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Comparison of different methods of assessing *in vitro* resistance of *Mycobacterium tuberculosis* to rifampicin

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Background & objectives: Definitions of *in vitro* resistance to rifampicin in. strains of *Mycobacterium tuberculosis* by different methods have not been consistent, leading to variations in the interpretation and validity of results. This study compared three methods of defining *in vitro* resistance to rifampicin.

Methods: (i) A total of 598 clinical isolates of *M. tuberculosis* were concurrently compared by the minimal inhibitory concentration (MIC) and the proportion method on Lowenstein-Jensen medium; (ii) 54 strains tested by the MIC method were retested by the proportion method and the BACTEC radiometric method; and (iii) 72 strains which yielded an MIC of 64 mg/l by the MIC method were retested by the same method.

Results: Out of 598 cultures tested by the MIC and the proportion methods, identical classification as susceptible or resistant was observed in 99.7 per cent. A 100 per cent agreement was observed when 54 strains were tested by the MIC, proportion and BACTEC radiometric methods. When 72 strains with an MIC of 64 mg/l were retested by the same method, 61 (85%) yielded a lower MIC, 9 (12%) gave the same MIC while **2** (3%) yielded a higher MIC of 128 mg/l, reflecting perhaps the inherent limitations of the variations in the inoculum size.

Interpretation & conclusion: All 3 definitions of resistance, *viz.*, an MIC of 128 mg/l, a proportion of I per cent or more on 40 mg/l by the proportion method, both on L-J medium and a growth of 1 per cent or more on 2 mg/l by the radiometric method were found to be equally satisfactory.

Key words *In vitro* definitions - *Mycobacterium tuberculosis* - rifampicin

The standardization of drug susceptibility determinations has become important to formulate treatment policies for patients with drug resistant tuberculosis, especially those with multi-drug resistance (MDR-TB). A lack of standardization in the methodology and definitions of resistance may cause errors in the interpretation and validity of the tests and when results from different studies are compared. Although the definition of resistance to isoniazid has been uniform in most of the studies undertaken world-wide, it has not been so in the case of rifampicin resistance. Thus, while the Tuberculosis Research Centre, Chennai has been consistently using a minimal inhibitory concentration (MIC) of 128 mg/l or more on Lowenstein-Jensen (L-J) medium to define resistance to rifampicin', British workers have used MICs of both 64 and 128 mg/l as indicative of resistance[2,3]. The World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Diseases (IUATLD)

recommend a growth of 1 per cent or more on a concentration of 40 mg/l by the proportion method to indicate resistance[4,5]. For the BACTEC radiometric method a concentration of 2 mg/l has been used to define resistance to rifampicin[6].

Hence, a comparison of definitions of resistance to rifampicin by the MIC and proportion methods on L-J medium as well as by the radiometric method was undertaken, the results of which are presented.

Material & Methods

Strains: (i) A total of 598 consecutive clinical isolates of *Mycobacterium tuberculosis* were concurrently tested by the MIC and the proportion methods on L-J medium.

(ii) Fifty four strains of *M. tuberculosis,* comprising 35 strains which were sensitive and 19 strains which were resistant to rifampicin by the MIC method, were retested by the proportion and the BACTEC radiometric methods.

(iii) Seventy two clinical isolates, from patients admitted to controlled clinical trials at this Centre, which had shown a MIC of 64 mg/l by the MIC method, were retested by the same method to observe the consistency of the results obtained.

All the above investigations were undertaken during the period 1998-99.

Methods: L-J medium, without potato starch[7], prepared in the laboratory and BACTEC 12B medium obtained from Becton Dickinson Diagnostic Systems, USA were used for the study.

Susceptibility testing: For the MIC method, a 3 mm loopful of the bacterial suspension containing approximately 4 mg/ml was used to inoculate drug-free and drug-containing slopes (32, 64 and 128 mg/l). The standard strain, *M. tuberculosis* H_{37} Rv was included in every batch of tests as a check on the inoculum size as well as the drug concentrations in the medium. The slopes were incubated at 37°C and read at the end of 28 days. The lowest drug concentration which inhibited growth (defined as 20 colonies) was taken as the MIC[8].

The proportion tests were set up on a single concentration of 40 mg/l, using the neat and three serial ten-fold dilutions of the inoculum as recommended by Canetti and others[4]. The standard strain H_{37} Rv was tested with every new batch of medium. The tests were read at 42 days. The number of bacilli growing on the drug-containing slope was expressed as a proportion of the number of colonies on the drug-free slope.

The BACTEC radiometric method was performed as described earlier[9].

