Original Research

Comparative evaluation of *Mycobacterium tuberculosis* drug susceptibility testing between Direct Nitrate Reductase assay and Direct Proportion Method

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Received June 22, 2016. Accepted July 6, 2016

Abstract

Background: Drug resistance – multi-drug resistant (MDR) and extensive drug resistant (XDR) in tuberculosis (TB) is a matter of great concern for TB control programs. There is concern and need for early diagnosis of these multi-drug-resistant strains for better treatment.

Objective: To compare the usefulness of Nitrate Reductase Assay (NRA) and direct proportion as a tool for rapid and accurate detection of resistance to first line anti-tubercular drugs.

Material and Methods: A total of 120 sputum-positive AFB smears of pulmonary tuberculosis (PTB) patients were selected for the study. The samples were processed by NRA and direct proportion for assessment of drug susceptibility testing to first line anti-tubercular drugs.

Results: The sensitivity of NRA was 100%, 92.30%, 81.81%, and 72.72 % for RIF, INH, EMB, and STR, respectively. The specificity of NRA was 100%, 100%, 96.33%, and 89.79% for RIF, INH, EMB, and STR, respectively. The performance of NRA susceptibility testing was rapid and the median time of obtaining results was shorter using NRA (10 days) as compared to PM (28 days).

Conclusion: Direct NRA is simple, easy to perform, rapid, relatively less expensive, without requirement of expensive reagents and sophisticated equipments. It is useful tool suitable for early determination, first line anti-tubercular drugs namely rifampicin, isoniazid, ethambutol, and streptomycin with excellent concordance for INH and RIF resistance and relatively low accuracy for streptomycin, with good sensitivity and specificity.

KEY WORDS: Tuberculosis, multidrug resistant, Nitrate Reductase Assay

Introduction

Tuberculosis (TB) has been a major cause of suffering and death. India is the highest TB burden country in the world and accounts for nearly one-fifth (21%) of global burden of TB. Early diagnosis of TB and initiating optimal treatment with chemotherapy via multidrug therapy is recommended.[1] Drug resistance – multi-drug resistant (MDR) and extensive drug resistant (XDR) in TB is a matter of great concern for TB control programs since there is no cure for some multidrug-resistant TB strains of *Mycobacterium tuberculosis*.[2] There is concern that these strains could spread around the world, stressing the need for additional control measures, such as new diagnostic methods, better drugs for treatment, and a more effective vaccine. Hence there is need for diagnosis the resistant strains early. Hence, among different methods for detection of TB drug resistance, the usefulness of Nitrate Reductase Assay (NRA) and direct proportion as a tool for rapid and accurate detection of resistance to first line anti-tubercular drugs was compared.

Access this article online	
Website: http://www.ijmsph.com	Quick Response Code:
DOI: 10.5455/ijmsph.2017.22062016563	

Material and Methods

The prospective study was conducted at our institute after the ethical clearance. All sputum positive (3+) according to RNTCP (Revised National Tuberculosis Control Programme)[3] grading of AFB smears of pulmonary tuberculosis (PTB) patients were included in the study. Patients with HIV-TB, co-infection, and other immune-compromised patients as evident from history, clinical examination and investigations were excluded from the study.

After taking written consent and noting of demographic details, the sputum samples of the patient were subjected to microscopy by ZN (Ziehl–Neelsen) stain and graded according to the RNTCP guidelines.[3] Those samples showing 3+ positivity for AFB on smear were taken and subjected to digestion and decontamination by using Modified Petroff's method.[4] The sediment obtained after Modified Petroff's method was re-suspended in 3 ml of sterile distilled water. Out of 3 ml suspension, 1.5 ml was used for direct NRA and remaining 1.5 ml for the direct proportion method.

Direct NRA

The NRA was performed as described by Musa et al.[5] Of the 1.5 ml suspension, 0.2 ml was inoculated into four tubes of Lowenstein–Jensen (LJ) medium each, containing potassium nitrate (KNO_3 – 1000 μg/ml) and anti-tubercular drugs of specific concentration. The critical concentrations were 0.2, 40, 4.0, and 2.0 μg/ml for INH (Isoniazid), RIF (Rifampicin), STR (streptomycin), and EMB (ethambutol), respectively. Remaining 0.7 ml suspension was diluted 1:10 with sterile distilled water, from which 0.2 ml was used to inoculate 3 tubes of drug free LJ media with KNO_3 to serve as growth control. All inoculated tubes were incubated at 37°C. The tubes were observed daily for up to 7 days to rule out bacterial contamination and rapidly growing mycobacteria.

On 10th day, 0.5 ml of Griess reagent was added to one drug-free control tube to observe any color change (strong or weak pink). If color change occurred in the control tube, Griess reagent was added to drug containing tubes and susceptibility results were read. If there was no color change in the control tube, the remaining control and drug containing tubes were kept back for further incubation. The same procedure was repeated at day 14 and if needed at day 18 using the last, i.e., the 3rd growth control tube.

