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Evaluation of various methods of susceptibility to ofloxacin in strains of *Mycobacterium tuberculosis*.

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A comparison of three methods of susceptibility testing was undertaken on 30 susceptible and 25 resistant strains of *Mycobacterium tuberculosis* to determine an acceptable *in vitro* definition of resistance of ofloxacin. The strains were tested by the proportion method on Lowenstein Jensen (L-J) and 7H11 media and also by the BACTEC radiometric method. Using a criterion of 1 per cent or more growth at a concentration of 2 mg/1, there was a 100 per cent agreement with the conventional MIC method by the proportion tests on L-J as well as on 7H11 media. The BACTEC radiometric method, at the same concentration, yielded 98 per cent agreement. Thus, any of these methods could be used depending upon the infrastructure available.

Key Words : *Mycobacterium tuberculosis* - Ofloxacin susceptibility test - tuberculosis

It has been shown in clinical trials that the currently available anti-tuberculosis drugs and treatment regimens provide almost 100 per cent cure rate and reduce mortality to as low as 1 per cent¹. However, these regimens under programme conditions result in a less favourable response, the reasons being several and most importantly, the difficulty in delivering the available regimens and poor compliance shown by the patients. Even the introduction of short course chemotherapy (SCC) under the National Tuberculosis Control Programme has not brought in any reasonable change to this situation. Due to this almost a third of the patients harbour tubercle bacilli resistant to one or more drugs. Combined resistance, especially to rifampicin and isoniazid (MDR-TB) which was virtually non existing until the early eighties started emerging as a serious thrat. For example, in a study reported from the Tuberculosis Research Centre (TRC), Chennai, among tuberculosis patients of the Raichur District,

Karnataka, combined resistance to rifampicin and isoniazid was found to an extent of 6 per cent². To contain this situation directly observed treatment short course (DOTS) is being advocated and at present, the Government of India is vigorously implementing this strategy in many districts of this country. In addition, new chemotherapeutic drugs are needed and new treatment regimens required to shorten the duration of treatment and also to combat drug resistant tuberculosis including MDR-TB. Among the new drugs, some of the quinolones in general and ofloxacin, ciprofloxacin and sparfloxacin in particular had shown good antituberculosis activity under *in vitro* conditions¹. For proper usage of these drugs in treatment regimens, invitro determination of their minimal inhibitory concentrations (MIC) is an important first step. However, studies to determine their MICs using different methods have not been undertaken concurrently so far. Hence, a study has been

undertaken to compare the susceptibility pattern of ofloxacin using different susceptibility testing methods.

MATERIAL & METHODS

Strains : Fifty five strains of *Mycobacterium tuberculosis* consisting of 30 strains susceptible and 25 resistant to ofloxacin as determined by conventional MIC method³, isolated from as many patients were included in this study. The strains were coded to conceal their identity before testing.

Methods : The strains were tested by three methods, viz., (i) the proportion sensitivity (PST) method on Lowenstein Jensen (L-J) medium; (ii) the proportion sensitivity method on 7H11 agar medium; and (iii) BACTEC radiometric method.

Media : L-J medium, without potato starch⁴, prepared in the laboratory; Middlebrook 7H11 agar⁵ prepared from Difco dehydrated powder as per the manufacturer's instructions and enriched with oleic acid-albumin-dextrose-catalase (OADC) supplement (prepared in the laboratory from ingredients obtained from Sigma Chemical Co., USA), and BACTEC 12B medium obtained from Becton Dickinson Diagnostic systems, USA were used for this study.

Drug Concentrations : A stock solution of ofloxacin (Sigma Chemical Co., USA Cat. No.0-8757, Lot. No.103H0083) was prepared in sterile N/10 NaOH. Subsequent dilutions were made with sterile distilled water and aseptically added to the media to give the desired final concentrations of 0.25, 0.5, 1, 2, 4 and 8 mg/l in L-J, 1, 2 and 4 mg/l in 7H11 and 2 mg/l in BACTEC 12B.

