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Evaluation of Nitrate Reduction Assay as a Simple Procedure for Drug Susceptibility Testing of *Mycobacterium tuberculosis*

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The reliability of nitrate reduction assay (NRA) as a growth indicator of the *Mycobacterium tuberculosis* was evaluated as rapid antimicrobial susceptibility testing (AST). Seventy two isolates were examined to critical concentration of Isoniazid (INH), Streptomycin (STR), Rifampin (RMP), and Ethambutol (EMB). The results of NRA were compared to the results obtained by radiometric method (BACTEC 460), which served as reference method. According to our results, NRA method can be used as reliable, rapid, free radioactive waste, and inexpensive method for testing the susceptibility of *M. tuberculosis* isolates to mentioned antibiotics. Compatibility values between NRA and BACTEC reference method for Isoniazid, Rifampicin, Ethambutol and Streptomycin were 98.6%, 97.2%, 98.6%, and 100% respectively. 94.4% of NRA results agreed with those obtained by BACTEC 460.

Key words: *Mycobacterium tuberculosis*, Antimicrobial susceptibility testing, Nitrate reduction, BACTEC 460. NRA, AST.

Treatment of bacterial infection depends up mainly on its identification and suitability towards appropriate antibiotic. This process should be performed at short time, especially with bacteria which have epidemic dissemination. One of the most risky bacteria in our life is *Mycobacterium tuberculosis*. It cause Tuberculosis which kills 3,000,000 people in the world every year, more than AIDS, malaria, and other tropical disease combined[9]. Tuberculosis is a global epidemic; it affects mainly the poor countries, where 98% of all TB deaths occur[5].

Spread of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* has become a major public health concern, since it often cause incurable disease. MDR defined as TB showing resistance, at least to isoniazid and rifampin. World Health Organization's[12], reported that, 52 countries have a median prevalence of 1.8% (range, 0 to 18.1%) for MDR strains and 11.1% (range 2.9 to 40.8%) for strains with any drug resistance.

The reemergence of tuberculosis and the spread of drug-resistant tuberculosis have emphasized the need for rapid diagnosis. However, the standard culture methods currently in use are quite slow, since detection of mycobacterial growth on conventional Löwenstein-Jensen solid medium requires 4 to 8 weeks.

The time is not only significant threat to the patient, but to the community; health care workers as well. For these reasons, the BACTEC 460 method which is a quick and effective method in identification of MDR strains, but it need costly equipment and substrate; produces radio active

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waste; and is consequently not feasible in most resource-poor settings[2].

Another recently method is the nitrate reductase assay on LJ medium, which is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite. The presence of nitrite can easily be detected with specific reagents that produce a change in color

This method depends on the metabolic pathway of the bacteria, which indicate bacterial growth, depending on the change of this substrate "nitrate".

The aim of this work is to evaluate nitrate reductase as a novel, rapid, low-cost methods for performing DST for Isoniazid "INH", Rifampicin "RMP", Ethambutol "EMP", Streptomycin "STR" on *M. tuberculosis* isolates originating from different areas in Egypt. The results were compared to those obtained by the BACTEC 460.

MATERIALSAND METHODS

Strains

Seventy two clinical isolates of *M. tuberculosis*, from 20 different geographic regions in Egypt were used[1]. All strains were stored at -70°C and cultured on standard Löwenstein-Jensen medium before use.

Drug Susceptibility Test (DST)

Antibiotic stock solutions for INH, RIF, STR, and EMB were prepared in advance and kept at-70°C until use. Drug susceptibility was determined by standard procedures in a BACTEC 460 instrument (Becton Dickinson, Sparks, Md.) in Tuberculosis reference Lab, Cairo, Egypt. The following antibiotics concentrations are used: 0.2 µg/ml for INH, 2.0 µg/ml for RIF, 4.0 µg/ml for STR, and 5.0 µg/ml for EMB[2].

Nitrate Reduction Assay (NRA)

Standard Löwenstein-Jensen medium was used, with 1,000 g of potassium nitrate /ml. and with or without antimicrobials incorporated. The drug concentrations were prepared in Löwenstein-Jensen medium according to Canetti *et al.,*[3] as following: 0.2g/ml for INH, 40 g/ml for RMP, 4.0 g/ml for STR, and 2.0g/ml.

The method described according to Angeby *et al.,*[2] was usedf or NRA testing. 10 ml of Löwenstein-Jensen medium was prepared portions in 75- by 25-mm screw-cap glass tubes. Two 1µl loops of bacteria, from fresh cultures on Löwenstein-Jensen medium, were dispensed and vortexed in 3 ml of phosphate-buffered saline (pH 7.4).

Part of the suspension was diluted 1:10 in phosphate buffered saline. For each strain, 0.2 ml of the undiluted suspension was inoculated into the tubes containing Lowenstein-Jensen medium with KNO_3 and theantibiotics; 0.2 ml of the 1:10 dilution was inoculated into three drug-free tubes containing Löwenstein-Jensen medium with KNO_3 incorporated. The latter tubes served as growth controls. The tubes were incubated at 37°C.

