Indian J Med Res 120, November 2004, pp 463-467

Evaluation of three methods to determine the antimicrobial susceptibility of *Mycobacterium tuberculosis*

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Received October 13, 2003

***Background & objectives*: Tuberculosis continues to be a major public health problem in India, especially with the emergence of drug resistance. A study was carried out to establish a rapid and accurate method of susceptibility testing for *Mycobacterium tuberculosis* using three methods *viz.*, proportion method by agar dilution on Middlebrook 7H11 agar, proportion method using the conventional Lowenstein-Jensen (L-J) medium and E test strip method.**

***Methods*: A total of seventy five clinical isolates from pulmonary and extrapulmonary sites were characterised and speciated by biochemical tests, growth and other standard parameters, and eight random isolates, also by polymerase chain reaction (PCR). Antimicrobial susceptibility of *M.tuberculosis* was performed by proportion method on L-J medium and Middlebrook 7H11 agar medium for isoniazide (INH), rifampicin (RIF), ethambutol (EMB), streptomycin (STM) and ciprofloxacin (CIP) using recommended critical concentrations. The two methods were compared with the E test method.**

***Results*: The 75 *M.tuberculosis* strains were isolated from sputum (47), pus (23), aspirate fluid (2), skin tissue (2) and gastric aspirate (1). Of these 49 (65.3%) isolates were sensitive and one (1.3%) was resistant to all the five drugs tested and by all the three methods. Eleven (14.7%) isolates were resistant to INH alone by the three methods. The E test method detected one isolate resistant to INH and 2 to RIF which were missed by the other two methods. The results obtained by all the three methods compared well.**

***Interpretation & conclusion*: The three methods *viz.*, proportion methods with L-J, Middlebrook 7H11 agar and the E test concurred fully in 57 isolates (76%). Association between L-J and Middlebrook 7H11 agar methods was 59 per cent. E test and the L-J methods did not differ significantly for all the drugs. The finding show that the E test method is superior to the other two methods in terms of simplicity of performance and the rapidity of results. Another advantage is that the MIC values can also be obtained simultaneously by this method.**

Key words Agar dilution - drug susceptibility - E-test - *M.tuberculosis*

Tuberculosis in India is one of the largest public health problems of immense consequence, with an estimated one death per minute[1]. For control measures to succeed, early detection and treatment of patients and contacts is very essential. At times the patients fail to respond to treatment with anti tubercular drugs, the reasons for this could be many, one of them being drug resistance, which is increasingly being reported from both developed and developing countries[2-5]. Tuberculosis resistant to treatment with the two most

important antitubercular drugs (isoniazid and rifampicin) is known as multi drug resistant tuberculosis (MDR-TB). MDR-TB is reported both from India and other parts of the world[6]. Different methods for testing drug susceptibility of the tubercle bacilli have been used in the past and the most widely accepted method is the proportion method using Lowenstein-Jensen (L-J) medium. Other non-conventional methods used in many laboratories include E test, bioluminescence, polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) *etc*. The limitations in the adequate assessment of drug resistance may be due to non-uniformity and lack of standardization of laboratory procedures, which is also an important factor, resulting in misleading reports and thus a poor knowledge of the true incidence of drug resistance[7]. A rapid and standardised method for drug susceptibility which is easy to perform and does not require expensive equipment, is desirable. In the present study three methods *viz*., proportion method using L-J medium, proportion method using Middlebrook 7H11 agar and the E test strip method were evaluated in order to reach a consensus on the optimal method that can be routinely used for drug susceptibility of *M. tuberculosis*[8].

Material & Methods

Seventy five clinical isolates of *M.tuberculosis* from various specimens [Sputum (47), pus (23), gastric aspirate (1), aspirate fluids (2) and skin tissue (2)], were speciated using the standard methods, including growth parameters and biochemical tests[9]. Eight of the 75 isolates were randomly selected and confirmed by PCR. All work was carried out in a Class II Biological Safety Cabinet as per recommendations[10,11].

Drug susceptibility: All isolates were tested for susceptibility to the following antituberculous drugs-isoniazide (INH), rifampicin (RIF), ethambutol (EMB), streptomycin (STM) and ciprofloxacin (CIP). The concentrations used in the proportion method on L-J medium and Middlebrook 7H11 agar medium were as recommended by the Tuberculosis Research Centre, Chennai[12] (Table).

M.tuberculosis $H_{37}Rv$, the reference strain (obtained from National Tuberculosis Institute, Bangalore) sensitive to all the antituberculosis agents, was used as the control strain in each batch of tests put up.

The proportion methods using L-J medium and the Middlebrook 7H11 agar were performed as per established standard procedures and calculations for resistance also made as recommended in standard literature[13-16].

E test susceptibility: Susceptibility by E test method (AB Biodisk, Sweden) was performed on Middlebrook 7H11 agar with Oleic acid-albumin-dextrose-catalase (OADC) supplement. Bacterial suspension was made in sterile distilled water and matched with MacFarland No.3. The surface of the agar was swabbed with the suspension. The plates were pre-incubated at 37°C in CO_2 jars for 24 h. E test strips were placed on the surface of the agar, the plates were sealed with cling films and again incubated at 37°C in CO_2 jar for 7-10 days. E test strips containing gradient concentrations of the INH, RIF, EMB, STM and CIP were used. Minimum inhibitory concentrations (MICs) were recorded and reported as sensitive or resistant as per AB Biodisk manufacturer's guidelines[17,18].