Sensitivity testing : For L-J media, 2-4 wk old subculture was selected and a standard method was followed for setting up of sensitivity testing⁶. In brief, 4 mg (moist weight) of growth was homogenised in 1 ml of water. From this 4 serial ten-fold dilutions were made. One 3 mm loopful of these suspensions was used to inoculate drug free and drug containing slopes, incubated at 37°C and read at 28 days and again at 40 days and the proportions of mutants grown were calculated⁶.

For 7H11 medium a large loopful of representative growth from a 2 wk old subculture was suspended

Table I. Comparison of ofloxacin sensitivity test results by the proportion method of Lowenstein - Jensen medium at different concentrations compared with the conventional method

Conventional (L-J)	Proportions resistant on L-J medium at						Total
	1 mg/l		2 mg/l		4 mg/l		
	<1%	≥1%	<1%	≥1%	<1%	≥1%	
Sensitive	25	5	30	0	30	0	30
Resistant	0	25	0	25	2	23	25
Total	25	30	30	25	32	23	55

L-J, Lowenstein-Jensen medium

in sterile distilled water. This was vortexed for 5 min and allowed to settle. The supernatant was diluted with sterile distilled water to match MacFarland#1. Serial further dilutions 10⁻² to 10⁻⁴ were made with distilled water. These dilutions were inoculated in 10ml quantities on each quadrant of drug free and drug containing plates, and incubated at 37°C for 3 wk at the end of which, readings were taken. Micro colonies, if any, were counted using a colony counter and the proportions of resistant mutants were calculated⁶.

The BACTEC radiometric method was performed as described earlier⁷. Ofloxacin solution was added to give a final concentrations of 2 mg/l. Data were statistically analysed using McNemar's test.

Results

Proportion method on L-J medium : The results of PST on L-J medium at different concentrations compared with the results of the conventional test results are given in Table I. At a concentration of 1 mg/l, out of 30 strains sensitive by the conventional method, 25 were susceptible by PST. However, all 25 resistant by the conventional method were also resistant by PST at 1 mg/l. The difference was not significant (P>0.1), the overall agreement being 91 per cent. At a concentration of 4 mg/l, while all 30 strains sensitive by the conventional method were also sensitive by PST, 23 of 25 resistant by the conventional method, were resistant by PST. The difference was not significant (P>0.1), the overall agreement being 96 per cent. At 2 mg/l, there was a 100 per cent agreement between sensitive and resistant strains by both methods. Thus using the discrimination approach⁸, a criterion of 1 per cent or more at

2 mg/1 concentration of ofloxacin was found to be the best definition of resistance with 100 per cent agreement with the conventional method.

Proportion method on 7H11 medium : The results of PST method using 7H11 medium are given in Table II. At a concentration of 1 mg/1, only 18 of 30 strains sensitive by the conventional method were sensitive by PST, a statistically significant difference ($P < 0.01$). However, all resistant strains gave identical readings, the overall agreement being 78 per cent. At 2 mg/1, all 30 strains sensitive by the conventional method were sensitive by PST on 7H11. Further, all 25 resistant by conventional method, were also resistant by 7H11 method, the agreement being 100 per cent. At 4 mg/1, while all 30 strains sensitive by conventional method were also sensitive by PST on 7H11, one resistant strain by the conventional method was misclassified by PST on 7H11 medium, the agreement being 98 per cent. Therefore a criterion of 1 per cent or more as resistant at 2 mg/1 concentration of ofloxacin was found to be the best definition with 100 per cent agreement with the conventional method.

BACTEC radiometric method : The results of BACTEC radiometric method in 12B medium at

2 mg/1 concentration are given in Table III. Out of 30 strains sensitive by the conventional method, 29 were susceptible by BACTEC radiometric method. Twenty five strains resistant by the conventional method were all resistant by BACTEC method. Thus the overall agreement with the conventional method were all resistant by BACTEC method. Thus the overall agreement with the conventional method was 98 per cent, the difference being not significant statistically.

Discussion

The standardisation of drug susceptibility determinations to newer drugs has become important to formulate treatment policies for patients with drug resistant tuberculosis, especially MDR-TB. Dual infection of HIV and TB is likely to enhance the actual number of MDR-TB cases globally. This has resulted in studies on several rapid and sensitive methods of drug susceptibility testing employing newer methods. However, lack of standardisation in the methodology as well as in the definition of resistance used in various studies may cause errors in the interpretations and validity of the results.

