Direct NRA by using KNO_3			
		NO.		%	
		R	S	Sensitivity	Specificity
RIF	R	60	5	93	-
	S	2	24	-	96
INH	R	64	4	90	-
	S	1	22	-	97
STR	R	67	3	80	-
	S	1	20	-	94
EMB	R	67	4	71	-
	S	2	18	-	90
Total	R	258	16	80	-
	S	6	84	-	97

R= Resistant ; S= Susceptible

Sensitivity = reflects the ability to detect (true resistant).

Specificity = reflects the ability to detect (true susceptibility).

Table 2. Sensitivity and specificity of the NRA using KNO_3 and $NaNO_3$ compared to the DST method for *M. tuberculosis* in sputum samples.

Drug	Direct proportion method (DST) determination	NRA – KNO_3				NRA- $NaNO_3$			
		No		%		NO		%	
		R	S	Sensitivity	Specificity	R	S	Sensitivity	Specificity
RIF	**R**	60	5	93	-	61	3	100	-
	S	2	24	-	96	2	25	-	96
INH	**R**	64	4	90	-	65	5	93	-
	S	1	22	-	97	1	20	-	100
STR	**R**	67	3	80	-	63	4	85	-
	S	1	20	-	94	2	22	-	98
EMB	**R**	67	4	71	-	60	6	76	-
	S	2	18	-	90	1	24	-	97
Total	**R**	258	16	80	-	250	18	86	-
	S	6	84	-	97	6	91	-	98

R = Resistant ; S = Susceptible

Sensitivity = reflects the ability to detect true resistant.

Specificity = reflects the ability to detect true susceptibility.

