Original Article

Drug susceptibility test of *Mycobacterium tuberculosis* by nitrate reductase assay

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Abstract

Background: Drug susceptibility testing for *Mycobacterium tuberculosis* (*M. tuberculosis*) is especially required in difficult cases of tuberculosis (TB) chemotherapy and in cases of multidrug resistance (MDR-TB; combined resistance to isonizid and rifampicin with or without resistance to any other drug). The methods for *in vitro* cultivation and drug susceptibility testing (DST) of *M. tuberculosis* are cumbersome and not readily adaptable in most routine laboratories, particularly those in the developing world due to limited resources and lack of political will in those countries. A simple and cost effective method, the nitrate reductase assay (NRA), was compared with the gold standard proportion (egg bases Lowenstein Jensen's [LJ]) method for DST of *M. tuberculosis* in order to substantiate its suitability for routine use in Nigeria and in other countries of the developing world with high TB endemicity.

Method: Drug susceptibility test was performed for 70 pulmonary isolates of *M tuberculosis* (Indirect DST) and 20 sputum (10 acid fast bacilli [AFB] positive and 10 AFB negative) specimens (direct DST) by the NRA and the proportion method using 0.2µg isoniazid (INH), 2µg ethambutol (EMB), 40 µg rifampicin (RIF) and 4 µg streptomycin STR).

Results: The indirect NRA showed sensitivity and specificity for INH: 100% and 100%, EMB: 75% and 100% RIF: 90% and 96.6%, STR: 66.6% and 91.8%. The results of direct NRA and proportion method for INH, EMB RIF and STR agreed 10/10 (100%) for AFB negative specimens and 9/10 (90%) with AFB positive specimens.

Conclusion: Drug susceptibility test of *M. tuberculosis* by the NRA is simple and sensitive with shorter turn around time of 10 to 1 4 days compared to 42 days by the LJ proportion method. The direct use of AFB positive sputum specimens is likewise reproducible and excludes about 3 – 8 weeks period required for isolation of *M. tuberculosis.*

Key Words: *Mycobacterium tuberculosis*, drug susceptibility test, proportion method, nitrate reductase assay

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Introduction

Reports of *M. tuberculosis* resistant to antituberculosis (TB) drugs are increasing globally and routine laboratories are becoming increasingly aware of the need of drug susceptibility testing (DST), especially for treatment failures, proper therapeutic monitoring, and documentation purposes. In Sub-Saharan Africa where co-infection by *M. tuberculosis* and the human immunodeficiency virus (HIV) is associated with high morbidity and mortality, the prevailing high rates of multidrug resistant tuuberculosis (MDR-TB) have become a concern for global intervention [1] thus highlighting the need for effective treatment and control of TB through early diagnosis and exclusion of MDR-TB.

Drug susceptibility testing for *M. tuberculosis* is a difficult procedure. The slow-growing nature of the bacillus by conventional methods (about three to eight weeks for isolation and 28 to 42 days for susceptibility tests) [2-3], the technological expertise required, and the high cost of the nonconventional automated and molecular techniques which have shorter turnaround times (1- $\geq$ 21 days) [3-4] are some reasons that most routine laboratories are unable to perform DST for *M. tuberculosis*. Comprehensive studies on *M. tuberculosis* in Nigeria are therefore scarce and published reports are scanty. A study on drug susceptibility testing in the southwestern region of the country reported an overall resistance of 53% [5]. While this prevalence may be rated as high, more studies are needed for appropriate assessment of MDR-TB in Nigeria. Less expensive and

simple DST methods for *M tuberculosis* are ideal and highly relevant for use in resource-limited laboratories and nations with high TB endemicity. Studies on the use of the nitrate reductase assay (NRA), a simple and inexpensive technique based on the characteristic ability of *M. tuberculosis* to reduce nitrate to nitrite, have been reported and the results were found comparable to gold standard methods [6-8].