Definition of resistance: For the MIC method, a MIC of 128 mg/l or more was interpreted as indicative of resistance. For the proportion method, a growth of 1 per cent or more on the drug-containing slope (40 mg/l) when compared to the growth on the control slope was interpreted as resistant. In the BACTEC radiometric method, growth of a proportion of 1 per cent or more in the drug-containing vial (2 mg/l) indicated resistance. Thus, a strain was reported as resistant when the difference in growth index (DGI) between two consecutive daily readings in the drug-containing vial (inoculated with the neat suspension) was more than the DGI in the drug-free vial (inoculated with a 1:100 diluted inoculum) during the same period.

Statistical methods: The significance of data was analysed using the Chi-square test.

Results

Of the 598 clinical isolates tested by the MIC and proportion methods, 572 (95.6%) were classified as sensitive by the MIC method while 26 (4.3%) were resistant (Table I). By the proportion method, 570 (95.3%) were classified as sensitive and 28 (4.7%) as resistant. Thus, the agreement between the two methods was 99.7 per cent. Two strains sensitive by the MIC method were observed as resistant by the proportion method. The difference in classification (0 vs 2) was not statistically significant.

When 54 strains tested by the MIC method were retested by the proportion and the BACTEC radiometric methods, there was a 100 per cent

agreement in the classification as sensitive or resistant by both methods (Table 11).

Amalgamating Tables 1and-11, out of 652 strains tested by the MIC and the proportion methods, as many as 650 (99.7%) yielded an identical classification; an excellent agreement. When the 72 strains with an MIC of 64 mg/l in the first test were retested, as many as 61(85%) showed an MIC of 32 or less, 2 strains (3%) were resistant (MIC 128) while 9 strains (12%) showed the same MIC of 64 mg/l (results not tabulated).

Discussion

Standardization of definitions of resistance to anti-tuberculosis drugs helps in the comparison of results between laboratories and also between various methods. A uniform definition for resistance is not being employed by laboratories reporting drug susceptibility results for rifampicin by the MIC method. This method is known to be affected by the inoculum size based both on the bacterial content in the suspension as well as the volume used to inoculate the slopes[10].

Table 1. Rifampicin susceptibility test results clinical isolates of *M.tuberculosis* by the MIC and proportion methods

MIC method	Proportion method		Total
	Sensitive	Resistant	
Sensitive	570	2	572
Resistant	0	26	26
Total	570	28	598

Table II. Comparison of rifampicin susceptibility test results by the proportion method and BACTEC radiometric method with the MIC method in strains of *M.tuberculosis*

MIC method	Proportion method		BACTEC method		Total
	Sensitive	Resistant	Sensitive	Resistant	
Sensitive	35	0	35	0	35
Resistant	0	19	0	19	19
Total	35	19	35	19	54

The Tuberculosis Research Centre (TRC), Chennai, India, has been using the definition of an MIC of 128 mg/l or more on L-J medium for defining resistance to rifampicin'. This definition was arrived at by correlating the number of unfavourable responses among patients following treatment with rifampicin-containing regimens using the MICs of 128 and 64 mg/l as used by British workers[2,3]. Thus, two major chemotherapy studies were undertaken at this Center involving 2161 patients[11,12]. Of these, 1692 had organisms sensitive to all drugs initially and 78 had organisms with rifampicin MIC of 64 mg/l, none of whom had an unfavourable response. On the other hand, of the 71 patients (who were also resistant to isoniazid) with pretreatment rifampicin MIC of 128 mg/l or more, 66 (93%) had an unfavourable response, a highly significant difference ($P<0.001$) (Tuberculosis Research Centre, unpublished observations). Further, our definition has correlated well with the BACTEC definition in a series of ongoing external quality assessment studies being undertaken at this Centre by the WHO supra-regional laboratories in Australia and Belgium. Out of 77 strains tested for susceptibility to rifampicin till date, identical classification as sensitive or resistant was obtained in 76 (98.7%) strains (unpublished data). Further, this Centre has also been regularly implementing internal quality control in drug susceptibility tests using a panel of 10 standard strains, comprising known resistant as well as sensitive strains. The rifampicin-resistant strain was tested on 181 occasions and the sensitive strain on 149 occasions over a two year period. Correct classifications were obtained on 180 occasions (99.4%) for the resistant strain and on 148 occasions for the sensitive strain (99.3%). Thus, the reproducibility of results has been excellent over the two year period.