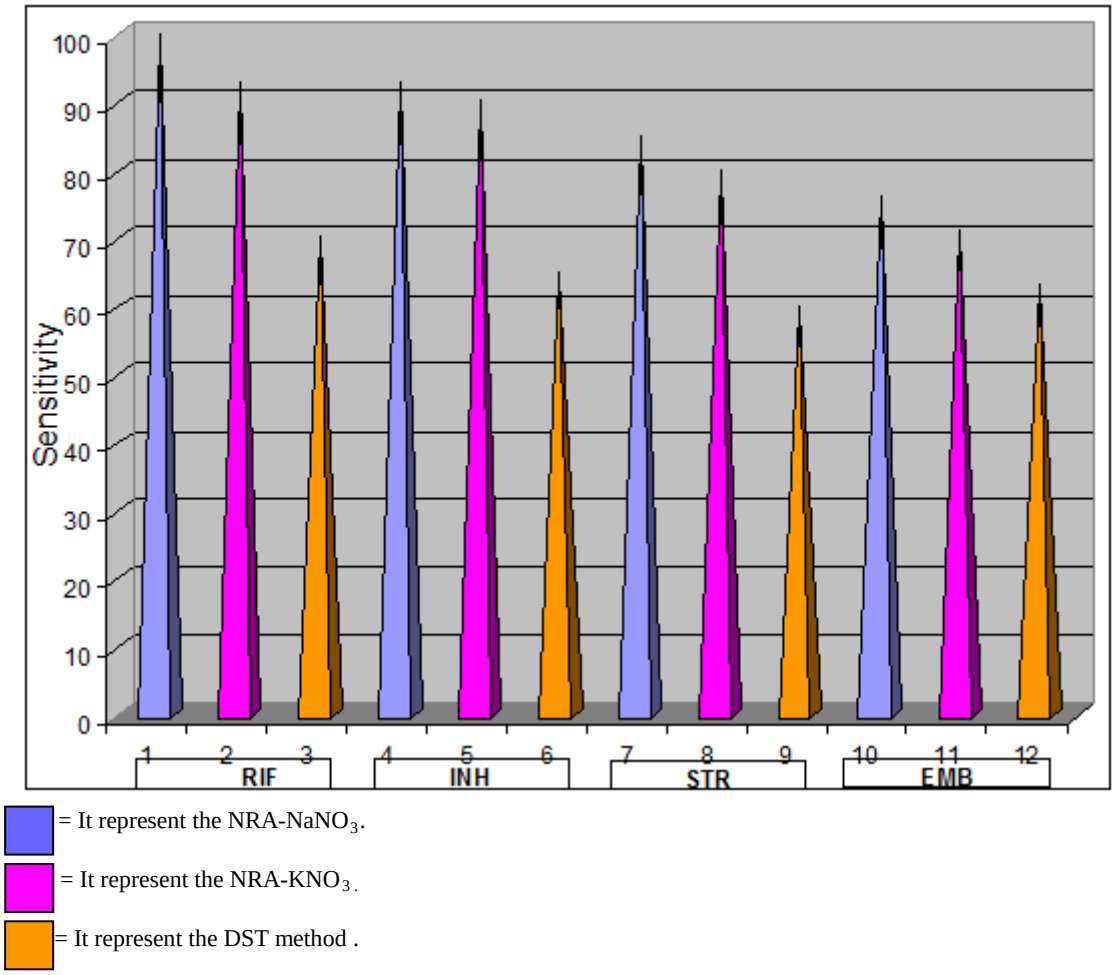

Figure 1. Results the sensitivity of NRA using KNO_3, $NaNO_3$ compared to DST method.

4. Discussion

To our knowledge, this is the first evaluation of the direct NRA in Iraq. The most worrisome trend during recent years is an increase in multidrug-resistant MDR (for example resistant to RIF and INH) TB strains. Rapid detection of MDR strains is very important to restrict their spread in the population. Current method for DST of MDR-TB are either costly or very slow. So, a cost-effective and rapid drug-susceptibility method is required to guide the treatment of TB (Coban *et al.*, 2004 ; Mishra *et al.*, 2009). Complete agreement between the results of the direct NRA and DST method was found for RIF, which is important since RIF, together with INH, is the most important antituberculosis drug. Resistance to RIF is also almost always associated with multidrug resistance (Vareldzis *et al.*,1994) and can thus serve as a marker of MDR of *M. tuberculosis* strains if resources are limited.

The direct NRA was comparable to the direct DST method regarding susceptibility testing of INH (sensitivity to detect resistant was 90% with specificity was 97%). In addition, the sensitivity to detect resistance to STR and EMB were low to be acceptable (80% and 71%), but the specificity were 94% and 90% respectively. Results for RIF and INH susceptibility were similar to indirect NRA method (Sethi *et al.*, 2004 ; Musa *et al.*, 2005). These results may be need to adjusting the critical drug concentrations used in the NRA test, although the susceptibility of *M. tuberculosis* to STR and EMB is more complicated to determine the antibiotic sensitivity (Maira *et al.*, 2012) .

The NRA method utilizes the detection of nitrate reduction as an indication of growth, and therefore, results can be obtained faster than by visual detection of colonies. The ability to reduce nitrate is typical for *M. tuberculosis*, although some other mycobacterial species, like *Mycobacterium kansasii*, and most rapid growers share this characteristic, nitrate reductase-negative strains of *M. tuberculosis* are rare (< 1%)(Rosales *et al.*, 2009).

In the other hand, this study showed that the NRA gave similar results using KNO_3 or $NaNO_3$ as nitrate source. NRA using $NaNO_3$ showed high sensitivity and specificity for RIF (100% and 96%) and INH (93% and 100%). These results are in agreement with previous studies presented in a meta-analysis that evaluated the accuracy of the NRA for the detection of MDR .

According to that meta-analysis; the sensitivity and specificity were more than 94% and 92% for RIF and INH (Maire *et al.*, 2012). Another important finding in this study was that 97% of the isolates showed results in 10 days with NRA using $NaNO_3$ whereas 88% of the isolates gave results in 10 days with NRA using KNO_3 in the previous studies (Coban *et al.*, 2004). Our study suggests the use of $NaNO_3$ as the source of nitrate for NRA.

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