This study is the first to be conducted in Jos, Plateau State, North Central region of Nigeria. Seventy pulmonary isolates of *M. tuberculosis* and 20 sputum samples (10 acid fast bacilli [AFB] positive and 10 AFB negative), all from patients diagnosed for pulmonary tuberculosis in Jos, Nigeria, were examined for susceptibility to first-line anti tuberculosis drugs (INH, EMB RIF and STR) by NRA and the gold standard proportion method.

Materials and Methods

Specimens

The NRA was performed on a total of 70 isolates of *M. tuberculosis* and 20 sputum specimens (10 AFB positive and 10 AFB negative). The isolates and sputum specimens were from cases reported for pulmonary TB in four TB centres in Jos, Nigeria, between August and December 2006.

Sputum samples were treated in cetyl pyridinum chloride (CPC) for digestion and decontamination, then washed in sterile distilled water by centrifugation at 3,500 g for 10 minutes to remove CPC. Centrifugation was repeated and the supernatant decanted. The deposit was resuspended in 1 ml distilled water and prepared ready for Mycobacterial isolation on LJ medium [3] or for direct DST on drug-containing LJ slopes [3]. Isolates phenotypically identified as *M. tuberculosis* [3] were prepared ready for DST by the NRA and proportion methods.

Drug susceptibility test (DST)

First-line anti tuberculosis drugs, comprised of 0.2µg INH, 2µg EMB, 40µg RIF and 4µg STR, were incorporated into LJ medium for the proportion method or LJ medium containing 1000 µg potassium nitrate for the NRA, and then steamed at 80^0 C for 50 minutes. After preparation, the media were incubated for 48 hours at room temperature for sterility check before use.

DST by proportion method

Bacterial suspensions for drug susceptibility testing were made by scraping about 4 mg of freshly grown *M. tuberculosis* colonies from LJ medium using a 3 mm internal diameter (24 standard wire gauge [SWG]) wire loop, into 500 µl of sterile distilled water in a bijou bottle with 5 glass beads, then vortexed for about 30 seconds to homogenise. The suspensions (S1-S4) were made up to 4 ml volume by adding 3.5 ml sterile distilled water and allowed to settle for about 30 minutes before gently aspirating the upper portion (1mg/ml) into a fresh bijou bottle (S1 suspension). S1 was further diluted 10 fold to obtain S2-S4.

S1-S4 bacterial concentrations were respectively inoculated into drug-free and drug-containing LJ slopes using a 3 mm internal diameter wire loop and incubated at 37^0 C.

Growth was recorded at 28 days and at 42 days as follows: +++ for confluent growth, ++ for more than 100 colonies, and 1-100 actual number of colonies. Susceptibility or resistance was recorded when the proportion of bacteria in drug-containing medium to that of drug free medium is < 1 or ≥ 1 respectively.

DST by NRA (indirect)

Bacterial suspension for NRA was treated as the proportion method but sterile phosphate buffered saline (PBS) instead of distilled water was used as diluent. The NRA was performed as previously described [7] with slight modifications. In brief, S1 bacterial suspension was inoculated into each drug containing LJ slope while 1/10 dilution of S1 in PBS was inoculated into paired drug-free LJ slopes and incubated at 37^0 C for 10 to 14 days.

DST by NRA (Direct)

Deposits from CPC treated sputum samples diluted to 1:5 and 1:10 with sterile PBS were respectively inoculated onto each of the drug-containing and paired drug-free NRA LJ slope and incubated at 37^0C for 10 to 14 days.

After 10 days' incubation for the indirect and direct NRAs, 50 µl mixture of freshly prepared solutions of 1% ethylene diamine dihydrochloride (50 µl), 2% sulphanilamide; (50µl) and concentrated hydrochloric acid; (25 µl), was added to one of each paired drug-free LJ slopes and observed for reddish/violet coloration on the surface of the slants indicative of a positive NRA and growth of *M. tuberculosis* or absence of colouration as negative NRA.

