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A direct rifampicin sensitivity test for tubercle bacilli

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A direct sensitivity test for rifampicin has been standardised for early detection of resistance for the mycobacterium tuberculosis smear positive sputum samples. Indirect sensitivity tests set up from primary cultures of the same samples served as controls. The direct test showed 95 per cent agreement with the standard indirect test and as such 74 per cent and 90 per cent of the resistant strains were detected by the fourth week and fifth week, respectively, with an overall gain of 4-5 wks time. Resistance could be detected earlier for multibacillary specimens. This direct sensitivity test on Lowenstein Jensen (LJ) medium offers a feasible alternative for laboratories which lack facilities to perform drug susceptibility tests by the rapid but sophisticated and costly BACTEC method. The method is simple to perform, economic, reliable and amenable to confirmation by the indirect test, if needed.

Key words Direct sensitivity test - *Mycobacterium tuberculosis* - rifampicin resistance

In the developing countries, most laboratories study drug susceptibility for Mycobacterium tuberculosis by the indirect test method from the growth appearing in primary culture. Thus, to obtain the results, at least an additional 4-5 wk are required over the time taken for the primary culture. Although rapid methods such as BACTEC radiometric systems bioluminescence assay, use of DNA probes, and HPLC have been described they are costly, require highly sophisticated equipment and technical skill and are therefore beyond the reach of most laboratories in these regions. Among the simpler methods available, direct sensitivity test on Lowenstein Jensen (LJ) medium which yields results along with he primary culture has been shown to be as good as the indirect tests for isoniazid and streptomycin[2]. The method for direct sensitivity test using sputum swab culture was described in 1970[3].

Rigampicin is one of the key drugs in the short course regimens for tuberculosis. Despite the availability of very effective regimens, treatment failure and emergence of drug resistance are on the rise in the community- because of inadequate and irregular treatment[4]. It has been shown that patients harbouring rifampicin resistant organisms very often do not respond to treatment perhaps because resistance to rifampicin is most often accompanied by resistance to other antituberculous drugs, particularly isoniazid[5,6].

The objective of the present study was to standardise a direct sensitivity test for rifampicin from smear positive sputum samples and to compare the same with the indirect test done on primary cultures obtained from the same samples.

Material and Methods

Samples : It was intended to include 100 samples each of sensitive and resistant cultures for standardising the test. All smear positive sputum samples from treated as well as untreated patients, serially processed in the laboratory were obtained taking care not to include more than two specimens

from each patient. As the proportion of rifampicin resistant cultures was expected to be very low, in order to reduce the intake period, samples were also collected from patients known to excrete resistant organisms and thus a total of 243 samples obtained over a period of 10 wk were tested by the two methods.

Sputum smears were graded by fluorescent microscopy[7].

LJ medium with rifampicin : Rifampicin was added to LJ medium to give pre-inspissation concentrations of 32, 64 and 128 mg/l for the indirect test and 32 and 64 mg/l for the direct test.

Direct sensitivity test : Sputum samples were processed by the modified Petroff 's method[8]. The deposit was inoculated with a 5 mm loop on two slopes each of LJ medium without drugs (plain LJ) and with 32 and 64 mg/l of rifampicin (R32 and R64, respectively) for the direct test. The six slope were randomised, incubated and were examined weekly for eight weeks. Growth was recorded as 3+ (confluent), 2 + (≥ 100 colonies) or as 1-99 colonies.

Indirect sensitivity test : Two further plain LJ slopes were inoculated from the same deposit as the primary culture for the indirect test. These slopes were incubated and examined weekly for 8 wk. The indirect tests were set up from them using the standard procedure[9] as and when positive cultures were obtained. The tests were randomised and read at 4 wk.

The standard strain *M. tuberculosis* H37Rv was included in each batch of indirect sensitivity test as control.

The criteria adopted for defining resistance are as shown in Table I.

Table 1. Criteria used for defining resistance to rifampicin

	Growth on plain LJ medium	Growth on drug containing LJ medium
indirect test	≥ 2+ < 100 colonies	≥ 20 colonies on R64 (MIC > 64) test repeated
Direct test	≥ 2+	≥ 20 colonies on R32 or R64 (MIC > 32 or > 64)
	1 - 99 colonies	≥ 1 colony on R32 or R64 (i.e., > 1% resistance)

MIC, minimal inhibitory concentration
LJ. Lowenstein Jensen

Analyses : In the direct test, cultures were classified as sensitive or resistant using the criteria based on both R 32 and R 64, and each of these results were correlated with results of the indirect test. The Chi-square test was applied to determine the statistical significance of the comparisons made.