An isolate was considered resistant to a particular anti-tubercular drug if there was a color change in the drug containing tube in question greater than that in the 1:10 diluted growth control tube observed on the same day. If there was no color change, then the drug was considered susceptible.

Direct Proportion Method (PM)

The technique was carried out on LJ medium according to the standard laboratory's procedure as given by Canetti et al.[6] Of 1.5 ml suspension, 0.2 ml was inoculated into each tube containing LJ medium with anti-tubercular drugs. The remaining 0.7 ml suspension was diluted in 1:100 and 0.2 ml of this dilution was inoculated into two tubes of LJ medium without anti-tubercular drug. It was labeled as a control medium. The tubes were incubated at 37°C. The first reading was taken on 28th day of incubation and the second on 40th day as follows: +++ for confluent growth, ++ for more than 100 colonies, and 1–100 actual numbers of colonies.

Growth was identified on the basis of their cell morphology, growth rate, pigmentation, colony morphology and niacin test.

Statistical analysis was done by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy by taking direct proportion method as the gold standard.

Results

A total of 127 smear-positive (3+) sputum samples were included in the study. Out of 127 smear-positive (3+) sputum samples, 6 samples showed contaminated growth and 1 sample was niacin negative, thus 7 samples were excluded from data analysis and the 120 samples constituted the sample size. The mean age of patients was 32 years (range 11–70 years). Out of 120 patients 87 were male and 33 female.

Direct NRA

All the results were obtained with in eighteen days duration. The results were obtained on 10th, 14th and 18th day in 34 (28.3%), 69 (85%), and 17 (100%) patients, respectively. The mean duration of detection was 13.4 days. Out of 120 samples, RIF showed 15 resistant and 105 susceptible, INH showed 12 resistant and 108 susceptible, EMB showed 13 resistant and 107 susceptible and STR showed 26 resistant and 94 susceptible.

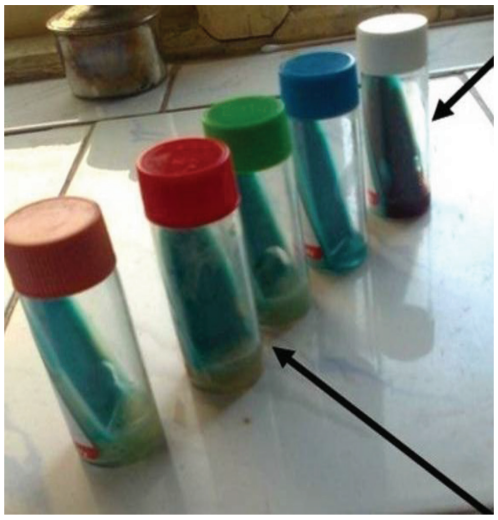

Figure 1: DNA result showing change in color (positive test) no color change (negative result).

Direct Proportion Method

All results were obtained by 40 days with 37(30.83%) samples showing results in 28 days and 83 (100%) samples showing the results in 40 days. The mean duration of detection was 36.3 days. Out of 120 samples, RIF showed 15 resistant and 105 susceptible, INH showed 13 resistant and 107 susceptible, EMB showed 11 resistant and 109 susceptible and STR showed 22 resistant and 98 susceptible.

Overall

For RIF, 15 and 105 strains were detected to be resistant and susceptible, respectively, by both the methods. Thus accuracy, PPV and NPV of NRA for RIF was 100%.

For INH, both the methods detected 12 resistant samples and 107 susceptible samples but 1 sample gave discordant results, being susceptible by the NRA but resistant by the PM. The accuracy, PPV and NPV of NRA for INH was 99.1%, 100%, and 99.07%, respectively.

For EMB, 9 and 105 samples were identified as true positive and true negative, respectively, by both the methods, 6 samples gave discordant results, 2 of which were false negative (i.e., resistant by PM while being susceptible by NRA) and 4 of which were false positive (i.e., susceptible by PM but resistant by NRA). The accuracy, PPV, and NPV of NRA for EMB was 95.5%, 69.23%, and 98.13%, respectively.

For STR, 16 isolates were correctly detected as resistant and 88 as susceptible by both the methods; 16 samples gave discrepant results, 6 of which were false-negative and 10 samples were false positive. The accuracy, PPV, and NPV of NRA for STR was 86.6%, 61.53%, and 93.61%, respectively.

The sensitivity of NRA was 100%, 92.30%, 81.81%, and 72.72% for RIF, INH, EMB, and STR, respectively. The specificity of NRA was 100%, 100%, 96.33%, and 89.79% for RIF, INH, EMB, and STR, respectively.

The overall sensitivity of NRA compared to that of the proportion method was found to be 85.24% (52/61), and the specificity was 96.65% (405/419). There was full concordance in the results of RIF (100%), INH (99.1%), EMB (95.5%), and STR (86.6%). However, 23 samples gave discordant results. The overall accuracy of NRA was 95.2%, and PPV and NPV was 78.78% and 97.82%, respectively (Table 1).