After 7 days, 0.5 mlof a mixture of three reagents was added to one drug-free control tube. If any color change (strong or weak) could be seen, the corresponding antibiotic-containing tubes were also tested and susceptibility results were read. If no color change was seen in the growth control tube, this tube was discarded and the other two control tubes and the antibiotic tubes were re-incubated. The procedure was then repeated at day 10, using the second growth control, and if needed, also at day 14, using the last growth control tube. The reagents consisted of 1 part 50% (vol/vol) concentrated hydrochloric acid (HCl), 2 parts 0.2% (wt/vol) sulfanilamide, and 2 parts 0.1% (wt/vol) *n*-1-naphthylethylenediaminedi hydrochloride. They were mixed shortly before use. The results were classified as negative (no color change) or (pale pink) to 5 (deep red to violet). An isolate was considered resistant to a certain drug if there was a color change in the antibiotic tube in question greater than that in the 1:10-diluted growth control on the same day. All strains were coded and tested blindly. Comparison of NRA and BACTEC 460 results were done after decoding.

RESULTS

Seventy two clinical isolates of *M. tuberculosis* were analysed for their drug susceptibility by the NRA, and BACTEC 460 methods. Table 1 shows the results obtained with NRA compared to BACTEC 460 method.

For isoniazid, 31 isolates were found resistant and 41 were susceptible by nitrate reduction method whereas, 32 isolates were resistant and 40 were susceptible by Bactec 460 method. Thus, 1 isolate gave discordant results

Table 1. Agreement between the results of used antibiotics susceptibility method (BM, NRA)

Antibiotic	Isoniazid "INH"		Rifampicin "R"		Ethambutol "E		Streptomycin "S"	
Method	BM	NRA	BM	NRA	BM	NRA	BM	NRA
Susceptible (-)	40	41	41	43	39	40	44	44
Resistant (+)	32	31	31	29	33	32	28	28
Total Number	72	72	72	72				
Discordant results	1	2	1	0				
Agreement ratio	98.6%	97.2%	98.6%	100%				
Average of total Agreement ratio				94.4%				

since it was resistant by BM but susceptible by NRA. For rifampicin, 2 isolates gave discordant results, since 29 isolates were resistant and 43 were susceptible according to NRA method, whereas 31 isolates were resistant and 41 were susceptible according to BM.

For Ethambutol, 32 isolates were resistant and 39 were susceptible by both methods; one straingave a discordant result being susceptible by BM but resistant byNRA. For streptomycin, 28 isolates were resistant and 44 susceptibleby both methods and no isolates gave discordant results (table 1, fig. 1).

As shown in table 1, compatibility values for Isoniazid, Rifampicin, Ethambutol and Streptomycin were 98.6%, 97.2%, 98.6%, and 100% respectively and specificity value of NRA were 94.4%.

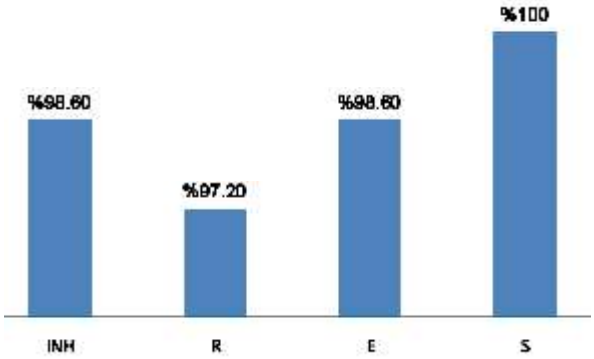

Fig. 1. Agreement between the results of used antibiotics susceptibility method (BM, NRA)

DISCUSSION

Many workers have examined the efficiency of NRA method as a inexpensive and fast method in detection of drug susceptibility of *Mycobacterium tuberculosis* isolates, since 7 -10 days are only needed to obtain results of this test comparing with LJ proportion method, which need up to 6 weeks to report the results. Golyshevskaia *et al.*,[4] has determined TB drug resistance based on nitrate reductaseactivity which indicates resistance of *M. tuberculosis* on liquid media in 4-7 days.Of course there arefast methods that clarify the sensitivity of TB isolates towards the used drugs, but it needsexpensive tools and conditions prepared for radioactive wastes (Bactec 460). Sethi *et al.,*[10] have concluded that NRA and proportion method agreed with 99 percent for isoniazid and ethambutol., in addition to complete agreement (100%) was found for rifampicin and streptomycin. There results were available in 7-14 days by NRA as compared to proportion method (PM) which takes 4-6 wk.The same results were obtained by Lemus*et al.,*[6, 7] where they found that the overall agreement between the NRA and PM was 98.8%.

The accuracy of NRA method comparing with technical method (Bactec 460) for detection of TB drug susceptibility was performed

byÄngeby*et al.,*[2]. They were the first whom reported NRA as a novel method for drug susceptibility test of TB. They tested a panel of 57 *M. tuberculosis* strains with various resistance patterns by the NRA method and the BACTEC 460 method. They concluded that the susceptibilities to INH, RMP, STR, and EMB gave an overall agreement of 94% between the NRA and BACTEC 460 techniques.

Shikama*et al.,*[11] concluded that the results of 117 TB strains susceptibility test by using NRA and Bactec 960 do not differ significantly. On average, they reported that NRA results were available after 10 days. These results also support these which obtained by Rosales *et al.,*[8]. They found good agreement and excellent specificity between NRA and the BACTEC 460TB reference method for INH, RMP and OFX.

Our results agree with the mentioned results, where the compatibility values between NRA and Bactec 460 methods were 98.6%, 97.2%, 98.6%, and 100% for isoniazid, rifampicin, ethambutol and streptomycin respectively and the overall specificity value of NRA were 94.4%. This method is simple to perform; permit visual and simple reading of results; practicable for laboratories with limited resources to discover drug susceptibilities of TB.

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