The present study compares the drug susceptibility patterns of known sensitive and resistant strains of *M. tuberculosis* to ofloxacin by the proportion method on L-J as well as on 7H11 media and also by the BACTEC radiometric method, with a view to determine an acceptable definition for ofloxacin resistance.

For the proportion method on L-J medium, a critical concentration of 2 mg/1 was found to give perfect agreement between the sensitive and resistant populations. Using this criterion of resistance, viz., 1 per cent or more at 2 mg/1, 100 per cent agreement was found with the conventional method, indicating that the definition of 8 mg/1 or more on L-J by the absolute concentration method also could be valid. The disadvantage of the absolute concentration method, however, is that it is likely to be influenced both by the inoculum size as well as the viability of the organism. The proportion method is not affected by either of these. However, it takes as long as 6 wk for the results to become available.

With the proportion method on 7H11 agar medium also, the best agreement of 100 per cent

Table II. Comparison of ofloxacin sensitivity test results by the proportion method on 7H11 at different concentrations compared with the conventional method.

Conventional (L-J)	Proportions resistant on L-J medium at						Total
	1 mg/1		2 mg/1		4 mg/1		
	<1%	≥1%	<1%	≥1%	<1%	≥1%	
Sensitive	25	5	30	0	30	0	30
Resistant	0	25	0	25	2	23	25
Total	25	30	30	25	32	23	55

L-J, Lowenstein- Jensen medium; * $P < 0.01$

Table III. Comparison of ofloxacin (2 mg/1) sensitivity test results by the BACTEC radiometric method compared with the conventional method.

Conventional (L-J)	BACTEC radiometric method		Total
	Sensitive	Resistant	
Sensitive	29	1	30
Resistant	0	25	25
Total	29	26	55

L-J, Lowenstein-Jensen medium

with the conventional method on L-J was found to be with 2 mg/1, using a criterion of 1 per cent or more as resistance. This is in agreement with earlier observations made by other workers (Siddiqi, SH-personal communication). The major advantage of using this medium is that the results become available by 3 wk. Further this medium being transparent, permits early detection of colonies. Also because of its lower protein content, there is less amount of binding of the drugs to proteins.

The agreement with the BACTEC radiometric method was found to be 98 per cent. The major advantage of this method is the early availability of results, usually within 8-10 days. This would be very beneficial to patients harbouring drug resistant organisms as effective treatment with appropriate regimens could be initiated earlier. Further, the BACTEC method being an automated technique, has a potential for introducing standardisation in the laboratory. The only constraint with this technique is the high cost, both initial and recurring. However, this constraint could be partially offset by the ease and early availability of results in a region with a high prevalence rate of the disease.

Since all the 3 methods studied along with the conventional absolute concentration method have been shown to be equally efficient, any of these methods could be used in a routine laboratory setup depending upon the availability of trained manpower and other infrastructure.

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References

1. Paramasivan C.N. Newer antimycobacterial drugs and their role in the treatment of tuberculosis patients. *Indian J Tuberc*. 1994; 41: 7-16.
2. Paramasivan CN. An overview on drug resistant tuberculosis in India. *Indian J Tuberc* 1998; 45: 73-81.
3. Venkataraman P, Paramasivan CN, Prabhakar R. *In vitro* activity of ciprofloxacin and ofloxacin against south Indian isolates of *Mycobacterium tuberculosis*. *Indian J Tuberc* 1994; 41: 87-90.
4. Jensen KA. Towards a standardisation of laboratory methods. Second report of the Sub-committee of Laboratory Methods of International Union Against Tuberculosis. *Bull Int Union Tuberc* 1955; 25: 89-104.
5. Gohn ML, Waggoner RF, McClatchy JK. The 7H11 medium for the cultivation of Mycobacteria. *Am Rev Respir Dis* 1968; 98: 295-6.
6. 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 WHO* 1969; 41: 21-43.
7. 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.
8. Radhakrishna S. Discrimination analysis in medicine. *Statistician* 1964; 14: 147-67.

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