Results

Of the 243 smear positive samples (10 of 3+, 57 of 2+ and 176 of 1+ grade, respectively) included in the study, 193 were culture positive for *M. tuberculosis,* 41 were negative for culture, 2 yielded non tuberculous mycobacteria (NTM) and 7 specimens were contaminated. The standard indirect test on the 193 *M. tuberculosis* isolates showed that 131 were sensitive and 62 were resistant to rifampicin (Table II). It was observed that 127 of 131 sensitive and 57 of 62 resistant cultures were identically classified by the direct test based on R32. Four sensitive and five resistant cultures were misclassified. Similar proportions of agreement and disagreement were seen in the test based on R64 also and there was no statistical difference between the disagreements occurring among the sensitive and the resistant cultures ($P > 0.05$).

In the direct test, results were available for 136 samples (70.5%) by the third week of setting up the cultures, for 173 (89.6%) by the fourth week, for 185 (95.9%) by the fifth week and for the rest by the eighth week (Table III). The direct test results from the third week based on R32 showed agreement with the indirect test classification in a high proportion (92.6%). Further analysis (not tabulated) showed that among the few cultures showing disagreement in

Table II. Agreement in the classification of cultures of *M. tuberculosis* by the indirect and direct tests for rifampicin sensitivity

Direct test based on		Indirect test Sensitive N= 131	Resistant N=62	Total
R32	Sens.	127	5	132
	Resis.	4	57	61
R64	Sens.	129	6	135
	Resis.	2	56	58

classification, more number of resistant cultures were classified as sensitive than vice versa (P< 0.02) up to the fourth week when the direct test was based on R32 and up to the fifth week when based on R64.

Table IV presents the results analysed on the grade of growth on the plain medium in the direct test to see whether the agreement was similar with all grades of positives. The extent of agreement at 5 wk based on R32 ranged from 97 per cent with 3+ growth to 89 per cent with growth of 20 colonies or less, the difference being nonsignificant (P > 0.2). When based on R64, it ranged from 99 to 89 per cent and the extent of agreement was significantly higher with 3+ growth than with all the other grades (P = 0.01).

Detection of resistant cultures by the direct test week by week is presented in Table V. About 74 per cent of them were detected by the fourth week and 90 per cent by the fifth week, on R32. The detection rate was slower, though not significantly, on R64. Analysis (not tabulated) showed that 45 (73.8%) of the resistant cultures on R32 and 43 (74.1%) of those on R64 had equal growth on the plain and drug containing slopes indicating that the majority were fully resistant to rifampicin.

Table III. Agreement between the rifampicin sensitivity classification of *M. tuberculosis* based on the indirect test and the direct tests read at different weeks

Direct test based on		Agreement with the indirect test at			
		3 wk	4 wk	5 wk	8 wk
R32	No.	126	159	175	184
	%	92.6	91.9	94.5	95.3
R64	No.	122	155	173	186
	%	89.9	91.2	93.5	96.3
No. of positive culture		136	173	185	193

Table V. Rate of detection of rifampicin resistance by the direct sensitivity test

Weeks after setting up	Based on			
	R32		R64	
	No.	%	No.	%
2	6	9.8	4	6.9
3	28	45.1	24	41.4
4	45	73.8	42	72.4
5	55	90.2	49	84.5
6	60	98.4	55	94.8
7	61	100.0	58	100.0

To determine the optimum time required to classify the cultures based on the direct test, analysis was done to compare the time taken for emergence of growth on drug free and drug containing slopes. In 40 of 61 (65.6%) cultures resistant on R 32, growth emerged simultaneously, in 12 (19.7%), growth emerged in the drug containing slopes after one week, in 8 (13. 1%) after two weeks and in 1 (1.6%) after three weeks (data not tabulated). The corresponding figures for growth on R 64 were 33 of 58 (56.9%) simultaneously, 15 (25.9%) after one week, 4 (6.9%) after two weeks and 6 (10.3%) after three weeks. Thus a proportion of resistant cultures took a longer time to appear on the drug containing slopes, raising the possibility of misclassifying such cultures as sensitive if the classification were to be done immedi-

Table IV. Agreement between the indirect and direct tests for rifampicin sensitivity of *M. tuberculosis* with different grades of positivity

Grades of growth	Agreement (%) based on											
	R32 read at						R62 read at					
	3 wk		5 wk		8 wk		3 wk		5 wk		8 wk	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
3+	51	100	77	97	86	98	49	96	78	99	88	100
2+	59	89	52	93	48	94	57	86	50	89	48	94
20-99 cols	10	(83)	29	93	30	94	10	(83)	28	90	29	91
1-19 cots	6	(86)	17	(89)	20	(91)	6	(86)	17	(89)	20	(91)

Numbers in parentheses are percentages based on less than 25 numbers

ately after growth appeared on the drug free slopes.