Discussion

TB continues to be a major cause of morbidity and mortality throughout the world. The most worrisome trend during recent years is an increase in multidrug-resistant (i.e., resistant to RIF and INH) TB strains. Rapid detection of MDR strains is very important to restrict their spread in the population. Current methods for drug sensitivity testing (DST) of MTB are either costly or very slow. Hence, a cost-effective and rapid drug susceptibility method is required to guide the treatment of TB.[1,2,7] Comparison between direct the NRA and direct Proportion Method for first-line anti-tubercular drugs was done, i.e. RIF, INH, STR, and EMB in 120 sputum samples (3+).

Musa et al,[5] Mishra,[8] Sethi et al,[9] Gupta et al,[10] and Anamika et al[11] all concluded that on comparing NRA with the standard proportion method for four first line anti-tubercular drugs, the performance of NRA susceptibility testing was rapid and the median time of obtaining results was shorter using NRA (10 days) as compared to PM (28 days). These results are quite similar to our study where it was found that the mean duration of detection was 13.4 days by the NRA method and 36.3 days by direct proportion method the overall delay was 23 days.

RIF and INH are the most important anti-tubercular drugs. Resistance to RIF is almost always associated with multidrug resistance and thus can serve as a marker of

Table 1: Comparison of susceptibility results by direct NRA and direct proportion method

Antitubercular drug	Direct PM	Direct NRA Method			
		No.		%	
		Resistant	Susceptible	Sensitivity	Specificity
RIF	Res	15	0	100	
	Sus	0	105		100
INH	Res	12	1	92.30	
	Sus	0	107		100
EMB	Res	9	2	81.81	
	Sus	4	105		96.33
STR	Res	16	6	72.72	
	Sus	10	88		89.79
Total	Res	52	9	85.24	
	Sus	14	405		96.65

MDR-TB if resources are limited.[12] Hence it is important to know the resistant to RIF. In our study, complete agreement between the results of direct NRA and direct proportion method for rifampicin, and there was a good concordance for INH (99.1%) and EMB (95.5%) as well. However, the concordance for STR was found to be lower (86.6%) in our study. The accuracy of NRA, in our study was higher for all the drugs used, except STR, which was below the standard level, according to the criteria established by the WHO (99.0% and 97.0% for RIF and INH, respectively, and 92.0% for EMB and STR) as reasonable performance goals for reference laboratories. This is because STR is a difficult drug to test even by standard methods. This was also seen with other various studies comparing NRA with the proportion method.[5,9–19] In series by, Gupta,[10] Coban,[13] Anamika,[11] Agatha,[15] and Angeby[17] all had the sensitivity of the STR below the established criteria by WHO i.e below 92%, whereas the series by Sethi et al[9] and Lemus et al[16] had acceptable limit with sensitivity for STR of 100% and 96%, respectively.

Newer methods like real-time PCR for DST are rapid but costly, require equipment and reagents, need skilled technical personnel and are not available everywhere and are restricted to reference laboratories only. Conventional methods like the proportion method are cumbersomeness and take long-turn-around time.

NRA can be performed in the classical LJ medium, routinely used in TB laboratories, with the addition of KNO_3 without need of any sophisticated equipment or expensive reagent, making it a widely used method. Results are easy to observe by a color change in the medium. Some strains (<1%) of MTB lack nitrate reductase and would create false results since the control would be negative and the test would therefore be invalid.[11] However, no such strains were encountered in our study. Secondly, strains of *Mycobacterium bovis* do not reduce nitrate, for which reason the NRA technique is not applicable.[12] Further, the ability to reduce nitrate is typical for *M. tuberculosis*, but some other mycobacterial species, like *M. kansasii*, *M. smegmatis*, also possess this enzyme, but these are not frequently encountered in humans and they can be easily identified by morphological and biochemical tests.[9] A very uncommon limitation is that nitrite might be further reduced to nitric oxide, which cannot be detected by the Griess reagents.

Conclusion

To conclude, direct NRA is simple, easy to perform, rapid, relatively less expensive, without requirement of expensive reagents and sophisticated equipments useful tool suitable for early determination, first line anti-tubercular drugs namely rifampicin, isoniazid, ethambutol, and streptomycin with excellent concordance for INH and RIF resistance and relatively low accuracy for streptomycin, with good sensitivity and specificity with overall accuracy of NRA was 95.2%. Further, extending the scope of utilizing direct NRA for susceptibility testing of second line drugs for *M. tuberculosis* is recommended.

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How to cite this article: Jain N, Jain N, Jain V. Comparative evaluation of *Mycobacterium tuberculosis* drug susceptibility testing between Direct Nitrate Reductase assay and Direct Proportion Method. Int J Med Sci Public Health 2017;6:86-90

Source of Support: Nil, **Conflict of Interest:** None declared.