Table. Concentration of various antituberculosis drugs used in three methods

Drug	Concentration (µg/ml) in the medium	E test strip (µg/ml)
INH	0.2	0.016 -256
EMB	2.0	0.016 -256
STM	4.0	0.016-1024
RIF	40.0	0.016 -256
CIP	2.0	0.016 -256

INH, isoniazid; EMB, ethambutol; STM, streptomycin; RIF, rifampicin; CIP, ciprofloxacin

Statistical analysis: Chi square test was used for statistical analysis.

Results

Of the 75 samples, 52 (69.3%) were sensitive to all the 5 drugs by the L-J medium proportion method, while 54 (72 %) and 55 (73.3%) were sensitive by the Middlebrook 7H11 agar and E-test methods, respectively; 49 (65.3%) were sensitive to all the five drugs by all the three methods. One (1.3%) isolate alone was resistant to all the five drugs by the three methods. A total of 56 (74.7%) isolates were sensitive and 11 (14.7%) were resistant to INH by the three methods. Similarly, 70 (93.3%), 58 (77.3%), 67 (89.3%) and 64 (85.3%), isolates were sensitive to EMB, STM, RIF and CIP by the three methods respectively. The highest number of resistant isolates was seen for INH-11 (14.7%), followed by STM-7 (9.3%), RIF-6 (8.0%), CIP- 3 (4.0%) and EMB-2 (2.7%) by all the three methods.

The E test picked up one isolate resistant to INH, one to EMB and 2 resistant to RIF, all of which were recorded as sensitive by the L-J medium proportion method. The Middlebrook 7H11 method has also shown more resistance to INH (3), EMB (2) and STM (3) individually, as compared to L-J method (INH-0, EMB-0 and STM-1). Resistance to CIP alone by the L-J method was 5 (6.7%), while both the other methods did not show any such resistance.

Five isolates were resistant both to INH and RIF (MDR), by the L-J and agar dilution methods, while by E test method, 6 isolates were seen to be MDR. Overall, all three methods agreed fully in 57 cases (76 %).

Agreement between L-J and E test methods was 65 (87 %) while, between L-J and M7H11 methods it was 59 (79%). E test and L-J methods did not differ significantly (P>0.05 for all drugs).

It was also observed that the results were available in about 10 days by the E test method, whereas the agar dilution methods results took 10 days to 3 wk. As for the L-J method, the results took a minimum of 28 days after isolation.

Discussion

There are a few studies in which different methods to determine the antimicrobial susceptibility of *M. tuberculosis* have been compared[16-18]. We tested 75 isolates of *M.tuberculosis* and found good agreement between the three methods used in the present study. L-J method yielded results only after 4-8 wk, while M7H11 methods gave results in 10-14 days. E test method was comparatively a rapid method which took least amount of time *i.e.*, 7-10 days. E test method has been established[17,18] to be an accurate and rapid MIC determining method. It is especially of great value in drug resistant cases where the MIC is important. Wanger *et al*[19,20] demonstrated more than 90 per cent correlation between the E test and the agar proportion method for isoniazid, ethambutol, rifampicin and streptomycin. Hausdorfer *et al*[21] have also recorded over 90 per cent concordance between the results of E test and the agar proportion methods for all the four first line antituberculous drugs. Hoffner *et al*[17] has shown good association between these two techniques. The overall agreement between the three methods was 76 per cent which may seem low in comparison to 90 per cent and above by other authors[19-21], may be because we compared three methods of antimicrobial susceptibility testing while others did only two methods *viz*., Middlebrook 7H11 agar and the E test. In the present study, the overall percentage of agreement has been lowered by the Middlebrook 7H11 agar method. If only L-J and E test methods were compared the agreement was 87 per cent which is almost similar to other studies.

The conventional methods, like L-J method, Middlebrook 7H11 agar method and even the BACTEC radiometric method, have all been standardized mainly for the first line antituberculous drugs[18-22]. The E test method on the other hand, gives MIC values in a very simple, yet accurate way and thus may be applicable to second line and some experimental drugs. We have demonstrated its efficacy with one such second-line drug *viz*.,

ciprofloxacin. It was seen that none of the isolates showed resistance to CIP when tested by the E test and Middlebrook methods, while 5 isolates were resistant by the L-J proportion method. The fact that the ciprofloxacin drug powders used in the L-J and Middlebrook 7H11 methods were from different batches/brands may have contributed to this result.

Another advantage with the E test method is that it yields results in 7-10 days, unlike most other methods which take anywhere between 10 days (Middlebrook7H11 agar method) and 6 wk (L-J proportion method). This has been corroborated by several studies[18-22]. E test has also been shown to be a promising method for the susceptibility testing of more slowly growing and even the rapidly growing mycobacteria.

Tuberculosis is emerging as a global problem especially MDR-TB and associated HIV/AIDS. It would be best to seek newer and better methods to establish sensitivity patterns. Growing *M. tuberculosis* in culture with the drug incorporated is important to study the drug resistance pattern. L-J proportion method, though considered the gold standard earlier is time consuming and diagnostic delays may increase resistance to newer drugs. For this reason, it is necessary to invest on newer, more accurate and rapid methods of diagnosis and susceptibility testing. The present study showed that the E test method is rapid and superior to L-J proportion method and the Middlebrook7H11 agar proportion method, which are time-consuming. Thus, the E test seems promising as a rapid, reliable and simple method as reported by many[23].

Acknowledgment

The authors thanks the Council of Scientific and Industrial Research (CSIR), New Delhi for financial assistance in carrying out this study.

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