Out of the 652 isolates tested by the MIC method and the proportion method in the present study, ,agreement in results was observed in 650 (99.7%) strains. In 54 strains compared by the MIC method and the radiometric method, 100 per cent agreement in results was obtained. An earlier study from this Centre, on 78 strains comparing the radiometric method with the MIC method, yielded 99 per cent agreement between the two methods[9]. This shows

that the definition of 128 mg/l for resistance as used in the MIC method is valid.

It is known that the development of resistance in mycobacteria to rifampicin is a single-step process and that there is no borderline or doubtful resistance to rifampicin unlike in the case of drugs like streptomycin or isoniazid[13]. Thus, most of the cultures are either fully sensitive (MIC 32 or less) or resistant (MIC 128 or more) and strains with a MIC 64 mg/l are rare. An analysis of over 8000 strains tested before treatment at this Centre in a 10-year period from more than 4000 patients showed that the proportion of strains with a MIC of 64 mg/l was of the order of 1.6 per cent (unpublished observations). When a sample of these strains was retested in the present study, 85 per cent had a MIC of 32 mg/l. It is likely that growth on 32 mg/l, resulting in a MIC of 64 mg/l, could have been due perhaps to a heavier inoculum, which is an inherent limitation of the MIC method.

It is, therefore, recommended that the proportion test, being more precise, and as recommended by the WHO and IUATLD[4,5], may be employed using the criterion of 1 per cent or more growth on a single concentration of 40 mg/l as reported in this study. If, for any reason, only the MIC method is employed due to non-availability of technical infrastructure, a MIC of 128 mg/l or more would be the best definition of resistance to rifampicin. A further advantage of the MIC method is that it is the least expensive and far less complicated than the other method. The BACTEC radiometric method yields results within a few days, but requires considerable technical experience and involves high costs, both initial and recurring[9].

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References

1. Tuberculosis Research Centre, Madras. Study of chemotherapy regimens of 5 and 7 months duration and the role of corticosteroids in the treatment of sputum-positive patients with pulmonary tuberculosis in south India. *Tubercle* 1983; 64 :73-91.

2. Singapore Tuberculosis Service/British Medical Research Council. Clinical trial of three 6-month regimens of chemotherapy given intermittently in the continuation phase in the treatment of pulmonary tuberculosis. Am *Rev, Respir Dis* 1985; *132* : 374-8.

3. A controlled clinical trial of daily and intermittent regimens of rifampicin plus ethambutol in the I-e-treatment of patients with pulmonary tuberculosis in Hong Kong. A Hong Kong Tuberculosis Treatment Services/Brompton Hospital/British Medical Research Council Investigation. *Tubercle* 1974; *55* : 1-25.

4. WHO/IUATLD Global Working Group on Antituberculosis Drug Resistance Surveillance. *Guidelines for surveillance of drug resistance in tuberculosis.* WHO/TB/96.216. Geneva: World Health Organization, 1997.

5. The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network - Minimum requirements, role and operation in a low income country. Paris, France: International Union Against Tuberculosis and Lung Disease (IUATLD); 1998.

6. Siddiqi SH. In: *BACTEC TB system : Product and procedure manual,* section IV. Maryland USA : Becton Dickinson Diagnostic Instrument Systems; 1989 p. 2.

7. Jensen KA. Towards a standardization of laboratory methods. Second report of the sub-committee of laboratory methods of the International Union Against Tuberculosis. *Bull Int Union Tuberc* 1955; *25* : 89-104.

8 Canetti G, Fox W, Khomenko A, Mahler HT, Menon NK, Mitchison DA, *et al.* Advances in techniques of testing mycobacterial drug sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bull World Health Organ* 1969; *41* : 21-43.

9. Venkataraman P, Herbert D, Paramasivan CN. Evaluation of the BACTEC radiometric method in the early diagnosis of tuberculosis. *Indian J Med Res* 1998; *108* : 120-7.

10. Collins FM, Mackaness GB, Montalbine V, Smith MM. A comparison of the Steenken minimal inhibitory concentration and the Canetti proportion methods for determining levels of drug resistance in cultures of *Mycobacterium tuberculosis. Am Rev Respir Dis* 1968; *98* : 189-200.

11. Tuberculosis Research Centre. Interim findings on the evaluation of split drug regimens for pulmonary tuberculosis

- A randomized controlled clinical trial. *IndianJ Tuberc* 1995: *42* :201-6.

12. Tuberculosis Research Centre. A controlled clinical trial of oral short-course regimens in the treatment of sputum-positive pulmonary tuberculosis. *Int J Tuberc Lung Dis* 1997; l : 509-17.
13. Tsukamura M. The pattern of resistance development to rifampicin in *Mycobacterium tuberculosis*. *Tuberculue* 1972; 53 : 111-7.

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