The drug-containing LJ slopes corresponding to the drug-free and positive NRA were likewise treated and observed for colour indication of positive NRA (drug resistance) or negative NRA (drug susceptible).

The remaining drug-free and drug-containing slopes with negative NRAs at 10 days were incubated further for 14 days and treated as above (Figure 1).

Figure 1. NRA showing resistance in INH, RIF (MDR-TB) and STR. From Left to Right: Drug-free 02 µg INH, 2 µg EMB, 40 µg RIF and 4 µg STR (LJ slopes). Drug-free, INH, RIF and STR = positive NRA, EMB=Negative NRA.

Results

The results of drug susceptibility testing with *M. tuberculosis* isolates showed susceptibility in 88.6% INH, 94.3% EMB; 85.7% RIF and 87.1% STR by the proportion method and 88.6% INH, 95.7% EMB, 84.3% RIF and 84.3% STR by NRA. The sensitivity (ability to detect true resistance) and specificity (ability to detect true susceptibility) for NRA is 100% and 100%, 75% and 100%, 90% and 96.6%, and 66.6% and 98.2% for INH, EMB, RIF and STR respectively (Table 1). MDR-TB was detected in a total of 8/70 (11.4%) by both proportion method and NRA. Resistance occurred in all four drugs in 2/70 (2.9%) by proportion method and 3/70 (4.3%) by the NRA.

Table 1. Comparison of drug susceptibility testing of *Mycobacterium tuberculosis* by proportion method and nitrate reductase assay.

Drug		RES	SUS	Sensitivity (%)	NPV (%)	Specificity (%)	PPV (%)
INH	RES	7	0	100	100	100	100
	SUS	0	62				
EMB	RES	3	1	75	98.5	100	100
	SUS	0	66				
RIF	RES	9	1	90	98.3	96.6	81.8
	SUS	2	58				
STR	RES	8	4	66.6	93.4	98.2	88.8
	SUS	1	57				

NRA- Nitrate reduductase assay, Res= Resistant, Sus= susceptible, INH= isoniazid, EMB=Ethambutol, RIF= Rifampicin, STR= Streptomycin, PPV= Positive predictive value, NPV= negative predictive value Prop= proportion method.

All 10 AFB positive sputum samples inoculated directly on nitrate reductase media showed positive indication of growth in the drug-free medium and the results of drug susceptibility testing concurred 100% with the proportion method in each case for INH, EMB and RIF, and in 9/10 (90%) for STR only. The AFB negative sputum samples showed no evidence of Mycobactrial growth in the plain drug-free and nitrate reductase LJ slopes.

Discussion

In our experience, the DST of *M. tuberculosis* by NRA using culture isolates or by direct inoculation of AFB positive sputum is simple and reproducible. Young *M. tuberculosis* colonies yielded results in about 10 days compared to older colonies requiring 14 or more days. Though the total sample size for this study is small, the results obtained compared with previous reports and supports the use of DST, particularly for INH and RIF [8].

The shorter turnaround time (10 to 14 days) is an advantage over the proportion method, which requires ≥ 28 days for culture and 42 days for DST, while the direct NRA excludes a period of 3 to 8 weeks of mycobacterial cultivation. The simple, cost effective and rapid profile of the NRA is suitable for large-scale surveillance studies in resource-limited settings.

Reports on drug susceptibility testing by NRA are generally favourable for INH and RIF (8-11). Noting that both drugs are major first-line anti-tuberculosis agents and considering that rifampicin is a surrogate marker for MDR- *M. tuberculosis* (12-13), the NRA could be used to screen for resistance to both drugs to enable prompt assessment of MDR prevalence particularly in highly endemic regions.

The rather low sensitivity rates for EMB and STR observed in this study require further evaluation. Previous studies reporting similar findings have suggested a review of the drug concentrations [8-10]. Considering that streptomycin is generally used for the treatment of other clinical conditions and therefore subject to abuse, the NRA needs to be adequately controlled and doubtful results confirmed by a gold standard method.

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Conflict of interest: No conflict of interest is declared.