The time taken for detection of resistance was related to the smear grade of the samples. Of 16 resistant cultures from specimens with smear grade of 2+, 13 were detected on R32 and 11 on R64 by the third week; but out of 45 with smear grade of 1+, only 15 and 13 were detected by three weeks on R32 and R64 respectively. Thus detection of resistance was earlier for specimens with more organisms in them.

Discussion

The direct test described here has the advantage of providing susceptibility results for rifampicin within 5 wk or less, with a gain of four weeks or more over the indirect method. By the direct test, results were available for 96 per cent of the samples in five weeks and the agreement of results with the indirect test was of the order of 94-95 per cent. The extent of agreement was unaffected by the grade of growth of the cultures for classification based on R32. The standard indirect test in use in most of the laboratories defines resistance to rifampicin as a 20 colony minimal inhibitory concentration (MIC) of > 64 mg/l on LJ medium provided that the test was based on growth of 100 colonies or more on the plain medium. In the direct test too, the definition of a 20 colony MIC of > 32 and > 64 mg/l was used for cultures showing more than 100 colonies. However, this definition leaves those cultures with < 100 colonies on the plain medium without a definitive result. For such cultures, a second definition was employed which considered growth of 1 colony or more on the drug containing medium as the mark of resistance which is in effect equivalent to > 1 per cent resistance to 32 or 64 mg/l in the population tested. This criterion is similar to that laid down by Conetti and as cited by Heifets[10] and again by Vareldzis *et al* [11] in the WHO recommendations on measurement of drug resistance. The latter considered the absolute concentration method (MIC), the resistance ratio method (RR) and the proportion method as being good measures for detecting resistance to isoniazid and rifampicin because there is a large *in vitro* difference between susceptible and resistant strains; and further suggested that the proportion method was the best of the three. In this investigation it was seen that, by employing a dual definition (MIC and proportion method) all grades of cultures could be classified by the direct test with more than 90 per cent accuracy.

A note of caution is called for, regarding classifying cultures as sensitive when the growth on the plain medium is very poor. It was seen in the present study that 73 per cent of the resistant cultures had identical grades of growth on the plain and drug containing slopes in the direct test. In this method there is a potential risk for a few of the cultures being misclassified as sensitive if the growth on the plain medium is too low. This was seen only in one culture in our study, where there was only one colony on the plain medium alone and that was resistant by the indirect test. Hence, it is recommended that direct test results based on fewer than 10 colonies be confirmed by an indirect test especially if it appears to be sensitive.

The misclassification of a small proportion of resistant cultures as sensitive in the earlier weeks of incubation of the direct test could be attributed to the observed delay in the emergence of growth on the drug containing slopes as compared to the plain slopes. Therefore, while resistance can be reported as soon as adequate growth is seen on the drug containing slopes, a culture may be reported as sensitive only 2-3 wk after growth is first observed on the plain slopes. However, this does not affect the value of the test aimed primarily to report resistance as early as possible.

Four cultures classified as sensitive by the indirect test were classified as resistant by the direct test. Three of these cultures came from patients known to excrete resistant organisms and the fourth one missed being considered as resistant in the indirect test as there were only 19 colonies instead of the mandatory 20 colonies on the R64 slope.

Four of six resistant cultures misclassified as sensitive in the direct test came from three patients who had evidence of harbouring a mixed population of resistant and sensitive organisms, a phenomenon usually observed as a transitional phase among patients before they produce sputum with organisms fully resistant during the subsequent period of follow up. In the fifth, the classification based on growth of only one colony as cited earlier was unreliable. There

was no justifiable explanation for the misclassification by the direct test of the sixth culture.

Based on the findings of this investigation, R32 is recommended as preferable to R64 if only on&concentration is to be used in the direct test because, on R32 the growth was better, earlier detection of resistance was possible on it, classification was more precise when related to the patients' resistance status and it was less affected by the grade of growth *i.e.,* bacterial load of the sample. Further, 32 mg/l is closer to 40 mg/l, the critical preinspissation concentration in LJ medium, used to define rifampicin resistance".

The practical advantages of the direct sensitivity test are many. The results are available earlier, the test is more representative of the sample as it is based on the primary culture itself; losses due to contamination or failure to grow on subculture are avoided and administrative delays in setting up the indirect test are eliminated. Further, among the seven cultures excluded from the analyses because all the primary plain slopes were contaminated and indirect tests could not be set up from them, four could be classified as resistant by the direct test having shown 2+ growth on all the drug containing slopes.

Thus the direct sensitivity test described here can be set up in any mycobacteriology laboratory using existing culture facilities and resistance can be reported from the third week onwards. It requires just 2 slopes each of LJ medium, plain and with 32 mg/1 of rifampicin. The test is simple to perform, cost effective and reliable and therefore suitable for the developing countries.

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